ANALOGS OF CHLORAMPHENICOL AS MECHANISM-BASED INACTIVATORS
OF RAT LIVER CYTOCHROMES P-450

BY

Natalie Elizabeth Miller

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1987
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Analogs of chloramphenicol as mechanism-based inactivators of rat liver cytochromes P-450

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Natalie Elizabeth Miller entitled Analogs of Chloramphenicol as Mechanism-based Inactivators of Rat Liver Cytochromes P-450 and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: Natalie E. Mullis
DEDICATION

I would like to dedicate this work to my parents and to Kevin Ryan for their love and support.
ACKNOWLEDGMENTS

I wish to express my sincere gratitude
to my advisor and mentor Dr. James Halpert, who has provided
the support, guidance and encouragement which has made this
work possible,
to the members of my advisory committee, Dr. Dean Carter,
Dr. Jay Gandolfi, Dr. David Nelson and Dr. Glenn Sipes, who
have provided much helpful advice and guidance, and
to my colleagues in the lab, for their help and friendship.
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ABSTRACT

The cytochrome P-450 dependent monooxygenase system plays a key role in the bioactivation and detoxication of xenobiotics. Isozyme-specific inhibitors of cytochrome P-450 may be useful in elucidating the role of particular isozymes in xenobiotic metabolism or in suppressing the bioactivation of xenobiotics and enhancing detoxication. The antibiotic chloramphenicol is a selective mechanism-based inactivator of rat liver cytochromes P-450, inactivating 6 of the 12 isozymes monitored, including the major phenobarbital-inducible isozyme PB-B. Analogs of chloramphenicol have been tested to determine the importance of various functional groups in regulating the effectiveness and isozyme selectivity of chloramphenicol as a mechanism-based inactivator of cytochromes P-450. This information will aid in the design of more effective and isozyme specific mechanism-based inactivators. The dihalomethyl group and the propanediol moiety were found to be important in determining the efficacy of inactivation and the ability to inactivate the enzyme by virtue of the modification of the protein as opposed to the modification of the heme moiety. The propanediol side chain also plays a role in the isozyme selectivity. Unlike chloramphenicol,
N(2-p-nitrophenethyl) dichloroacetamide (pNO₂ DCA), which contains an ethyl group in place of the propanediol side chain of chloramphenicol, is an effective inactivator of BNF-B, the major beta-naphthoflavone-inducible isozyme, as well as PB-B, in vitro and in vivo. Alkaline hydrolysis and enzymatic digestion of the covalently modified isozymes has shown that chloramphenicol and pNO₂ DCA are both metabolized by cytochromes P-450 to oxamyl chlorides which bind to lysine and other amino acid residues of the enzyme. However, the mechanism by which pNO₂ DCA inactivates BNF-B differs significantly from that by which chloramphenicol inactivates PB-B, although both involve an impairment of the transfer of electrons from NADPH-cytochrome P-450 reductase, suggesting that there are differences in the active sites of these two isozymes.
CHAPTER 1

INTRODUCTION

Cytochrome P-450

The cytochrome P-450-dependent monooxygenase system is an extremely versatile enzyme system which catalyzes the metabolism of a wide range of xenobiotics and endogenous compounds. Among the thousands of compounds metabolized by cytochrome P-450 are such diverse compounds as hexane, benzo(a)pyrene, 7-ethoxycoumarin, amphetamines, chlorpromazine and aldrin (Masters, 1980). The cytochrome-P-450 mediated metabolism of xenobiotics may result in their detoxification, activation or conversion to pharmacologically active forms.

Detoxification of xenobiotics by cytochrome P-450 is largely the result of the metabolism of relatively lipophilic compounds to more hydrophilic products which may then be excreted. Cytochrome P-450 enzymes also catalyze the addition or exposure of functional groups such as hydroxyl and carboxyl groups. These compounds can then undergo conjugation and subsequent excretion (Conney, 1986; Sipes and Gandolfi, 1986).

Cytochrome P-450 also plays a key role in the bioactivation of xenobiotics. Many drugs, industrial
chemicals, pesticides and other compounds are relatively inert until they are metabolized by this enzyme system in vivo to products which cause cellular dysfunction, neoplasia or mutagenesis (Guengerich and Liebler, 1985; Estabrook, Werringloer, and Peterson, 1979). Benzo(a)pyrene, for example, is metabolized by cytochrome P-450 to an epoxide which is mutagenic. Other compounds which undergo bioactivation include chloroform, carbon tetrachloride, procainamide, hydralazine and parathion (Nelson, 1982; Halpert, Hammond and Neal, 1980). Many drugs are also metabolized by cytochrome P-450 to the therapeutically active forms. The analgesic effect of codeine, for example, is primarily due to the metabolite morphine rather than to codeine itself (Welch, 1979).

Cytochrome P-450 is also involved in the metabolism of numerous endogenous compounds. It is an important enzyme system in the biosynthesis and catabolism of cholesterol and the biosynthesis of steroid hormones, prostaglandins and bile salts (Parke and Ioannides, 1982). It is also important in the production of active forms of vitamin D (Waterman, John and Simpson, 1986).

Cytochrome P-450 is ubiquitous in nature, being found in plants and microorganisms as well as in animals (White and Coon, 1980). Although cytochrome P-450 is present to some degree in most tissues in mammals, except skeletal muscle and erythrocytes, it is most abundant in the
liver (Guengerich and Liebler, 1985). Cytochrome P-450 is localized primarily in the smooth endoplasmic reticulum of the liver cells. It is also found, to a lesser extent, in the mitochondria of the kidney, liver, corpus luteum and adrenal cortex. In mammals, the cytochromes P-450 found in the adrenal cortex mitochondria are important in the synthesis of steroid hormones, while the cytochromes P-450 found in the endoplasmic reticulum of the liver are involved primarily in the oxidation of xenobiotics (Weiner, 1986).

The cytochrome P-450-dependent monooxygenase system consists of three major components: two enzymes (cytochrome P-450 and NADPH-cytochrome P-450 reductase) and phospholipid. Cytochrome P-450 is the terminal oxidase of the monooxygenase system. NADPH-cytochrome P-450 reductase is important in the transfer of electrons from NADPH to cytochrome P-450. The cytochrome P-450 and NADPH-cytochrome P-450 reductase are embedded in the phospholipid membrane of the endoplasmic reticulum. The phospholipid apparently facilitates the interaction of NADPH-cytochrome P-450 reductase and cytochrome P-450 (Lu and West, 1982; Welch, 1979).

The cytochrome P-450-dependent monooxygenase system catalyzes many types of reactions. Monooxygenases in general catalyze the insertion of one atom of molecular oxygen into a substrate while the other atom is reduced to
water (Lehninger, 1975). The general monooxygenase reaction can be written as:

\[ \text{RH} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{ROH} + \text{H}_2\text{O} \quad \text{Equation 1.} \]

(Weiner, 1986)

The oxidative reactions catalyzed by cytochrome P-450 can be classified as 1) carbon hydroxylation 2) heteroatom oxygenation 3) heteroatom dealkylation 4) epoxidation 5) oxidative denalogenation (Guengerich, 1984; Sipes and Gandolfi, 1986). Examples of these reactions are given in Table 1. A scheme for the catalytic cycle of cytochrome P-450 is shown in Figure 1. The generally accepted sequence of events includes:

1. substrate binding to the ferric enzyme

2. transfer of electrons from reduced pyridine nucleotide to the cytochrome catalyzed by NADPH-cytochrome P-450 reductase

3. reduction of cytochrome P-450 to the ferrous form

4. binding of molecular oxygen to give a ferrous cytochrome P-450-dioxygen complex

5. transfer to the ferrous cytochrome P-450 complex of a second electron from reduced NADPH-cytochrome P-450 reductase or of an electron from cytochrome b₅
Table 1. Oxidative Reactions Catalyzed by Cytochromes P-450.

(Adapted from Sipes and Gandolfi, 1986)

<table>
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<th>Reaction</th>
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<td>1. Carbon hydroxylation</td>
<td>R-CH₂CH₂-CH₂→R-CH₂-CHOH-CH₃</td>
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<tr>
<td>2. Heteroatom oxygenation</td>
<td>R-S-R'→R-SO-R'</td>
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<td>3. Heteroatom dealkylation</td>
<td>R-(NH₃,O,S)-CH₃→R-(NH₂,OH,SH) + CH₂O</td>
</tr>
<tr>
<td>4. Epoxidation</td>
<td>R-CH-CH-R'→R-CH-CH-R'</td>
</tr>
<tr>
<td>5. Oxidative dehalogenation</td>
<td>R-C-H→R-C-OH→R-C-H + HX</td>
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Figure 1. Catalytic cycle of cytochrome P-450
(From Ingelman-Sundberg, Haaparanta and Rydström, 1981)
6. cleavage of the oxygen-oxygen bond with incorporation of one oxygen atom into a molecule of water

7. insertion of the second oxygen atom into the substrate and dissociation of the oxygenated product (Ortiz de Montellano, 1986; Guengerich and Liebler, 1985)

Despite the name, the cytochrome P-450 monooxygenase system can also catalyze reductive reactions involving direct electron transfer. These include the reduction of nitro, azo, N-oxide and epoxide groups (Coon and Persson, 1980).

The term cytochrome P-450 actually refers to a family of heme-containing proteins. At least thirteen different cytochrome P-450 isozymes have been identified in rat liver, thirteen from rabbit liver and nine from human liver (Guengerich and Liebler, 1986). Many of these isozymes are induced by agents such as phenobarbital, beta-naphthoflavone and pregnenolone-16-alpha-carbonitrile (Ryan et al., 1979; Guengerich et al., 1982). The isozymes differ in many respects, including spectral and immunological properties, molecular weight (ranging from 45,000 to 60,000), terminal amino acid sequence and proteolytic peptide patterns (Levin et al., 1977; Ortiz de Montellano et al., 1981). The isozymes also have different but overlapping substrate specificities. Different isozymes may also metabolize the same substrate at different positions.
(Guengerich and Liebler, 1986). The substrate specificity of each isozyme is of particular importance, as one isozyme might metabolize a substrate to a toxic metabolite while another isozyme might be involved in the detoxification of the same compound. The major beta-naphthoflavone-inducible isozyme of rat liver cytochrome P-450 (BNF-B), for example, is believed to play a key role in the activation of many toxic chemicals and carcinogens such as benzo(a)pyrene. The major phenobarbital-inducible isozyme, PB-B, on the other hand is relatively inactive at metabolizing benzo(a)pyrene to the mutagenic form (Lu and West, 1980). Thus, the balance between the activation and detoxification of xenobiotics is largely determined by the relative amounts and activities of different cytochrome P-450 isozymes.

The numerous cytochrome P-450 isozymes also have many characteristics in common. The active site of each isozyme contains a protoporphyrin IX prosthetic group within its active site (Weiner, 1986). The ferrous form of the cytochrome binds carbon monoxide forming a complex which has a characteristic absorbance maximum around 450 nm, thus the name "cytochrome P-450" (Sato and Omura, 1978). The heme moiety is located in a large, relatively open hydrophobic depression in the surface of the apoprotein. The iron is always penta- or hexa-coordinate. Four of the ligands are contributed by the planar porphyrin ring. The fifth ligand is believed to be a thiolate anion contributed by a cysteine
residue of the apoprotein. The sixth ligand, in the substrate-free form, is believed to be an easily displaceable ligand such as the hydroxyl group of water or tyrosine (White and Coon, 1980). Little is known about the importance of other amino acid residues in the apoprotein (Guengerich et al., 1982a).

Cytochrome P-450 Inhibitors

While a great deal has been learned about the cytochrome P-450 dependent monooxygenase system in the last thirty years, a number of questions still remain. The elucidation of the structure, catalytic mechanism and function of an enzyme system as complicated as the cytochrome P-450 monooxygenase system is very difficult. However, some of the most valuable tools in the study of cytochromes P-450 have been, and continue to be, enzyme inhibitors.

An enzyme inhibitor is basically defined as any compound which reduces the observed activity of an enzyme (Whitaker, 1972). Inhibitors of cytochrome P-450 can act by many different mechanisms and at many different steps in the catalytic cycle. These inhibitors can be classified into the following general categories:

1. **Inhibitors which bind reversibly with the enzyme.**

   Inhibitors in this category primarily act on
cytochrome P-450 before the oxygen transfer step. Included in this category is the inhibition observed when different substrates compete for the substrate binding site. An example of this is the mutual inhibition of toluene and benzene metabolism observed in vivo and in vitro (Ortiz de Montellano and Reich, 1986). This category also includes the inhibitor carbon monoxide, which inhibits cytochrome P-450 by binding to the ferrous prosthetic heme group thus preventing the binding of oxygen (Testa and Jenner, 1981). Also included in this category is metyrapone, one of the most commonly used cytochrome P-450 inhibitors. Metyrapone apparently inhibits cytochrome P-450 by binding simultaneously to the apoprotein and the heme iron (Ortiz de Montellano and Reich, 1986).

2. **Inhibitors which bind quasi-irreversibly with the heme iron atom.**

These inhibitors bind reversibly but so tightly that they can only be displaced under extreme experimental conditions. The methylenedioxybenzyl compound isosafrole is an example of this class of inhibitor. Isosafrole is metabolized by cytochrome P-450 to a reactive metabolite which forms a stable complex with the ferrous heme iron (Ortiz de Montellano and Reich, 1986).
3. **Inhibitors which bind irreversibly to the apoprotein or heme moiety.**

2-isopropyl-4-pentenamide (AIA), for example, is metabolized by cytochrome P-450 to a reactive species which inactivates the enzyme by covalently binding to the heme prosthetic group (Ortiz de Montellano and Mico, 1981). Irreversible inactivators may also inactivate the enzyme by binding to the apoprotein. The alkylating agent 2-bromo-4'-nitroacetophenone binds covalently to amino acid residues within the apoprotein and inactivates the enzyme (Parkinson et al., 1986). Some inhibitors such as the insecticide parathion modify both the heme and the protein moieties of cytochrome P-450 (Halpert and Neal, 1981).

4. **Inhibitors which accelerate generalized degradation of the heme moiety without apparently binding to the enzyme.**

This category includes the peroxides and those compounds which lead indirectly to the production of lipid peroxides. The peroxides degrade the heme to pyrrole and dipyrrole fragments (Ortiz de Montellano and Reich, 1986).

