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Selective inactivation of four rat liver microsomal androstenedione hydroxylases by chloramphenicol analogs

Stevens, Jeffrey Charles, M.S.
The University of Arizona, 1988
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This thesis has been approved on the date shown below:

JAMES HALPERT
Associate Professor of Pharmacology and Toxicology
ACKNOWLEDGEMENTS

I wish to express my gratitude to my advisor Dr. James Halpert for his personal support and scientific guidance and also to Dr. I. Glenn Sipes and Dr. Dean Carter for their constructive criticism of this project.

A simple acknowledgement to my family for the support that they have shown for my personal goals seems inadequate. Their continual encouragement during this project provided the confidence I needed to pursue a scientific career.
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Abstract

The steroid androstenedione has been shown to be a valuable tool for the study of selective inactivation of rat liver cytochrome P-450 isozymes. The validity of this method was investigated using microsomes, purified cytochromes P-450, cytochrome P-450 antibodies, and the mechanism-based inactivator chloramphenicol. Enzyme inactivation and antibody inhibition studies show that microsomes from phenobarbital- and non-phenobarbital-treated rats are needed to accurately monitor the inactivation of the major phenobarbital-inducible P-450 isozyme (PB-B) and of the major constitutive androstenedione 16-alpha hydroxylase (UT-A). Enzyme inactivation studies showed that the antibiotic chloramphenicol caused different rates of NADPH-dependent enzyme inactivation among four androstenedione hydroxylases (16-beta > 6-beta > 16-alpha > 7-alpha). The results with twelve chloramphenicol analogs show that their selectivity as cytochrome P-450 inactivators is dependent upon at least three structural features: 1) the number of halogen atoms, 2) the presence of a para-nitro group on the phenyl ring, and 3) substitutions on the ethyl side chain.
Chapter 1
Introduction

Cytochrome P-450

The cytochrome P-450-dependent monooxygenase system catalyzes the biotransformation of many endogenous compounds and xenobiotics ranging from pharmaceuticals to environmental pollutants and carcinogens (Lu and West, 1980). The name cytochrome P-450 was first used by Omura and Sato in 1964 to describe an iron-containing protein that in the reduced form bound carbon monoxide to give a characteristic absorption maximum at 450 nm (Omura and Sato, 1964). Later studies established that this protein is the terminal oxidase of the liver microsomal drug-metabolizing enzyme system (Cooper et al., 1965; Omura et al., 1965). In general, this enzyme system is responsible for the metabolism of lipophilic compounds to more water-soluble products that are more readily excreted by mammals in the bile or urine.

The liver contains by far the highest amount of cytochrome P-450. Lesser amounts are located in the kidney, lung, gastrointestinal tract, gonads, and skin, as well as other tissues. Mammalian anatomy and physiology are well suited to complement this enzyme distribution and maximize metabolism and detoxification. Compounds administered to animals via oral or intraperitoneal routes may be transported by the portal circulation to the liver before distribution to other parts of the body. Some drugs such as the
anesthetic lidocaine are extensively metabolized by the hepatic cytochrome P-450 system before reaching the systemic circulation. The parent compound therefore has reduced pharmacologic activity due to this so called first-pass effect (Boyce, Adams and Duce, 1970).

At the cellular level, cytochrome P-450 is found anchored in the phospholipid bilayer of the endoplasmic reticulum. The second major component of this enzyme system, the flavoprotein NADPH-cytochrome P-450 reductase, is found in close proximity to cytochrome P-450. Concentrated samples of enzymatically active cytochrome P-450 are obtained by first mechanically shearing the liver tissue. Microvesicles of endoplasmic reticulum membrane are formed, and these microsomes are then isolated by differential centrifugation. Research in drug metabolism often utilizes the in vitro microsomal metabolism of compounds as a simple, preliminary indicator of biotransformation reactions in vivo.

Cytochrome P-450-catalyzed oxidative metabolism involves the reaction of an enzymatically activated oxygen atom with particular chemical bonds of the substrate. The catalytic cycle of cytochrome P-450 reactions has been studied by several research groups, and the currently accepted mechanism is shown in Figure 1. The six sequential catalytic steps are as follows (Estabrook and Werringloer, 1977):

1) the oxidized ferric (Fe\textsuperscript{3+}) form of the P-450 hemoprotein forms a complex with the substrate,

2) the enzyme-substrate complex accepts one electron from
NADPH via the cytochrome P-450 reductase, resulting in the ferrous (Fe$^{+2}$) form of the enzyme,

3) the reduced iron binds oxygen to form oxycytochrome P-450. This is the step where carbon monoxide may also bind to give the characteristic absorbance,

4) another electron is then donated by the reductase or from cytochrome b$_{5}$ to the complex,

5) the oxygen–oxygen bond is cleaved, and one oxygen atom is incorporated into the substrate and the other forms a molecule of water,

6) the metabolic product dissociates from the regenerated form of the enzyme.

**Cytochrome P-450 Mediated Reactions**

The reactions involved in drug metabolism have been subdivided into two categories, which distinguish not only the chemical reactions but also the subcellular location of the enzymes involved. Cytochromes P-450 are generally considered the predominant phase I enzymes which catalyze the oxidation, reduction, or hydrolysis of substrates to a more water-soluble metabolite. This phase I product may then be conjugated to an endogenous hydrophilic compound by means of one or more biosynthetic phase II enzymes usually found in the cytosol. Glucuronidation, sulfation, glutathione conjugation, amino acid conjugation, and acetylation reactions occur preferentially at various functional groups on the compound (as reviewed by Sipes and Gandolfi, 1986). (The enzyme responsible for glucuronidation, uridine diphosphate glucuronyltransferase, is a
Figure 1. Catalytic cycle of cytochrome P-450.
(From Benet and Sheiner, 1985)
non-cytosolic, membrane-bound enzyme of the endoplasmic reticulum). The classic approach to drug metabolism has been to consider the integration between the phase I and phase II reactions of drug metabolism: phase I enzymes add or expose chemical functional groups necessary for phase II conjugation reactions. The ultimate metabolite contains one or more water soluble ionizable groups which enable rapid elimination of the compound. However, this oversimplification has lead to the emergence of many exceptions which suggest that phase I is not a prerequisite for or more predominant than phase II conjugation reactions in the metabolism of xenobiotics. For example, in normal individuals only 4% of a dose of acetaminophen undergoes phase I reactions while 94% is excreted as either the product of glucuronidation or sulfation (Rumack and Lovejoy, 1986). This is because acetaminophen has a pre-existing functional groups.

The cytochrome P-450 system catalyzes the reactions shown in Table 1 (Gillette, 1966). One reaction of interest to this work is oxidative dehalogenation. The ability of mammals to effectively remove halogen atoms from xenobiotics is critical in light of the continuous introduction of these compounds into the modern environment. Insecticides, pesticides, antibacterial agents, anesthetics, cleaning agents, propellants, and organic solvents represent the major threat of halogenated compounds to man. One method of metabolism of these compounds is oxidative dehalogenation. The cytochrome P-450 activated oxygen attacks at a carbon-hydrogen bond to produce a reactive halohydrin which is then dehalogenated.
Table 1. Reactions Catalyzed by Liver Microsomal Cytochrome P-450

(From a review by Gillette. 1966)

<table>
<thead>
<tr>
<th>Aromatic hydroxylation</th>
<th>N-Oxidation</th>
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<tr>
<td>Aliphatic hydroxylation</td>
<td>Sulfoxidation</td>
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<td>N-Dealkylation</td>
<td>Dehalogenation</td>
</tr>
<tr>
<td>S-Dealkylation</td>
<td>Azoreduction</td>
</tr>
<tr>
<td>O-Dealkylation</td>
<td>Nitroreduction</td>
</tr>
<tr>
<td>Deamination</td>
<td>Peroxidation</td>
</tr>
<tr>
<td>Desulfuration</td>
<td>Epoxidation</td>
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As will be explained later, mechanistic studies have shown that the dichloroacetamide antibiotic chloramphenicol undergoes oxidative dehalogenation to an oxamyl chloride intermediate which then can modify the protein portion of the cytochrome P-450 enzyme (Pohl, Nelson and Krishna, 1978; Pohl and Krishna, 1978).

