Isozyme-Selective Modulation of Cytochrome P450 by Mechanism-Based Inactivation and Pathobiological Stress

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Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario, CANADA

June, 1998

Abstract

The mechanisms and kinetics of the isozyme- and tissue-selective mechanism-based (MB) inactivation of cytochrome P450 (CYP) by *N*-benzyl- (BBT), *N*-α-methylbenzyl- (αMB) or *N*-α-ethylbenzyl-1-aminobenzotriazole (αEB) were assessed using hepatic and pulmonary microsomes prepared from phenobarbital treated guinea pigs. Metabolic intermediate complexation was identified as a novel mechanism by which these inhibitors interact with CYP, however it was not found to contribute to CYP2B inactivation by these compounds. The tissue selectivity of BBT and αMB for pulmonary *vs.* hepatic CYP2B inactivation could be rationalized in large part by the very rapid and potent inactivation of this isozyme in guinea pig lung compared with liver microsomes. Similarly, the more rapid inactivation of CYP2B by αMB compared with BBT in guinea pig lung microsomes is consistent the greater potency and selectivity of the former. The chirality of the α-carbon substituent of αMB was also shown to be an important mechanistic determinant of the rate of CYP2B inactivation. Pathobiological stress was also associated with isozyme- and tissue-selective modulation of CYP. For example, administration of β-naphthoflavone to rats caused a direct aryl hydrocarbon receptor (AHR)-mediated induction of CYP1A1 in all tissues studied, while at the same time, the toxicity associated with this treatment indirectly caused tissue-selective changes in the expression and catalytic activity of CYP2E1. Orthotopic liver transplantation was shown to differentially depress hepatic CYP-dependent catalytic activity, and to selectively increase CYP1A1 catalytic activity in rat lung, and to a lesser extent in kidney. A similar response to acute sodium arsenite treatment was observed where hepatic CYP was decreased and pulmonary CYP1A1 mRNA and catalytic activity were selectively increased. Studies were performed to determine if bilirubin accumulation may contribute to the induction of extrahepatic CYP1A1 during pathobiological states. In cell culture, bilirubin was shown to directly induce CYP1A1 expression and catalytic activity in an AHR-dependent manner. These data represent the

first report of an endogenous compound that is both a ligand for the AHR and a mediator of CYP1A1 expression.

Keywords:

aryl hydrocarbon receptor

bilirubin

cytochrome P450

guinea pig

kidney

liver

liver transplantation

lung

mechanism-based inactivation

pathobiological stress

rat

sodium arsenite

Co-Authorship

The following thesis contains material that was previously published in manuscripts co-authored by Christopher Sinal, Maurice Hirst, Christopher Webb, M. George Cherian, Lin-Fu Zhu, Robert Zhong and John Bend. All of the textual material and figures presented in this thesis were authored by Christopher J. Sinal. The majority of the experimental work presented in this thesis was also performed solely by Christopher J. Sinal. However, in some cases special procedures and technical assistance were required to perform the required experiments. The author would like to take this time to gratefully acknowledge the contributions and assistance provided by the following colleagues: Dr. Maurice Hirst for preparing the individual enantiomers of *N*-α-methylbenzyl-1-aminobenzotriazole that were used in Chapter 4; Christopher Webb for his assistance in performing the assays presented in Figures 4.3 and 4.4, as well as the assays presented in Figures 5.4 and 5.5; Dr. M. George Cherian for coordinating the larger study, of which the data in Chapter 6 represents a small part; Dr. Lin-Fu Zhu and Dr. Robert Zhong for performing the liver transplant surgeries presented in Chapter 6. Original manuscripts, versions of which appear in Chapters 2-5 and 7-8 of this thesis, were written by Christopher Sinal and John R. Bend. For copyright releases see Appendices A-D.

Acknowledgments

I would like to thank Chris Webb, Gord McCallum, Bradley Gillesby, Dr. Tim Zacharewski, Dr. Janine Clemons, Dr. Zhi-Fen Wu, and Bryan Bishop for their excellent technical assistance. I would also like to thank these people for their companionship, critical discussions and helpful suggestions. I would like to acknowledge the Ontario Government Scholarship program for four years of personal support and the Medical Research Council of Canada for providing the primary source of research funding to our laboratory during my tenure. I would like to thank my family for their support over the years and in particular my wife Tara Perrot-Sinal for her support, encouragement and the inspiration that she provided. Finally, I am indebted to my supervisor Dr. John R. Bend whose encouragement, guidance, advocacy and confidence were greatly appreciated. I would also like to thank Dr. Bend for the example of integrity and work ethic that he provided to me on a daily basis.

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3MC	3-methylcholanthrene
AA	arachidonic acid
ABT	1-aminobenzotriazole
αEB	*N*-α-ethylbenzyl-1-aminobenzotriazole
AFB_1	aflatoxin B_1
AHR	aryl hydrocarbon receptor
αMB	*N*-α-methylbenzyl-1-aminobenzotriazole
αNF	α-naphthoflavone
ARNT	aryl hydrocarbon nuclear translocator protein
BaP	benzo[*a*]pyrene
BBT	*N*-benzyl-1-aminobenzotriazole
BD	1,3-benzodioxole
bHLH	basic helix-loop-helix
βNF	β-naphthoflavone
BTE	basic transcription element
DEC	diethyldithiocarbamate
DRE	dioxin responsive element
EET	epoxyeicosatrienoic acid
GAPDH	glyceraldehyde 6-phosphate dehydrogenase
GSH	reduced glutathione
GTF	general transcription factor
HETE	monohydroxyeicosatetraenoic acid
HIF	hypoxia inducible factor
HO	heme oxygenase
HPLC	high performance liquid chromatography

Hsp90	heat shock protein 90
IL-1	interleukin-1
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
LPS	lipopolysaccharide
MB	mechanism-based
MI	metabolic intermediate
MROD	7-methoxyresorufin *O*-demethylation
ORD	optical rotatory dispersion
P450 reductase	NADPH-cytochrome P450 reductase
PAH	polycyclic aromatic hydrocarbon
PB	phenobarbital
PCB	polychlorinated biphenyl
PNP	*p*-nitrophenol hydroxylation
PROD	7-pentoxyresorufin *O*-dependentylation
ROS	reactive oxygen species
RT-PCR	reverse transcription-coupled polymerase chain reaction
SDS	sodium dodecyl sulfate
TBP	TATA binding protein
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TNFα	tumour necrosis factor α
UDP-GT	UDP-glucuronosyltransferase
XRE	xenobiotic responsive element

Chapter 1

Introduction

1.1 Biotransformation and Cytochromes P450

1.1.1 Biotransformation

In general terms, biotransformation refers to the enzymatic conversion of lipophilic compounds of endogenous (endobiotic) or exogenous (xenobiotic) origin to more polar, water soluble metabolites. Depending upon the substrate, biotransformation can serve to: (1) reduce the biological half-life of the compound, (2) reduce the accumulation of the compound within the organism, (3) alter the biological activity of the compound, and/or (4) alter the duration of the biological activity of the compound. Since the products of biotransformation are generally more water-soluble and thus, more readily eliminated, this process affords protection against the toxicity of xenobiotics. While biotransformation is first and foremost a detoxication mechanism, some xenobiotics which are not inherently toxic, can be biotransformed to toxic reactive intermediates, a process termed metabolic activation or bioactivation (Guengerich and Liebler, 1987). For example, carcinogenic polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (BaP) undergo a highly stereo- and regioselective multistep bioactivation to epoxides and diols to generate the ultimate carcinogen 7*R*,8*S*-dihydrodiol-BaP-9*S*,10*R*-epoxide (Gelboin, 1980). Similarly, the relatively simple alkene, vinyl chloride is bioactivated to its toxic chloroethylene metabolite by similar mechanisms (Guengerich *et al.*, 1991). Thus, biotransformation plays a dual detoxication and toxication role in the metabolism and elimination of

xenobiotics. The occurrence of a toxic effect due to a particular compound or its reactive metabolites is dependent upon the balance between metabolic detoxication and toxication within a cell, tissue, organ or organism.

The liver is quantitatively the most important site of biotransformation, although other tissues and organs also contribute depending upon the expression of biotransformation enzymes, the route of exposure, the nature of the xenobiotic and cell/tissue/organ-specific factors. For example, the pneumotoxin, 4-ipomeanol causes a cell-selective necrosis of Clara cells, due in large part to the relatively high level of epoxidation of this compound in this lung cell type compared with other pulmonary cell types (Boyd, 1980; Devereux *et al.*, 1989). Biotransformation reactions can be divided into phase I, II and III metabolism. Phase I metabolic reactions include a variety of reduction, oxidation and hydrolysis reactions which result in the introduction or unmasking of functional groups that can participate in subsequent phase II or phase III reactions (Williams, 1959). Phase II reactions most commonly involve conjugation of parent xenobiotics or phase I metabolites with inorganic sulfate, amino acids, glutathione (GSH) or glucuronic acid. These conjugation reactions increase the hydrophilicity of the substrates and facilitate elimination via renal and hepatic biliary routes. Phase III reactions involve further metabolism of phase I and/or phase II metabolites and may utilize the same enzymes as phase I and phase II biotransformations. A number of excellent reviews have been published regarding the mechanisms and detoxication/toxication aspects of these phase I, phase II and phase III metabolic transformations (Armstrong, 1997; Cashman *et al.*, 1996; Gonzalez, 1997; Park *et al.*, 1995). This thesis is concerned primarily with the regulation of expression and catalytic activity of the most important enzyme system involved in phase I metabolic reactions – the cytochrome P450 monooxygenase system.

1.1.2 Cytochromes P450

Cytochrome P450 (CYP) is a general term that refers to a gene superfamily of heme-containing monooxygenases that first appeared 3.5 billion years ago (Degtyarenko and Archakov, 1993). The CYPs responsible for xenobiotic metabolism first appeared 400-500 million years ago, presumably to enable animals to detoxify plant-derived chemicals (Degtyarenko and Archakov, 1993). Collectively, CYPs are capable of the oxidative biotransformation of a wide variety of chemically and biologically unrelated compounds of both endogenous and exogenous origin. Substrates include drugs, carcinogens, solvents, dyes, vitamins, steroids and fatty acids including eicosanoids (Graham-Lorence and Peterson, 1996; Negishi *et al.*, 1996; Porter and Coon, 1991). Most commonly, CYP-dependent metabolism of xenobiotics results in the formation of metabolites with increased polarity that are more readily excreted either directly, or subsequent to phase II conjugation. However, biotransformation by CYP can also bioactivate xenobiotics to more biologically active and/or toxic metabolites. For example, the pro-drug terfenadine, requires CYP-dependent biotransformation to a carboxylic acid metabolite, the active antihistamine (Honig *et al.*, 1992). Similarly, pro-carcinogens such as BaP are bioactivated via multi-step mechanisms, many of which are CYP-dependent, to products with much greater toxicity, mutagenicity or carcinogenicity (Gelboin, 1980; Guengerich *et al.*, 1991). Many of the biotransformations of endogenous compounds by CYP are important components of essential physiological processes. For example, CYPs participate in the biosynthesis of a number of sex steroids (Omura and Morohashi, 1995; Warner and Gustafsson, 1995). Some CYPs can also bioactivate endogenous arachidonic acid (AA) to a series of isomeric epoxyeicosatrienoic acids (EETs) and monohydroxyeicosatetraenoic acids (HETEs) that have a number of potent biological effects (Capdevila *et al.*, 1992; Knickle and Bend, 1994; McCallum *et al.*, 1996; McGiff *et al.*, 1996). Hence, CYPs are critical in: (1) pharmacology, due to catalysis, in many cases, of the rate-limiting steps in drug elimination and/or bioactivation, (2) physiology, due to

participation in the biosynthesis and/or catabolism of biologically active endogenous compounds, and (3) toxicology, due to a role in the toxication and/or detoxication of numerous xenobiotics.

In mammals, the highest CYP content is found in the liver, although lower levels are also found in most tissues of the body (Gonzalez, 1992; Park *et al.*, 1995). As a result of the multitude of CYP isoforms and tissue-specific patterns of CYP expression, tissue-selective toxicological responses to certain xenobiotics can occur. Within a cell, CYPs which metabolize xenobiotics are found predominantly within the endoplasmic reticulum, or microsomal fraction obtained by differential centrifugation (Black, 1992). However, a number of steroidogenic CYPs are localized preferentially in the mitochondria (Omura and Morohashi, 1995). In addition to multiple CYP isozymes, the complete microsomal cytochrome P450 monooxygenase system requires the flavoprotein NADPH-cytochrome P450 reductase (P450 reductase) which mediates the transfer of electrons from NADPH to CYP during catalysis (Waterman and Estabrook, 1983). In the analogous mitochondrial system, the electron transfer system consists of a unique iron-sulfur protein known as adrenodoxin, and the associated flavoprotein, NADPH-adrenodoxin reductase (Hanukoglu *et al.*, 1987).

1.1.3 Metabolic Reactions of Cytochromes P450

Collectively, CYPs catalyze thousands of individual reactions utilizing a large number of structurally diverse substrates of both endogenous and exogenous origin. This diversity arises from: (1) the existence of a large number of closely related isoforms, (2) high catalytic efficiency, (3) low and frequently overlapping substrate specificities and, (4) commonly, low substrate affinity. Furthermore catalysis is driven by the activation of oxygen rather than substrate binding, which implies that, in many cases, the protein structure determines catalytic specificity through complementation of the transition state rather than substrate binding (Koymans *et al.*, 1993a). Most of the reactions catalyzed by

CYPs are oxidative in nature and result in the incorporation of a single atom of oxygen, derived from O_2, into the substrate (Koymans *et al.*, 1993b; Porter and Coon, 1991). The basic reaction scheme of CYP oxygen activation and substrate oxidation is outlined in Fig. 1.1. Briefly, the generally accepted sequence of events is: (1) binding of the substrate to ferric CYP, (2) NADPH-dependent 1-electron reduction of the CYP iron by P450 reductase, (3) binding of O_2 with the ferrous iron to form an unstable $[FeO_2]^{2+}$ complex, (4) donation of a second electron by P450 reductase (or in some cases cytochrome b_5), (5) heterocyclic scission of the FeO-O bond to generate H_2O and the $[FeO]^{3+}$ complex, (6) oxidation of the substrate to regenerate the ferric form of CYP, and (7) release of the oxidized substrate to complete the cycle. In some cases, CYP can oxidize substrates without the requirement for P450 reductase, NADPH or O_2 (8) by utilizing peroxy compounds such as alkyl hydroperoxides as a source of atomic oxygen and reducing equivalents (Coon *et al.*, 1996; Hollenberg, 1992).

The most common CYP-dependent reactions can be broadly classified as aliphatic and aromatic hydroxylation, *N*-, *O*- and *S*-dealkylations, deamination, *N*-oxidation, *N*-hydroxylation, dehalogenation and epoxidation (Guengerich and Macdonald, 1990; Koymans *et al.*, 1993a; 1993b). CYPs are also able to catalyze a variety of reductive reactions by the transfer of electrons from Fe^{2+} to the substrate. These reactions occur predominantly in regions of the liver and other organs where O_2 tension is low (Goeptar *et al.*, 1995). One of the best studied examples of this type of reaction is the participation of CYP-dependent reduction in the generation of phosgene from carbon tetrachloride (Mico and Pohl, 1983).

1.1.4 Nomenclature of Cytochromes P450

At the time of publication of the most recent update on sequences and recommended nomenclature (Nelson *et al.*, 1996), in excess of 480 CYP genes had been described in numerous eukaryotes (plants and animals) and prokaryotes. CYP genes are referred to by

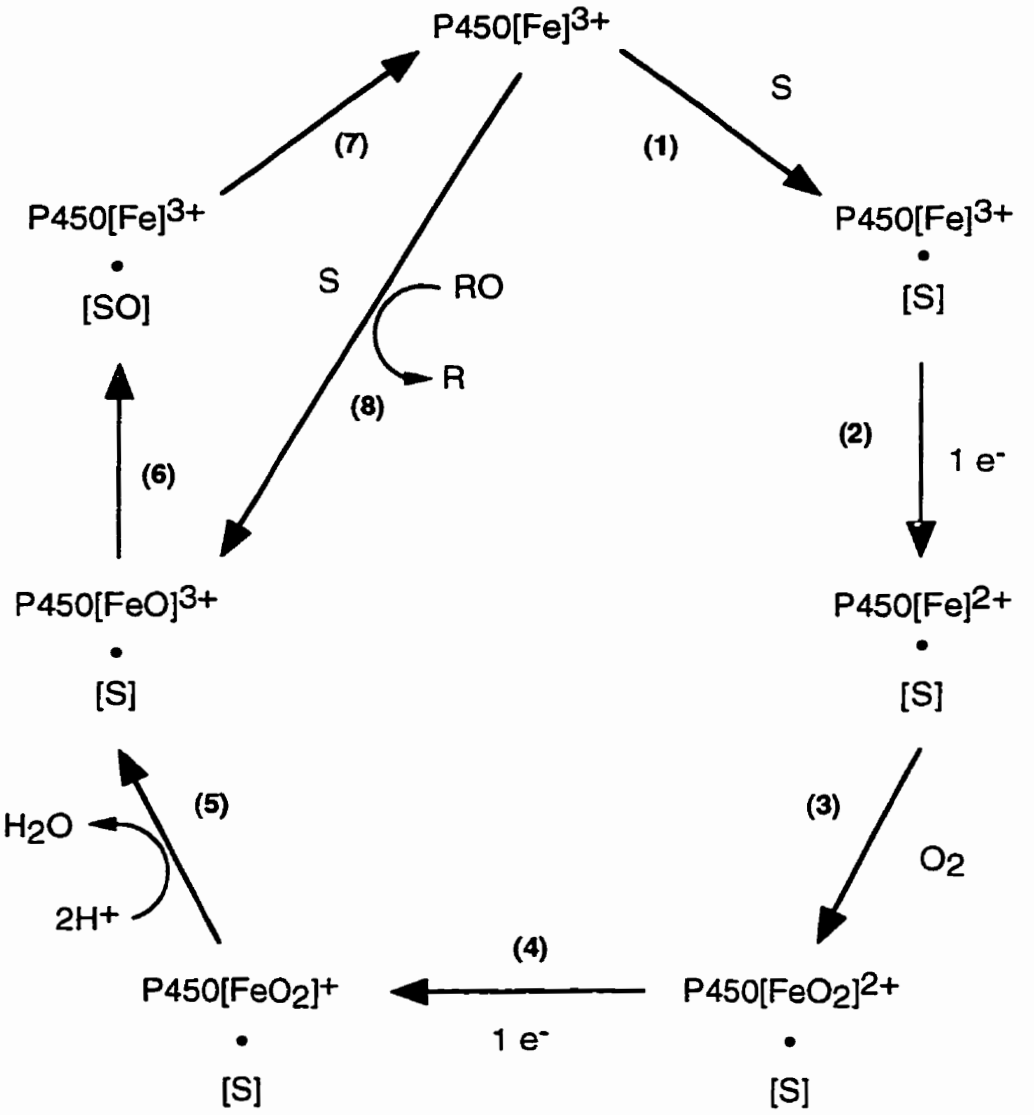


Figure 1.1
General reaction cycle for the oxidation of substrates by CYP.

a systematic nomenclature based upon the evolution of the superfamily, although a number of common names based upon prototypical substrates or inducers still persist. The italicized root symbol "*CYP*" ("*Cyp*" for mouse and *Drosophila*), representing cytochrome P450, followed by an Arabic numeral denoting the family, a capital letter (lower case for mouse and *Drosophila*) indicating the subfamily, and an Arabic numeral designating the individual gene within the subfamily (*i.e. CYP1A1*; *Cyp1a1* for mouse and *Drosophila*) is recommended for CYP gene nomenclature (Nelson *et al.*, 1996). *CYPs* having the same gene number in different species are considered as orthologous. The corresponding gene products (mRNA, cDNA or enzyme) are named with the same non-italicized numbers and capital (including mouse and *Drosophila*) letters (*i.e.* CYP1A1 for mouse, *Drosophila* and all others). CYPs having the same catalytic function in different species are considered as homologous. To improve clarity and consistency with the gene symbol nomenclature, gene products originating from mouse genes will be referred to using lower case letters (*i.e.* Cyp1a1) throughout this thesis.

In general, CYPs within the same gene family (*i.e.* CYP1A1 and CYP1B1) exhibit >40% amino acid sequence similarity, while sequences with the same subfamily (*i.e.* CYP1A1 and CYP1A2) exhibit >55% amino acid sequence identity (Nelson *et al.*, 1996). Also of note, CYP genes often exist as clusters in the genome of mammals, with all members of a subfamily located at the same point on a chromosome. In some cases, different subfamilies within a single gene family also exhibit such clustering as is the case with the CYP2A, CYP2B and CYP2F subfamilies (Bale *et al.*, 1991).

1.2 Overview of Major Cytochromes P450

1.2.1 Hepatic and Extrahepatic Cytochrome P450

Most of the CYPs characterized to date have been obtained from liver tissue, however many of the same isozymes are also expressed extrahepatically. Furthermore, a

number of CYPs exhibit characteristic tissue-specific or -selective patterns of expression and regulation. Examples include CYP2G1 which occurs only in olfactory tissue (Ding and Coon, 1994), a prostaglandin ω-hydroxylase present only in the lung of pregnant animals (Williams *et al.*, 1984), and CYP2J2, a human AA epoxygenase highly expressed in heart (Wu *et al.*, 1997). The liver is quantitatively the most important site of xenobiotic metabolism in the body owing to its large mass and high CYP concentration. This substantial metabolic capacity provides a great potential for hepatotoxicity as a result of CYP-dependent bioactivation of xenobiotics to toxic metabolites. However, the liver is relatively well protected against reactive metabolites by a large number of chemical detoxication systems including glutathione *S*-transferases, glucuronosyltransferases, and epoxide hydrolases, among others (Armstrong, 1997; De Waziers *et al.*, 1990; Forrester *et al.*, 1992; Gonzalez, 1997; Lu and Tappel, 1992; Prestera *et al.*, 1993). Tissues such as the kidney (Krishna and Klotz, 1994), small intestine (Watkins, 1992), vascular endothelium (Stegeman *et al.*, 1995) and brain (Ravindranath and Boyd, 1995) also express significant amounts of CYP and therefore, are potential sites for chemical toxicity and carcinogenesis. In particular, the lung expresses relatively high levels of a number of CYPs implicated in xenobiotic oxidation (Buckpitt *et al.*, 1995; Cho *et al.*, 1995; Shimada *et al.*, 1992). However, in contrast to the liver, the lung exhibits several characteristics that render it more susceptible to xenobiotic toxicity. These include: (1) exposure to xenobiotics via the circulation and inspired air, (2) exposure to all of the cardiac output and hence, all blood-borne substances, (3) differences in the spectrum and relative level of expression of individual CYPs compared with liver which may favour bioactivation of some classes of xenobiotics, (4) heterogeneity of cell types, each with different levels of CYPs and chemical detoxication enzymes, (5) high O_2 tension which favours formation of reactive oxygen species (ROS) and drives CYP-dependent oxidations, and (6) a relatively low and heterogeneous distribution of detoxication systems compared with liver. As mentioned previously, the fungal pneumotoxin, 4-ipomeanol is an example of a xenobiotic

that requires metabolic activation for lung-selective toxicity. Various lines of evidence have implicated CYP in this process, including the absence of 4-ipomeanol toxicity in humans which lack the pulmonary isozyme (CYP2F) required for bioactivation (Guengerich, 1994). Thus, due to a number of factors, the lung is a particularly important site of CYP-mediated toxicity and/or carcinogenesis.

Mammalian CYP isozymes belonging to families 1-4, which include at least 15 subfamilies (Nelson *et al.*, 1996), are localized primarily in the endoplasmic reticulum. These CYPs are the primary isozymes concerned with xenobiotic metabolism and exhibit quite broad and overlapping substrate specificities. CYPs of the remaining families metabolize endogenous compounds and exhibit rather rigid substrate selectivities. These include CYPs involved in the metabolism of thromboxane, prostacyclin, bile acids, steroids and vitamin D_3 (Kagawa and Waterman, 1995; Miyata *et al.*, 1994; Omura and Morohashi, 1995; Yokoyama *et al.*, 1993). The studies presented in this thesis are concerned with the regulation of expression and catalytic activity of CYPs involved in xenobiotic metabolism, and in particular, the CYP1 and CYP2 families. A summary of experimentally useful inducers and marker catalytic activities of these isoforms is presented in Table 1.1.

1.2.2 The CYP1 Family

The CYP1 family is composed of two subfamilies - CYP1A and CYP1B. Within a species, CYP1A1 and CYP1A2 exhibit, on average, 70% amino acid sequence identity (Nelson *et al.*, 1996). Across species, these CYP1As are among the most highly conserved with complete or nearly complete coding sequences for one or both reported for human (Jaiswal *et al.*, 1985; Quattrochi and Tukey, 1989), mouse (Kimura *et al.*, 1984a), rat (Yabusaki *et al.*, 1984a; 1984b), guinea pig (Black *et al.*, 1997; Ohgiya *et al.*, 1993), hamster (Sagami *et al.*, 1991), monkey (Komori *et al.*, 1992) and dog (Uchida *et al.*, 1990). The evolutionary conservation of the CYP1A subfamily across mammals is consistent with a critical role in the metabolism of environmental chemicals. Furthermore,

Table 1.1 - Summary of experimentally useful inducers and marker substrates for xenobiotic metabolizing CYPs in rodents.

Family/Subfamily/Isozyme	Marker Substrates	Inducers
CYP1A1	7-ethoxyresorufin	TCDD
	benzo[*a*]pyrene	βNF
CYP1A2	7-methoxyresorufin	isosafrole
	caffeine	βNF
CYP2A	coumarin	PB (CYP2A1, weakly)
	testosterone (7α- and	TCDD (CYP2A3, weakly)
	15α-hydroxylation)	none (CYP2A2)
CYP2B	7-pentoxyresorufin	PB
	benzphetamine	
CYP2C	mephenytoin	PB (weakly)
	tolbutamide	
CYP2D	debrisoquine	not inducible
	codeine	
CYP2E1	p-nitrophenol	ethanol
	chlorzoxazone	diabetes
		starvation
CYP3A	nifedipine	dexamethasone
	erythromycin	phenobarbital
		pregnenolone 16α-carbonitrile
CYP4A	lauric acid	clofibrate

the neonatal lethality associated with mice homozygous for deletions of the *Cyp1a2* gene (Pineau *et al.*, 1995) indicates that this isoform also performs essential developmental or physiological functions.

The catalytic activity of all members of the CYP1 family is increased in the liver after treatment of animals with 3-methylcholanthrene (3MC), β-naphthoflavone (βNF) and other PAHs, but most potently by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a halogenated PAH (Denison and Whitlock, 1995). Induction occurs primarily at the level of gene transcription, and is mediated by the aryl hydrocarbon receptor (AHR), a novel, ligand-activated transcription factor (Rowlands and Gustafsson, 1997; Schmidt and Bradfield, 1996). The mechanism of AHR-mediated induction of gene expression is discussed in more detail later in this thesis (section 1.4). Although the transcriptional regulation of all members of the CYP1 family shares many common characteristics, most notably AHR-mediated induction, several notable differences exist. First, while CYP1A1 is inducibly expressed in most tissues of the body, constitutive and inducible expression is confined primarily to the liver (Kimura *et al.*, 1984b). For example, human CYP1A1 expression is increased in the lung (McLemore *et al.*, 1990; Omiecinski *et al.*, 1990b), placenta (Okey *et al.*, 1997; Song *et al.*, 1985) and lymphocytes (Song *et al.*, 1985) of smokers, while CYP1A2 is induced only in the liver. Second, a number of inducers selective for CYP1A1 or CYP1A2 have been described. For example, isosafrole administration to animals preferentially induces hepatic CYP1A2 expression, with only minor effects on CYP1A1 (Adams *et al.*, 1993). Third, a number of immortalized hepatocyte cell lines exhibit inducible CYP1A1 expression similar to that seen in the liver (Fujii-Kuriyama *et al.*, 1992). In contrast, such cell lines with stable constitutive or inducible CYP1A2 expression have not been reported. Finally, while CYP1A1 expression is virtually absent in the livers of untreated animals, CYP1A2 expression is readily detected (Kimura *et al.*, 1984b). CYP1B1 also exhibits a number of unique regulatory characteristics. In addition to exhibiting tissue-specific (kidney, prostate, adrenal, breast)

patterns of expression, the basal and inducible expression of CYP1B1 is also influenced by the sex and hormonal status of the animal (Christou *et al.*, 1995; Walker *et al.*, 1995).

In general, the CYP1A subfamily oxidatively biotransforms lipophilic xenobiotics, including a number of pro-carcinogenic PAHs, heterocyclic amines and aromatic amines (Josephy *et al.*, 1995; McManus *et al.*, 1990). Collectively, these CYPs metabolize approximately 90% of known carcinogens. The best characterized example for CYP1A1 is its participation in the bioactivation of BaP to the potent mutagen 7*R*,8*S*-dihydrodiol-BaP-9*S*,10*R*-epoxide (Gelboin, 1980). A number of studies and convergent lines of evidence indicate that increased levels of CYP1A1 expression, particularly in the lung, is a risk factor for chemical carcinogenesis (Spivack *et al.*, 1997). Human CYP1A1 also metabolizes a number of drugs such as warfarin (Darbyshire *et al.*, 1996) and tamoxifen (Williams *et al.*, 1994).

CYP1A2 bioactivates a number of pro-carcinogens, and in particular, aromatic amines such as 2-acetylaminofluorene, 2-aminoanthracene, 2-aminofluorene and 4-aminobiphenyl (Aoyama *et al.*, 1989; McManus *et al.*, 1990). The potent hepatic carcinogen and dietary contaminant aflatoxin B_1 (AFB_1) is also bioactivated to mutagenic epoxide metabolites by CYP1A2 (Aoyama *et al.*, 1989). However, CYP1A2 also catalyzes the 4-hydroxylation of AFB_1 to aflatoxin M_1, a detoxication pathway (Faletto *et al.*, 1988; Gurtoo *et al.*, 1978). CYP1A2 is the major catalyst of caffeine and phenacetin metabolism in humans (Bloomer *et al.*, 1995; Butler *et al.*, 1989), and contributes to propanolol (Ching *et al.*, 1996), warfarin (Darbyshire *et al.*, 1996), clozapine and theophylline (Rendic and Di Carlo, 1997) metabolism.

CYP1B1 catalyzes the oxidation of PAHs and aryl amines and is able to bioactivate several PAH dihydrodiols, an activity that may contribute to PAH-induced mammary carcinogenesis in rodents (Savas *et al.*, 1994). Little information is available regarding the metabolism of drugs by this isoform, however the hydroxylation of 17β-estradiol by human CYP1B1 is suggestive of an endogenous function (Christou *et al.*, 1995).

1.2.3 The CYP2 Family

Orthologues of the CYP2A subfamily include CYP2A1, 2A2 and 2A3 in the rat, Cyp2a4 and 2a5 in the mouse and CYP2A6 and 2A7 in humans (Nelson *et al.*, 1996). Of the rat isoforms, CYP2A1 is weakly inducible by phenobarbital (PB) treatment, CYP2A2 is non-inducible and CYP2A3 is weakly induced by PAHs (Fernandez-Salguero and Gonzalez, 1995). CYP2A1 and 2A2 are expressed primarily in liver while CYP2A3 is expressed in lung and intestine, but not liver (Chang and Waxman, 1996). Additionally, CYP2A1 is a female-dominant isoform while CYP2A2 appears to be male-specific (Waxman *et al.*, 1995). CYP2A1 catalyzes the 3-hydroxylation of coumarin and the 7α-hydroxylation of testosterone, while CYP2A2 hydroxylates testosterone at the 15α-position, but does not metabolize coumarin (Guengerich, 1997). Human CYP2A6 possesses coumarin 7-hydroxylation activity and has been shown to bioactivate AFB_1 and diethylnitrosamine to mutagenic metabolites (Gonzalez and Gelboin, 1994).