**Uses of Cytochrome P-450 Inhibitors**

The importance of cytochrome P-450 inhibitors is perhaps most readily illustrated by their contribution to the current understanding of cytochrome P-450. Cytochrome P-450 inhibitors have played key roles in:
1. the discovery and determination of the basic structure of cytochrome P-450

2. the determination of the physiological function of cytochrome P-450

3. the determination of the nature of the active site and the mechanism of catalytic action of cytochrome P-450

4. the discovery of the presence of multiple isozymes of cytochrome P-450

5. the determination of the role of particular isozymes in the metabolism of different substrates

1. Discovery and determination of the basic structure of cytochrome P-450

The discovery of cytochrome P-450 was the result of the use of the inhibitor carbon monoxide. As mentioned earlier, carbon monoxide inhibits cytochrome P-450 by binding to the ferrous heme moiety of the enzyme. In 1955, Williams observed an unusual carbon monoxide binding spectrum in reduced rat liver microsomes. This phenomenon was subsequently reported by Klingenberg (1958) and Garfinkel (1958). This unusual carbon monoxide binding spectrum was the first indication of the presence of the novel heme protein, cytochrome P-450 (Omura and Sato, 1978). The very name cytochrome P-450 was derived from the fact
that it is a pigment with a carbon monoxide-binding spectrum with an absorbance peak at 450 nm (Omura and Sato, 1964). The binding of carbon monoxide also provided information about the nature of cytochrome P-450. The ability of carbon monoxide to bind to this unknown pigment suggested that a heavy metal ion was present in the chromophore of the pigment. Although the total heme content of the microsomes suggested that the unknown pigment might contain heme, the carbon monoxide difference spectrum was not entirely consistent with a heme-containing protein. The hemoprotein nature of the pigment was first confirmed with the use of another inhibitor, ethylisocyanide (Sato and Omura, 1978). Ethylisocyanide was found to bind to reduced microsomal cytochrome P-450 producing an absorbance spectrum which was characteristic of a heme protein (Omura and Sato, 1964).

2. Determination of the physiological function of cytochrome P-450

Cytochrome P-450 inhibitors have also played key roles in the elucidation of the physiological function of cytochrome P-450. Although Conney et al. (1957) had noted in the late 1950's that carbon monoxide inhibited the hydroxylation of many substrates, the enzyme involved in the hydroxylation was not identified. Estabrook et al. (1963) were the first to discover that cytochrome P-450 was the terminal oxidase in many monoxygenase reactions. They
demonstrated that the inhibition of the steroid C_{21}-
hydroxylase activity of rat adrenal cortex microsomes by
carbon monoxide could be reversed by light. The light
reversal of this inhibition was most effective around 450
nm. This photochemical action spectrum corresponded with
the spectrum of the carbon monoxide complex of cytochrome P-
450, implicating cytochrome P-450 in the hydroxylation of
this steroid (Estabrook, Cooper and Rosenthal, 1963; Cooper
et al., 1979). The same technique was subsequently used to
show the involvement of rat liver microsomal cytochrome P-
450 in the hydroxylation of many substrates, such as
codeine, acetanilide and monomethylaminopyrine (Cooper et
al., 1979; Omura and Sato, 1978).

3. Determination of the nature of the active site and the
mechanism of catalytic action of cytochrome P-450

It has been very difficult to elucidate the nature
of the active site of cytochrome P-450. X-ray
crystallography is currently the only direct method for
visualizing the active site of an enzyme. Unfortunately,
however, this method cannot yet be used to visualize the
active site of mammalian cytochromes P-450 because no
crystals have yet been produced (Miwa and Lu, 1986). Indirect methods have therefore been resorted to in order to obtain information about the function of different amino
acid residues and about the size and conformation of the
active site. Cytochrome P-450 inhibitors of various types have been used to gain much of the information currently available about the active site. The inhibitor 22-amino-23,24-bisnor-5-chole-3-beta-ol, for example, has been shown to form a stable complex with the ferric form of P-450scc, an isozyme involved in the metabolism of cholesterol. The side chain amine was found to bind to the heme iron while the steroid ring bound to the cholesterol-ring binding site of the apoprotein. Analogs of this inhibitor in which the amine was located further from the ring were unable to bind at both sites, suggesting that the heme and substrate binding sites were fixed. This allowed an estimation of the distance between the substrate binding site and the heme catalytic site of this isozyme (Sheets and Vickery, 1983).

Inhibitors which bind to specific amino acid residues within the active site have been used to begin to determine the role of these amino acid residues in regulating such functions as substrate binding and interaction with NADPH-cytochrome P-450 reductase. The results of the modification of lysine residues in the rabbit isozyme LM2 with fluorescein isothiocyanate, for example, suggested that lysine residues in this isozyme function in the interaction of cytochrome P-450 and reductase (Bernhardt et al., 1984).
Inhibitors of cytochrome P-450 have also been used to study the catalytic mechanism of cytochrome P-450. The mechanism by which cytochrome P-450 oxidizes substrates has been difficult to study due in large part to the difficulty in isolating and identifying activated intermediates involved in the catalytic mechanism. Mechanism-based inactivators have been invaluable as a means of "trapping" and identifying these intermediates. The mechanism-based inactivators are catalytically converted to reactive intermediates which then bind to the heme or protein moiety of the enzyme (Walsh, 1984). This heme- or protein-bound metabolite can frequently then be identified, thus providing information about the mechanism by which the inhibitor was metabolized. For example, one of the key questions which has yet to be fully resolved is whether the activated oxygen is transferred from the enzyme to the substrate in a stepwise or concerted fashion. The majority of the evidence suggests that oxygen transfer occurs in a stepwise fashion by single electron steps. Some of the important evidence for this mechanism was provided by studies done with various P-450 inhibitors. For example, substituted olefins are metabolized by cytochrome P-450 to reactive intermediates which alkylate the heme moiety. The regiospecificity of the binding suggested the formation of radical intermediates, which provided evidence for a stepwise, single electron mechanism. (Ortiz de Montellano et al., 1982).
4. Discovery of the presence of multiple isozymes of cytochrome P-450

When the physiological role of cytochrome P-450 in monooxygenase reactions was first being examined, it was generally believed that only one molecular form of cytochrome P-450 existed. However, evidence soon began to accumulate suggesting that more than one enzyme was responsible for the broad range of substrates metabolized by cytochrome P-450. Enzyme inhibitors provided some of the early evidence for the existence of multiple isozymes of cytochrome P-450. If only one form of cytochrome P-450 existed, inhibitors would be expected to inhibit equally all reactions catalyzed by this enzyme. However, not all reactions were inhibited to the same extent, as was demonstrated by studies on the metabolism of the steroid hormone testosterone. Testosterone is hydroxylated at several different positions by rat liver microsomes, including the 6-beta, 7-alpha and 16-alpha positions. The insecticide chlorthion was found to strongly inhibit the hydroxylation of testosterone by these microsomes at the 16-alpha position, but this inhibitor did not greatly inhibit the hydroxylation of testosterone at the 6-beta or 7-alpha positions. Carbon monoxide also differentially inhibited the hydroxylation of testosterone at these positions (Lu and West, 1980). This differential inhibition provides indirect
evidence for the existence of multiple isozymes with different substrate specificities.

5. Determination of the role of particular isozymes in the metabolism of different substrates

The ability of cytochrome P-450 inhibitors to differentially inhibit different isozymes has also been useful in the attempt to determine the presence of different isozymes in different tissues. Simply monitoring the metabolism of a given substrate is not sufficient, as many isozymes may contribute to the observed activity. However, the ability of an isozyme-specific inhibitor to decrease the observed catalytic activity would demonstrate the presence of that isozyme. Metyrapone, for example, which is an effective inhibitor of the isozyme PB-B but not of the isozyme BNF-B, and alpha-naphthoflavone, which inhibits BNF-B, were used to determine the effect of the antioxidant ethoxyquin on certain P-450 isozymes in rat liver microsomes. Ethoxyquin was found to induce the isozyme PB-B but not BNF-B as demonstrated by the ability of metyrapone but not alpha-naphthoflavone to inhibit the ethoxycoumarin metabolism of the microsomes (Netter, 1980).

Cytochrome P-450 inhibitors have also been used to determine the contribution of different isozymes to the metabolism of particular substrates in different tissues and in different individuals. This process is crucial to the
understanding of the exact role of different isozymes in the activation and detoxification of particular xenobiotics. Once this is known, it may be possible to direct the metabolism towards detoxification by inhibiting only those isozymes involved in bioactivation, leaving any isozymes which play a role in the detoxification intact (Gelboin and Friedman, 1985).

The use of cytochrome P-450 inhibitors to determine the presence of different isozymes and the role of these isozymes in the metabolism of particular substrates requires that the inhibitors inactivate only a single cytochrome P-450 isozyme (isozyme-specific). Inhibitors which inhibit several isozymes can obviously not be used to distinguish between these isozymes. Most of the cytochrome P-450 inhibitors currently available are not isozyme-specific. Carbon monoxide for example, inhibits nearly all cytochrome P-450 isozymes. Many inhibitors, such as metyrapone are selective but not isozyme-specific. Metyrapone inhibits several phenobarbital-inducible isozymes even though it does not inhibit the major beta-napthoflavone-inducible isozyme (Testa and Jenner, 1981). It is necessary, therefore, to discover or design more specific inhibitors of cytochromes P-450.
Mechanism-based Inactivators of Cytochromes P-450

Among the most specific inactivators of cytochrome P-450 are the mechanism-based inactivators. As mentioned previously, mechanism-based enzyme inactivators are compounds which first bind to the active site of the enzyme and are then converted to a reactive metabolite. This reactive species can then covalently bind to the enzyme or a prosthetic group and thus inactivate the enzyme (Abeles, 1978; Walsh, 1984). The specificity of mechanism-based inactivators resides in the fact that the inactivator must not only bind to the enzyme but must also be metabolized at the active site of the enzyme to generate the reactive intermediate. This is illustrated in the following scheme for mechanism-based inactivation:

\[
E + I \xrightarrow{K_D} E\cdot I \xrightarrow{k_{cat}} E\cdot I^* \rightarrow E-X \quad \text{Equation 2}
\]

(Sjoerdsmma, 1981)

where \(E\) = enzyme, \(I\) = inhibitor, \(I^*\) = transformed inhibitor and \(E-X\) = irreversibly inactivated enzyme.

Three features contribute to the selectivity of mechanism-based inactivators. First, the inactivator must be able to bind to the enzyme at the substrate binding site. Thus a certain degree of selectivity is due to the binding specificity, the \(K_D\) term in Equation 2. Second, the enzyme must mistake the inactivator for a substrate.
The inactivator must be metabolized in the active site of the enzyme to generate the reactive species (the $k_{\text{cat}}$ term). Mechanism-based inactivators are also referred to as $k_{\text{cat}}$ inhibitors since they exploit the catalytic specificity as well as the binding specificity of the enzyme. The third factor which contributes to the selectivity of mechanism-based inactivators is the fact that the reactive species is generated within the active site of the enzyme. The reactive intermediate is thus more likely to covalently bind to amino acid residues within the active site of the same enzyme rather than binding to other enzymes (Walsh, 1980).

Prior to the discovery of mechanism-based enzyme inactivators, the active-site directed inactivation of enzymes was carried out with affinity-labeling agents. These are compounds which resemble the substrate of an enzyme but which contain an additional chemically reactive functional moiety. Thus, these compounds can specifically bind to the active site like a true substrate, while the reactive group can then bind at or near the active site (Lehninger, 1975). Affinity-labeling agents differ from mechanism-based inactivators in that catalysis is not required for inactivation to occur. The selectivity of affinity-labeling agents is dependent on the binding specificity alone. While these agents can be useful in vitro, they are not generally selective enough to be used in vivo. Affinity-labeling agents already have their
functional group exposed and are reactive free in solution. Thus, they can react nonspecifically with random biomolecules in addition to the target enzyme (Rando, 1974). Mechanism-based inactivators, on the other hand, are not reactive until they are metabolized by the target enzyme.

The process of mechanism-based inactivation was first recognized by Helmkamp et al. in 1969. They determined that the acetylenic compound 3-decynyl-5-pantetheine was a mechanism-based inactivator of beta-hydroxydecanoyl thiolester dehydrase, a key enzyme in the bacterial biosynthesis of unsaturated fatty acids (Walsh, 1984; Helmkamp et al., 1969). Since this time numerous mechanism-based inactivators have been discovered or designed. Many of these are therapeutically active agents. For example, the antibiotic clavulanate (which inactivates plasmid RTEM beta-lactamase), phenylhydrazine (the monoamine oxidase inhibitor) and allopurinol (a xanthine oxidase inhibitor) are all mechanism-based inactivators (Walsh, 1980).

There are several criteria which can be used to characterize mechanism-based inactivators. Mechanism-based inactivators should demonstrate:

1. loss of enzyme activity which is time-dependent and pseudo-first order. The time dependence suggests, although it does not prove, that covalent modification has occurred.
The first-order inactivation provides evidence that inactivation occurs before the inactivator is released from the enzyme.

2. substrate protection against inactivation. This provides evidence for the involvement of the active site of the enzyme in the inhibitory process. There is competition between substrate and the inactivator at the active site.

3. irreversible inactivation. This is demonstrated by showing that the enzyme is not reactivated by extensive dialysis or gel filtration (Abeles and Maycock, 1976; Sjoerdsma, 1981; Rando, 1984; Walsh, 1977).

There are at least three general mechanisms by which mechanism-based inactivators of cytochrome P-450 can inactivate the enzyme. First, following cytochrome P-450 catalyzed metabolism of the inhibitor into a reactive intermediate, inactivation can occur by the subsequent covalent binding of the intermediate to the protein moiety of the enzyme (Halpert and Neal, 1980). A second mechanism involves the covalent binding of the intermediate to the prosthetic heme group, frequently producing abnormal porphyrins, or "green pigments" (Ortiz de Montellano and Correia, 1980). Recently, a third mechanism was discovered in which the reactive metabolite mediates the activation of
the heme group into a product that binds irreversibly to the protein moiety of the enzyme (Davies, Britt and Ponl, 1986).

Many mechanism-based inactivators have been described which inactivate P-450 by covalently binding to the heme. Among the most frequently studied of these compounds are the olefins and acetylenes, including 2-isopropyl-4-pentenamide (also called allylisopropylacetamide, AIA) which is metabolized by cytochrome P-450 to a reactive intermediate which alkylates the heme. The destructive potential of these compounds lies in the unsaturated carbon-carbon bond, as evidenced by the ability of ethylene and acetylene themselves to alkylate the prosthetic heme (Ortiz de Montellano and Correia, 1983).

Compounds which covalently modify the heme group can produce hepatic porphyrias by disrupting normal heme metabolism, thus limiting their use in vivo. For example, 2-isopropyl-4-pentenamide (AIA), alkylates the heme prosthetic group of cytochrome P-450 and forms green pigments. These green pigments then migrate from the intact apoprotein, allowing newly synthesized heme to be accepted by the free apoprotein and to again be converted into green pigments. Thus, newly synthesized heme would be destroyed and diverted from its normal inhibitory role on 5-aminolevulinate synthetase, the rate limiting step in heme synthesis, allowing the buildup of heme products. In addition, some green pigments, such as those produced by
3,5-diethoxycarbonyl-1,4-dihydrocollidine, can directly inhibit the enzyme ferrochelatase, which catalyzes the incorporation of iron into protoporphyrin (De Matteis, 1978; Ortiz de Montellano, Beilan and Kunze, 1981), thus causing a further derangement in normal heme metabolism.