Because of the progress made during the last ten years in the isolation and characterization of multiple cytochrome P-450 isozymes, investigations into cytochrome P-450-related biochemical toxicology have been forced to move beyond characterizing the biotransformation of compounds as simply "cytochrome P-450 mediated". At least fifteen distinct rat hepatic cytochrome P-450 isozymes of varying substrate specificity and catalytic activity have been isolated by different laboratories (Waxman, 1986). An example of the complexity of these multiple enzyme forms can be seen with two related isozymes induced by phenobarbital. Isozymes P-450b and P-450e (corresponding to P-450 PB-B and P-450 PB-D, Guengerich et al., 1982) have identical N-terminal amino acid sequences for the first 396 amino acid residues (overall > 97% homologous), similar molecular weights by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and exhibit cross reactivity in immunological studies (reviewed in Conney, 1986). Yet the fact that there are 13 amino acid differences between these two proteins indicates that they are distinct gene products. In addition, the homologous structure of these proteins does not correspond to similar catalytic activities. Purified cytochrome P-450 PB-D has been shown to have only a fraction of
the catalytic activity of P-450 PB-B toward classical P-450 substrates when studied in a reconstituted system (Conney, 1986).

**Induction of Cytochromes P-450**

As was previously mentioned, different cytochrome P-450 isozymes can be induced by administration of various xenobiotics or steroid hormones (as reviewed by Guengerich and Liebler, 1985). The most extensively studied inducing agents such as phenobarbital, 3-methylcholanthrene, isosafrole, beta-naphthoflavone, clofibrate, and pregnenolone-16-alpha-carbonitrile can increase the levels of a particular isozyme from nearly undetectable levels to a significant percentage of total tissue P-450. These changes in specific P-450 isozyme levels may drastically alter the rate of metabolism of their corresponding substrates. Clinical pharmacologists must be knowledgeable of enzyme induction, since a patient receiving the anticoagulant warfarin or dicoumarol may exhibit a decreased response to a given drug dose if the enzyme inducer phenobarbital (therapeutically used as a sedative or anticonvulsant) is also a part of the patients drug regimen (Robinson and MacDonald, 1966).

Enzyme induction is considered an environmental factor which can partially account for certain individual variations in microsomal drug metabolism. However, the fact that there are differences in the susceptibility of individuals to induction and differences of six-fold or more in individual rates of metabolism suggests a genetic cause (Vesell, 1977). Vogel is credited with defining the study of the genetic basis of drug metabolism as pharmacogenetics (Weinshilbaum, 1984). Research in the study of genetic polymorphism usually begins
with a clinical observation of significant differences in any of three
determinations; a) steady-state plasma drug levels, b) individual
differences in drug response, and/or c) unforeseen drug toxicity.
For example, Mahgoub and coworkers (1977) found a bimodal
distribution of the metabolic ratio (amount of drug/ amount of drug
metabolite in urine) for the adrenergic-blocking drug debrisoquine in
a clinical study of 94 British volunteers. Soon after, it was reported
that those people having the "poor metabolizer" phenotype had an
increased drug response and incidence of side effects to debrisoquine
(Idle and Smith, 1979). The clinical question recently received a
biochemical answer with the purification and characterization of the
human liver cytochrome P-450 isozyme involved in debrisoquine 4-
hydroxylation (Disterlath et al., 1985; Meyer et al., 1985). The next
question to be answered is whether this genetic polymorphism is due
to different enzyme levels or expression of a 'defective' form of the
enzyme. One potential application of a detailed pharmacogenetic
study is to screen patients for phenotype in order to insure the
administration of a pharmacologically effective yet non-toxic dose of
a particular drug.

Inactivation of Cytochrome P-450 Isozymes

The multiplicity of mammalian hepatic cytochrome P-450
isozymes presents a challenge to investigations into the role of the
different P-450 isozymes in the metabolism of various compounds in
vivo. One approach to this problem has been the development of
chemical inhibitors of cytochrome P-450 enzymes. The
pharmacological use of specific inhibitors of other enzyme systems is
an integral part of modern patient care and therefore the object of intense research and development by the pharmaceutical industry. For example, the drug allopurinol inhibits the enzyme xanthine oxidase and thereby controls the production of uric acid. The process ultimately alleviates a painful arthritic condition known as gout (Meyers, Jawetz, and Goldfien, 1980). In an analogous manner, the objective of this work is to develop isozyme-specific cytochrome P-450 inhibitors as probes which the toxicologist may use in studying and possibly altering cytochrome P-450 mediated metabolism.

**Chloramphenicol.** The antibiotic chloramphenicol (Figure 2) was first isolated by Burkholder in 1947. The substance was identified, prepared synthetically, and given the trade name chloromycetin to signify that it contained chlorine and was produced by an actinomycete mold (Sande and Mandell, 1985). Parke Davis obtained the pharmaceutical patent and marketed the drug as a broad spectrum antibiotic. However, results from the first three years of usage showed an unusually high incidence of serious hematologic disorders in patients administered chloramphenicol. Consequently, other antibiotics were substituted in drug regimens, and research began into the mechanism by which chloramphenicol causes aplastic anemia. Studies suggest that the nitro group of the compound may be metabolized to an intermediate ultimately responsible for the hematological toxicity. Microflora of the gastrointestinal tract may produce this metabolite via reductive metabolism (as discussed in Sande and Mandell, 1985). Although a definitive answer to this problem has evaded scientists, other interesting questions regarding
Figure 2. Structure of chloramphenicol.
pharmacologic and toxicologic properties of chloramphenicol have been investigated.

Chloramphenicol is administered orally and has a large volume of distribution, which includes crossing of the blood/brain and placental barriers. The compound is metabolized by hepatic cytochromes P-450 and excreted mainly as the glucuronide conjugate. Infants and children must be given reduced chloramphenicol dosages and plasma drug levels closely monitored since glucuronidation ability is not fully developed. "Grey-baby syndrome" is the clinical term which describes this serious intoxication. Generally, chloramphenicol is only used in the adult in situations where the benefits of eradicating a drug resistant bacterial infection outweigh any potential harmful effects.

Another clinical side effect of chloramphenicol is the inhibition of oxidative drug metabolism (Dixon and Fouts, 1962). The plasma concentrations of tolbutamide, dicoumarol, and diphenylhydantoin were shown to be increased by a single dose of chloramphenicol (Christensen and Skovsted, 1969). Later, several studies using rat liver microsomes showed that chloramphenicol inhibits cytochromes P-450 by acting as a suicide substrate (Halpert and Neal, 1980; Halpert, 1982; Halpert, Naslund and Betner, 1983). The selectivity of this cytochrome P-450 enzyme inactivation by chloramphenicol and structural analogs is the focus of this project.

Among chemical inhibitors, mechanism-based inactivators or suicide substrates are potentially the best candidates for selective cytochrome P-450 inhibition (Abeles, 1978). This statement is based
Specifically, the compound must first bind to the active site of the enzyme and then be converted to a reactive species. This intermediate then forms a covalent bond with the enzyme or prosthetic group thus inactivating the enzyme (Walsh, 1984). As shown in Figure 3, (Ortiz de Montellano and Reich, 1987; Halpert, 1981) the dichloromethyl moiety of chloramphenicol undergoes oxidative dechlorination by cytochrome P-450 to yield a reactive oxamyl chloride intermediate. This product may then undergo hydrolysis to give the oxamic acid or acylate one or more lysine amino acids of the cytochrome P-450 protein (Halpert, 1981; Halpert, 1982; Halpert, Naslund and Betner, 1983). A subsequent study showed that this acylation renders the enzyme inactive by impairing the cytochrome P-450/NADPH-cytochrome P-450 reductase electron transfer necessary for catalytic activity (Halpert, Miller and Gorsky, 1985).

These mechanistic studies clearly prompted further investigations into the effect of chloramphenicol on different cytochrome P-450 isozymes both in vivo and in vitro. Halpert et al. (1985) utilized four different methods for monitoring particular P-450 isozymes and concluded that chloramphenicol administration in vivo inactivates four out of nine rat liver cytochromes P-450. Then in an attempt to identify which structural features are required for inactivation, chloramphenicol analogs were synthesized. It was found that the rate and selectivity of isozyme inactivation in vitro can be altered by modifying structural features other than the dichloromethyl
Figure 3. Proposed mechanism for the inactivation of cytochrome P-450 PB-B by chloramphenicol (Halpert, 1981; reviewed by Ortiz de Montellano and Reich, 1987).
group, the site of metabolism (Halpert et al., 1986; Miller and Halpert, 1986).