CYP2B1 and CYP2B2 are the rat members of the CYP2B subfamily. While these isoforms exhibit extensive (91%) sequence identity (Nelson *et al.*, 1996), regulation of their expression is quite different. For example, while CYP2B1 is not constitutively expressed in liver, it is highly induced by PB treatment (Funae and Imaoka, 1993). In contrast, hepatic CYP2B2 is basally expressed, but is only moderately induced after PB treatment. Tissue-specific regulation of CYP2Bs is also observed in the rat with constitutive CYP2B1 expression occurring both in the lung and testis, while CYP2B2 is not expressed in these tissues (Soucek and Gut, 1992). Neither isoform is inducible extrahepatically. While PB is the prototypical inducer of CYP2Bs, non co-planar polychlorinated biphenyls (PCBs) such as 2,4,5,2',4',5;-hexachlorobiphenyl also increase hepatic expression in the rat (Ikegwuonu *et al.*, 1996). The orthologous rabbit isoforms (CYP2B4 and 2B5) are also inducible in the liver by PB and some PCBs (Domin *et al.*, 1986). Two human CYP2Bs which are expressed in liver (CYP2B6) and lung (CYP2B7)

have been identified (Gonzalez, 1992). Although CYP2Bs preferentially bioactivate some pro-carcinogens including cyclophosphamide (Chang *et al.*, 1993), 6-aminochrysene (Mimura *et al.*, 1993) and a number of *N*-nitrosodialkylamines (Kawanishi *et al.*, 1992), this subfamily is generally associated with detoxication. Known substrates for CYP2B5 include PB and other barbiturates, phenytoin, phenylbutazone, benzphetamine, nicotine, 7-pentoxyresorufin and a number of organochlorine pesticides such as DDT, aldrin, endrin, heptachlor and chlordane (Nims and Lubet, 1996).

The CYP2C subfamily is the largest in mammals with 8, 10 and 4 cDNAs cloned to date from rat, rabbit and human, respectively. There is considerable overlap between the substrate specificity of CYP2Bs and CYP2Cs, particularly with respect to the metabolism of barbiturate drugs (Richardson and Johnson, 1996). Also in common, CYP2Cs are inducible, albeit more weakly than CYP2Bs, by PB (Funae and Imaoka, 1993). CYP2C expression in rodents appears to be predominantly hepatic, although low level expression occurs in the kidney, lung and brain (Karara *et al.*, 1993; Ryan *et al.*, 1993; Warner and Gustafsson, 1994). CYP2Cs catalyze the formation of EETs from endogenous AA, with CYP2C3 representing the major AA epoxygenase of rat kidney (Karara *et al.*, 1993). These AA metabolites exert a number of potent biological effects and are important in kidney regulation and function (Capdevila *et al.*, 1981; McGiff *et al.*, 1996). CYP2Cs are also an important subfamily for drug metabolism due to their high hepatic content (approximately 16% of total liver CYP in humans) and their oxidation of many drugs (Shimada *et al.*, 1994). Clinically important drugs metabolized by CYP2Cs include mephenytoin, tolbutamide, warfarin, phenytoin and a number of barbiturates (Richardson and Johnson, 1996). Although generally not involved in toxication, CYP2C isozymes bioactivate tienilic acid to a reactive epoxide metabolite capable of alkylating and inactivating the CYP2C isozyme which catalyzes its formation (Lopez-Garcia *et al.*, 1993). The covalently modified CYP may then be recognized by the immune system and initiate an

autoimmune response, potentially leading to autoimmune hepatitis (Wrighton and Stevens, 1992).

Six CYP2D isoforms have been cloned and sequenced from rat, while 5 and 1 have been identified in mouse and human, respectively (Nelson *et al.*, 1996). Two human pseudogenes (*CYP2D7P* and *CYP2D8P*) have been described (Kimura *et al.*, 1989), although no cDNA has been cloned that corresponds to these genes and their expression has not been documented. No known xenobiotic inducers of the CYP2D subfamily have been identified, although sex-dependent and developmental patterns of constitutive expression have been described (Gonzalez, 1996). There is considerable interest in human CYP2D6 because although this isoform represents only a small proportion of total hepatic CYP (approximately 2%), about 30% of currently used drugs are metabolized by this isoform (Shimada *et al.*, 1994). Furthermore, CYP2D6 exhibits genetic polymorphisms that can cause accumulation of unchanged parent drug owing to deficient metabolism, leading to potential toxicity and adverse drug reactions in humans (Inaba *et al.*, 1995). Examples of drugs affected by this CYP2D6 polymorphism include debrisoquine, sparteine and codeine (Rannug *et al.*, 1995).

The CYP2E orthologues found in different species are highly homologous, resulting in the designation of CYP2E1 for the single isoform of rat, mouse and human (Nelson *et al.*, 1996). Rabbit is the exception, with two closely related isoforms designated as CYP2E2 and CYP2E3 (Nelson *et al.*, 1996). CYP2E1 expression is regulated in a complex manner with independent mechanisms of transcriptional, post-transcriptional and post-translational modulation identified. For example, acute ethanol treatment induces CYP2E1 catalytic activity and protein levels by protein stabilization, possibly through inhibition of site-specific phosphorylation which targets CYP2E1 for degradation (Roberts *et al.*, 1995). On the other hand, CYP2E1 levels are also increased at the transcriptional level by pathobiological states such as starvation, chronic ethanol exposure and diabetes, most likely as part of a global gluconeogenesis response (Hu *et al.*,

1995). Constitutive expression of CYP2E1 is highest in the liver with lower levels occurring in the kidney, lung, brain, lymphocytes and small intestine (Chen *et al.*, 1996; Forkert, 1995; Soh *et al.*, 1996; Tindberg and Ingelman-Sundberg, 1996). Toxicologically, CYP2E1 is a very important isozyme due to two important factors: First, CYP2E1 bioactivates a number of low molecular weight pro-carcinogens such as benzene, nitrosamines and azoxymethane, as well as the hepatotoxicants acetaminophen, halothane and enflurane (Anundi and Lindros, 1992; Lee *et al.*, 1996b; Lieber, 1997; Valentine *et al.*, 1996). Secondly, CYP2E1 exists predominantly in the high-spin form, an unusual property that allows the enzyme to activate oxygen by the transfer of one or more electrons (Fig. 1.1) in the absence of substrate (Ingelman-Sundberg and Johansson, 1984). Depending upon the conditions, a number of cytotoxic and potentially mutagenic ROS can be generated by this process of futile cycling (Koop *et al.*, 1996). Thus, induction of CYP2E1 has the potential to contribute to toxicity and carcinogenesis both in the presence and absence of substrate. CYP2E1 has also been reported to metabolize endogenous AA to toxic metabolites that are apoptotic and may be important in alcoholic liver disease (Chen *et al.*, 1997; French *et al.*, 1997). Other drug and xenobiotic substrates of CYP2E1 include *p*-nitrophenol, aniline, chlorzoxazone, ethanol and acetone (Ronis *et al.*, 1996).

The remaining CYP2s belong to the CYP2F-2K subfamilies. CYP2F1 and Cyp2f2 have been identified in human and mouse lung, respectively, and are able to bioactivate the pro-carcinogen β-naphthylamine (Gonzalez and Gelboin, 1994). This isozyme is not expressed in rat lung and is responsible for the species-selective (mouse *vs.* rat) toxicity of β-naphthylamine toxicity to murine Clara cells (Buckpitt *et al.*, 1992; 1995). CYP2G1 is an olfactory-specific CYP of rat and rabbit (Ding and Coon, 1994) and CYP2J2 is an AA epoxygenase highly expressed in human heart (Wu *et al.*, 1996). Little information is available regarding the toxicological relevance of these CYPs.

1.2.4 The CYP3 Family

Presently, CYP3A represents the only CYP3 subfamily that has been identified. In rat and mouse, two CYP3A isoforms have been identified (CYP3A1, CYP3A2 and Cyp3a11, Cyp3a13), while in humans three distinct cDNAs (CYP3A4, 3A5 and 3A7) have been cloned and sequenced (Nelson *et al.*, 1996). As a group, CYP3As are induced by a large number of synthetic steroids (pregnenolone 16α-carbonitrile, spironolactone and dexamethasone), glucocorticoids, macrolide antibiotics (triacetyloleandomycin, erythromycin and rifampicin) and PB (Denison and Whitlock, 1995; Okey, 1990; Wrighton and Stevens, 1992). The mechanism of induction has not been fully elucidated, although for most of these compounds induction appears to be primarily transcriptional and dependent upon the inducer used (Okey, 1990). Human CYP3A isoforms constitute on average 30% of the total hepatic CYP and are associated with >40% of drug metabolism (Shimada *et al.*, 1994). CYP3As, and in particular CYP3A4, are also expressed at relatively high levels in human small intestine and are an important determinant of the first-pass metabolism of a number of drugs (De Waziers *et al.*, 1990; Debri *et al.*, 1995; Watkins, 1992). Generally, CYP3As mediate the metabolic deactivation of xenobiotic substrates or in some cases, bioactivation of pro-drugs to the active pharmacological agent (Wrighton and Stevens, 1992). However, the pro-carcinogens AFB_1, 6-aminochrysene and 1-nitropyrene can all be bioactivated by CYP3As (Gonzalez and Gelboin, 1994). Furthermore, the 9,10-epoxidation of BaP-7,8-diol to yield the ultimate carcinogen is catalyzed by a CYP3A (Shimada *et al.*, 1989). Drug substrates of human CYP3As include cyclosporin, erythromycin, estradiol, felodipine, FK-506, nifedipine, omeprazole, terfenadine and troleandomycin (Maurel, 1996).

1.2.5 The CYP4 Family

The CYP4 family consists of 6 subfamilies (CYP4A-4F) of which, CYP4A has been the most extensively studied. In rat and rabbit, the cDNAs for 4 CYP4As have been

cloned and sequenced, while 3 and 2 CYP4As have been identified in mouse and human, respectively (Nelson *et al.*, 1996). Members of the CYP4A subfamily are constitutively expressed in the liver and represent the major CYPs present in kidney (Imaoka *et al.*, 1988). Clofibrate and other peroxisome proliferators induce the expression of CYP4As via a signal transduction pathway mediated by the peroxisome proliferator activated receptor (Johnson *et al.*, 1996). CYP4As are efficient catalysts of the ω and ω-1 hydroxylation of a number of fatty acid substrates including lauric acid, palmitic acid, prostaglandins, leukotrienes and AA (Lake and Lewis, 1996). The metabolism of xenobiotics by CYP4As appears to be limited, although CYP4A1 catalyzes the hydroxylation of the plasticizer and weak rodent carcinogen mono-2-ethyl hexyl phthalate (Lake and Lewis, 1996). Rabbit and human lung CYP4B1 has been shown to be involved in the bioactivation of the pro-carcinogens 2-aminofluorene and 4-aminobiphenyl (Gonzalez and Gelboin, 1994; Vanderslice *et al.*, 1987) although the recombinant human isozyme is much less active in this regard (Czerwinski *et al.*, 1991).

1.3 Inhibition of Cytochrome P450

1.3.1 Classification of Cytochrome P450 Inhibitors

Many inhibitors of CYP-dependent catalysis, some with selectivity for particular subfamilies or individual isoforms, have been identified and can be categorized based upon their mechanism(s) of action. Three steps of the CYP catalytic cycle (Fig. 1.1) are particularly vulnerable to inhibition: (1) substrate binding, (2) binding of O_2, and (3) oxidation of the substrate. In general, inhibitors that act prior to oxidation of the substrate are classified as reversible competitive inhibitors. Agents that act during, or subsequent to oxygenation of the substrate fall into the category of quasi-irreversible or irreversible inhibitors and act through the formation of metabolic intermediate complexes or by mechanism-based inactivation. While these designations are useful for categorizing the

various types of CYP inhibitors, in reality, many of these compounds exhibit multiple mechanisms of inhibition depending upon the isozymes involved and the metabolic fate of the inhibitor. An example of such multiplicity of inhibition mechanisms is provided by *N*-aralkylated derivatives of 1-aminobenzotriazole (ABT), a class of CYP inhibitors that are examined in detail in Chapters 2, 3 and 4 of this thesis.

1.3.2 Competitive Inhibition

Competitive inhibitors of CYP bind reversibly to the heme iron and/or elsewhere in the active site and subsequently compete with O_2 for substrate binding. Such competitive CYP inhibitors, as a group, exhibit only a small degree of selectivity for individual isoforms, particularly those that ligate with the heme iron only. For example, CO is a general inhibitor of all CYP isoforms by virtue of its ability to effectively compete with O_2 for ligation to the ferrous heme iron, a common feature of all CYPs (Testa and Jenner, 1981). Other small molecules such as cyanide and NO are also able to inhibit CYP in a manner similar to CO (Stadler *et al.*, 1994). Recently, an irreversible mechanism of inhibition, which promotes heme loss from CYP, has also been identified for NO (Minamiyama *et al.*, 1997). Agents that simultaneously bind to CYP heme and lipophilic regions of CYP protein are inherently more effective and selective than agents that bind to heme alone. Among the most potent and selective reversible competitive inhibitors of CYP are nitrogen heterocyclic compounds, including imidazoles, quinolines, pyridines and ellipticines (Murray, 1987; Testa and Jenner, 1981). The potency of inhibition by these compounds arises from an ability to block both oxygen and substrate binding. Thus, the relative selectivity of these inhibitors depends upon the tightness of fit to the substrate binding site and how well this binding allows coordination of the inhibitor nitrogen with the heme iron (Murray, 1987). An example of a useful selective inhibitor of CYP is quinidine which inhibits human CYP2D6 (Ono *et al.*, 1996). Ketoconazole is a reversible CYP inhibitor that has been used extensively in experimental studies and has also been

associated with numerous drug-drug interactions in humans involving CYP3A-dependent metabolism (Bourrié *et al.*, 1996).

1.3.3 Metabolic Intermediate Complexation

The selectivity of enzyme inhibitors is increased substantially by including a requirement for metabolism by the target enzyme in the mechanism of inhibition. The presence of a latent functional group within an inhibitor can restrict inhibition to those CYPs which are capable of binding the compound, metabolism to generate the active inhibitor and orientation of the metabolic intermediate such that interaction with CYP heme is possible. Inhibitors that act in this manner are said to form metabolic intermediate (MI) complexes with CYP. Such inhibitors coordinate very tightly with CYP heme, but can be displaced under specific experimental conditions and thus, inhibit CYP in a quasi-irreversible manner. Chemical structures associated with MI complexation include methylenedioxy derivatives (Marcus *et al.*, 1985; Murray *et al.*, 1983), alkyl and aromatic amines (Franklin, 1991; Lindeke and Paulsen-Sörman, 1988) and hydrazines (Battioni *et al.*, 1983; Hines and Prough, 1980).

Aryl and alkyl methylenedioxy compounds, such as 1,3-benzodioxole, undergo CYP-dependent oxidation to intermediates that coordinate tightly with both the ferrous and ferric forms of the heme iron (Marcus *et al.*, 1985). The ferrous complexes can be visualized in the absorbance difference spectrum of CYP in the presence of NADPH by characteristic maxima at λ = 427 and 455 nm (Dahl and Hodgson, 1979). Ferric complexes have a single absorbance maximum at 437 nm (Dahl and Hodgson, 1979). The peaks at 427 and 455 nm probably represent two distinct complexes, however the relationship between the two presently is not clear (Murray, 1997; Murray and Reidy, 1989). Catalytically active CYP can be regenerated from the ferric complex by incubation with lipophilic compounds that displace the inhibitor from the active site (Dickins *et al.*, 1979). In contrast, the ferric complexes are not displaced by lipophilic compounds, but

require irradiation at 400-500 nm for dissociation (Ullrich and Schnabel, 1973). The weaker nature of the ferric *vs.* ferrous complex, combined with extensive structure-activity studies, indicates that the former represents coordination of a reactive intermediate with the heme iron only, while the latter reflects concurrent binding with CYP heme and the substrate binding site (Murray, 1997; Murray *et al.*, 1985). The majority of experimental evidence supports the formation of a carbene-iron complex for methylenedioxy compounds (Mansuy *et al.*, 1979) and structural resemblance of carbenes to CO readily rationalizes the resemblance of the 455 nm ferrous complex to that formed with CO and CYP heme.

Aryl and alkyl amines are a large class of compounds that form MI complexes with CYP. This class includes a number of clinically useful macrolide antibiotics such as troleandomycin and erythromycin that form MI complexes with CYP3A isozymes (Bensoussan *et al.*, 1995; Franklin, 1991; Lindstrom *et al.*, 1993), psychomotor stimulant amphetamines that form MI complexes with CYP2B isozymes (Franklin, 1974; 1995), as well as the antidepressant nortryptiline and the H_2-receptor antagonist cimetidine that form MI complexes with CYP2C11 (Faux and Combes, 1994; Murray, 1992). Evidence is presented in Chapter 2 of this thesis for the inclusion of *N*-aralkylated derivatives of ABT in this class of MI complex forming amines. Oxidation of such amines, via multiple CYP-dependent steps, yields intermediates that coordinate tightly to ferrous CYP heme, and consequently, interfere with O_2 binding and inhibit CYP-dependent catalysis. The resultant MI complexes give rise to a characteristic absorbance maximum in the 455-460 nm range (Franklin, 1982). The MI complexes formed with many amines, such as SKF-525A (Murray, 1988) or troleandomycin (Pessayre *et al.*, 1982), are of sufficient stability that intact MI complexes can be isolated from animals treated *in vivo* with the inhibitor. However, dissociation of the MI complex and restoration of catalytic activity can be achieved by conversion of CYP heme to the ferric state using oxidizing agents such as potassium ferricyanide (Murray, 1988).

One of the best characterized classes of MI complex forming amines, with respect to the metabolic steps required for generation of the inhibitory intermediate, is the amphetamines. Studies with these compounds have shown that the amine group is first *N*-hydroxylated to yield the corresponding hydroxylamine derivative (Franklin, 1974; Mansuy *et al.*, 1977). The hydroxylamines are more effective at MI complexation than the parent compound, but require further oxidation to generate the species that coordinates with the heme iron. This species is most likely produced by a two electron oxidation of the hydroxylamine to generate a nitroso derivative which binds to the ferrous CYP heme via coordination of the nitrogen with ferrous iron (Franklin, 1974). This mechanism of activation and the nature of the MI complex ligand have proven to be a valid model for MI complexation of CYP by most alkyl and aryl amines. Interestingly, the ability of some amines to act as apparent inducers of the CYPs they inhibit can also be rationalized by MI complex formation. For example, *in vivo* MI complexation of triacetyloleandomycin with CYP3A isoforms stabilizes the enzymes and prolongs their half-life in the hepatocyte (Watkins *et al.*, 1986; Wrighton *et al.*, 1986). The net effect is to increase the concentration of CYP protein (*i.e.* induction) which does not result in a corresponding increase of catalytic activity without dissociation of the MI complex (Pessayre *et al.*, 1981a; 1981b). A similar response is seen with the antibiotic, tiamulin (Witkamp *et al.*, 1995).

1.3.4 Mechanism-Based Inactivation

The most selective inhibitors of CYP generally fall into the class of mechanism-based (MB) inactivators. Similar to MI complexation, selectivity for the inhibition of individual CYP subfamilies or individual isoforms arises from the requirement for highly specific binding and metabolic activation to generate the ultimate inhibitory species. In contrast to MI complexation, MB inactivation is truly irreversible (hence the term inactivation) and the loss of function that results can only be replaced by *de novo* synthesis of the target enzyme (Halpert, 1995; Halpert *et al.*, 1994). MB inactivation is a time-

dependent, pseudo first-order process that obeys saturation kinetics and can be characterized by a maximal rate constant (k_{inact}) and by an inhibitor constant (K_I) that reflects the concentration of inactivator required to achieve the half-maximal rate of inactivation (Silverman, 1988). A difference in either of these constants for inactivation of two different CYPs can result in selective inactivation of one (Halpert, 1995). In Chapters 3 and 4 of this thesis, experimental evidence is presented to rationalize the selectivity of *N*-aralkylated derivatives of ABT as MB inactivators of CYP2B *vs.* CYP1A isoforms in terms of inactivation kinetics. Several irreversible MB inactivators of CYP have been reported to be highly selective for a particular CYP isozyme(s). Examples of some experimentally useful MB inactivators that exhibit relative selectivity towards xenobiotic metabolizing CYPs in rats are given in Table 1.2.

Irreversible MB inactivation of CYP occurs by three different processes, all of which produce covalent modifications. The first involves alkylation of a pyrrole nitrogen in the prosthetic heme group by reactive intermediates produced by CYP-dependent metabolism of the inhibitor. A number of terminal acetylenes, olefins and dihydropyridines inactivate CYP by this mechanism (Ortiz de Montellano, 1991; Ortiz de Montellano and Correia, 1983; Ortiz de Montellano and Reich, 1984). A second mechanism involves covalent modification by alkylation or arylation of the CYP apoprotein. One of the best characterized examples of this mechanism is the conversion of chloramphenicol to a reactive oxamyl intermediate that inactivates CYP2B1 by modifying one or more lysine residues in the protein and thereby prevents transfer of electrons from P450 reductase (Halpert *et al.*, 1985). A third mechanism involves the generation of metabolic intermediates that are capable of activating the CYP heme moiety to a reactive species that can then bind covalently with the apoprotein. A well characterized example of this mechanism is illustrated by cumene hydroperoxide which promotes attachment of the activated heme group of CYP2B1 to a cysteine residue in the protein moiety (Yao *et al.*, 1993). Any particular MB inactivator may utilize one or more of these mechanisms to

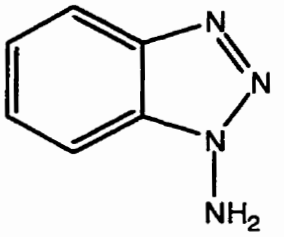
Table 1.2 - Summary of experimentally useful relatively selective mechanism-based inactivators of xenobiotic metabolizing CYPs in rat.

Family/Subfamily/Isozyme	Inhibitor	Reference
CYP1A1	1-ethynylpyrene	(Hopkins *et al.*, 1992)
CYP1A2	furafylline	(Sesardic *et al.*, 1990)
CYP2A	8-methoxypsoralen	(Mays *et al.*, 1990)
CYP2B	αMB	(Woodcroft *et al.*, 1990)
	secobarbital	(He *et al.*, 1996a)
CYP2C6	pregn-4,20-diene-3-one	(Halpert *et al.*, 1988)
CYP2D	4-allyloxymetamphetamine	(Lin *et al.*, 1994)
CYP2E1	disulfiram	(Brady *et al.*, 1988)
	diallyl sulfide	(Brady *et al.*, 1991)
CYP3A	gestodene	(Guengerich, 1990a)
CYP4A1	10-undecynoic acid	(CaJacob *et al.*, 1988)

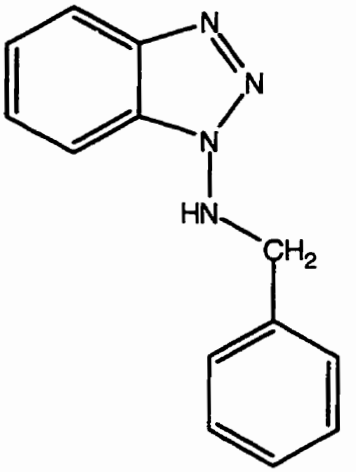
inhibit CYP, depending upon the specific CYP isoform considered and the number of different isozymes affected.

One of the best characterized MB inactivators of CYP is ABT. *In vitro*, ABT has been shown to irreversibly inactivate hepatic (Ortiz de Montellano and Mathews, 1984; Woodcroft and Bend, 1990), pulmonary (Mathews and Bend, 1986; Woodcroft *et al.*, 1990), renal (Mugford *et al.*, 1992) and adrenal (Xu *et al.*, 1994) CYP. ABT is also an effective MB inactivator of CYP in these tissues *in vivo* (Knickle and Bend, 1992; Knickle *et al.*, 1993; Mugford *et al.*, 1992; Xu *et al.*, 1995). Following CYP-dependent oxidation of the amino group, ABT undergoes rearrangement to form a highly reactive benzyne intermediate and two molecules of N_2. *N,N*-bridged phenylene-protoporphyrin IX adducts have been isolated from the livers of rats treated with ABT (Ortiz de Montellano, 1991; Ortiz de Montellano and Mathews, 1984). Based upon this, it is believed that the mechanism by which ABT inactivates CYP involves *N*-arylation of the prosthetic heme moiety by addition of the benzyne intermediate across two vicinal nitrogens. Unlike most MB inactivators, ABT inactivates a wide variety of CYP isozymes and exhibits very little selectivity. Examination of changes in substrate oxidations diagnostic for CYP1A, CYP2B, CYP2E, CYP4A and CYP4B subfamilies has revealed that all are inactivated to some degree by ABT (Ortiz de Montellano and Costa, 1986; Tierney *et al.*, 1992; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990). A limited degree of selectivity for guinea pig pulmonary CYP4B *vs.* CYP1A and CYP2B isoforms has been achieved using low concentrations of ABT (Woodcroft *et al.*, 1990).

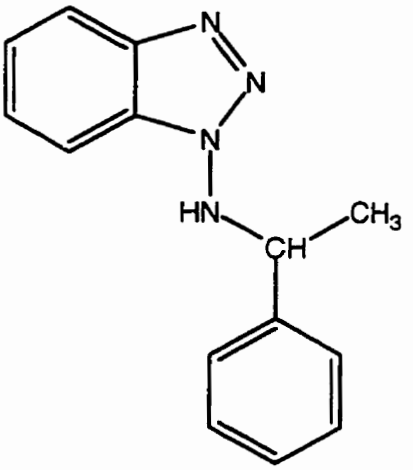
In an effort to produce a MB inactivator of CYP that exhibits selectivity for individual isoforms in concert with the effective inactivation exhibited by ABT, a series of *N*-aralkylated derivatives was designed and synthesized in our laboratory (Fig. 1.2). *N*-benzyl-1-aminobenzotriazole (BBT), *N*-α-methylbenzyl-1-aminobenzotriazole (αMB) and *N*-α-ethylbenzyl-1-aminobenzotriazole (αEB) have since been shown to be potent isozyme- (CYP2B) and lung-selective MB inactivators of CYP both *in vitro* (Grimm *et al.*,



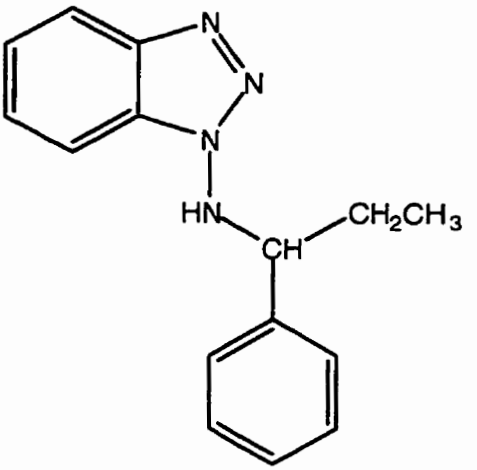
ABT



BBT



αMB



αEB

Figure 1.2
Chemical structures of *N*-aralkylated derivatives of ABT.

1995; Mathews and Bend, 1986; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990) and *in vivo* (Knickle *et al.*, 1994; Mathews and Bend, 1993). These compounds contain the ABT nucleus attached to *N*-aralkylated substituents designed to structurally mimic benzphetamine, a substrate oxidized by CYP2B isoforms (Guengerich, 1997; Serabjit-Singh *et al.*, 1979). The ABT derivatives are also expected to be concentrated in the lung *in vivo* via the pulmonary facilitated uptake system for basic lipophilic amines (Bend *et al.*, 1985). Of the ABT derivatives, αMB exhibits the greatest selectivity and potency for pulmonary CYP2B. For example, using guinea pig lung microsomes *in vitro*, 1 μM αMB inactivated >90% of the CYP2B-dependent 7-pentoxyresorufin *O*-deethylation (PROD) activity, compared with <15% loss of CYP1A- and CYP4B-dependent catalytic activities (Woodcroft *et al.*, 1990). The ability of these compounds to inhibit rabbit pulmonary CYP was investigated *in vivo* by Mathews and Bend (1993). One hour following i.v. administration of 1 μmol/kg αMB, 80% inactivation of pulmonary CYP2B4 *vs.* 5% inactivation of CYP1A1 was observed. In a similar study with guinea pigs (Knickle *et al.*, 1994), 0.075 μmol/kg αMB inactivated 80% of pulmonary CYP2B-dependent catalytic activity with only a very small effect on CYP1A1- or CYP4B-dependent catalysis. Additionally, the same dose of αMB did not inactivate any of the hepatic CYP-dependent catalytic activities examined by more than 15%. Taken together with the aforementioned *in vitro* studies, these data verify the ability of *N*-aralkylated derivatives of ABT, and in particular αMB, to act as potent lung-selective MB inactivators of CYP2B. Data presented in Chapters 3 and 4 of this thesis provides additional evidence and rationale in terms of inactivation kinetics, for the selectivity of these compounds.

A number of studies with BBT have provided evidence for the inactivation of CYP by at least three mechanisms. Firstly, an abnormal porphyrin pigment with physical properties identical to the phenylene-porphyrin adduct formed by ABT has been isolated from the liver of PB-induced rats treated with BBT (Mathews and Bend, 1986). This is indicative of heme arylation caused by the liberation of a reactive benzyne intermediate via

N-debenzylation of BBT in a manner similar to that described for ABT (Ortiz de Montellano and Mathews, 1984). Secondly, incubation of guinea pig hepatic or pulmonary microsomes with either of two forms of radiolabeled BBT, *N*-benzyl-1-amino-[^{14}C]2,3-benzotriazole or *N*-[^{14}C]7-benzyl-1-aminobenzotriazole, in the presence of NADPH, results in the covalent attachment of radioactive label to proteins that migrate in SDS-PAGE to the molecular weight range of CYP proteins (Woodcroft *et al.*, 1997). This suggests that BBT may be activated to at least two reactive species capable of covalent modification of CYP apoprotein. Recent studies have identified ABT, benzotriazole and benzaldehyde as major metabolites of guinea pig hepatic and pulmonary microsomal (Woodcroft *et al.*, 1997) or recombinant rat CYP2B1-dependent (Kent *et al.*, 1997a) metabolism of BBT. The precise relationship of these metabolites to the inactivation of CYP by BBT remains to be elucidated. Taken together, these data indicate that *N*-aralkylated derivatives of ABT can irreversibly inactivate CYP by covalent modification of the heme moiety and CYP apoprotein. Finally, data presented in Chapter 2 of this thesis demonstrates the ability of these compounds to form MI complexes with CYP. Additional data is presented in Chapters 3 and 4 which describes MB and competitive inhibition of CYP by the ABT derivatives.

1.3.5 Uses and Implications of Cytochrome P450 Inhibition

Inhibitors of CYP have been employed as sensitive biochemical probes to determine the role of individual isozymes in the metabolism of endogenous and exogenous compounds. For example, αMB was used in combination with other MB inhibitors, an inhibitory antibody, and isozyme-selective CYP inducers, to show that CYP2B18 is solely responsible for the formation of EETs from AA in guinea pig lung (Knickle and Bend, 1994). MB inactivators, in contrast to inhibitory antibodies, can be used to inhibit CYP in intact cells and thus, are useful both *in vivo* and *in vitro*. Additionally, as clearly demonstrated with the *N*-aralkylated derivatives of ABT, rational design can be used to

produce inhibitors with tissue- and isozyme-selectivity. Thus, the potential exists for the use of selective MB inhibitors *in vivo* for therapeutic purposes, especially for steroidogenic CYPs that participate in essential physiological processes and exhibit very narrow substrate selectivities (Halpert *et al.*, 1988; Kurokohchi *et al.*, 1992; Xu *et al.*, 1995). The various therapeutic applications of CYP inhibitors have been summarized in excellent review articles (Correia and Ortiz de Montellano, 1993; Vanden Bossche *et al.*, 1995) and will not be considered further here. MB inactivators can also be used to gain information about CYP structure and function. For example, MB inactivation by secobarbital, in combination with site-directed mutagenesis studies, has been used to probe the active site topology and amino acid determinants of substrate selectivity of CYP2B1 (He *et al.*, 1996a; 1996b).

CYP inhibition is also the basis of a number of clinically relevant drug-drug interactions, particularly with drugs that are metabolized by a single isozyme. For example, individuals deficient in CYP2D6-dependent metabolism due to a polymorphism are susceptible to toxicity from debrisoquine, a drug which is not metabolized by other CYP isoforms (Inaba *et al.*, 1995; Rannug *et al.*, 1995). A similar outcome would be expected with normal individuals concomitant with administration of a CYP2D6 inhibitor such as quinidine (Ono *et al.*, 1996). Inhibition of CYP-dependent metabolism of drugs can also occur as a result of exposure to certain constituents of the diet. A number of naturally occurring flavonoids are potent inhibitors of CYP, and in particular of CYP3As, which metabolize the majority of drugs in humans (Siess *et al.*, 1995; Tsyrlov *et al.*, 1994). Concomitant ingestion of grapefruit juice is known to increase felodipine and terfenadine bioavailability substantially in humans (Bailey *et al.*, 1995; 1996; Rau *et al.*, 1997), thereby increasing the effective dose of the drug and increasing the potential for undesirable pharmacodynamic effects (Benton *et al.*, 1996). This interaction is thought to result from MB inactivation of intestinal CYP3A4-dependent biotransformation by flavonoids contained within grapefruit juice (Lown *et al.*, 1997). In summary, studies of CYP inhibition by a number of compounds has provided valuable information about the

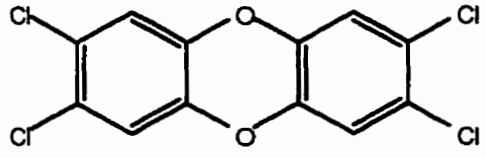
structure and function of CYP enzymes, the potential for development of novel therapeutics and the basis of drug-drug and drug-food interactions.

