0026-895X/86/010019-06$02.00/0

MOLECULAR PHARMACOLOGY, 30:19-24

Dichloromethyl Compounds as Mechanism-Based Inactivators of Rat Liver Cytochromes P-450 *In Vitro*

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Received February 25, 1986; Accepted April 11, 1986

SUMMARY

Twenty dichloromethyl compounds have been tested as potential mechanism-based inactivators of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 (PB-B) in a reconstituted system. With the exception of dichloromethane and dichloroacetamide, all the compounds decreased the ethoxycoumarin deethylase activity of the enzyme in a time- and NADPH-dependent manner. The inhibitory compounds could be divided into two classes according to whether the loss of monooxygenase activity was accompanied by a decrease in spectrally detectable cytochrome P-450. *N*-Monosubstituted dichloroacetamides in which the side-chain consisted of a phenyl or *n*-octyl group were able to mimic the action of chloramphenicol and inactivate the PB-B without destroying the heme moiety. In contrast, dichloroacetamides containing an *n*-hexyl, *n*-butyl, or methyl substituent caused a significant loss of heme, as did the five non-amides tested: 1,1,2,2-tetrachloroethane, 1,1-dichloroacetone, methyl dichloroacetate, α,α-dichlorotoluene, and α,α-dichloroacetophenone. Representative compounds were also examined as inactivators of the major β-naphthoflavone-inducible isozyme of rat liver cytochrome P-450 (BNF-B), using a reconstituted system, as well as of constitutive cytochromes P-450, using intact liver microsomes from untreated rats. These studies suggested a marked difference in isozyme selectivity between certain of the compounds. For example, of the isozymes monitored, only the PB-B was affected by α,α-dichlorotoluene in an NADPH-dependent manner, whereas *N*-octyl dichloroacetamide inactivated not only the PB-B and BNF-B, but also certain constitutive cytochromes, as evidenced by decreases in microsomal *S*-warfarin hydroxylase activities. These studies help delineate the structural requirements for the use of dichloromethyl compounds as probes of cytochrome P-450 function and as potential isozyme-selective inhibitors.

The cytochrome P-450-dependent monooxygenase system plays a key role in the metabolism of a wide variety of endogenous compounds and xenobiotics. Despite the impressive progress in recent years with regard to understanding the multiplicity, regulation, and catalytic mechanism of cytochrome P-450, relatively little information is available about the nature of the binding site for substrate or for NADPH-cytochrome P-450 reductase, or about the roles of various isozymes of cytochrome P-450 in metabolizing specific compounds *in vivo* (1). A major reason for the slow progress in these areas has been the lack of specific irreversible inhibitors which could be used to map the various binding regions on the cytochromes P-450 and to probe their function *in vivo*.

This work was supported by United States Public Health Service Grants ES 03619 (J. H.) and ES 03516 (L. S. K.) and by a Starter Grant from the Pharmaceutical Manufacturers Association Foundation. J. R. H. is the recipient of a Faculty Development Award in Pharmacology from the Pharmaceutical Manufacturers Association Foundation and of Research Career Development Award ES 00151 from the National Institutes of Health. N. E. M. was supported by National Institutes of Health Grant T32 ES 07091.

Among the potentially most specific enzyme inhibitors are the mechanism-based inactivators, also known as suicide substrates. These are substrate molecules for the target enzyme which, in the process of catalytic conversion, are changed into intermediates or products that inactivate the enzyme (2). The requirement for catalysis adds an extra degree of potential selectivity compared to reversible inhibitors, which rely solely on binding (3). The most thoroughly studied suicide substrates of cytochromes P-450 are the olefins and acetylenes. These compounds inactivate cytochromes P-450 mainly as the result of the alkylation of the heme prosthetic group. The destructive potential is inherent in the double or triple bond, as evidenced by the ability of ethylene and acetylene themselves to inactivate cytochrome P-450 (4). Certain acetylenic fatty acid derivatives offer great promise as specific probes of cytochrome P-450 function *in vivo* (5).

Our own interest has centered on the antibiotic chloramphenicol (Fig. 1). Chloramphenicol is unusual among mechanism-based inactivators of the major phenobarbital-inducible

ABBREVIATIONS: PB-B, the major isozyme of rat liver cytochrome P-450 induced by phenobarbital; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BNF, β-naphthoflavone; BNF-B, the major isozyme of rat liver cytochrome P-450 induced by β-naphthoflavone; Ph, phenyl; EDTA, ethylenediaminetetraacetic acid.

