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Selective Inactivation of Four Rat Liver Microsomal Androstenedione Hydroxylases by Chloramphenicol Analogs

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SUMMARY

The steroid androstenedione has been shown to be a valuable tool for the study of the selective inactivation of cytochrome P-450 isozymes in intact rat liver microsomes. The validity of this approach was investigated using microsomes, purified cytochrome P-450 isozymes, antibodies to particular cytochromes P-450, and the known mechanism-based inactivator chloramphenicol. Enzyme inactivation and antibody inhibition studies show that microsomes from both phenobarbital- and non-phenobarbital-treated rats are needed to accurately monitor the inactivation of the major phenobarbital-inducible cytochrome P-450 isozyme (PB-B) and of the major constitutive androstenedione 16α-hydroxylase (UT-A). Similar experiments indicate that, although isozyme P-450g does catalyze the 6β-hydroxylation of androstenedione in a reconstituted system, this cytochrome appears to make only a minimal contribution to microsomal 6β-hydroxylase activity, which reflects instead the activity of pregnenolone-16α-carbonitrile-induced isozymes. With these parameters investigated, initial enzyme inactivation studies showed that the antibiotic chloramphenicol caused different rates of NADPH-dependent enzyme inactivation among the four androstenedione hydroxylases monitored (16β > 6β > 16α > 7α). Based on these data, 12 chloramphenicol analogs were examined, and the results with these compounds show that their selectivity as cytochrome P-450 inactivators is a function of at least three structural features: 1) the number of halogen atoms, 2) the presence of a *para*-nitro group on the phenyl ring, and 3) substitutions on the ethyl side chain. For example, the compound *N*-(2-phenethyl)dichloroacetamide was shown to reversibly inhibit but not inactivate the cytochrome(s) P-450 responsible for androstenedione 6β-hydroxylase activity, whereas *N*-(2-*p*-nitrophenethyl) and *N*-(1,2-diphenethyl)dichloroacetamide rapidly inactivated the 6β-hydroxylase. The ability to monitor the activity of multiple isozymes with a single substrate should allow the development of a systematic approach to the design of selective inactivators of rat liver cytochromes P-450.

The cytochrome P-450-dependent monooxygenase system catalyzes the metabolism of many endogenous and xenobiotic compounds, usually to less lipophilic products which can then be excreted from the body. At least 13 distinct rat hepatic cytochrome P-450 isozymes of varying substrate specificity have been isolated by different laboratories (1). The multiplicity of rat hepatic cytochromes P-450 presents a challenge to investigations into the roles of the different P-450 isozymes in the metabolism of various compounds *in vivo*. One approach to this problem has been the development of chemical inhibitors, among which mechanism-based inactivators or suicide substrates are potentially the best candidates for selective cytochrome P-450 inhibition due to the requirement for metabolism (2). In previous studies we have found that chloramphenicol administration *in vivo* inactivates four of nine rat liver cytochromes P-450 monitored and that the rate and selectivity of isozyme inactivation *in vitro* can be altered by modifying structural features other than the dichloromethyl group, the site of metabolism (3, 4).

By definition, selective enzyme inactivation can be accurately studied and characterized only when an appropriate biochemical assay has been developed for each isozyme of interest. The metabolism of endogenous steroid hormones such as androstenedione has been studied in several laboratories using both microsomal preparations and purified cytochromes P-450 in a reconstituted system. Many of the constitutive and xenobiotic-inducible P-450 isozymes shown to have broad specificity with classical substrates such as 7-ethoxycoumarin and benzphetamine (5) have been shown to hydroxylate androstenedione regio- and stereoselectively (6, 7). Whereas others have noted the usefulness of this correlation between the formation of specific steroid metabolites and the presence of particular P-450 isozymes in aiding the comparison of P-450 preparations

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ABBREVIATIONS: androstenedione or AD, androst-4-ene-3,17-dione; PCN, pregnenolone-16α-carbonitrile; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; BNF-B, the major isozyme of rat liver cytochrome P-450 induced by β-naphthoflavone; UT, untreated.

isolated from different laboratories or in studying the contribution of different isozymes to overall microsomal activity (6), we hypothesized that the monitoring of certain androstenedione hydroxylase activities might be an effective and appropriate method for testing a large number of compounds as potentially selective enzyme inactivators. The present report describes the application of this approach to the design of chloramphenicol analogs with enhanced isozyme selectivity.

Experimental Procedures

Materials

Androst-4-ene-3,17-dione <4-^{14}C> (52.0 mCi/mmol) was purchased from New England Nuclear Research Products (Boston, MA). Unlabeled androstenedione and 6β-OH androstenedione were purchased from Steraloids (Wilton, NH). NADPH, β-naphthoflavone, chloramphenicol, 16α-OH androstenedione or (AD), and dilauryl L-3-phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). HEPES, sodium cholate, and sodium deoxycholate were purchased from Calbiochem-Behring (La Jolla, CA). The compounds *N*-methylphenethylamine, 1,2-diphenethylamine, 2,2-diphenethylamine, phenethylamine, 3-phenyl-1-propylamine, 4-nitrobenzylamine hydrochloride, chloroacetyl chloride, dichloroacetyl chloride, 7-hydroxycoumarin, and 7-ethoxycoumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). PCN and 7α-OH androstenedione were gifts from The Upjohn Co. (Kalamazoo, MI) and Dr. David Waxman (Harvard Medical School, Boston, MA), respectively. *N*-[2-Hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]chloroacetamide (deschlorochloramphenicol) was a gift from Dr. Lance Pohl (National Institutes of Health, Bethesda, MD). DEAE-Sephacel and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Hypatite C was purchased from Clarkson Chemical Co. (Williamsport, PA). The reagents and molecular weight standards for gel electrophoresis and immunochemical studies were purchased from Bio-Rad Laboratories (Richmond, CA).