1.4 CYP1A1 and the Aryl Hydrocarbon Receptor

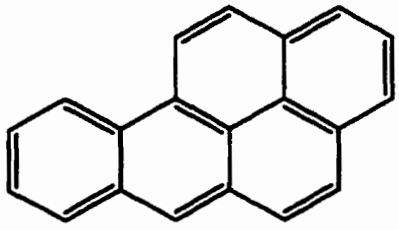
1.4.1 Induction of CYP1A1

Studies of the metabolites generated by CYP1A1-dependent oxidation of PAHs have revealed the importance of these compounds in PAH-induced chemical toxicity including carcinogenesis (Gelboin, 1980; Kawajiri *et al.*, 1993). Early in these studies, it became apparent that PAHs also caused an induction of CYP1A1-dependent catalytic activity, illustrating the ability of a cell to respond to chemicals in the external environment (Nebert *et al.*, 1976). Induction of CYP1A1 presumably represents a protective response which acts to prevent the accumulation of toxic levels of lipophilic xenobiotics within the cell. The fact that many CYP1A1 inducers are also substrates for this enzyme establishes a sensitive biochemical feedback loop where increased levels of inducer/substrate leads to increased synthesis of CYP1A1, which in turn, acts to reduce the level of substrate/inducer and subsequently depress synthesis of CYP1A1 protein.

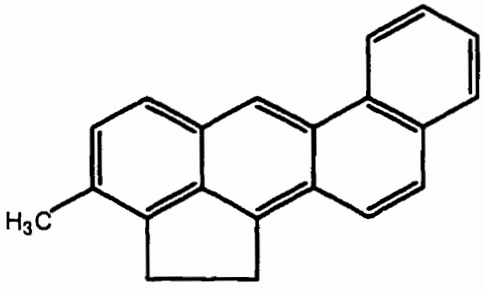
The carcinogenic PAHs, 3MC and BaP (Fig. 1.3) are well established inducers of CYP1A1 and are also metabolized by CYP1A1 (Conney, 1982). In contrast, the halogenated PAH, TCDD is a much more potent inducer of CYP1A1 possibly because of its resistance to metabolism by virtue of the placement of chlorine atoms in the molecule (DeVito and Birnbaum, 1995). Thus, exposure to TCDD leads to accumulation of the compound and a more sustained induction of CYP1A1. Early on, genetic studies in mice established that the inductive effects of PAHs and TCDD are mediated by the AHR, a novel, ligand-activated transcription factor (Okey *et al.*, 1994; Rowlands and Gustafsson, 1997; Whitlock *et al.*, 1997). It is now well established that ligand activation of the AHR leads to its heterodimerization with the AHR nuclear translocator (ARNT) protein,



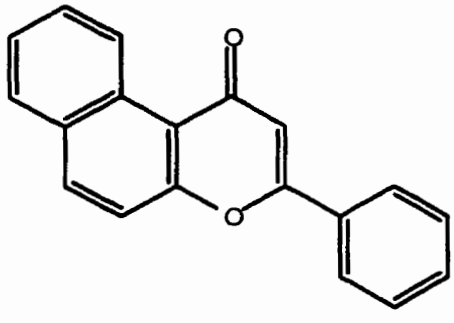
2,3,7,8-tetrachlorodibenzo-*p*-dioxin
(TCDD)



benzo[*a*]pyrene
(B[*a*]P)



3-methylcholanthrene
(3-MC)



ß-naphthoflavone
(ßNF)

Figure 1.3
Chemical structures of some prototypical AHR ligands.

transcriptional activation of the *CYP1A1* gene, increased accumulation of CYP1A1 mRNA and an increase in CYP1A1 protein and catalytic activity (Schmidt and Bradfield, 1996; Whitlock *et al.*, 1996).

The potency of TCDD for AHR-mediated CYP1A1 induction reflects, in part, the lack of metabolism of this compound in cells, but primarily results from high affinity (pM) binding with the AHR (Landers and Bunce, 1991). The reason that cells contain a receptor that exhibits extremely high affinity binding with a compound that only very recently has accumulated to significant concentrations in the environment as a result of anthropogenic actions remains speculative. It has been suggested that the evolutionary pressure to maintain the AHR derives from the presence of naturally occurring AHR ligands, which are mimicked by TCDD, present within some edible plants (Gonzalez and Nebert, 1990). An alternative, and not necessarily exclusive suggestion, is that TCDD mimics some endogenous AHR ligand(s) that serve as physiological mediators or signalling molecules. Indirect evidence for the existence of such ligands is inferred from the presence of transcriptionally active AHR/ARNT heterodimers in activated leukocytes (Crawford *et al.*, 1997) and Hepa 1c1c7 mouse hepatoma cells (Chang and Puga, 1998) in the absence of exogenous ligand. Similarly, AHR-deficient knockout mice have decreased spleen and lymph node lymphocyte numbers, decreased liver size, severe hepatic fibrosis and approximately 50% lethality shortly after birth (Fernandez-Salguero *et al.*, 1995) indicating that the AHR plays an important endogenous role in the development of the liver and immune system. However, putative endogenous AHR ligands remain unidentified. Recently, Chang and Puga (1998) have reported that transient transfection of AHR and ARNT expression plasmids into AHR- and CYP1A1-deficient African Green monkey kidney cells leads to high level expression of an AHR/ARNT-dependent luciferase reporter gene in the absence of exogenously added agonist. Decreased expression of the reporter gene could be achieved by expression of a functional CYP1A1 enzyme, competition with chimeric or truncated AHR proteins containing the AHR ligand binding domain or

treatment with the AHR antagonist, α-naphthoflavone (αNF). The authors interpret these data as evidence for an endogenous CYP1A1 substrate, which accumulates in cells lacking CYP1A1 catalytic activity, and can function as an endogenous AHR ligand. In Chapters 6, 7 and 8 of this thesis, experimental evidence is presented which supports the existence of endogenous AHR ligands, one of which has been identified as bilirubin.

1.4.2 Aryl Hydrocarbon Receptor-Dependent CYP1A1 Induction

A number of genetic, biochemical and molecular biological approaches have led to the identification of *cis*-acting regulatory sequences present within the 5'-regulatory region of the *CYP1A1* gene (Denison and Deal, 1990; Denison *et al.*, 1988; Fujisawa-Sehara *et al.*, 1987; Gonzalez and Nebert, 1985; Israel and Whitlock, 1983; 1986; Jones *et al.*, 1985; Sogawa *et al.*, 1986). The sequences responsible for AHR-mediated induction and binding of the AHR/ARNT heterodimer are designated as dioxin responsive elements (DREs) or xenobiotic responsive elements (XREs) and occur as multiple copies within the 5'-regulatory region of the mouse (Denison and Deal, 1990), rat (Robertson *et al.*, 1994) and human (Hines *et al.*, 1988) *CYP1A1* genes. Mutational analysis and electrophoretic mobility shift experiments have identified inducible AHR/ARNT protein-DNA interactions at a core nucleotide sequence 5'-TNGCGTG-3' of the DRE (Shen and Whitlock, 1992; Yao and Denison, 1992). Protein-DNA crosslinking studies revealed the presence of two proteins inducibly bound to DREs, eventually leading to the realization that the liganded AHR binds to DNA as a heterodimer with ARNT (Reyes *et al.*, 1992; Whitelaw *et al.*, 1993).

A second type of promoter sequence has also been identified just upstream of the transcriptional start site of the *CYP1A1* gene. This promoter sequence contains binding sites for a number of general transcription factors (GTFs) such as the TATA binding protein (TBP) and exhibits inducible AHR/ARNT-dependent binding with its cognate proteins (Jones and Whitlock, 1990). A third sequence, termed the basic transcription

element (BTE), that is required for basal and fully inducible *CYP1A1* expression, has also been identified (Imataka *et al.*, 1992). The BTE sequence is also found in other CYP genes and is now regarded as a GC box where Sp1 and the novel BTE binding protein bind. The presence of negative regulatory silencer elements in the 5'-regulatory region of the mouse *Cyp1a1* (Gonzalez and Nebert, 1985; Jones *et al.*, 1985) and human CYP1A1 (Boucher and Hines, 1995; Boucher *et al.*, 1993) gene has been reported. These negative regulatory sequences may account, in part, for the very low level of constitutive expression of CYP1A1 in most tissues and cell types.

The AHR was originally cloned from mouse after numerous problems with receptor instability during purification and the low cell content were overcome (Burbach *et al.*, 1992; Ema *et al.*, 1992). To date, additional cDNA sequences and primary amino acid structures of the AHR from multiple species, including human (Dolwick *et al.*, 1993a; Eguchi *et al.*, 1994; Ema *et al.*, 1994) and rat (Carver *et al.*, 1994) have been deduced. Despite the similarity of molecular mechanism to steroid nuclear receptors, structural analysis of the AHR has demonstrated only minor resemblance. Such studies have revealed the presence of two distinct and characteristic structural domains - the basic helix-loop-helix (bHLH) and PAS domains in the *N*-terminal half of the AHR (Burbach *et al.*, 1992; Dolwick *et al.*, 1993b; Fukunaga *et al.*, 1995). ARNT also contains these domains which form the interface for interactions between these two proteins during heterodimerization (Hoffman *et al.*, 1991). The PAS domain of the AHR also functions in the interaction with heat shock protein 90 (Hsp90) and ligands prior to heterodimerization with ARNT (Antonsson *et al.*, 1995; Burbach *et al.*, 1992). The transcriptional activation domains of the AHR and ARNT are believed to reside in the *C*-terminal portion of the proteins (Dong and Whitlock, 1994; Sogawa *et al.*, 1995; Whitelaw *et al.*, 1994). Two putative phosphorylation sites have been identified in the *C*-terminal half of the AHR (Mahon and Gasiewicz, 1995). *In vitro* treatment of the AHR with phosphatases and treatment of cultured cells with protein kinase C and tyrosine kinase inhibitors have been

shown to inhibit DNA binding of the AHR/ARNT heterodimer and to inhibit CYP1A1 induction (Berghard *et al.*, 1993; Carrier *et al.*, 1992; Reiners *et al.*, 1993). These studies indicate that phosphorylation of the AHR and/or ARNT may be of functional relevance.

1.4.3 A Model for Aryl Hydrocarbon Receptor-Dependent CYP1A1 Induction

Combined evidence from numerous studies of the structure of the *CYP1A1* promoter, the structure of the AHR and ARNT and the interaction of the AHR/ARNT heterodimer with DNA control sequences, both *in vitro* and *in vivo*, has led to the development of a general model for CYP1A1 induction. A simplified representation of this model is presented in Fig. 1.4. Lipophilic ligands passively diffuse into the cell and bind to the AHR, which is normally maintained in the inactive state in the cytosol as a complex with two molecules of Hsp90. Upon ligand binding, the AHR dissociates from Hsp90 and translocates to the nucleus by a process which remains largely uncharacterized. Within the nucleus, the liganded AHR heterodimerizes with ARNT, a process which may be modulated by, or require protein phosphorylation (Berghard *et al.*, 1993; Carrier *et al.*, 1992). Upon binding to DREs, gross alterations of chromosomal structure within the *CYP1A1* promoter region occur, which may allow accessibility of GTFs such as the TBP to their cognate binding sites (Ko *et al.*, 1996; Wu and Whitlock, 1992). An initiation complex then forms at the promoter and transcription of CYP1A1 RNA ensues, leading to an increase in CYP1A1 mRNA, protein and catalytic activity. If the inducer is also a substrate for CYP1A1, eventual depletion of cellular concentration of the ligand leads to a loss of activation of *CYP1A1* gene transcription.

While the current working model for AHR-dependent gene induction was developed primarily based upon studies of CYP1A1 induction, it is likely that it is also relevant to other genes which contain DRE sequences. For example, CYP1A2 is induced upon exposure to PAHs and DRE-like sequences have been identified in the regulatory

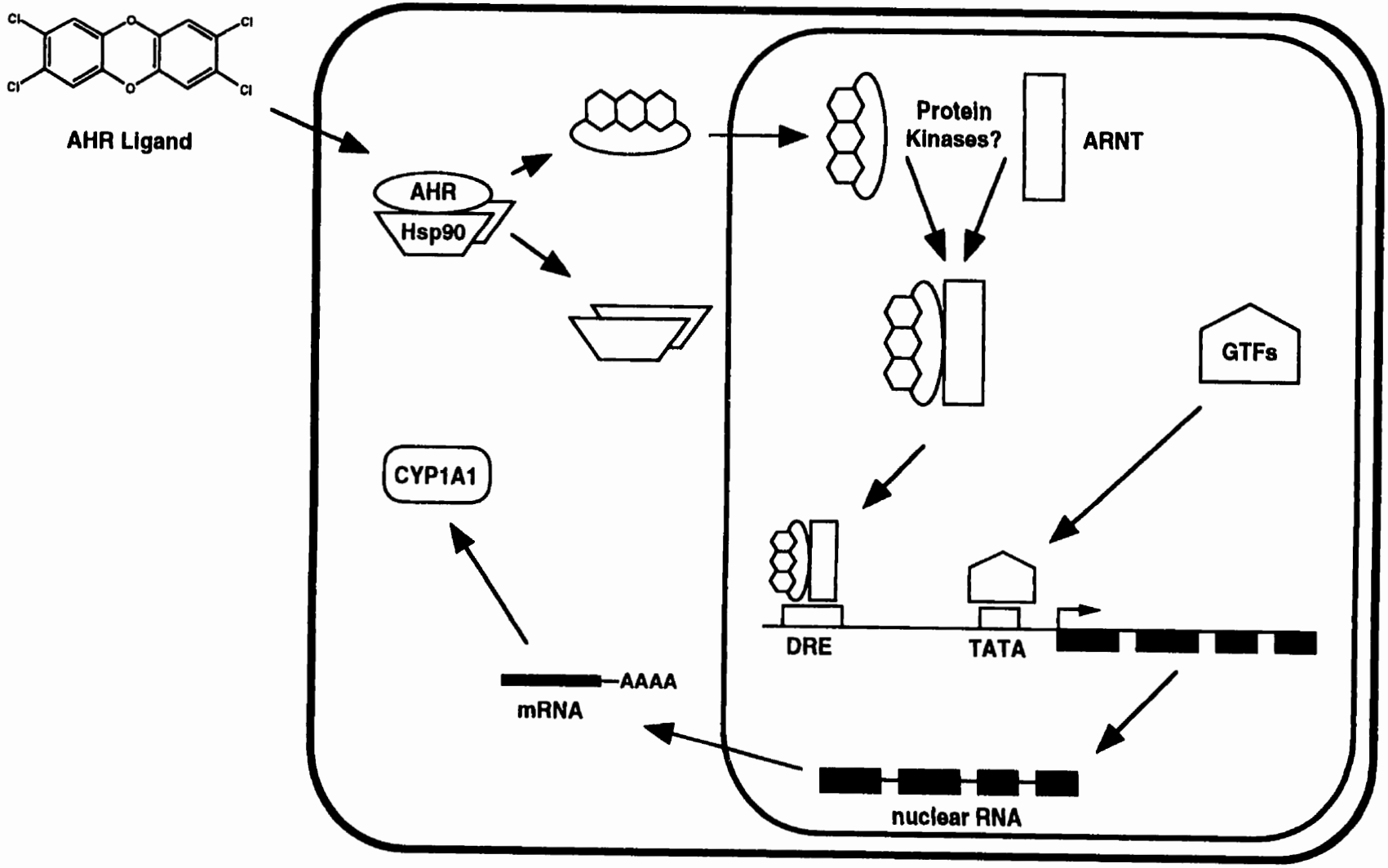


Figure 1.4
Simplified model of AHR-mediated induction of CYP1A1.

region of the human *CYP1A2* gene (Quattrochi and Tukey, 1989; Quattrochi *et al.*, 1994). Furthermore, AHR knockout mice have decreased inducibility and constitutive expression of hepatic CYP1A2, supporting a role for the AHR in CYP1A2 regulation (Fernandez-Salguero *et al.*, 1995). However, hepatic induction of CYP1A2 by methylenedioxyphenyl compounds in these mice (Ryu *et al.*, 1996) and the lack of extrahepatic induction of this isoform in normal mice (Kimura *et al.*, 1984b) indicates that other regulatory mechanisms are also important. CYP1B1 is also induced by AHR ligands such as TCDD, however induction of this CYP is sex-, tissue- and steroidogenic cell-dependent, indicating a linkage to ovarian hormone-regulated pathways (Walker *et al.*, 1995). A DRE core sequence that is required for AHR-dependent induction by PAHs has been identified in the 5'-regulatory region of the glutathione *S*-transferase-Ya gene (Pimental *et al.*, 1993). This DRE is contained within a composite site which also binds the transcriptional enhancer C/EBPα, and allows cooperative interactions with the AHR (Pimental *et al.*, 1993). Other genes, such as aldehyde 3-dehydrogenase, NADPH:quinone oxidoreductase and some UDP glucuronosyltransferases (UDP-GTs) are also induced by AHR ligands, in concert with exposure to a variety of other stimuli associated with oxidative stress (Nebert, 1989; Nebert *et al.*, 1990; 1996; Vasiliou *et al.*, 1995a; 1995b).

Two recent reports have described putative transcriptional coactivators for the AHR (Carver and Bradfield, 1997; Ma and Whitlock, 1997). Further studies will help to determine if these proteins fulfill the criteria of "true" coactivators that can modify the actions of the AHR in a manner similar to that described for steroid nuclear receptor coactivators (Glass *et al.*, 1997). The recent finding that the nuclear receptor coactivator CBP/P300 interacts with the transactivation domain of ARNT supports this possibility (Kobayashi *et al.*, 1997). Thus, while the simplified model put forth addresses many aspects of the regulation of CYP1A1 by AHR ligands, substantial additions and modifications are required to fully describe the complex interplay among multiple

transcription factors and cellular stimuli involved in the regulation of this and other AHR regulated genes.

1.5 Modulation of Cytochrome P450 during Pathobiological Stress

1.5.1 Infection and Inflammation

As discussed previously, many drugs are dependent upon hepatic CYP-dependent metabolism for clearance from the circulation, bioactivation to the active pharmacological agent or termination of pharmacological activity. Furthermore, the production of toxicologically or biologically active or inactive metabolites from xenobiotics or endogenous substrates is also catalyzed by a number of CYPs. Similar to induction or inhibition by xenobiotics, infections, inflammation and other pathobiological stresses, defined here as any influence (*ie.* disease state, injury, toxicant) that perturbs normal homeostatic mechanisms within a cell, organ or organism as a whole, can cause changes in CYP expression and/or catalytic activity. Modulation of the CYP-dependent formation of metabolites from endogenous compounds, drugs and other xenobiotics thus, may be a consequence of these pathobiological states and/or may contribute to their progression.

During episodes of infectious disease (bacterial, parasitic, viral), CYP metabolism is depressed and the capacity of the liver to metabolize drugs can be compromised (Renton and Knickle, 1990). One of the earliest reports in humans documented the prolongation of theophylline elimination in children infected with influenza (Chang *et al.*, 1978; Koren and Greenwald, 1985). Renton (1978) proposed that these effects were due to a decrease of hepatic CYP content. Since then, it has become clear that depression of hepatic CYP-dependent metabolism is a common property of a number of viral infections (Renton and Knickle, 1990). Similarly, infection of mice with the gram-positive bacteria *Listeria monocytogenes* results in large decreases of hepatic CYP content, CYP-dependent catalytic

activity and an increase of theophylline half-life (Azri and Renton, 1987; 1991). Many studies have also shown that administration of lipopolysaccharide (LPS), a major cell wall constituent of gram-negative bacteria, causes decreases of hepatic CYP content and catalytic activity in humans (Shedlofsky *et al.*, 1994) and experimental animals (Morgan, 1993b; Sewer *et al.*, 1997). Importantly, the effect of LPS injection has been shown to be selective for individual constitutive hepatic isozymes with CYP2C6, 2C11, 2C12, 2E1 and 3A2 exhibiting particular sensitivity (Morgan, 1989; 1993b; Sewer *et al.*, 1996). Inflammation produced by stimulation of the murine reticuloendothelial system *in vivo* with dextran sulfate or latex beads (Peterson and Renton, 1986), as well as subcutaneous turpentine administration to rabbits (Proulx and du Souich, 1995) also causes depression of hepatic CYP content and catalytic activity. Similarly, partial hepatectomy, a model of inflammation and liver regeneration, selectively depresses CYP1A2 and CYP2E1 mRNA and catalytic activity, while CYP3A and CYP2A1 are largely unaffected (Trautwein *et al.*, 1997). Interestingly, direct stimulation of Kupffer cells with *Corynebacterium parvum* reduces CYP2E1 catalytic activity in rat liver and subsequently diminishes acetaminophen or carbon tetrachloride-induced injury mediated by this isoform (Raiford and Thigpen, 1994).

Increases of CYP content and catalytic activity, although not as common, have also been reported during pathobiological states. For example, intraperitoneal injection of LPS or particulate irritants to rats was recently shown to suppress hepatic CYP2C11 mRNA and protein and increase CYP4A1, 4A2 and 4A3 mRNAs, while having no effect on CYP2E1 or CYP3A2 (Sewer *et al.*, 1997). CYP4A2, 4A3 and 2E1 mRNAs were also increased in the kidney of treated animals (Sewer *et al.*, 1997). Renal CYP4As hydroxylate endogenous AA to 20-HETE, a vasoconstrictor and regulator of kidney function (Fitzpatrick and Murphy, 1989). Whether increased formation of this metabolite or other eicosanoids contributes to, or attenuates the pathobiological state, remains to be elucidated. Interestingly, the induction of renal CYP2E1 observed in the kidney of LPS- or particulate-

treated animals (Sewer *et al.*, 1997) occurred at the pre-translational level. This more closely resembles the induction of CYP2E1 that occurs in response to pathobiological states such as starvation (Leakey *et al.*, 1989; Ueng *et al.*, 1993) and diabetes (Chen *et al.*, 1996; Hu *et al.*, 1995) than it does to the stabilization of CYP2E1 protein by chemical inducers such as ethanol (Roberts *et al.*, 1995). A selective increase of CYP1A or 2C *vs.* CYP2A, 2B, 2E1 or 4A has also been reported in the brain of rats after partial hepatectomy (Warner *et al.*, 1993). Thus, a number of pathobiological states are associated with differential suppressive or inductive effects on individual CYPs resulting in the potential for altered responses to xenobiotic exposure and endogenous substrate metabolism.

1.5.2 Regulation of Cytochromes P450 by Cytokines

The liver responds to inflammation or infection with a cytokine-mediated induction of the synthesis and secretion of acute phase proteins with various regulatory roles in the inflammatory response (Huston, 1997). Studies of the regulation of CYPs during these pathobiological states has focused largely on mediation by interleukin-1 (IL-1), tumour necrosis factor-α (TNFα) and IL-6, the major proinflammatory cytokines (Watkins *et al.*, 1995). Down-regulation of constitutive CYP2C mRNA, protein and catalytic activity by IL-1 in primary cultures of rat hepatocytes (Chen *et al.*, 1995) and in rat liver *in vivo* (Morgan *et al.*, 1994; Wright and Morgan, 1991) has been described in detail. Similarly, administration of IL-1 to rats significantly decreases constitutive lung expression of CYP2B1 mRNA and catalytic activity (Sakai *et al.*, 1992). In addition to inhibiting constitutive CYP2B and 1A catalytic activity in primary cultures of rat hepatocytes, IL-1 also blocks induction of these isozymes in response to PB or TCDD, respectively (Abdel-Razzak *et al.*, 1995; Barker *et al.*, 1992). This suggests transcriptional modulation of these CYPs, similar to that for CYP2Cs (Wright and Morgan, 1991). Treatment with IL-1 or TNFα also decreases CYP1A1, CYP2E1 and CYP3A mRNA and catalytic activity in primary cultures of human hepatocytes (Abdel-Razzak *et al.*, 1993a). IL-6 inhibits the

expression of CYP1A1 in primary cultures of human or rat hepatocytes (Abdel-Razzak *et al.*, 1993a; 1995) and rat liver *in vivo* (Chen *et al.*, 1992b); CYP2B in primary cultures of rat hepatocytes (Abdel-Razzak *et al.*, 1995), but not rat liver *in vivo* (Chen *et al.*, 1992b); CYP2C in primary cultures of human or rat hepatocytes (Abdel-Razzak *et al.*, 1993a; 1995) and rat liver *in vivo* (Morgan *et al.*, 1994); and, CYP3A in primary cultures of human or rat hepatocytes (Abdel-Razzak *et al.*, 1993a; 1995) and rat liver *in vivo* (Morgan *et al.*, 1994). Most of these studies have also reported IL-6 inhibition of the induction of these CYPs by model compounds. On the other hand, IL-4 has been reported to increase CYP2E1 mRNA 5-fold in primary cultures of human hepatocytes (Abdel-Razzak *et al.*, 1993a), and IL-2 has been reported to cause smaller increases of hepatic CYP2D mRNA, protein and catalytic activity in the rat (Kurokohchi *et al.*, 1993). It is important to note that most of the studies cited above employed rather high supraphysiologic doses of cytokine treatment and caution must be exercised when extrapolating these results to the *in vivo* situation.

A profound immunological response and cytokine release is associated with a variety of pathobiological states (Huston, 1997). Thus, the differential effects of these states on the expression, catalytic activity and inducibility of CYPs can be attributed, at least in part, to the actions of cytokines. Other factors may also contribute. For example, induction of nitric oxide synthase and NO release also occurs in many pathobiological states (Barnes and Belvisi, 1993; Berdaux, 1993; Nathan, 1992; Sessa, 1994; Stuehr and Griffith, 1992) and may also modulate CYP. NO has been shown to reversibly inhibit CYP1A catalytic activity by ligation with the heme iron and prevention of O_2 binding (Stadler *et al.*, 1994). NO can also irreversibly inhibit CYP by the formation nitrosyl complexes which promotes its conversion to the denatured P420 form and leads to heme loss (Kim *et al.*, 1995b; Minamiyama *et al.*, 1997). NO has also been suggested as the mediator of the down-regulation of hepatic CYP at the transcriptional level *in vivo* by inflammatory mediators (Khatsenko *et al.*, 1993), and in particular interferon (Hodgson and Renton, 1995; Khatsenko *et al.*, 1993). Oxidative stress provoked by H_2O_2 treatment

of primary cultures of rat hepatocytes has been shown to block CYP1A1 and 1A2 mRNA induction in response to βNF (Barker *et al.*, 1994). Induction of microsomal heme oxygenase is another response commonly associated with pathobiological states. The function and regulation of this enzyme is considered further in the next section.

1.6 Heme Oxygenase and the Regulation of Cytochromes P450

1.6.1 Heme Oxygenase

Microsomal heme oxygenase (HO) catalyzes the initial and rate-limiting step of heme catabolism (Fig. 1.5) - the oxidative cleavage of the α-meso carbon bridge of type b heme molecules to yield equimolar quantities of biliverdin, CO and iron (Maines, 1988). Biliverdin is subsequently converted to bilirubin by the cytosolic enzyme, biliverdin reductase (Maines and Trakshel, 1993). Under normal physiological conditions, HO activity is highest in the spleen where senescent erythrocytes are sequestered and destroyed (Maines, 1984). Three isoforms of HO have been identified: HO-1, an inducible isoform expressed in virtually all tissues and cell types (Choi and Alam, 1996; Maines, 1997), and the closely related constitutive isoforms HO-2 and HO-3 that are also widely expressed, but may have particular functional relevance in the central nervous system and vascular endothelium (McCoubrey *et al.*, 1997; Sun *et al.*, 1990; Zakhary *et al.*, 1996). Heme is the prototypical inducer of HO-1, although transcriptional activation of the gene also occurs in response to heavy metals, cytokines, H_2O_2, UV-radiation, ischemia-reperfusion, hypoxia, hyperoxia, hyperthermia and sulfhydryl reagents (Applegate *et al.*, 1991; Ewing *et al.*, 1994; Keyse and Tyrrell, 1989; Lee *et al.*, 1997; Rizzardini *et al.*, 1993; Smith *et al.*, 1993; Taketani *et al.*, 1989). A common feature of these inducers is that they all may facilitate or initiate the production of ROS and/or cause depletion of cell GSH levels (Halliwell and Cross, 1994; Kehrer, 1993). These common properties have led to the hypothesis that

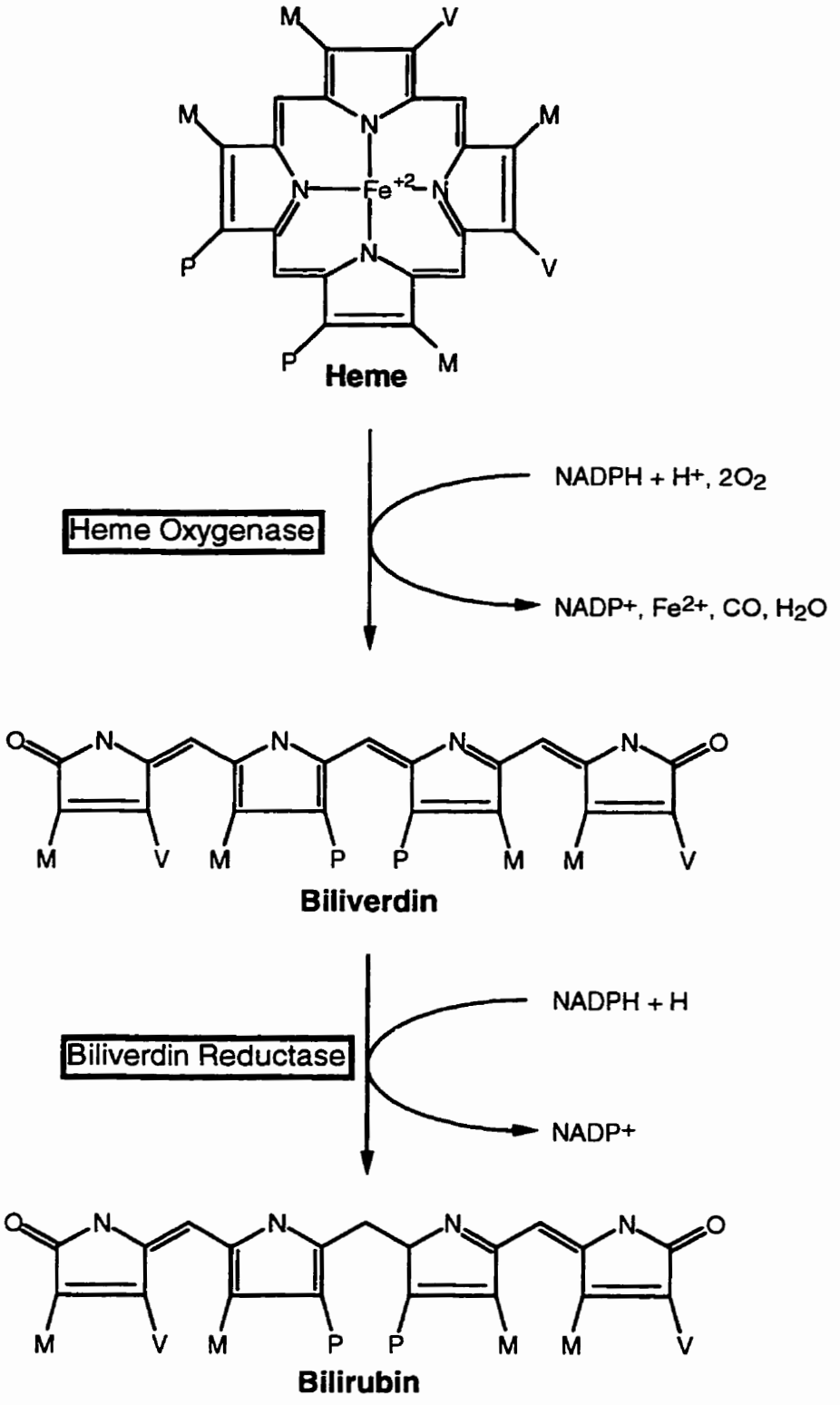


Figure 1.5
Reaction scheme for the enzymatic conversion of intracellular heme to bilirubin.
M : $-CH_3$, V : $-CH=CH_2$, P : $-CH_3-CH_2-COOH$

HO-1 induction is part of the generalized cellular response to oxidative stress and plays a protective role during such conditions (Lee *et al.*, 1996a).

