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# Analogues of Chloramphenicol as Mechanism-Based Inactivators of Rat Liver Cytochrome P-450: Modifications of the Propanediol Side Chain, the *p*-Nitro Group, and the Dichloromethyl Moiety

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**SUMMARY**

The importance of the *p*-nitro group, the propanediol side chain, and the dichloromethyl moiety of chloramphenicol in regulating its effectiveness and selectivity as a mechanism-based inactivator of rat liver cytochromes P-450 has been examined. 1-*p*-Nitrophenyl-2-dichloroacetamidoethane, 1-*p*-nitrophenyl-2-dibromoacetamidoethane, and 1-phenyl-2-dichloroacetamidoethane were as effective as chloramphenicol at inactivating the major phenobarbital-inducible isozyme of rat liver cytochrome P-450, whereas 1-*p*-nitrophenyl-2-difluoroacetamidoethane caused no enzyme inactivation. Unlike chloramphenicol, 1-*p*-nitrophenyl-2-dichloroacetamidoethane and 1-phenyl-2-dichloroacetamidoethane also inactivated the major β-naphthoflavone-inducible isozyme of rat liver cytochrome P-450.

Alkaline hydrolysis of the adducts formed upon *in vitro* incubation of liver microsomes from phenobarbital- and β-naphthoflavone-induced rats with [$^{14}$C]-1-*p*-nitrophenyl-2-dichloroacetamidoethane resulted in the release of 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide and oxalic acid. Enzymatic digests of the radiolabeled protein produced by incubation of a reconstituted system containing the major isozymes induced by β-naphthoflavone or phenobarbital with [$^{14}$C]-1-*p*-nitrophenyl-2-dichloroacetamidoethane led to the release of 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide and 4-nitro-1-phenethyl oxamyl lysine. These results suggest that a single oxamyl chloride intermediate is responsible for the covalent modification and, hence, inactivation of both isozymes by 1-*p*-nitrophenyl-2-dichloroacetamidoethane.

The antibiotic chloramphenicol has been shown to be a mechanism-based inactivator, or suicide substrate, of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 both *in vitro* and *in vivo* (1, 2). Chloramphenicol is unusual among mechanism-based inactivators of cytochrome P-450 in that it inactivates the enzyme by virtue of the covalent modification of the protein rather than the heme moiety. The major reactive metabolite responsible for the enzyme inactivation is chloramphenicol oxamyl chloride, which is formed during the oxidative dechlorination by cytochrome P-450 of the dichloromethyl moiety of chloramphenicol (3, 4). The major covalently bound species has been identified as an adduct of chloramphenicol oxamic acid and the ε-amino group of one or more lysine residues in the enzyme (5).

Specific mechanism-based inactivators such as chloramphenicol, which bind to the protein moiety of cytochrome P-450, are potentially useful in determining the role of particular amino acid residues in the enzyme in regulating such functions as substrate binding or interaction with NADPH-cytochrome P-450 reductase. In fact, we have recently found that the loss of monooxygenase activity of the major phenobarbital-inducible P-450 isozyme following its modification by chloramphenicol metabolites results from an impaired ability to accept electrons from NADPH-cytochrome P-450 reductase (6). However, it was unclear whether this was due to 1) the modification of amino acid residues which are directly involved in interactions with the reductase or 2) to the introduction of bulky chloramphenicol metabolites into sensitive sites on the enzyme. Although the dichloromethyl moiety of chloramphenicol has been shown to be important in the covalent binding of chloramphenicol to the protein, the importance of the remainder of the chloramphenicol molecule in the inactivation of cytochrome P-450 has not been determined. The remaining functional groups of the molecule could affect either the binding of reactive intermediates to the enzyme or the loss of activity of the enzyme

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**ABBREVIATIONS:** IP, intraperitoneal; $pNO_2DCAE$, 1-*p*-nitrophenyl-2-dichloroacetamidoethane; MeOH, methanol; TLC, thin layer chromatography; TMS, tetramethylsilane; $pNO_2DFAE$, 1-*p*-nitrophenyl-2-difluoroacetamidoethane; $pNO_2DBAE$, 1-*p*-nitrophenyl-2-dibromoacetamidoethane; PB-B, the major isozyme of rat liver cytochrome P-450 induced by phenobarbital; BNF-B, the major isozyme of rat liver cytochrome P-450 induced by β-naphthoflavone; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

once the reactive intermediates are bound. Alterations in these functional groups could also affect the isozyme selectivity of chloramphenicol.

Mechanism-based inactivators are thought to be among the most selective enzyme inhibitors due to their added catalytic requirement (7). Effective and selective inhibitors of different cytochrome P-450 isozymes are potentially useful *in vivo* for therapeutic purposes and as physiological probes into the importance of different isozymes in xenobiotic metabolism (8, 9). It has recently been determined that, although chloramphenicol is selective, it is not a totally specific inactivator of rat liver cytochromes P-450. Thus, when administered at a dose of 300 mg/kg, IP, to control rats or rats treated with various inducers, chloramphenicol inactivated four of nine major cytochrome P-450 isozymes monitored (10). One possible means of increasing the selectivity as well as the effectiveness of this inhibitor as a mechanism-based inactivator is to synthesize structural analogues. The present investigation has focused on the effectiveness of analogues of chloramphenicol containing modifications of the propanediol side chain, the *p*-nitro group, and the dichloromethyl moiety in order to determine whether these functional groups play a role in the inactivation of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450. The analogue, $pNO_2DCAE$, which contains an ethyl group in place of the propanediol group of chloramphenicol, was also further examined in regard to its isozyme selectivity and covalent interactions with cytochromes P-450.