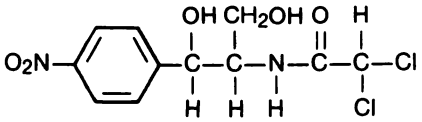

Fig. 1. Structure of chloramphenicol.

isozyme of rat liver cytochrome P-450 (PB-B) in that it inactivates the enzyme by virtue of the covalent modification of the protein rather than the heme moiety (6, 7). The major reactive metabolite responsible for the enzyme inactivation is chloramphenicol oxamyl chloride, which is formed during the oxidative dechlorination by the cytochrome P-450 of the dichloromethyl moiety of chloramphenicol (8, 9). Binding of this reactive metabolite to one or more lysine residues in the protein leads to a loss of monooxygenase function, apparently as the result of an impaired ability of the cytochrome to accept electrons from NADPH-cytochrome P-450 reductase (10). At present, however, it is unclear whether this is due to the modification of amino acid residues which are directly involved in interactions with the reductase, steric hindrance by the bulky bound chloramphenicol metabolites, or conformational changes in the enzyme. With regard to the isozyme selectivity of chloramphenicol, we have recently found evidence for the inhibition of four isozymes upon administration of chloramphenicol to rats *in vivo*, whereas no inhibition was observed of five other isozymes monitored (11).

As a means of enhancing the isozyme selectivity of chloramphenicol and of refining the use of chloramphenicol as a tool for probing the catalytic sites of cytochromes P-450, we have begun to synthesize structural analogs. The results to date confirm the importance of the dichloromethyl moiety and suggest that no other metabolic alterations other than oxidative dechlorination need occur in order to cause inactivation of the cytochrome P-450. The remainder of the molecule does, however, alter isozyme selectivity and inhibitory potency (12). In the present investigation we have examined a series of dichloromethyl compounds in order to determine the minimal structure required to mimic the inhibitory effect of chloramphenicol on cytochrome P-450 PB-B. We have also examined the effect of representative compounds on other isozymes as a means of identifying structural features capable of conferring potential isozyme selectivity.

Experimental Procedures

Materials. Dilauryl L-3-phosphatidylcholine, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, NADPH, and cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, MO. Sodium cholate, sodium deoxycholate, and HEPES were purchased from Calbiochem-Behring, La Jolla, CA. Dichloromethane (T) was obtained from Fisher Scientific, Fair Lawn, NJ, and dichloroacetamide (R) was from Pfaltz and Bauer, Waterbury, CT. α,α-Dichloroacetophenone (I), α,α-dichlorotoluene (J), methyl dichloroacetate (P), 1,1-dichloroacetone (Q), 1,1,2,2-tetrachloroethane (S), *n*-octylamine, *n*-hexylamine, *n*-butylamine, *t*-butylamine, methylamine hydrochloride, 3-phenyl-l-propylamine, 4-phenyl-l-butylamine, *p*-nitroaniline, *p*-chloroaniline, aniline, *p*-nitrobenzylamine hydrochloride, 7-ethoxycoumarin, BNF, and dichloroacetyl chloride were purchased from Aldrich Chemical Co., Milwaukee, WI.

Synthesis of dichloracetamides. Dichloroacetamides were prepared by the method of Rebstock (13) by reacting the respective primary amine with dichloracetyl chloride in aqueous alkali as described previously (12) for the preparation of *N*-(2-*p*-nitrophenethyl) dichloroacetamide (A) and *N*-(2-phenethyl) dichloroacetamide (B). The ethyl acetate extract containing the product was washed with dilute acid, sodium bicarbonate, and water and was then dried under nitrogen. The product was crystallized from aqueous methanol. All dichloroacetamides were characterized by melting point (uncorrected) and by NMR (^1H, CD_3COCD_3, TMS internal standard) δ(ppm) as follows: *N*-(3-phenylpropyl) dichloroacetamide (C), 73° (lit. 75–76°), 2.0 (m, 2H), 2.7 (m, 2H), 3.4 (m, 2H), 5.9 (s, 1H), 7.3 (s, 5H); *N*-(4-phenylbutyl) dichloroacetamide (D), oil, 1.6 (m, 4H), 2.7 (m, 2H), 3.4 (m, 2H), 6.0 (s, 1H), 7.2 (s, 5H); *N*-(*p*-nitrobenzyl) dichloroacetamide (E), 140° (lit. 139–140°), 4.6 (m, 2H), 6.0 (s, 1H), 7.8 (dd, 4H); *N*-(*p*-nitrophenyl) dichloroacetamide (F), 128° (lit. 127°), 6.1 (s, 1H), 8.0 (dd, 4H); *N*-(*p*-chlorophenyl) dichloroacetamide (G), 132–133° (lit. 132°), 6.0 (s, 1H), 7.4 (dd, 4H); *N*-phenyl dichloroacetamide (H), 117° (lit. 118°), 6.0 (s, 1H) 7.4 (m, 5H); *N*-octyl dichloroacetamide (K), 36° (lit. 31–32°), 0.9 (m, 3H), 1.3 (s, 12H), 3.3 (m, 2H), 6.1 (s, 1H); *N*-hexyl dichloroacetamide (L), 36–37° (lit. 31–32°), 0.9 (m, 3H), 1.3 (s, 8H), 3.4 (m, 2H), 6.1 (s, 1H); *N*-butyl dichloroacetamide (M), 44° (lit. 43°), 1.0 (m, 3H), 1.5 (m, 4H), 3.4 (m, 2H), 6.1 (s, 1H); *N*-(*t*-butyl) dichloroacetamide (N), 158–159°, 1.4 (s, 9H), 5.8 (s, 1H); *N*-methyl dichloroacetamide (O), 76–77° (lit. 79°), 2.9 (d, 3H), 5.9 (s, 1H). Literature values for the melting points were taken from the following sources: C, E (14); F (15); G (16); H (17); K, L, M, O (18).