Synthesis of Chloramphenicol Analogs

Synthesis of dichloro- and chloroacetamides was conducted by the method of Rebstock (8). The acetylated products were isolated from the organic phase of the reaction mixture and recrystallized from aqueous methanol as previously described for the synthesis of *N*-(2-*p*-nitrophenethyl)dichloroacetamide and *N*-(2-phenethyl)dichloroacetamide (4). Compounds were analyzed for purity by gas-liquid chromatography using a Hewlett-Packard 5790A gas chromatograph equipped with a flame ionization detector. Purity was determined by peak area analysis with the assumption that the analyte and any impurity responded similarly to the detector. Analyte purity of greater than 97% was considered acceptable. All chloramphenicol analogs were characterized by uncorrected melting point and by NMR (^{1}H, 60 or 250 MHz, $CDCl_3$, tetramethylsilane internal standard) δ (ppm) as follows: *N*-(2-phenethyl)chloroacetamide (C), 63–64.5° (lit. 67–67.5°) (9), 2.9 (*t*, 2H, J = 7.0 Hz), 3.6 (*q*, 2H, J = 6.7 Hz), 4.0 (*s*, 2H), 7.2–7.4 (*m*, 5H); *N*-(2-*p*-nitrophenethyl)chloroacetamide (E), 93–95° (lit. 100°) (10), 3.0 (*t*, 2H, J = 7.0 Hz), 3.6 (*q*, 2H, J = 7.0 Hz), 4.0 (*s*, 2H), 7.4 (*d*, 2H), 8.2 (*d*, 2H); *N*-(3-phenylpropyl)chloroacetamide (F), 54–55° (lit. 58–59°) (11), 1.9 (*m*, 2H), 2.7 (*t*, 2H, J = 7.6 Hz), 3.3 (*q*, 2H, J = 6.7 Hz), 4.0 (*s*, 2H), 7.2–7.3 (*m*, 5H); *N*-(2,2-diphenethyl)chloroacetamide (H), 75–77° (lit. 73–74°) (12), 4.0 (*m*, 2H), 4.0 (*s*, 2H), 4.2 (*t*, 1H, J = 7.9 Hz), 7.2–7.4 (*m*, 10H); *N*-(1,2-diphenethyl)chloroacetamide (J) 143–145° (lit. CA85(1):5705y), 3.1 (*m*, 2H), 4.0 (*s*, 2H), 5.3 (*q*, 1H, J = 7.7 Hz), 7.1–7.4 (*m*, 10H); *N*-methyl-*N*-(2-phenethyl)dichloroacetamide (K) 61–63° (lit. U.S. Patent No. 4208203), 2.9 (*m*, 2H), 3.0 and 3.1 (two singlets, 3H), 3.6 (*m*, 2H), 5.9 and 6.2 (two singlets, 1H), 7.2–7.4 (*m*, 5H); *N*-(2-*p*-nitrobenzyl)chloroacetamide (L) 106–108° (lit. 110–112°) (9), 4.2 (*s*, 2H), 4.6 (*d*, 2H, J = 6.2 Hz), 7.4 (*d*, 2H, J = 8.1 Hz), 8.2 (*d*, 2H, J = 9.1 Hz). Two of the analogs represent novel compounds with no previously reported melting points. Therefore, the structure of each was confirmed using carbon and proton NMR and elemental analysis. Elemental analyses were performed by Desert Analytics (Tucson, AZ). Carbon-13 magnetic resonance spectra were recorded at 62.9 MHz on a Bruker WM-250 spectrometer. For these spectra, chemical shifts are reported as δ values in ppm from the center line of the $CHCl_3$-d triplet (77.0 ppm). For *N*-(2,2-diphenethyl)dichloroacetamide (G), m.p. 112–114°, ^{1}H NMR ($CDCl_3$) δ 3.9 (*m*, 2H), 4.2 (*m*, 1H), 5.8 (*s*, 1H), 7.3 (*m*, 10H); ^{13}C NMR ($CDCl_3$) δ 44.4, 50.1, 66.3, 127.1, 127.9, 128.8, 141.1, 164.0. Analysis Calculated $C_{16}H_{15}Cl_2NO$: C, 62.35; H, 4.91; N, 4.54; Cl, 23.01. Found: C, 62.39; H, 4.79; N, 4.52; Cl, 22.71. For *N*-(1,2-diphenethyl)dichloroacetamide (I), 138–139°, ^{1}H NMR ($CDCl_3$) δ 3.2 (*d*, 2H, J = 6.6 Hz), 5.2 (*q*, 1H, J = 7.5 Hz), 5.8 (*s*, 1H), 7.0–7.5 (*m*, 10H); ^{13}C NMR ($CDCl_3$) δ 42.3, 55.1, 66.4, 126.3, 126.9, 127.8, 128.5, 128.7, 129.3, 136.3, 140.1, 163.2. Analysis Calculated for $C_{16}H_{15}Cl_2NO$: C, 62.35; H, 4.91; N, 4.54; Cl, 23.01. Found: C, 62.53; H, 4.80; N, 4.63; Cl, 22.83. The analogs *N*-(2-phenethyl)dichloroacetamide (B) and *N*-(2-*p*-nitrophenethyl)dichloroacetamide (D) were available from previous syntheses (4).

Animal Treatment and Preparation of Microsomes

Adult male Sprague-Dawley rats (150–250 g) were pretreated with PCN or phenobarbital. PCN was administered at 100 mg/kg by gastric intubation once daily for 4 days in 1 ml of a 1% Tween 80-corn oil suspension. On the fourth day food was withheld, animals were killed on day 5, and liver microsomes were prepared as described previously (13). Animals pretreated with phenobarbital were administered 0.1% (w/v) sodium phenobarbital for 5 days in the drinking water. Protein was determined by the method of Lowry *et al.* (14) and cytochrome P-450 content by the method of Omura and Sato (15).