## Materials and Methods

Chloramphenicol, NADPH, dilauryl L-3-phosphatidylcholine, prolidase, carboxypeptidase-A, leucine aminopeptidase, protease (*Streptomyces griseus*, type XIV), and *N*-α-t-butyloxycarbonyl-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO). β-Naphthoflavone, 7-ethoxycoumarin, *p*-nitrophenethylamine hydrochloride, phenethylamine, dibromoacetic acid, dichloroacetic acid, *N,N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, dichloroacetyl chloride, and methyl oxalyl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). [$^{14}$C]Chloramphenicol was purchased from New England Nuclear (Boston, MA). Difluoroacetic acid was purchased from Alfa Products (Danvers, MA). Deschlorochloramphenicol was a gift from Dr. Lance Pohl, National Institutes of Health (Bethesda, MD).

**Synthesis of $pNO_2DCAE$.** *p*-Nitrophenethylamine hydrochloride (2.0 g, 10 mmol) was dissolved in an excess of 1 N NaOH (40 ml) and added to 80 ml of ethyl acetate. Dichloroacetyl chloride (30 mmol) was added dropwise to the reaction mixture, which was stirred for 10 min at room temperature. The mixture was transferred to a separatory funnel, and the water phase was discarded. The ethyl acetate phase was washed first with HCl (10 ml of 1 N HCl once, 10 ml of 0.1 N HCl twice) and then with 5% sodium bicarbonate (10 ml, three times), and finally with water (10 ml, two times). The series of washes was repeated once more. The ethyl acetate phase was then rotary evaporated to dryness. The product was redissolved in $CHCl_3$/MeOH (90:10) and applied to Analtech Silica Gel GF 500-μm TLC plates, which were developed in $CHCl_3$/MeOH (90:10). The product ($R_f$ = 0.83) was scraped from the plates and eluted with $CHCl_3$/MeOH (90:10) and dried under $N_2$. The resulting light yellow solid had an m.p. of 97.5–99°. The product was >99% pure of contaminants absorbing at 254 nm when spotted on analytical TLC plates which were developed in $CHCl_3$/MeOH (90:10). The product also yielded one spot on TLC with two other solvent systems: propanol/water (70:30) ($R_f$ = 0.96), and isopropanol/ammonium hydroxide/water (85:5:10) ($R_f$ = 0.91). The identity of the product was confirmed by NMR ($^1$H, $CD_3COCD_3$, TMS internal standard) δ (ppm) 3.3 (*m*, 4H, —$CH_2$—$CH_2$—), 6.3(*s*, 1H, —$CHCl_2$), 7.9 (*dd*, 4H, aromatic).

1-Phenyl-2-dichloroacetamidoethane was synthesized in a similar manner, with phenethylamine used in place of *p*-nitrophenethylamine. The product had an m.p. of 74–75°. The identity of the compound was confirmed by NMR. (1H, $CD_3COCD_3$, TMS internal standard) δ(ppm) 3.2 (*m*, 4H, —$CH_2$—$CH_2$—), 6.3 (*s*, 1H, $CHCl_2$), 7.3 (*s*, 5H, aromatic).

**Synthesis of [$^{14}$C]$pNO_2DCAE$.** Radiolabeled $pNO_2DCAE$ was synthesized using a modification of the method of Sonenberg *et al.* (11) for the preparation of bromamphenicol. As the [$^{14}$C]dichloroacetic acid which was necessary for the synthesis was not available, 200 μl of 1 N NaOH was added to 10 μmol of [$^{14}$C]chloramphenicol (1mCi/mmol), and the chloramphenicol was allowed to hydrolyze for 2 hr at room temperature. After acidifying the mixture with 200 μl of 2 N HCl, the [$^{14}$C]dichloroacetic acid formed was extracted into ethyl acetate and evaporated under nitrogen. Dioxane (200 μl), 2.1 mg of dicyclohexylcarbodiimide, and 1.4 mg of *N*-hydroxysuccinimide (27.8 μl of a 0.05 mg/μl solution in dioxane) were then added to the [$^{14}$C]dichloroacetic acid, and the reaction mixture was allowed to sit at room temperature. After 1 hr, 10 μmol of neutralized *p*-nitrophenethylamine were added, and the reaction mixture was again allowed to sit for 1 hr. Ethyl acetate (4 ml) was added, and the dicyclohexylurea was removed by centrifugation. The supernatant was then washed with 2% sodium bicarbonate (2 ml, three times), 0.1 N HCl (2 ml, three times), and water (2 ml, three times), and was dried under nitrogen. The sample was redissolved in 200 μl of MeOH and purified by preparative TLC as described in the synthesis of unlabeled $pNO_2DCAE$. The product was found to be >98% radiochemically pure by reverse phase TLC (MeOH/$H_2O$, 70:30) ($R_f$ = 0.43).

$pNO_2DFAE$ and $pNO_2DBAE$ were prepared in a similar manner, except on a larger scale, using difluoroacetic acid and dibromoacetic acid, respectively. $pNO_2DFAE$ had an m.p. of 103–104° and $pNO_2DBAE$ had an m.p. of 134–135°. The identity of these products was confirmed by NMR. 1) $pNO_2DBAE$: ($^1$H,$CD_3COCD_3$, TMS internal standard) δ (ppm) 3.4 (*m*, 4H, —$CH_2$—$CH_2$, 6.2 (*s*, 1H, $CHBr_2$), 7.9 (*dd*, 4H, aromatic). 2) $pNO_2DFAE$: ($^1$H,$CD_3COCD_3$, TMS internal standard) δ (ppm) 3.3 (*m*, 4H, —$CH_2$—$CH_2$), 6.1 (*t*, 1H, $CHF_2$), 7.9 (*dd*, 4H, aromatic).

**Preparation of microsomes.** Adult male Sprague-Dawley rats (150–200 g) were pretreated with phenobarbital or β-naphthoflavone. Phenobarbital was administered as a 0.1% (w/v) sodium phenobarbital solution for 5 days in the drinking water. For *in vivo* studies of cytochrome P-450 inhibition, phenobarbital-induced rats were starved and the phenobarbital was replaced with water 24 hr before sacrifice. These rats were then injected IP with 10 or 100 mg/kg of $pNO_2DCAE$ or 300 mg/kg of chloramphenicol in 0.7 ml of propylene glycol and were sacrificed by cervical dislocation after 1 hr. Rats induced with β-naphthoflavone were injected IP once daily with 40 mg/kg of β-naphthoflavone in 0.5 ml of corn oil for 3 days before sacrifice. All liver microsomes were prepared as described previously (2).

