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Effects of 4-Alkyl Analogues of 3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine on Hepatic Cytochrome P-450 Heme, Apoproteins, and Catalytic Activities following *In Vivo* Administration to Rats

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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) via heme destruction. We have examined the time course of effects of DDC analogues on the catalytic activities and apoproteins of the major β-naphthoflavone-, dexamethasone-, and phenobarbital-inducible isozymes of rat liver P-450 following *in vivo* administration. In β-naphthoflavone-treated rats, all DDC analogues examined caused loss of the P-450 chromophore and dramatic loss of 7-ethoxyresorufin *O*-deethylase activity, a catalytic marker for P-450c. The isopropyl, hexyl, and isobutyl analogues caused the most pronounced loss/alteration of P-450c apoprotein levels, as revealed by two monoclonal antibodies (MAbs), 1-31-2 and 1-7-1. The apoprotein of P-450d was not altered. In dexamethasone-treated rats, all analogues except 4-hexyl-DDC caused loss of the P-450 chromophore and erythromycin *N*-demethylase activity, a catalytic marker for P-450p-related isozymes. Only 4-isopropyl-DDC caused significant loss/alteration of the apoprotein of P-450p-related forms, as revealed by MAb 2-13-1. In phenobarbital-treated rats, all analogues reduced the level of the P-450 chromophore, whereas only 4-hexyl-DDC and 4-isopropyl-DDC lowered 7-pentoxyresorufin *O*-dealkylase activity, a catalytic marker for P-450b. MAbs 2-66-3 and 2-8-1 revealed no change in the level of phenobarbital-inducible apoproteins recognized by these probes. In agreement with our previous *in vitro* studies [*Mol. Pharmacol.* **35**;626–634 (1989)], P-450 c and p are targets for mechanism-based inactivation by DDC analogues. However, unlike the situation *in vitro*, loss of enzyme activity *in vivo* is, at least in some instances, accompanied by loss/alteration of the corresponding P-450 apoprotein.

The dihydropyridine DDC (Fig. 1, compound **a**) and various 4-alkyl analogues (Fig. 1, compounds **b**–**e**) cause mechanism-based inactivation of P-450 (1–3). As a result of one-electron oxidation of the DDC analogue's nitrogen atom, catalyzed by P-450, the 4-alkyl group is ejected as a radical (1, 4), which is capable of alkylating one or more of the pyrrole nitrogens of the heme prosthetic group (5). In most cases, the resulting *N*-alkylPP regioisomers (which differ with respect to the pyrrole ring nitrogen that is alkylated, N_A, N_B, N_C, N_D) inhibit ferrochelatase (EC 4.99.1.1) (2, 3, 6), resulting in protoporphyrin IX accumulation. For *N*-alkylPPs with *N*-alkyl groups larger than methyl, ferrochelatase-inhibitory activity resides preferentially in the N_A and N_B regioisomers (5, 6). In addition to *N*-alkylation, an alternate fate of the P-450 heme has recently been described, in which P-450 suicide substrates such as 4-ethyl-DDC (Fig. 1, compound **b**) and AIA cause activation of the heme to a reactive species that forms a covalent adduct with the apoprotein (7, 8). With various DDC analogues, the fate of the prosthetic heme and the regioselectivity of heme alkylation differ (6, 9, 10), thus affecting the ferrochelatase-lowering activity of these compounds. It seems likely that the DDC analogue itself and/or the P-450 isozyme target may play a role in determining the fate of the prosthetic heme and the regioselectivity of heme *N*-alkylation.

Recent studies have been aimed at elucidating the P-450 isozyme selectivities of 4-ethyl-DDC (8, 11) and other 4-alkyl analogues of DDC (12). Correia *et al.* (8) demonstrated that administration of 4-ethyl-DDC to rats resulted in loss of cata-

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

lytic markers selective for P-450 h, k, and p.[2] The steroid-inducible P-450p was shown to be a key target, the inactivation of which leads mainly to heme-derived protein adducts. Tephly *et al.* (11) were the first to demonstrate that 4-ethyl-DDC was capable of causing loss/alteration of the apoprotein of P-450 c, h, k, and p *in vivo*. Our *in vitro* studies (12) revealed that a series of 4-alkyl analogues of DDC caused mechanism-based loss of the catalytic activities of the major βNF-inducible isozyme P-450c and the major DEX-inducible isozyme P-450p, with only minor apoprotein alterations. The major PB-inducible isozyme P-450b was not a target for inactivation.

These and other studies suggest that differences exist in the *in vitro* and *in vivo* effects of P-450 suicide substrates. The major differences are seen in the effect of heme-destructive agents on the apoproteins of P-450 isozymes. First, for both 4-ethyl-DDC and AIA, the formation of heme-derived protein adducts is greater in microsomes than following *in vivo* administration (8, 22). Second, *in vivo* administration of these agents results in loss/alteration of selected P-450 apoproteins (8, 11, 23), whereas microsomal incubation does not (12, 23).