Purification of Cytochrome P-450 Isozymes

The major phenobarbital-inducible isozyme of rat liver cytochrome P-450 (PB-B[1]) was isolated as described by Guengerich *et al.* (5) using modifications described by Graves *et al.* (16). The same basic procedure was used to isolate isozymes BNF-B, UT-A, UT-F, and P-450g.[2] The specific cytochrome P-450 contents of the preparations used were: PB-B (16 nmol/mg), BNF-B (15 nmol/mg), UT-A (14 nmol/mg), UT-F (15 nmol/mg), and P-450g (19 nmol/mg). NADPH-cytochrome P-450 reductase was purified as described previously (17). One unit of reductase is defined as the amount which reduces 1 μmol of cytochrome *c*/min when assayed in 300 mM potassium phosphate buffer (pH 7.7) at 25°.

Inactivation Studies

Microsomal system. Liver microsomes from PCN- or phenobarbital-treated rats were incubated with inhibitor at 37° for 2 min. Reactions were started by the addition of NADPH and allowed to proceed for indicated times up to 12.5 min, at which point 80-μl aliquots were taken and added to 20 μl of [^{14}C]AD in buffer. The reaction proceeded for an additional 1.5 min before quenching with 50 μl of tetrahydrofuran. Incubation conditions after addition of the AD were: 0.25 mg/ml microsomal protein, 24 μM AD, 1 mM NADPH, 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA. Inhibitors were added in methanol. Fifty-μl aliquots were spotted on the preadsorbent loading zone of a Baker silica gel thin layer chromatography plate [250 μm, Si250F (19c)], and the plate was developed twice in chloroform/ethyl acetate (1:2). Localization of AD metabolites and quantification of enzyme activity were performed as previously described (16).

Reconstituted system. The purified cytochrome P-450 isozymes

[1] Since there is no generally accepted nomenclature applied to rat liver cytochromes P-450 (1), in this publication preparations of cytochrome P-450 from different laboratories are referred to according to the nomenclature utilized in each respective laboratory. References for cytochromes P-450 mentioned in this report but not specifically identified in Table 1 are given in the text.

[2] J. Halpert, J.-Y. Jaw, L. Cornfield, C. Balfour, and E. Mash, Jr., manuscript in preparation.

UT-A, UT-F, PB-B, and P450g were assayed for residual enzyme activity after incubation with an inhibitor in a reconstituted system using [^{14}C]AD as the substrate. Incubation mixtures contained 0.05 nmol/ml P-450, 0.30 units/ml NADPH-cytochrome P-450 reductase, 30 μg/ml dilauryl L-3-phosphatidylcholine, 0.1 mg/ml sodium deoxycholate, 0.05 M HEPES buffer (pH 7.6), 15 mM $MgCl_2$, 0.1 mM EDTA, 0.36 μM NADPH, and inhibitor added in methanol. The 0.1-ml reaction mixture was incubated at 37° for 2 min, and the reaction was started by the addition of NADPH. The procedure was identical to that for the microsomal system with the exception that the incubation of enzyme and inhibitor was only allowed to proceed for a maximum of 6 min. Incubation conditions for purified BNF-B were identical to those for UT-F, UT-A, PB-B, and P-450g except that 7-ethoxycoumarin replaced AD as the substrate used to assay enzymatic activity. 7-Hydroxycoumarin formation was monitored on an Aminco-Bowman spectrofluorometer (excitation 366 nm, emission 454 nm) as described previously (4, 18). Antibody inhibition studies using previously available anti-PCNb IgG (16), anti-PB-B IgG (19), and control IgG were conducted using both microsomes and purified cytochromes P-450. In each case, antibody was added to the incubation mixture and then incubated for 30 min at 25°. The tubes were then returned to ice, [^{14}C] AD was added, and the tubes were preincubated for 3 min at 37°. The reaction was started by the addition of NADPH and was allowed to proceed for 3 min at 37°. AD metabolites were identified and enzyme activity was determined as described earlier.

Immunochemical Methodology

SDS-polyacrylamide gel electrophoresis was conducted as described by Laemmeli (20) and proteins were then transferred electrophoretically to nitrocellulose according to the Bio-Rad Trans-Blot Kit instructions. Following incubation of the nitrocellulose sheets in 3% gelatin for 30 min to block all non-protein-bound sites, the nitrocellulose was incubated with anti-PCNb IgG (20 μg/ml) for 2 hr in 1% gelatin. Immunoreactive proteins were then visualized and identified using a horseradish peroxidase Immuno-blot Assay Kit purchased from Bio-Rad.

Results

AD hydroxylase activities in microsomes and reconstituted systems. Results from several laboratories have indicated that the formation of each of the four major hydroxylated AD metabolites (7α-OH AD, 6β-OH AD, 16β-OH AD, and 16α-OH AD) primarily reflects the activity of a single cytochrome P-450 isozyme (Table 1). Experiments conducted during the initial phase of this investigation were devoted toward confirming these assignments using our own preparations of microsomes and purified cytochromes P-450. Rates of formation of the four major hydroxylated metabolites in intact liver microsomes were found to be linear under the following conditions regardless of the source of microsomal protein: 24 μM AD, 25 μg of microsomal protein, and a 3.0-min incubation period. Liver microsomes from PCN- and phenobarbital-treated rats exhibited the expected metabolite profiles compared to control microsomes (21), i.e., 6β-hydroxylase activity was increased by both treatments and 16β-hydroxylase activity was increased only after the phenobarbital treatment (data not shown).