Preparation of microsomes. Adult male Sprague-Dawley rats (150–200 g) were pretreated with phenobarbital or BNF. Phenobarbital was administered as a 0.1% (w/v) sodium phenobarbital solution for 5 days in the drinking water. Rats induced with BNF were injected intraperitoneally once daily with 40 mg/kg of BNF in 0.5 ml of corn oil for 3 days prior to sacrifice. All liver microsomes were prepared as described previously (7).

Preparation of enzymes. PB-B was isolated as described by Guengerich and Martin (19) using modifications described by Halpert *et al.* (10). These modifications allowed the separation of PB-B from PB-D, a closely related isozyme also induced by phenobarbital. The major β-naphthoflavone inducible isozyme (BNF-B) was also purified as described by Guengerich and Martin (19) with modifications as described by Haaparanta *et al.* (20). NADPH-cytochrome P-450 reductase was purified by chromatography on Whatman DE-52 and on 2′,5′-ADP-agarose (19) as described previously (10). 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°.

Incubations of a reconstituted system with inhibitors. Incubation mixtures consisted of 1 nmol/ml of PB-B, 2 units/ml of NADPH-cytochrome P-450 reductase, 30 μg/ml of dilauryl L-3-phosphatidyl choline, 100 μg/ml of sodium deoxycholate, 0.05 M HEPES buffer (pH 7.5), 15 mM $MgCl_2$, 0.1 mM EDTA, 1 mM NADPH, and inhibitor added in 10 μl of methanol. The 1-ml mixture was preincubated for 3 min at 37°, and the reaction was started with the addition of the NADPH and allowed to proceed for 15 min. In some cases the reaction was stopped by the addition of an equal volume of ice-cold 100 mM potassium phosphate butter (pH 7.25) containing 20% glycerol, 0.5% sodium cholate, 0.4% Emulgen 913, and 1 mM EDTA. Samples were then assayed for cytochrome P-450 by the method of Omura and Sato (21). In other cases, samples were quenched by the addition of sodium cholate to a final concentration of 0.6%, placed on ice, and then assayed for pyridine hemachromagen (21). Finally, some samples were placed on ice and then dialyzed for 24 hr at 4° against two 1-liter portions of 50 mM Tris-acetate (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Aliquots were then removed and assayed for cytochrome P-450 content and 7-ethoxycoumarin deethylase activity as described previously (6).

Analytical methods. Protein was determined by the method of Lowry *et al.* (22). Melting points were determined on a Mel-Temp melting point apparatus and are uncorrected. NMR spectra were recorded on a JEOL FX-90Q instrument. Assays of warfarin hydroxylase activity were performed as described previously (23).

Results

Inactivation of cytochrome P-450 PB-B by dichloromethyl compounds. The experimental protocol used to screen dichloromethyl compounds as potential inactivators of PB-B is illustrated in Fig. 2. Essentially, the method involves preincubating a complete reconstituted system with the compound in question for times ranging from 0 to 6 min, and then adding 7-ethoxycoumarin to the same tube in order to measure residual monooxygenase activity. The time-dependent loss of enzyme activity (inactivation) is superimposed upon a competitive (reversible) inhibition due to the presence of unmetabolized inhibitor during the subsequent assay of ethoxycoumarin deethylase activity (25). The extent of the reversible inhibition is most clearly evident at the intercept on the ordinate and increases with increasing inhibitor concentration (12). Pseudo-first order kinetics were observed for the inactivation of the PB-B by four of the compounds shown in Fig. 2. With one of the compounds, α,α-dichloroacetophenone (I), biphasic kinetics were observed, the second phase becoming noticeable when about two-thirds of the enzyme activity had been lost. Similar biphasic kinetics are observed with chloramphenicol and certain of its analogs, and possible reasons for such behavior have been discussed previously (12).