With these observations in mind, we felt it essential to extend our previous *in vitro* findings (12) to a consideration of the *in vivo* effects of a series of 4-alkyl analogues of DDC. As in the previous study (12), we have employed DDC analogues representative of three groups. The first group, typified by 4-ethyl-DDC (Fig. 1, compound **b**) and 4-hexyl-DDC (Fig. 1, compound **e**), destroys P-450 heme, resulting in the formation of an *N*-alkylPP that inhibits ferrochelatase. In the case of 4-ethyl-DDC, pathways of heme destruction in addition to *N*-alkylation have been documented (7, 8). 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 (1), 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 essentially lacks ferrochelatase-lowering activity (3, 6). The goal of the present work was to examine the effects of these DDC analogues on the catalytic activities and apoprotein moieties of the major βNF-, DEX-, and PB-inducible isozymes of rat hepatic P-450 following *in vivo* administration and to compare the results with those previously obtained in microsomal systems (12).

Materials and Methods

Source of compounds. DDC analogues were synthesized as described previously (1, 3). MAbs were prepared by the hybridoma method described previously (24–26). All other compounds were obtained from the sources indicated previously (12).

Animals and treatment. Male Sprague-Dawley rats (250–300 g) were obtained from Charles River Canada, Inc. (St. Constant, Quebec, Canada). The rats were housed and pretreated with P-450-inducing agents as described (12). Twenty-four hours after the last inducer pretreatment, rats received a single intraperitoneal injection of a DDC analogue (0.4 mmol/kg in DMSO). Controls received an equivalent volume (0.5 ml) of the vehicle, DMSO. Rats were sacrificed by decapitation at 0, 2, 4, and 8 hr after DDC analogue administration.

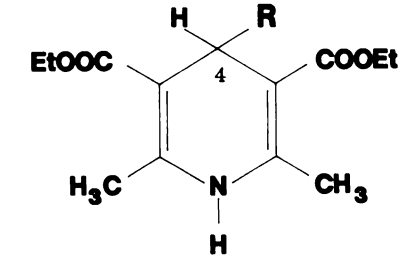

Fig. 1. Structure of dihydropyridine analogues. **a**, R = —CH_3, DDC; **b**, R = —CH_2CH_3, 4-ethyl-DDC; **c**, R = —$CH(CH_3)_2$, 4-isopropyl-DDC; **d**, R = —$CH_2CH(CH_3)_2$, 4-isobutyl-DDC; **e**, R = —$(CH_2)_5CH_3$, 4-hexyl-DDC.

Preparation and storage of hepatic microsomes. 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 (2). The microsomal pellets were stored at −70° for less than 2 weeks.

Analytical methods. Microsomal P-450 and heme content were determined spectrophotometrically by the methods of Omura and Sato (27). Protein was assayed by the method of Lowry *et al.* (28). In microsomes from PB- and βNF-treated rats, 7PRFOD and 7ERFOD activities, respectively, were assayed by the method of Burke *et al.* (29), as described previously (12). In microsomes from DEX-treated rats, ERND activity was measured by the method of Wrighton *et al.* (30), as described previously (12).

For the immunodetection of P-450 apoproteins, microsomal samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (31) and transferred to nitrocellulose (32), and individual P-450 isozymes were localized and quantitated by laser densitometry as described (12). Gel slots were loaded with 2, 1, and 6 μg of protein for microsomes from βNF-, DEX-, and PB-treated rats, respectively. MAbs 1-31-2, 1-36-1, and 1-7-1, directed toward MC-inducible rat liver P-450, were used at a 1:200 dilution. MAbs 1-31-2 and 1-36-1 recognize P-450c, whereas MAb 1-7-1 recognizes P-450 c and d (15). With the electrophoretic conditions employed, P-450 c and d are fully resolved, allowing for their independent densitometric analysis. MAbs 2-66-3 and 2-8-1, directed toward PB-inducible rat liver P-450, were used at 1:40 and 1:60 dilutions, respectively. 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 (15). MAb 2-13-1, directed toward a member of the steroid-inducible P450IIIA subfamily, P-450 2a/PCN-E, was used at a 1:2000 dilution. This MAb recognizes the constitutive male-specific P-450 2a form in addition to the PCN- and/or PB-inducible isozyme PB-2a/PCN-E (15). Other members of the P450IIIA subfamily may be identified by this MAb also and, thus, we have used this MAb as a probe for apoprotein moieties representative of the P-450p-related family of isozymes. MAb HyHel-9, directed toward chicken lysozyme, was used to control for nonspecific binding in Western blots. These MAbs have been characterized previously (24–26). A goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate was used at a 1:2000 dilution, except following MAb 2-13-1, in which case a 1:5000 dilution was used.

Statistical analysis. In all cases, a randomized design two-way analysis of variance was employed to identify significant ($p \leq 0.05$) drug and time effects. *Post hoc* randomized design one-way analyses of variance with Newman-Keuls test were used to identify significantly different ($p \leq 0.05$ or 0.01) groups along the drug and time dimensions.

[2] Designations given to apparently equivalent rat liver 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) (13–15). 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 (16) is equivalent to PCN1 (17) and PCNa (18), and this isozyme has the gene designation IIIA1 (14). It appears that a second PCN- and/or PB-inducible isozyme exists, which has been referred to as PB/PCN-E (19), PB-2a (20), and PCNb (18). In addition, closely related constitutive male forms such as PCN2 (17), PCNc (21), and 2a (15) 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 P450IIIA subfamily.