Preparations of four purified cytochrome P-450 isozymes were also assayed for their ability to metabolize AD in a reconstituted system. Each of the isozymes produced one major metabolite with the following activities in nmol of metabolite formed/min/nmol of cytochrome P-450: UT-F, 7α-OH (11.5); PB-B, 16β-OH (9.8); UT-A, 16α-OH (4.5); and P-450g, 6β-OH (3.3). We reported previously that a major PCN-inducible isozyme of rat liver cytochrome P-450 termed PCNb was essentially inactive in metabolizing AD in a reconstituted system but that polyclonal antibodies to this protein, when added to microsomes from PCN-treated male rats at a concentration of 6 mg of IgG/nmol of total cytochrome P-450, inhibited more than 80% of the AD 6β-hydroxylase activity (16). In the present investigation we confirmed that these antibodies caused a similar or greater extent of inhibition of AD 6β-hydroxylation in microsomes from phenobarbital-treated or control rats. We also confirmed that these antibodies had no effect on the 6β-hydroxylase activity of purified P-450g in a reconstituted system and did not recognize P-450g on Western blots (data not shown). Thus, in agreement with recent results based on studies with antibodies to P-450g (22), this cytochrome appears to make only a minimal contribution to microsomal AD 6β-hydroxylase activities. Rather, as indicated in Table 1, AD 6β-hydroxylase activity in microsomes from control and PCN- or phenobarbital-treated rats appears to primarily reflect the major cytochrome(s) P-450 of the PCN family.[3]

The final set of preliminary experiments was designed to identify an appropriate source of microsomes for monitoring the activities of isozymes UT-A and PB-B. Because of the low AD 16β-hydroxylase activities in microsomes from control or PCN-treated rats, it was necessary to use microsomes from phenobarbital-induced animals to accurately measure the activity of isozyme PB-B. However, as reported by us (19) and others (6, 7), isozyme PB-B is not completely stereoselective at the 16-position and produces 16β-OH and 16α-OH AD in a ratio of 10:1. Because of the high levels of PB-B in microsomes from phenobarbital-induced animals, concern arose that the 16α-hydroxylase activity in such microsomes might not solely reflect the activity of isozyme UT-A. Therefore, antibody inhibition experiments were carried out in which microsomes from phenobarbital- or PCN-treated rats were incubated with 6 mg of anti-PB-B IgG/nmol of cytochrome P-450 prior to assays of AD metabolism. Under these conditions, a 71% decrease in AD 16β-hydroxylase activity and a 27% decrease in 16α-hydroxylase activity were observed in the microsomes from the phenobarbital-treated animals, whereas only an 11% decrease in 16β-hydroxylase activity and no decrease in 16α-hydroxylase activity were observed in the microsomes from the PCN-treated rats. Based on these and other results to be

TABLE 1

Cytochrome P-450 isozymes responsible for androstenedione metabolism

Androstenedione hydroxylase activity	Nomenclatures applied to cytochromes P-450 responsible	
	Levin[a]	Guengerich[a]
7α	P-450a[b]	UT-F[c]
6β	P-450p	PB/PCN-E[c]
16β	P-450b[b]	PB-B[d]
16α	P-450h[e]	UT-A[d]

[a] The cytochrome P-450 nomenclatures used here represent those adopted by W. Levin and F. P. Guengerich.
[b] Ref. 7.
[c] Ref. 21.
[d] Ref. 24.
[e] Ref. 27.

[3] We have recently obtained evidence that liver microsomes from PCN- and phenobarbital-treated rats contain at least two immunochemically related cytochrome P-450 isozymes referred to as PCNa and PCNb, both of which may be involved in AD 6β-hydroxylation (16).

described later, only microsomes from phenobarbital-treated rats were subsequently used to monitor the activity of PB-B, whereas only microsomes from PCN-treated rats were used to monitor the activity of UT-A.

Inhibition and inactivation of microsomal AD hydroxylases by chloramphenicol. Having confirmed the isozyme assignments of the AD hydroxylase activities indicated in Table 1, and having identified an appropriate source of microsomes for measuring these activities, subsequent experiments were devoted toward determining the appropriate inhibitor concentrations and incubation times for monitoring the *in vitro* inactivation of the isozymes in question. Initial experiments were carried out with chloramphenicol, which has been shown previously to inactivate isozymes UT-A, PB-B, and the PCN-inducible isozyme(s) responsible for AD 6β-hydroxylation but not to inactivate UT-F (3, 4, 16). We hypothesized that a concentration of chloramphenicol sufficient to *inhibit* AD hydroxylation when added together with the substrate ought to be sufficient to *inactivate* susceptible isozymes if preincubated with the microsomes and NADPH before the addition of AD. Chloramphenicol was therefore added to microsomal incubations at concentrations ranging from 0 to 500 μM. Hydroxylase activity was plotted versus the log of the chloramphenicol concentrations and nonlinear regression least squares analysis of the curves was used to determine the concentration of compound which produces 50% inhibition. For example, the I_{50} value for inhibition of the 6β-hydroxylase was 250 μM in microsomes from both PCN- and phenobarbital-treated rats, whereas the I_{50} value for inhibition of the 16β-hydroxylase was 40 μM in microsomes prepared from phenobarbital-treated rats but 120 μM in microsomes prepared from PCN-treated rats.

Based on the above results, the ability of chloramphenicol to inactivate the various AD hydroxylases was examined by preincubating microsomes from PCN- or phenobarbital-treated rats with 50 or 250 μM chloramphenicol in the presence of NADPH for times ranging from 0.5 to 12.5 min and then transferring an aliquot of the incubation mixture to a separate tube containing AD to measure residual monooxygenase activity (Fig. 1). Incubations with AD were carried out for 1.5 min based on experiments which indicated that formation of all four hydroxylated metabolites was linear for this time even in the presence of 250 μM chloramphenicol. Analysis of the data shows both a time-dependent loss of enzyme activity corresponding to enzyme inactivation and a reversible inhibition component due to the presence of unmetabolized inhibitor during the AD hydroxylase assay. Reversible inhibition is represented by the decrease in the y intercept value relative to the methanol control and increases in a dose-dependent fashion (4). As shown in Fig. 1, 250 μM chloramphenicol produces approximately 30% reversible inhibition of the 6β-hydroxylase compared to the methanol control as measured by the y intercept of the plots. As was also observed in our initial I_{50} experiment, the AD 16β-hydroxylase is more sensitive to reversible inhibition by chloramphenicol than the AD 6β-hydroxylase. It should also be noted that the time course of AD 16β-hydroxylase inactivation becomes biphasic when approximately three-fourths of the initial activity is lost. Biphasic kinetics were also observed for compounds other than chloramphenicol and were not found to be isozyme dependent. Generally, the phenomenon was observed whenever at least two-thirds of a particular hydroxylase activity was lost during the course of the experiment.