**Monitoring Selective Enzyme Inactivation**

By definition, selective enzyme inactivation can be accurately studied and characterized only when an appropriate biochemical assay has been developed for each isozyme of interest. The substrates shown in Table 2 are traditionally used to monitor cytochrome P-450 activity. However, these compounds are general cytochrome P-450 substrates and may be metabolized to different extents by many isozymes. For example, benzphetamine demethylation is catalyzed by cytochrome P-450 PB-B and also the UT-A isozyme to a lesser extent (Astrom and DePierre, 1986). In contrast, two beta-naphthoflavone-inducible and immunochemically similar cytochrome P-450 isozymes, designated ISF-G and BNF-B, can be distinguished by their ability to regiospecifically hydroxylate the isozyme-specific P-450 substrate 17-beta-estradiol (Dannan et al., 1986).

As mentioned earlier in "Cytochrome P-450 Mediated Reactions", various inducing agents can be used to increase the levels of certain cytochrome P-450 isozymes while repressing the expression of others. This technique is critical to measuring the activity of certain isozymes expressed at low constitutive levels such as the inducible cytochromes PB-B and BNF-B (induced by phenobarbital and beta-naphthoflavone, respectively). Although the overall catalytic activities of particular cytochrome P-450 isozymes in microsomes can be altered by the judicious use of inducing agents, this enhances but
Table 2. Some Compounds Metabolized by Cytochromes P-450

- 7-ethoxycoumarin
- p-nitroanisole
- d-benzphetamine
- aminopyrine
- ethylmorphine
- acetanilide
- benzo[a]pyrene
- aniline
does not replace the need for the detection of these activities using isozyme specific assays.

The metabolism of endogenous steroid hormones such as androstenedione (Figure 4) has been studied in several laboratories using both microsomal preparations and purified cytochromes P-450 in a reconstituted system. Early investigations on steroid metabolism were largely qualitative. Conney and Klutch (1963) reported an increase in the hydroxylation of testosterone and androstenedione in liver microsomes of rats treated with phenobarbital and chlorcyclizine. More descriptive kinetic studies found that hepatic cytochrome P-450 has an approximately thousand-fold higher affinity for endogenous steroids relative to xenobiotic substrates. This led to speculation that endogenous steroids could be the physiological substrates of liver cytochromes P-450 (Kuntzman, Lawrence and Conney, 1965). However, in their review article on microsomal steroid metabolism, Conney and Kuntzman (1971) recognize that the specific biochemical questions of which cytochromes are involved in each reaction can only be answered after the microsomal system has been solubilized and purified.

Advancements in cytochrome P-450 purification and characterization brought an increase in the number of investigations into the in vitro metabolism of foreign and endogenous substrates by reconstituted cytochrome P-450 preparations. Many of the constitutive and xenobiotic-inducible P-450 isozymes shown to have broad specificity with classical substrates (Table 2)(Guengerich et al., 1982), have been shown to regio- and stereoselectively hydroxylate
Figure 4. Structure of androstenedione showing the major sites of cytochrome P-450 catalyzed hydroxylation.
androstenedione (Table 3) (Waxman, Ko and Walsh, 1983; Wood et al., 1983). In addition to the examples mentioned earlier, the constitutive rat hepatic cytochrome P-450 UT-F was reported to carry out the isozyme specific formation of 7-alpha-OH androstenedione. This enables the monitoring of an enzyme which did not metabolize any of the classical P-450 substrates to any appreciable extent (Ryan et al., 1979, Guengerich et al., 1982).

Further studies have shown that specific steroid hydroxylation reactions can be altered by factors such as species, sex, age, hormone levels, diet, and the administration of cytochrome P-450 inducers. Phenobarbital and other cytochrome P-450 inducing agents were shown to decrease the pharmacologic effectiveness of a synthetic glucocorticoid used in treating steroid-dependent asthmatics (Brooks et al., 1972). Examples of unwanted pregnancies occurring with women using oral contraceptives (synthetic estrogens and progestational steroids) in combination with anticonvulsants (i.e. phenobarbital) or the antibiotic, rifampin, have also been noted (Levin, Kuntzman and Conney, 1979). Human cigarette smokers have been shown to have reduced serum testosterone levels compared to nonsmokers (Shaarawy and Mahmoud, 1982), presumably because of inducing agents known to be present in smoke.

Statement of Problem. One approach to studying the effects of potential enzyme inactivators is to monitor residual enzyme activity with a substrate which is metabolized by multiple isozymes, each at a specific site. For this purpose, we have used androstenedione since several laboratories have shown that hydroxylation of this substrate
Table 3.
Cytochrome P-450 Isozymes Responsible for Androstenedione Metabolism.

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<th>Androstenedione Hydroxylase Activity</th>
<th>Nomenclatures Applied to Cytochromes P-450 Responsible</th>
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<tr>
<td></td>
<td>Levin&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>7-alpha</td>
<td>P-450&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>6-beta</td>
<td>P-450&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td>16-beta</td>
<td>P-450&lt;sup&gt;bb&lt;/sup&gt;</td>
</tr>
<tr>
<td>16-alpha</td>
<td>P-450&lt;sup&gt;hc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> The cytochrome P-450 nomenclatures used here represents those adopted by W. Levin and F. P. Guengerich.

<sup>b</sup> (Wood et al, 1983)

<sup>c</sup> (Ryan et al, 1984)

<sup>d</sup> (Waxman, Dannan, and Guengerich, 1985)

<sup>e</sup> (Waxman, 1984)
in each of four positions (7-alpha, 6-beta, 16-alpha, and 16-beta) can largely be attributed to a single cytochrome P-450 isozyme. Others have noted the usefulness of the correlation between the formation of specific steroid metabolites and the presence of particular P-450 isozymes in aiding the comparison of P-450 preparations isolated from different laboratories or in studying the contribution of different isozymes to overall microsomal activity (Waxman, Ko and Walsh, 1983). However, we hypothesized that the monitoring of certain androstenedione hydroxylase activities might be an effective and appropriate method for testing a large number of compounds as potentially selective enzyme inactivators. The present report describes the application of this approach to the design of chloramphenicol analogs with enhanced isozyme selectivity.
Materials and Methods

Materials.

Androst-4-ene-3,17-dione <4-14C> (52.0 mCi/mmol) was purchased from NEN Research Products (Boston, MA). The compound was determined to be greater than 98% radiochemically pure. Unlabelled androstenedione and 6-beta-OH-androstenedione were purchased from Steraloids (Wilton, NH). NADPH, beta-naphthoflavone, chloramphenicol, 16-alpha-OH-androstenedione, and dilauryl L-3-phosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid), sodium cholate, and sodium deoxycholate were purchased from Calbiochem-Behring, La Jolla, CA. The compounds N-methylphenethylamine, 1,2-diphenethylamine, 2,2-diphenethylamine, phenethylamine, 3-phenyl-1-propylamine, 4-nitrobenzylamine hydrochloride, chloroacetyl chloride, dichloroacetyl chloride, 7-hydroxycoumarin, and 7-ethoxycoumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pregnenolone-16-alpha-carbonitrile (PCN) and 7-alpha-OH-androstenedione were gifts from the Upjohn Co. (Kalamazoo, MI) and Dr. David Waxman (Harvard Medical School, Boston, MA), respectively. N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenethyl] chloroacetamide (deschlorochloramphenicol) was a gift from Dr. Lance Pohl (National Institutes of Health, Bethesda, MD). DEAE-Sephasel and Sepharose 4B were purchased from Pharmacia Fine Chemicals (Piscataway, NJ).
Hypatite C was purchased from Clarkson Chemical Co. (Williamsport, PA). The reagents and molecular weight standards for gel electrophoresis and immunochemical studies were purchased from Bio-Rad (Richmond, CA).