Although the precise mechanisms by which HO-1 may confer protection against cellular stress have not been definitively established, the substrate and products of HO-dependent metabolism provide some clues. Intracellular H_2O_2 and superoxide can be converted to the highly reactive and toxic hydroxyl radical via the Fenton reaction, which is efficiently catalyzed by free iron and free heme (Kappus, 1987). HO-1, in combination with ferritin, may be part of a protective response in which free heme is degraded and is thus, not available to catalyze ROS generation (Balla *et al.*, 1993). Ferritin synthesis induced by the release of iron from the degradation of heme may then act to sequester free iron and prevent its participation in ROS generation (Balla *et al.*, 1992; Cairo *et al.*, 1995). Bilirubin, a product of heme degradation, may also combat the effects of oxidative stress as a result of its potent antioxidant properties (Stocker *et al.*, 1987a; 1987b; Yamaguchi *et al.*, 1995; 1996). Finally, CO is rapidly becoming accepted as a member of a new class of gaseous cellular messengers that also includes NO (Maines, 1997). Similar to NO, CO can increase cellular cGMP levels via guanylate cyclase activation, leading to vascular smooth muscle relaxation (Kharitonov *et al.*, 1995). This vasodilatory effect of CO may improve tissue oxygenation and perfusion during pathobiological states and thereby reduce the severity of damage (Suematsu *et al.*, 1994). Thus, HO may provide protection both through the removal of a potentially harmful compound (free heme), and by the production of beneficial products (bilirubin and CO). Due to the central role of HO in heme homeostasis, it is also possible that this enzyme has some regulatory function with respect to the synthesis and/or degradation of heme containing proteins such as CYP.

1.6.2 Heme Oxygenase and Cytochrome P450 Degradation

As mentioned previously, HO activity is elevated dramatically during a number of pathobiological states in which hepatic CYP content and catalytic activity are decreased.

This relationship has led to the proposal that the decline of CYP under these conditions may be due to the direct actions of HO on the heme of CYP. In one of the earliest studies to address this proposal, rat hepatic heme was labeled *in vivo* by administration of a tracer pulse of the heme precursor [^{14}C]δ-aminolevulinic acid such that heme degradation by HO could be monitored by [^{14}C]CO expiration (Bissell and Hammaker, 1976). A marked increase of [^{14}C]heme degradation, derived primarily from CYP proteins, was observed subsequent to treatment of the animals with compounds (heme, LPS) known to induce hepatic HO-1. In a more recent study, the temporal relationship between hepatic HO-1 induction and CYP loss following intraperitoneal administration of aluminum to rats was examined (Fulton and Jeffery, 1994). HO-1 activity was induced significantly by 6 h post-dosing, while significant loss of CYP did not occur until 12 h, indicating that HO induction occurs prior to, and may be a requirement for, CYP loss. In a more direct approach to the question, Kutty *et. al* (1988) used purified preparations of rat CYP1A1 or CYP2B1 as substrates in a reconstituted HO/biliverdin reductase system. Near quantitative conversion, albeit at very low rates (3-5% of hematin metabolism), of CYP derived heme to bilirubin was observed. However, the P420 form of CYP1A1 was most susceptible to degradation, suggesting that denaturation of CYP and heme release may be required prior to HO-dependent metabolism of CYP-derived heme. Thus, evidence exists to support the role of HO in the regulation of CYP catalytic activity either by direct degradation of CYP-bound heme, or subsequent to its release from the protein.

1.6.3 Regulation of Cytochrome P450 by Heme

Heme is a versatile molecule known to regulate a variety of cell processes such as differentiation, transcription and post-translational modification of a number of enzymes (Maines, 1984). Thus, it is likely that HO can regulate heme-regulated and/or heme-dependent genes by exerting control over the levels of free heme within the cell. At least three potential mechanisms have been identified by which heme can regulate the expression

and catalytic activity of CYP. The first derives from early work (Sardana *et al.*, 1976) showing that biosynthesis of complete CYP apoprotein occurs without heme insertion, such that subsequent incorporation of heme into nascent protein is required for assembly of the functional protein. Heme incorporation does not appear to be tightly coupled to apoprotein synthesis, for up to 30% of total CYP protein is present as non-functional apoprotein in the livers of PB or 3MC treated rats (Sadano and Omura, 1985). The absence of substantial changes in functional hepatic CYP content after administration of 3MC to Gunn rats has been suggested to be related to limited hepatic heme availability in this strain of rat (Celier and Cresteil, 1991). Similarly, inhibition of heme synthesis and depletion of the free heme pool in liver by treatment of mice with lead acetate has been shown to decrease CYP catalytic activity as a result of decreased heme saturation of apoprotein, a condition that can be counteracted by injection of exogenous hematin (Jover *et al.*, 1996). Furthermore, the addition of hemin to culture media has been shown to cause a marked increase in the levels of enzyme activity and heme-containing CYP purified from cells used for heterologous overexpression of some CYPs (Asseffa *et al.*, 1989; Gonzalez and Korzekwa, 1995). Taken together, these data indicate that the amount of functional CYP resulting from *de novo* synthesis is dependent on the level of free heme available for incorporation into apoprotein. Thus, HO may indirectly regulate CYP function by influencing the cellular levels of free heme.

A second mechanism may represent a more dynamic approach to the control of functional CYP levels via reversible association with heme. For example, [^{3}H]heme administered to rats is rapidly incorporated into functional hepatic CYP, indicating that exogenously supplied heme gains access to a free heme pool within the hepatocyte which dynamically exchanges heme with CYP and/or provides heme for incorporation into newly synthesized apoprotein (Correia *et al.*, 1989). The concept of reversible heme association/dissociation with CYP is also supported by the finding that CYP which has been rendered functionally inactive as a result of heme alkylation by

allylisopropylacetamide, can be reconstituted by the addition of exogenous heme *in vitro* (Sardana *et al.*, 1976), in perfused rat liver (Farrell *et al.*, 1981) or *in vivo* (De Matteis *et al.*, 1986; Farrell and Correia, 1980). These data indicate that changes in free heme levels caused by the action of HO have the potential to affect the function of CYP by shifting the prevailing equilibrium between heme association and dissociation.

The third mechanism by which HO-mediated changes in free heme levels might affect CYP regulation is based upon studies that provide evidence that heme can modulate CYP expression at the level of gene transcription. Administration of heme biosynthesis inhibitors such as cobalt chloride or 3-amino-1,2,4-triazole to rats has been reported to attenuate the induction of CYP1A1/2 and CYP2B1/2 mRNAs by 3MC and PB, respectively, as well as to block the transcriptional activation of the corresponding CYP genes in isolated nuclei prepared from livers of these animals (Bhat and Padmanaban, 1988a; 1988b; Dwarki *et al.*, 1987). Addition of exogenous heme to the rats used in these studies, or to the isolated nuclei, reversed the effects of the heme biosynthesis inhibitors. In a subsequent study, transcription of an exogenously added fragment of the CYP2B1/2 gene containing a 179 bp segment of the 5'-regulatory region and a small portion of the coding region, was monitored in isolated nuclei, as was the binding of proteins to the regulatory region of the gene (Upadhya *et al.*, 1992). When isolated nuclei and nuclear extracts (as a source of DNA binding proteins) prepared from PB-treated rats were used, transcription of the gene fragment and protein binding were increased dramatically when compared to nuclei and nuclear extracts, respectively, from untreated animals. Similar to the previous studies, transcription and protein binding could be blocked by the co-treatment of PB-induced animals with heme biosynthesis inhibitors, and subsequently, could be restored by the addition of heme *in vitro* to the isolated nuclei and nuclear protein extracts. Recently, a nuclear protein has been isolated which may mediate the positive modulation of inducible CYP2B1/2 gene transcription by heme (Sultana *et al.*, 1997). These results

suggest that a cellular free heme pool, which may be regulated in part by HO, can modulate the expression of some CYP genes at the transcriptional level.

1.6.4 Regulation of Cytochrome P450 by Heme Degradation Products

The products of HO-mediated heme degradation may also be regulators of CYP expression and catalytic activity. As discussed in Section 1.3, CO binds efficiently to ferrous heme and thus, can inhibit CYP-dependent catalysis in a non-selective manner (Testa and Jenner, 1981). Evidence also exists which indicates that bilirubin, generated by the action of biliverdin reductase on heme-derived biliverdin (Fig. 1.5), may affect the expression and catalytic activity of CYP in an isozyme-selective manner, and may also serve as a substrate for CYP-dependent metabolism. Most of the normal physiological production of bilirubin occurs in the spleen via the HO-mediated catabolism of heme derived from the hemoglobin of senescent erythrocytes (Maines, 1988). Bilirubin is also produced in the liver in Kupffer cells and hepatocytes, as well as in the bone marrow and by tissue macrophages (Jansen *et al.*, 1995). Serum bilirubin levels must be maintained at low levels for at concentrations >450 μM in the human adult and >340 μM in the neonate, bilirubin can enter the brain and cause bilirubin encephalopathy and kernicterus (Amato, 1995). The normal route for bilirubin elimination involves conjugation by UDP-GTs to form mono- and di-glucuronides which are excreted in the bile (Chowdhury *et al.*, 1994). In humans with Crigler-Najjar syndrome, and in the corresponding congenitally jaundiced Gunn rat model, bilirubin conjugation is absent due to mutations in the bilirubin UDP-GT gene (Iyanagi *et al.*, 1989; Jansen, 1996). As a result, plasma levels of unconjugated bilirubin can reach 50-350 μM and 300-800 μM in the Gunn rat and humans with Crigler-Najjar syndrome, respectively (Chowdhury *et al.*, 1994). Although severe hyperbilirubinemia is a hallmark of these conditions, plasma levels of unconjugated bilirubin remain relatively constant, indicative of a balance between the formation and

elimination of bilirubin. This raises the possibility that alternate routes for bilirubin elimination become functionally relevant in the absence of conjugation.

Gunn rat bile contains colourless hydroxylated products of bilirubin, consistent with an enzyme-mediated oxidation of bilirubin (Berry *et al.*, 1972). A mitochondrial bilirubin oxidase activity has also been identified in rat liver (Cardenas *et al.*, 1982; Cardenas-Vazquez *et al.*, 1986), intestine and kidney (Yokosuka and Billing, 1980). This enzyme activity does not require NADP, NAD, or ATP and is inhibited by KCN, thiol reagents, NADH and albumin (Cardenas-Vazquez *et al.*, 1986). Administration of the CYP1A1 inducer TCDD to Gunn rats stimulates microsomal bilirubin catabolism in the livers of Gunn rats 7-fold, concomitant with significant decreases of unconjugated bilirubin levels in the plasma (Kapitulnik and Ostrow, 1978). In contrast, treatment with inducers of other CYPs such as PB, do not have a similar effect (Cohen *et al.*, 1985). A PAH inducible bilirubin oxidase activity has been identified in hepatic microsomes prepared from Gunn rats or chick embryos. (De Matteis *et al.*, 1989). This reaction requires NADPH and O_2 and is inhibited by an antibody that recognizes both CYP1A1 and CYP1A2 (De Matteis *et al.*, 1991a). Taken together, these data indicate that microsomal and mitochondrial bilirubin oxidation systems exist, and may become functionally relevant to bilirubin elimination in the absence of conjugation activity.

With respect to microsomal bilirubin oxidation, it appears that PAH inducible CYPs, and specifically, CYP1A1 and/or CYP1A2, are of particular importance. Regulation of P450 genes, especially *CYP1A1*, by their substrates is very common (Denison and Whitlock, 1995). Thus, if bilirubin is in fact a substrate for CYP1A1, it is reasonable to expect that increased levels of this CYP would be associated with increased plasma and/or tissue levels of unconjugated bilirubin. Evidence for such a relationship has been provided by the observation that elevated levels of CYP1A1 mRNA and protein have been detected in the liver of Gunn rats (Kapitulnik and Gonzalez, 1993; Kapitulnik *et al.*, 1987). Furthermore, pathobiological conditions which increase the production of bilirubin

would also be expected to cause increases of CYP1A1 expression. Arsenite (As^{+3}), a metal that has been linked to an increased risk of human cancer (Hopenhayn-Rich *et al.*, 1996; Liu and Chen, 1996; Simonato *et al.*, 1994), is a potent inducer of multiple stress proteins and chemical detoxication enzymes (Bauman *et al.*, 1993; Falkner *et al.*, 1993a; Ovelgönne *et al.*, 1995; Wiegant *et al.*, 1994). In contrast, As^{+3} treatment decreases hepatic CYP concentrations and catalytic activity (Albores *et al.*, 1992a; 1995). As^{+3} treatment also causes a profound induction of hepatic HO-1 activity (Brown and Kitchin, 1996; Sardana *et al.*, 1981) and corresponding increases of bilirubin production and excretion in the bile (Albores *et al.*, 1989). Acute sodium As^{+3} administration (75 μmol/kg; sc.) has been shown to potentiate the induction of CYP1A1-dependent EROD activity by βNF in guinea pig lung (Falkner *et al.*, 1993b). Subsequently, As^{+3} treatment alone was shown to be sufficient to cause a selective, transient increase of rat lung EROD activity (Albores *et al.*, 1995). Similarly, partial hepatectomy is associated with increased CYP1A1 protein levels in brain (Warner *et al.*, 1993) and total CYP content and CYP1A1-dependent catalytic activity in kidney (Solangi *et al.*, 1988). Substantial depression of hepatic CYP-dependent catalytic activity, as well as profound hepatic, but not extrahepatic, induction of HO-1 was also observed. These data indicate that pathobiological stresses associated with heavy metal exposure or partial hepatectomy share the common traits of hepatic P450 depression and selective elevation of extrahepatic CYP1A1 catalytic activity. This suggests that the initial tissue insult is not the cause of the effect, but more likely, initiates a series of physiological and cellular alterations that ultimately contribute to this response. The induction of hepatic HO-1 activity observed in these models, in combination with studies that associate bilirubin levels with CYP1A1 expression, raise the possibility that perturbations in heme metabolism leading to elevated levels of heme metabolites in the circulation and possibly some extrahepatic tissues, contribute or are primarily responsible for the selective up-regulation of extrahepatic CYP1A1 during and after conditions of pathobiological stress.

In summary, induction of hepatic HO-1 activity has the potential to affect the expression and catalytic activity of CYPs by a variety of mechanisms including (1) direct degradation of heme associated with CYP proteins, (2) regulation of free heme available for incorporation into newly synthesized P450, (3) regulation of free heme available for reversible association/dissociation with functional CYP, (4) regulation of the effects of free heme on CYP gene transcription, (5) reversible inhibition of CYP by heme-derived CO, and (6) regulation of CYP expression and catalytic activity by the production of bilirubin. Experiments are presented in Chapters 6, 7 and 8 of this thesis that provide both indirect and direct evidence for the last mentioned mechanism - regulation of CYP1A1 by bilirubin.

1.7 Aims and Approaches

1.7.1 Aims

The CYP monooxygenase system catalyzes a wide variety of oxidative reactions important for the metabolism of a number of compounds of endogenous and exogenous origin. Due to the variety and importance of the metabolic transformations performed by this enzyme system, changes in the catalytic activity of individual isozymes can not only alter the oxidation of xenobiotics, but may also result in alterations of essential physiological processes. The long term goals of the research conducted in this area are to define the contributions of individual CYP isozymes to the *in vivo* metabolism of endogenous and xenobiotic compounds and to address the toxicological, pharmacological and physiological implications of changes in their expression and catalytic activity. The experiments presented in this thesis focus on two main areas of direct relevance to these goals - modulation of CYP by selective MB inactivation and by pathobiological stress induced by exposure to toxic compounds and/or acute tissue injury.

The first aspect of the work presented in this thesis considers modulation of CYP by isozyme-selective MB inactivation. *N*-aralkylated derivatives of ABT (BBT, αMB and

αEB) are potent isozyme- (CYP2B) and tissue-selective (lung) MB inactivators of rabbit and guinea pig CYP *in vitro* and *in vivo* (Grimm *et al.*, 1995; Knickle and Bend, 1992; Mathews and Bend, 1986; 1993; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990). These compounds irreversibly inactivate CYP by at least two mechanisms: covalent modification of the prosthetic heme moiety (Mathews and Bend, 1986) and covalent modification of the CYP apoprotein moiety (Woodcroft *et al.*, 1997). The isozyme selectivity of the ABT derivatives most likely derives in large part from a structural resemblance to amphetamines, known substrates of CYP2Bs (Guengerich, 1997; Serabjit-Singh *et al.*, 1983). Amphetamines also inhibit CYP in a quasi-irreversible manner through the of formation MI complexes, raising the possibility that the ABT derivatives may also interact with CYP in this manner. Among the ABT derivatives, αMB exhibits the greatest isozyme selectivity and has proven to be a useful and sensitive biochemical probe for the identification of CYP isoforms catalyzing endogenous AA bioactivation (Knickle and Bend, 1994). It is believed that the greater selectivity of αMB, and the isozyme- and tissue-selectivity of the ABT derivatives as a group, can be rationalized in terms of structure using sensitive measures of CYP binding and inactivation kinetics.

The goals of the research in our laboratory are to further examine the utility of the ABT derivatives as sensitive biochemical probes for the study of CYP-dependent oxidation and to characterize the mechanisms by which these inhibitors operate. Investigations in this area will provide important contributions to the knowledge of how xenobiotics and endogenous substrates interact with and are oxidized by CYP. Furthermore, these studies are vital to the development of biochemical probes to define the roles of individual CYP isozymes in the metabolism of xenobiotics and endogenous substrates of physiological importance, as well as for the development of therapeutic agents. Consistent with these goals, the work presented in this thesis was undertaken to test the following hypotheses:

(1) Other mechanisms, such as MI complexation, are also involved in the inhibitory interactions of the ABT derivatives with CYP.

(2) Structural differences are the basis of the selectivity (tissue and isozyme) of the ABT derivatives and the increased potency and selectivity of αMB *vs.* BBT.

(3) Consideration of the metabolites generated and prediction of intermediates that might be involved in the metabolism of the ABT derivatives can provide a rationale for their differential selectivity and potency for CYP inhibition.

The second major aspect of the work presented in this thesis describes modulation of CYP by conditions of pathobiological stress. Infectious disease, inflammation, ischemia-reperfusion, oxidative stress and exposure to toxic chemicals can modulate CYP expression and catalytic activity in an isozyme- and tissue-selective manner. For example, acute exposure to the heavy metal salt, sodium arsenite (As^{+3}) causes profound induction of stress proteins (*i.e.* metallothionein, HO-1, NADPH:quinone oxidoreductase, glutathione *S*-transferases) in the liver concomitant with decreased CYP content and catalytic activity (Albores *et al.*, 1992a; 1992b; 1993a; Falkner *et al.*, 1993b). In contrast, As^{+3} potentiates the induction of CYP1A1-dependent EROD activity by βNF in guinea pig lung (Falkner *et al.*, 1993b), and causes an increase of EROD activity in rat lung in the absence of co-treatment with CYP1A1 inducers (Albores *et al.*, 1995). The AHR, a ligand-activated transcriptional activator of CYP1A1, binds large lipophilic ligands and is not known to be directly activated by As^{+3}. Instead, it is more likely that accumulation of an endogenous compound(s) that is capable of binding with the AHR, occurs secondary to acute As^{+3} toxicity.

The long term goals of research by our laboratory in this area are concerned with examining the mechanisms responsible for the isozyme- and tissue-selective modulation of CYP-dependent catalytic activity and gene expression during conditions of pathobiological

stress. Such stress may result from exposure to toxic chemicals, tissue injury and/or inflammation. It is believed that such conditions can have profound effects on the metabolism of a number of endogenous and exogenous compounds of pharmacological, toxicological and/or physiological relevance. Consistent with these goals, the work presented in this thesis was undertaken to test the following hypotheses:

(1) Common features can be identified for the effects of pathobiological stress on the CYP monooxygenase system in a number of model systems.

(2) Some isozyme-selective alterations in CYP-dependent catalytic activity and gene expression during pathobiological stress occur secondary to the initial stress.

(3) Changes in the levels of endogenous compounds mediate the modulation of CYP in response to pathobiological stress.

1.7.2 Approaches

A detailed account of the methodologies used for the experiments presented in this thesis is given for each of the studies presented in Chapters 2-8. However, a number of aspects common to all of the experimental approaches are worth discussing briefly here. First, the guinea pig was used for all of the experiments examining MB inactivation of CYP by *N*-aralkylated derivatives of ABT. A major reason for this choice was that most of the detailed characterizations of these compounds has been carried out in our laboratory using this experimental animal (Knickle and Bend, 1992; Knickle *et al.*, 1994; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1997). Also, the guinea pig lung contains relatively high concentrations of CYP, and in particular CYP2B, compared with other rodent species (Yamada *et al.*, 1992). This, combined with the high inducibility of this isoform in liver in response to PB treatment, substantially increases the sensitivity available for studies of MB inactivation by BBT, αMB or αEB.

Second, in some of the studies presented in this thesis, experimental animals were pre-treated with βNF or PB to selectively induce the expression of certain CYP isoforms. For example, for the studies of MB inactivation by the ABT derivatives, PB treatment was used to increase the hepatic content of CYP2B, thus allowing better assessment of the inhibition of these isozymes. CYP2Bs are not induced extrahepatically by PB treatment (Domin *et al.*, 1984; Yamada *et al.*, 1992). For some experiments, βNF was used to induce CYP1A1 in lung, kidney and heart, and CYP1A1 and CYP1A2 in liver. Very low levels of CYP1A2 mRNA expression have been detected by reverse transcriptase coupled polymerase chain reaction (RT-PCR) in rat kidney after 3MC treatment (Kim *et al.*, 1995a), however functional induction of the catalytic activity of this isoform in extrahepatic tissues does not occur.

Third, a number of relatively selective substrates were used to assess the catalytic activity of CYP isozymes. EROD was used for CYP1A1, although CYP1A2 is a major contributor to this activity in the livers of untreated animals (Burke and Mayer, 1974; Burke *et al.*, 1985; Tassaneeyakul *et al.*, 1993; Weaver *et al.*, 1994). Microsomal 7-pentoxyresorufin *O*-depentylation activity (PROD) activity was used for the CYP2B subfamily, although this activity is primarily catalyzed by CYP2B1 and CYP2B2 in rat tissues (Burke *et al.*, 1985; Lubet *et al.*, 1985; Weaver *et al.*, 1994). Microsomal 7-methoxyresorufin *O*-demethylation was used for CYP1A2 (Nerurkar *et al.*, 1993) and *p*-nitrophenol hydroxylation for CYP2E1 (Mishin *et al.*, 1996; Reinke and Moyer, 1985).

Finally, mouse hepatoma Hepa 1c1c7 cells were used to examine the mechanisms and mediators underlying the induction of CYP1A1 in response to conditions of pathobiological stress. These cells are particularly suited to studies of CYP1A1 regulation because of their faithful reproduction of the expression of this isoform *in vivo* - very low constitutive expression which is highly induced by treatment with PAHs. Also, a number of variant lines of these cells are available which lack functional AHR, ARNT or CYP1A1 due to the production of defective proteins or due to an absence of expression of the normal

protein (Hankinson, 1991; Kärenlampi *et al.*, 1986; Kimura *et al.*, 1987). Furthermore, as demonstrated in Chapter 8, these cells also express basal and inducible HO activity, thereby permitting studies of functional and regulatory interactions between this enzyme and CYP.

Chapter 2

Isozyme Selective Metabolic Intermediate Complexation of Guinea Pig Hepatic Cytochrome P450 with *N*-Aralkylated Derivatives of 1-Aminobenzotriazole[1]

2.1 Objectives

Many alkylamine and arylamine compounds including orphenadrine (Bast and Noordhoek, 1982), macrolide antibiotics such as troleandomycin (Pessayre *et al.*, 1982), the tricyclic antidepressants imipramine, desipramine, amitriptyline and nortriptyline (Murray and Field, 1992) and the prototypical CYP inhibitor, proadiphen (SKF 525-A) (Murray, 1988), form MI complexes and effectively inhibit CYP in an isozyme selective/specific manner. Three *N*-aralkyl derivatives (Fig. 2.1) of ABT, BBT, αMB and αEB have previously been demonstrated to be potent and isozyme selective MB inactivators of rabbit and guinea pig hepatic and pulmonary CYP (Knickle *et al.*, 1994; Mathews and Bend, 1986; 1993; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990), both *in vitro* and *in vivo*. The objective of this study was to test the hypothesis that these arylamine compounds are also able form MI complexes with CYP. To this end, the capacity and specificity of BBT, αMB and αEB for the *in vitro* formation of MI complexes with CYP of hepatic microsomes from untreated, PB-induced and βNF-induced guinea pigs was determined. The concentration dependence of complex formation was

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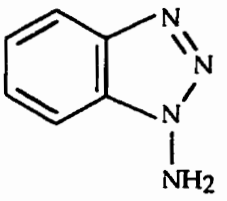
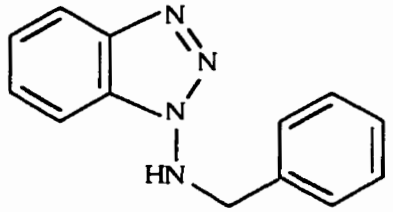
1 A version of this chapter has been published.

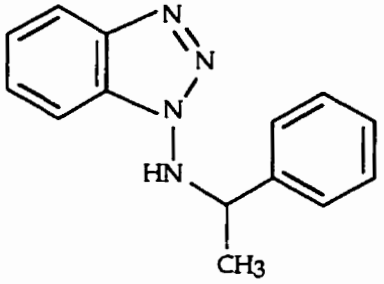
Sinal CJ and Bend JR (1995) Isozyme-selective metabolic intermediate complex formation of guinea pig hepatic cytochrome P450 by *N*-aralkylated derivatives of 1-aminobenzotriazole. *Chem. Res. Toxicol.* **8**: 82-91.



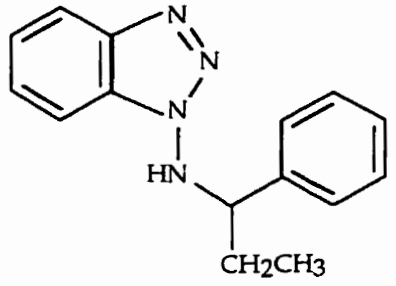
ABT
1-aminobenzotriazole



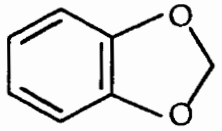
BBT
N-benzyl-1-aminobenzotriazole



αMB
N-α-methylbenzyl-1-aminobenzotriazole



αEB
N-α-ethylbenzyl-1-aminobenzotriazole



BD
1,3-benzodioxole

Figure 2.1
Chemical structures of the MB inactivators of CYP used in this study.

utilized as an index to determine the apparent K_m and $V_{max(obs)}$ of complex formation. In addition, the spectral characteristics of the interaction of BBT, αMB and αEB with guinea pig hepatic microsomes were also compared with those obtained for the interaction of BD, a compound which forms MI complexes with CYP, and ABT, a known MB inactivator of CYP monooxygenase activities.

2.2 Materials and Methods

2.2.1 Materials

ABT, BBT, αMB and αEB were synthesized and purified as previously described (Mathews and Bend, 1986; Woodcroft *et al.*, 1990). NADPH was purchased from Sigma Chemical Co. (St. Louis, MO); βNF and BD from Aldrich Chemical Co. (Milwaukee, WI); and reduced glutathione (GSH), potassium ferricyanide, PB, Me_2SO, sodium dithionite and all other chemicals (reagent grade or better) from BDH (Toronto, ON).

2.2.2 Animal Treatment and Preparation of Microsomes

Male Hartley guinea pigs or male rabbits (250-300 g or 2.5 kg, respectively; Charles River Laboratories, St. Constant, PQ) were used. Some of the animals were treated intraperitoneally with 80 mg/kg PB (2% in saline) or 80 mg/kg βNF (2% in corn oil) daily for 4 days and sacrificed 24 h following the last injection by asphyxiation with CO_2. All animals were allowed free access to food (Purina guinea pig chow) and water throughout the treatment period.

Washed hepatic and pulmonary microsomes were prepared by differential centrifugation as previously described (Bend *et al.*, 1972). Briefly, the tissues were homogenized in homogenization buffer (1.15% KCl in 50 mM potassium phosphate buffer, pH 7.4; 3 mL per g tissue mass) using a motor driven Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 12 500 x g for 20 min followed by centrifugation of the supernatant at 125 000 x g for 50 min (Beckman Ti50 rotor). The

resulting pellet was resuspended in homogenization buffer using a hand-held homogenizer and centrifuged again at 125 000 x g for 50 min. The microsomal pellet was then resuspended in 100 mM potassium phosphate buffer (pH 7.4) using a hand-held homogenizer. All of the preceding steps were performed on ice, or at 4°C. Aliquots of resuspended microsomal protein were stored at -80°C until assay. Microsomal protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. Specific CYP content was determined from the dithionite difference spectrum of carbon monoxide-saturated microsomes using a Beckman DU-65 spectrophotometer with $\varepsilon = 100\ mM^{-1} \cdot cm^{-1}$ (Estabrook *et al.*, 1972).

2.2.3 Time Course of Complex Formation

All spectral determinations were performed at 25° C. Experimental samples contained 1 mg/mL (unless otherwise noted in text) of hepatic microsomal protein from untreated, βNF-treated or PB-treated guinea pigs. The test compounds (structures in Fig. 2.1) were dissolved in either Me_2SO (BD, BBT, αMB and αEB) or distilled water (ABT) and 5 μL added to the microsomal suspension to give a final inhibitor concentration of 200 μM. The final cuvette volume was made up to 1 mL with 0.1M potassium phosphate buffer (pH 7.4). A baseline scan of zero absorbance was recorded between 400 and 500 nm using a Beckman DU-8 single beam spectrophotometer (reference). In some experiments, GSH (1 mM final concentration) was also included in the incubation mixtures. Complex formation was started by the addition of 0.1 M NADPH (10 μL) in distilled water (final concentration = 1 mM). In some experiments, the concentration of NADPH was varied between 0.05 - 1.0 mM by the addition of 10 μL of an appropriate stock solution. Repetitive scans were recorded at intervals of approximately 90 s for a period of 15 - 30 min. The amount of complex formed was calculated by measuring the absorbance change at the wavelength of maximal absorption of each complex (455 - 460 nm) relative to the absorbance change at 490 nm (an isosbestic reference point). Estimates

of the proportion of total CYP complexed were derived using an extinction coefficient of $\varepsilon = 64\ mM^{-1} \cdot cm^{-1}$ (Franklin, 1991).

Dissociation of the ferrous-CYP complexes was followed spectrophotometrically (Beckman DU-8) after the addition of potassium ferricyanide (50 μM) to microsomes from PB-treated guinea pigs (2 mg/mL) that had been pre-incubated with 200 μM inhibitor and 1 mM NADPH (1 mL final volume) for 45 min at 37° C. An initial baseline scan (430 - 500 nm) of zero absorbance for the ferrous-complexed microsomes was recorded as a reference. After the addition of ferricyanide (10 μL of 5 mM) followed by a 5 min incubation period, a second scan was recorded over the same wavelengths. Complex dissociation was followed as a loss of maximal absorbance in the 455 nm range.

2.2.4 Determination of Spectral Binding To Cytochrome P450

All determinations were performed at 25°C. Experimental samples contained 2 mg of guinea pig hepatic microsomal protein in a volume of 1.0 mL in 0.1 M potassium phosphate buffer (pH 7.4) in a semi-micro spectrophotometer cuvette. Various dilutions of the inhibitors (BBT, αMB and αEB) were prepared in Me_2SO. After a baseline scan was recorded between 350 and 500 nm, sufficient volumes of these dilutions were added sequentially to the cuvette to give the desired inhibitor concentrations (2.5 - 400 μM). The total final volume of inhibitor/solvent added was 20 μL. Scans of 350-500 nm were recorded immediately after each addition. The magnitude of spectrally assayable inhibitor/CYP binding was quantitated as the change in absorbance at 387 nm minus the change in absorbance at 425 nm relative to the initial reference scan. The apparent spectral K_S and ΔA_{max} values were determined by double reciprocal plots.