The rate constant for inactivation of the AD 16β-hydroxylase

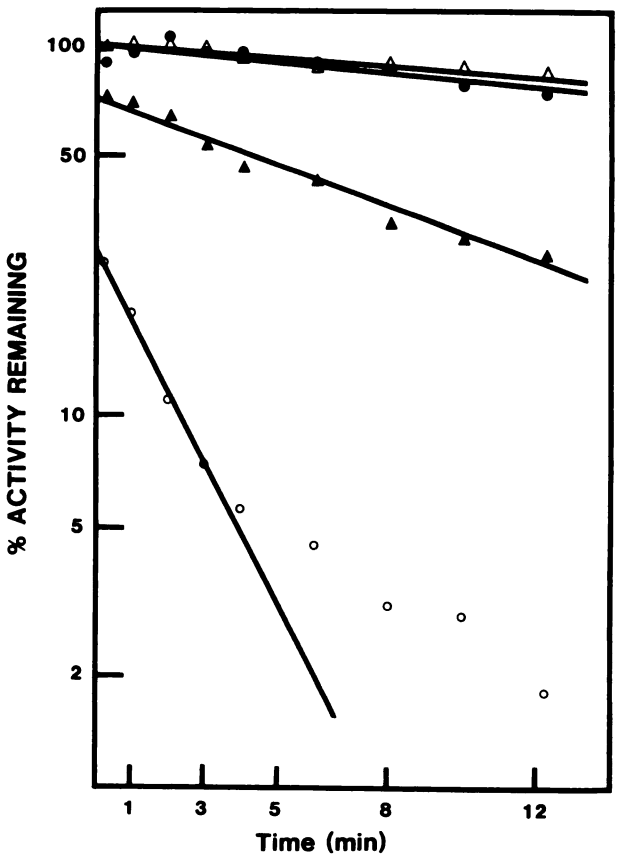

Fig. 1. Effect of preincubation with 250 μM chloramphenicol on the androstenedione 6β- and 16β-hydroxylase activities of liver microsomes from phenobarbital-treated male rats. Microsomes were incubated at 37° for 2 min, and reactions were started by the addition of NADPH and allowed to proceed for the times indicated, at which point 80-μl aliquots were removed and added to 20 μl of [^{14}C]androstenedione in buffer. The reactions were allowed to proceed for an additional 1.5 min to determine residual monooxygenase activity. Incubation conditions and identification and quantitation of androstenedione metabolites are described under Experimental Procedures. ●, 16β-hydroxylase activity after preincubation with NADPH alone; △, 6β-hydroxylase activity after preincubation with NADPH alone; ○, 16β-hydroxylase activity after preincubation with NADPH plus 250 μM chloramphenicol; ▲, 6β-hydroxylase activity after preincubation with NADPH plus 250 μM chloramphenicol.

in the presence of 250 μM chloramphenicol was 0.47 min^{-1} for microsomes prepared from phenobarbital-induced animals and 0.10 min^{-1} for microsomes from PCN-treated rats. The loss of AD 16β-hydroxylase activity in microsomes from PCN-treated rats is therefore likely due to the inactivation of cytochrome P-450 isozymes other than PB-B. In contrast to results obtained with the AD 16β-hydroxylases, rate constants for loss of AD 6β-hydroxylase activity with 250 μM chloramphenicol were similar regardless of the source of microsomal protein (0.09 min^{-1} and 0.10 min^{-1} for microsomes obtained from phenobarbital- and PCN-induced animals). These data are cited as evidence that valid rate constants for inactivation of the AD 6β-hydroxylase could be obtained by averaging the results of identical experiments done with the two kinds of microsomal samples (Table 2). No inactivation of the AD 7α-hydroxylase was observed in either type of microsomes.

Correlation of inactivation of purified cytochromes P-450 with inactivation experiments using a microsomal system. To further validate the use of the microsomal system, rate constants for inactivation by chloramphenicol observed in

TABLE 2

Compounds tested as potentially selective inactivators of cytochromes P-450.

The R, n, and X designations refer to substituents on the parent structure shown in Fig. 1.

Compound	R_1	R_2	R_3	n	X
CAP[a]	CH_2OH	OH	NO_2	0	Cl
A	CH_2OH	OH	NO_2	0	H
B	H	H	H	0	Cl
C	H	H	H	0	H
D	H	H	NO_2	0	Cl
E	H	H	NO_2	0	H
F	H	H	H	1	H
G	H	phenyl	H	0	Cl
H	H	phenyl	H	0	H
I	phenyl	H	H	0	Cl
J	phenyl	H	H	0	H

[a] CAP, chloramphenicol.

microsomes were compared with results obtained with purified isozymes in a reconstituted system. Purified isozyme UT-F showed no NADPH-dependent loss of AD 7α-hydroxylase activity when incubated with 250 μM chloramphenicol, consistent with the microsomal data. Purified cytochrome P-450g was also resistant to inactivation by 250 μM chloramphenicol, suggesting that the time-dependent loss of AD 6β-hydroxylase activity caused by 250 μM chloramphenicol in intact microsomes is due to the inactivation of PCN-inducible cytochrome(s) P-450. Also, rate constants for inactivation of the AD 16α-hydroxylase by chloramphenicol were in agreement regardless of whether microsomes from PCN-treated animals ($k_{inactivation} = 0.08$ min^{-1}) or purified UT-A ($k_{inactivation} = 0.11$ min^{-1}) were used as the enzyme source. For the major phenobarbital-inducible enzyme in rat liver, PB-B, the rate constant of inactivation of 0.47 min^{-1} determined for microsomes in the presence of 250 μM chloramphenicol is in good agreement with the reported $k_{inactivation}$ of 0.40 min^{-1} observed previously in a reconstituted system (4).