In all cases, analyses were performed on the raw data and not the percentage of control data presented in the figures.

Results

In order to examine the isozyme selectivities of DDC analogues, we have performed time-course analyses of the effects of these compounds on P-450 isozyme catalytic activities and apoproteins following *in vivo* administration to βNF-, DEX-, and PB-treated rats. Results are expressed as mean ± SD.

Effect of DDC analogues in βNF-treated rats. With the exception of DDC, all compounds significantly lowered P-450 levels at all time points examined (Fig. 2). The various 4-alkyl analogues had similar effects, destroying 63–79% of the P-450 at the 8-hr time point. Microsomal heme was lowered in a similar pattern by these compounds (data not shown). Over the same time course, these analogues reduced 7ERFOD activity by 93–99% (Fig. 3), indicating that P-450c catalytic activity is a target for inhibition by these compounds. Using MAb 1-31-2, we have shown that after 8-hr of treatment, the isopropyl, hexyl, and isobutyl analogues reduced P-450c immunoreactivity by 72–91% (Fig. 4). DDC and 4-ethyl-DDC were less effective in this regard, reducing P-450c immunoreactivity by only 29 and 40%, respectively. This differentiation of DDC analogues into two groups is clear at the 8-hr time point but less pronounced at earlier times. MAb 1-36-1 revealed that only 4-isobutyl-DDC significantly lowered P-450c immunoreactivity (data not shown), whereas MAb 1-7-1 revealed significant reductions caused by the isopropyl, isobutyl, and hexyl analogues (Fig. 5). MAb 1-7-1, which also recognizes P-450d, revealed that DDC analogues do not affect P-450d apoprotein levels over an 8-hr time course (data not shown). A previous study (11) revealed that the P-450d apoprotein was not a major target for destruction by 4-ethyl-DDC.

Effect of DDC analogues in DEX-treated rats. Following DEX treatment, DDC and 4-hexyl-DDC did not significantly lower P-450 levels (Fig. 6). The isobutyl analogue reduced P-450 levels by 48% after 8 hr. 4-Ethyl-DDC caused

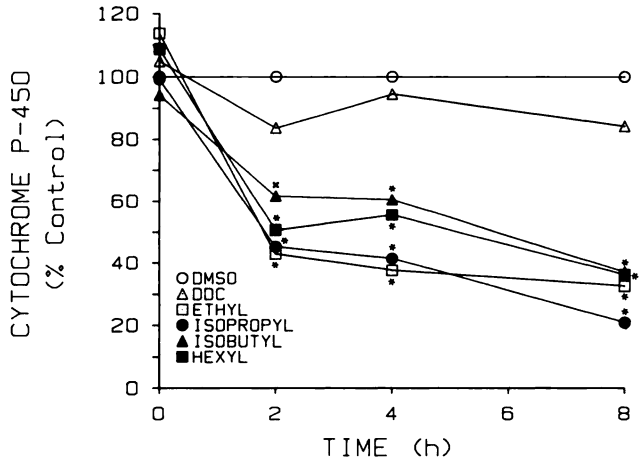

Fig. 2. Time course of the effect of DDC analogues on microsomal P-450 levels in βNF-treated rats. Data are expressed as a percentage of the vehicle (DMSO) control at each time point. Each *point* represents the mean of determinations made on four different rats. Control P-450 values were as follows: 0 hr, 1.22 ± 0.20; 2 hr, 1.52 ± 0.19; 4 hr, 1.40 ± 0.07; and 8 hr, 1.46 ± 0.28 nmol/mg of protein. ˣSignificantly different ($p \leq 0.05$) from DMSO and DDC; *significantly different ($p \leq 0.01$) from DMSO and DDC, as determined by a randomized design one-way analysis of variance and Newman-Keuls test.

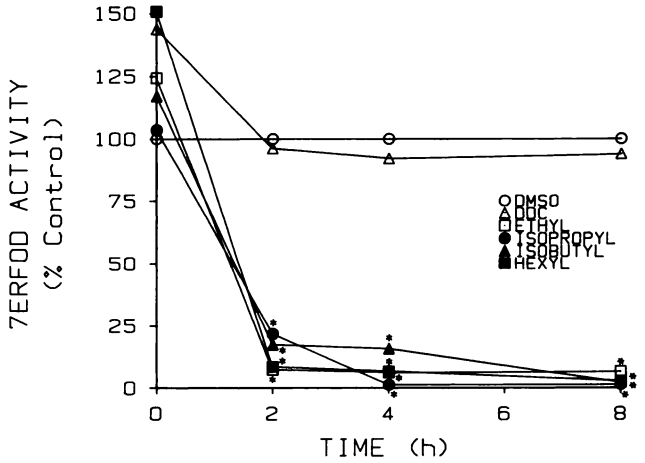

Fig. 3. Time course of the effect of DDC analogues on microsomal 7ERFOD activity in βNF-treated rats. Legend as in Fig. 2, except control 7ERFOD activity values were as follows: 0 hr, 1.82 ± 1.2; 2 hr, 2.47 ± 0.27; 4 hr, 2.95 ± 1.4; and 8 hr, 2.30 ± 1.4 nmol of resorufin/min/mg of protein.