The isozyme selectivity of inactivators which modify the heme has not been well investigated. However, the mechanism-based inactivators which bind to the protein are potentially more isozyme selective since it is the apoprotein which distinguishes the different isozymes, while the heme is common to all of them. Mechanism-based inactivators which bind to the protein are also potentially useful in identifying functional amino acid residues within the active site. The mechanism-based inactivator 6-beta-bromopenicillanic acid, for example, was used to identify in the active site of Bacillus cereus beta-lactamase a serine residue which is involved in the turnover of the enzyme (Loosemore, Cohen and Pratt, 1980; Cohen and Pratt, 1980).

Cytochrome P-450 inactivators which bind to the heme are obviously of no use for this purpose. The antibiotic chloramphenicol (Fig. 2) is one of the few mechanism-based inactivators which inactivates the cytochrome P-450 by virtue of the covalent modification of the protein (Ponl and Krishna, 1978).
Figure 2. Structure of chloramphenicol
**Chloramphenicol as a Mechanism-based Inactivator**

The metabolic pathway by which chloramphenicol inactivates the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 is shown in Figure 3. Chloramphenicol is oxidatively metabolized by this isozyme to the putative halohydrin (Fig. 3, compound 1). HCl is spontaneously eliminated to form the reactive oxamyl chloride (compound 2). This oxamyl chloride can then hydrolyze to form chloramphenicol oxamic acid or can covalently bind to the protein moiety of the cytochrome (Pohl and Krishna, 1978; Pohl, Nelson and Krishna, 1978). The major covalently bound species has been identified as an adduct of chloramphenicol oxamic acid and the epsilon-amino group of one or more lysine residues in the cytochrome. Modification of the lysine appears to be primarily responsible for the enzyme inactivation (Halpert, 1981; Halpert, 1982; Halpert, Naslund and Betner, 1983). The modification of lysine residues does not inhibit substrate binding or hydroxylation, but rather impairs electron transport from NADPH-cytochrome P-450 reductase to the heme of cytochrome P-450 (Halpert, Miller and Gorsky, 1985).

While chloramphenicol is an isozyme selective inactivator, it is not a totally specific inactivator of rat liver cytochromes P-450. When administered at a dose of 300 mg/kg i.p. to control rats or rats treated with various inducers, chloramphenicol inactivated six of the twelve
Figure 3. Metabolic pathway by which chloramphenicol inactivates cytochrome P-450 PB-B. Based on the scheme proposed by Pohl, Nelson and Krishna (1981) and Halpert (1981).
major isozymes monitored. The isozymes inhibited by chloramphenicol are designated UT-A, PB-B, PB-D and PB-C according to the nomenclature of Guengerich et al. (1982a). The pregnenolone-16-alpha-carbonitrile-inducible isozyme responsible for the 6-beta-hydroxylation of androstenedione and a constitutive warfarin R-8 hydroxylase were also inhibited (Halpert et al., 1986).

Chloramphenicol is one of the few mechanism-based inactivators of cytochromes P-450 that has been shown to be effective in vivo as well as in vitro. Chloramphenicol prolongs the duration of action of many drugs which are normally metabolized by cytochrome P-450 to inactive and excretable forms. For example, the duration of action of pentobarbital is prolonged in mice treated with the antibiotic (Adams, Isaacson and Masters, 1977). Chloramphenicol can also inhibit the toxic effects of compounds which require bioactivation by cytochrome P-450. The hepatotoxicity of carbon tetrachloride in rats, for example, is decreased by the administration of chloramphenicol soon after the administration of carbon tetrachloride (Dolci and Brabec, 1978). Chloramphenicol also alters drug metabolism in man. Diphenylhydantoin levels, for instance, have been shown to increase four- to five-fold and tolbutamide levels nearly two-fold in the plasma of patients who received two grams of chloramphenicol
daily (Remmer, 1974). As a result, the levels of drugs administered with chloramphenicol must be monitored carefully to prevent overdosage. The ability of chloramphenicol to inhibit drug metabolism is largely the result of the chloramphenicol-mediated inactivation of cytochrome P-450. While this inhibition of drug metabolism was first observed as a side effect of the use of chloramphenicol as an antibiotic, it is not related to the antibacterial action of chloramphenicol.

**Chloramphenicol Background**

The antibacterial activity of chloramphenicol is due to the binding of chloramphenicol to the 50S subunit of bacterial ribosomes thus preventing the attachment of a complete aminoacyl transfer RNA to the ribosome and inhibiting the formation of a peptide bond (Powell, 1982). While chloramphenicol is a broad spectrum antibiotic, effective in diseases such as bacterial meningitis, its use in man has been severely limited by the side effects.

As already mentioned, one of the side effects of chloramphenicol is the inhibition of drug metabolism. However, chloramphenicol produces more severe side effects which are not related to the inactivation of cytochrome P-450. One of the more common side effects is the dose-related bone marrow suppression which is associated with the antibiotic activity. The suppression is believed to be due
to the binding of chloramphenicol to 70S ribosomes, resulting in a decrease in mitochondrial protein synthesis and ultimately in cessation of cellular proliferation. The bone marrow cells return to normal after termination of chloramphenicol exposure (Yunis, 1980; Yunis, 1973). The most serious side effect of chloramphenicol, however, is an irreversible bone marrow aplasia. Aplastic anemia is a rare, but frequently fatal disease which occurs in approximately 1 in 40,000 patients treated with the antibiotic. The manifestation of aplastic anemia is not related to the dose of chloramphenicol, the duration of administration or the length of time since the last administration (Krishna, Aykac and Siegall, 1981). Although the mechanism by which chloramphenicol produces aplastic anemia is unknown, it is believed to involve reductive products such as the hydroxylamino or nitroso metabolites (Fig. 4) (Yunis et al., 1980). Thus aplastic anemia is associated with metabolism of the p-nitro group of chloramphenicol. In contrast, however, it is the dichloromethyl moiety of chloramphenicol which is oxidatively metabolized by cytochrome P-450 to produce the reactive intermediates which covalently bind to and inactivate cytochrome P-450.
Figure 4. Reductive products of chloramphenicol.

(Yunis et al., 1980)
Objectives

While chloramphenicol is an effective mechanism-based inactivator of cytochrome P-450, it possesses several characteristics which limit its usefulness. Many of these limitations arise from the fact that chloramphenicol is not an isozyme-specific inactivator, which makes it more difficult to use as a tool to determine the role of specific isozymes in metabolizing xenobiotics and endogenous compounds \textit{in vitro} and \textit{in vivo} and as a means of redirecting the metabolism of xenobiotics towards detoxification. In addition, chloramphenicol cannot be used in man due to the potential problems with aplastic anemia. However, one means of increasing the selectivity of chloramphenicol and perhaps in the process also eliminating the potential for causing aplastic anemia is by synthesizing analogs of chloramphenicol. The first objective of this research therefore, was to determine if chloramphenicol analogs can be designed that are effective and isozyme-specific inactivators of cytochromes P-450. The primary approach to this objective has been to first determine the role of different structural features of the chloramphenicol molecule in regulating its effectiveness and isozyme selectivity. This information will provide a rational basis for the design of more effective and more specific inactivators of cytochromes P-450.
As mentioned previously, chloramphenicol is one of the only mechanism-based inactivators of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 (PB-B) which inactivates the enzyme by covalently modifying only its protein moiety. Chloramphenicol has therefore been used to probe the importance of particular amino acid residues within the active site of the isozyme in its catalytic activity. However, chloramphenicol binds to more than one amino acid residue of PB-B, which limits the use of chloramphenicol in determining the role of particular amino acid residues. In addition, since chloramphenicol is isozyme selective it cannot be used to study the active site of all isozymes. It may be possible, however, to design analogs of chloramphenicol which bind to single amino acid residues and to different cytochrome P-450 isozymes. The second objective of this research, therefore, was to determine if chloramphenicol analogs can be used as tools to probe the active site of cytochromes P-450. The mechanism by which analogs of chloramphenicol inactivate PB-B and other isozymes will be examined and compared to that by which chloramphenicol inactivates PB-B, as an initial means of characterizing the active site. The relation of this inhibition to the covalent modification of particular amino acid residues will also be examined.
CHAPTER 2

ANALOGS 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

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 (Halpert and
Neal, 1980; Halpert, Nåslund and Betnér, 1983). 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
dehlorination by cytochrome P-450 of the dichloromethyl
moiety of chloramphenicol (Pohl and Krishna, 1978; Pohl,
Nelson and Krishna, 1978). The major covalently bound
species has been identified as an adduct of chloramphenicol
oxamic acid and the epsilon-amino group of one or more
lysine residues in the enzyme (Halpert, 1981).
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 (Halpert, Miller and Gorsky, 1985). 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 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 (Abeles, 1978). Effective and selective inhibitors of different P-450 isozymes are potentially useful in vivo for therapeutic purposes and as physiological probes into the importance of different isozymes in xenobiotic metabolism (Naslund and Halpert, 1984; Rando, 1984). 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 i.p.\(^1\) to control rats or rats treated with various inducers, chloramphenicol inactivated six of twelve major P-450 isozymes monitored (Halpert et al., 1985). One possible means of increasing the selectivity, as well as the effectiveness of this inhibitor as a mechanism-based inactivator is to synthesize structural analogs. The present investigation has focused on the effectiveness of analogs of chloramphenicol containing modifications of the propanediol side chain, the p-nitro

\(^{1}\) Abbreviations used are: pNO\(_2\)DCA, N-(2-p-nitrophenethyl) dichloroacetamide; i.p., intraperitoneal; MeOH, methanol; TLC, thin layer chromatography; TMS, tetramethyilsilane; pNO\(_2\)DFA, N-(2-p-nitrophenethyl) difluoroacetamide; pNO\(_2\)DBA, N-(2-p-nitrophenethyl) dibromoacetamide; 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 beta-naphthoflavone; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.
group and the dichloromethyl moiety in order to determine if these functional groups play a role in the inactivation of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450. The analog N-(2-p-nitrophenethyl) dichloroacetamide (pNO₂DCA) (Figure 5), 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

Materials.

Chloramphenicol, NADPH, dilauryl L-3-phosphatidylcholine, prolidase, carboxypeptidase-A, leucine aminopeptidase, protease (Streptomyces griseus, type XIV), and N-alpha-t-butyloxycarbonyl-L-lysine were purchased from Sigma Biochemicals, Saint Louis, Missouri. Beta-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 the Aldrich Chemical Co., Milwaukee, Wisconsin. (¹⁴C) Chloramphenicol was purchased from New England Nuclear, Boston, Massachusetts. Difluoroacetic acid was purchased from Alfa Products,
Figure 5. Structure of $p$NO$_2$DCA.
Danvers, Massachusetts. Deschlorochloramphenicol was a gift from Dr. Lance Pohl, NIH, Bethesda, Maryland.

**Synthesis of p-NO₂DCA.**

p-Nitrophenethylamine-HCl (2.0g, 10 mmoles) was dissolved in an excess of 1 N NaOH (40 ml) and added to 80 ml ethyl acetate. Dichloroacetyl chloride (30 mmoles) was added dropwise to the reaction mixture, which was stirred for ten minutes 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 1 N HCl once, 10 ml 0.1N HCl twice) then with 5% sodium bicarbonate (10 ml, 3 times) and finally with water (10 ml, 2 times). The series of washes was repeated once more. The ethyl acetate phase was then rotary evaporated to dryness. The product was redissolved in CHCl₃-MeOH (90:10) and applied to Analtech Silica Gel GF 500 micron TLC plates, which were developed in CHCl₃-MeOH (90:10). The product (Rₐ =0.83) was scraped from the plates and eluted with CHCl₃-MeOH (90:10) and dried under N₂. The resulting light yellow solid had a melting point of 97.5-99⁰. The product was >99% pure of contaminants absorbing at 254 nm when spotted on analytical TLC plates which were developed in CHCl₃-MeOH (90:10). The product also yielded one spot on TLC with two other solvent systems: propanol-water (70:30) (Rₐ =0.96), and isopropanol-ammonium hydroxide-water (85:5:10)
(R<sub>f</sub>=0.91). The identity of the product was confirmed by NMR (<sup>1</sup>H, CD<sub>3</sub>COCD<sub>3</sub>, TMS internal standard) δ (ppm) 3.3 (m, 4H, -CH<sub>2</sub>-CH<sub>2</sub>-), 6.3 (s, 1H, -CHCl<sub>2</sub>), 7.9 (dd, 4H, aromatic).

N-(2-phenethyl) dichloroacetamide was synthesized in a similar manner, with phenethylamine used in place of p-nitrophenethylamine. The product had a melting point of 74-75°. The identity of the compound was confirmed by NMR. (<sup>1</sup>H, CD<sub>3</sub>COCD<sub>3</sub>, TMS internal standard) δ (ppm) 3.2 (m, 4H, -CH<sub>2</sub>-CH<sub>2</sub>-), 6.3 (s, 1H, CHCl<sub>2</sub>), 7.3 (s, 4H, aromatic).

N-(2-p-nitrophenethyl) chloroacetamide (pNO<sub>2</sub>MCA) was also synthesized in the same manner with chloroacetyl chloride used in place of dichloroacetyl chloride. The identity of the compound was confirmed by NMR. (<sup>1</sup>H, CDCl<sub>3</sub>, TMS internal standard) δ (ppm) 3.3 (m, 4H, -CH<sub>2</sub>-CH<sub>2</sub>-), 4.0 (s, 2H, CH<sub>2</sub>Cl), 7.8 (dd, 4H, aromatic). The melting point of pNO<sub>2</sub>MCA was 98-99°C.

Synthesis of (14C) pNO<sub>2</sub>DCA.

Radiolabeled pNO<sub>2</sub>DCA was synthesized using a modification of the method of Sonenberg et al. (1973) for the preparation of bromamphenicol. As the (14C) dichloroacetic acid which was necessary for the synthesis was not available, 200 ul 1N NaOH was added to 10 umoles (14C) chloramphenicol (1mCi/mmole), and the chloramphenicol was allowed to hydrolyze for two hours at room temperature. After acidifying the mixture with 200 ul 2N HCl, the (14C)
dichloroacetic acid formed was extracted into ethyl acetate and evaporated under nitrogen. Dioxane (200 ul), 2.1 mg dicyclohexylcarbodiimide, and 1.4 mg N-hydroxysuccinimide (27.8 ul of a 0.05 mg/ul solution in dioxane) were then added to the (14C) dichloroacetic acid, and the reaction mixture was allowed to sit at room temperature. After one hour, 10 umoles neutralized p-nitrophenethylamine were added, and the reaction mixture was again allowed to sit for 1 hour. Ethyl acetate (4 ml) was added, and the dicyclohexylurea was removed by centrifugation. The supernatant was then washed with 2% sodium bicarbonate (2 ml, 3 times), 0.1N HCl (2 ml, 3 times), and water (2 ml, 3 times), and was dried under nitrogen. The sample was redissolved in 200 ul MeOH and purified by preparative TLC as described in the synthesis of unlabeled pN02DCA. The product was found to be >98% radiochemically pure by reverse-phase TLC (MeOH-H2O 70:30) (Rf = 0.43).