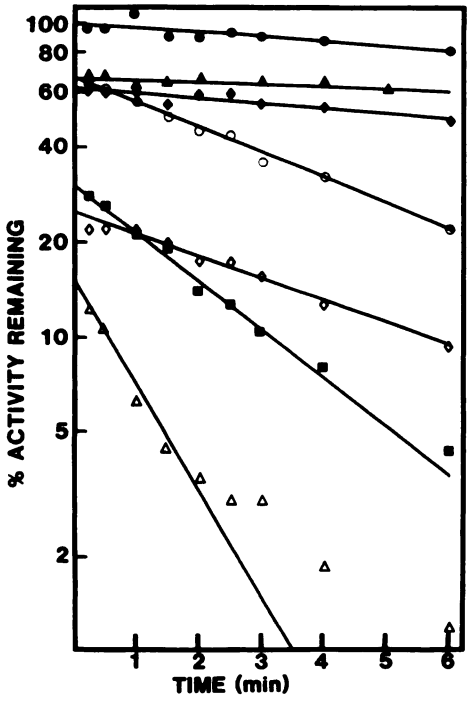

Fig. 2. Inactivation of purified cytochrome P-450 PB-B by representative dichloromethyl compounds. The residual enzymatic activity of purified cytochrome P-450 was assayed by monitoring the metabolism of 7-ethoxycoumarin as described previously (12). The reconstituted system contained 0.05 nmol of cytochrome P-450 PB-B, 0.3 unit of NADPH-cytochrome P-450 reductase, 30 μg of dilauryl L-3-phosphatidylcholine, 100 μg of sodium deoxycholate, 0.05 M HEPES buffer (pH 7.5), 15 mM $MgCl_2$, 0.1 mM EDTA, 0.36 μmol of NADPH, and inhibitor (added in 10 μl of methanol) in a final volume of 1.0 ml. Reactions were started with the addition of the NADPH after a 3-min preincubation at 37°. Incubations were continued at 37° for specified times before the addition of 0.3 μmol of 7-ethoxycoumarin. Incubations were again continued for 1 min. Formation of 7-hydroxycoumarin was monitored (24) on an Aminco-Bowman spectrofluorometer (excitation 366 nm, emission 454 nm). ●, no inhibitor; ▲, 10 mM CH_2Cl_2 (T); ◆, 10 mM NH_2-CO-$CHCl_2$ (R); ○, 500 μM CH_3CO-$CHCl_2$ (Q); ■, 100 μM $CHCl_2$-$CHCl_2$ (S); ◇, 2.5 mM CH_3-NH-CO-$CHCl_2$ (O); △, 100 μM Ph-CO-$CHCl_2$ (I).

Although double-reciprocal plots of the rate of enzyme inactivation as a function of inhibitor concentration can be used to determine the maximal rate of inactivation and the inhibitor concentration required for half-maximal inactivation (K_I), it was not feasible to determine the kinetic constants for the large number of compounds of interest. Therefore, the following guidelines were followed in choosing the concentrations used. Initially, all compounds were tested at 5 or 100 μM.[1] Those compounds which caused no enzyme inactivation at these concentrations were tested at successively higher concentrations, until one was found which caused at least 30% *reversible* inhibition. As illustrated in Fig. 2, 500 μM dichloroacetone (Q), 10 mM dichloromethane (T), and 10 mM dichloroacetamide (R) all caused approximately the same amount of reversible inhibition whereas, of the three compounds, only dichloroacetone decreased enzyme activity with time. In fact, even at a concentration of dichloromethane of 50 mM, which caused 85% reversible inhibition, no enzyme inactivation was observed (data not shown). The time-dependent loss of enzyme activity caused by the various compounds occurred only in the presence of NADPH, confirming the requirement for metabolism of the inhibitors.