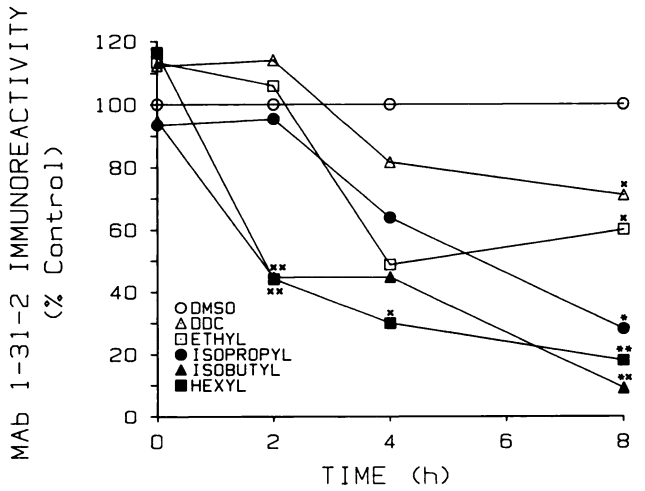

Fig. 4. Time course of the effect of DDC analogues on microsomal MAb 1-31-2 immunoreactivity in βNF-treated rats. Legend as in Fig. 2, except control immunoreactivity values were as follows: 0 hr, 49.4 ± 18.2; 2 hr, 53.1 ± 6.3; 4 hr, 74.5 ± 23.8; and 8 hr, 94.0 ± 24.7 arbitrary units. ˣSignificantly different ($p \leq 0.05$) from DMSO: *significantly different from DMSO ($p \leq 0.01$), DDC, and 4-ethyl-DDC ($p \leq 0.05$); ˣˣsignificantly different from DDC ($p \leq 0.01$), 4-ethyl-DDC, DMSO, and 4-isopropyl-DDC ($p \leq 0.05$); **significantly different from DMSO, DDC ($p \leq 0.01$), and 4-ethyl-DDC ($p \leq 0.05$); *ˣsignificantly different ($p \leq 0.01$) from DMSO, DDC, and 4-ethyl-DDC.

destruction of 63 and 83% of the total P-450 at 4 and 8 hr, respectively, following administration. The most active analogue was 4-isopropyl-DDC, destroying 83% of the total P-450 after only 2 hr of drug treatment. A similar pattern of heme loss was also observed (data not shown). DDC analogues differed with respect to their abilities to reduce P-450p catalytic activity (Fig. 7). DDC and 4-hexyl-DDC were inactive, whereas the isobutyl and ethyl analogues significantly lowered ERND activity. 4-Isopropyl-DDC caused the most rapid and dramatic lowering of this marker activity, inhibiting essentially 100% of this activity at the 2-hr time point. In contrast to these enzyme activity results, MAb 2-13-1 revealed that only 4-isopropyl-DDC caused significant loss/alteration of P-450p-related apoprotein (Fig. 8). Although this reduction in immunoreactivity

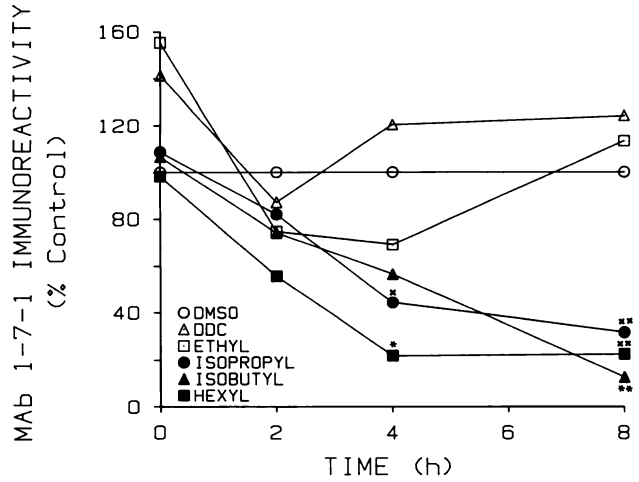

Fig. 5. Time course of the effect of DDC analogues on microsomal MAb 1-7-1 (P-450c) immunoreactivity in βNF-treated rats. Legend as in Fig. 2, except control immunoreactivity values were as follows: 0 hr, 44.1 ± 21.2; 2 hr, 97.6 ± 56.4; 4 hr, 74.2 ± 16.3; and 8 hr, 69.2 ± 22.0 arbitrary units. ˣSignificantly different ($p \leq 0.05$) from DDC; *significantly different from DDC ($p \leq 0.01$) and DMSO ($p \leq 0.05$); ˣˣsignificantly different from DDC, 4-ethyl-DDC ($p \leq 0.01$), and DMSO ($p \leq 0.05$); **significantly different ($p \leq 0.01$) from DDC, 4-ethyl-DDC and DMSO.