N-(2-p-nitrophenethyl) difluoroacetamide (pNO2DFA), N-(2-p-nitrophenethyl) dibromoacetamide (pNO2DBA) and N-(2-p-nitrophenethyl trichloroacetamide (pNO2TCA) were prepared in a manner similar to that described for the synthesis of (14C) pNO2DCA, except on a larger scale, using
difluoroacetic acid, dibromoacetic acid and trichloroacetic acid respectively. pNO$_2$DFA had a melting point of 103-104$^\circ$C, pNO$_2$DBA had a melting point of 134-135$^\circ$C, and pNO$_2$TCA had a melting point of 141-142$^\circ$C. The identity of these products was confirmed by NMR. (1) pNO$_2$DBA: ($^1$H,CD$_3$COCD$_3$, TMS internal standard) $\delta$ (ppm) 3.4 (m, 4H,-CH$_2$-CH$_2$), 6.2 (s, 1H, CHBr$_2$), 7.9 (dd, 4H, aromatic). (2) pNO$_2$DFA: ($^1$H,CD$_3$COCD$_3$, TMS internal standard) $\delta$ (ppm) 3.3 (m, 4H,-CH$_2$-CH$_2$), 6.1 (t, 1H, CHF$_2$), 7.9 (dd, 4H, aromatic). (3) pNO$_2$TCA: ($^1$H, CD$_3$COCD$_3$, TMS internal standard) $\delta$ (ppm) 3.4 (m, 4H,-CH$_2$-CH$_2$), 7.8 (dd, 4H, aromatic).

Preparation of Microsomes.

Adult male Sprague-Dawley rats (150-200g) were pretreated with phenobarbital or beta-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 hours prior to sacrifice. These rats were then injected with 10 or 100 mg/kg pNO$_2$DCA or 300 mg/kg chloramphenicol in 0.7 ml propylene glycol and were sacrificed by cervical dislocation after one hour. Rats induced with beta-naphthoflavone were injected i.p. once daily with 40 mg/kg beta-naphthoflavone in 0.5 ml corn oil for 3 days prior to sacrifice. All liver
microsomes were prepared essentially as described by van der Hoeven and Coon (1974). Livers were removed from the rats and rinsed with 1.15% potassium chloride. The livers were homogenized in three volumes of 0.1 M Tris-acetate buffer, pH 7.4, containing 0.1 M potassium chloride, 1.0 mM EDTA, and 20 uM butylated hydroxytoluene (BHT). The mixture was then centrifuged for 30 minutes at 10,000 x g. The supernatant was centrifuged at 105,000 x g for 40 minutes. The resulting pellet was suspended in 0.1 M potassium pyrophosphate buffer, pH 7.4, containing 1.0 mM EDTA and 20 uM BHT. The suspension was centrifuged at 105,000 x g for 40 minutes, and the resulting pellet was resuspended in 0.01 M Tris-acetate, pH 7.4, containing 20% (w/v) glycerol, 0.1 mM EDTA and 100 uM phenylmethylsulfonyl fluoride and stored at -70°C.

Preparation of enzymes.

Several isozymes of cytochrome P-450 were isolated from the microsomes of phenobarbital- or betanaphthoflavone-induced rats, including the major phenobarbital-inducible isozyme PB-B and the major betanaphthoflavone-inducible isozyme BNF-B. PB-B and BNF-B were isolated as described by Guengerich and Martin (1980) using modifications described by Halpert, Miller and Gorsky (1985) and Haaparanta et al (1983). These modifications allowed
the separation of PB-B from PB-D, a closely related isozyme also induced by phenobarbital.

A simplified scheme for the purification of several isozymes is shown in Figure 6. Microsomes from phenobarbital- or beta-naphthoflavone-induced rats were solubilized with 0.6% sodium cholate (w/v) and clarified by centrifugation. The solubilized microsomes were then applied to an n-octylamino-Sepharose column (3.2 x 30 cm) previously equilibrated with Buffer A (100 mM potassium phosphate, pH 7.25, 20% glycerol, 1 mM EDTA) containing 0.5% (w/v) sodium cholate. A crude cytochrome P-450 mixture was eluted with Buffer A containing 0.33% (w/v) sodium cholate and 0.06% (w/v) Lubrol. The cytochrome P-450 containing fractions from the n-octylamino-Sepharose column were then diluted three-fold with cold aqueous 20% (v/v) glycerol and applied to a hydroxylapatite column (5 x 12 cm). The column was washed sequentially with 3 bed volumes each of 40, 90, and 180 mM potassium phosphate, pH 7.25 containing 20% (v/v) glycerol, 0.1mM EDTA and 0.3% (w/v) Lubrol and fractions were collected. Each of these buffers elutes different mixtures of cytochrome P-450 isozymes. The fractions containing the desired P-450 isozymes were then concentrated using an Amicon Ultrafiltration cell and dialyzed against Buffer B (5 mM potassium phosphate, pH 7.7, 0.1% (w/v) Lubrol, 0.2% (w/v) sodium cholate, 20% (v/v) glycerol and 0.1 mM EDTA). The sample was then applied to a 1.5 x 60 cm
DEAE-Sephacel column, and fractions were collected. The column was washed first with Buffer B, followed by Buffer C (which contains 10 mM potassium phosphate instead of the 5 mM potassium phosphate of Buffer B). Several isozymes were eluted with these two buffers. Those which were not were eluted from the column with a 1.0 liter linear gradient of 0 to 100 mM sodium chloride in Buffer C. A further purification step was necessary for several of the isozymes obtained, such as PB-C and UT-A. Those fractions which required further purification were dialyzed against CM Sepharose Equilibration Buffer (5 mM potassium phosphate, 20% glycerol, 0.2% (w/v) Emulgen 911 and 0.1 mM EDTA). The samples were then applied to a 1-3 ml CM-Sepharose column. The various P-450 isozymes were eluted with 30, 50, or 100 mM potassium chloride in CM-Sepharose Equilibration buffer.

The nomenclature used for most of the isozymes purified is that of Guengerich et al. (1982b). There are several other names for each of these isozymes. For example, BNF-B appears to correspond to the P-450c of Ryan et al (1979) and P-448 of Harada and Omura (1981). PB-B corresponds to P-450b of Ryan et al (1979).

NADPH-cytochrome P-450 reductase was purified by chromatography on Whatman DE52 as described by Yasukochi and Masters (1976) followed by chromatography on 2',5'-ADP-agarose (Guengerich and Martin, 1980) as described previously (Halpert, Miller and Gorsky, 1985). One unit of
Figure 6. Scheme for the purification of cytochrome P-450 isozymes from rat liver microsomes. All isozymes were purified from the microsomes of phenobarbital-induced rats, except those in parentheses which were purified from the microsomes of beta-naphthoflavone-induced rats. (Scheme adapted from Guengerich et al, 1982).
NADPH-cytochrome P-450 reductase is defined as the amount of enzyme which reduces 1 umole of cytochrome c per minute when assayed in 300 mM potassium phosphate buffer, pH 7.7, at 25°C.

Incubation of purified PB-B with chloramphenicol analogs.

The enzymatic activity of purified cytochrome P-450 before and after incubation with chloramphenicol and analogs was assayed by monitoring the metabolism of 7-ethoxycoumarin essentially as described by Waxman and Walsn (1982). The reconstituted system contained 0.05 nmol cytochrome P-450 PB-B, 0.3 units NADPH-cytochrome P-450 reductase, 30 ug dilauryl L-3-phosphatidylcholine, 100 ug sodium deoxycholate, 0.05 M HEPES buffer (pH 7.5), 15 mM MgCl₂, 0.1 mM EDTA, 0.36 umoles NADPH, and inhibitors (added in 10 ul MeOH) in a final volume of 1.0 ml. Reactions were started with the addition of the NADPH after a 3-minute preincubation at 37°C. Incubations were continued at 37°C for specified times before the addition of 0.3 umoles 7-ethoxycoumarin. Incubations were again continued for 1 minute. Under these conditions product formation was linear with time for all concentrations of inhibitor used. The metabolism of 7-ethoxycoumarin to 7-hydroxycoumarin was monitored as described by Greenlee and Poland (1978). 7-Hydroxycoumarin was extracted from the incubation mixture first into 2 ml of chloroform and then into 3 ml of 30 mM
sodium borate. The 7-hydroxycoumarin in the borate phase was monitored on an Aminco-Bowman spectrofluorometer (excitation 366 nm, emission 454 nm).

Microsomal incubations.

The microsomal metabolism of 7-ethoxycoumarin was assayed essentially as described previously (Halpert, Naslund and Betner, 1983). The 1 ml incubation mixture contained 0.05 mg microsomal protein, 0.5 mM NADP, 1 unit glucose-6-phosphate dehydrogenase, 0.3 mM 7-ethoxycoumarin, 15 mM MgCl₂, 0.1 mM EDTA, 0.05 M HEPES buffer (pH 7.5), and 10 mM glucose-6-phosphate. After a 3 minute preincubation at 37°C, the reaction was started by the addition of the glucose-6-phosphate and allowed to proceed for 10 minutes.

Incubations of a reconstituted system with (¹⁴C) chloramphenicol or (¹⁴C) pNO₂DCA).

Incubation mixtures consisted of 1 nmol/ml PB-B or BNF-B, 2 units/ml NADPH-cytochrome P-450 reductase, 30 ug/ml dilauryl L-3-phosphatidyl choline, 100 ug/ml sodium deoxycholate, 0.05 M HEPES buffer (pH 7.5), 15 mM MgCl₂, 0.1 mM EDTA, 1 mM NADPH, and 10 uM (¹⁴C) pNO₂DCA (1.1 mCi/mmol) or 100 uM (¹⁴C) chloramphenicol (1.8 mCi/mmol). The mixture was preincubated for 3 minutes at 37°C, and the reaction was started with the addition of the NADPH. The samples were incubated for 15 minutes and then dialyzed for 48 hours at 4°C against 4 x 1-L 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, P-450 content and ethoxycoumarin deethylase activity, as described by Halpert and Neal (1980). Some incubation mixtures (containing 10 nmol P-450 in 10 ml) were dialyzed for 24 hours against distilled water. The protein precipitated, and was 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% SDS and 57.5 ug 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 hours. Prolidase (25 ug), carboxypeptidase (25 ug), and leucine aminopeptidase (25 ug) were added, and the digest was allowed to proceed overnight at 37°.

Microsomal incubations with ($^{14}$C) pNO$_2$DCA.

Microsomal protein (1 mg/ml) was incubated with 20 uM ($^{14}$C) pNO$_2$DCA (1.1mCi/mmol), 0.5 mM NADP, 15 mM MgCl$_2$, 0.1 mM EDTA, 10 mM glucose-6-phosphate, and 1 unit glucose-6-phosphate dehydrogenase in a final volume of 1 ml of 0.05 M HEPES buffer. After a preincubation of 3 minutes at 37°, the reaction was started with the addition of the glucose-6-phosphate and allowed to proceed for 30 minutes. Incubations were terminated by the addition of 2 ml ethyl acetate. After vortexing and centrifuging, the ethyl
acetate phase was discarded, and the samples were washed 3 more times with 2 ml 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 water and precipitated with 3 ml methanol. NaOH (1 N, 200 ul) was added, and the samples were allowed to sit at room temperature for 2 hours. Samples from phenobarbital-induced rats were then neutralized with 2N HCl. Samples from BNF induced rats were heated at 600 for 60 minutes before neutralizing with HCl. The microsomal protein was removed by centrifugation, and the supernatants were applied to a Bio-Rad P-2 column (1.5 x 45 cm) equilibrated with 0.05 M N-ethyl morpholine acetate buffer (pH 7.5). The column was eluted with the same buffer at a rate of 10 ml/hour, and 1-ml fractions were collected and monitored by liquid scintillation counting and UV spectroscopy.

Sleeping Times.

Male Sprague-Dawley rats (160-200g) were pretreated with 0.1% (w/v) phenobarbital in the drinking water for 5 days. The animals were then fasted for the next 24 hours, and the phenobarbital was replaced with fresh drinking water. Rats were injected i.p. with 10, 100 or 300 mg/kg chloramphenicol or pNO2DCA in 0.5 ml propylene glycol or with the vehicle alone. After 1 hour, sodium pentobarbital
(100 mg/kg) was administered i.p. 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 (1964), and protein was determined by the method of Lowry et al. (1951). 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 chloramphenicol analogs.

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 pNO₂DCA is shown in Fig. 7. Pseudo-first-order kinetics were observed for the initial phase of inactivation, which is consistent with inactivation proceeding via a suicide mechanism. From the plot of the reciprocal of the initial rate constant of inactivation as a function of the reciprocal of the inhibitor concentration, the maximal rate constant of inactivation by pNO₂DCA (k_{inactivation}) was determined to be \(0.6 \text{ min}^{-1}\), and the
inhibitor concentration required for half-maximal inactivation ($K_I$) was 0.8 uM. (Fig. 8). The $k_{inactivation}$ for chloramphenicol was 0.4 min$^{-1}$ and the $K_I$ was found to be 15 uM.

The inactivation of the 7-ethoxycoumarin deethylase activity obtained with 5 uM pNO$_2$DCA, 5 uM pNO$_2$DBA, 5 uM N-(2-phenethyl) dichloroacetamide and 100 uM pNO$_2$DFA is shown in Fig. 9. The rate constant obtained with N-(2-phenethyl) dichloroacetamide ($k = 0.53$ min$^{-1}$) was similar to that obtained with pNO$_2$DCA ($k = 0.59$ min$^{-1}$), while the rate constant with pNO$_2$DBA was slightly greater ($k = 0.70$ min$^{-1}$). pNO$_2$DFA, however, showed no evidence of time-dependent inactivation at a concentration of 100 uM.

The monochloromethyl analog of chloramphenicol, deschlorochloramphenicol, and the trichloromethyl analog of pNO$_2$DCA, pNO$_2$TCA, were also unable to inactivate PB-B at a concentration of 100 uM (Appendix A, Fig. 17). However, N-(2-p-nitrophenethyl) chloroacetamide, pNO$_2$MCA, the monochloromethyl analog of pNO$_2$DCA inactivated PB-B with a $k_{inactivation}$ of 0.3 min$^{-1}$ and a $K_I$ of 18 uM. Unlike the other analogs examined thus far, the inactivation of PB-B by 100 uM pNO$_2$MCA was accompanied by a decrease in spectrally-detectable cytochrome P-450 (Figure 10). pNO$_2$MCA caused a 47% decrease in cytochrome P-450 content and a 63% decrease in ethoxycoumarin deethylase activity.

To determine whether the isozyme selectivity of
pNO$_2$DCA differed from that of chloramphenicol, the major beta-naphthoflavone-inducible isozyme was incubated with 100 uM chloramphenicol or 5 uM pNO$_2$DCA. As seen in Fig. 11, chloramphenicol did not inactivate this isozyme, whereas pNO$_2$DCA inactivated the enzyme with a rate constant (k = .19 min$^{-1}$) approximately one-third that at which it inactivated the major phenobarbital-inducible isozyme. N-(2-phenethyl) dichloroacetamide inactivated the major beta-naphthoflavone induced enzyme at approximately the same rate as pNO$_2$DCA (k = 0.14 min$^{-1}$).

Covalent binding of metabolites of chloramphenicol and pNO$_2$DCA in a reconstituted system.