Inactivation of AD hydroxylases by chloramphenicol analogs. Previous work from this laboratory has focused on the ability of chloramphenicol analogs to inactivate PB-B and the major β-naphthoflavone-inducible cytochrome P-450 isozyme, BNF-B (4). Comparison of rate constants for inactivation of these isozymes by chloramphenicol and the analog *N*-(2-phenethyl)dichloroacetamide gave preliminary indications of which structural features might enhance or dramatically decrease the ability of the compounds to cause enzyme inactivation. Based on these results, the utility of the AD hydroxylase assay in aiding in the design of selective cytochrome P-450 inactivators was examined using a series of compounds (Table 2) of the general structure shown in Fig. 2. The choice of compounds synthesized was dictated by the ready availability of the starting amines as well as by the presence of substituents which might be expected to have a major effect on the ability of the compounds to act as substrates for and potentially inactivate particular isozymes.

Due to the large number of analogs of interest and the varied sensitivity of the different isozymes to reversible inhibition, it was impractical to determine the I_{50} values for each inhibitor/enzyme pair. Therefore, analogs were tested initially at a low concentration (5–50 μM) and at a 5-fold higher concentration. The AD 7α-hydroxylase, UT-F, was consistently resistant to reversible inhibition by any analog. The inhibition of the other AD hydroxylases by chloramphenicol analogs paralleled that seen for chloramphenicol itself in that the 6β-hydroxylase was the most resistant to inhibition. Therefore, a concentration of each analog which gave approximately 30% reversible inhibition of the 6β-hydroxylase was chosen for analysis of the inactivation[4] kinetics.

Fig. 3 illustrates the ability of different chloramphenicol analogs to inactivate the cytochrome(s) P-450 responsible for AD 6β-hydroxylase activity. These results, in addition to the data obtained for other AD hydroxylases, are shown in Table 3. The major findings can be summarized as follows. Chloramphenicol was shown to inactivate the four AD hydroxylases in the following order: 16β > 6β > 16α > 7α. (Since the AD 7α-hydroxylase was not inactivated by any of the analogs tested, including chloramphenicol, future reference will be limited to the remaining three hydroxylase activities.) Regarding the dichloroacetamides, the removal of the functional groups at all three *R* positions (Fig. 2) resulted in the compound *N*-(2-phenethyl)dichloroacetamide (**B**), which inactivated the 16β-hydroxylase more rapidly than the 16α-hydroxylase and did not inactivate the 6β-hydroxylase. The addition of a *para*-nitro group on the phenyl ring results in the analog *N*-(2-*p*-nitrophenethyl)dichloroacetamide (**D**), which is an efficient 16β-hydroxylase inactivator but does not select between inactivation of the AD 6β- and 16α-hydroxylases. Addition of a phenyl group at the R_2 position of compound **B** resulted in a compound (**G**) which inactivated the three hydroxylases in the following order: 16β > 6β > 16α. By simply moving the phenyl group from the R_2 position to the R_1 position on the ethyl side chain (**I**), the selectivity was altered (6β > 16β > 16α). Preliminary results with the only tertiary amide synthesized, *N*-methyl-*N*-(2-phenethyl)dichloroacetamide (**K**), indicate that this compound is a comparatively nonselective and slow inactivator.

The corresponding chloroacetamides to the above mentioned dichloroacetamides were also synthesized and tested (Table 3). *N*-(2-Phenethyl)chloroacetamide (**C**) was a relatively poor inactivator, and neither the effectiveness nor the selectivity of this analog was greatly enhanced by the addition of a phenyl group at the R_1 or R_2 positions (compounds **J** and **H**). In contrast, the analog *N*-(2-*p*-nitrophenethyl)chloroacetamide (**E**) was the only compound that effectively inactivated the 16β- and 6β-hydroxylases while selecting against inactivation of the 16α-hydroxylase. This analog was shown previously to inactivate the purified isozyme PB-B via destruction of the heme moiety of the enzyme and not by protein modification.[5] We investigated the selectivity of the analog *N*-(2-*p*-nitrophenethyl)chloroacetamide further by testing structurally related analogs. The compound with one carbon removed from the ethyl chain, *N*-(*p*-nitrobenzyl)chloroacetamide (**L**), was a poor and nonselective inactivator. Finally, the analog having a propyl chain but lacking the *para*-nitro group, *N*-(3-phenylpropyl)chloroacetamide (**F**), was also found to be a less effective inactivator than *N*-(2-*p*-nitrophenethyl)chloroacetamide.

Inactivation of the major β-naphthoflavone-inducible cytochrome P-450 isozyme. The selectivity of certain of the compounds was investigated further by examining the ability to inactivate the major β-naphthoflavone-inducible cytochrome

[4] That the observed time-dependent loss of enzyme activity caused by chloramphenicol and certain analogs was actually NADPH dependent and irreversible was confirmed by *in vitro* experiments in which microsomes were preincubated with inhibitor in the presence or absence of NADPH and then recovered from the incubation mixture by ultracentrifugation as described previously (3).

[5] N. E. Miller, unpublished data.

TABLE 3

Rate constants for inactivation of androstenedione hydroxylases by chloramphenicol analogs

Various chloramphenicol analogs were incubated with rat liver microsomes as described in the legends to Figs. 1 and 3. Rate constants for inactivation were calculated by linear regression analysis of the natural logarithm of the residual androstenedione hydroxylase activity as a function of time. In the case of compounds which showed kinetics consistent with biphasic inactivation, the values shown represent only the rapid, initial phase. Since the androstenedione 7α-hydroxylase was consistently resistant to inactivation by any of the compounds tested, only the remaining three hydroxylase activities are reported.