**Preparation of enzymes.** The major phenobarbital-induced isozyme of rat liver cytochrome P-450 (PB-B) was isolated as described by Guengerich and Martin (12) using modifications described by Halpert *et al.* (6). 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 (12) with modifications as described by Haaparanta *et al.* (13).

**Microsomal incubations.** The microsomal metabolism of 7-ethoxycoumarin was assayed essentially as described previously (2). The 1-ml incubation mixture contained 0.05 mg of microsomal protein, 0.5 mM NADP, 10 units of glucose-6-phosphate dehydrogenase, 0.3 mM 7-ethoxycoumarin, 15 mM $MgCl_2$, 0.1 mM EDTA, 0.05 M HEPES buffer (pH 7.5), and 10 mM glucose-6-phosphate. After a 3-min preincubation at 37°, the reaction was started by the addition of the glucose-6-phosphate and allowed to proceed for 10 min.

**Incubations of a reconstituted system with [$^{14}$C]chloramphenicol or [$^{14}$C]$pNO_2DCAE$.** Incubation mixtures consisted of 1 nmol/ml of PB-B or BNF-B, 2 units/ml of NADPH-cytochrome P-450

reductase, 30 μg/ml 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 10 μM [$^{14}$C]$pNO_2DCAE$ (1.1 mCi/mmol) or 100 μM [$^{14}$C]chloramphenicol (1.8 mCi/mmol). The mixture was preincubated for 3 min at 37°, and the reaction was started with the addition of the NADPH. The samples were incubated for 15 min and then dialyzed for 48 hr at 4° against four 1-liter portions of 50 mM Tris-acetate (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. In some instances, aliquots were then removed and assayed for $^{14}$C, cytochrome P-450 content and ethoxycoumarin deethylase activity, as described by Halpert and Neal (1). Some incubation mixtures (containing 10 nmol of cytochrome P-450 in 10 ml) were dialyzed for 24 hr against distilled water. The protein precipitated and was then collected by centrifugation.

**Enzymatic hydrolysis of the $^{14}$C-labeled protein.** *N*-Ethylmorpholine acetate buffer (0.05 M, 0.125 ml, pH 7.5) containing 0.1% sodium dodecyl sulfate and 57.5 μg of protease was added to the protein precipitate obtained from dialysis of the $^{14}$C-labeled reconstituted system. The mixture was incubated at 37° for 4 hr. Prolidase (25 μg), carboxypeptidase (25 μg), and leucine aminopeptidase (25 μg) were added, and the digest was allowed to proceed overnight at 37°.

**Microsomal incubations with [$^{14}$C]$pNO_2DCAE$.** Microsomal protein (1 mg/ml) was incubated with 20 μM [$^{14}$C]$pNO_2DCAE$ (1.1 mCi/mmol), 0.5 mM NADP, 15 mM $MgCl_2$, 0.1 mM EDTA, 10 mM glucose-6-phosphate, and 10 units of glucose-6-phosphate dehydrogenase in a final volume of 1 ml of 0.05 M HEPES buffer. After a preincubation of 3 min at 37°, the reaction was started with the addition of the glucose-6-phosphate and allowed to proceed for 30 min. Incubations were terminated by the addition of 2 ml of ethyl acetate. After vortexing and centrifuging, the ethyl acetate phase was discarded, and the samples were washed three more times with 2 ml of ethyl acetate. Methanol (3 ml) was added, the protein precipitate was spun down, and the supernatant was removed. The precipitate was washed once more with 1 ml of water and precipitated with 3 ml of methanol. NaOH (1 N, 200 μl) was added, and the samples were allowed to sit at room temperature for 2 hr. Samples from phenobarbital-induced rats were then neutralized with 2 N HCl. Samples from BNF-induced rats were heated at 60° for 60 min before neutralizing with HCl. The microsomal protein was removed by centrifugation, and the supernatants were applied to a Bio-Rad P-2 column (1.0 × 45 cm) equilibrated with 0.05 M *N*-ethylmorpholine acetate buffer (pH 7.5). The column was eluted with the same buffer at a rate of 10 ml/hr, and 1-ml fractions were collected and monitored by liquid scintillation counting and UV spectroscopy.

**Sleeping times.** Male Sprague-Dawley rats (160–200 g) were pretreated with 0.1% (w/v) phenobarbital in the drinking water for 5 days. The animals were then starved for the next 24 hr and the phenobarbital was replaced with fresh drinking water. Rats were injected IP with 10, 100, or 300 mg/kg of chloramphenicol or $pNO_2DCAE$ in 0.5 ml of propylene glycol or with the vehicle alone. After 1 hr, sodium pentobarbital (100 mg/kg) was administered IP. Sleeping time was measured as the time between the loss and the regaining of the righting reflex.

**Analytical methods.** Cytochrome P-450 content was monitored spectrally by the method of Omura and Sato (14), and protein was determined by the method of Lowry *et al.* (15). Melting points were determined on a Mel-Temp melting point apparatus and are uncorrected. NMR spectra were recorded on a JEOL FX-90Q instrument.