Incubation of ($^{14}$C) pNO$_2$DCA (10 uM) with a reconstituted system containing the major isozyme of 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 $^{14}$C/nmol 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 of 1.5 nmol $^{14}$C/nmol P-450 (Halpert and Neal, 1980). Incubation of the major beta-naphthoflavone-inducible isozyme with ($^{14}$C) pNO$_2$DCA (10 uM) resulted in the covalent binding of $^{14}$C to the proteins of
Figure 7. Inactivation of purified cytochrome P-450 by pNO₂DCA as a function of inhibitor concentration. The enzymatic activity of cytochrome P-450 PB-B was monitored following incubation with the inhibitor for various times as described in Materials and Methods. The rate constants for inactivation were determined from the rapid initial phase of inactivation by linear regression analysis of the natural logarithm of the residual ethoxycoumarin deethylase activity as a function of time. The suicide inactivation of PB-B by pNO₂DCA 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 pNO₂DCA concentrations. (●) no inhibitor; (○) 0.5 μM; (▲) 1 μM; (△) 5 μM.
Figure 8. Double reciprocal plot of the rate constant of the initial phase of inactivation as a function of pNO₂DCA concentration. $K_I$ is determined from the intercept on the abscissa and $k_{inactivation}$ from the intercept on the ordinate.
Figure 9. Inactivation of purified cytochrome P-450 PB-B by analogs of chloramphenicol. The assay was carried out as described in Materials and Methods. Cytochrome P-450 PB-B was incubated with 100 µM pNO₂DFA (△), 5 µM pNO₂DCA (O), 5 µM pNO₂DBA (▲) and 5 µM N-(2-phenethyl) dichloroacetamide (■) and (●) no inhibitor added.
Figure 10. Cytochrome P-450 content of purified cytochrome P-450 PB-B following preincubation with 100 μM pNO₂DCA (——), 100 μM pNO₂MCA (●●●●) or (——) no inhibitor PB-B was incubated for 10 minutes with pNO₂DCA or pNO₂MCA and a complete reconstituted system, placed on ice and dialyzed as described in Materials and Methods. Aliquots were then taken for the determination of the 7-ethoxycoumarin deethylase activity and the cytochrome P-450 content (Halpert and Neal, 1980).
Figure 11. Effect of preincubation with chloramphenicol and pNO₂DCA on the ethoxycoumarin deethylase activity of purified cytochrome P-450 BNF-B in a reconstituted system. The assay was carried out as described in Materials and Methods except the incubation time after the addition of the 7-ethoxycoumarin was extended to 2 minutes in the case of chloramphenicol. (●) no inhibitor; (□) 100 μM chloramphenicol; (△) 5 μM pNO₂DCA.
the system with a stoichiometry of inactivation of 1 nmol $^{14}$C/nmol 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) $p$NO$_2$DCA were run on a Bio-Rad P-2 column. The enzymatic digest of protein from incubation of BNF-B and ($^{14}$C) $p$NO$_2$DCA yielded two major peaks upon chromatography (Fig. 12). Peak A ($V_e = 33$ ml) co-chromatographed with the synthetic standard 4-nitro-l-phenethyl-1,2-dicarboxylic acid amide (Fig. 14, compound e) while peak B ($V_e = 43$ ml) corresponded to the synthetic 4-nitro-l-phenethyl oxamyl lysine (Fig. 14, compound f). The identity of the products of the enzymatic digests was confirmed by HPLC (Fig. 13). The enzymatic digest of protein from the incubation of PB-B and $p$NO$_2$DCA 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 $p$NO$_2$DCA 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) $p$NO$_2$DCA.

Microsomes from rats pretreated with phenobarbital
or beta-naphthoflavone were incubated with \(^{14}\text{C}\) pNO\(_2\)DCA as described in Materials and Methods and treated with 1N NaOH. Chromatography of the alkaline hydrolysate of the \(^{14}\text{C}\)-labeled microsomes from phenobarbital-induced rats on a Bio-Rad P-2 column yielded 2 major peaks containing approximately the same amount of radiolabel (Appendix A, Fig. 18). One peak \((V_e = 36 \text{ ml})\) was identified as 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide (Fig. 14, Compound e) and the other peak \((V_e = 21 \text{ ml})\) as oxalic acid (Fig. 14, Compound d) by co-chromatography with the respective synthetic standards. Chromatography of the \(^{14}\text{C}\) labeled microsomes from beta-naphthoflavone induced rats also resulted in two similar peaks \((V_e=21 \text{ ml and } V_e=37 \text{ ml})\). In a similar fashion, alkaline hydrolysis of microsomes from phenobarbital-induced rats to which \(^{14}\text{C}\) chloramphenicol had been covalently bound was previously found to yield two approximately equal peaks corresponding to chloramphenicol oxamic acid (Fig. 14, Compound 5) and oxalic acid (Fig. 14, Compound 4) (Halpert, Naslund and Betner, 1983).

Effect of in vivo treatment with pNO\(_2\)DCA on rat liver microsomal enzymes.

Treatment of phenobarbital-induced rats with 10 or 100 mg/kg pNO\(_2\)DCA or 300 mg/kg chloramphenicol one hour prior to their termination resulted in significant inhibition of 7-ethoxycoumarin deethylase activity as
Figure 12. Chromatography of an enzymatic digest of $^{14}$C-labeled BNF-B after incubation with pNO$_2$DCA. The digest was run on a Bio-Rad P-2 column (1.5 x 45 cm) in 0.05 M N-ethylmorpholine acetate buffer (pH 7.5) as described in Materials and Methods. 4-Nitro-l-phenethyl-1,2-dicarboxylic acid amide and 4-nitro-l-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 (Pohl and Krishna, 1978; Halpert, 1981). The synthetic standards were monitored by the absorbance at 280 nm (---) and the labeled adducts by scintillation counting (O). Peak A co-chromatographs with 4-nitro-l-phenethyl-1,2-dicarboxylic acid amide, whereas peak B co-chromatographs with 4-nitro-l-phenethyl-oxamyl lysine.
Figure 13. Reverse phase HPLC of peaks A and B obtained from chromatography on a Bio-Rad P-2 column of the enzymatic digest of $^{14}\text{C}$-labeled BNF-B after incubation with pN0$_2$DCA (Fig. 12). HPLC was performed on a Beckman 421A instrument with Beckman 110A pumps and an Altex 5 micron, 4.6 mm 25 cm C-18 column. The column was eluted with a 15 minute (peak B) or 30 minute (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 chromatogram of peak A from the P-2 column. Graph B is the HPLC chromatogram obtained from peak B from the P-2 column. This was monitored at 300 nm in order to eliminate background absorbance.
Figure 14. Proposed scheme for the metabolic activation of chloramphenicol (top) and pNO₂DCA (bottom) by cytochrome P-450. The proposed scheme for chloramphenicol is based on the scheme proposed by Pohl et al. (Pohl and Krishna, 1978; Pohl, Nelson and Krishna, 1978) and previous work of Halpert (1981).
compared to the vehicle control \( (p<0.05) \). The ethoxycoumarin deethylase activities remaining (as a percent of the vehicle control) in the microsomes of rats treated with 10 mg/kg pNO\(_2\)DCA and 300 mg/kg chloramphenicol were not significantly different (50% and 47% respectively), while the activity remaining in the microsomes of rats treated with 100 mg/kg pNO\(_2\)DCA was significantly lower than both the 10 mg/kg pNO\(_2\)DCA and 300 mg/kg chloramphenicol groups (only 32% of the vehicle control) \( (p<0.05) \). The P-450 content of the microsomes did not differ between untreated rats and rats treated with chloramphenicol or pNO\(_2\)DCA. In order to determine which isozymes of cytochrome P-450 within these microsomes were inhibited, the metabolism of warfarin was monitored as described previously (Halpert et al., 1985). The structure of warfarin is shown in Fig. 15. Warfarin has an asymmetric carbon and can be hydroxylated at several different positions by different cytochrome P-450 isozymes. The regio- and stereoselective metabolism of warfarin can be used to monitor the levels of several isozymes present in microsomes and the ability of inhibitors, such as pNO\(_2\)DCA, to decrease these levels. The same order of isozyme inhibition was observed with pNO\(_2\)DCA as was found previously with chloramphenicol (Halpert et al., 1985). Isozyme PB-C

2. Results were analyzed by Student's t-test.
(an isozyme induced 2 to 3-fold by phenobarbital) was inhibited to the greatest extent by pNO₂DCA, whereas the isozyme responsible for the R-10 hydroxylation of warfarin was not inhibited. Isozyme PB-B was intermediate in its susceptibility to pNO₂DCA. For example, at the dose of 10 mg/kg pNO₂DCA 60% inhibition of PB-C but only 36% inhibition of PB-B was observed.

The greater inhibitory potency of pNO₂DCA 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 pNO₂DCA (117 ± 25 min) was significantly greater than that of rats given 10 or 100 mg/kg 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 chloramphenicol was not significantly different from 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
Figure 15. Structure of warfarin.
enzyme for the analog; 2) the maximal rate at which the analog 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 the covalent binding on the activity of the enzyme. In the present study, pNO₂ DCA, in which the propanediol side chain of chloramphenicol was replaced with an ethyl group, was shown to be nearly twenty-fold more potent than chloramphenicol, both in vivo and in vitro. N-(2-phenethyl) dichloroacetamide was also shown to inactivate the major phenobarbital- and beta-naphthoflavone-inducible isozymes of cytochrome P-45₀ with a rate constant approximately equal to that obtained with pNO₂ DCA. Thus, neither the pNO₂ 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 (Halpert, Miller and Gorsky, 1985). The present results would suggest that the presence of the pNO₂ 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 pNO₂ 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 pNO₂ DCA with a dibromomethyl group actually increased the rate constant of inactivation of PB-B, whereas replacement with a difluoromethyl moiety resulted in a compound which did not inactivate the enzyme at a concentration twenty times that of the other inhibitors tested. The monochloromethyl analog of chloramphenicol was also tested and found to be incapable of inactivating the enzyme.

Although deschlorochloramphenicol does not inactivate PB-B, the monochloromethyl analog of pNO₂ DCA, pNO₂ MCA, is an effective inactivator of PB-B. However, the inactivation was largely due to the modification of the heme rather than to protein modification. Analogs of chloramphenicol with alterations in other functional groups can also inactivate PB-B by heme modification. N-octyl
dichloroacetamide, for example, inactivates PB-B without the loss of spectrally detectable cytochrome P-450. However, as the length of the alkyl side chain was decreased from the n-octyl to n-hexyl and n-butyl, an increasing ability to modify the heme moiety was observed. N-methyl dichloroacetamide, in fact, inactivated PB-B primarily as a result of heme modification rather than protein modification (Halpert et al., 1986). These results indicate that alterations in the functional groups of chloramphenicol affect the target of the inactivator.

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, pNO₂DCA was shown to inactivate this isozyme with a rate constant 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 pNO₂DBA, N-(2-phenethyl)dichloroacetamide and pNO₂DCA exhibited biphasic kinetics (Fig. 7, Fig. 9). The rate constants for each analog 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 probably demonstrate more clearly the biphasic nature of the inactivation, particularly for those analog 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 with 5 μM pNO₂ DCA did not alter the kinetics. The addition of 1 mM dithiothreitol as a trapping agent to incubations of PB-B and 5 μM pNO₂ DCA 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 (Halpert, Miller and Gorsky, 1985). In addition, biphasic kinetics are also seen for the inactivation of BNF-B by 5 μM pNO₂ DCA (Fig. 11), 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 P-450 isozymes by
other mechanism-based inactivators (Waxman and Walsh, 1982; MacDonald et al., 1982). 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 (MacDonald et al., 1982). It is not yet known if a similar explanation could account for the biphasic kinetics observed for the inactivation of PB-B and BNF-B by chloramphenicol analogs.

The metabolic pathway by which chloramphenicol inactivates PB-B has previously been determined (Pohl and Krishna, 1978; Pohl, Nelson and Krishna, 1978; Halpert, 1981). Since pNO₂DCA 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 pNO₂DCA by PB-B and BNF-B. Alkaline hydrolysis of the adducts formed between phenobarbital and beta-naphthoflavone induced microsomes and pNO₂DCA led to the release of oxalic acid (Fig. 14, Compound d) and 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide (Fig. 14, Compound e), while an enzymatic digest produced 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide and 4-nitro-1-phenethyl oxamyl lysine (Fig. 14, Compound f). These results suggest that the metabolic pathway by which pNO₂DCA 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 (Pohl and Krishna, 1978; Pohl, Nelson and Krishna, 1978; Halpert, 1981) (Fig. 14). The major difference between chloramphenicol and pNO₂DCA metabolism is the formation of oxalic acid upon the enzymatic digestion of the radiolabeled protein obtained from incubation of PB-B with (¹⁴C) chloramphenicol (Halpert, 1981). 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 (Halpert, 1982). However, the present investigation has shown that in addition to the stable lysine adduct, 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide instead of oxalic acid is produced upon enzymatic digestion of the protein from the incubation of PB-B and BNF-B with pNO₂DCA. The oxalic acid is only produced under the harsher conditions of alkaline hydrolysis. This would suggest that there is only one reactive intermediate of pNO₂DCA 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-l-phenethyl-oxamyl lysine adduct, could be derived from the same oxamyl chloride intermediate.

The pathways by which pNO₂DCA 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. pNO₂DCA 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 if the mechanisms of inactivation are actually the same for these two isozymes. Future studies with other structural analogs of chloramphenicol should also aid in the design of mechanism-based inactivators of cytochrome P-450 with enhanced rather than diminished isozyme-selectivity.
CHAPTER 3

MECHANISM-BASED INACTIVATION OF THE MAJOR BETA-NAPHTHOFLAVONE-INDUCIBLE ISOZYME OF RAT LIVER CYTOCHROME P-450 BY THE CHLORAMPHENICOL ANALOG N-(2-P-NITROPHENETHYL) DICHLOROACETAMIDE

The cytochrome P-450 dependent monooxygenase system is generally associated with the detoxification of xenobiotics and endogenous compounds. However, this system is also involved in the activation of many chemicals to more toxic forms. The major beta-naphtoflavone-inducible isozyme of rat liver cytochrome P-450 (BNF-B)$^1$ in particular is believed to play a key role in the activation of many toxic chemicals and carcinogens (Lu and West, 1980). The metabolism of benzo(a)pyrene and other polycyclic aromatic hydrocarbons to mutagenic forms, for example, is preferentially catalyzed by BNF-B (Levin et al., 1977). However, little is really known about the active site of

1. Abbreviations used are: BNF-B, the major beta-naphtoflavone-inducible isozyme of rat liver cytochrome P-450; PB-B, the major phenobarbital-inducible isozyme of rat liver cytochrome P-450; DLPC, dilauryl L-3-phosphatidyl choline, HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
BNF-B and how it differs from that of other cytochrome P-450 isozymes. In particular, the role of different amino acid residues of the enzyme in regulating such functions as substrate binding and interaction with NADPH-cytochrome P-450 reductase is relatively unknown (Guengerich et al., 1982a). One means of elucidating this kind of information is with the use of specific, irreversible inhibitors which modify particular amino acid residues.