**Synthesis of Chloramphenicol Analogs**

Synthesis of dichloro- and chloroacetamides was conducted by the method of Rebstock (1950) and is represented schematically in Figure 5. The acetylated products were isolated from the organic phase of the reaction mixture and recrystallized from aqueous methanol as previously described for the synthesis of N-(2-p-nitrophenethyl) dichloroacetamide and N-(2-phenethyl) dichloroacetamide (Miller and Halpert, 1986). Compounds were analyzed for purity by gas-liquid chromatography using a Hewlett Packard 5790A gas chromatograph equipped with a flame ionization detector. The instrument was fitted with a glass column (1/4" x 6') packed with Chromosorb W 100/120 coated with a stationary phase of 1.5% OV-17. Nitrogen served as the carrier gas and was adjusted to a flow rate of 30 ml/min. The detector gas flow rates were 30 ml/min and 240 ml/min for H₂ and air, respectively. Oven temperature was kept at 80° for 1 min. and increased at a rate of 20°/min to a final temperature of 270°. The injector and detector temperatures were 220° and 235° respectively. Purity was determined by peak area analysis with the assumption that the analyte and any impurity responded similarly to the detector. Analyte purity of greater than 97% was considered acceptable pending results of other methods of analysis. The general analog structure is shown in Figure
Figure 5. Method for the synthesis and isolation of chloramphenicol analogs.
6 with the specific structural substitutions given in Table 4. All chloramphenicol analogs were characterized by uncorrected melting point and by NMR (\(^1\)H, 60 or 250 MHz, CDCl\(_3\), TMS internal standard) S (ppm) as follows: N-(2-phenethyl) chloroacetamide (C), 63-64.5° (lit. 67-67.5°) (Mukaiyama, 1972), 2.9 (t, 2H, J=7.0Hz), 3.6 (q, 2H, J=6.7Hz), 4.0 (s, 2H), 7.2-7.4 (m, 5H); N-(2-p-nitrophenethyl) chloroacetamide (E), 93-95° (lit. 100°) (Isshiki and Kuwata, 1951), 3.0 (t, 2H, J=7.0Hz), 3.6 (q, 2H, J=7.0Hz), 4.0 (s, 2H), 7.4 (d, 2H), 8.2 (d, 2H); N-(3-phenylpropyl) chloroacetamide (F), 54-55° (lit. 58-59°) (Elorriaga et al., 1976), 1.9 (m, 2H), 2.7 (t, 2H, J=7.6), 3.3 (q, 2H, J=6.7Hz), 4.0 (s, 2H), 7.2-7.3 (m, 5H); N-(2,2-diphenethyl) chloroacetamide (H), 75-77° (lit. 73-74°) (Griffith et al., 1984), 4.0 (m, 2H), 4.0 (s, 2H), 4.2 (t, 1H, J=7.9Hz), 7.2-7.4 (m, 10H); N-(1,2-diphenethyl) chloroacetamide (J) 143-145° (lit. Chemical Abstracts CA85(1):5705y), 3.1 (m, 2H), 4.0 (s, 2H), 5.3 (q, 1H, J=7.7Hz), 7.1-7.4 (m, 10H); N-methyl-(2-phenethyl) dichloroacetamide (K) 61-63° (lit. U.S. Patent #4208203), 2.9 (m, 2H), 3.0 and 3.1 (two singlets, 3H), 3.6 (m, 2H), 5.9 and 6.2 (two singlets, 1H), 7.2-7.4 (m, 5H); N-(2-p-nitrobenzyl) chloroacetamide (L) 106-108° (lit. 110-112°) (Mukaiyama et al., 1972), 4.2 (s, 2H), 4.6 (d, 2H, J=6.2Hz), 7.4 (d, 2H, J=8.1), 8.2 (d, 2H, J=9.1Hz). Two of the analogs represent novel compounds with no previously reported melting points. Therefore, the structure of each was confirmed using carbon and proton nuclear magnetic resonance and elemental analysis. Elemental analyses were performed by Desert Analytics, Tucson, Arizona. Carbon-13 magnetic resonance spectra were recorded at 62.9 MHz on a Brucker WM-250
Figure 6. Structure of chloramphenicol analogs with R, X, and n positions representing sites of structural alteration.
Table 4.
Compounds tested as potentially selective inactivators of cytochromes P-450. The R, n, and X designations refer to substituents on the parent structure shown in Figure 6.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>n</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP</td>
<td>CH₂OH</td>
<td>OH</td>
<td>NO₂</td>
<td>0</td>
<td>Cl</td>
</tr>
<tr>
<td>A</td>
<td>CH₂OH</td>
<td>OH</td>
<td>NO₂</td>
<td>0</td>
<td>H</td>
</tr>
<tr>
<td>B</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>0</td>
<td>Cl</td>
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<tr>
<td>C</td>
<td>H</td>
<td>H</td>
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<td>H</td>
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<tr>
<td>D</td>
<td>H</td>
<td>H</td>
<td>NO₂</td>
<td>0</td>
<td>Cl</td>
</tr>
<tr>
<td>E</td>
<td>H</td>
<td>H</td>
<td>NO₂</td>
<td>0</td>
<td>H</td>
</tr>
<tr>
<td>F</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>1</td>
<td>H</td>
</tr>
<tr>
<td>G</td>
<td>H</td>
<td>phenyl</td>
<td>H</td>
<td>0</td>
<td>Cl</td>
</tr>
<tr>
<td>H</td>
<td>H</td>
<td>phenyl</td>
<td>H</td>
<td>0</td>
<td>H</td>
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<tr>
<td>I</td>
<td>phenyl</td>
<td>H</td>
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<td>0</td>
<td>Cl</td>
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<tr>
<td>J</td>
<td>phenyl</td>
<td>H</td>
<td>H</td>
<td>0</td>
<td>H</td>
</tr>
</tbody>
</table>
spectrometer. For these spectra, chemical shifts are reported as \( S \) values in parts per million (ppm) from the center line of the CHCl\(_3\)-d triplet (77.0 ppm). For N\-(2,2-diphenethyl) dichloroacetamide (G), m.p. 112-114\(^\circ\); \(^1\)H NMR (CDCl\(_3\)) S 3.9 (m, 2H), 4.2 (m, 1H), 5.8 (s, 1H), 7.3 (m, 10H); \(^13\)C NMR (CDCl\(_3\)) S 44.4, 50.1, 66.3, 127.1, 127.9, 128.8, 141.1, 164.0. Anal. Calcd for C\(_{18}\)H\(_{15}\)Cl\(_2\)NO: C, 62.35; H, 4.91; N, 4.54; Cl, 23.01. Found: C, 62.39; H, 4.79; N, 4.52; Cl, 22.71. For N\-(1,2-diphenethyl) dichloroacetamide (I), m.p. 138-139\(^\circ\); \(^1\)H NMR (CDCl\(_3\)) S 3.2 (d,2H, J=6.6Hz), 5.2 (q, 1H, J=7.5Hz), 5.8 (s, 1H), 7.0-7.5 (m, 10H); \(^13\)C NMR (CDCl\(_3\)) S 42.3, 55.1, 66.4, 126.9, 127.8, 128.5, 128.7, 129.3, 136.3, 140.1, 163.2. Anal. Calcd for C\(_{18}\)H\(_{15}\)Cl\(_2\)NO: C, 62.35; H, 4.91; N, 4.54; Cl, 23.01. Found: C, 62.53; H, 4.80; N, 4.63; Cl, 22.83. The analogs N\-(2-phenethyl) dichloroacetamide (B) and N\-(2-p-nitrophenethyl) dichloroacetamide (D) were available from previous syntheses (Miller and Halpert, 1986).

**Animal Treatment and Microsome Preparation.** Adult male Sprague-Dawley rats (150-250g) were pretreated with PCN or phenobarbital. PCN was administered at 100 mg/kg by gastric intubation once daily for four days in 1 ml of a 1% Tween 80-corn oil suspension. On the fourth day food was withheld. Animals were killed on day five. Liver microsomes were prepared as described previously (Halpert, Naslund, and Betner, 1983). Animals pretreated with phenobarbital were administered 0.1% (w/v) sodium phenobarbital for 5 days in the drinking water. Protein was determined by the method of Lowry et al. (1951) and cytochrome P-450 content by the method of Omura and Sato (1964).
Purification of Cytochrome P-450 Isozymes

The major phenobarbital-inducible isozyme of rat liver cytochrome P-450 (PB-B) was isolated as described by Guengerich et al. (1982) using modifications described by Graves and Halpert (1987). The same basic procedure was used to isolate isozymes BNF-B (Miller and Halpert, 1987), UT-A, UT-F, and P-450g (Halpert et al., to be published). The specific cytochrome P-450 contents of the preparations used were: PB-B (16 nmol/mg), BNF-B (15 nmol/mg), UT-A (14 nmol/mg), UT-F (15 nmol/mg), and P-450g (19 nmol/mg). NADPH-cytochrome P-450 reductase was purified as described previously (Miller and Halpert, 1985). One unit of reductase is defined as the amount which reduces 1 micromole of cytochrome c per minute when assayed in 300 mM potassium phosphate buffer (pH 7.7) at 25°.