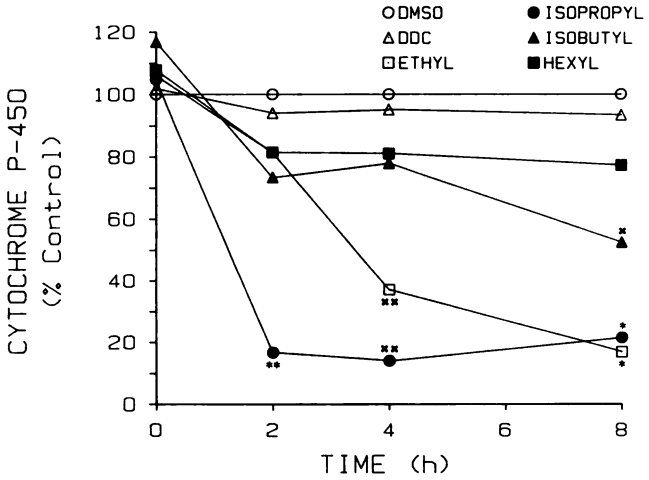

Fig. 6. Time course of the effect of DDC analogues on microsomal P-450 levels in DEX-treated rats. Legend as in Fig. 2, except control P-450 values were as follows: 0 hr, 1.67 ± 0.23; 2 hr, 1.99 ± 0.06; 4 hr, 1.63 ± 0.09; and 8 hr, 1.81 ± 0.31 nmol/mg of protein. ˣSignificantly different from DMSO ($p \leq 0.01$) and DDC ($p \leq 0.05$); *significantly different from DMSO, DDC, 4-hexyl-DDC ($p \leq 0.01$), and 4-isobutyl-DDC ($p \leq 0.05$); ˣˣsignificantly different ($p \leq 0.01$) from DMSO, DDC, 4-hexyl-DDC, and 4-isobutyl-DDC; **significantly different ($p \leq 0.01$) from DMSO, DDC, 4-hexyl-DDC, 4-ethyl-DDC, and 4-isobutyl-DDC.

was statistically significant, the finding is less dramatic than that seen for P-450c apoprotein levels.

Effect of DDC analogues in PB-treated rats. All compounds except DDC lowered P-450 levels following PB treatment (Fig. 9). However, in general, PB induction tends to reduce the percentage of P-450 inactivation caused by DDC analogues, in comparison with the βNF- and DEX-induced states. The hexyl and isopropyl analogues reduced P-450 levels by 41 and 68%, respectively, following 8 hr of drug treatment. However, 4-isobutyl-DDC and 4-ethyl-DDC caused maximal P-450 destruction at 2 and 4 hr, respectively; these analogues reduced P-450 levels by only 20 and 37%, respectively. All analogues caused patterns of heme loss that paralleled P-450

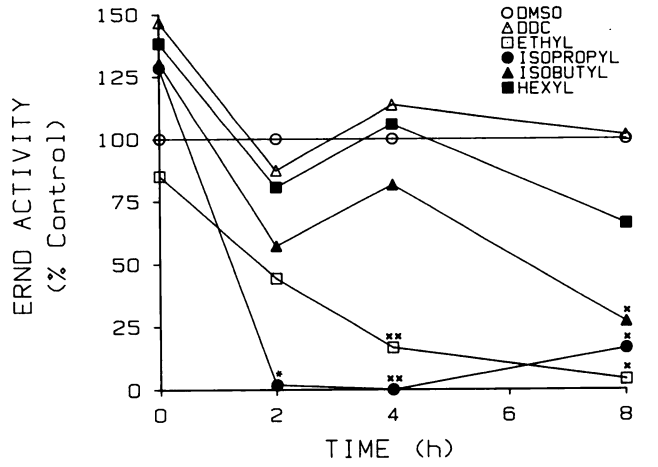

Fig. 7. Time course of the effect of DDC analogues on microsomal ERND activity in DEX-treated rats. Legend as in Fig. 2, except control ERND activity values were as follows: 0 hr, 4.29 ± 0.63; 2 hr, 6.77 ± 1.4; 4 hr, 3.85 ± 1.1; and 8 hr, 5.28 ± 2.6 nmol of HCHO/min/mg of protein. *Significantly different from DMSO, DDC ($p \leq 0.01$), and 4-hexyl-DDC ($p \leq 0.05$); ˣˣsignificantly different ($p \leq 0.01$) from DDC, 4-hexyl-DDC, DMSO, and 4-isobutyl-DDC.