Previous experiments have shown that the chloramphenicol analog N-(2-p-nitrophenethyl) dichloroacetamide (pN02DCA), unlike chloramphenicol itself, is a mechanism-based inactivator of BNF-B with a stoichiometry of 1.0 nmol pN02DCA metabolites bound/nmol cytochrome P-450 inactivated (Miller and Halpert, 1986). pN02DCA is oxidatively dechlorinated by BNF-B and by the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 (PB-B) to a reactive oxamyl chloride which covalently binds to lysine and other amino acid residues (Miller and Halpert, 1986). The importance of lysine residues in several cytochrome P-450 isozymes has previously been demonstrated. The chloramphenicol-mediated covalent modification of lysine residues of PB-B apparently impairs electron transport from NADPH-cytochrome P-450 reductase to the heme (Halpert, 1981; Halpert, Miller and Gorsky, 1985). Lysine residues in rabbit P-450 LM2 seem to function in the interaction between the cytochrome P-450 and the reductase
(Bernhardt et al., 1984), while lysine residues in rat P-450b have been implicated in substrate binding (Kunz and Richter, 1983). However, the mechanism by which the covalent modification of lysine and other amino acid residues in BNF-B by pNO₂DCA inactivates the enzyme is unknown. The effect of pNO₂DCA on BNF-B *in vivo* is also unknown. A selective inactivator of BNF-B *in vivo* would be potentially useful for therapeutic purposes and as a probe of the importance of this isozyme in xenobiotic metabolism. The primary goals of this investigation therefore were 1) to determine if pNO₂DCA is indeed a potent and selective inactivator of BNF-B and 2) to determine the mechanism by which the modification of particular amino acid residues with pNO₂DCA inactivates BNF-B.

**Materials and Methods**

**Materials.**

NADPH, dilauryl L-3-phosphatidyl choline, catalase, glucose and glucose oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). Beta-naphthoflavone, 7-hydroxycoumarin, and iodosobenzene diacetate were purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium deoxycholate and HEPES were obtained from Calbiochem-Behring (La Jolla, CA). Isosafrole was from ICN (Plainview, NY). Ethoxyresorufin and resorufin were purchased from Molecular Probes, Inc. (Junction City, OR). N-(2-p-nitrophenethyl)
dichloroacetamide \((\text{pN0}_2 \text{DCA})\) was synthesized as described in Chapter 2 (Miller and Halpert, 1986).

**Preparation of microsomes.**

Adult male Sprague-Dawley rats \((150-220 \text{ g})\) were injected i.p. with 40 mg/kg beta-naphthoflavone in 0.5 ml corn oil once daily for 3 days. For *in vivo* studies of cytochrome \(\text{P-450}\) inhibition, beta-naphthoflavone-induced rats were fasted for 24 hours after the last injection. The rats were then injected i.p. with 0, 10 or 100 mg/kg \(\text{pN0}_2 \text{DCA}\) in 0.5 ml corn oil and were killed one hour later by cervical dislocation. All liver microsomes were prepared as described previously (Halpert, Näslund and Betnér, 1983).

**Purification of enzymes.**

BNF-B, PB-B and NADPH-cytochrome \(\text{P-450}\) reductase were purified as described in Chapter 2. One unit of NADPH-cytochrome \(\text{P-450}\) reductase is defined as the amount of enzyme which reduces 1 umole of cytochrome c per minute when assayed in 300 mM potassium phosphate buffer, \(\text{pH} \, 7.7\), at 25°C.

**Modification of BNF-B with \(\text{pN0}_2 \text{DCA}\).**

Incubation mixtures consisted of 0.6–1 nmol/ml BNF-B or PB-B, 2 units/ml NADPH-cytochrome \(\text{P-450}\) reductase, 30 ug/ml DLPC, 100 ug/ml sodium deoxycholate, 0.05 M HEPES
buffer (pH 7.5), 15 mM MgCl₂, 0.1 mM EDTA, 1 mM NADPH, and 10 uM pNO₂DCA. Controls contained NADPH but no pNO₂DCA. The mixtures were preincubated for 3 minutes at 37°C and the reactions were started with the addition of the NADPH. The samples were incubated for 15 min and then dialyzed for 48 hours at 4°C against 4 one-liter volumes of 50 mM Tris-acetate (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. The BNF-B treated with pNO₂DCA is referred to as the modified enzyme, while the control is referred to as the native enzyme.

Substrate binding assays.

Reference and sample cuvettes contained 0.12 nmol BNF-B and 30 ug DLPC in 0.6 ml 50 mM potassium phosphate buffer (pH 7.4). Ethoxycoumarin (50 uM) in a small volume of methyl sulfoxide was then added to the sample cuvette, and an equal volume of solvent was added to the reference cuvette. The percent conversion of low-spin ferric cytochrome P-450 to the high-spin form was determined from the peak-to-trough absorbance difference \( \Delta A(390\text{nm}-420\text{nm}) \), based on an extinction coefficient of 126 mM⁻¹cm⁻¹ (Cinti et al., 1979).

Assays of monooxygenase activity.

The 7-ethoxyoumarin deethylase activity of the native and modified enzyme was determined in the presence of different concentrations of NADPH-cytochrome P-450
reductase. Incubation mixtures containing 0.05 nmol BNF-B, 30 ug DLPC, 100 ug deoxycholate, 300 uM ethoxycoumarin, 0.05M HEPES buffer (pH 7.5), 15 mM MgCl₂, 0.1 mM EDTA, and reductase (in a final volume of 1 ml) were preincubated for 3 minutes at 37°C. NADPH (0.2 mM) was then added, and the incubation was continued for 5 minutes at 37°C. The reductase added to the incubations ranged from 3 to 48 units/nmol P-450. The formation of 7-hydroxycoumarin was determined as described in Chapter 2 (Miller and Halpert, 1986).

Results

Effect of in vivo treatment with pNO₂DCA on rat liver microsomes.

In order to determine if pNO₂DCA is a potent and selective inactivator of BNF-B in vivo, beta-naphthoflavone-induced rats were injected with 10 or 100 mg/kg pNO₂DCA, and liver microsomes were prepared one hour later. The ethoxyresorufin deethylase activity of the resulting microsomes was monitored because the deethylation of ethoxyresorufin to resorufin is mediated specifically by BNF-B (Burke et al., 1985). As shown in Table 2, the microsomes from beta-naphthoflavone-induced rats treated in vivo with 10 or 100 mg/kg pNO₂DCA exhibited a significant dose-dependent decrease in ethoxyresorufin deethylase activity as compared to the vehicle control (72% and 95%
decrease respectively) \((p<0.02)^2\). The cytochrome \(P-450\) contents of the microsomes from the \(10\) \(mg/kg\) and \(100\) \(mg/kg\) groups were also slightly lower than the vehicle control (86\% and 68\% of the control respectively) \((p<0.05)\).

In order to determine which other isozymes of cytochrome \(P-450\) within these microsomes might be inhibited, the metabolism of warfarin was monitored as described previously (Halpert et al, 1985). This assay can be used to monitor the activity of several different cytochrome \(P-450\) isozymes on the basis of the hydroxywarfarin metabolites formed. Decreases in the rates of formation of R-6 and R-8 hydroxywarfarin are used to monitor the inhibition of BNF-B in beta-naphthoflavone-induced rat liver microsomes. As can be seen in Fig. 16, both the R-6 and R-8 hydroxylase activities were decreased by 64\% in the microsomes of rats treated with \(10\) \(mg/kg\) \(p\text{NO}_2\text{DCA}\) as compared to the vehicle control. The rates of formation of dehydro-, 4'- and 10-hydroxywarfarin were not decreased, suggesting that there is some degree of isozyme selectivity at this lower dose of \(p\text{NO}_2\text{DCA}\). At the \(100\) \(mg/kg\) dose, however, all of the hydroxylase activities monitored were decreased, with the R-6 and R-8 hydroxylase activities decreased by 95\%.

2. Results were analyzed by Student's t-test.
Table 2. Effect of pNO$_2$DCA administration on rat liver microsomes.

Animals were treated as described in Materials and Methods. Incubation mixtures consisting of 0.05-0.10 mg microsomes, 1.5 µM ethoxyresorufin, 0.05 M HEPES buffer (pH 7.5), 15 mM MgCl$_2$ and 0.1mM EDTA in a final volume of 2 ml were preincubated for 3 min at 25°C. The reaction was started with the addition of 0.4 mM NADPH and the rate of formation of resorufin was determined from the linear portion of the curve (excitation= 550nm, emission= 600nm) on an Aminco-Bowman spectrofluorometer. All reaction rates were linear with protein concentration under the conditions used. Cytochrome P-450 content was monitored spectrally by the method of Omura and Sato (1964) and protein was determined by the method of Lowry et al (1951). Results are expressed as the mean ± SD of four microsomal samples per treatment group. Values in parentheses represent the activity remaining in the microsomes from the pNO$_2$DCA- treated rats expressed in per cent of the activity in the microsomes from untreated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ethoxyresorufin Deethylase Activity (nmol/min/mg)</th>
<th>Cytochrome P-450 Content (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.60 ± 0.63</td>
<td>1.65 ± 0.16</td>
</tr>
<tr>
<td>10 mg/kg pNO$_2$DCA</td>
<td>0.45 ± 0.15 (28%)</td>
<td>1.45 ± 0.12 (86%)</td>
</tr>
<tr>
<td>100 mg/kg pNO$_2$DCA</td>
<td>0.08 ± 0.01 (5%)</td>
<td>1.13 ± 0.11 (68%)</td>
</tr>
</tbody>
</table>
Inactivation of purified BNF-B by pNO$_2$DCA.

Previous studies suggested that pNO$_2$DCA is also able to inactivate BNF-B \textit{in vitro} (Miller and Halpert, 1986). The kinetics of the inactivation were determined in the current investigation. To do this, the inactivation of the 7-ethoxycoumarin deethylase activity of BNF-B was monitored at various concentrations of pNO$_2$DCA. Biphasic kinetics were observed for the inactivation, with the second phase becoming apparent only after approximately 60% of the initial enzyme activity was lost. Biphasic kinetics have also been observed for the inactivation of PB-B by pNO$_2$DCA and chloramphenicol as well as for other mechanism-based inactivators of cytochrome P-450 (Miller and Halpert, 1986; Waxman and Walsh, 1982; MacDonald et al., 1982). Although the reasons for the biphasic nature of the kinetics is not known, possible explanations have been described in Chapter 2 (Miller and Halpert, 1986). The rate constants for the inactivation of BNF-B by pNO$_2$DCA were determined from the initial more rapid phase of inactivation. Pseudo-first order kinetics were observed for this initial phase of inactivation, which is consistent with mechanism-based inactivation. The maximal rate constant for inactivation of BNF-B by pNO$_2$DCA ($k_{\text{inactivation}}$) and the apparent Km for the inactivator ($K_I$) were determined from a double reciprocal plot of the rate constant for enzyme inactivation as a function of the inhibitor concentration. As seen in Table
Figure 16. Effect of in vivo pNO₂DCA administration to beta-naphthoflavone-induced rats on in vitro rates of hepatic microsomal metabolism of R-warfarin. Beta-naphthoflavone-induced rats were injected i.p. with 0, 10 or 100 mg/kg pNO₂DCA and liver microsomes were prepared one hour later as described in Materials and Methods. Incubations with warfarin and identification and quantitation of metabolites by HPLC were prepared as described previously (Halpert et al., 1985). The metabolism of R-warfarin to dehydrowarfarin (dehydro), 4'-hydroxywarfarin (4'-OH), 6-hydroxywarfarin (6-OH), 7-hydroxywarfarin (7-OH), 8-hydroxywarfarin (8-OH), and 10-hydroxywarfarin (10-OH) was monitored. Results are the means of triplicate analyses of rates from 4 individual rats.
3, pNO$_2$DCA is a very effective inactivator of BNF-B as well as PB-B. In fact, the $K_I$ obtained for the inactivation of BNF-B by pNO$_2$DCA is 6-fold lower than that obtained previously for the inactivation of PB-B by chloramphenicol, while the $k_{\text{inactivation}}$ values are similar (Miller and Halpert, 1986).

Modification of BNF-B with pNO$_2$DCA.

The results presented so far demonstrate that pNO$_2$DCA is an effective mechanism-based inactivator of BNF-B in vivo and in vitro. The remaining experiments were designed to elucidate the mechanism by which pNO$_2$DCA inactivates BNF-B. For this purpose, BNF-B was preincubated with pNO$_2$DCA and a complete reconstituted system. The pNO$_2$DCA-modified enzyme thus obtained was then compared in various ways to native BNF-B which had been incubated in the absence of pNO$_2$DCA. There was no apparent decrease in cytochrome P-450 content in the modified enzyme, suggesting that heme was not modified, nor was there a shift in the wavelength maximum for the dithionite-reduced ferrous carbonyl complex following modification with pNO$_2$DCA (Appendix A, Fig. 19).

Substrate binding.

Spectral binding assays were performed in order to determine if the binding of ethoxycoumarin to the modified BNF-B is impaired. Similar Type I spectra were produced by
Table 3. Kinetics of inactivation of BNF-B and PB-B by pNO₂DCA.

The kinetics of inactivation of BNF-B by pNO₂DCA were determined as described previously for the inactivation of PB-B by pNO₂DCA (Miller and Halpert, 1986). The reconstituted system contained 0.05 nmol of cytochrome P-450 BNF-B, 0.3 U fresh NADPH-cytochrome P-450 reductase, 30 μg DLPC, 100 μg sodium deoxycholate, 0.05 M HEPES buffer (pH 7.5), 15 mM MgCl₂, 0.1 mM EDTA, 0.36 umoles NADPH, and 0, 1, 5 and 25 μM pNO₂DCA in a final volume of 1 ml. Reactions were started with the addition of the NADPH after a 3 min preincubation at 37°C. Incubations were continued at 37°C for varying lengths of time before the addition of 0.3 umol 7-ethoxycoumarin. Incubations were again continued for 1 min. Formation of 7-hydroxycoumarin was monitored as described previously (Halpert, Naslund and Betner, 1983).

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>kinactivation(min⁻¹)</th>
<th>K_I(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNF-B</td>
<td>0.52</td>
<td>2.7</td>
</tr>
<tr>
<td>PB-B</td>
<td>0.60</td>
<td>0.8</td>
</tr>
</tbody>
</table>

a The kinactivation is the rate constant for inactivation under conditions of saturating inactivator, and the K_I is the apparent Km for the inactivator.

b The kinetics of inactivation of PB-B by pNO₂DCA were determined in Chapter 2 (Miller and Halpert, 1986).
50 uM ethoxycoumarin for the native and modified enzyme, and an 8% low spin to high spin-transition was observed in both cases. Higher concentrations of ethoxycoumarin could not be used for the binding assays as the absorbance of the substrate interferes with the spectra. However, when expressed as a percent of the activity of the native enzyme, the residual ethoxycoumarin deethylase activity of the modified enzyme is the same at 50 uM, 100 uM and 300 uM ethoxycoumarin (28%).