## Results

**Kinetics of inactivation of PB-B by analogues of chloramphenicol.** The rapid inactivation of the 7-ethoxycoumarin deethylase activity of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 observed at various concentrations of $pNO_2DCAE$ is shown in Fig. 1. Pseudo-first order kinetics were observed for the initial phase of inactivation, which is consistent with inactivation proceeding via a suicide

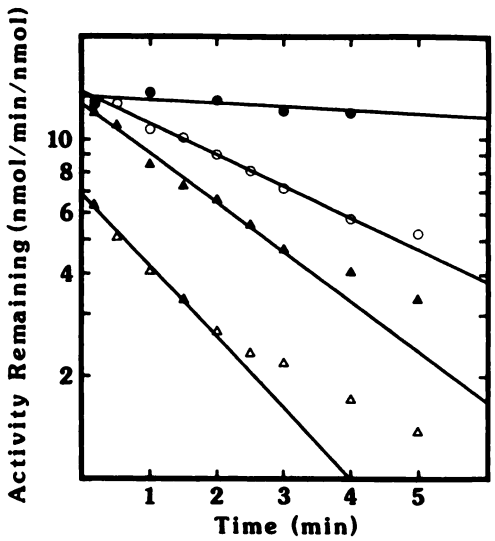


**Fig. 1.** Inactivation of purified cytochrome P-450 PB-B by $pNO_2DCAE$ as a function of inhibitor concentration. The enzymatic activity of purified cytochrome P-450 before and after incubation with chloramphenicol and analogues was assayed by monitoring the metabolism of 7-ethoxycoumarin essentially as described by Waxman and Walsh (16). The reconstituted system contained 0.05 nmol of cytochrome P-450 PB-B, 0.3 units 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 0, 0.5, 1, and 5 μM analogue 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. Under these conditions product formation was linear with time for all concentrations of inhibitor used. Formation of 7-hydroxycoumarin was monitored (19) on an Aminco-Bowman spectrofluorometer (excitation 366 nm, emission 454 nm). The suicide inactivation of PB-B by $pNO_2DCAE$ is superimposed upon the competitive inhibition of 7-ethoxycoumarin deethylase activity. The competitive inhibition is most noticeable at the *ordinate* intercept and increases with increasing $pNO_2DCAE$ concentrations. ●, no inhibitor; ○, 0.5 μM; ▲, 1 μM; △, 5 μM.

mechanism. From the plot of the reciprocal of the initial rate of inactivation as a function of the reciprocal of the inhibitor concentration, the maximal rate of inactivation by $pNO_2DCAE$ ($k_{inactivation}$) was determined to be 0.6 $min^{-1}$ and the inhibitor concentration required for half-maximal inactivation ($K_I$) was 0.8 μM (Fig. 2). The $k_{inactivation}$ value for chloramphenicol was 0.4 $min^{-1}$ and the $K_I$ was found to be 15 μM (data not shown).

The inactivation of the 7-ethoxycoumarin deethylase activity obtained with 5 μM $pNO_2DCAE$, 5 μM $pNO_2DBAE$, and 5 μM 1-phenyl-2-dichloroacetamidoethane and with 100 μM $pNO_2DFAE$ is shown in Fig. 3. The rate obtained with 1-phenyl-2-dichloroacetamidoethane ($k = 0.53$ $min^{-1}$) was similar to that obtained with $pNO_2DCAE$ ($k = 0.59$ $min^{-1}$), whereas the rate with $pNO_2DBAE$ was slightly greater ($k = 0.70$ $min^{-1}$). $pNO_2DFAE$, however, showed no evidence of time-dependent inactivation at a concentration of 100 μM.

To determine whether the isozyme selectivity of $pNO_2DCAE$ differed from that of chloramphenicol, the major β-naphthoflavone-inducible isozyme was incubated with 100 μM chloramphenicol or 5 μM $pNO_2DCAE$. As seen in Fig. 4, chloramphenicol did not inactivate this isozyme, whereas $pNO_2DCAE$ inactivated the enzyme at a rate ($k = 0.19$ $min^{-1}$) approximately one-third that at which it inactivated the major phenobarbital-inducible isozyme. 1-Phenyl-2-dichloroacetamidoethane also inactivated the major β-naphthoflavone-induced enzyme at

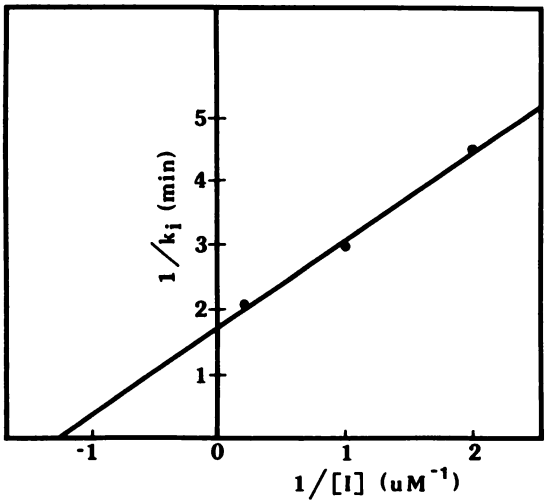


**Fig. 2.** Double reciprocal plot of the rate of the initial phase of inactivation of P-450 PB-B by $pNO_2DCAE$ as a function of inhibitor concentration. $K_i$ is determined from the intercept on the *abscissa* and $k_{inactivation}$ from the intercept on the *ordinate*.

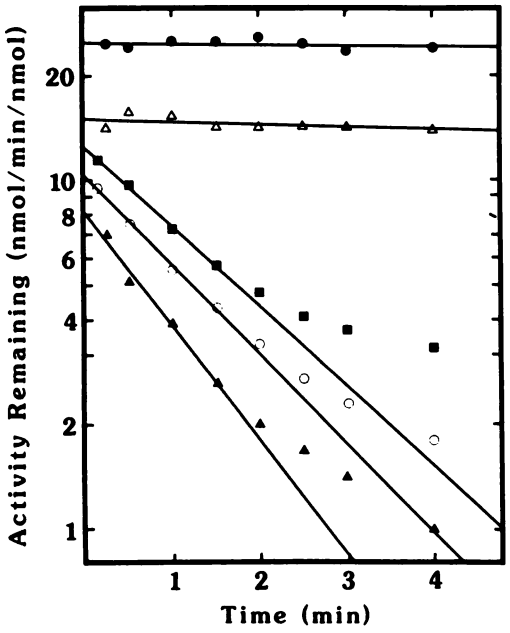


**Fig. 3.** Inactivation of purified cytochrome P-450 PB-B by analogues of chloramphenicol. The assay was carried out essentially as described in Fig. 1 except that 100 μM $pNO_2DFAE$ (△), 5 μM $pNO_2DCAE$ (○), 5 μM $pNO_2DBAE$ (▲), and 5 μM 1-phenyl-2-dichloroacetamidoethane (■) were incubated with the PB-B in place of the varying concentrations of $pNO_2DCAE$. ●, no inhibitor added.

approximately the same rate as $pNO_2DCAE$ ($k$ = 0.14 min$^{-1}$) (data not shown).