Inactivation Studies

Microsomal System. Liver microsomes from PCN- or phenobarbital-treated rats were incubated with inhibitor at 37° for two minutes (Figure 7). Reactions were started by the addition of NADPH and allowed to proceed for indicated times up to 12.5 minutes, at which point 80-ul aliquots were taken and added to 20 ul of (14C) androstenedione (1.154 x 10^6 total dpm) in dilute HEPES buffer. The reaction proceeded for an additional 1.5 minutes before quenching with 50 ul tetrahydrofuran. Incubation conditions after addition of the androstenedione were: 0.25 mg/ml microsomal protein, 24 uM androstenedione, 1 mM NADPH, 50 mM HEPES buffer (pH 7.6), 15 mM MgCl₂, and 0.1 mM EDTA. Inhibitors were added in methanol.
Fifty-microliter aliquots were spotted on the preadsorbent loading zone of a Baker silica gel thin-layer chromatography plate [250u, Si250F (19c)], and the plate was developed twice in chloroform:ethyl acetate (1:2). Localization of androstenedione metabolites and quantification of enzyme activity was performed as previously described (Graves, Kaminsky and Halpert, 1987).

**Reconstituted System.** The purified cytochrome P-450 isozymes UT-A, UT-F, PB-B, and P450g were assayed for residual enzyme activity after incubation with an inhibitor in a reconstituted system using (14C) androstenedione as the substrate. Incubation mixtures contained 0.05 nmol/ml of P-450, 0.30 units/ml of NADPH-cytochrome P-450 reductase, 30 ug/ml dilauryl L-3-phosphatidyl choline, 0.1 mg/ml of sodium deoxycholate, 0.05 M HEPES buffer (pH 7.5), 15 mM MgCl2, 0.1 mM EDTA, 0.36 uM NADPH, and inhibitor added in methanol. The 0.1-ml reaction mixture was incubated at 37° for 2 minutes, and the reaction was started by the addition of NADPH. The procedure was identical to that for the microsomal system with the exception that the incubation of enzyme and inhibitor was only allowed to proceed for a maximum of 6 minutes. Incubation conditions for purified BNF-B were identical to those for UT-F, UT-A, PB-B, and P-450g except that 7-ethoxycoumarin replaced androstenedione as the substrate used to assay enzymatic activity.

7-Hydroxycoumarin formation was monitored on an Aminco-Bowman spectrofluorometer (excitation 366 nm, emission 454 nm) as described previously (Miller and Halpert, 1986; Greenlee and Poland, 1978). Antibody inhibition studies using previously available anti-PCNb IgG
Figure 7. Methodology used for monitoring the inactivation of rat liver microsomal androstenedione hydroxylases.
(Graves, Kaminsky and Halpert, 1987), anti-PB-B IgG (Duignan et al., 1987), and control IgG were conducted using both microsomes and purified cytochromes P-450. In each case, antibody was added to the incubation mixture and then incubated for 30 minutes at 25° to allow formation of any antibody-enzyme complex. The tubes were then returned to ice, (14C)-androstenedione was added, and the tubes were preincubated for 3 minutes at 37°. The reaction was started by the addition of NADPH and allowed to proceed for 3 minutes at 37°. Androstenedione metabolites were identified and enzyme activity was determined as described earlier.

**Immunochromatological Methodology**

SDS-polyacrylamide gel electrophoresis was conducted as described by Laemmeli (1970) and proteins were then transferred electrophoretically to nitrocellulose according to the Bio-Rad Trans-Blot Kit instructions. Following incubation of the nitrocellulose sheets in 3% gelatin for 30 minutes to block all non-protein bound sites, the nitrocellulose was incubated with anti-PCNb IgG (20 µg/ml) for 2 hr. in 1% gelatin. Immunoreactive proteins were then visualized and identified using a horseradish peroxidase Immuno-blot Assay Kit purchased from Bio-Rad, Richmond, CA.
CHAPTER 3

RESULTS

Androstenedione Hydroxylase Activity in Microsomes and Reconstituted Systems.

Results from several laboratories have indicated that the formation of each of the four major hydroxylated androstenedione metabolites (7-alpha-OH AD, 6-beta-OH AD, 16-beta-OH AD, and 16-alpha-OH AD) primarily reflects the activity of a single cytochrome P-450 isozyme (Table 3). Experiments conducted during the initial phase of this investigation were devoted towards confirming these assignments using our own preparations of microsomes and purified cytochromes P-450. Rates of formation of the four major hydroxylated metabolites in intact liver microsomes were found to be linear under the following conditions regardless of the source of microsomal protein: 24 μM androstenedione, 25 μg microsomal protein, and a 3.0 minute incubation period. Liver microsomes from PCN- and phenobarbital-treated rats exhibited the expected metabolite profiles compared to control microsomes (Waxman, Dannan, and Guengerich, 1985), i.e. 6-beta hydroxylase activity was increased by both treatments and 16-beta hydroxylase activity was increased dramatically only after the phenobarbital pretreatment (Table 5).

Preparations of four purified cytochrome P-450 isozymes were also assayed for their ability to metabolize androstenedione in a reconstituted system. Each of the isozymes produced one major
Table 5. Androstenedione hydroxylase activities of male rat liver microsomes obtained from animals treated with phenobarbital or PCN. The values in parentheses are published values to be used for comparison (Waxman, Dannan, and Guengerich, 1985).

<table>
<thead>
<tr>
<th>Rat Liver Microsomes</th>
<th>Androstenedione Hydroxylase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol metabolite/min⁻¹/mg protein⁻¹</td>
</tr>
<tr>
<td></td>
<td>7-alpha</td>
</tr>
<tr>
<td>control</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>(0.23)</td>
</tr>
<tr>
<td>+ phenobarbital</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>(0.45)</td>
</tr>
<tr>
<td>+ PCN</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>(0.28)</td>
</tr>
</tbody>
</table>
metabolite with the following activity in nmol metabolite formed/min/nmol cytochrome P-450: UT-F, 7-alpha-OH (11.5); PB-B, 16-beta-OH (9.8); UT-A, 16-alpha-OH (4.5); and P-450g, 6-beta-OH (3.3). It has been reported previously that a major PCN-inducible isozyme of rat liver cytochrome P-450 termed PCNb was essentially inactive in metabolizing androstenedione in a reconstituted system but that polyclonal antibodies to this protein, when added to microsomes from PCN-treated male rats at a concentration of 6 mg IgG/nmol total cytochrome P-450 inhibited more than 80% of the androstenedione 6-beta hydroxylase activity (Figure 8) (Graves, Kaminsky, and Halpert, 1987). In the present investigation we confirmed that these antibodies caused a similar or greater extent of inhibition of androstenedione 6-beta hydroxylation in microsomes from phenobarbital-treated or control rats. We also confirmed that these antibodies had no effect on the 6-beta hydroxylase activity of purified P-450g in a reconstituted system and did not recognize P-450g on Western blots. Thus, in agreement with recent results based on studies with antibodies to P-450g (McClellan-Green et al., 1987), this cytochrome appears to make only a minimal contribution to microsomal androstenedione 6-beta hydroxylase activities. Rather, as indicated in Table 3, androstenedione 6-beta hydroxylase activity in microsomes from control and PCN- or phenobarbital-treated rats appears to primarily reflect the major cytochrome(s) P-450 of the PCN-family. (We have recently obtained evidence that liver microsomes from PCN- and phenobarbital-treated rats contain at least two immunochemically related cytochrome P-450 isozymes referred to
Figure 8. Inhibition of androstenedione 6-beta hydroxylation by anti-PCN IgG. The experimental procedure is described in METHODS.
as PCNa and PCNb, both of which may be involved in androstenedione 6-beta hydroxylation (Graves, Kaminsky and Haipert, 1987).

The final set of preliminary experiments was designed to identify an appropriate source of microsomes for monitoring the activities of isozymes UT-A and PB-B. Because of the low androstenedione 16-beta hydroxylase activities in microsomes from control or PCN-treated rats, it was necessary to use microsomes from phenobarbital-induced animals to accurately measure the activity of isozyme PB-B. However, as reported by us (Duignan et al., 1987) and others (Waxman, Ko and Walsh, 1983; Wood et al., 1983), isozyme PB-B is not completely stereoselective at the 16-position, and produces 16-beta-OH and 16-alpha-OH androstenedione in a ratio of 10:1. Because of the high levels of PB-B in microsomes from phenobarbital-induced animals, concern arose that the 16-alpha-hydroxylase activity in such microsomes might not solely reflect the activity of isozyme UT-A. Therefore antibody inhibition experiments were carried out in which microsomes from phenobarbital- or PCN-treated rats were incubated with 6 mg anti-PB-B IgG/nmol cytochrome P-450 prior to assays of androstenedione metabolism. Under these conditions, a 71% decrease in androstenedione 16-beta hydroxylase activity and a 27% decrease in 16-alpha hydroxylase activity was observed in the microsomes from the phenobarbital-treated animals, whereas only an 11% decrease in 16-beta hydroxylase activity and no decrease in 16-alpha hydroxylase activity was observed in the microsomes from the PCN-treated rats (Figure 9).
Figure 9. Inhibition of androstenedione 16-beta and 16-alpha hydroxylation by anti-PB-B IgG in microsomes obtained from phenobarbital and PCN treated rats.