2.2.5 Kinetics of Complex Formation

Experimental samples contained 1 mg/mL of hepatic microsomal protein from PB-treated guinea pigs in 0.1M potassium phosphate buffer (pH 7.4). The test compounds

(BBT, αMB and αEB) were dissolved in Me_2SO and 5-10 μL added to the samples (final concentration = 10 - 500 μM; 10 - 1000 μM for αMB). A baseline scan was recorded between 400 and 500 nm using a Beckman DU-8 single beam spectrophotometer (reference). Complex formation was started by the addition of 0.1 M NADPH (10 μL) in distilled water (final concentration = 1 mM). The rate of complex formation was determined by least-squares linear regression of the linear portion of the absorbance change *vs.* time curve. For BBT, αMB and αEB, the amount of complex formed after 25 s was recorded. The rate of complex formation was determined by dividing the change in absorbance by 25 s. Apparent kinetic constants [K_m, $V_{max(obs)}$] were determined using double-reciprocal plots.

2.2.6 Washing Protocol for Inhibited Microsomes

Experimental samples contained 2 mg/mL guinea pig hepatic microsomal protein. The test compounds (BD, BBT, αMB and αEB) were dissolved in Me_2SO and 15 μL added to the samples to give a final inhibitor concentration of 200 μM. The final volume was made up to 2.97 mL with 0.1M potassium phosphate buffer (pH 7.4). Complex formation was started by the addition of 0.1 M NADPH (30 μL) in distilled water (final concentration = 1 mM) and was allowed to proceed for 10 min at 37°C. At the end of the incubation time, aliquots of the samples were cooled on ice, followed by either spectrophotometric determination of spectral complex (unwashed) or washing by ultracentrifugation (washed). Briefly, washed samples were sedimented at 100 000 RPM (412 160 x g) for 10 min at 4°C (Beckman TL-100 ultracentrifuge, TLA 100.3 rotor) followed by resuspension and resedimentation in 0.1M potassium phosphate buffer (pH 7.4). In some experiments, the microsomal samples were reduced by the addition of 10 mg sodium dithionite prior to washing. Control samples for each group were treated identically to the samples but did not contain NADPH. Washed and unwashed inhibited microsomal samples were added to a spectrophotometric cuvette to give a final

concentration of 2 mg/mL. A baseline scan of zero absorbance was recorded between 400 and 500 nm. Control samples were used to obtain reference scans. Repetitive scans were recorded and the amount of complex present was calculated measuring the absorbance change at the wavelength maximal absorption of each complex (455 - 460 nm) relative to the absorbance change at 490 nm.

2.2.7 Mechanism-Based Inactivation Assays

Incubation mixtures contained 5 mg/mL of hepatic microsomal protein and 10 μM inhibitor in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. Inactivation reactions were started by the addition of NADPH (1 mM final) and were maintained at 37°C in a shaking water bath. Control incubations contained an equal volume of Me_2SO in place of the inhibitor solution. After 30 min, the incubations were subjected to a washing protocol prior to the enzyme assays. Washing of the microsomes to remove excess inhibitor was accomplished by sedimentation of the inhibition mixtures at 100 000 x g for 60 min at 4°C (Beckman XL-90 ultracentrifuge; 50Ti rotor), and resuspension of the microsomal pellet in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. Subsequently, the microsomes were centrifuged at 412 160 x g for 15 min at 4°C (Beckman TL-100 ultracentrifuge; TLA 100.3 rotor) followed by resuspension in the same buffer. After determination of protein concentration, the washed microsomes were stored at -80°C until further use.

The rates of PROD and EROD activity were determined based on the methods of Burke and Mayer (1974) and Lubet *et al.* (1985), respectively. Measurements were performed in fluorimeter cuvettes maintained at 37°C, with stirring, in a Perkin-Elmer fluorescence spectrophotometer (model LS-5B). Incubations contained 0.3 mg hepatic microsomal protein and were made up to a final volume of 1.42 mL with 0.1 M potassium phosphate buffer, pH 7.8. After 5 min of temperature equilibration, 5 μL of 7-pentoxyresorufin or 7-ethoxyresorufin (in Me_2SO; final concentration of 4 and 1 μM,

respectively) were added and a baseline fluorescence scan was recorded (excitation = 525 nm; emission = 585 nm). Reactions were started by the addition of 75 μL NADPH dissolved in incubation buffer to obtain a final incubation concentration of 1 mM and were allowed to proceed for 10 min. The rate of resorufin formation was calculated by comparison to known amounts (5 pmol) added in 5 μL Me_2SO to the reaction mixture. All assays were verified to proceed linearly with respect to time and protein concentration.

2.2.8 Statistics

Data were analyzed by analysis of variance (ANOVA), followed by the Dunnett's test for multiple comparisons where appropriate (Tables 2.1, 2.2 and 2.4) using SuperANOVA v1.11 for Macintosh software (Abacus Concepts, Berkeley, CA).

2.3 Results

2.3.1 Metabolic Intermediate Complex Formation

Formation of MI complexes was observed upon incubation of BD, BBT, αMB or αEB, but not ABT, with guinea pig hepatic microsomes containing CYP and NADPH. The maximal absorbance of the spectral complexes of the *N*-aralkylated derivatives of ABT occurred in the 455-457 nm region (Fig. 2.2). Representative scans were recorded from incubations with hepatic microsomes from PB-treated guinea pigs, as these provided the greatest rate and extent of complex formation for these inhibitors. With BD, the peak absorbance occurred at approximately 460 nm.

Induction with PB or βNF had an affect on both the rate and maximal extent of complex formation observed for BBT, αMB, αEB or BD. Hepatic microsomes from PB-treated animals had the fastest rate and greatest extent of complex formation in all cases (Fig. 2.3). At equimolar concentrations (200 μM), the ABT analogues formed complexes at a faster rate than was observed for BD. Complex formation was minimal with BBT and

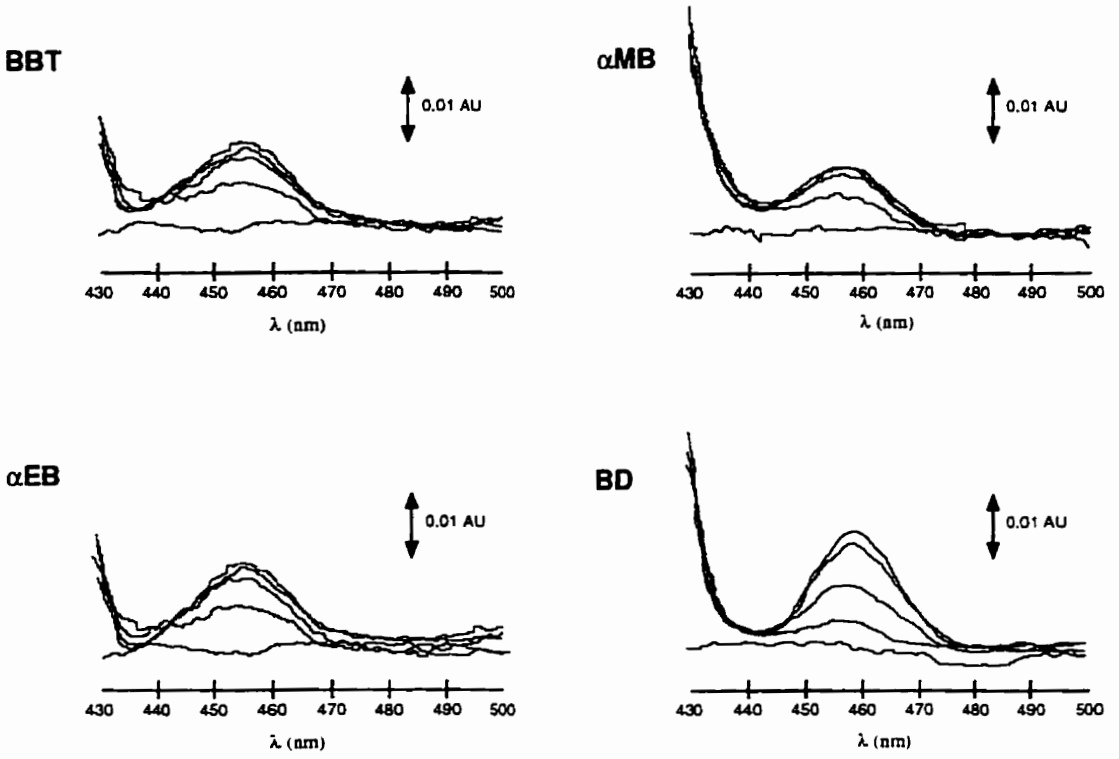


Figure 2.2
Representative spectra obtained upon the incubation of 200 μM BBT, αMB, αEB or BD with 1 mM NADPH in PB-treated guinea pig hepatic microsomes. Repetitive scans were recorded at approximately 90 s intervals.

αEB, and was not detected with αMB, using microsomes from βNF-treated guinea pigs (Fig. 2.3). MI complex formation with BD was fastest and greatest in magnitude with microsomes from PB- and βNF-induced guinea pigs. The extent of complex formation with BBT, αMB and αEB apparently reflected a selectivity for complexation with a PB-inducible CYP isozyme(s) in guinea pig liver. In contrast, the extent of complex formation with BD reflected the total CYP content (PB > βNF > untreated) of the microsomal samples.

The order of magnitude of NADPH-dependent MI complex formation with BBT, αMB and αEB in hepatic microsomes was PB-treated > untreated > βNF-treated (Table 2.1). In hepatic microsomes from PB-treated guinea pigs, 2.4- (BBT), 3.0- (αMB) and 3.9-fold (αEB) increases in the total (% complexed • [CYP]) amount of MI complex formed were noted. On the other hand, βNF induction markedly decreased MI complex formation with all three compounds.

2.3.2 Spectral Binding with Cytochrome P450

The inhibitors BBT, αMB and αEB produced a type I spectral change, characterized by the appearance of an absorption peak at approximately 387 nm and a trough near 425 nm, when added to hepatic microsomes from PB-treated guinea pigs. The magnitude of the spectral change was dependent upon the concentration of the added compounds (Fig. 2.4). Apparent spectral dissociation constants (K_s) were calculated from the data by double-reciprocal plots yielding values of 22 ± 5, 16 ± 2 and 5 ± 1 μM for BBT, αMB and αEB respectively (Table 2.2). The corresponding ΔA_{max} values were determined to be 0.0047 ± 0.002, 0.0049 ± 0.004 and 0.0058 ± 0.005, respectively.

2.3.3 Kinetics of Complex Formation

To compare the kinetics of NADPH-dependent MI complex formation for BBT, αMB and αEB, various concentrations (10 - 1000 μM) were incubated with NADPH and

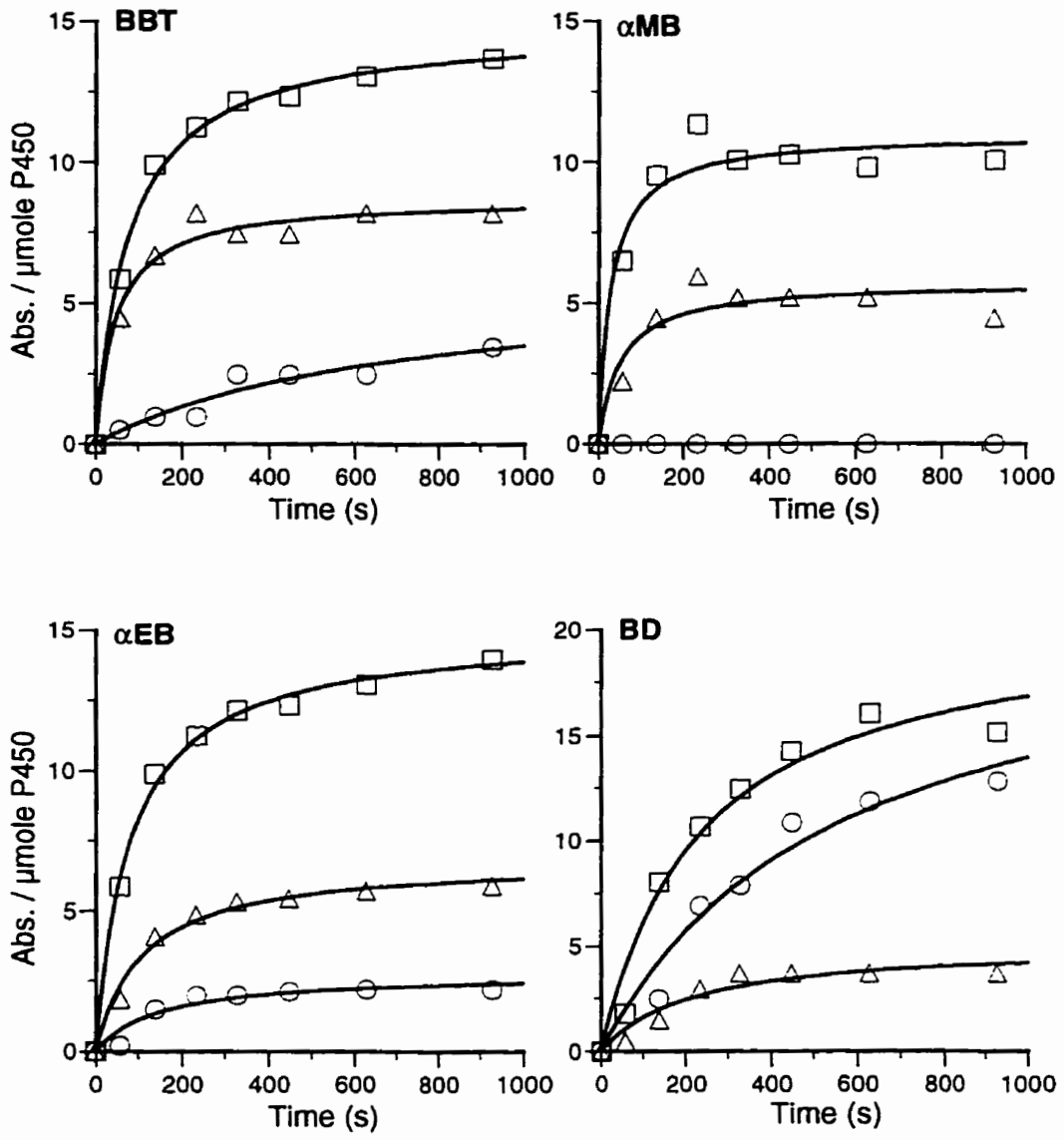


Figure 2.3
Time course of MI complex formation by 200 µM BBT, αMB, αEB or BD with PB-treated (□), β-NF-treated (○) and control (△) guinea pig hepatic microsomes. The change in absorbance was measured as $\Delta A_{455-490}$ for BBT, αMB and αEB and $\Delta A_{460-490}$ for BD. Each point is representative of the mean of at least three experiments with microsomes pooled from 4-5 animals.

Table 2.1 - Percentage of total CYP involved in NADPH-dependent MI complex formation with BBT, αMB and αEB in hepatic microsomes from untreated, βNF-treated and PB-treated guinea pigs. Values represent the mean ± S.D. (N=3-4)

	% of total CYP complexed [a]		
Inhibitor	Untreated	βNF-induced	PB-induced
BBT	13 ± 1	5 ± 2 [b]	20 ± 3 [b]
αMB	9 ± 4	ND [c]	16 ± 2 [b]
αEB	9 ± 1	3 ± 1 [b]	21 ± 1 [b]

[a] Determined from the absorbance change at 455 nm relative to 490 nm using $\varepsilon = 64\ mM^{-1} \cdot cm^{-1}$; microsomal CYP content (nmol/mg) was determined as 0.69 ± 0.06, untreated; 1.01 ± 0.09, βNF; 1.12 ± 0.09, PB.

[b] Significantly different from untreated.

[c] ND = not detected ($\Delta A < 0.001$)

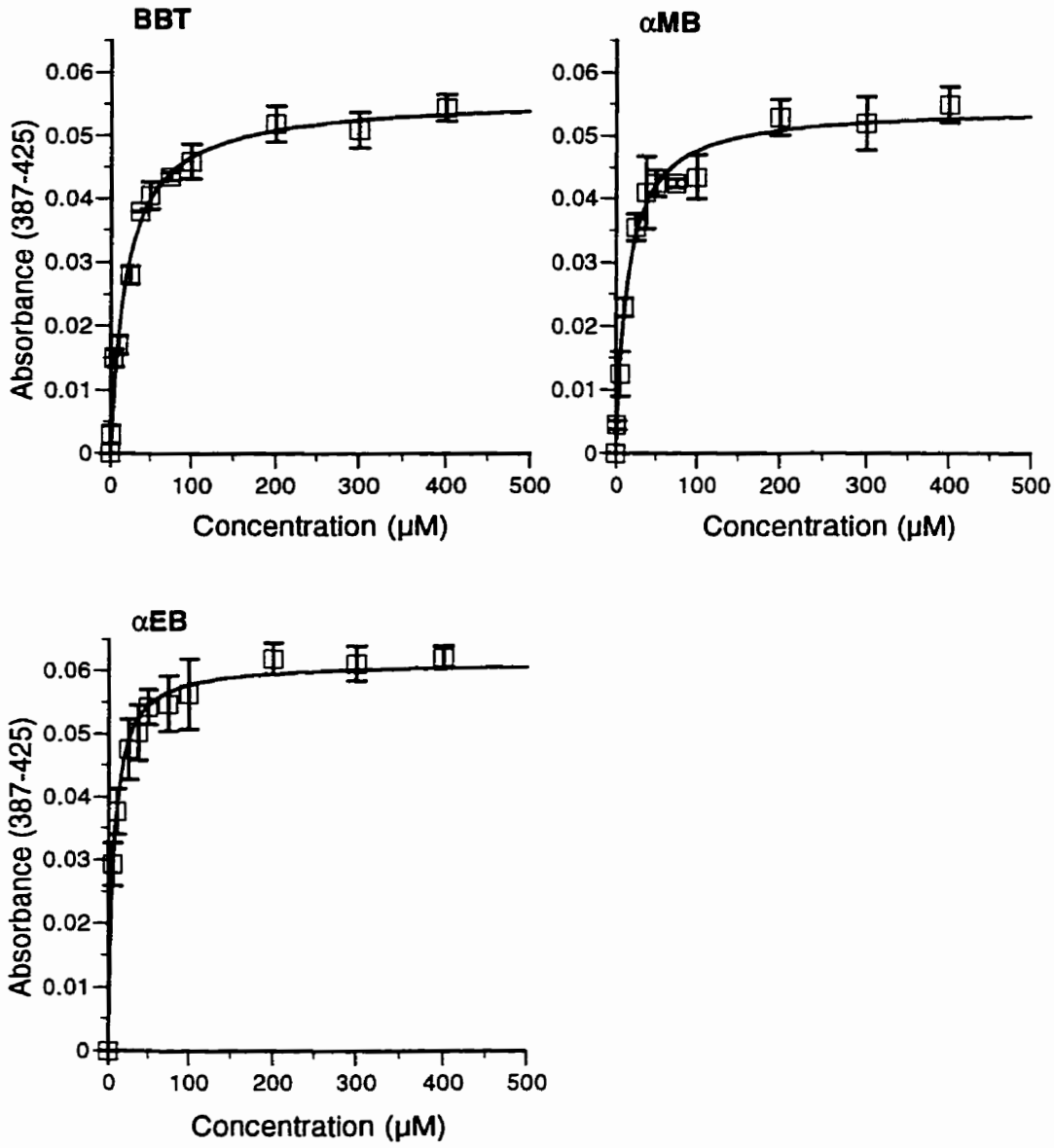


Figure 2.4
Spectral binding curves of BBT, αMB and αEB with CYP of hepatic microsomes from PB-treated guinea pigs in the absence of NADPH. Each point is representative of the mean ± SD of at least three experiments with microsomes pooled from 4-5 animals.

Table 2.2 - Apparent spectral constants for binding to microsomal CYP in the absence of NADPH (K_S and ΔA_{max}) and for MI complex formation in the presence of NADPH (K_m and $V_{max(obs)}$) for BBT, αMB and αEB using hepatic microsomes from PB-treated guinea pigs. Reported values are mean ± SD (N=3-4)

Compound	K_S (μM)	ΔA_{max}	K_m (μM)	$V_{max(obs)}$ (ΔA/min/μmol CYP)
BBT	22 ± 5	0.049 ± 0.004	108 ± 44	25 ± 3
αMB	16 ± 2	0.047 ± 0.002	338 ± 96 [a]	21 ± 4
αEB	5 ± 1 [b]	0.058 ± 0.005	97 ± 48	21 ± 2

[a] Significantly different from BBT and αEB.

[b] Significantly different from αMB and BBT.

hepatic microsomes from PB-treated guinea pigs. Initial rates of complex formation were observed to be concentration dependent and appeared to approach near maximal values over the concentration range studied. Values for the apparent kinetic constants of complex formation were derived from double-reciprocal plots (Fig. 2.5). All of the test compounds displayed linear relationships over the moderate to high concentration values studied. At very low inhibitor concentrations, the relationships became curvilinear, with large decreases in the rate of complexation associated with relatively small changes in concentration (data not shown). As a consequence of this behaviour, the apparent kinetic constants (Table 2.2) were derived using those concentrations at which linearity was observed. The apparent K_m values decreased in the order αMB > BBT > αEB. The smaller apparent K_m values for BBT and αEB when compared with αMB suggest a higher substrate affinity or efficiency of complex formation.

2.3.4 Dissociation of Metabolic Intermediate Complexes

The addition of 50 μM potassium ferricyanide to microsomes from PB-treated guinea pigs that had been incubated for 45 min with 200 μM inhibitor and 1 mM NADPH resulted in a loss in absorbance which was maximal at approximately 455 nm for BBT, αMB and αEB (not shown). This indicates that the MI complexes formed with these compounds are more stable with the ferrous form of the heme than with its ferric form. In contrast, the addition of ferricyanide to samples that had been incubated with BD and NADPH did not result in a loss of absorbance in the region in which the complex displays maximum absorption (approximately 460 nm).

2.3.5 Effect of Washing and NADPH Concentration

To further investigate the stability of MI complexes of the inhibitors with CYP, samples of guinea pig hepatic microsomal protein were incubated with NADPH and the inhibitors at a concentration of 200 μM for 10 min. At the end of the incubation period,

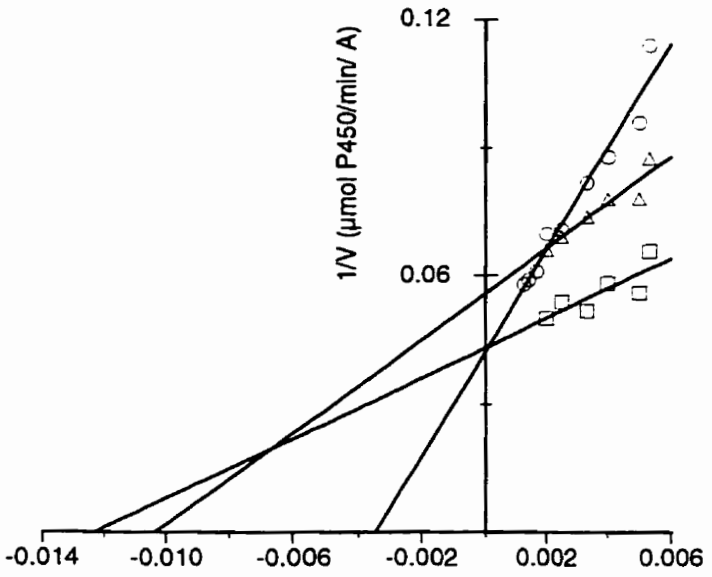


Figure 2.5
Double-reciprocal plot of the initial rates of NADPH-dependent MI complex formation as a function of inhibitor concentration for BBT (□), αMB (○) and αEB (△) in hepatic microsomes of PB-treated guinea pigs. Each point is representative of the mean of at least three experiments with microsomes pooled from 4-5 animals.

half of the sample volume was washed to remove unreacted inhibitor and NADPH and was compared with the unwashed sample volume. As shown in Table 2.3, the washing protocol had no appreciable effect on the complex formed from BD. In contrast, the MI complexes formed with BBT and αEB were decreased in magnitude significantly by washing. In fact, with αMB, no complex was found subsequent to washing. When samples incubated with 200 μM BBT and 1 mM NADPH were reduced with sodium dithionite prior to washing, the persistence of the formed MI complexes was improved dramatically compared with samples washed in the absence of sodium dithionite (Table 2.4). Additionally, the unwashed samples had the largest magnitude of MI complexation and the lowest CYP content. In samples that were not reduced prior to washing, the greatest loss of MI complex was observed concomitant with the highest CYP content.

The magnitude and persistence of MI complexation with 200 μM BBT and hepatic microsomes from PB-treated guinea pigs was decreased dramatically with decreasing NADPH concentration (Fig. 2.6). With 1 mM NADPH, the magnitude of MI complex formation increased rapidly up to 5 min, followed by a slower increase up to 30 min. In contrast, with 0.05 μM NADPH the initial and rapid increase up to 5 min was observed followed by a steady loss that was essentially complete after 20 min.

2.3.6 Effect of Glutathione

Incubations were also carried out in the presence or absence of 1 mM GSH. MI complex formation was followed spectrophotometrically in a manner identical to the time course experiments using a final inhibitor concentration of 200 μM, and 1 mM NADPH. GSH had a substantial attenuating effect with BBT, αMB and αEB, resulting in losses of 50-70 % of maximal complex formation (Fig. 2.7). Inclusion of 1 mM GSH did not have a significant attenuating effect upon complex formation when BD was used as the inhibitor. In order to assess the effect of GSH on the MB inactivation of CYP by BBT, αMB or ABT, hepatic microsomes from PB-treated guinea pigs were incubated with 10 μM

Table 2.3 - Effect of resuspension and resedimentation of microsomes on the stability of MI complexes formed by 200 μM BBT, αMB, αEB and BD with guinea pig hepatic microsomal protein (2 mg) incubated with 1 mM NADPH.

Inhibitor	Condition	ΔA / mg protein	% Loss [a]
BBT	unwashed	0.012 ± 0.001	
	washed	0.002 ± 0.001 [b]	81
αMB	unwashed	0.010 ± 0.002	
	washed	ND [c]	> 95
αEB	unwashed	0.009 ± 0.002	
	washed	0.001 ± 0.001 [b]	89
BD	unwashed	0.010 ± 0.003	
	washed	0.009 ± 0.003	10

[a] % loss of mean.

[b] Significantly different from unwashed.

[c] Not detected (< 0.001 A / mg of protein)

Table 2.4 - Effect of washing and dithionite reduction on CYP content and MI complexes formed from 200 μM BBT with hepatic microsomes from PB-treated guinea pigs. Values are mean ± SD (N = 3)

Sample	ΔA / mg protein	% Loss of MI Complex	[CYP] [a] (nmol / mg of protein)
Unwashed			
(-) dithionite	0.013 ± 0.001	0	0.46 ± 0.02
Washed			
(-) dithionite	0.002 ± 0.001 [b]	85	0.74 ± 0.04 [b]
(+) dithionite	0.010 ± 0.003	23	0.58 ± 0.07 [b]

[a] Control CYP content = 1.34 ± 0.08 nmol/mg of microsomal protein.

[b] Significantly different from unwashed

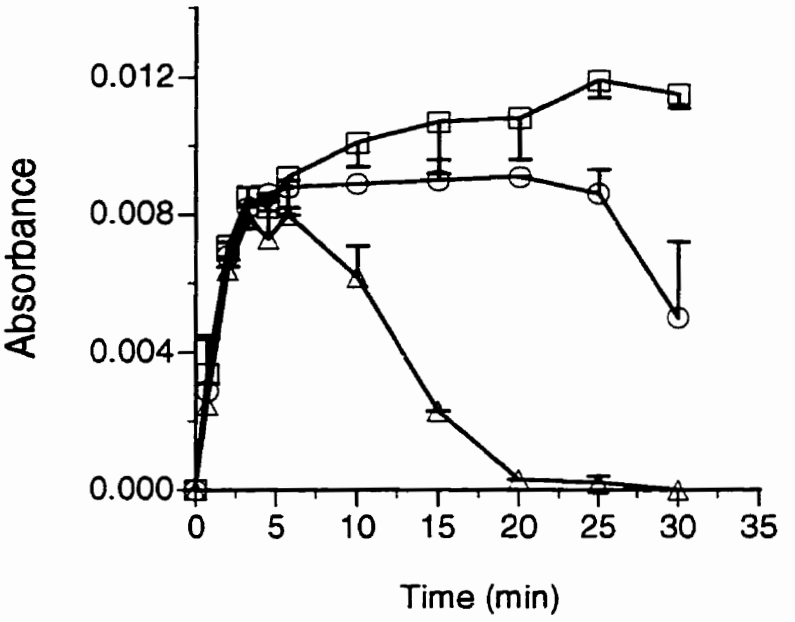


Figure 2.6
Time course of MI complex formation with 200 μM BBT using hepatic microsomes from PB-treated guinea pigs in the presence of 1 mM (□), 0.1 mM (○) or 0.05 mM (△) NADPH. Each point is representative of the mean ± SD of at least three experiments with microsomes pooled from 4-5 animals.

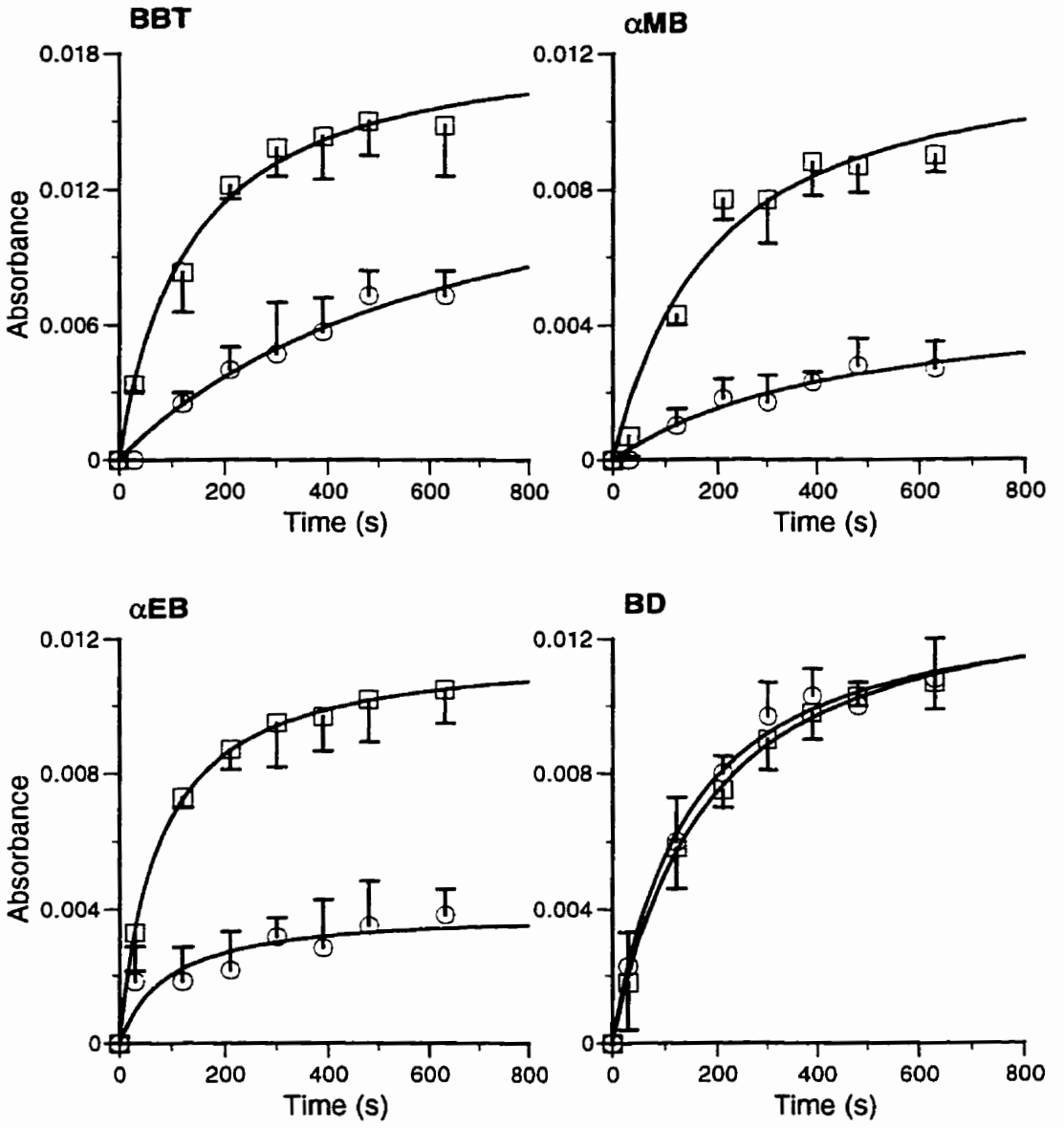


Figure 2.7
Time course of NADPH-dependent complex formation with 200 μM BBT, αMB, αEB or BD using 2 mg/mL of hepatic microsomes from PB-treated guinea pigs in the absence (□) or presence (○) of 1 mM GSH. Each point is representative of the mean ± SD of at least three experiments with microsomes pooled from 4-5 animals.

inhibitor and 1 mM NADPH for 30 min in the absence or presence of 1 mM GSH. Following two cycles of sedimentation and resuspension to remove excess, unreacted inhibitor, the microsomes were assayed for PROD and EROD, enzyme activities selective for CYP2B1/2 and CYP1A isoforms in rat (Burke *et al.*, 1985; Lubet *et al.*, 1985). Both BBT and αMB caused substantial losses of PROD activity with comparatively smaller losses of EROD activity and CYP concentration (Table 2.5). Equimolar ABT caused a smaller decrease of PROD activity and larger decrease of CYP content when compared with the *N*-aralkylated derivatives. Inclusion of 1 mM GSH was without effect on the inactivation of EROD and PROD activities, as well as CYP content, by any of the inhibitors. These data clearly distinguish MB inactivation and MI complexation by the *N*-aralkylated derivatives of ABT.