**Covalent binding of metabolites of chloramphenicol and $pNO_2DCAE$ in a reconstituted system.** Incubation of [$^{14}$C]$pNO_2DCAE$ (10 μM) with a reconstituted system containing the major isozyme of cytochrome P-450 induced by phenobarbital led to the binding of $^{14}$C to the proteins of the reconstituted system. The stoichiometry of the inactivation of the 7-ethoxycoumarin deethylase activity was 1.5 nmol of $^{14}$C/nmol of cytochrome P-450. Previous studies have shown that incubation of the major phenobarbital-inducible isozyme with [$^{14}$C] chloramphenicol also resulted in a stoichiometry of inactivation

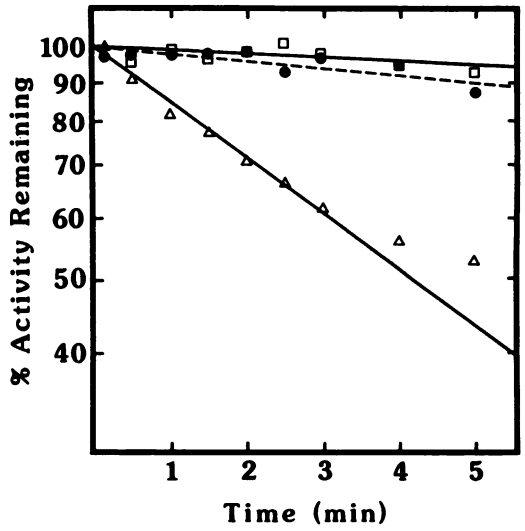


**Fig. 4.** Effect of preincubation with chloramphenicol and $pNO_2DCAE$ on the ethoxycoumarin deethylase activity of purified cytochrome P-450 BNF-B in a reconstituted system. The assay was carried out as described in Fig. 1 except that the incubation time after the addition of the ethoxycoumarin was extended to 2 min in the case of chloramphenicol. ●, no inhibitor; (□) 100 μM chloramphenicol; △, 5 μM $pNO_2DCAE$.

of 1.5 nmol of $^{14}$C/nmol of cytochrome P-450 (1). Incubation of the major β-naphthoflavone-inducible isozyme with [$^{14}$C] $pNO_2DCAE$ (10 μM) resulted in the covalent binding of $^{14}$C to the proteins of the system with a stoichiometry of inactivation of 1 nmol of $^{14}$C/nmol of cytochrome P-450.

**Enzymatic hydrolysis of the $^{14}$C-labeled proteins of a reconstituted system.** The enzymatic digests of the $^{14}$C-labeled proteins produced after incubation of a reconstituted system containing either PB-B or BNF-B with [$^{14}$C] $pNO_2DCAE$ were run on a Bio-Rad P-2 column. The enzymatic digest of protein from incubation of BNF-B and [$^{14}$C] $pNO_2DCAE$ yielded two major peaks upon chromatography (Fig. 5). Peak A ($V_e$ = 33 ml) co-chromatographed with the synthetic standard 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide (see Fig. 7, compound *e*), whereas peak B ($V_e$ = 43 ml) corresponded to the synthetic 4-nitro-1-phenethyl oxamyl lysine (see Fig. 7, compound *f*). The identity of the products of the enzymatic digests were confirmed by HPLC (Fig. 6). The enzymatic digest of protein from the incubation of PB-B and $pNO_2DCAE$ also yielded the same two peaks ($V_e$ = 33 ml and 42 ml), the identity of which was also confirmed by HPLC (data not shown). Thus, both isozymes form a stable lysine adduct of $pNO_2DCAE$ as well as a labile adduct, which hydrolyzes to the free acid metabolite under the conditions of the enzymatic digest.

**Alkaline hydrolysis of microsomes incubated with [$^{14}$C]$pNO_2DCAE$.** Microsomes from rats pretreated with phenobarbital or β-naphthoflavone were incubated with [$^{14}$C] $pNO_2DCAE$ as described in Materials and Methods and treated with 1 N NaOH. Chromatography of the alkaline hydrolysate of the $^{14}$C-labeled microsomes from phenobarbital-induced rats on a Bio-Rad P-2 column yielded two major peaks containing approximately the same amount of radiolabel. One peak ($V_e$ = 36 ml) was identified as 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide (Fig. 7, compound *e*) and the other peak ($V_e$ = 21 ml) as oxalic acid (Fig. 7, compound *d*) by co-chromatography with the respective synthetic standards. Chromatography of the $^{14}$C-labeled microsomes from β-naphthoflavone-induced