Based on experiments similar to those shown in Fig. 2, 20 dichloromethyl compounds were tested for their ability to inactivate cytochrome P-450 PB-B. Two of the compounds, *N*-(2-*p*-nitrophenethyl) dichloroacetamide (A) and *N*-(2-phenethyl) dichloroacetamide (B), have been reported previously (12) and are included here for purposes of comparison. The data obtained are shown in Table 1. The major findings can be summarized as follows. First, compounds of disparate structure containing the dichloromethyl group as the only common structural element are all capable of inactivating the cytochrome P-450 PB-B. These include an aliphatic compound (S), a compound with a benzylic dichloromethyl group (J), two dichloromethyl ketones (I, Q), and a dichloroacetate (P), as well as a number of dichloroacetamides (A–H, K–O). Second, among the dichloroacetamides, based on a phenylalkyl side-chain $Ph(CH_2)_{n-}$, maximal inactivation occurs when $n = 2$ (cf. B, C, D or A, E, F). However, the methylene groups are clearly not essential since, at higher concentrations, *N*-phenyl dichloroacetamide (H) is an effective inactivator of the PB-B, and is made even more so when a nitro- (F) or chloro- (G) substituent is introduced in the *para*-position. Finally, the ability of chloramphenicol to inactivate the PB-B without causing a loss of spectrally detectable cytochrome can be mimicked not only by dichloroacetamides containing a phenyl group in the side-chain but also by *N*-octyl dichloroacetamide (K). In contrast, as the alkyl group is shortened, not only does the effectiveness of the compound as an inactivator decrease (L, M, O), but heme modification ensues. In fact, *N*-methyl dichloroacetamide (O) appears to inactivate the enzyme mainly by virtue of heme modification (Fig. 3).

Inactivation of cytochrome P-450 BNF-B by dichloromethyl compounds. We have reported previously (12) that, although chloramphenicol does not inactivate BNF-B, this isozyme is inactivated by *N*-(2-*p*-nitrophenethyl) dichloroa-

[1] The K_I values for *N*-(2-*p*-nitrophenethyl) dichloroacetamide (A) and for chloramphenicol are 0.8 μM and 15 μM, respectively (12).

TABLE 1

Rate constants for inactivation of purified cytochrome P-450 PB-B by dichloromethyl compounds

Cytochrome P-450 PB-B was incubated with the various dichloromethyl compounds as described in the legend to Fig. 2. Rate constants for inactivation were calculated by linear regression analysis of the natural logarithm of the residual ethoxycoumarin deethylase activity as a function of time. For those compounds which yielded biphasic kinetics of inactivation, the rate constants shown were calculated from the rapid, initial phase. The second phase was observed only after approximately two-thirds of the enzyme activity had been lost.

	Compound	Concentration	$k_{inactivation}$	Percentage of cytochrome P-450 lost[a]
			min^{-1}	
	Control (methanol)		0.02 ± 0.01 (n = 19)	
A	p-NO_2-Ph-$(CH_2)_2$-NH-CO-$CHCl_2$	5 μM	0.59[b]	0[c]
B	Ph-$(CH_2)_2$-NH-CO-$CHCl_2$	5 μM	0.54[b]	16 ± 4[d]
C	Ph-$(CH_2)_3$-NH-CO-$CHCl_2$	5 μM	0.10[b]	16 ± 4[d]
D	Ph-$(CH_2)_4$-NH-CO-$CHCl_2$	5 μM	0.08[b]	13 ± 9[d]
E	p-NO_2-Ph-CH_2-NH-CO-$CHCl_2$	5 μM	0.17	
		100 μM	0.50	1 ± 1
F	p-NO_2-Ph-NH-CO-$CHCl_2$	5 μM	0.08	
		100 μM	0.54	0 ± 0
G	p-Cl-Ph-NH-CO-$CHCl_2$	5 μM	0.08	
		100 μM	0.42	0 ± 2
H	Ph-NH-CO-$CHCl_2$	5 μM	0.03	
		100 μM	0.21	8 ± 3
I	Ph-CO-$CHCl_2$	5 μM	0.47	
		100 μM	0.75	70 ± 0
J	Ph-$CHCl_2$	100 μM	0.35	38 ± 2
K	$CH_3(CH_2)_7$-NH-CO-$CHCl_2$	5 μM	0.48	0 ± 3[d]
L	$CH_3(CH_2)_5$-NH-CO-$CHCl_2$	20 μM	0.27	
		100 μM	0.42	19 ± 0
M	CH_3-$(CH_2)_3$-NH-CO-$CHCl_2$	20 μM	0.10	
		100 μM	0.19	33 ± 0
N	$(CH_3)_3$-C-NH-CO-$CHCl_2$	100 μM	0.13	37 ± 2
O	CH_3-NH-CO-$CHCl_2$	1000 μM	0.12	
		2500 μM	0.16	48 ± 4
P	CH_3O-CO-$CHCl_2$	100 μM	0.09	
		500 μM	0.21	43 ± 2
Q	CH_3-CO-$CHCl_2$	100 μM	0.05	
		500 μM	0.18	41 ± 2
R	NH_2-CO-$CHCl_2$	10 mM	0.03	0 ± 10
S	$CHCl_2$-$CHCl_2$	100 μM	0.35	51 ± 0
T	CH_2Cl_2	10 mM	0.01	ND[e]

[a] Duplicate incubations were carried out as described in Experimental Procedures. Cytochrome P-450 was assayed as the reduced carbon monoxide complex (21). Values are given as the percentage of cytochrome P-450 lost ± the standard deviation compared to controls incubated with NADPH but no inhibitor.