2.3.7 Metabolic Intermediate Complexation with Pulmonary Microsomes

No evidence for the *in vitro* formation of MI complexes was found upon incubation of 200 μM BBT or αMB with 1 mM NADPH using lung microsomes from untreated guinea pigs (Fig. 2.8). Complex formation did occur however, with incubations containing 200 μM BD, to a degree comparable to that observed in liver, when adjusted for the CYP concentration of the two tissues. To investigate the possibility that the failure to observe spectral complexes with the ABT analogues was a consequence of the low CYP concentrations (typically 0.10 - 0.18 nmol/mg of microsomal protein) in guinea pig lung samples, the experiments were repeated using samples of untreated rabbit lung microsomes. Rabbit lung CYP has previously been demonstrated to exhibit a significant amount of CYP2B4-dependent monooxygenase activity and to be susceptible to isozyme selective, irreversible MB inactivation by BBT and αMB, both *in vitro* and *in vivo* (Knickle *et al.*, 1994; Mathews and Bend, 1986; 1993). No MI spectral complex was observed upon incubation of 200 μM BBT or αMB with 1 mM NADPH using rabbit lung

Table 2.5 - Effect of 1 mM GSH on the MB inactivation of guinea pig hepatic microsomal PROD and EROD activities and CYP content by 10 μM BBT, αMB or ABT. Values are mean ± SD (N = 3)

Sample	PROD (pmol/min/mg)	EROD (pmol/min/mg)	[CYP] (nmol/mg)
Control			
(-)GSH	103.5 ± 9.2	153.0 ± 7.2	0.90 ± 0.02
(+)GSH	99.8 ± 3.6	146.1 ± 6.7	0.88 ± 0.03
BBT			
(-)GSH	38.8 ± 8.0	115.8 ± 15.2	0.83 ± 0.02
(+)GSH	44.4 ± 2.6	125.2 ± 7.6	0.85 ± 0.05
αMB			
(-)GSH	7.4 ± 2.6	125.5 ± 6.3	0.79 ± 0.04
(+)GSH	9.0 ± 1.0	119.1 ± 6.4	0.82 ± 0.08
ABT			
(-)GSH	52.0 ± 2.9	138.2 ± 7.0	0.69 ± 0.05
(+)GSH	56.8 ± 2.6	141.3 ± 8.3	0.70 ± 0.07

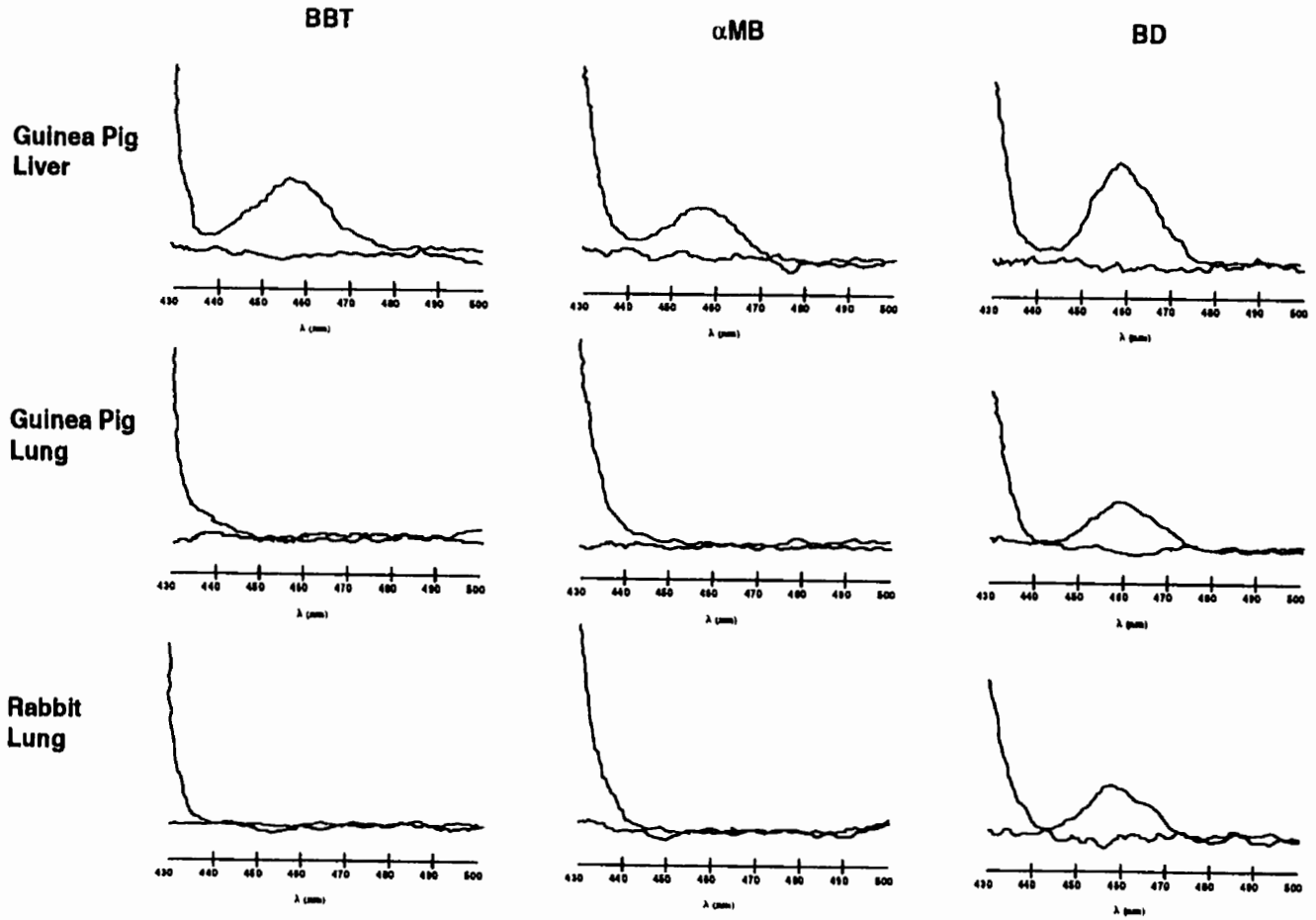


Figure 2.8
Representative spectra obtained upon the incubation of 200 μM BBT, αMB or BD with 1 mM NADPH and guinea pig liver, guinea pig lung or rabbit lung microsomes. Scans were recorded 10 min after NADPH addition.

microsomes, although complex formation was observed in these microsomes with incubations containing 200 μM BD and 1 mM NADPH.

2.4 Discussion

Previous work from this laboratory has demonstrated the potent and isozyme selective, irreversible MB inactivation of microsomal CYP-dependent activity in rabbit or guinea pig liver and lung (Knickle and Bend, 1994; Knickle *et al.*, 1994; Mathews and Bend, 1986; 1993; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990) by BBT, αMB or αEB, *N*-aralkylated derivatives of ABT. The loss of catalytic activity is accompanied by some loss of spectrally assayable CYP as well as the generation of modified porphyrin pigment(s) in liver identical to that obtained with ABT (Mathews and Bend, 1986). This suggests that benzyne-dependent heme arylation plays a role in the NADPH-dependent inactivation of CYP by these compounds. Additionally, we have identified binding of ^{14}C-BBT metabolites, derived from the benzyl ring of the inhibitor, to guinea pig hepatic microsomal protein (Woodcroft *et al.*, 1997), suggesting that another inhibitory mechanism may involve covalent modification of CYP apoprotein. A further mechanism of BBT, αMB and αEB inhibition of CYP may involve generation of MI complexes. The objective of this study was to determine if BBT, αMB and αEB are capable of forming NADPH-dependent MI complexes in guinea pig hepatic microsomes, and if so, to characterize and compare the three compounds in this regard. The MI complex forming compound BD was also used in some experiments to validate our methodology. Due to the mechanistic differences between complex forming amines and methylenedioxyphenyl compounds, the choice of BD also allowed us to compare the behaviour of the ABT analogues with a well characterized CYP inhibitor.

BBT, αMB and αEB all generated MI complexes when hepatic microsomes from guinea pigs were incubated with NADPH (Fig. 2.2). Complex formation was maximal in

microsomes from PB-treated animals (Fig. 2.3), suggesting a selectivity for a PB-inducible CYP isozyme(s) in guinea pig liver. In contrast, MI complex formation with BBT, αMB and αEB was found to be least efficient in microsomes from animals treated with βNF (Fig. 2.3). Competitive metabolic pathways, catalyzed by βNF-inducible isozymes such as CYP1A1 and CYP1A2 in guinea pig liver, may result in the conversion of BBT, αMB and αEB, to metabolites which are not active as MI complexing ligands. Alternatively, guinea pig hepatic CYP1A isozymes may be induced by βNF treatment at the expense of CYP isozymes involved in MI complex formation with the ABT derivatives. This is consistent with the concept that MI complex formation is dependent upon the proportion of total CYP isozymes that are capable of metabolism of the parent compound to the active complex forming ligand (Franklin, 1991). The effect of CYP induction on MI complex formation observed in the present study was similar to that observed by Woodcroft and Bend (1990) for the inactivation of guinea pig hepatic PROD activity. While this most likely primarily reflects a loss of the catalytic activity of CYP2B18, the guinea pig homologue of rat CYP2B1 and rabbit CYP2B4 (Nelson *et al.*, 1996), other closely related isoforms may also contribute to this activity (*i.e.* CYP2B2 or CYP2B5 in rat or rabbit liver, respectively). Thus, PROD activity is most correctly referred to as CYP2B-dependent in rat, rabbit and most likely, guinea pig liver as well. In the study by Woodcroft and Bend (1990), inhibition of hepatic CYP2B-dependent PROD activity by the ABT derivatives was most effective in microsomes from PB-treated guinea pigs > untreated > βNF-treated. Specifically, 10 μM αMB was shown to irreversibly inactivate 88 ± 3% of PROD activity in hepatic microsomes from PB-treated guinea pigs. Therefore, the ability of the ABT derivatives, at a concentration of 200 μM, to form MI complexes with CYP2B isoforms is most likely limited by competition with mechanisms of irreversible, MB inactivation that proceed at lower inhibitor concentrations.

The apparent spectral dissociation constants (K_s) of 22 ± 5, 16 ± 2 and 5 ± 1 μM for BBT, αMB and αEB respectively, represent a substrate-enzyme interaction of relatively

high affinity and the significantly smaller K_s and larger ΔA_{max} values observed for αEB relative to BBT are consistent with its higher lipophilicity. The apparent K_m values for MI complex formation with BBT and αEB were significantly lower than that observed for αMB. The inverse relationship of apparent K_s with respect to α-substituent chain length indicates that the lipophilicity of the inhibitor is important in binding. Since the spectral binding of BBT, αMB or αEB is to a type I binding site on the apoprotein, while CYP-dependent oxidation occurs at the heme (protoporphyrin IX), at least two distinct steps are required. Factors affecting substrate binding to CYP are likely to affect the overall kinetic profile of catalysis. Thus, it appears that the increased lipophilic character due to the presence of the α-ethyl group significantly improves binding, resulting in a lower K_m value.

A lag-phase occurs prior to the initiation of MI complex formation with amphetamine and benzphetamine (Franklin, 1974; Werringloer and Estabrook, 1973). Interestingly, no lag phase was apparent for complex formation with BBT, αMB or αEB. Based upon previous work (Lindeke *et al.*, 1982) showing that the lag phase of a series of amine analogues of amphetamine for MI complex formation decreases with increasing lipophilicity, it is likely that our failure to observe this phenomenon is a reflection of the high lipophilicity of BBT, αMB and αEB.

The spectral dissociation constants (K_s) for the inhibitors most likely reflect type I binding to CYP isozymes that are subject to both and reversible inhibition and irreversible MB inactivation. On the other hand, the apparent K_m value represents an interaction with the isozyme(s) capable of forming MI complexes with BBT, αMB or αEB. Since this value is expected to be affected by competitive metabolic pathways, as well as the proportion of total CYP capable of forming MI complexes, the discrepancy between the apparent K_s and K_m values is not surprising.

The significant loss of MI complex formed from BBT, αMB or αEB in response to the microsomal washing indicates a low stability of this complex, compared with that

formed by BD. This observation contrasts with the stability of other amine derived MI complexes of SKF 525-A (Murray, 1988) or troleandomycin (Pessayre *et al.*, 1982) formed *in vivo*, subsequent to microsome preparation involving similar washing protocols to those employed in the present study. The persistence of the MI complexes of BBT, αMB or BBT with sodium dithionite reduction prior to washing, coupled with their dissociation by potassium ferricyanide oxidation, indicates the requirement of ferrous heme for maintenance of these complexes. The loss of MI complex in the presence of low concentrations of NADPH supports this conclusion and also suggests that formation of an MI complex is not sufficient to maintain CYP heme in the reduced state. Thus, it appears that in the presence of excess NADPH, an equilibrium state exists in which the complex is continually formed and dissociated. Maintenance of this equilibrium is dependent upon the continued presence of free, metabolically unmodified inhibitor, as well as an adequate concentration of reducing equivalents. Elimination of one or both of these conditions by washing of the incubated microsomes results in a disruption of this equilibrium. Due to the inclusion of similar washing protocols in previous *in vitro* (Mathews and Bend, 1986; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990) and *in vivo* (Knickle *et al.*, 1994; Mathews and Bend, 1993) studies involving these inhibitors, it is highly unlikely that MI complex formation contributed measurably to the observed irreversible inactivation of CYP1A- and CYP2B-dependent monooxygenase activities.

In the unwashed samples, approximately 66% of the control CYP content (1.34 ± 0.08 nmol/mg) was lost (Table 2.4). This most likely represents the combined effects of irreversible MB inactivation and reversible MI complex formation. The samples washed in the absence of sodium dithionite displayed a 45% loss of CYP when compared to the control value. Virtually all of the CYP loss in this case can be accounted for by irreversible mechanisms, due to the almost complete loss of MI complex. Therefore, the difference of CYP loss between the washed and unwashed samples in the absence of sodium dithionite, indicates that approximately 21% of the total CYP in hepatic microsomes from PB-treated

guinea pigs existed as a MI complex prior to washing. This is consistent with the estimate of 20 ± 3% given in Table 2.1.

Inclusion of 1 mM GSH in the incubation mixtures containing BBT, αMB or αEB with hepatic microsomes and NADPH had a profound attenuating effect upon formation of the 455 nm MI complex (Fig. 2.7), suggesting that a reactive, electrophilic intermediate is generated and released from CYP during formation of the 455 nm complex. GSH, a potent nucleophile, has previously proven effective in decreasing the magnitude of MI complex formation *in vitro* with the cyclic orphenadrine analogue nefopam (Leurs *et al.*, 1989). Published data also indicate that the nitrone intermediate produced during metabolism of norbenzphetamine is capable of conjugation with cellular GSH in intact hepatocytes (Hirata *et al.*, 1979). However, subsequent work (Franklin *et al.*, 1980) suggests that while a nitrone intermediate is involved in amphetamine metabolism, it is unlikely that this species is released from its CYP isozyme of origin. Thus it is likely that differences exist in the metabolism and/or product properties/stability of BBT, αMB and αEB and complex forming amphetamines. An important aspect of our results is the suggestion that more than one hepatic CYP isozyme may be involved in generation of the 455 nm MI complex. However, consistent with recent work (Kent *et al.*, 1997a), our data have demonstrated that 1 mM GSH does not have an effect on the rate or maximum extent of irreversible, MB inactivation of hepatic microsomal CYP2B-dependent PROD or CYP1A-dependent EROD activities by BBT or αMB. This shows conclusively that formation of MI complexes with CYP is not required for NADPH-dependent inactivation of monooxygenase activity by these inhibitors.

Previous demonstrations of the increased potency and selectivity of MB inactivation of pulmonary CYP2B monooxygenase activities by αMB or BBT (Mathews and Bend, 1986; 1993; Woodcroft *et al.*, 1990) when compared to hepatic CYP2B provided the rationale for extension of the present study to the lung. Complex formation was not detected in lung microsomes from untreated guinea pigs or rabbits with 200 μM BBT,

αMB or αEB. In contrast, complexes were easily detected when BD was used at the same concentration. Published data indicate that MI complex formation by amphetamines with CYP of rabbit lung does occur (Buening and Franklin, 1976). However, in the same study, complexes of pulmonary CYP were not observed with SKF 525-A or propoxyphene. This demonstrates that BBT, αMB and αEB are more similar to SKF 525-A and propoxyphene than to amphetamines with regard to MI complex formation with pulmonary CYP isozymes. It is apparent that significant differences exist between the hepatic and pulmonary metabolism of the ABT analogues with respect to MI complex formation.

Approximately 60 - 80% of total CYP in lung microsomes from untreated rabbits can be accounted for by two isozymes, CYP2B4 and CYP4B1 (Guengerich, 1990b) and all of the isozymes known to be expressed in rabbit lung are also found in the liver. However many other hepatic CYPs are not expressed at significant levels in lung. Therefore, it is likely that a specific CYP isoform that is present in guinea pig liver, but not in lung, plays a significant role in generating MI complexes from BBT, αMB or αEB. Since CYP2B18 represents approximately 25% of the total CYP of PB-treated guinea pig liver microsomes (Yamada *et al.*, 1992), it is possible that MI complexation occurs in competition with irreversible mechanisms of inactivation, with this isozyme. However, the constitutive expression of this isoform in guinea pig lung, coupled with a lack of MI complex formation with the ABT derivatives in lung microsomes, it is unlikely that complexation involves CYP2B18 to a significant degree. Recently, *N*-aralkylated derivatives of ABT have been shown to form MI complexes at a greater rate with liver microsomes from dexamethasone treated rats, when compared to microsomes from PB treated rats (Pappas and Franklin, 1996). This finding is consistent with the involvement of dexamethasone inducible guinea pig homologue(s) of rat CYP3A isozymes, and a lack of expression of this isozyme in lung. Furthermore, CYP2C6, 2C7 and 3A2 are all inducible by PB-treatment in rat liver (Guengerich *et al.*, 1982) and are not known to be

expressed at a significant level in rat lung (Baron and Voigt, 1990). Therefore it is likely that the PB-inducible CYP isozyme(s) involved in MI complex formation with BBT, αMB or αEB may be the guinea pig hepatic homologues of these rat isoforms. However, it is important to note that MI complexation of purified recombinant rat CYP2B1 with BBT has been reported (Kent *et al.*, 1997a), indicating the capacity of this isozyme to form MI complexes with the ABT derivatives. At present, it is unclear whether the apparent disparity between these data, and the absence of MI complexation with the ABT derivatives in guinea pig or rabbit lung microsomes reported here reflects microheterogeneity between homologous rodent CYP2B isoforms, or complex interactions among multiple isozymes in an intact microsomal system.

A potential metabolic pathway for the *N*-aralkylated ABT analogues is given in Fig. 2.9. An initial *N*-hydroxylation of the parent compound (A) to form a secondary hydroxylamine (B) is known to be essential for the formation of MI complexes from many secondary amines (Lindeke and Paulsen-Sörman, 1988) and is a common reaction in the metabolism of many aromatic amines (Koymans *et al.*, 1993b). A subsequent oxidation yields a nitroxide radical (C) which due to the presence of α-hydrogens, may be further converted to a nitrone (D). Subsequent conversion to benzotriazole and a phenyl-nitroso species (E) is also possible. Indeed, ^{14}C-benzotriazole has been identified as a metabolite of ^{14}C-BBT in guinea pig hepatic microsomes (Woodcroft *et al.*, 1997). Either the nitroxide or nitroso species may be candidates for the MI complex forming ligand. The absence of an appreciable lag phase in the formation of MI complexes from the ABT analogues suggests that the ultimate complex forming ligand is close to the parent compound, thus favouring the nitroxide as the ligand. Additionally, the attenuation of MI complex formation in the presence of 1 mM GSH suggests the production and release of a reactive intermediate, such as a nitroxide or nitrone, capable of conjugation with GSH. This also raises the possibility that a reactive metabolite is formed by one CYP isozyme, is released and forms a complex with a second.

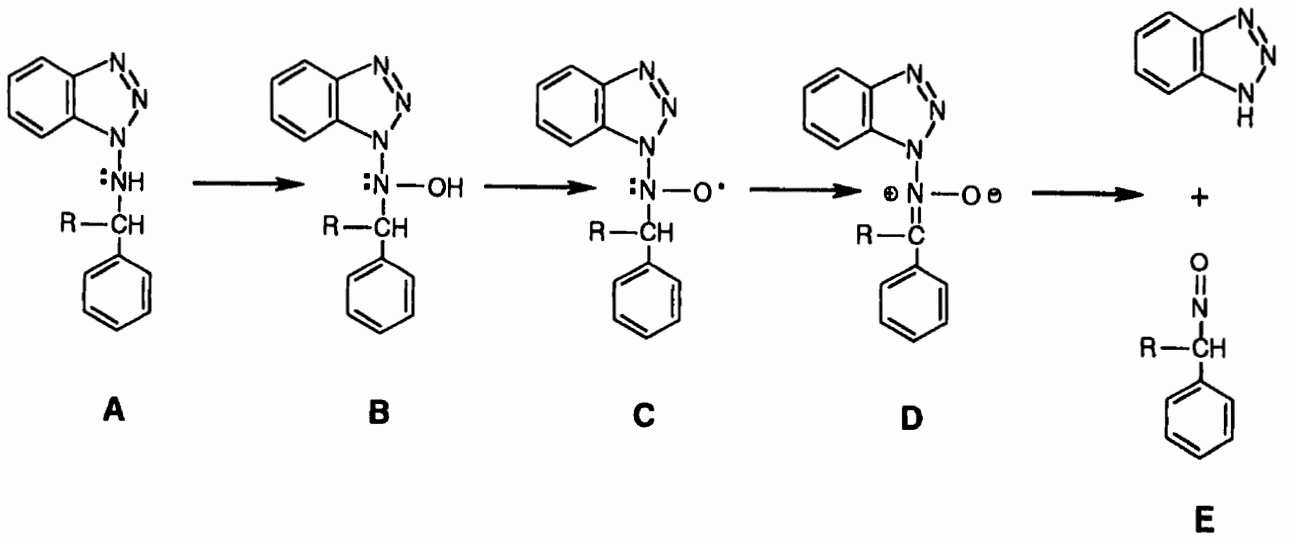


Figure 2.9
Proposed metabolism of *N*-aralkylated derivatives of ABT leading to MI complex formation with CYP. (R = H, BBT; R = CH_3, αMB; R = C_2CH_5, αEB)

A nitroxide as the MI complexing ligand, potassium ferricyanide dissociation of the complex, a 455 nm maximal MI complex absorbance and increased MI complex formation in PB-treated animals are all features associated with amphetamines (Franklin, 1974; Hirata *et al.*, 1979; Lindeke *et al.*, 1979). Additionally, Lindeke *et al.* (1979) have demonstrated that double bond formation in secondary nitrones from amphetamines occurs preferentially with a less substituted carbon. This may in part explain the increased complex formation of BBT relative to αMB. These investigators have also described an approximate 8-fold decrease in the K_m for MI complex formation in progressing from a methyl to ethyl α-carbon substituent with a series of substituted phenylethylamines (Lindeke *et al.*, 1982). Bast *et al.* (1990) have reported greater than 5-fold increases in the MI complexation of CYP in progressing from a methyl to ethyl alkyl substituent in a series of diphenhydramine derivatives. In both cases, the improvements in MI complex formation were found to correlate well with the octanol/buffer partition properties of the compound. The decrease in MI complex formation with αMB relative to BBT, and the increase with αEB relative to αMB are therefore not surprising.

The absence of MI complex formation in the lung by both the ABT analogues and SKF 525-A also suggests some similarities in metabolism. For example, it is likely that both classes of compounds are metabolized by a CYP isoform(s) that is present in liver but not lung. However, while PB-induction results in a faster rate of SKF 525-A MI complex formation relative to untreated rat liver, this treatment has no effect on the maximum extent of complex formation (Buening and Franklin, 1974), which is regarded as a more accurate indicator of changes in isozyme subpopulations (Franklin, 1982). Our work has demonstrated an increase in rate and extent of complex formation with the ABT analogues in PB-treated *vs.* untreated guinea pig liver. Thus, while comparisons with other MI complex forming species are valuable, they only remain so when differences are taken into account.

In conclusion, BBT, αMB, and αEB are potent and isozyme-selective MB inactivators of guinea pig hepatic and pulmonary CYP2B18. MI complexation is not likely to contribute to the MB inactivation of guinea pig hepatic CYP2B18, the homologue of rabbit CYP2B4, due to the irreversible destruction of this isoform at very low inhibitor concentrations, the lack of GSH attenuation of this destruction, the instability of formed complexes and the absence of MI complex formation with guinea pig pulmonary CYP.

Chapter 3

Kinetics and Selectivity of Mechanism-Based Inactivation of Guinea Pig Hepatic and Pulmonary Cytochrome P450 by *N*-Benzyl-1-Aminobenzotriazole and *N*-α-Methylbenzyl-1-Aminobenzotriazole[2]

3.1 Objectives

Two *N*-aralkyl derivatives of ABT, BBT and αMB, are potent MB inactivators of microsomal CYP that were synthesized in our laboratory (Mathews and Bend, 1986). Previous studies have demonstrated that these compounds are isozyme (CYP2B) and tissue (lung) selective inhibitors of rabbit and guinea pig CYP both *in vitro* and *in vivo* (Grimm *et al.*, 1995; Knickle *et al.*, 1994; Knickle *et al.*, 1993; Mathews and Bend, 1986; 1993; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990). BBT and αMB are known to exhibit multiple inhibition mechanisms including heme alkylation (Mathews and Bend, 1986) and covalent binding to CYP protein (Woodcroft *et al.*, 1997), which appears to be quantitatively the most important. Published data (Sinal and Bend, 1995) and the experiments presented in Chapter 2 have also identified MI complexation as a further mechanism by which these compounds interact with CYP. The objective of this study was to test the hypothesis that the isozyme- and tissue-selectivity of these inhibitors is due, at

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2 A version of this chapter has been published.

Sinal CJ and Bend JR (1996) Kinetics and selectivity of mechanism-based inhibition of guinea pig hepatic and pulmonary cytochrome P450 by *N*-benzyl-1-aminobenzotriazole and *N*-α-methylbenzyl-1-aminobenzotriazole. *Drug Metab. Dispos.* **24**: 996-1001.

least in part, to the more rapid inactivation of CYP2B *vs.* CYP1A isoforms. To this end, mechanistic information pertaining to the basis of the tissue and isozyme selectivity of the ABT derivatives was obtained through an *in vitro* examination of their kinetics of CYP inactivation in guinea pig hepatic and pulmonary microsomes. An important aspect of this work involved dissociation of the MB and non-MB effects of these inhibitors on individual CYP1A- and 2B-dependent catalytic activities.

3.2 Materials and Methods

3.2.1 Materials

BBT and αMB were synthesized and purified as previously described (Mathews and Bend, 1986; Woodcroft *et al.*, 1990). NADPH was purchased from Sigma Chemical Co., St. Louis, Mo.; 7-ethoxyresorufin, 7-pentoxyresorufin, 7-methoxyresorufin and resorufin from Molecular Probes Inc., Eugene, OR.; and PB, Me_2SO and all other chemicals (reagent grade or better) from BDH, Toronto, Canada.

3.2.2 Animal Treatment and Preparation of Microsomes

Male Hartley guinea pigs (250-300g) were treated intraperitoneally with 80 mg/kg PB (2% in saline) for 4 days and sacrificed 24 hr following the last injection by asphyxiation with CO_2. All animals were allowed free access to food (Purina guinea pig chow) and water throughout the treatment period. Hepatic and pulmonary microsomes were prepared by differential centrifugation as previously described (Bend *et al.*, 1972). Microsomal protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Microsomes were stored at -80°C until use.

3.2.3 Preparation of Inhibitor Solutions

BBT and αMB were initially made as stock solutions (0.3 M) in methanol. The concentrations of these solutions were verified by UV-absorbance spectrophotometry (λ = 280 nm) against standard curves. Volumes of 100 μL were aliquoted into 1 mL volumetric flasks and the methanol was evaporated under a gentle stream of N_2. The remaining residue was made up to 1 mL with Me_2SO to give a final concentration of 0.03 M. Serial dilutions of this stock solution were used to obtain all of the inhibitor concentrations used.

3.2.4 Dynamic Inhibition Assays

Incubation mixtures containing hepatic or pulmonary protein (0.3 mg) and 5 μL of the appropriate inhibitor dilution, were made up to a volume of 1.5 mL with 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, in a fluorimeter cuvette maintained at 37°C. The reactions were started by the addition of NADPH (final concentration = 1 mM) and were allowed to preincubate, with stirring, for periods of 15 sec to 45 min. At the end of the pre-incubation time, either 7-pentoxyresorufin or 7-ethoxyresorufin was added to a final concentration of 5 μM. An additional 25 μL of 6 mM NADPH in 0.1 M buffer, was also added to the cuvette at this time. The rates of PROD and EROD activity were determined based on the methods of Burke and Mayer (1974) and Lubet *et al.* (1985), respectively. All measurements were carried out at an excitation wavelength of 525 nm and an emission wavelength of 585 nm on a Perkin-Elmer fluorescence spectrophotometer (model LS-5B). The reaction was allowed to proceed at 37°C and the rate of formation of resorufin was calculated by comparison to known amounts (5 pmol) added in 5 μL Me_2SO to the reaction mixture. All assays were verified to proceed linearly with respect to time and protein concentration. The apparent half-life for inactivation ($t_{1/2}$) was calculated by curve-peeling of the data points for pre-incubation times of 15 sec to 2 min with respect to those obtained for 10 - 45 min. Linear regression

of the natural logarithm of the residual values with respect to time was used to obtain an equation from which $t_{1/2}$ was calculated.

3.2.5 Static Inhibition Assays

Incubation mixtures contained 0.2 mg/mL of hepatic microsomal protein and 10 μM of inhibitor in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. For the amount of inhibition at 0 time (initial), 1.5 mL aliquots were removed just prior to the addition of NADPH and were assayed for PROD and EROD activity as described above. MROD activity was also measured in the same manner at a final substrate concentration of 5 μM. Reactions were started by the addition of NADPH (1 mM final) and were maintained at 37°C in a shaking water bath. Control incubations contained an equal volume of Me_2SO in place of the inhibitor solution. After 45 min, the incubations were split into one half which was assayed for PROD, EROD or MROD activity directly, and a second half that was subjected to a washing protocol prior to the enzyme assays. Washing of the microsomes to remove excess inhibitor was accomplished by sedimentation of the inhibition mixtures at 100 000 x g for 60 min at 4°C (Beckman XL-90 ultracentrifuge; 50Ti rotor), and resuspension of the microsomal pellet in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. Subsequently, the microsomes were centrifuged at 412 160 x g for 15 min at 4°C (Beckman TL-100 ultracentrifuge; TLA 100.3 rotor) followed by resuspension in the same buffer. After determination of protein concentration, these washed microsomes were re-assayed for PROD, EROD and MROD activities as described above.

3.2.6 Statistical Analysis

Data were analyzed by one-way ANOVA, followed by the Tukey-Kramer multiple comparisons test using SuperANOVA v1.11 for Macintosh software (Abacus Concepts, Berkeley, CA).