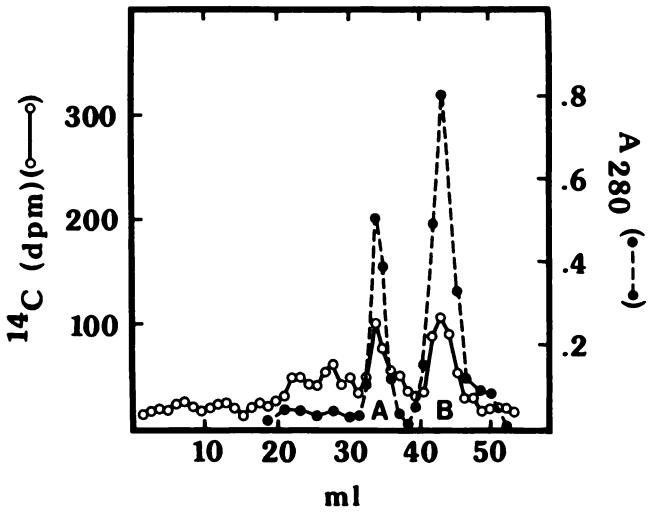


**Fig. 5.** Chromatography on a Bio-Rad P-2 column (1.0 × 45 cm) in 0.05 M *N*-ethylmorpholine acetate buffer (pH 7.5) of an enzymatic digest of $^{14}$C-labeled BNF-B after incubation with $pNO_2DCAE$ as described in Materials and Methods. 4-Nitro-1-phenethyl-1,2-dicarboxylic acid amide and 4-nitro-1-phenethyl oxamyl lysine were added prior to chromatography. These synthetic standards were synthesized by a method directly analogous to the synthesis of chloramphenicol oxamic acid and chloramphenicol oxamyl lysine (3, 5). The synthetic standards were monitored by the absorbance at 280 nm (●– – –●) and the labeled adducts were monitored by scintillation counting (○—○). Peak A co-chromatographs with 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide, whereas peak B co-chromatographs with 4-nitro-1-phenethyl-oxamyl lysine.

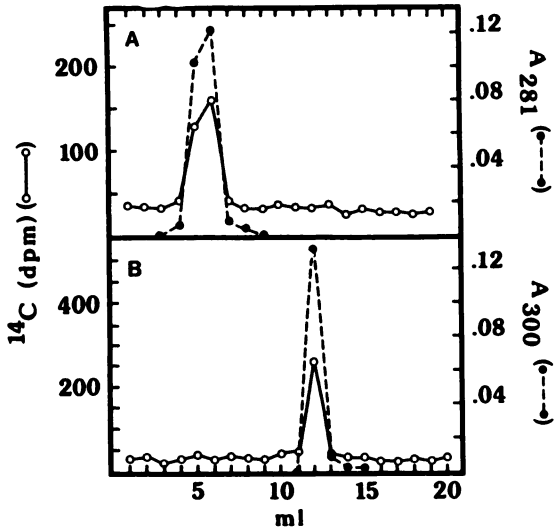


**Fig. 6.** Reverse phase HPLC of peaks A and B obtained from chromatography on a Bio-Rad P-2 column of the enzymatic digest of $^{14}$C-labeled BNF-B after incubation with $pNO_2DCAE$ (Fig. 5). HPLC was performed on a Beckman 421A instrument with Beckman 110A pumps and an Altex 5-μm, 4.6-mm, 25-cm C-18 column. The column was eluted with a 15-min (peak B) or 30-min (peak A), 25–100% MeOH-water gradient at a rate of 0.5 ml/min, and 1-ml fractions were collected. The fractions were monitored by UV spectroscopy (●– – –●) and by liquid scintillation counting (○—○). Graph A is the HPLC chromatograph of peak A from the P-2 column. Graph B is the HPLC chromatograph obtained from peak B from the P-2 column. This was monitored at 300 nm in order to eliminate background absorbance.

rats also resulted in two similar peaks ($V_e$ = 21 ml and $V_e$ = 37 ml). In a similar fashion, alkaline hydrolysis of microsomes from phenobarbital-induced rats to which [$^{14}$C]chloramphenicol had been covalently bound was previously found to yield two approximately equal peaks corresponding to chloramphenicol oxamic (Fig. 7, compound *5*) and oxalic acid (Fig. 7, compound *4*) (2).

**Effect of *in vivo* treatment with $pNO_2DCAE$ on rat liver microsomal enzymes.** Treatment of phenobarbital-induced rats with 10 or 100 mg/kg of $pNO_2DCAE$ or 300 mg/kg of chloramphenicol one hr before sacrifice resulted in significant inhibition of 7-ethoxycoumarin deethylase activity as compared to the vehicle control ($p < 0.05$). The ethoxycoumarin deethylase activities remaining (as a percentage of the vehicle control) in the microsomes of rats treated with 10 mg/kg of $pNO_2DCAE$ and 300 mg/kg of chloramphenicol were not significantly different (50% and 47%, respectively), whereas the activity remaining in the microsomes of rats treated with 100 mg/kg of $pNO_2DCAE$ was significantly lower than both the 10-mg/kg $pNO_2DCAE$ and 300-mg/kg chloramphenicol groups (only 32% of the vehicle control) ($p < 0.05$). The cytochrome P-450 content of the microsomes did not differ between untreated rats and rats treated with chloramphenicol or $pNO_2DCAE$.

In order to determine which isozymes of cytochrome P-450 within these microsomes were inhibited, the metabolism of warfarin was monitored as described previously (10). The same order of isozyme inhibition was observed with $pNO_2DCAE$ as that found previously with chloramphenicol (10). Isozyme PB-C (an isozyme induced 2- to 3-fold by phenobarbital) was inhibited to the greatest extent by $pNO_2DCAE$, wherease PB/PCN-E, an isozyme induced by pregnenolone 16α-carbonitrile as well as phenobarbital, was not inhibited. Isozyme PB-B was intermediate in its susceptibility to $pNO_2DCAE$. For example, at the dose of 10 mg/kg of $pNO_2DCAE$, 60% inhibition of PB-C but only 36% inhibition of PB-B was observed.

The greater inhibitory potency of $pNO_2DCAE$ compared to chloramphenicol *in vivo* was also demonstrated by the sleeping time assay. The pentobarbital-induced sleeping time for phenobarbital-treated rats given 10 mg/kg of $pNO_2DCAE$ (117 ± 25 min) was significantly greater than that of rats given 10 or 100 mg/kg of chloramphenicol (49 ± 8 min and 63 ± 21 min, respectively) or the vehicle control (60 ± 24 min) ($p < 0.02$). The sleeping time of rats given 10 or 100 mg/kg of chloramphenicol was not significantly different from that of the vehicle control.