Functional interaction of modified BNF-B and NADPH-cytochrome P-450 reductase.

Previous studies have shown that the loss of monooxygenase activity following the modification of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 (PB-B) with chloramphenicol is the result of an impaired ability of the enzyme to accept electrons from NADPH-cytochrome P-450 reductase (Halpert et al., 1986). There are several ways to determine whether the inactivation of BNF-B by pN02DCA proceeds by a similar mechanism. In order to circumvent the steps involving electron transfer, the iodosobenzene- supported metabolism of 7-ethoxycoumarin was monitored. As seen in Table 4, the NADPH-supported metabolism of ethoxycoumarin by the modified enzyme was only 18% of the activity of the native enzyme. However, there was no difference in the iodosobenzene-
supported metabolism of 7-ethoxycoumarin with the native and modified BNF-B. Although there is some question as to the exact mechanism of the iodosobenzene-supported oxygenation, there is good evidence to suggest that iodosobenzene produces an oxoiron species which is similar or identical to that obtained with NADPH (Ortiz de Montellano, 1986). Thus, modified BNF-B is apparently fully capable of binding and metabolizing ethoxycoumarin provided an activated iron-coordinated oxygen species can be formed.

The decrease in the NADPH-supported metabolism of ethoxycoumarin is apparently not dependent upon the NADPH-cytochrome P-450 reductase concentration. The residual activity of the modified BNF-B, expressed as a percent of the activity of the native enzyme, is not altered over an added reductase to cytochrome P-450 ratio of 3 to 48 units per nmol of P-450.

Another means of examining the functional interaction between cytochrome P-450 and P-450 reductase is by monitoring the NADPH oxidase activity of the native and modified BNF-B. The same rate of NADPH oxidation was observed with the native and modified enzyme in the absence of added substrate (Table 5). However, when the NADPH oxidase activity was monitored in the presence of 0.1 mM ethoxycoumarin, the rate of NADPH oxidation by the modified enzyme was only 34% of that by the native enzyme. This loss of NADPH oxidase activity was accompanied by a 75% decrease
Table 4. Effect of pNO$_2$DCA on the NADPH and iodosobenzene-supported metabolism of ethoxycoumarin.

Incubations contained 0.05 nmol BNF-B, 300uM ethoxycoumarin, 0.05 M HEPES (pH 7.5), 15 mM MgCl$_2$, 0.1mM EDTA, 60 ug DLPC, 200 ug sodium deoxycholate and 0.3 U fresh reductase in a final volume of 2 ml. The reaction was started by the addition of 0.2 mM NADPH or 0.5 mM iodosobenzene diacetate. The rate of formation of 7-hydroxycoumarin was determined from the linear portion of the curve (excitation=370, emission = 460nm) as described previously (Halpert, Naslund and Betner, 1983). Results represent the mean ± SD of the individual values from 4 separate experiments. Values in parentheses represent the residual activity of the modified enzyme expressed in per cent of the native enzyme.

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>NADPH-supported (nmoles/min/nmol cytochrome P-450)</th>
<th>iodosobenzene-supported (nmoles/min/nmol cytochrome P-450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-pNO$_2$DCA</td>
<td>20.8 ± 1.6</td>
<td>35.6 ± 5.4</td>
</tr>
<tr>
<td>+pNO$_2$DCA</td>
<td>3.7 ± 0.6 (18%)</td>
<td>34.9 ± 3.5</td>
</tr>
</tbody>
</table>
in ethoxycoumarin deethylase activity. The residual NADPH oxidase activity of the modified BNF-B was slightly greater than the residual ethoxycoumarin deethylase activity, suggesting that the modified enzyme is less efficient in utilizing NADPH for monooxygenation.

Formation of reduced cytochrome P-450.

The steady-state level of enzymatically-reducible cytochrome P-450 BNF-B was examined next. As seen in Table 6, there was no difference in the level of cytochrome P-450 reducible by NADPH-cytochrome P-450 reductase in the modified BNF-B as compared to the native enzyme. However, for both the native and modified enzyme the amount of cytochrome P-450 reducible enzymatically under aerobic conditions was only about 33% of the dithionite-reducible cytochrome P-450. The rate of reduction of BNF-B in the absence of substrate is extremely slow compared to that of other cytochrome P-450 isozymes (Parkinson et al., 1986), which favors autooxidation under aerobic conditions. Under more anaerobic conditions, the steady-state level of the ferrous carbonyl complex was increased to 75% of the dithionite-reducible enzyme for the native enzyme and to 90% for the modified enzyme. The fact that the level for the native BNF-B was lower than that seen with the modified BNF-B is probably not significant since untreated BNF-B (BNF-B which was never incubated at 37°C) had steady-state levels
Table 5. NADPH oxidase activity of native and pNO$_2$DCA modified BNF-B.

Incubation mixtures containing 0.05 nmol/ml BNF-B, 0.05 M HEPES buffer (pH 7.5), 15 mM MgCl$_2$, 0.1 mM EDTA, 0.3 U/ml fresh reductase, 30 ug/ml DLPC and 100 ug/ml sodium deoxycholate were preincubated for 3 min at 37°C. NADPH (0.1 mM) was added and the rate of NADPH oxidation was monitored at 37°C by monitoring the decrease in absorbance at 340 nm. The NADPH oxidase activity in the presence of ethoxycoumarin was determined as described above with the addition of 0.1 mM ethoxycoumarin to the cuvette before the preincubation. The reaction was terminated after 5 min by transferring the contents of the cuvette to a tube containing 0.1 ml 2N HCl, and 7-hydroxycoumarin was determined as described earlier (Halpert, Naslund and Betner, 1983). Results represent the mean ± SD of the individual values from 4 separate experiments. The number in parentheses represents the activity remaining in the modified enzyme expressed as a per cent of the activity of the native enzyme.

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>-ethoxycoumarin</th>
<th>+ethoxycoumarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>- pNO$_2$DCA</td>
<td>34 ± 5</td>
<td>150 ± 35</td>
</tr>
<tr>
<td>+ pNO$_2$DCA</td>
<td>34 ± 5</td>
<td>51 ± 9 (34%)</td>
</tr>
</tbody>
</table>
of enzymatically-reducible BNF-B which were 86% of the dithionite-reducible enzyme.

The steady-state level of enzymatically-formed ferrous carbonyl cytochrome P-450 measures the formation of reduced cytochrome P-450 in the absence of substrate metabolism. The metabolism of isosafrole can be used to monitor the formation of reduced cytochrome P-450 under turnover conditions. Isosafrole is first metabolized by BNF-B to an activated species, possibly a carbene, which can form a stable metabolite-P-450 complex only with the ferrous form of the enzyme. Enzymatic turnover and oxidation of isosafrole are apparently necessary for the formation of the stable complex (Ortiz de Montellano and Reich, 1986). The rate of formation of the metabolite-P-450 complex was rapid and quickly deviated from linearity. Since it was not possible to determine the true initial rate, complex formation was monitored at two different time points, as shown in Table 7. At one minute complex formation is still increasing, while at 15 minutes a plateau level of complex formation is reached for both the native and modified enzyme. At the early time point, the amount of metabolite complex formed by the modified BNF-B was only about half that formed with the native enzyme. Although this does indicate that the rate of complex formation is lower for the modified BNF-B, the true extent of the decrease in rate may be even greater than 50%. The maximal level of complex
Table 6. Steady-state levels of the enzymatically-formed ferrous carbonyl complex of native and modified BNF-B under aerobic and anaerobic conditions.

A reconstituted system containing 0.12 nmol/ml BNF-B, 0.72 U/ml fresh NADPH-cytochrome P-450 reductase, and 30 μg/ml DLPC in 50 mM potassium phosphate buffer, pH 7.6, was bubbled with carbon monoxide for 5 min. A base-line was recorded on a Beckman DU-7 scanning spectrophotometer. The steady-state level of the ferrous carbonyl complex of cytochrome P-450 was then determined after the addition of 0.2 mM NADPH. Sodium dithionite was then added and additional scans run until no further increase in the ΔA448-490 was observed. The same experiments were performed under more anaerobic conditions by adding an oxygen scavenging system consisting of 6.7 mM glucose, 13 U/ml glucose oxidase, and 590 U/ml catalase to the cuvette after bubbling with carbon monoxide, but before adding the NADPH (Backes, Sligar and Schenkman, 1982). Results represent the mean ± SD of the individual values from 4 separate experiments.

<table>
<thead>
<tr>
<th>Preincubation Conditions</th>
<th>Cytochrome P-450 (% of dithionite-reducible)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
</tr>
<tr>
<td>no treatment</td>
<td>34 ± 6</td>
</tr>
<tr>
<td></td>
<td>86 ± 13</td>
</tr>
<tr>
<td>- pNO2DCA</td>
<td>33 ± 4</td>
</tr>
<tr>
<td></td>
<td>75 ± 4</td>
</tr>
<tr>
<td>+ pNO2DCA</td>
<td>32 ± 9</td>
</tr>
<tr>
<td></td>
<td>90 ± 3</td>
</tr>
</tbody>
</table>
formation obtainable with the modified enzyme was identical to that of the native enzyme, as observed at the 15-minute time point.

Several experiments have also been done with pNO\textsubscript{2}DCA-modified PB-B in order to determine if the mechanism by which pNO\textsubscript{2}DCA inactivates PB-B is the same as the mechanism by which it inactivates BNF-B. NADPH oxidase activities and the steady-state levels of the ferrous carbonyl complex were monitored as described for pNO\textsubscript{2}DCA-modified BNF-B. The results obtained suggest that pNO\textsubscript{2}DCA inactivates PB-B by a somewhat different mechanism than that by which it inactivates BNF-B. pNO\textsubscript{2}DCA-modified PB-B exhibited a decreased rate of NADPH oxidation in the absence as well as in the presence of ethoxycoumarin, there was a shift in the wavelength maximum for the dithionite-reduced ferrous carbonyl complex (Appendix A, Fig. 20), and the steady-state level of enzymatically reduced PB-B was decreased for the pNO\textsubscript{2}DCA-modified enzyme under aerobic conditions (Appendix A, Table 9).

**Discussion**

Previous studies have demonstrated that, like chloramphenicol, pNO\textsubscript{2}DCA is an effective mechanism-based inactivator of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 (PB-B) (Miller and Halpert, 1986). The present investigation has shown that, unlike
Table 7. Cytochrome P-450-isosafrole metabolite complex formation by native and pNO\textsubscript{2}DCA-modified BNF-B.

Formation of a cytochrome P-450-isosafrole metabolite complex was assayed spectrally essentially as described by Ryan, Thomas and Levin (1980). Incubations consisted of 0.2 nmol/ml native or modified BNF-B, 0.05 M HEPES buffer (pH 7.5), 15 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 1.2 U/ml fresh NADPH cytochrome P-450 reductase, 30 μg/ml DLPC, and 0.2 mM isosafrole. A baseline was recorded after a 3 min preincubation at 25°C. NADPH (0.2 mM) was then added to start the reaction. Scans of the metabolite complex were recorded at several time points, including 1 min and 15 min after addition of NADPH. Results are the mean ± SD of the individual values from 4 separate experiments.

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Δ A\textsubscript{454-490}/nmol cytochrome P-450/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditions</td>
<td>1 min</td>
</tr>
<tr>
<td>- pNO\textsubscript{2}DCA</td>
<td>0.037 ± 0.003</td>
</tr>
<tr>
<td>+ pNO\textsubscript{2}DCA</td>
<td>0.017 ± 0.005</td>
</tr>
</tbody>
</table>
chloramphenicol, pNO₂DCA is also an effective and at least somewhat selective inactivator of the major beta-naphthoflavone-inducible isozyme (BNF-B). Earlier studies have shown that pNO₂DCA is oxidatively metabolized by BNF-B to a reactive oxamyl chloride which covalently binds to lysine and other amino acid residues of the enzyme. The present investigation has concentrated on elucidating the mechanism by which the modification of these amino acids inactivates BNF-B.

The binding of chloramphenicol metabolites to lysine residues close to the heme moiety of PB-B has previously been shown to inactivate the enzyme by blocking electron transport from NADPH-cytochrome P-450 reductase to the PB-B. We have shown in the present investigation that the mechanism by which pNO₂DCA inactivates BNF-B is in some respects similar to that by which chloramphenicol inactivates PB-B, in that an impairment in the transfer of electrons is also involved. The major finding in this regard is that the modification of BNF-B by pNO₂DCA results in the inhibition of the NADPH- but not the iodosobenzene-supported metabolism of ethoxycoumarin.

As with chloramphenicol-modified PB-B, the decrease in NADPH-dependent ethoxycoumarin deethylase activity with the pNO₂DCA modified BNF-B is probably not due to a decreased affinity of the modified enzyme for reductase (Miller and Halpert, 1986). The percentage loss of activity
of the modified enzyme was not altered over a wide range of reductase concentrations. If the affinity of the reductase for the modified enzyme were decreased, the inhibition would probably be overcome somewhat at the higher reductase concentrations.

There are also significant differences, however, between the effects of pNO$_2$DCA and chloramphenicol on the two enzymes. First of all, there is no shift in the wavelength maximum for the dithionite-reduced ferrous carbonyl complex of the pNO$_2$DCA-modified BNF-B, suggesting that the binding of pNO$_2$DCA metabolites to BNF-B does not perturb the heme absorbance. The pNO$_2$DCA-modified BNF-B also differs from chloramphenicol-modified PB-B in that the NADPH oxidase activity of the modified BNF-B in the absence of substrate is not decreased as compared to that of the native BNF-B. As seen in Table 5, the rate of NADPH oxidation catalyzed by the native BNF-B is greatly enhanced upon the addition of ethoxycoumarin. An increase in NADPH oxidase activity is frequently seen with the addition of certain substrates to cytochrome P-450, possibly due to the associated change in spin state of the cytochrome and metabolism of the substrate (Nordbloom and Coon, 1977). The rate of NADPH oxidation with the modified BNF-B, however, increases only slightly in the presence of ethoxycoumarin. Although this could be due to impaired ethoxycoumarin binding to the modified BNF-B, we do not feel that this is
the case for three reasons. First, the spectral binding studies demonstrate that ethoxycoumarin binds to the same extent to the native and modified enzyme. Second, the iodosobenzene-supported metabolism of ethoxycoumarin is not decreased with the modified enzyme. Third, experiments in which the effect of ethoxycoumarin on isosafrole metabolism was monitored suggest that ethoxycoumarin binds to a similar extent to the native and modified enzyme (data not shown). Another possibility might be that pNO₂-DCA and ethoxycoumarin bind in such a way that their combined presence causes steric disturbances which impair the interaction of the reductase with the cytochrome P-450.