- PB microsomes: 71% inhibition
- PCN microsomes: 19% inhibition
- 16-beta: 71%
- 16-alpha: 27%
- No decrease
Based on these and other results to be described later, only microsomes from phenobarbital-treated rats were subsequently used to monitor the activity of PB-B, whereas only microsomes from PCN-treated rats were used to monitor the activity of UT-A.

**Inhibition and Inactivation of Microsomal Androstenedione Hydroxylases by Chloramphenicol.**

Having confirmed the isozyme assignments of the androstenedione hydroxylase activities indicated in Table 3 and having identified an appropriate source of microsomes for measuring these activities, subsequent experiments were devoted towards determining the appropriate inhibitor concentrations and incubation times for monitoring the *in vivo* inactivation of the isozymes in question. Initial experiments were carried out with chloramphenicol, which has been shown previously to inactivate isozymes UT-A, PB-B, and the PCN-inducible isozyme(s) responsible for androstenedione 6-beta hydroxylation but not to inactivate UT-F (Halpert et al., 1985; Miller and Halpert, 1986; Graves, Kaminsky and Halpert, 1987). We hypothesized that a concentration of chloramphenicol sufficient to inhibit androstenedione hydroxylation when added together with the substrate ought to be sufficient to inactivate susceptible isozymes if pre-incubated with the microsomes and NADPH prior to the addition of androstenedione. For example, the $I_{50}$ value for inhibition of the 6-beta hydroxylase was 250 uM in microsomes from both PCN- and phenobarbital-treated rats (Table 6), whereas the $I_{50}$ value for inhibition of 16-beta hydroxylase was 40 uM in microsomes prepared from phenobarbital-treated rats but 120 uM in microsomes prepared
Chloramphenicol Concentrations Required to Obtain 50% Reversible Inhibition

Chloramphenicol was added to microsomal incubations at concentrations ranging from 0 to 500 μM. Hydroxylase activity was plotted vs. the log of the Chloramphenicol concentrations and non-linear regression least squares analysis of the curves was used to determine the concentration of compound which produces 50% inhibition.

<table>
<thead>
<tr>
<th>Hydroxylase</th>
<th>PB microsomes</th>
<th>PCN microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-beta</td>
<td>253 ± 8</td>
<td>228 ± 24</td>
</tr>
<tr>
<td>16-beta</td>
<td>37 ± 5</td>
<td>142 ± 25</td>
</tr>
<tr>
<td>16-alpha</td>
<td>113 ± 10</td>
<td>214 ± 29</td>
</tr>
</tbody>
</table>
from PCN-treated rats. Based on the above results, the ability of chloramphenicol to inactivate the various androstenedione hydroxylases was examined by pre-incubating microsomes from PCN- or phenobarbital-treated rats with 50 or 250 uM chloramphenicol in the presence of NADPH for times ranging from 0.5 to 12.5 minutes and then transferring an aliquot of the incubation mixture to a separate tube containing androstenedione to measure residual monooxygenase activity (Figure 7). Incubations with androstenedione were carried out for 1.5 minutes based on experiments which indicated that formation of all four hydroxylated metabolites was linear for this time even in the presence of 250 uM chloramphenicol. Analysis of the data show both a time-dependent loss of enzyme activity corresponding to enzyme inactivation and a reversible inhibition component due to the presence of unmetabolized inhibitor during the androstenedione hydroxylase assay. Reversible inhibition is represented by the decrease in the y-intercept value relative to the methanol control and increases in a dose-dependent fashion (Halpert et al., 1985). As shown in Figure 10, 250 uM chloramphenicol produces approximately 30 per cent reversible inhibition of the 6-beta hydroxylase compared to the methanol control as measured by the y-intercept of the plots. As observed in our initial iso experiment, the androstenedione 16-beta hydroxylase is more sensitive to reversible inhibition by chloramphenicol than the androstenedione 6-beta hydroxylase. It should also be noted that the time course of androstenedione 16-beta hydroxylase inactivation becomes biphasic when approximately three-fourths of the initial activity is lost.
Figure 10. Effect of preincubation with 250 μM chloramphenicol on the androstenedione 6-beta and 16-beta hydroxylase activities of liver microsomes from phenobarbital-treated male rats. Incubation conditions and identification and quantitation of androstenedione metabolites are described in Chapter 2. 

(●) 16-beta hydroxylase activity after preincubation with NADPH alone. (△) 6-beta hydroxylase activity after preincubation with NADPH alone. (○) 16-beta hydroxylase activity after preincubation with NADPH plus 250 μM chloramphenicol. (▲) 6-beta hydroxylase activity after preincubation with NADPH plus 250 μM chloramphenicol.
Biphasic kinetics were also observed for compounds other than chloramphenicol and were not found to be isozyme dependent. Generally, the phenomenon was observed whenever at least two-thirds of a particular hydroxylase activity was lost during the course of the experiment.

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

**Correlation of Inactivation of Purified Cytochromes P-450 with Inactivation Experiments Using a Microsomal System.**

To further validate the use of the microsomal system, rate constants for inactivation by chloramphenicol observed in microsomes
were compared with results obtained with purified isozymes in a reconstituted system. Purified isozyme UT-F showed no NADPH-dependent loss of androstenedione 7-alpha hydroxylase activity when incubated with 250 μM chloramphenicol, consistent with the microsomal data. Purified cytochrome P-450g was also resistant to inactivation by 250 μM chloramphenicol, suggesting that the time-dependent loss of androstenedione 6-beta hydroxylase activity caused by 250 μM chloramphenicol in intact microsomes is due to the inactivation of PCN-inducible cytochrome(s) P-450. Also, rate constants for inactivation of androstenedione 16-alpha hydroxylase by chloramphenicol were in agreement regardless of whether microsomes from PCN-treated animals (kinactivation = 0.08 min⁻¹) or purified UT-A (kinactivation = 0.11 min⁻¹) were used as the enzyme source. For the major phenobarbital-inducible enzyme in rat liver, PB-B, the rate constant of inactivation of 0.47 min⁻¹ determined for microsomes in the presence of 250 μM chloramphenicol is in good agreement with the reported kinactivation of 0.40 min⁻¹ observed previously in a reconstituted system (Miller and Halpert, 1986).

Inactivation of Androstenedione Hydroxylases by Chloramphenicol Analogs.

Previous work from this laboratory has focused on the ability of chloramphenicol analogs to inactivate PB-B and the major beta-naphthoflavone-inducible cytochrome P-450 isozyme, BNF-B (Miller and Halpert, 1986). Comparison of rate constants for inactivation of these isozymes by chloramphenicol and the analog N-(2-phenethyl) dichloroacetamide gave preliminary indications of which structural
features might enhance or dramatically decrease the ability of the compounds to cause enzyme inactivation. Based on these results, the utility of the androstenedione hydroxylase assay in aiding in the design of selective cytochrome P-450 inactivators was examined using a series of compounds of the general structure shown in Figure 6. The choice of compounds synthesized was dictated by the ready availability of the starting amines as well as by the presence of substituents which might be expected to have a major effect on the ability of the compounds to act as substrates for and potentially inactivate particular isozymes.

Due to the large number of analogs of interest and the varied sensitivity of the different isozymes to reversible inhibition, it was impractical to determine the $I_{50}$ values for each inhibitor/enzyme pair. Therefore, analogs were tested initially at a low concentration (5 - 50 uM) and at a 5-fold higher concentration. The androstenedione 7-alpha hydroxylase, UT-F, was consistently resistant to reversible inhibition by any analog. The inhibition of the other androstenedione hydroxylases by chloramphenicol analogs paralleled that seen for chloramphenicol itself in that the 6-beta hydroxylase was the most resistant to inhibition. Therefore, a concentration of each analog which gave approximately 30% reversible inhibition of the 6-beta hydroxylase was chosen for analysis of the inactivation kinetics. That the observed time-dependent loss of enzyme activity caused by chloramphenicol and certain analogs was actually NADPH-dependent and irreversible was confirmed by in vitro experiments in which microsomes were preincubated with inhibitor in
the presence or absence of NADPH and then recovered from the incubation mixture by ultracentrifugation as described previously (Halpert et al., 1985).