## Discussion

The present investigation has examined the importance of three structural features of the chloramphenicol molecule in regulating its effectiveness and selectivity as a mechanism-based inactivator of rat liver cytochromes P-450. Changes in the various functional groups of chloramphenicol could alter: 1) the affinity of the enzyme for the analogue; 2) the maximal rate at which the analogue is metabolized to reactive intermediates; 3) the extent to which the reactive intermediates covalently bind as opposed to being converted to stable products; and 4) the effect of covalent binding on the activity of the enzyme. In the present study, $pNO_2DCAE$, in which the propanediol side chain of chloramphenicol was replaced with an ethyl group, was shown to be nearly 20-fold more potent than chloramphenicol, both *in vivo* and *in vitro*. 1-Phenyl-2-dichloroacetamidoethane was also shown to inactivate the major phenobarbital- and β-naphthoflavone-inducible isozymes of cytochrome P-450 at a rate approximately equal to that obtained

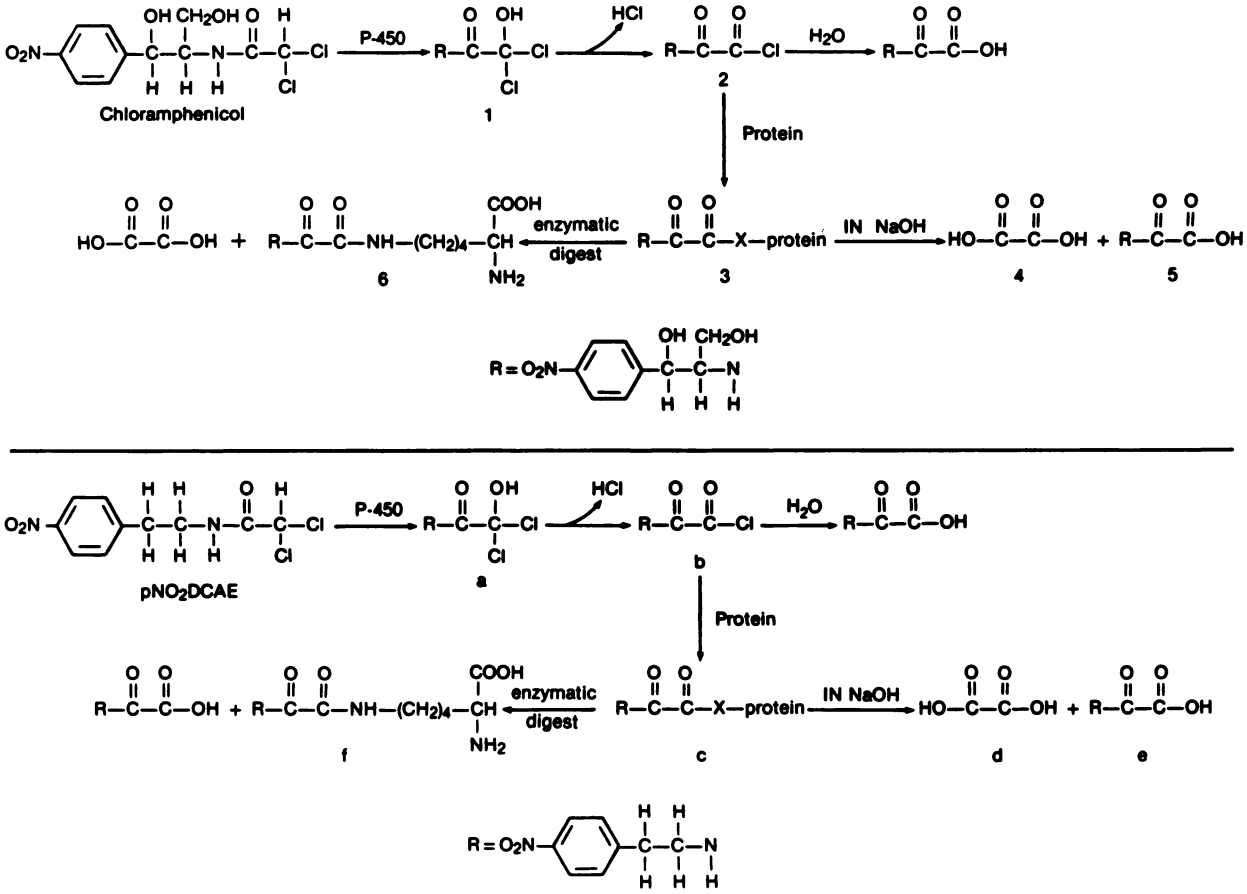


**Fig. 7.** Proposed scheme for the metabolic activation of chloramphenicol (*top*) and $pNO_2DCAE$ (*bottom*) by cytochrome P-450. The proposed scheme for chloramphenicol is based on the scheme proposed by Pohl and colleagues (3, 4) and previous work by Halpert (5).

with $pNO_2DCAE$. Thus, neither the $pNO_2$ group nor the intact propanediol side chain is required for the suicide inactivation of cytochrome P-450 PB-B or BNF-B. These functional groups and their possible metabolites are therefore not necessary for binding to occur and are apparently not contributing to the inactivation once the metabolite is bound. Previous studies have indicated that the loss of enzyme activity following the covalent binding of chloramphenicol metabolites to cytochrome P-450 PB-B results from the inhibition of electron transport from NADPH-cytochrome P-450 reductase (6). The present results would suggest that the presence of the $pNO_2$ group and the intact propanediol side chain at critical sites on the enzyme is not responsible for the loss of enzyme activity following the covalent binding of chloramphenicol metabolites to PB-B. However, the remainder of the chloramphenicol molecule could still be bulky enough to create steric disturbances when introduced into sensitive sites in the enzyme. Alternatively, the covalent binding of chloramphenicol metabolites (with or without the $pNO_2$ group or the propanediol side chain) to amino acid residues essential for electron transport from cytochrome P-450 reductase could be responsible for the inactivation. Alterations of the dihalomethyl moiety have shown that the nature of this group is of importance. Replacement of the dichloromethyl moiety of $pNO_2DCAE$ with a dibromomethyl group actually increased the rate of inactivation of PB-B, whereas replacement with a difluoromethyl moiety resulted in a compound which did not inactivate the enzyme at a concentration 20 times that of the other inhibitors tested. The monochloromethyl analog of chloramphenicol was also tested and found to be incapable of inactivating the enzyme.[1]

Alteration of the propanediol side chain also resulted in a change in the isozyme selectivity. Although chloramphenicol does not inactivate the major $\beta$-naphthoflavone-inducible isozyme, $pNO_2DCAE$ was shown to inactivate this isozyme at a rate approximately one-third that at which it inactivates the phenobarbital-inducible isozyme. Although this is an example of a decrease in isozyme selectivity, it does illustrate that the selectivity can be altered by changing the structure of the inhibitor. The results also suggest that it is not the dichloromethyl moiety of chloramphenicol which is responsible for the isozyme selectivity, but rather some element of the remainder of the molecule.