The fact that the NADPH oxidase activity of pNO₂-DCA-modified BNF-B in the absence of substrate is the same as that of the native enzyme also indicates that the mechanism of inactivation differs from that by which 2-bromo-4'-nitroacetophenone inactivates P-450c (which corresponds with BNF-B). 2-bromo-4'-nitroacetophenone, one of very few known specific, irreversible inactivators of the enzyme, alkylates cysteine residues and apparently acts as an uncoupler in that it increases the rate of NADPH oxidation and oxygen consumption without substrate metabolism (Parkinson et al., 1986). If pNO₂-DCA were acting in the same way, the NADPH oxidase activity of the pNO₂-DCA-modified BNF-B would have been greater than that of the native enzyme in the absence of substrate.
While the functional interaction between the modified BNF-B and the reductase seems to be impaired, the steady-state level of the enzymatically-formed ferrous carbonyl complex of modified BNF-B is not lower than that observed for native BNF-B under either aerobic or anaerobic conditions. Furthermore, the modified BNF-B is able to form the same maximal level of isosafrole metabolite complex as the native enzyme, even though the rate of complex formation is slower. These two findings clearly indicate that the modified BNF-B is capable of undergoing reduction by NADPH-cytochrome P-450 reductase in contrast to what has been observed with chloramphenicol-modified PB-B which appears to be completely inactivated and refractory to enzymatic reduction (Halpert et al., 1985). The data further suggest that the incubation of BNF-B with pN02DCA produces modified but partially active cytochrome P-450 (so called wounded enzyme) and that the defect is an impairment in the transfer of electrons from NADPH-cytochrome P-450 reductase. The mechanism by which pN02DCA inactivates PB-B resembles that by which chloramphenicol inactivates PB-B, rather than the mechanism by which pN02DCA inactivates BNF-B. This suggests that the mechanism by which chloramphenicol analogs inactivate cytochromes P-450 is isozyme-dependent.

In conclusion, pN02DCA has been shown to be an effective and potent mechanism-based inactivator of BNF-B in *vitro* and *in vivo*. pN02DCA is therefore one of the few
mechanism-based inactivators of BNF-B described to date and has the potential to be used in vivo to monitor the metabolism of xenobiotics by BNF-B. The mechanism by which pNO₂DCA inactivates BNF-B was also examined and shown to be significantly different from that by which chloramphenicol inactivates PB-B, although both involve an impairment in electron transfer from NADPH-cytochrome P-450 reductase. With further research into the mechanism of inactivation, it may be possible to use pNO₂DCA to gain further insight into the nature of the active site of BNF-B.
CHAPTER 4

CONCLUSION

The research which has been described here stems largely from the need for effective and isozyme-specific mechanism-based inactivators of cytochromes P-450. While many new enzyme inhibitors have been discovered by the traditional method of screening large numbers of compounds for inhibitory activity, this process is both time consuming and expensive. A more effective means of developing new enzyme inhibitors is by actually designing inhibitors based on a knowledge of the structure of other inhibitors and the mechanisms by which they inactivate the enzyme. Chloramphenicol has previously been shown to be a mechanism-based inactivator of several isozymes of cytochrome P-450, but chloramphenicol is not isozyme specific, which limits its use in determining the role of particular isozymes in xenobiotic metabolism and in redirecting metabolism of certain compounds. It was hypothesized, therefore, that by determining the importance of different functional groups of chloramphenicol in regulating its effectiveness and isozyme specificity, it might be possible to design more effective and more specific inactivators.
One of the major findings of the data described here is that only the metabolism of the dichloromethyl moiety of chloramphenicol is necessary for the mechanism-based inactivation of cytochromes P-450. This has been further borne out in recent studies in which a large number of compounds which contained the dichloromethyl moiety as the common structural element were examined for their ability to inactivate the major phenobarbital-inducible isozyme PB-B (Table 8). These studies have all suggested that like certain other functional groups such as the triple-bond (Ortiz de Montellano and Kunze, 1980), the double-bond (Ortiz de Montellano and Mico, 1980), and the thiono-sulfur group (Neal and Halpert, 1982) the dichloromethyl group confers upon a molecule the ability to inactivate cytochromes P-450 (Halpert, Balfour, Miller and Kaminsky, 1986).

The isozyme specificity and inhibitory potency of chloramphenicol is not dependent upon the dichloromethyl group but rather upon the remainder of the molecule. Several analogs, such as pNO_2 DCA, have been synthesized which are much more effective than chloramphenicol as inactivators of PB-B in vitro and in vivo. In addition, it has been demonstrated that the pNO_2 group is not necessary for the inactivation of PB-B. This is important, as the pNO_2 group is believed to be primarily responsible for the ability of chloramphenicol to produce aplastic anemia in
Table 8. 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 for pNO₂DCA in Materials and Methods, Chapter 1. (From Halpert et al., 1986).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>$k_{\text{inact}}$</th>
<th>Percentage of cytochrome P-450 lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (methanol)</td>
<td>0.02 ± 0.01 (n = 19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>p-NO₂-Ph(CH₂)₂-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.59 ± 0.05</td>
</tr>
<tr>
<td>B</td>
<td>Ph(CH₂)₂-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.54 ± 0.01</td>
</tr>
<tr>
<td>C</td>
<td>Ph(CH₂)₂-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>D</td>
<td>Ph(CH₂)₂-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>E</td>
<td>p-NO₂-Ph-CH₂-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>F</td>
<td>p-NO₂-Ph-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>G</td>
<td>p-Cl-Ph-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>H</td>
<td>Ph-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>I</td>
<td>Ph-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>J</td>
<td>Ph-CHCl₂</td>
<td>100 μM</td>
<td>0.75 ± 0.01</td>
</tr>
<tr>
<td>K</td>
<td>CH₂(CH₂)₂-NH-CO-CHCl₂</td>
<td>5 μM</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>L</td>
<td>CH₂(CH₂)₂-NH-CO-CHCl₂</td>
<td>20 μM</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>M</td>
<td>CH₂(CH₂)₂-NH-CO-CHCl₂</td>
<td>100 μM</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>N</td>
<td>CH₂(CH₂)₂-C-NH-CO-CHCl₂</td>
<td>100 μM</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>O</td>
<td>CH₂-NH-CO-CHCl₂</td>
<td>1000 μM</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>P</td>
<td>CH₂CO-CO-CHCl₂</td>
<td>500 μM</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Q</td>
<td>CH₂CO-CHCl₂</td>
<td>1000 μM</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>R</td>
<td>NH₂-CO-CHCl₂</td>
<td>100 μM</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>S</td>
<td>CHCl₂-CO-CHCl₂</td>
<td>100 μM</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>T</td>
<td>CH₂Cl₂</td>
<td>10 mM</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

*ND* indicates not determined.
man (Yunis et al., 1980). While no analog of chloramphenicol has yet been found to be completely isozyme specific, the data described here do demonstrate that the isozyme selectivity can be changed by altering functional groups such as the propanediol side chain. Some promising results have been found recently with chloramphenicol analogs containing additional phenyl rings. For example, N-(2-phenethyl) dichloroacetamide does not significantly inactivate the isozyme responsible for the 6-beta-hydroxylation of androstenedione, but does inactivate the isozyme BNF-B. However, the addition of a second phenyl group to the methylene carbon adjacent to the amide nitrogen of N-(2-phenethyl) dichloroacetamide greatly enhances the rate of inactivation of the 6-beta-hydroxylase, but abolishes the ability to inactivate BNF-B (Jeff Stevens, personal communication).

Alterations in different functional groups of chloramphenicol have also been shown to affect the ability to inactivate the isozyme PB-B by covalent modification of the protein moiety as opposed to heme modification. The target of the inactivation is important for several reasons. First, inhibitors which bind to the protein moiety are potentially more isozyme selective. In addition, compounds which modify the heme may not be useful in vivo. As mentioned in Chapter 1, hepatic porphyrias may develop with many of these compounds. Thus, chloramphenicol analogs
which inactivate cytochrome P-450 by protein modification may be preferable for use \textit{in vivo}.

The information which has been described concerning the importance of the different functional groups of chloramphenicol in determining its effectiveness, isozyme selectivity and mechanism of inactivation has provided a foundation for the design of even more effective and isozyme specific mechanism-based inactivators of cytochromes P-450. Perhaps the most promising approach to the design of isozyme-specific inhibitors involves the attachment of the dichloromethyl moiety to substrates which are only metabolized by a single isozyme. Progesterone, for example, is hydroxylated at the 21-position by only a single rabbit liver cytochrome P-450 isozyme, P-450 1. The analog 21,21 dichloroprogesterone, which contains a dichloromethyl group in place of the methyl group at the 21-position of progesterone is currently being examined as a specific inactivator of the P-450 1 isozyme.

The second objective of this research was to determine if chloramphenicol analogs could be used to probe the active site of different cytochrome P-450 isozymes. This required an understanding of the mechanism by which certain chloramphenicol analogs inactivate different P-450 isozymes. The analog N-(2-p-nitrophenethyl) dichloroacetamide (\textit{pNO}_2\textit{DCA}) was chosen as a prototype for
these studies for several reasons:

1. $p\text{NO}_2\text{DCA}$ is even more effective than chloramphenicol as a mechanism-based inactivator of the isozyme PB-B

2. $p\text{NO}_2\text{DCA}$ differs from chloramphenicol in its isozyme selectivity in that it inactivates BNF-B in addition to PB-B.

3. $p\text{NO}_2\text{DCA}$ inactivates BNF-B with a stoichiometry of one nmol $p\text{NO}_2\text{DCA}$ metabolites bound per nmol of BNF-B inactivated, which suggested that $p\text{NO}_2\text{DCA}$ might bind to a single amino acid residue within the active site of BNF-B.

The results have shown that $p\text{NO}_2\text{DCA}$ inactivates PB-B and BNF-B by a pathway similar to that by which chloramphenicol inactivates PB-B, with formation of a lysine adduct as well as a labile adduct. The formation of more than one amino acid adduct with $p\text{NO}_2\text{DCA}$, as with chloramphenicol, makes it difficult to determine the role of particular amino acid residues in the catalytic functions of cytochrome P-450, such as the interaction with cytochrome P-450 reductase. In addition, the inactivation of both PB-B and BNF-B could result from steric disturbances due to the presence of the bulky $p\text{NO}_2\text{DCA}$ metabolite rather than to the modification of particular amino acid residues. Attempts to use much smaller analogs to help answer the question of steric hindrance have been unsuccessful, however, as the compounds examined destroy the heme rather than binding to the apoprotein.
While pNO$_2$DCA metabolites covalently bind to lysine and other amino acid residues of both PB-B and BNF-B, the mechanism by which such adduct formation leads to enzyme inactivation differs for the two isozymes. The differences in the mechanism of inactivation demonstrate that there are significant differences in the catalytic sites of these isozymes, even though both isozymes are able to metabolize pNO$_2$DCA to the same reactive intermediate, p-nitrophenethyl oxamyl chloride. With further research it should be possible to determine how the catalytic sites differ.

In conclusion, with the knowledge of the importance of the different functional groups of chloramphenicol in determining the effectiveness and isozyme selectivity of chloramphenicol as a mechanism-based inactivator of cytochromes P-450, it should soon be possible to design isozyme specific inactivators of cytochromes P-450 which can be used \textit{in vivo}. The diagnostic and therapeutic value of such compounds in man is potentially quite high. This is particularly true as recent studies have indicated that the amounts and activities of different cytochrome P-450 isozymes play key roles in the polymorphisms of drug metabolism observed in man (Gut et al, 1986). These polymorphisms may be associated with the variations in susceptibility to carcinogens observed in different populations. Isozyme-specific inactivators would be particularly useful in determining the presence of different
isozyms in different tissues and in different populations and relating these to particular polymorphisms. In addition, it may eventually be possible to use analogs of chloramphenicol in vivo to direct the metabolism of xenobiotics in susceptible populations towards detoxication.
APPENDIX A

SUPPLEMENTAL FIGURES AND TABLES
Figure 17. Inactivation of purified cytochrome P-450 by pN02TCA, pN02MCA and deschloramphenicol. The assay was carried out as described in Materials and Methods, Chapter 2. Cytochrome P-450 was incubated with 20 uM pN02TCA (O), 100 uM pN02MCA (△), 100 uM deschlorochloramphenicol (△) and no inhibitor (●).
Figure 18. Chromatography of the alkaline hydrolysate of \( ^{14}\text{C} \)-labeled microsomes from phenobarbital-induced rats after incubation with \( \text{pNO}_2\text{DCA} \). The hydrolysate was run on a Bio-Rad P-2 column (1.5 \( \times \) 45 cm) in \( 0.05 \text{ M N-ethylmorpholine buffer (pH 7.5) as described in Materials and Methods, Chapter 2. The synthetic standard 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide was added prior to chromatography. This standard was monitored at an absorbance of 280 nm (\( \text{A}_{280} \)) and the labeled adducts by scintillation counting (\( \text{A}_{280} \)). Peak B co-chromatographed with 4-nitro-1-phenethyl-1,2-dicarboxylic acid amide. Peak A was identified as oxalic acid by co-chromatography with authentic oxalic acid on a Bio-Rad P-2 column with another \( ^{14}\text{C} \)-labeled microsome preparation.
Figure 19. Cytochrome P-450 content of pNO₂DCA-modified BNF-B. Cytochrome P-450 content was monitored as described in Materials and Methods, Chapter 2. (-----) pNO₂DCA-modified BNF-B, (-----) native BNF-B.
Figure 20. Cytochrome P-450 content of pNO₂DCA-modified PB-B. Cytochrome P-450 content was monitored as described in Materials and Methods, Chapter 2. (-----) pNO₂DCA-modified PB-B, (----) native PB-B.
Table 9. Comparison of characteristics of pNO$_2$DCA-modified PB-B and pNO$_2$DCA-modified BNF-B. pNO$_2$DCA-modified PB-B and BNF-B are compared to native PB-B and BNF-B, respectively.

<table>
<thead>
<tr>
<th>Parameter monitored</th>
<th>pNO$_2$DCA-modified PB-B</th>
<th>pNO$_2$DCA-modified BNF-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength maximum for \ dithionite-reduced ferrous carbonyl complex</td>
<td>shift</td>
<td>no shift</td>
</tr>
<tr>
<td>NADPH-supported metabolism of ethoxycoumarin</td>
<td>↓ a</td>
<td>↓</td>
</tr>
<tr>
<td>Iodosobenzene-supported metabolism of ethoxycoumarin</td>
<td>b n.d.</td>
<td>c no change</td>
</tr>
<tr>
<td>NADPH oxidase activity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- ethoxycoumarin</td>
<td>↓</td>
<td>no change</td>
</tr>
<tr>
<td>- ethoxycoumarin</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>Steady-state level of enzymatically-formed ferrous carbonyl complex</td>
<td>↓</td>
<td>no change</td>
</tr>
<tr>
<td>Stoichiometry of inactivation (nmol 14C bound/nmol P-450 inactivated)</td>
<td>1.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

a decreased compared to the native enzyme
b not determined
c not significantly different from the native enzyme
APPENDIX B

PUBLICATIONS


6. Miller, N.E. and J.R. Halpert. Mechanism-based inactivation of the major beta-naphthoflavone-inducible isozyme of rat liver cytochrome P-450 by the chloramphenicol analog N-(2-p-nitrophenethyl) dichloroacetamide. Accepted for publication in Drug Metab. and Disp.

Papers Presented:


115

LIST OF REFERENCES