3.3 Results

3.3.1 Inhibition of Pulmonary PROD Activity

In these studies, PROD (Lubet *et al.*, 1985) was used as a marker for CYP2B18, the guinea pig orthologue of CYP2B1 (Nelson *et al.*, 1996), the isozyme primarily responsible for this catalytic activity in liver microsomes prepared from PB-treated rats. To account for the possibility that other closely related isoforms (*i.e.* CYP2B2 in rat) may also contribute to PROD activity, this activity is henceforth considered as CYP2B-dependent. Both BBT and αMB produced a time- and NADPH-dependent loss of pulmonary PROD activity in microsomes from PB-treated guinea pigs (Fig. 3.1). The reaction appeared to be first-order and monophasic under the conditions used in this experiment. An apparent half-life for inactivation ($t_{1/2}$) was determined from the initial rate of inactivation by BBT or αMB under identical reaction conditions. These values are "apparent" in the sense that they may not reflect the actual time for 50% loss of the initial activity, but rather, were derived as a useful means for comparing the rates of inactivation of BBT and αMB at a particular concentration. The initial rate of inactivation was very sensitive to small changes in concentration. For example, a >50-fold decrease in the magnitude of the calculated $t_{1/2}$ for BBT in progressing from 0.025 to 0.1 μM was observed (Table 3.1). Similarly, the rate of PROD inactivation by αMB was also observed to decrease in a concentration-dependent manner but, the increase of $t_{1/2}$ was not as marked as for BBT. At equimolar concentrations (0.025 μM) the rate of PROD inactivation was clearly much faster for αMB when compared with BBT as indicated by the approximately 35-fold shorter apparent half-life for inactivation (0.9 *vs.* 32.2 min). No comparison between inactivation by αMB and BBT was possible at 0.1 μM as the inactivation by the former was too rapid to obtain reliable measurements using our methods.

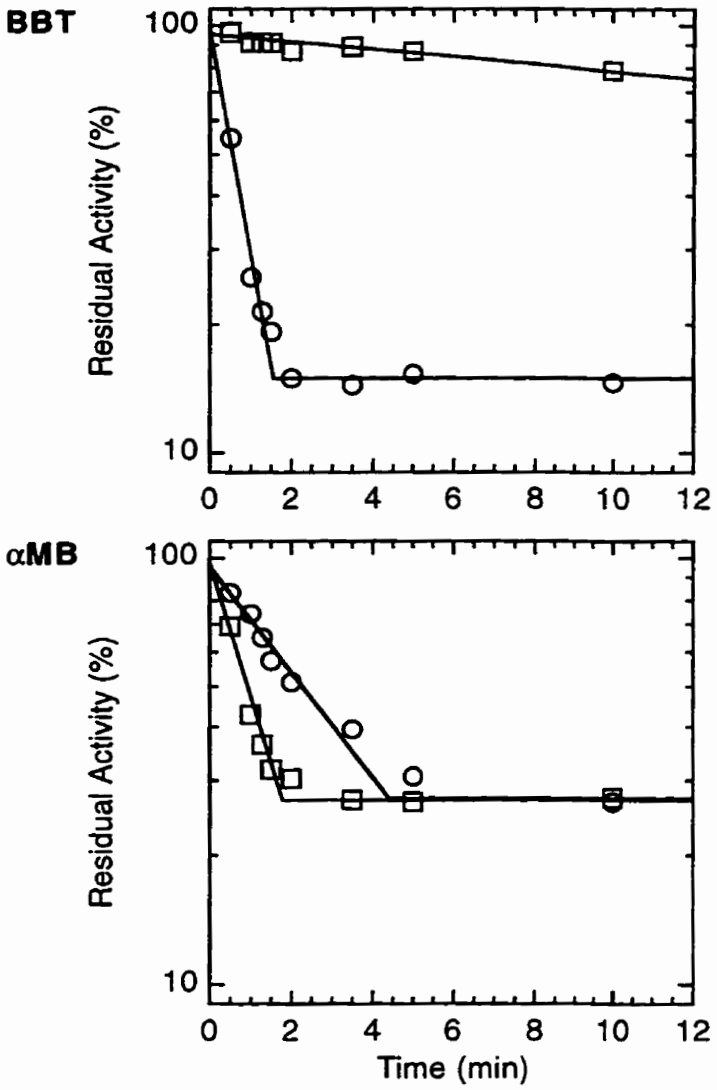

Figure 3.1
Time course of NADPH-dependent inactivation of PROD activity in pulmonary microsomes from PB-treated guinea pigs by BBT and αMB. Values represent the mean of the data obtained for duplicate experiments using microsomes from 4 individual animals. The control PROD value in the absence of any inhibitor was 21.3 ± 1.0 pmol/min/mg protein. The 100% residual activity values measured in the presence of the inhibitor, but without pre-incubation (t = 0 min) were 7.3 ± 0.1, and 14.3 ± 0.2 pmol/min/mg protein for 0.1 (○) and 0.025 μM (□) BBT, respectively. The 100% residual values for 0.025 (□) and 0.010 μM αMB (○) were 3.4 ± 0.2 and 7.1 ± 1.0 pmol/min/mg protein, respectively. The CYP concentration of the microsomes was 0.18 ± 0.03 nmol/mg protein (36 nM final in incubation). For purposes of clarity, some data points and the standard error bars are not shown. All data points were used for determination of the apparent half-life for inactivation ($t_{1/2}$) and the standard error did not exceed 12% for any data point.

Table 3.1 - Apparent half-life for inactivation and initial inhibition of pulmonary and hepatic microsomal PROD or EROD activity by BBT and αMB. Data represent the mean ± SE of duplicate experiments using microsomes from 4 individual animals.

CYP Activity	Inhibitor	Concentration (μM)	Half-life for Inactivation (min)	% Inhibition at Zero Time[a]
Lung PROD				
	BBT	0.1	0.6 ± 0.1	66
		0.025	32.2 ± 3.8	33
	αMB	0.025	0.9 ± 0.1	84
		0.01	2.6 ± 0.4	67
Liver PROD				
	BBT	10.0	5.0 ± 0.7	93
		1.0	2.1 ± 0.3	89
		0.1	154.8 ± 39.7	55
	αMB	10.0	4.5 ± 0.5	94
		1.0	5.2 ± 0.5	90
		0.1	13.2 ± 3.2	77
Liver EROD				
	BBT	1.0	3.1 ± 0.2	19
		0.1	8.1 ± 0.8	5
	αMB	1.0	6.2 ± 0.1	12
		0.1	11.0 ± 1.5	11

[a] Measured in the presence of inhibitor, but in the absence of incubation with NADPH. Control values in the absence of any inhibitor were: 21.3 ± 1.0 (lung PROD), 108.9 ± 4.7 (liver PROD) and 132.3 ± 5.8 pmol/min/mg protein (liver EROD).

3.3.2 Inhibition of Hepatic PROD Activity

Incubation of hepatic microsomes from PB-treated guinea pigs with BBT or αMB caused a time- and NADPH-dependent loss of CYP2B-dependent PROD activity (Fig. 3.2). In contrast to the pulmonary data, the inactivation in this case appeared to be biphasic with an initial fast phase and a secondary slower phase. However, with 0.1 μM BBT, the slow phase was apparently abolished. For the purposes of this study we chose to determine $t_{1/2}$ values for the initial rapid phase only. In general, the apparent half-life for inactivation decreased in a concentration-dependent manner (Table 3.1) with the magnitude of $t_{1/2}$ greater for BBT than αMB at all concentrations, with the exception of 1 μM. Decreasing the BBT concentration from 10 to 1 μM resulted in a substantial, and reproducible increase in the rate of inactivation as well a decrease in the magnitude of $t_{1/2}$. However, a similar effect was not observed for αMB at the concentrations used in this study.

Compared with the data obtained for inactivation of hepatic PROD activity, BBT and αMB were clearly more effective inhibitors of lung CYP in terms of potency and rate of inactivation. For example, at equimolar concentrations (0.1 μM), BBT exhibited an approximately 200-fold longer $t_{1/2}$ for liver compared with lung PROD inactivation. Similarly, the apparent half-life for inactivation of liver PROD was consistently longer at all concentrations studied when compared with the lowest concentration (0.01 μM) studied for αMB in lung microsomes.

3.3.3 Inhibition of Hepatic EROD Activity

In these experiments, EROD activity was used as a marker of CYP1A-dependent metabolism in liver microsomes from PB-treated guinea pigs (Burke *et al.*, 1985). Both BBT and αMB also caused a time- and NADPH-dependent loss of CYP1A-dependent EROD activity in hepatic microsomes from PB-treated guinea pigs (Fig. 3.3). Similar to the inactivation of liver PROD activity, the inactivation in this case also appeared to be

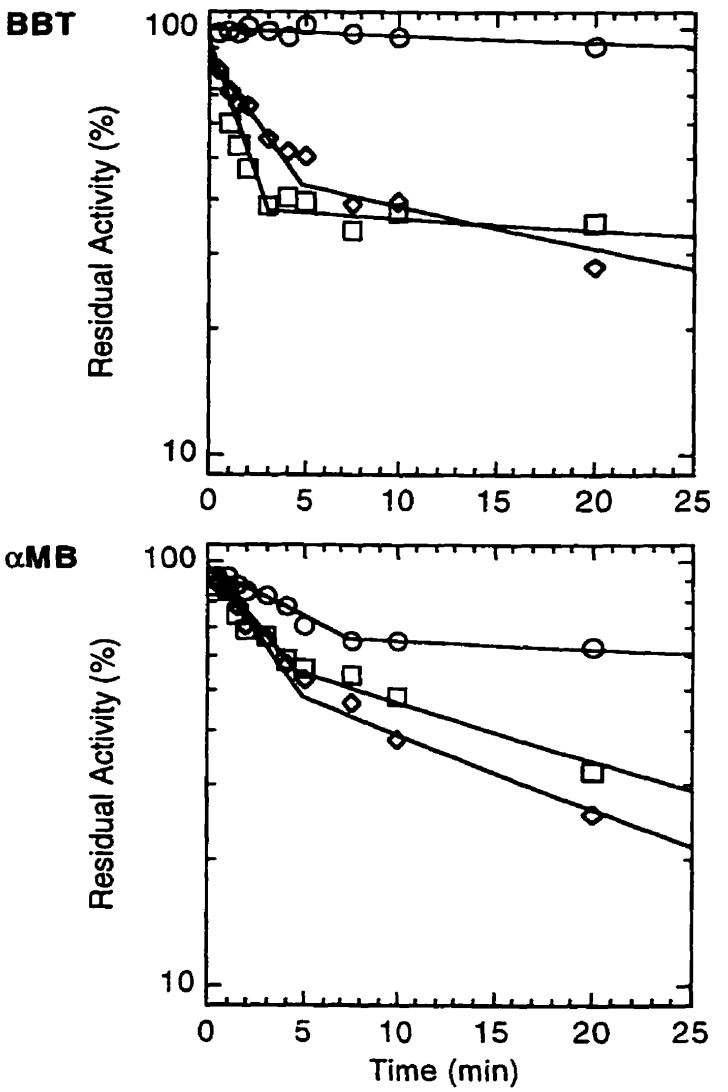

Figure 3.2
Time course of NADPH-dependent inactivation of PROD activity in hepatic microsomes from PB-treated guinea pigs by BBT and αMB. Values represent the mean of the data obtained for duplicate experiments using microsomes from 4 individual animals. The control PROD value in the absence of any inhibitor was 108.9 ± 4.7 pmol/min/mg protein. The 100% residual activity values measured in the presence of the inhibitor, but without pre-incubation (t = 0 min) were 7.1 ± 2.8, 11.9 ± 1.0 and 48.7 ± 4.6 pmol/min/mg protein for 10.0 (◇), 1.0 (□) and 0.1 μM (○) BBT , respectively. The 100% residual values for 10.0, 1.0, and 0.1 μM αMB were 6.3 ± 0.4, 10.6 ± 1.8 and 24.6 ± 5.0 pmol/min/mg protein, respectively. The CYP concentration of the microsomes was 1.20 ± 0.09 nmol/mg protein (240 nM final in incubation). For purposes of clarity, some data points and the standard error bars are not shown. All data points were used for determination of the apparent half-life for inactivation ($t_{1/2}$) and the standard error did not exceed 12% for any data point.

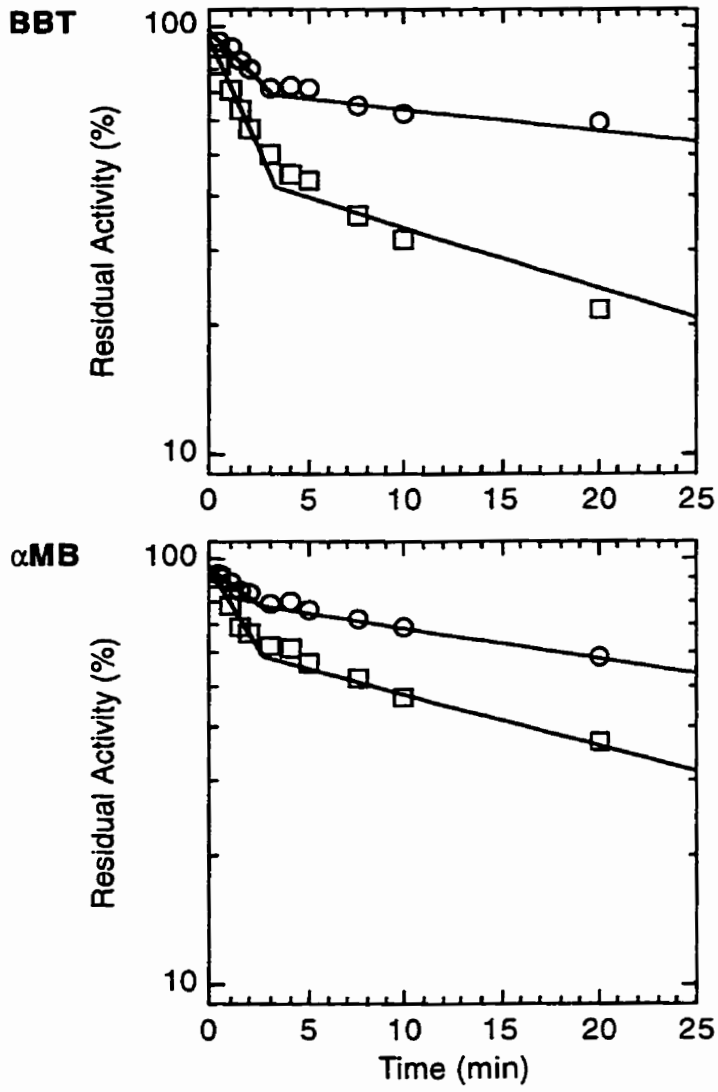

Figure 3.3
Time course of NADPH-dependent inactivation of EROD activity in hepatic microsomes from PB-treated guinea pigs by BBT and αMB. Values represent the mean of the data obtained for duplicate experiments using microsomes from 4 individual animals. The control EROD value in the absence of any inhibitor was 132.3 ± 5.8 pmol/min/mg protein. The 100% residual activity values measured in the presence of the inhibitor, but without pre-incubation (t = 0 min) were 107.3 ± 8.7, and 126.2 ± 2.8 pmol/min/mg protein for 1.0 (□) and 0.1 (○) μM BBT, respectively. The 100% residual values for 1.0 and 0.1 μM αMB were 116.9 ± 16.5 and 118.4 ± 9.2 pmol/min/mg protein, respectively. The CYP concentration of the microsomes was 1.20 ± 0.09 nmol/mg protein (240 nM final in incubation). For purposes of clarity, some data points and the standard error bars are not shown. All data points were used for determination of the apparent half-life for inactivation ($t_{1/2}$) and the standard error did not exceed 12% for any data point.

biphasic with an initial fast phase and a secondary slower phase. The rate of inactivation changed in a concentration-dependent manner and the apparent half-life for inactivation at equimolar concentrations, was generally shorter for BBT than αMB (Table 3.1). Furthermore, at the lowest BBT concentration studied (0.1 μM) the calculated $t_{1/2}$ was generally shorter for EROD inactivation compared with PROD inactivation. In contrast, the opposite was true with 1 μM BBT. The same comparisons for αMB show that only small differences exist for the calculated $t_{1/2}$ values for PROD and EROD inactivation at equimolar concentrations.

3.3.4 Dissociation of NADPH-Dependent and -Independent Inhibition

In order to determine the contribution of NADPH-independent inhibition to the actions of BBT and αMB, the initial level of CYP inhibition, in the absence of pre-incubation with NADPH, was measured. BBT and αMB both inhibited pulmonary and hepatic CYP catalytic activity in a concentration-dependent manner in the absence of incubation with NADPH (Table 3.1). Consistent with the inactivation rates, non-catalytic inhibition was more potent for pulmonary PROD activity. In contrast, NADPH-independent inhibition of hepatic PROD activity by both inhibitors was substantially more potent and complete than for EROD activity. To further investigate this relationship, liver microsomes that were incubated with NADPH and BBT or αMB for 45 min were assayed for enzymatic activity before and after washing, by repeated sedimentation and resuspension, to remove any inhibitor not irreversibly bound to CYP. Both inhibitors produced approximately 90% initial NADPH-independent inhibition of CYP2B-dependent PROD activity at a concentration of 10 μM (Table 3.2). In contrast, the initial level of inhibition of CYP1A-dependent EROD activity was much lower at 41% and 34%, respectively. Because EROD activity is generally attributed to CYP1A1 and CYP1A2 isozymes in liver (Burke *et al.*, 1985; Weaver *et al.*, 1994), we also examined the effect of the inhibitors on MROD activity, a selective marker for CYP1A2 (Nerurkar *et al.*, 1993).

Table 3.2 - Inhibition of guinea pig hepatic CYP-dependent PROD, EROD and MROD activities by 10 μM BBT or αMB after incubation times of 0 or 45 min. Samples incubated for 45 min were assayed directly or following washing by sedimentation and resuspension. Control samples were incubated with Me_2SO. Data represent the mean ± SE of duplicate experiments using microsomes from 4 individual animals.

	Initial (t = 0 min)		Unwashed (t = 45 min)		Washed (t = 45 min)	
Sample	Activity[a]	% Inhibition	Activity[a]	% Inhibition	Activity[a]	% Inhibition
PROD						
Control	96.1 ± 4.3		88.2 ± 3.1		86.9 ± 4.4	
BBT	10.0 ± 1.1[b]	90	2.2 ± 0.5[b]	98	19.4 ± 1.0[b,c]	78
αMB	8.9 ± 1.1[b]	91	2.2 ± 0.6[b]	98	6.0 ± 0.4[b]	93
EROD						
Control	136.2 ± 4.6		129.9 ± 2.4		123.5 ± 4.0	
BBT	80.4 ± 6.0[b]	41	4.7 ± 0.2[b,c]	96	24.4 ± 1.0[b,c]	80
αMB	89.6 ± 4.2[b]	34	12.6 ±0.6[b]	90	52.5 ± 1.7[b]	57
MROD						
Control	74.1 ± 3.6		72.8 ± 3.7		66.8 ± 1.6	
BBT	63.0 ± 3.5	15	2.2 ± 0.6[b]	97	57.1 ± 2.5[d]	15
αMB	67.9 ± 2.6	8	1.8 ± 0.4[b]	98	64.6 ± 1.9	3

[a] pmol/min/mg microsomal protein

[b] significantly different from control, $p<0.01$

[c] significantly different from αMB, $p<0.05$

[d] significantly different from control, $p<0.05$

The lowest level of initial NADPH-independent inhibition was recorded for this activity at 15% and 8%, respectively. After a 45 min incubation time with NADPH and either BBT or αMB, all CYP-dependent catalytic activities monitored were uniformly inhibited to >90%. However, after washing to remove excess inhibitor, only those microsomes incubated with αMB and NADPH retained >90% inhibition of PROD activity. In contrast, inhibition of CYP1A-dependent EROD activity by αMB was reduced to 57%, while inhibition of CYP1A2-dependent MROD activity was almost entirely reversed to 3% of control. On the other hand, washing microsomes that had been incubated with NADPH and 10 μM BBT for 45 min resulted in less inhibition of PROD activity (78%) than was observed for αMB (93%). However, BBT-mediated inhibition of EROD and MROD activity was greater than for αMB at 80% and 15%, respectively.

3.4 Discussion

One hallmark characteristic of MB inactivation of CYP is a time-and NADPH-dependent decrease in enzyme activity. As shown in Figs. 3.1-3.3, both BBT and αMB inhibited pulmonary and hepatic CYP catalytic activity in a time-, NADPH- and concentration-dependent manner. The biphasic inactivation of hepatic CYP2B-dependent PROD and CYP1A-dependent EROD activity by both inhibitors may be indicative of inactivation of more than one isozyme that is capable of supporting the measured catalytic activity. Abolition of the slow phase with decreasing inhibitor concentration and the absence of a slow phase for lung microsomal PROD inactivation is also consistent with inactivation of more than one isozyme in liver microsomes. However, studies of the inactivation of recombinant or purified rabbit CYP2B4-dependent 7-ethoxycoumarin *O*-deethylation activity have also revealed distinct fast and slow inactivation rates (Grimm *et al.*, 1995; Osawa and Coon, 1989). Thus, biphasic inactivation kinetics can occur with a single CYP isozyme, possibly as a result of multiple inhibition mechanisms.

An interesting aspect of this study was the extremely rapid and potent NADPH-dependent inactivation of pulmonary microsomal, compared with hepatic, PROD activity. Additionally, a marked concentration-dependence of the lung PROD inactivation rates for BBT and αMB was observed with a >50-fold increase of the $t_{1/2}$ value for BBT in response to a 4-fold concentration decrease (Table 3.1). The very rapid inactivation of pulmonary CYP by BBT is consistent with the well characterized tissue selectivity of these compounds *in vivo* (Knickle *et al.*, 1994). However, the basis of this very rapid and potent inactivation of pulmonary compared with hepatic PROD activity is not apparent from our data. It is possible that the lower CYP concentration of lung (0.18 ± 0.03 nmol/mg protein) when compared with liver microsomes (1.20 ± 0.09 nmol/mg protein), resulted in a greater effective inhibitor concentration. Alternatively, the presence of fewer distinct CYP isoforms in lung compared with liver may reduce the capacity of pulmonary microsomes to convert BBT and αMB to non-inhibitor metabolites.

It is clear from the $t_{1/2}$ values that αMB is a much more potent and rapid inhibitor of pulmonary microsomal CYP activity than BBT. This is also the case for inactivation of hepatic PROD activity at low concentrations. For example, at a concentration of 0.1 μM, the apparent half-life for inactivation for BBT was more than 10-fold greater than for equimolar αMB. However, at a concentration of 10 μM, there was no marked difference between the inactivation rates for either inhibitor. In general, the rates for inactivation of guinea pig hepatic PROD activity determined here were consistent with those previously measured for inactivation of rabbit hepatic CYP2B4 and CYP2B5 benzyloxyresorufin *O*-debenzylation and androstenedione 15α-hydroxylation activities (Grimm *et al.*, 1995), respectively, and for rat CYP2B1 7-ethoxy-4-trifluoromethyl coumarin *O*-deethylation activity (Kent *et al.*, 1997a), by similar concentrations of BBT or αMB . An unexpected result however, was the dramatic and reproducible increase in the inactivation rate for hepatic PROD activity by BBT at a concentration of 1 μM. The reason for this result is unclear, however it does suggest the existence of an optimal concentration for inactivation

of guinea pig hepatic CYP2B isozymes by BBT. A similar effect was not observed for αMB indicating that either the optimal concentration for αMB was not achieved in these experiments, or that a similar characteristic is not exhibited by this inhibitor.

BBT and αMB were also found to be effective NADPH-dependent inhibitors of hepatic CYP1A-dependent EROD activity. At equimolar concentrations, BBT was in general, a more rapid inactivator of this activity when compared with αMB. While inactivation of hepatic PROD and EROD activities by 1 or 0.1 μM αMB proceeded with similar rates, the $t_{1/2}$ for inactivation by 0.1 μM BBT was 20-fold longer for PROD compared with EROD activity. However, this situation was reversed for 1 μM BBT Again, the reason for the relatively high PROD inactivation rate for 1 μM BBT is not clear from our data. Thus, the characteristic selectivity for inhibition of CYP2B isoforms by BBT and αMB is not adequately explained by the inactivation rates. However, the relatively greater potency and selectivity of αMB *vs.* BBT for PROD inactivation is reflected in the shorter $t_{1/2}$ values at low concentrations with liver (0.1 μM) and lung (0.025 μM) microsomes. Furthermore, the selectivity and potency of both compounds for inactivation of pulmonary *vs.* hepatic CYP2B isozymes is clearly consistent with the very rapid rate of inactivation by BBT or αMB.

The substantial degree of PROD, and to a lesser extent EROD inhibition, by BBT or αMB in the absence of pre-incubation with NADPH is consistent with a requirement for high affinity binding of the parent compound prior to metabolic transformation to the active inhibitor. As shown in Table 3.2, 10 μM BBT and αMB inhibited hepatic CYP2B-dependent PROD activity (>90%) to a much greater extent than for CYP1A-dependent EROD activity (30-40%) or hepatic CYP1A2-dependent MROD activity (8-15%) in the absence of pre-incubation with NADPH. These data indicate that both inhibitors bind with high affinity to guinea pig hepatic CYP2B isoforms, and to a lesser extent with CYP1A isozymes. Since MROD activity is generally attributed to hepatic CYP1A2 catalytic activity, it is likely that BBT and αMB do not bind effectively to this isoform at a

concentration of 10 μM. Additionally, since EROD activity is generally attributed to the collective actions of hepatic CYP1A1 and CYP1A2 isoforms, it is likely that the initial inhibition of this activity primarily reflects binding of the inhibitors to CYP1A1.

The degree of inhibition of CYP-dependent catalytic activity after incubation with NADPH and BBT or αMB for 45 min reflects both NADPH-dependent and NADPH-independent inhibition. The NADPH-dependent component increases over time and represents irreversible, MB inactivation, as well as metabolic transformation of the parent compound to metabolites capable of producing reversible, non-MB inhibition. The NADPH-independent component reflects reversible non-MB inhibition by the parent compound, the magnitude of which should not increase over time in the presence or absence of NADPH. Thus, the increase in magnitude of inhibition to >90% for guinea pig hepatic PROD, EROD and MROD activities after a 45 min incubation with BBT or αMB in the presence of NADPH reflects both reversible inhibition and irreversible MB inactivation. After washing the microsomes to remove the reversible NADPH-independent inhibition, the amount of irreversible, MB inactivation remaining was greatest for PROD activity and less for EROD and MROD activities. In contrast, the amount of irreversible inactivation of PROD activity by BBT was less than for αMB, while inhibition of EROD and MROD activities was greater. Furthermore, BBT produced a greater magnitude of initial, NADPH-independent inhibition of EROD and MROD activity when compared with equimolar αMB. It is probable that this is largely a result of a higher degree of binding and MB inactivation of CYP1A2-dependent MROD activity by BBT that contributes to the observed inhibition of CYP1A-dependent EROD activity. In contrast, it appears that αMB is not as efficiently bound by CYP1A2 or transformed to an irreversible inhibitor. This may partially account for the increased potency and selectivity of αMB compared with BBT for MB inactivation of CYP2B isozymes demonstrated in this and other studies (Knickle *et al.*, 1994; Mathews and Bend, 1986; 1993; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990).

The data presented here indicate that BBT and αMB inactivate CYP2B and CYP1A1 isozymes in hepatic microsomes from PB-induced guinea pigs by initially binding with high affinity followed by a rapid, NADPH-dependent conversion to a metabolite(s) capable of irreversible inactivation. In contrast, binding of BBT and αMB to CYP1A2 is of relatively low affinity. However, NADPH-dependent conversion of the parent compound most likely results in production of a metabolite(s) that is capable of reversible inhibition of CYP1A2. Thus, the rate constants determined for PROD inactivation primarily reflect MB inactivation of CYP2B isozymes, while the rate constants for EROD inactivation reflect MB inactivation and NADPH-dependent reversible inhibition of CYP1A isozymes. The tissue selectivity of BBT and αMB for pulmonary *vs.* hepatic CYP2B isozymes is consistent with the very potent and rapid PROD inactivation in guinea pig lung compared with liver microsomes observed in this study. Finally, the relative selectivity of αMB compared with BBT for PROD inactivation *vs.* EROD inactivation is not explained by differences of inactivation rates for CYP2B *vs.* CYP1A isozymes. Instead, it is more likely that the relatively lower selectivity of BBT results from its availability for binding and metabolic oxidation by other CYP isoforms such as CYP1A2.

Chapter 4

Enantioselective Mechanism-Based Inactivation of Guinea Pig Hepatic Cytochrome P450 by *N*-α–Methylbenzyl-1-Aminobenzotriazole[3]

4.1 Objectives

N-aralkylated derivatives of ABT can inhibit CYP by at least three mechanisms: covalent modification of the prosthetic heme group (Mathews and Bend, 1986), covalent modification of the apoprotein moiety (Woodcroft *et al.*, 1997) and MI complexation (Chapter 2 and Sinal and Bend, 1995). Of these, protein modification appears to be the most important for CYP2B inactivation (Kent *et al.*, 1997a; Woodcroft *et al.*, 1997). These studies and others (Sinal and Bend, 1995; 1996), in addition to the experiments presented in Chapters 2 and 3 of this thesis, have also shown that the identity of the alkyl substituent present on the α-carbon is an important determinant of the inhibition mechanism, isozyme/tissue-selectivity and inactivation kinetics exhibited by ABT derivatives. The goal of this study was to test the hypothesis that the stereochemistry of the α-carbon substituent is also an important determinant of the isozyme-selectivity and inactivation kinetics exhibited by these compounds. To this end, individual enantiomers of

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3 A version of this chapter has been accepted for publication.

Sinal CJ, Webb CD, Hirst M and Bend JR (1998) Enantioselective mechanism-based inactivation of guinea pig hepatic cytochrome P450 by *N*-α-methylbenzyl-1-aminobenzotriazole. *Drug Metab. Dispos. (in press)*.

αMB (Fig. 4.1) were prepared and compared with respect to the *in vitro* kinetics of inactivation of CYP2B and CYP1A isoforms in guinea pig hepatic microsomes.

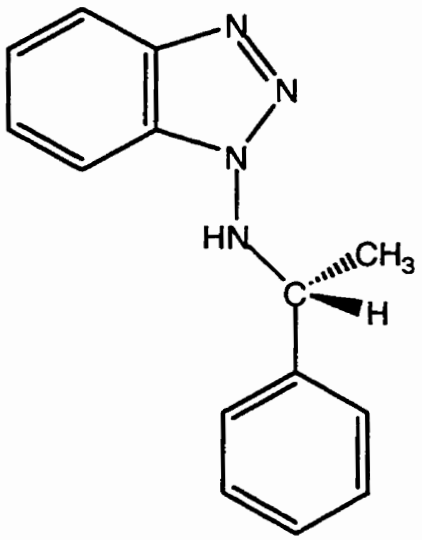
4.2 Materials and Methods

4.2.1 Materials

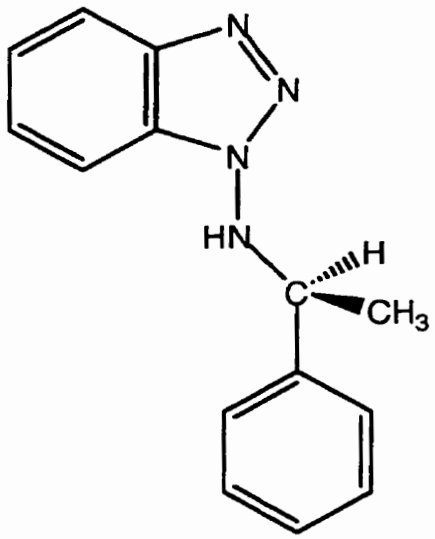
NADPH was purchased from Sigma Chemical Co., St. Louis, MO.; 7-ethoxyresorufin, 7-pentoxyresorufin, and resorufin from Molecular Probes Inc., Eugene, OR.; di-*p*-toluoyl-D-tartaric acid monoaldehyde and di-*p*-toluoyl-L-tartaric acid monoaldehyde from Aldrich Chemical Co, Milwaukee, WI; and PB, Me_2SO and all other chemicals (reagent grade or better) from BDH, Toronto, Canada.

4.2.2 Preparation and Characterization of αMB Enantiomers

Diastereoisomeric di-*p*-toluoyl-tartrate salts of αMB were prepared from racemic αMB in the following manner. αMB (250 mg; 0.66 mM) was mixed with di-*p*-toluoyl-D-tartaric acid monohydrate (305 mg; 0.75 mM) and the mixture dissolved in a small volume (2 mL) of diethyl ether. Following dissolution the ether was removed by evaporation and the residue dissolved with warming in a minimal volume of redistilled ethyl acetate. The solution (1.0 mL) was maintained in the cold at 4°C. Rosette crystals formed slowly. Three recrystallizations from warm ethyl acetate (0.6 - 0.8 mL) were conducted, yielding the D-tartrate salt (55 mg). Basification and extraction of the combined mother liquors yielded an αMB residue (187.7 mg; 0.50 mM) which was mixed with di-*p*-toluoyl-L-tartaric acid (201 mg; 0.52 mM). This mixture was dissolved, as before, in diethyl ether (2 mL). The solvent was then removed by evaporation. The residue was dissolved in and recrystallized slowly three times from ethyl acetate (0.4 - 0.6 mL), yielding the L-tartrate salt (35 mg).