Figure 11 illustrates the ability of different chloramphenicol analogs to inactivate the cytochrome(s) P-450 responsible for androstenedione 6-beta hydroxylase activity. These results in addition to the data obtained for other androstenedione hydroxylase activities are shown in Table 7. The major findings can be summarized as follows. Chloramphenicol was shown to inactivate the four androstenedione hydroxylases in the following order: 16-beta > 6-beta > 16-alpha > 7-alpha. (Since the androstenedione 7-alpha hydroxylase was not inactivated by any of the analogs tested, including chloramphenicol, future reference will be limited to the remaining three hydroxylase activities.) Regarding the dichloroacetamides, the removal of the functional groups at all three R positions (Figure 11) resulted in the compound N-(2-phenethyl) dichloroacetamide (B) which inactivated the 16-beta hydroxylase more rapidly than the 16-alpha hydroxylase and did not inactivate the 6-beta hydroxylase. The addition of a para-nitro group on the phenyl ring results in the analog N-(2-p-nitrophenethyl) dichloroacetamide (D) which is an efficient 16-beta hydroxylase inactivator but does not select between inactivation of the androstenedione 6-beta and 16-alpha hydroxylases. Addition of a phenyl group at the R2 position of compound B resulted in a compound (G) which inactivated the three hydroxylases in the following order - 16-beta > 6-beta > 16-alpha. By simply moving the phenyl group from the R2 position to the R1
Figure 11. Effect of preincubation with chloramphenicol analogs on the androstenedione 6-beta hydroxylase activity of liver microsomes prepared from PCN-treated male rats. The experimental conditions are as described in Chapter 2 and Figure 7.

(○) NADPH alone
(▲) NADPH + 50 μM N-(2-phenethyl) dichloroacetamide (B)
(■) NADPH + 250 μM N-(2-phenethyl) chloroacetamide (C)
(■) NADPH + 50 μM N-(2-p-nitrophenethyl) dichloroacetamide (D)
(△) NADPH + 25 μM N-(1,2-diphenethyl) dichloroacetamide (I)
position on the ethyl side chain (I), the selectivity was altered (6-beta > 16-beta > 16-alpha). Preliminary results with the only tertiary amide synthesized, N-methyl-(2-phenethyl) dichloroacetamide (K), indicate that this compound is a comparatively non-selective and slow inactivator.

The corresponding chloroacetamides to the above mentioned dichloroacetamides were also synthesized and tested (Table 7). N-(2-phenethyl) chloroacetamide (C) was a relatively poor inactivator, and neither the effectiveness nor selectivity of this analog was greatly enhanced by the addition of a phenyl group at the R1 or R2 positions (compounds J and H). In contrast, the analog N-(2-p-nitrophenethyl) chloroacetamide (E) was the only compound which inactivated the 16-beta and 6-beta hydroxylases while selecting against inactivation of the 16-alpha hydroxylase. This analog was shown previously to inactivate the purified isozyme PB-B via destruction of the heme moiety of the enzyme and not by protein modification (unpublished data from Dr. Natalie Miller). We investigated the selectivity of the analog N-(2-p-nitrophenethyl) chloroacetamide further by testing structurally related analogs. The compound with one carbon removed from the ethyl chain, N-(2-p-nitrobenzyl) chloroacetamide (L), was a poor and non-selective inactivator. Finally, the analog with a propyl chain but lacking the nitro group, N-(3-phenylpropyl) chloroacetamide (F), was also found to be a less effective inactivator than N-(2-p-nitrophenethyl) chloroacetamide.
Table 7.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>k&lt;sub&gt;inactivation&lt;/sub&gt; (min&lt;sup&gt;-1&lt;/sup&gt;) 6β-hydroxylase</th>
<th>16β-hydroxylase</th>
<th>16α-hydroxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (MEOH)</td>
<td></td>
<td>0.01 n = 13</td>
<td>0.01 n = 9</td>
<td>0.01 n = 9</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>250 µM</td>
<td>0.10</td>
<td>0.47</td>
<td>0.08</td>
</tr>
<tr>
<td>A Deschlorochloramphenicol</td>
<td>2.5 mM</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>B N-(2-phenethyl) dichloroacetamide</td>
<td>50 µM</td>
<td>0.01</td>
<td>0.21</td>
<td>0.05</td>
</tr>
<tr>
<td>C N-(2-phenethyl) chloroacetamide</td>
<td>250 µM</td>
<td>0.01</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>D N-(2-p-nitrophenethyl) dichloroacetamide</td>
<td>50 µM</td>
<td>0.16</td>
<td>0.52</td>
<td>0.11</td>
</tr>
<tr>
<td>E N-(2-p-nitrophenethyl) chloroacetamide</td>
<td>250 µM</td>
<td>0.09</td>
<td>0.13</td>
<td>0.00</td>
</tr>
<tr>
<td>F N-(3-phenylpropyl) chloroacetamide</td>
<td>250 µM</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>G N-(2,2-diphenethyl) dichloroacetamide</td>
<td>25 µM</td>
<td>0.13</td>
<td>0.72</td>
<td>0.04</td>
</tr>
<tr>
<td>H N-(2,2-diphenethyl) chloroacetamide</td>
<td>250 µM</td>
<td>0.01</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>I N-(1,2-diphenethyl) dichloroacetamide</td>
<td>25 µM</td>
<td>0.32</td>
<td>0.24</td>
<td>0.15</td>
</tr>
<tr>
<td>J N-(1,2-diphenethyl) chloroacetamide</td>
<td>250 µM</td>
<td>0.04</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>K N-methyl-N-(2-phenethyl) dichloroacetamide</td>
<td>250 µM</td>
<td>0.03</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>L N-(p-nitrobenzyl) chloroacetamide</td>
<td>250 µM</td>
<td>0.02</td>
<td>0.06</td>
<td>0.03</td>
</tr>
</tbody>
</table>

All compounds with the exceptions of A, F, H, J, K, and L were tested in two identical experiments with only the source of microsomal protein being changed for reasons described in Results. Rate constants for inactivation of the androstenedione 7α-hydroxylase and 6β-hydroxylase were determined by averaging the results from experiments using microsomes obtained from both phenobarbital- and PCN-treated rats. Rate constants for inactivation of the androstenedione 16β-hydroxylase and 16α-hydroxylase were obtained from experiments using microsomes from phenobarbital- and PCN-treated rats, respectively. Rate constants for inactivation shown for compounds A, F, H, J, K, and L were obtained using only microsomes obtained from phenobarbital-induced animals. These compounds were initially screened for their ability to affect androstenedione 6β- and 16β-hydroxylase activities.
Inactivation of the Major Beta-Naphthoflavone-Inducible Cytochrome P-450 Isozyme.

The selectivity of certain of the compounds was investigated further by examining the ability to inactivate the major beta-naphthoflavone-inducible cytochrome P-450 isozyme, BNF-B, in a reconstituted system (Table 8). These data corroborate a previous report (Miller and Halpert, 1986) that unlike chloramphenicol, the analogs N-(2-p-nitrophenethyl) dichloroacetamide (D) and N-(2-phenethyl) dichloroacetamide (B) inactivate the BNF-B isozyme. Lack of inactivation of BNF-B by N-(2-p-nitrophenethyl) chloroacetamide (E) indicates that the dichloromethyl function appears to be required, but not sufficient for, inactivation of BNF-B. Regarding substitutions made along the ethyl chain, the analog N-(2,2-diphenethyl) dichloroacetamide (I) was shown to inactivate the enzyme, whereas movement of the phenyl group from the R2 to R1 position resulted in a diphenyl compound unable to inactivate BNF-B.
Table 8.

Rate constants for inactivation of purified cytochrome P-450 BNF-B by chloramphenicol analogs. Residual monoxygenase activity was monitored by the conversion of 7-ethoxycoumarin to 7-hydroxycoumarin as described in Experimental Procedures. Rate constants for inactivation were calculated as described in Table 7.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>(k_{\text{inactivation}}) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (methanol)</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>100 (\mu)M</td>
<td>0.03</td>
</tr>
<tr>
<td>N-(2-phenethyl) dichloroacetamide</td>
<td>25 (\mu)M</td>
<td>0.40</td>
</tr>
<tr>
<td>N-(2-p-nitrophenethyl) dichloroacetamide</td>
<td>25 (\mu)M</td>
<td>0.39</td>
</tr>
<tr>
<td>N-(2-p-nitrophenethyl) chloroacetamide</td>
<td>250 (\mu)M</td>
<td>0.01</td>
</tr>
<tr>
<td>N-(1,2-diphenethyl) dichloroacetamide</td>
<td>25 (\mu)M</td>
<td>0.03</td>
</tr>
<tr>
<td>N-(2,2-diphenethyl) dichloroacetamide</td>
<td>25 (\mu)M</td>
<td>0.20</td>
</tr>
</tbody>
</table>
Chapter 4

Discussion

There are two possible approaches to solving the problem of metabolic activation of foreign chemicals and resultant toxicity. The first is to reduce or eliminate the production of toxic metabolites; the second is to enhance the production of non-toxic metabolites (Nelson, 1982). These objectives are the foundation for investigations into selective enzyme inhibitors. Targeting the cytochrome P-450 system for modification is critical due to the important role of these enzymes in the metabolism of xenobiotics (Chapter 1). Fortunately, these investigations are advancing because of progress in the purification of cytochrome P-450 isozymes and characterization of isozyme reactions. The traditional dogma that the cytochrome P-450 system has a broad substrate specificity is still true; but it can now be said that in certain cases, the production of a toxic metabolite is a function of a specific cytochrome P-450 isozyme.