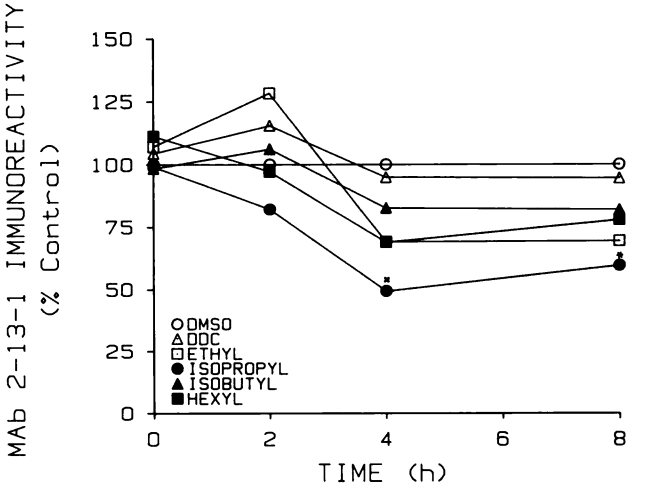

Fig. 8. Time course of the effect of DDC analogues on microsomal MAb 2-13-1 immunoreactivity in DEX-treated rats. Legend as in Fig. 2, except control immunoreactivity values were as follows: 0 hr, 80.9 ± 9.8; 2 hr, 96.2 ± 23.5; 4 hr, 119.4 ± 36.0; and 8 hr, 77.0 ± 16.3 arbitrary units. ˣSignificantly different ($p \leq 0.01$) from DMSO and DDC; *significantly different ($p \leq 0.05$) from DMSO.

loss (data not shown). With respect to P-450b function, only 4-hexyl-DDC and 4-isopropyl-DDC reduced 7PRFOD activity significantly, lowering this marker activity by 75–76% at 8 hr (Fig. 10). MAb 2-8-1 (Fig. 11) and MAb 2-66-3 (data not shown) revealed that DDC analogues did not alter the levels of apoproteins recognized by these MAbs over an 8-hr time course.

Discussion

Previous studies (8, 11) have examined the effects of 4-ethyl-DDC on P-450 isozymes following *in vivo* administration to rats. With respect to effects on total P-450 and selective catalytic activities, the current study generally confirms previous results for 4-ethyl-DDC and presents new findings for related 4-alkyl analogues of DDC.

Regardless of inducer pretreatment, DDC did not lower rat

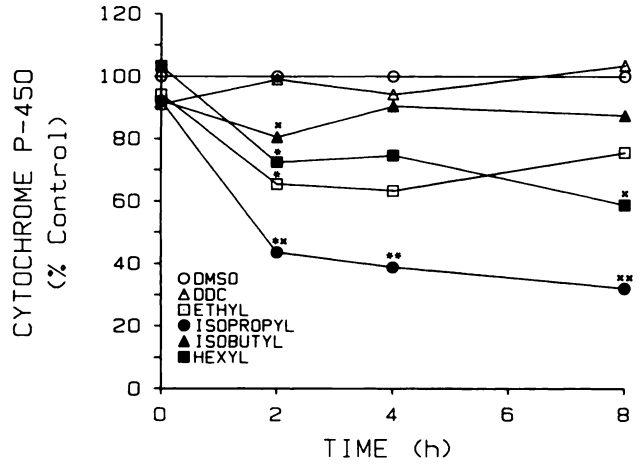

Fig. 9. Time course of the effect of DDC analogues on microsomal P-450 levels in PB-treated rats. Legend as in Fig. 2, except control P-450 values were as follows: 0 hr, 1.87 ± 0.36; 2 hr, 1.99 ± 0.22; 4 hr, 1.85 ± 0.42; and 8 hr, 1.72 ± 0.48 nmol/mg of protein. ˣˣSignificantly different from DDC, DMSO, 4-isobutyl-DDC, 4-ethyl-DDC ($p \leq 0.01$), and 4-hexyl-DDC ($p \leq 0.05$); **significantly different from DMSO, DDC, 4-isobutyl-DDC ($p \leq 0.01$), and 4-hexyl-DDC ($p \leq 0.05$); ˣˣsignificantly different ($p \leq 0.01$) from DDC, DMSO, 4-isobutyl-DDC, 4-ethyl-DDC, and 4-hexyl-DDC.

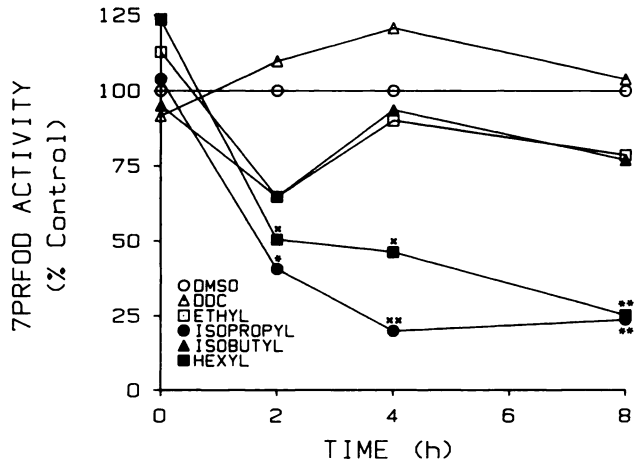

Fig. 10. Time course of the effect of DDC analogues on microsomal 7PRFOD activity in PB-treated rats. Legend as in Fig. 2, except control 7PRFOD activity values were as follows: 0 hr, 2.03 ± 0.58; 2 hr, 2.38 ± 0.25; 4 hr, 2.02 ± 0.72; and 8 hr, 1.86 ± 0.70 nmol of resorufin/min/mg of protein. ˣSignificantly different ($p \leq 0.05$) from DDC; *significantly different ($p \leq 0.05$) from DDC and DMSO; ˣˣsignificantly different from DDC ($p \leq 0.01$), DMSO, 4-isobutyl-DDC, and 4-ethyl-DDC ($p \leq 0.05$); **significantly different from DDC, DMSO ($p \leq 0.01$), 4-ethyl-DDC, and 4-isobutyl-DDC ($p \leq 0.05$).