	Compound	Concentration	$k_{inactivation}$ (min^{-1})[a]		
			6β-Hydroxylase[b]	16β-Hydroxylase	16α-Hydroxylase
	Control (methanol)		0.01 (n = 13)	0.01 (n = 9)	0.01 (n = 9)
	Chloramphenicol	250 μM	0.10	0.47	0.08
A	Deschlorochloramphenicol[a]	2.5 mM	0.03	0.01	0.01
B	*N*-(2-Phenethyl)dichloroacetamide	50 μM	0.01	0.21	0.05
C	*N*-(2-Phenethyl)chloroacetamide	250 μM	0.01	0.03	0.05
D	*N*-(2-*p*-Nitrophenethyl)dichloroacetamide	50 μM	0.16	0.52	0.11
E	*N*-(2-*p*-Nitrophenethyl)chloroacetamide	250 μM	0.09	0.13	0.00
F	*N*-(3-Phenylpropyl)chloroacetamide[a]	250 μM	0.00	0.00	0.01
G	*N*-(2,2-Diphenethyl)dichloroacetamide	25 μM	0.13	0.72	0.04
H	*N*-(2,2-Diphenethyl)chloroacetamide[a]	250 μM	0.01	0.06	0.05
I	*N*-(1,2-Diphenethyl)dichloroacetamide	25 μM	0.32	0.24	0.15
J	*N*-(1,2-Diphenethyl)chloroacetamide[a]	250 μM	0.04	0.05	0.02
K	*N*-Methyl-*N*-(2-phenethyl)dichloroacetamide[a]	250 μM	0.03	0.06	0.12
L	*N*-(*p*-Nitrobenzyl)chloroacetamide[a]	250 μM	0.02	0.06	0.03

[a] All compounds with the exceptions of A, F, H, J, K, and L were tested in two identical experiments with only the source of microsomal protein being changed for reasons described under Results. Rate constants for inactivation of the androstenedione 7α-hydroxylase and 6β-hydroxylase were determined by averaging the results from experiments using microsomes obtained from both phenobarbital- and PCN-treated rats. Rate constants for inactivation of the androstenedione 16β-hydroxylase and 16α-hydroxylase were obtained from experiments using microsomes from phenobarbital- and PCN-treated rats, respectively. Rate constants for inactivation shown for compounds A, F, H, J, K, and L were obtained using only microsomes obtained from phenobarbital-induced animals. These compounds were initially screened for their ability to affect androstenedione 6β- and 16β-hydroxylase activities, and the rate constants for inactivation of the 16α-hydroxylase only partially reflect isozyme UT-A.

[b] Male rat liver androstenedione hydroxylase activities (nmol of product formed/min/mg of microsomal protein) were calculated in the absence of any inhibitor. Microsomes obtained from phenobarbital-treated animals: 7α-OH AD, 0.6; 6β-OH AD, 2.7; 16β-OH AD, 10.5; 16α-OH AD, 1.9. Microsomes obtained from PCN-treated animals: 7α-OH AD, 0.5; 6β-OH AD, 5.1; 16β-OH AD, 0.6; 16α-OH AD, 1.6.

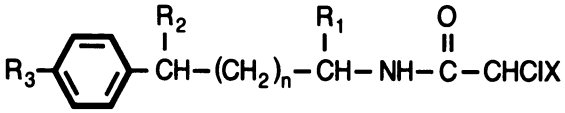

Fig. 2. Structure of chloramphenicol analogs with *R*, *X*, and *n* positions representing sites of structural alteration.

P-450 isozyme, BNF-B, in a reconstituted system (Table 4). These data corroborate a previous report (4) that, unlike chloramphenicol, the analogs *N*-(2-*p*-nitrophenethyl)dichloroacetamide (**D**) and *N*-(2-phenethyl)dichloroacetamide (**B**) inactivate the BNF-B isozyme. Lack of inactivation of BNF-B by *N*-(2-*p*-nitrophenethyl)chloroacetamide (**E**) indicates that the dichloromethyl function appears to be required, but is not sufficient, for inactivation of BNF-B. Regarding substitutions made along the ethyl chain, the analog *N*-(2,2-diphenethyl)dichloroacetamide (**I**) was shown to inactivate the enzyme, whereas movement of the phenyl group from the R_2 to the R_1 position resulted in a diphenyl compound unable to inactivate BNF-B.

Discussion

In order for significant progress to be made in the study of selective inactivation of cytochromes P-450, advances in the development and synthesis of appropriate compounds need to parallel the development of isozyme-specific assays. To our knowledge, this report documents the first use of four specific AD hydroxylase activities as probes for examining potentially selective inactivators. The methodology employed allowed us to begin to develop an alternative to the approach of Ortiz de Montellano and Reich (23), whereby isozyme-directed inactivators are designed by incorporating a double or triple bond into selected positions on substrates normally metabolized by a restricted number of cytochromes P-450. Our empirical approach to designing selective inactivators of cytochromes P-450 does not presuppose knowledge about the substrate specificities of the individual isozymes and may therefore be especially useful in those cases where no obvious means of targeting particular isozymes presently exists. In order to identify trends in the isozyme selectivity of our compounds, the use of a multiple-enzyme assay was essential.

The important methodological questions focused on confirming the validity of using microsomal activities to monitor particular cytochromes P-450, choosing the proper inhibitor concentrations and incubation conditions, and correlating data obtained with microsomes and purified enzymes. We used combinations of microsomal samples, purified cytochrome P-450 isozymes, and the corresponding anti-P-450 antibodies to identify in our laboratory and to compare to published reports the microsomal AD hydroxylase activity or activities attributable to certain cytochrome P-450 isozymes. A specific group of experiments addressed the question of the contribution of the constitutive cytochrome P-450 isozyme P-450g to AD 6β-hydroxylase activity in rat liver microsomes. Experiments with purified P-450g, including antibody inhibition studies, Western blot analysis, and inactivation experiments, indicate that, although this isozyme does have AD 6β-hydroxylase activity in a reconstituted system, it does not make a significant contribution to microsomal 6β-hydroxylase activity. A similar conclusion has been drawn in a recent study employing antibodies to P-450g (22). By utilizing microsomes prepared from phenobarbital- and PCN-treated rats, we believe that we were able to study the inactivation of the major phenobarbital- and PCN-inducible cytochrome P-450 isozymes as well as the two constitutive cytochrome P-450 isozymes, UT-F and UT-A.