[b] Results represent the mean of two independent experiments. The individual values were: A (0.59, 0.59); B (0.55, 0.53); C (0.11, 0.09); D (0.09, 0.07).

[c] See Ref. 12.

[d] The inhibitor concentration was 20 μM.

[e] ND, not determined.

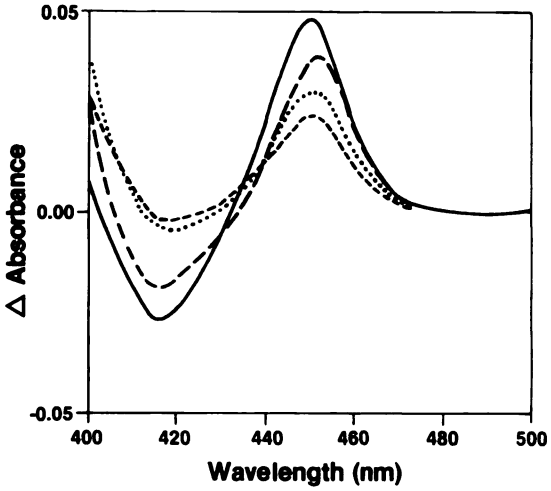

Fig. 3. Effect of preincubation with dichloroacetamides on cytochrome P-450 content of purified cytochrome P-450 PB-B. Cytochrome P-450 PB-B was incubated with a complete reconstituted system containing (– – –) 2.5 mM CH_3-NH-CO-$CHCl_2$ (O), (· · ·) 100 μM $CH_3(CH_2)_5$-NH-CO-$CHCl_2$ (M), (— — —) 100 μM CH_3 $(CH_2)_5$-NH-CO-$CHCl_2$ (L), or (—) no inhibitor. After 10 min, samples were placed on ice and then dialyzed as described in Experimental Procedures. Aliquots were then taken for cytochrome P-450 and for 7-ethoxycoumarin deethylase determination in the presence of a saturating amount of fresh reductase (6). Compound O caused a 50% decrease in cytochrome P-450 and a 70% decrease in 7-ethoxycoumarin deethylase activity, compound M a 30% decrease in cytochrome P-450 and a 70% decrease in deethylase activity, and compound L a 20% decrease in cytochrome P-450 and a 90% decrease in deethylase activity. Separate incubations confirmed that the loss of cytochrome P-450 caused by compounds O, L, and M was accompanied by a parallel loss of heme measured as pyridine hemachromagen (data not shown).

TABLE 2

Rate constants for inactivation of purified cytochrome P-450 BNF-B by dichloromethyl compounds

Incubations were carried out as described in the legend to Fig. 2 except for the use of the major BNF- rather than the major phenobarbital-inducible isozyme of rat liver cytochrome P-450. Rate constants for inactivation were calculated as described in Table 1.

Compound	Concentration	$k_{inactivation}$
	μM	min^{-1}
Control (methanol)		0.04
p-NO_2-Ph-$(CH_2)_2$-NH-CO-$CHCl_2$ (A)	5	0.19
p-NO_2-Ph-NH-CO-$CHCl_2$ (F)	100	0.69
Ph-CO-$CHCl_2$ (I)	100	0.45
Ph-$CHCl_2$ (J)	100	0.06
$CH_3(CH_2)_7$-NH-CO-$CHCl_2$ (K)	5	0.44
$CHCl_2$-$CHCl_2$ (S)	100	0.00