The inactivation of PB-B by $pNO_2DBAE$, 1-phenyl-2-dichloroacetamidoethane, and $pNO_2DCAE$ exhibited biphasic kinetics (Figs. 1 and 3). The rate constants for each analogue were determined from the initial, more rapid phase of inactivation. The second phase of inactivation became apparent only after approximately two-thirds of the initial enzyme activity was lost in each case. Extension of the incubation times would

[1] Deschlorochloramphenicol (100 $\mu$M) was incubated for various times with 0.05 nmol/ml of PB-B in the presence of a complete reconstituted system before the addition of ethoxycoumarin. The incubation was continued for 1 min. The residual ethoxycoumarin deethylase activity was determined as described for the other analogues of chloramphenicol in Materials and Methods.

probably demonstrate more clearly the biphasic nature of the inactivation, particularly for those analogue concentrations where two-thirds of the enzyme does not become inactivated during the time span of the experiment.

The reason for the biphasic nature of the kinetics is not clear. It does not appear to be the result of a depletion of NADPH or an inhibition of the cytochrome P-450 reductase by $NADP^+$ as the addition of an NADPH-regenerating system to incubations of PB-B and 5 $\mu$M $pNO_2DCAE$ did not alter the kinetics. The addition of 1 mM dithiothreitol as a trapping agent to incubations of PB-B and 5 $\mu$M $pNO_2DCAE$ also did not result in a conversion to monophasic kinetics. We do not feel that the biphasic kinetics are due to the presence of more than one isozyme in our PB-B preparation as we have removed the closely related PB-D from the PB-B in our purification procedure (6). In addition, biphasic kinetics are also seen for the inactivation of BNF-B by 5 $\mu$M $pNO_2DCAE$ (Fig. 4), and there is no evidence to suggest that more than one isozyme is present in this preparation. Biphasic kinetics have also been observed for the inactivation of cytochrome P-450 isozymes by other mechanism-based inactivators (16, 17). In the case of cyclopropylbenzylamine, the biphasic nature of the inactivation was tentatively attributed to the generation of a metabolite of cyclopropylbenzylamine which subsequently competed with the parent compound for metabolism, resulting in a second slower phase of enzyme inactivation (17). It is not yet known whether a similar explanation could account for the biphasic kinetics observed for the inactivation of PB-B and BNF-B by chloramphenicol analogues.

The metabolic pathway by which chloramphenicol inactivates PB-B has previously been determined (3–5). Since $pNO_2DCAE$ was not only more effective than chloramphenicol in the inactivation of PB-B but was also able to inactivate BNF-B, it was of interest to examine the pathway of metabolic activation of $pNO_2DCAE$ by PB-B and BNF-B. Alkaline hydrolysis of the adducts formed between phenobarbital- and $\beta$-naphthoflavone-induced microsomes and $pNO_2DCAE$ led to the release of oxalic acid (Fig. 7, compound *d*) and 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide (Fig. 7, compound *e*), whereas an enzymatic digest produced 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide and 4-nitro-1-phenethyl oxamyl lysine (Fig. 7, compound *f*). These results suggest that the metabolic pathway by which $pNO_2DCAE$ inactivates PB-B and BNF-B is similar to that reported for chloramphenicol, in which chloramphenicol is oxidatively dechlorinated by cytochrome P-450 to the reactive oxamyl chloride which covalently binds to the protein (3–5) (Fig. 7). The major difference between chloramphenicol and $pNO_2DCAE$ metabolism is the formation of oxalic acid upon the enzymatic digestion of the radiolabeled protein obtained from incubation of PB-B with [$^{14}$C]chloramphenicol (5). The formation of oxalic acid would require the cleavage of two linkages in the protein adducts of chloramphenicol oxamic acid. Since model studies with derivatives of chloramphenicol oxamic acid showed no evidence of the cleavage of more than one of the linkages susceptible to hydrolysis, it was hypothesized previously that the formation of oxalic acid was not consistent with an oxamyl chloride intermediate and that a different intermediate must also be produced (18). However, the present investigation has shown that, in addition to the stable lysine adduct, 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide is produced instead of oxalic acid upon enzymatic digestion of the protein from the incubation of PB-B and BNF-B with $pNO_2DCAE$. The oxalic acid is only produced under the harsher conditions of alkaline hydrolysis. This would suggest that there is only one reactive intermediate of $pNO_2DCAE$ and that both the oxalic acid and the 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide formed upon degradation of a labile adduct, as well as the 4-nitro-1-phenethyl-oxamyl lysine adduct, could be derived from the same oxamyl chloride intermediate.

The pathways by which $pNO_2DCAE$ inactivates PB-B and BNF-B appear to be similar. The same products are produced in approximately the same proportions upon the alkaline hydrolysis and enzymatic digestion of both isozymes. The stoichiometry of inactivation is lower for the inactivation of BNF-B than it is for PB-B, suggesting that binding might be more specific. $pNO_2DCAE$ was found to form lysine adducts with both isozymes. However, other amino acid adducts may also be formed to different extents with each of the two isozymes and with different effects on the activity of the isozymes. Further research into this area is necessary to determine whether the mechanisms of inactivation are actually the same for these two isozymes. Future studies with other structural analogues of chloramphenicol should also aid in the design of mechanism-based inactivators of cytochrome P-450 with enhanced rather than diminished isozyme selectivity.

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