Based on previous studies with chloramphenicol which showed that the initial step in enzyme inactivation is the conversion of the dichloromethyl moiety of the compound to a

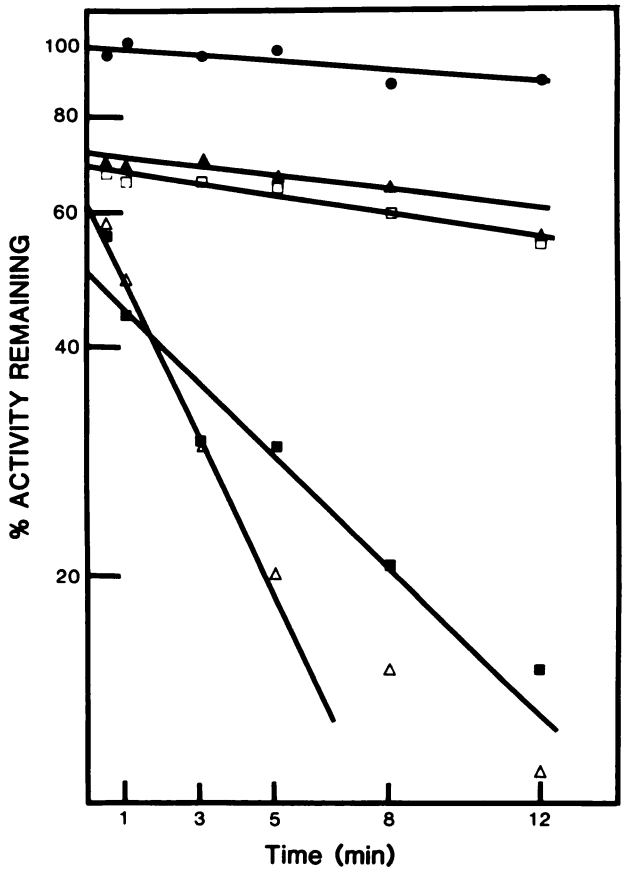

Fig. 3. Effect of preincubation with chloramphenicol analogs on the androstenedione 6β-hydroxylase activity of liver microsomes prepared from PCN-treated male rats. The experimental procedure was identical to that described for Fig. 1. ●, 6β-hydroxylase activity after preincubation with NADPH alone; ▲, 6β-hydroxylase activity after preincubation with NADPH plus 50 μM *N*-(2-phenethyl)dichloroacetamide (B); □, 6β-hydroxylase activity after preincubation with NADPH plus 250 μM *N*-(2-phenethyl)chloroacetamide (C); ■, 6β-hydroxylase activity after preincubation with NADPH plus 50 μM *N*-(2-*p*-nitrophenethyl)dichloroacetamide (D); △, 6β-hydroxylase activity after preincubation with NADPH plus 25 μM *N*-(1,2-diphenethyl)dichloroacetamide (I).

TABLE 4

Rate constants for inactivation of purified cytochrome P-450 BNF-B by chloramphenicol analogs

Residual monooxygenase activity was monitored by the conversion of 7-ethoxycoumarin to 7-hydroxycoumarin as described under Experimental Procedures. Rate constants for inactivation were calculated as described in Table 3.

Compound	Concentration	$k_{inactivation}$
	μM	min^{-1}
Control (methanol)		0.03
Chloramphenicol	100	0.03
N-(2-Phenethyl)dichloroacetamide	25	0.40
N-(2-*p*-Nitrophenethyl)dichloroacetamide	25	0.39
N-(2-*p*-Nitrophenethyl)chloroacetamide	250	0.01
N-(1,2-Diphenethyl)dichloroacetamide	25	0.03
N-(2,2-Diphenethyl)dichloroacetamide	25	0.20

reactive acyl chloride (25, 26), we began our investigation by concentrating on the dichloroacetamide analogs. Our hypothesis was that the remaining functional groups of the molecule may differentially affect its ability to be activated by or bind covalently to particular cytochromes P-450. The isozyme selectivity of the compound *N*-(2-phenethyl)dichloroacetamide is cited as evidence of this statement. Although this particular analog does not inactivate the AD 6β-hydroxylase, addition of a *para*-nitro group to the phenyl ring or the addition of a phenyl group at the R_2 position (Fig. 2) results in increasingly faster rates of 6β-hydroxylase inactivation (Table 3). In contrast, the presence or absence of an electrophilic *para*-nitro group on the phenyl ring of the molecule has no effect on the inactivation of the major β-naphthoflavone-inducible isozyme, BNF-B, whereas the addition of a phenyl group at the R_1 position of the ethyl chain renders the compound unable to inactivate this enzyme.

Based on the initial observation that the compound *N*-(2-*p*-nitrophenethyl)chloroacetamide (E) preferentially retained much of the ability of the corresponding dichloroacetamide (D) to inactivate the 6β-hydroxylase, a number of compounds containing a chloroacetamido group were subjected to preliminary examination to test the hypothesis that this functionality might confer selectivity toward the 6β-hydroxylase. However, all the other chloroacetamides tested either were poor inactivators of all the hydroxylases or exhibited no obvious advantages from the standpoint of selectivity compared to the dichloroacetamides.

The ability to screen a large number of related compounds for their ability to inactivate multiple cytochromes P-450 should now enable us to elaborate a set of predictive rules for which structural features of our chloramphenicol analogs favor the inactivation of particular isozymes. By incorporating all these features into a single molecule, it may therefore be possible to rationally design isozyme-selective inhibitors. It should also be noted that, even if totally specific inhibitors cannot be designed by this method, the availability of pairs of related compounds which differ from each other only in their effect on a single isozyme should still be of considerable utility.

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