cetamide (A) and N-(2-phenethyl) dichloroacetamide (B). Therefore, certain of the compounds shown in Table 1 were tested with the BNF-B in order to identify other structural features capable of conferring potential isozyme selectivity on dichloromethyl compounds. As seen in Table 2, marked differences in selectivity were observed with some of the compounds. For example, neither 1,1,2,2-tetrachloroethane (S) nor α,α-dichlorotoluene (J) inactivated the BNF-B, whereas both were effective inactivators of the phenobarbital enzyme. In contrast, both enzymes were inactivated at approximately the same rate by α,α-dichloroacetophenone (I), N-octyl dichloroacetamide (K), and N-(p-nitrophenyl) dichloroacetamide (F). In agreement with the above results using purified enzymes, incubation of intact liver microsomes with 100 μM dichlorotoluene for 10 min in the presence of NADPH caused a 10% decrease in spectrally detectable cytochrome P-450 in the case of microsomes from phenobarbital-treated rats, but no decrease in the case of BNF-treated animals. In contrast, similar incubations with dichloroacetophenone caused a 37% decrease in cytochrome P-450 in microsomes from phenobarbital-treated rats and a 25% decrease in microsomes from BNF-treated animals.

Inactivation of constitutive cytochromes P-450 by dichloromethyl compounds. As a further means of examining isozyme selectivity, liver microsomes from untreated rats were pre-incubated with three of the dichloromethyl compounds in the presence and absence of NADPH, and S-warfarin hydroxylase activities were monitored as described previously (11). In addition to the activities shown in Table 3, formation of S-dehydrowarfarin, S-8-hydroxywarfarin, and S-10-hydroxywarfarin was measured. However, these three activities were

TABLE 3

Effect of dichloromethyl compounds on *S*-warfarin hydroxylase activities of liver microsomes from untreated rats

Liver microsomes (1 mg/ml) were incubated in duplicate for 10 min at 37° in 5 ml of 50 mM HEPES buffer (pH 7.6) containing 15 mM $MgCl_2$, 1 mM EDTA, and 1 unit of glucose-6-phosphate dehydrogenase. When present, the NADPH-generating system consisted of 0.5 mM $NADP^+$ and 10 mM glucose 6-phosphate. Compounds were added in 50 μl of methanol. Controls received methanol only. After the incubation, the samples were placed on ice and then centrifuged at 100,000 × *g* for 50 min. The microsomal pellets were suspended in 1 ml of 10 mM Tris-acetate buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA and assayed for protein and for warfarin *S*-hydroxylase activity as described previously (11). *S*-Warfarin 8- and 10-hydroxylase activity as well as *S*-dehydrowarfarin formation were not affected by any of the treatments. The rates of formation of these metabolites in the microsomes incubated in the absence of NADPH and the absence of inhibitor were: 8-OH (0.02), 10-OH (0.04), and dehydrowarfarin (0.26).

Sample	Incubation conditions		Rate of metabolite formation		
	NADPH	Inhibitor[a]	4′OH	6-OH	7-OH
			nmol metabolite/mg protein/min		
1	–	–	0.23, 0.23	0.08, 0.09	0.06, 0.06
2	+	–	0.21, 0.21	0.09, 0.09	0.06, 0.06
3	–	F	0.19, 0.18	0.08, 0.07	0.05, 0.05
4	+	F	0.16, 0.14	0.04, 0.03	0.03, 0.03
5	–	J	0.18, 0.19	0.06, 0.07	0.05, 0.05
6	+	J	0.20, 0.17	0.08, 0.07	0.05, 0.05
7	–	K	0.17, 0.18	0.08, 0.07	0.05, 0.04
8	+	K	0.12, 0.13	0.04, 0.04	0.03, 0.03

[a] F, *p*-NO_2-Ph-NH-CO-$CHCl_2$ (100 μM); J, Ph-$CHCl_2$ (100 μM); K, $CH_3(CH_2)_7$-NH-CO-$CHCl_2$ (20 μM).

not affected by any of the compounds and are omitted to simplify the data presentation. As can be seen in Table 3, none of the activities was affected by incubation with NADPH alone, whereas all three activities were inhibited in an NADPH-dependent fashion by *N*-(*p*-nitrophenyl) dichloroacetamide (F) and by *N*-octyl dichloroacetamide (K), with 6-hydroxylation being the most sensitive and 4′-hydroxylation the least sensitive activity. In contrast, no NADPH-dependent decrease in any of the activities was noted with dichlorotoluene (J). *S*-4′-hydroxylation mainly reflects the activity of isozyme UT-A (11, 26), whereas *S*-7-hydroxylation reflects the activity of isozyme PB-C (26). Both isozymes contribute to *S*-6-hydroxylation.