In order for significant progress to be made in the study of selective inactivation of cytochromes P-450, advances in the development and synthesis of appropriate compounds need to parallel the development of isozyme-specific assays. To our knowledge, this investigation documents the first use of four specific androstenedione hydroxylase activities as probes for examining potentially selective inactivators. From the perspective of method
development, enzyme assays of this type are needed to reduce the need for researchers to purify and characterize each cytochrome P-450 isozyme of interest before assessing the selectivity of inactivation. However, the usefulness of purified cytochrome P-450 preparations in confirmatory experiments will not be eliminated as long as there are complicating factors such as animal strain differences and different but equally legitimate purification schemes.

Any assay designed for rapidly screening a biological parameter must not only be scientifically accurate but also resourceful and practical. Waxman et al. (1983) documented the separation and identification of fourteen monohydroxylated metabolites of androstenedione using thin-layer chromatography (eleven solvent systems), retention times on reverse-phase high performance liquid chromatography, and finally gas chromatography combined with mass spectral analysis to confirm the structure of the metabolites. Although rigorous analytical documentation is critical to the initial method development of any biochemical assay, duplicating such stringent procedures is obviously prohibitive. By obtaining the appropriate androstenedione metabolite standards, choosing a solvent system which separates the four metabolites of interest, and comparing both our chromatographic and metabolic profiles to those previously reported, we have simplified an enzymatic assay for screening cytochrome P-450 inhibitors.

The methodology employed allowed us to begin to develop an alternative to the approach of Ortiz de Montellano whereby isozyme-directed inactivators are designed by incorporating a double or triple
bond into selected positions on substrates normally metabolized by a restricted number of cytochromes P-450 (Ortiz de Montellano and Reich, 1984). Our empirical approach to designing selective inactivators of cytochromes P-450 does not presuppose knowledge about the substrate specificity of the individual isozymes. In order to identify trends in the isozyme selectivity of our compounds, the use of a multiple-enzyme assay was essential.

The important methodological questions focused on confirming the validity of using microsomal activities to monitor particular cytochromes P-450, choosing the proper inhibitor concentrations and incubation conditions, and correlating data obtained with microsomes and purified enzymes. We used combinations of microsomal samples, purified cytochrome P-450 isozymes, and the corresponding anti-P-450 antibodies to identify in our laboratory and to compare to published reports the microsomal androstenedione hydroxylase activity or activities attributable to certain cytochrome P-450 isozymes. A specific group of experiments addressed the question of the contribution of the constitutive cytochrome P-450 isozyme P-450g to androstenedione 6-beta hydroxylase activity in rat liver microsomes. Experiments with purified P-450g including antibody inhibition studies, Western blot analysis, and inactivation experiments indicate that although this isozyme does have androstenedione 6-beta hydroxylase activity in a reconstituted system, it does not make a significant contribution to microsomal 6-beta hydroxylase activity. A similar conclusion has been drawn in a recent study employing antibodies to P-450g (McClellan-Green et al., 1987). By utilizing microsomes
prepared from phenobarbital- and PCN-treated rats we believe that we were able to study the inactivation of the major phenobarbital and PCN-inducible cytochrome P-450 isozymes as well as the two constitutive cytochrome P-450 isozymes UT-F and UT-A.

Because previous studies with chloramphenicol showed that the initial step in enzyme inactivation is the conversion of the dichloromethyl moiety of the compound to a reactive acyl chloride (Halpert, 1981; Pohl and Krishna, 1978), we began our investigation by concentrating on the dichloroacetamide analogs. Our hypothesis was that the remaining functional groups of the molecule may differentially affect its ability to be activated by or bind covalently to particular cytochromes P-450. The isozyme selectivity of the compound N-(2-phenethyl) dichloroacetamide is cited as evidence of this statement. Although this particular analog does not inactivate the androstenedione 6-beta hydroxylase, addition of a para-nitro group to the phenyl ring or the addition of a phenyl group at the R2 position (Figure 3) results in increasingly greater rates of 6-beta inactivation (Table 4). In contrast, the presence or absence of an electrophilic para-nitro group on the phenyl ring of the molecule has no effect on the inactivation of the major beta-naphthoflavone-inducible isozyme, BNF-B, whereas the addition of a phenyl group at the R1 position of the ethyl chain renders the compound unable to inactivate this enzyme.

Based on the initial observation that the compound N-(2-p-nitrophenethyl) chloroacetamide (E) preferentially retained much of the ability of the corresponding dichloroacetamide (D) to inactivate
the 6-beta hydroxylase, a number of compounds containing a chloroacetamide group were subjected to preliminary examination to test the hypothesis that this functionality might confer selectivity towards the 6-beta hydroxylase. However, all the other chloroacetamides tested were either poor inactivators of all the hydroxylases or exhibited no obvious advantages from the standpoint of selectivity compared to the dichloroacetamides. One explanation for this lack of selectivity was alluded to in the RESULTS (Chapter 3); the chloroacetamides may be metabolized by cytochromes P-450 to a reactive intermediate which destroys the heme portion of the protein. Although mechanistic studies were not the focus of this study, this statement supports our hypothesis that the dichloromethyl moiety is required for protein modification leading to a highly selective mechanism of enzyme inactivation.

Unfortunately, it is easier to report the result of a particular inhibitor/isozyme interaction than to explain the finding in terms of molecular biochemistry. An intricate operational scheme for analog synthesis in drug design is proposed by one author (Topliss and Martin, 1975). Numeric values are listed for hydrophobic, electronic, and steric effects on structure-activity relationships. The ability of certain chloramphenicol analogs to inactivate the androstenedione 6-beta hydroxylase could be examined by considering these three parameters. For example, we have shown that substitutions made at the para position of the phenyl ring will effect the rate and selectivity of enzyme inactivation. The androstenedione 6-beta hydroxylase is not inactivated by N-(2-phenethyl) dichloroacetamide
(para-H substitution) but is inactivated by N-(2-p-nitrophenethyl) dichloroacetamide (para-NO₂ substitution). From examination of the hydrophobic constants for these functional groups, it can be stated that the para-nitro analog has a greater hydrophobic constant and rate of 6-beta hydroxylase inactivation than the para-hydrogen compound. Therefore, by testing two more analogs with increasing hydrophobic constants, para-Br and para-I, one could determine if the inhibitor/enzyme system is dependent upon the hydrophobic constant value of the para substituent.

Using the same rationale, the steric dependence of an enzyme/inhibitor system could be studied. The steric constant values for the para-Br and para-I compounds are similar and lie between those listed for hydrogen and nitro substitutions. Therefore, the 6-beta hydroxylase/inhibitor system would be considered to have a negative steric dependency if the rates of inactivation were shown to be para-NO₂ > para-Br = para-I > para-H. Integrating hydrophobic and steric dependency with electronic dependency would follow the same mode of reasoning. The electronic constants decrease in the order para-NO₂ > para-I = para-Br > para-H. If the rates of androstenedione 6-beta hydroxylase inactivation decreased in the same order, this system would be considered to have a positive electronic dependence.

The ability to screen a large number of related compounds for their ability to inactivate multiple cytochromes P-450 should now enable us to elaborate a set of predictive rules for which structural features of our chloramphenicol analogs favor the inactivation of
particular isozymes. By incorporating all these features into a single molecule it may therefore be possible to rationally design isozyme-selective inhibitors. It should be noted that even if totally specific inhibitors cannot be designed by this method, the availability of pairs of related compounds which differ from each other only in their effect on a single isozyme should still be of considerable utility.
Chapter 5

LIST OF REFERENCES