hepatic P-450 levels or catalytic activities selective for P-450 isozymes b, c, and p. This compound is similarly inactive in rat liver microsomal preparations (1, 4, 12). The ethyl analogue demonstrated preference for inactivation of βNF- and DEX-inducible forms of P-450, as compared with PB-inducible forms. Indeed, 4-ethyl-DDC effectively inactivated P-450 c and p in this study but not P-450b. Similar loss of P-450 c and p activities caused by 4-ethyl-DDC has been reported *in vivo* (8, 11) and *in vitro* (12). Also, the ethyl analogue previously caused only minimal loss of 7PRFOD activity (8) and testosterone 16β-hydroxylase activity (11), selective markers for P-450b function. In addition, our previous microsomal work (12) and studies with purified reconstituted P-450b (4) have indicated

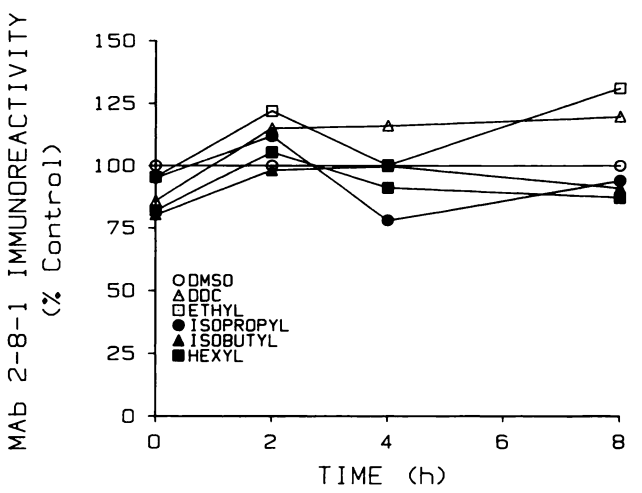

Fig. 11. Time course of the effect of DDC analogues on microsomal MAb 2-8-1 immunoreactivity in PB-treated rats. Legend as in Fig. 2, except control immunoreactivity values were as follows: 0 hr, 42.2 ± 8.4; 2 hr, 37.0 ± 7.4; 4 hr, 57.8 ± 8.3; and 8 hr, 45.8 ± 13.8 arbitrary units.

that this isozyme is immune to inactivation by 4-ethyl-DDC. The isopropyl analogue was the most effective inactivator of P-450 in all cases but demonstrated a preference for DEX-inducible isozymes.

The results of the present study for DDC, 4-ethyl-DDC, and 4-isopropyl-DDC are consistent with the recent work of Lee *et al.* (4). Their work demonstrated an excellent correlation between the free radical stabilities of the 4-alkyl groups, the 4-dealkyl to 4-alkyl pyridine metabolite ratio, the alkyl radical flux, and the P-450 chromophore destruction for these compounds. Thus, the expected rank order for P-450 destruction was observed in the present study: isopropyl > ethyl > DDC. However, factors other than free radical stability determine an analogue's *in vivo* P-450 destructive potential. The stability of the isobutyl radical is presumably similar to that of the ethyl radical; however, 4-isobutyl-DDC is a relatively poor inactivator of P-450 *in vivo*, especially in PB-treated rats. The relative inactivity of this analogue *in vivo* is surprising in light of *in vitro* findings that demonstrated 4-isobutyl-DDC to be an effective inactivator of P-450 in microsomes from PB-treated rats (12).

In agreement with our *in vitro* studies (12), 4-hexyl-DDC effectively inactivated P-450c but was unable to lower P-450 levels or ERND activity in DEX-treated rats. Clearly, the 4-hexyl substituent is incompatible with P-450p inactivation. Surprisingly, the hexyl analogue along with 4-isopropyl-DDC lowered 7PRFOD activity, reflecting loss of P-450b function (Fig. 10). Our *in vitro* studies (12) suggested that these analogues interact with the P-450b active site and inhibit 7PRFOD activity but do not cause mechanism-based inactivation of this isozyme. We propose two possible explanations, between which we cannot distinguish, for this discrepancy between *in vitro* and *in vivo* findings. First, these analogues may be mechanism-based inactivators of P-450b, and the *in vitro* results are difficult to interpret because of problems of competitive inhibition. Second, these compounds may be mechanism-based inactivators of P-450b *in vivo* but not *in vitro*. If indeed these analogues cause mechanism-based inactivation of P-450b, we believe that this isozyme is a relatively minor target.