Discussion

Although there are isolated reports in the literature of the inactivation of cytochromes P-450 by dichloromethyl compounds other than chloramphenicol analogs (27, 28), the present report to our knowledge describes the first systematic study of the inactivation of cytochrome P-450 isozymes by a large number of compounds of disparate structure containing a dichloromethyl group as the common structural element. The results indicate that the dichloromethyl group should be added to the list of functional groups such as the double-bond (29), triple-bond (30), and thiono-sulfur group (31), which are capable of conferring upon a molecule the ability to inactivate cytochromes P-450. There is one important difference between the dichloromethyl and the olefinic and acetylenic compounds, however, in that ethylene and acetylene themselves are capable of destroying cytochromes P-450, whereas we observed no inactivation of cytochrome P-450 PB-B by dichloromethane. This does not appear to be due to an inability of the enzyme to metabolize dichloromethane, since we confirmed that the known metabolite, carbon monoxide (32), did accumulate during the incubation of cytochrome P-450 PB-B with dichloromethane.[2]

One intriguing finding of the present investigation was the ability of *N*-octyl dichloroacetamide (K) to mimic the action of chloramphenicol and inactivate the cytochrome P-450 PB-B without causing any decrease in spectrally detectable cytochrome. This indicates that the presence of a dichloroacetamido group coupled to a hydrophobic side-chain may be sufficient for inactivation via protein modification to occur. As the length of the side-chain decreased in the series *n*-octyl to *n*-butyl, the effectiveness of dichloroacetamides as inactivators of the PB-B decreased, and a loss of spectrally detectable cytochrome became evident. A further decrease in the size of the side-chain as in the case of *N*-methyl dichloroacetamide (O), yielded a compound which inactivated the PB-B mainly as the result of heme rather than protein modification (Fig. 3).

The above results indicate that the apparent difference in the target of most acetylenic and olefinic suicide substrates of cytochromes P-450 (heme) as opposed to chloramphenicol (protein) may be at least in part a function of the structure of the remainder of the molecule to which the functional group activated by the P-450 is coupled. In fact, recently there have been several reports of acetylenic and olefinic compounds containing large hydrophobic side-chains which cause substantial inactivation of microsomal cytochrome P-450-dependent monooxygenase activities with minimal effects on spectrally detectable cytochrome (5, 33, 34). These data have been interpreted as indicating that suicide inactivation of cytochromes P-450 by such compounds need not involve heme alkylation and obligatory loss of cytochrome P-450 (33, 34). Similarly, our present results indicate that inactivation of cytochrome P-450 PB-B by dichloroacetamides need not be the result of modification of the protein moiety of the enzyme. Although we know that the presence of a dichloroacetamido function is not sufficient to ensure that enzyme inactivation by a dichloromethyl compound will be the result of protein modification, we do not yet know whether the dichloroacetamido group is a prerequisite for such protein-directed inactivation to occur. This is because all of the readily available non-amides caused a considerable loss of spectrally detectable cytochrome. At present, it is not evident whether the differential effects of α,α-dichloroacetophenone (I), and the *N*-phenyl dichloroacetamides (F–H), for example, reflect the difference in the reactivity of the intermediates formed from a dichloromethylketone as opposed to a dichloroacetamide, or the difference in distance between the dichloromethyl group and the benzene ring.

With regard to the isozyme selectivity of the dichloromethyl compounds tested, certain interesting differences were noted. For example, *N*-octyl dichloroacetamide (K) inactivated both purified PB-B and BNF-B and also caused NADPH-dependent decreases in three different constitutive warfarin *S*-hydroxylase activities in intact liver microsomes. In contrast, α,α-dichlorotoluene (J) inactivated only the PB-B and not the BNF-B and caused no NADPH-dependent decreases in any of the warfarin hydroxylase activities monitored. These results indicate that

[2] Cytochrome P-450 PB-B was incubated with dichloromethane (50 mM) in a reconstituted system as described in Experimental Procedures. An oxidized spectrum was recorded on a Beckman DU-7HS spectrophotometer immediately after addition of the NADPH to start the reaction, as well as 10 min later. Then, sodium dithionite was added, and a new spectrum was recorded. Formation of carbon monoxide was indicated by the increase in absorbance at 450 nm indicative of ferrous carboxycytochrome P-450.

dichloromethyl compounds may have broad utility as probes of cytochrome P-450 structure-function relationships. For example, from the standpoint of labeling potentially homologous regions in a large number of different cytochrome P-450 isozymes, a compound such as *N*-octyl dichloroacetamide might be very useful. In contrast, from the standpoint of developing isozyme-specific inhibitors for *in vivo* modulation of monooxygenase function, molecules based on a benzylic dichloromethyl group may be of considerable interest.

Acknowledgments

We wish to thank Mr. Brian Weck and Dr. Tim Wunz for the NMR analyses and Ms. Deborah Dunbar for expert technical assistance with the warfarin hydroxylase assays.

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