Concurrently with our functional studies, we examined the

effects of DDC analogues on the apoprotein moieties of the major inducible P-450 isozymes using the technique of Western blotting and laser densitometry. In βNF-treated rats, the isopropyl, hexyl, and isobutyl analogues caused dramatic decreases (72–91%) in P-450c immunoreactivity at 8 hr, demonstrated with MAb 1-31-2 (Fig. 4) and MAb 1-7-1 (Fig. 5). DDC and 4-ethyl-DDC caused minor apoprotein loss/alteration. Tephly *et al.* (11) previously demonstrated a similar but more pronounced loss/alteration of the P-450c apoprotein caused by 4-ethyl-DDC in 3-methylcholanthrene-treated rats. Increasing the size and bulkiness of the 4-alkyl group appears to increase the ability of the compound to cause P-450c apoprotein loss/alteration. The mechanism of apoprotein loss/alteration is unknown but, for all analogues, appears to lag behind the loss of P-450c catalytic activity (Fig. 3). This temporal relationship, also noted by Tephly *et al.* (11), indicates that loss of catalytic activity via heme modification precedes apoprotein loss/alteration. This observation is consistent with the fact that cellular proteases, such as the ubiquitin system, often demonstrate a preference for the degradation of denatured or "abnormal" proteins (33). In contrast to our results with MAb 1-31-2 and 1-7-1, MAb 1-36-1 detected a decrease in P-450c immunoreactivity caused only by 4-isobutyl-DDC. This discrepancy suggests that either altered P-450 apoproteins are not removed from the endoplasmic reticulum or the loss of P-450c apoprotein may not reflect proteolysis but rather conformational changes that result in loss of immunorecognition by two out of three MAbs.

In DEX-treated rats, only 4-isopropyl-DDC caused a lowering of P-450p-related immunoreactivity (Fig. 8). Once again, this effect on the apoprotein occurred after the loss of P-450p catalytic activity (Fig. 7). In contrast to previous studies (8, 11), 4-ethyl-DDC did not significantly lower P-450p apoprotein levels, as detected by MAb 2-13-1. Discrepancies in these studies may be due to differences in the specificities and sensitivities of the antibody probes employed. Our study employed MAb 2-13-1, which recognizes P-450 PB-2a/PCN-E and P-450 2a (15). Tephly *et al.* (11) used a polyclonal rabbit anti-rat P-450 PCN-E (19), which has been shown to recognize only its homologous antigen. Correia *et al.* (8) employed a polyclonal goat anti-rat P-450p (16, 30), which is apparently also monospecific. Because of the multiplicity of P-450p-related isozymes, it is difficult to make comparisons concerning the specificities of the different antibody preparations. Alternatively, DDC analogues may cause conformational changes in P-450p-related apoproteins that are detected only by certain antibody preparations.

We observed that the apoproteins recognized by MAbs 2-66-3 and 2-8-1 were not altered by DDC analogues (Fig. 11), in keeping with the only minor alteration in P-450b immunoreactivity caused by 4-ethyl-DDC shown previously (11).

The effects of DDC analogues on the P-450c and P-450p apoproteins *in vivo* differ from those seen *in vitro* (12). In microsomal incubations, inactivation of these isozymes is accompanied by only minor apoprotein alterations, resulting in the production of lower molecular mass fragments or high molecular mass aggregates (12); however, the main isozyme band does not decrease in density. In contrast, after *in vivo* administration, DDC analogues can cause loss/alteration of the apoprotein of selected isozymes, and neither immunoreactive fragments nor aggregates are detected. Such loss of immunoreactivity may be due to either conformational changes or accelerated proteolysis caused by DDC analogues. The study of Shiraki and Guengerich (34) examining the rates of degradation of P-450 apoproteins has demonstrated that, for seven rat liver P-450 isozymes (UT-A, PB-B, βNF-B, PB-C, PCN-E, ISF-G, UT-F), loss of the apoprotein is not accompanied by the appearance of microsomal immunoreactive intermediate fragments. This is consistent with the idea that P-450 isozymes are removed from the endoplasmic reticulum *in vivo*, before initiation of proteolysis by soluble proteases (33). The mechanism of apoprotein loss/alteration caused by DDC analogues may involve a direct attack by reactive metabolites, the production of heme-derived protein adducts, or destruction/alteration of the apoprotein promoted by loss of the heme prosthetic group. The temporal relationship between loss of P-450 heme and apoprotein observed in this study and by others (11, 23) suggests that the loss of heme and/or the covalent binding of heme to the apoprotein (8) may act as a signal for proteolytic degradation of the apoprotein. Clearly, mechanisms for apoprotein loss/alteration that are enhanced by suicide substrates *in vivo* (8, 11, 23) are not fully operative in microsomal systems (12, 23).

The present *in vivo* results demonstrate that the major βNF-inducible isozyme, P-450c, and the major DEX-inducible isozyme, P-450p, are important targets for inactivation by DDC analogues. Inactivation of these P-450 isozymes *in vivo* is, in some instances, followed by loss of apoprotein immunoreactivity due to either conformational changes or enhanced susceptibility to proteolytic degradation triggered by DDC analogues. These findings differ from our previous results (12), which demonstrated only minor apoprotein alterations caused by DDC analogues *in vitro*. We believe that a complete understanding of the interaction of DDC analogues with various P-450 isozymes, together with a knowledge of the fate of the P-450 heme moiety following such interaction, will provide an explanation for the differences in ferrochelatase-lowering activity and porphyrinogenicity observed with these compounds.

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