(*R*)-αMB



(*S*)-αMB

Figure 4.1
Structures of enantiomers of αMB.

After recrystallization, the individual αMB enantiomers were obtained after the salts were basified with 5% $NaHCO_3$ (2 mL) and extracted with diethyl ether (4 x 2 mL). The pooled organic phases were then taken to dryness under a gentle stream of N_2 and purified by isocratic normal phase high-performance liquid chromatography (HPLC) using a Waters C18 Resolve (5 μM, 8 x 100 mm) radial pak column. The mobile phase was hexane/ethyl acetate (6:1) at 1 mL/min with UV detection at 280 nm. The chemical identity of each enantiomer was established by comparison of HPLC retention time, NMR chemical shifts and mass spectra with authentic, racemic αMB. The stereochemical identity of the enantiomers was established by circular dichroism scans from 225 to 350 nm performed on a 0.1 mg/mL solution (in CH_2Cl_2) of the individual enantiomers. The instrument used was a Jasco J-500C spectropolarimeter with an IBM-9000 data system running CDSCAN software.

4.2.3 Animal Treatment and Preparation of Microsomes

Male Hartley guinea pigs (250-300g) were treated intraperitoneally with 80 mg/kg PB (2% in saline) for 4 days and sacrificed 24 hr following the last injection by asphyxiation with CO_2. All animals were allowed free access to food (Purina guinea pig chow) and water throughout the treatment period. Hepatic microsomes were prepared by differential centrifugation as previously described (Bend *et al.*, 1972). Microsomal protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Microsomal CYP content was determined from the dithionite difference absorption spectrum of carbon monoxide-saturated microsomes with $\varepsilon = 100$ $mM^{-1} \cdot cm^{-1}$ (Estabrook *et al.*, 1972). Microsomes were stored at -80°C until use.

4.2.4 Preparation of Inhibitor Solutions

The αMB enantiomers were initially dissolved as stock solutions (0.1 M) in methanol. The concentrations of these solutions were verified by UV-absorbance

spectrophotometry (λ = 280 nm) against standard curves. A racemic stock solution was produced by combing equal volumes of equimolar solutions of (+)αMB and (-)αMB. Volumes of 100 μL were aliquoted into 1 mL volumetric flasks and the methanol was evaporated under a gentle stream of N_2. The remaining residue was made up to 1 mL with Me_2SO to give a final concentration of 0.01 M. Serial dilutions of this stock solution were used to obtain all of the inhibitor concentrations used.

4.2.5 Inhibition Assays

Primary inhibition incubation mixtures contained hepatic microsomal protein (3.75 mg) and 5 μL of the appropriate inhibitor dilution made up to a final volume of 975 μL with 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The samples were allowed to pre-incubate for 5 min in a shaking water bath maintained at 37°C. Reactions were started by the addition of NADPH (final concentration = 1 mM) and were allowed to incubate, with shaking, for periods of 15 sec to 2.5 min. For the determination of competitive inhibition (t=0 min), identical inhibition incubations were performed in the absence of NADPH. Aliquots (15 μL) of the primary incubations were diluted 100-fold by transfer into pre-warmed secondary enzyme incubation mixtures containing substrate (5 μM 7-pentoxyresorufin or 1.3 μM 7-ethoxyresorufin), 0.1 M potassium phosphate buffer (pH 7.4), 1 mM EDTA and 1 mM NADPH. The secondary incubations were allowed to proceed for 3.5 min at 37°C prior to quenching with 2 volumes of ice-cold methanol. Precipitated protein was removed by centrifugation (Sorvall GLC-1, M rotor) for 5 min at 3000 rpm. The fluorescent product, resorufin, was measured at an excitation wavelength of 535 nm and an emission wavelength of 585 nm using a Perkin-Elmer fluorescence spectrophotometer (model LS-5B). The rate of PROD or EROD activity was calculated based on a standard curve of fluorescence *vs.* resorufin concentration. In some experiments the inhibition reaction was allowed to proceed for 30 min and then placed on ice. Following this, the microsomes were washed by sedimentation of the inhibition

mixtures at 412 160 x *g* for 15 min at 4°C (Beckman TL-100 ultracentrifuge; TLA 100.3 rotor), followed by resuspension in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. After determination of protein concentration, 50 μg of these washed microsomes were assayed for PROD or EROD activity as described above. All assays were verified to proceed linearly with respect to time and protein concentration.

4.2.6 Data Analysis

The apparent half-life for inactivation ($t_{1/2}$) was calculated by linear regression analysis of the natural logarithm of residual enzyme activity with respect to inhibitor incubation time. Apparent inactivation rate constants were derived from plots of $t_{1/2}$ *vs.* the reciprocal of inhibitor concentration by the method of Kitz and Wilson (1962). Data were analyzed by one-way ANOVA, followed by the Tukey-Kramer multiple comparisons test using SuperANOVA v1.11 for Macintosh software (Abacus Concepts, Berkeley, CA).

4.3 Results

4.3.1 Preparation and Identification of αMB Enantiomers

Individual enantiomers of αMB (Fig. 4.1) at the single chiral center, were obtained from racemic αMB by separation of diastereomeric salts and purification of the regenerated enantiomers. The HPLC retention time, NMR chemical shifts and mass spectral analysis (data not shown) of the purified enantiomers were identical to those obtained for an authentic αMB racemate (Mathews and Bend, 1986). The optical activity of the individual enantiomers was established through measurement of the optical rotatory dispersion (ORD; Fig. 4.2). A positive Cotton effect (peak - 288 nm, trough - 260 nm) was clearly seen for one compound while a negative Cotton effect (peak - 260 nm, trough - 288 nm) was observed for the other, indicating that the two compounds were indeed enantiomeric forms of the same compound. As such, the individual enantiomeric forms of αMB are henceforth

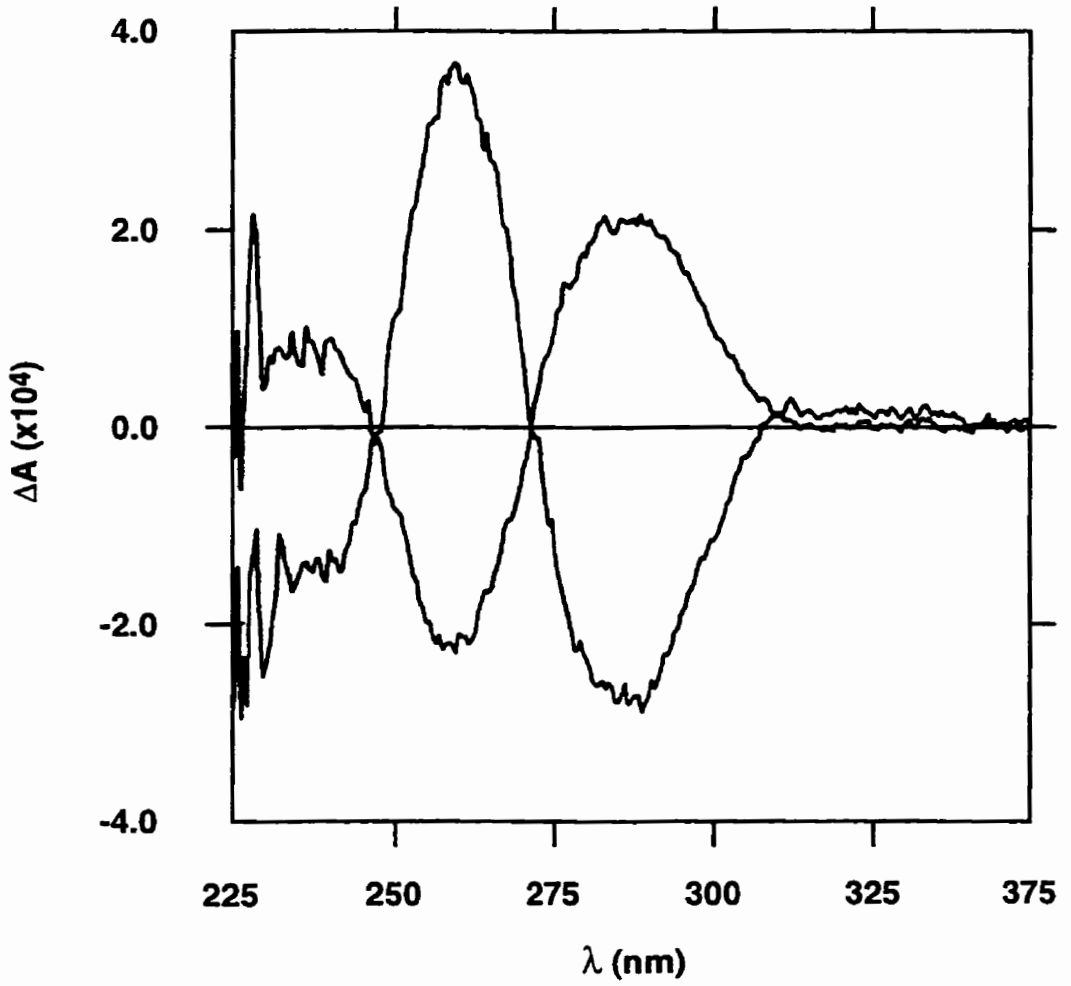


Figure 4.2
ORD profiles of αMB enantiomers. Individual ORD spectra were obtained from a 0.1 mg/ml solution (in CH_2Cl_2) of each enantiomer as detailed in Materials and Methods. The peak at 288 nm and trough at 260 nm indicates a positive Cotton effect while a negative Cotton effect is indicated by the peak at 260 nm and trough at 288 nm.

referred to as (+) or (-)αMB in reference to their respective Cotton effects. It is also possible to tentatively assign an absolute configuration to each of the enantiomers based upon their Cotton effect and comparison with similar compounds in the literature. The benzene chromophore shows three well-defined absorption bands associated with $\pi \rightarrow \pi^*$ transitions. If the benzene ring is substituted with a chiral group these transitions become optically active and produce Cotton effects (Johnson *et al.*, 1987). The B_{2U} transition at 240-270 nm, as observed by its Cotton effect, correlates to the absolute configuration of the chiral substituent attached to the benzene ring. (*S*)-α-phenylethylamine exhibits a positive B_{2U} Cotton effect and alkyl substitution on the amine group does not change the sign of this effect, since both (*S*)-*N*,*N*-dimethyl-α-phenylethylamine and its methyl iodide maintain a positive sign (Johnson *et al.*, 1987). In addition, for α-phenylethylamine a negative Cotton effect at 268 nm correlates with the (*R*) configuration (Smith *et al.*, 1968). Assuming that the benzotriazole substitution on the amino group of α-phenylethylamine also does not change the sign of the Cotton effect then the αMB enantiomer with a positive Cotton effect ((+)αMB) correlates with the (*S*) configuration and a negative Cotton effect ((-)αMB) correlates with the (*R*) configuration.

4.3.2 Mechanism-Based Inactivation of Hepatic PROD Activity

In these studies, PROD (Lubet *et al.*, 1985) was used as a marker for CYP2B18, the guinea pig orthologue of CYP2B1 (Nelson *et al.*, 1996), the isozyme primarily responsible for this catalytic activity in liver microsomes prepared from PB-treated rats. To account for the possibility that other closely related isoforms (*i.e.* CYP2B2 in rat) may also contribute to PROD activity, this activity is henceforth considered as CYP2B-dependent. The time course for inactivation of this activity by the individual αMB enantiomer preparations is shown in Fig. 4.3. All three preparations produced a rapid time- and NADPH-dependent loss of PROD activity, consistent with MB inactivation

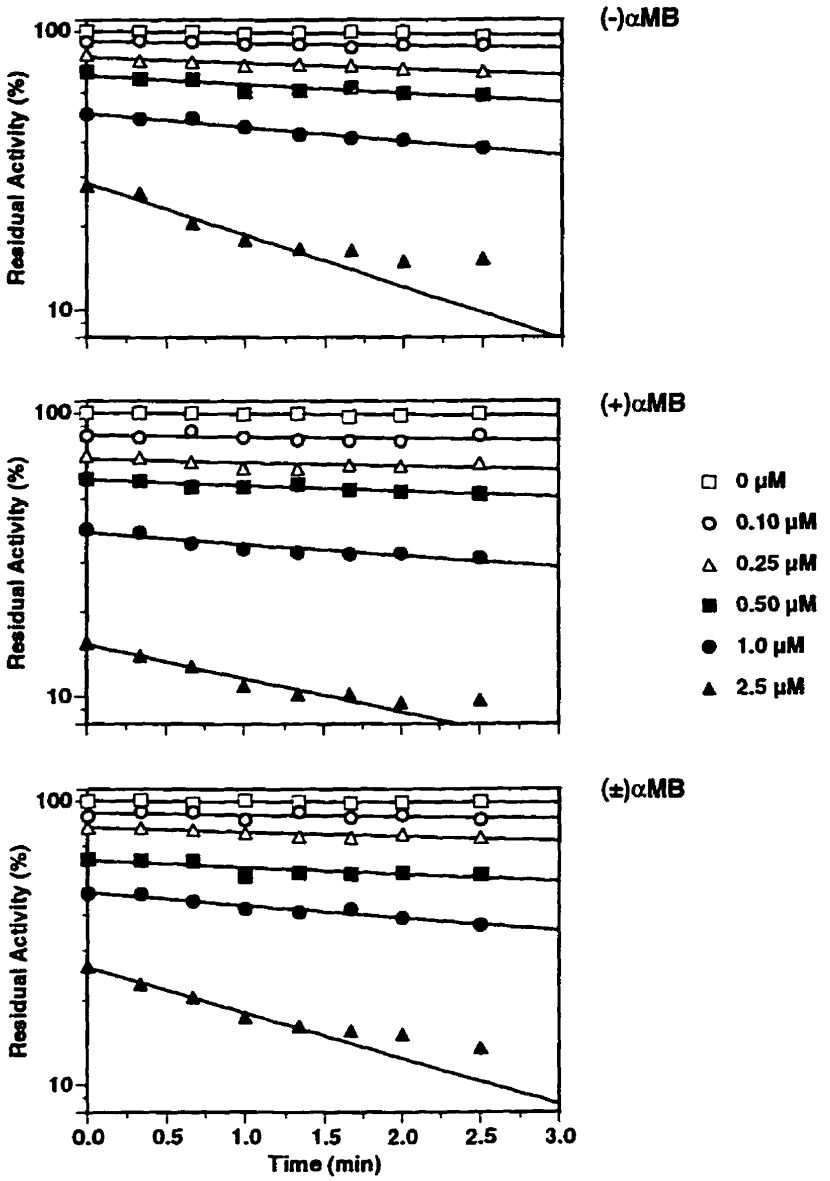


Figure 4.3
Time course of NADPH-dependent MB inactivation of PROD activity in hepatic microsomes from PB-treated guinea pigs by αMB. At the indicated times, aliquots of microsomes incubated in the presence of (-)αMB, (+)αMB or (±)αMB and 1 mM NADPH were transferred to secondary incubation mixtures and assayed for PROD activity as described under Materials and Methods. Each data point represents the mean of experiments performed in duplicate using microsomes prepared from 4 individual livers. The control PROD activity for the experiments with (-)αMB, (+)αMB or (±)αMB was 247.4 ± 19.7, 262.6 ± 27.9 and 260.0 ± 19.7 pmol/min/nmol CYP protein, respectively.

by these compounds. The highest concentrations of these inhibitors caused an extensive (90%) loss of total PROD activity within 3 min of incubation. The rates of inactivation were found to increase in a concentration-dependent manner, indicative of a pseudo-first order rate process (Table 4.1). At all concentrations studied, with the exception of 1 μM, the time required for a 50% loss of the initial PROD activity ($t_{1/2}$) was significantly shorter for (-)αMB when compared with (+)αMB. However, statistically significant differences in the rate of (-)αMB *vs.* (±)αMB inactivation of PROD activity were not observed.

Each of the αMB preparations also produced a substantial concentration-dependent degree of reversible inhibition, as indicated by the residual PROD activity after incubation of microsomes with various concentrations of the inhibitors in the absence of NADPH (Fig. 4.3, Table 4.1). It is important to note that the concentrations indicated in Table 4.1 represent the inhibitor concentration in the initial inhibition incubation. Thus, the actual inhibitor concentration in the secondary enzyme activity incubation mixture is expected to be on the order of 100-fold less due to dilution (see Materials and Methods). In contrast to the rates of MB inactivation, competitive PROD inhibition was significantly greater for 0.5, 1.0 or 2.5 μM (+)αMB than equimolar (-)αMB, however a significant difference from equimolar racemic (±)αMB was only found at 2.5 μM (+)αMB.

4.3.3 Mechanism-Based Inactivation of Hepatic EROD Activity

In these experiments, EROD activity was used as a marker of CYP1A-dependent metabolism in liver microsomes from PB-treated guinea pigs (Burke *et al.*, 1985). As with PROD inactivation, all three of the αMB preparations caused a time- and NADPH-dependent MB inactivation of hepatic EROD activity (Fig. 4.4). However, in this case, the maximum amount of inactivation that could be achieved within 3 min was approximately 50%. Furthermore, the rates for EROD inactivation were less than that observed for PROD (Table 4.2). For example, at a concentration of 2.5 μM, the $t_{1/2}$ for EROD inactivation was 3.5- to 7.3-fold longer than for PROD inactivation depending on the αMB preparation

Table 4.1 - Half-life for mechanism-based inactivation and extent of competitive inhibition of PROD activity in PB-induced guinea pig hepatic microsomes by enantiomers of αMB.

Concentration (μM)	(-)αMB		(+)αMB		(±)αMB	
	$t_{1/2}$ (min)	% of Control[a] (t=0 min)	$t_{1/2}$ (min)	% of Control[a] (t=0 min)	$t_{1/2}$ (min)	% of Control[a] (t=0 min)
0.10	36.5 ± 8.5[b]	91.7 ± 1.6	55.9 ± 10.5	83.4 ± 8.4	46.2 ± 6.3	88.8 ± 8.1
0.25	13.9 ± 1.9[b]	82.7 ± 8.3	23.9 ± 6.2	71.0 ± 8.0	19.3 ± 3.7	81.1 ± 7.2
0.50	9.3 ± 2.1[c]	71.7 ± 3.7[b]	14.7 ± 2.2	58.8 ± 6.4	12.4 ± 1.3	62.9 ± 7.1
1.00	6.0 ± 0.5	50.5 ± 5.2[b]	7.5 ± 1.0	39.1 ± 4.3	6.7 ± 0.7	48.7 ± 5.2
2.50	1.2 ± 0.1[b]	28.1 ± 3.5[c]	2.6 ± 0.6	15.6 ± 4.1	1.9 ± 0.7	26.4 ± 5.5[b]

[a] The extent of competitive inhibition as determined by incubation of microsomes with inhibitor for 5 min at 37°C in the absence of NADPH. The control PROD activity for the experiments with (-)αMB, (+)αMB or (±)αMB was 247.4 ± 19.7, 262.6 ± 27.9 and 260.0 ± 19.7 pmol/min/nmol of CYP, respectively. All values are the mean ± SD of independent experiments, performed in duplicate, using liver microsomes prepared from 4 individual animals (N=4).

[b] significantly different from (+)αMB, $p<0.05$

[c] significantly different from (+)αMB, $p<0.01$

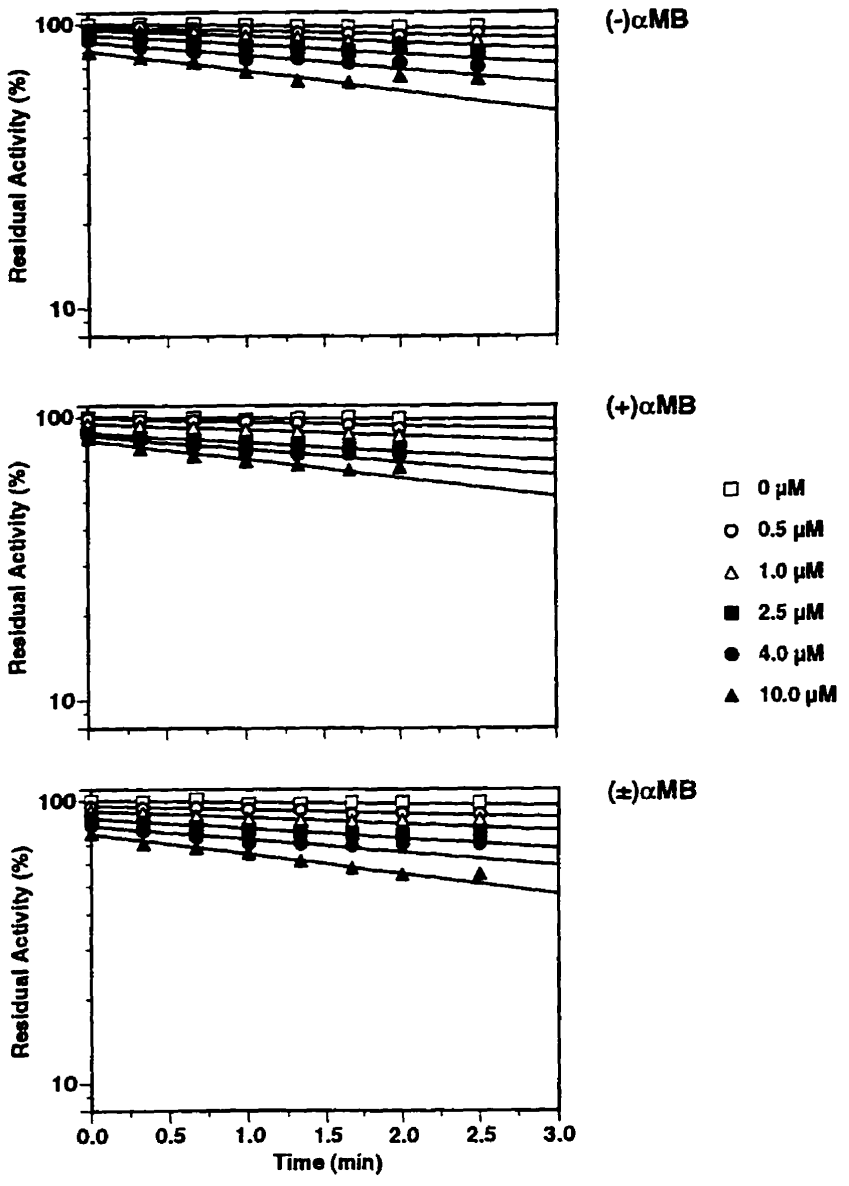


Figure 4.4
Time course of NADPH-dependent MB inactivation of EROD activity in hepatic microsomes from PB-treated guinea pigs by αMB. At the indicated times, aliquots of microsomes incubated in the presence of (-)αMB, (+)αMB or (±)αMB and 1 mM NADPH were transferred to secondary incubation mixtures and assayed for EROD activity as described under Materials and Methods. Each data point represents the mean of experiments performed in duplicate using microsomes prepared from 4 individual livers. The control EROD activity for the experiments with (-)αMB, (+)αMB or (±)αMB was 81.2 ± 12.7, 76.0 ± 6.9 and 74.0 ± 13.4 pmol/min/nmol CYP protein, respectively.

Table 4.2 - Half-life for mechanism-based inactivation and extent of competitive inhibition of EROD activity in PB-induced guinea pig hepatic microsomes by enantiomers of αMB.

Concentration (μM)	(-)αMB		(+)αMB		(±)αMB	
	$t_{1/2}$ (min)	% of Control[a] (t=0 min)	$t_{1/2}$ (min)	% of Control[a] (t=0 min)	$t_{1/2}$ (min)	% of Control[a] (t=0 min)
0.50	23.5 ± 6.4	96.5 ± 3.3	23.1 ± 2.3	99.5 ± 5.0	22.8 ± 6.0	95.5 ± 4.5
1.00	13.2 ± 1.8	95.2 ± 3.7	14.8 ± 0.6	94.7 ± 7.3	13.9 ± 1.7	92.7 ± 2.2
2.50	8.8 ± 0.7	91.9 ± 3.0	9.1 ± 0.5	88.9 ± 10.2	8.8 ± 0.7	87.3 ± 0.9
4.00	6.9 ± 0.8	88.5 ± 2.1	6.5 ± 1.0	87.2 ± 8.8	6.6 ± 0.9	82.9 ± 4.2
10.00	4.3 ± 0.4	80.5 ± 6.0	4.5 ± 0.5	85.3 ± 9.4	4.3 ± 0.2	77.4 ± 4.0

[a] The extent of competitive inhibition as determined by incubation of microsomes with inhibitor for 5 min at 37°C in the absence of NADPH. The control EROD activity for the experiments with (-)αMB, (+)αMB or (±)αMB was 81.2 ± 12.7, 76.0 ± 6.9 and 74.0 ± 13.4 pmol/min/nmol of CYP, respectively. All values are the mean ± SD of independent experiments, performed in duplicate, using liver microsomes prepared from 4 individual animals (N=4).

used. Although the rates of EROD inactivation increased in a concentration-dependent manner, there was no significant difference between the $t_{1/2}$ for any of the compounds at any of the concentrations used in this study.

All of the αMB preparations produced some degree of competitive inhibition of EROD activity, similar to that observed for PROD (Fig. 4.4, Table 4.2). However, the level of this type of inhibition was not as great as that observed for PROD at any equivalent inhibitor concentration. Furthermore, no significant differences were found between the level of reversible EROD inhibition caused by equimolar concentrations of the αMB enantiomers or racemate.

4.3.4 Inactivation Rate Constants

The maximal inactivation rate constant (k_{inact}) and the inhibitor concentration required for the half-maximal rate of inactivation (K_I) were determined for each of the αMB preparations using the obtained $t_{1/2}$ values (Tables 4.1,4.2). This was accomplished by the use of Kitz and Wilson (1962) plots of inactivation $t_{1/2}$ *vs.* the reciprocal of inhibitor concentration (Fig. 4.5). As indicated by the positive value of the point of intersection with the ordinate, the rate of inactivation of CYP2B or CYP1A isozymes was a saturable process with respect to inhibitor concentration. The k_{inact} value for PROD inactivation by (-)αMB (0.49 ± 0.06 min^{-1}) was significantly greater than that for (+)αMB (0.35 ± 0.03 min^{-1}; Table 4.3). In contrast, the K_I value for (-)αMB (2.4 ± 0.7 μM) was not significantly greater when compared to (+)αMB (2.7 ± 0.5 μM). No significant differences were found when the individual enantiomers were compared with the racemate, and no significant differences were found among the k_{inact} and K_I values for EROD inactivation by the αMB compounds. However, the k_{inact} values for PROD inactivation were on average, approximately 2.4-fold larger than those for EROD inactivation . In contrast, the K_I values were generally similar for EROD inactivation than those for PROD inactivation.

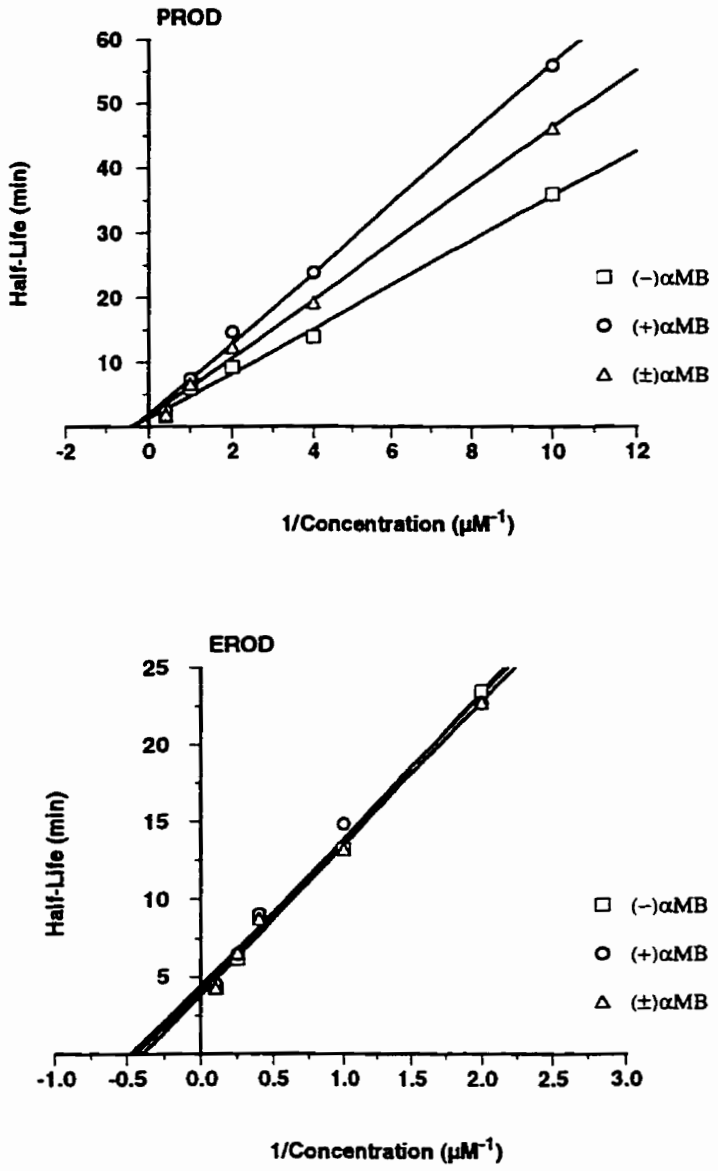


Figure 4.5
Kitz and Wilson plot of the half-life for MB inactivation of PROD or EROD activity as a function of the reciprocal of inhibitor concentration. The apparent kinetic constants for CYP inactivation by (-)αMB, (+)αMB or (±)αMB were derived from the intercepts of the straight lines with the x- and y-axes. k_{inact} = 0.693/y-intercept; K_I = 1/x-intercept. Each data point represents the mean of experiments performed in duplicate using microsomes prepared from 4 individual livers.

Table 4.3 - Apparent inactivation rate constants for MB inactivation of PROD and EROD activity in PB-induced guinea pig hepatic microsomes by enantiomers of αMB.

	PROD[a]		EROD[a]	
Inhibitor	k_{inact} (min^{-1})	K_I (μM)	k_{inact} (min^{-1})	K_I (μM)
(-)αMB	0.49 ± 0.06[b]	2.4 ± 0.7	0.18 ± 0.02	2.1 ± 0.4
(+)αMB	0.35 ± 0.03	2.7 ± 0.5	0.16 ± 0.02	2.5 ± 0.5
(±)αMB	0.39 ± 0.04	2.5 ± 0.6	0.17 ± 0.02	2.3 ± 0.5

[a] All values are the mean ± SD of independent experiments, performed in duplicate, using liver microsomes prepared from 4 individual animals (N=4).

[b] significantly different from (+)αMB, $p<0.05$

4.3.5 Maximum Extent of Inactivation

In order to determine if the differences in the rates of CYP inactivation between the αMB enantiomers had any effect on the maximum extent of inhibition, we performed extended inhibition reactions which were 30 min in length. Following a 30 min incubation, the inhibited microsomes were washed by sedimentation and resuspension in fresh buffer to minimize the effect of competitive inhibition due to excess inhibitor. As shown in Fig. 4.6, no significant differences in the extent of PROD inactivation were observed among any of the αMB preparations at any of the concentrations used. A similar result occurred with EROD inactivation, however, a significant difference between 10 μM (+)αMB *vs.* (-)αMB or (±)αMB was found. Comparatively little loss (approximately 20%) of spectrally assayable CYP occurred with the highest concentration of the αMB preparations in spite of the substantial (up to 95%) loss of PROD, and to a much lesser extent, EROD activity. No significant differences were found between the loss of CYP caused by equimolar (+)αMB, (-)αMB and (±)αMB.

4.4 Discussion

N-aralkylated derivatives of ABT are well established isozyme (CYP2B) and tissue (lung) selective inhibitors of guinea pig and rabbit CYP, both *in vivo* and *in vitro* (Knickle and Bend, 1992; Mathews and Bend, 1993; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990). The length of the alkyl substituent present at the α-carbon position is an important determinant of the actions of these compounds. For example, αMB and αEB are both substantially more potent and selective than BBT for MB inactivation of hepatic and pulmonary CYP2B isoforms *in vitro* (Mathews and Bend, 1986; Woodcroft *et al.*, 1990). Similarly, αMB is a more potent and selective inactivator of pulmonary CYP2B *in vivo* than BBT (Knickle *et al.*, 1994; Mathews and Bend, 1993). In contrast, MI complexation of CYP, an inhibition mechanism not relevant to CYP2B inactivation, exhibits a 3-fold

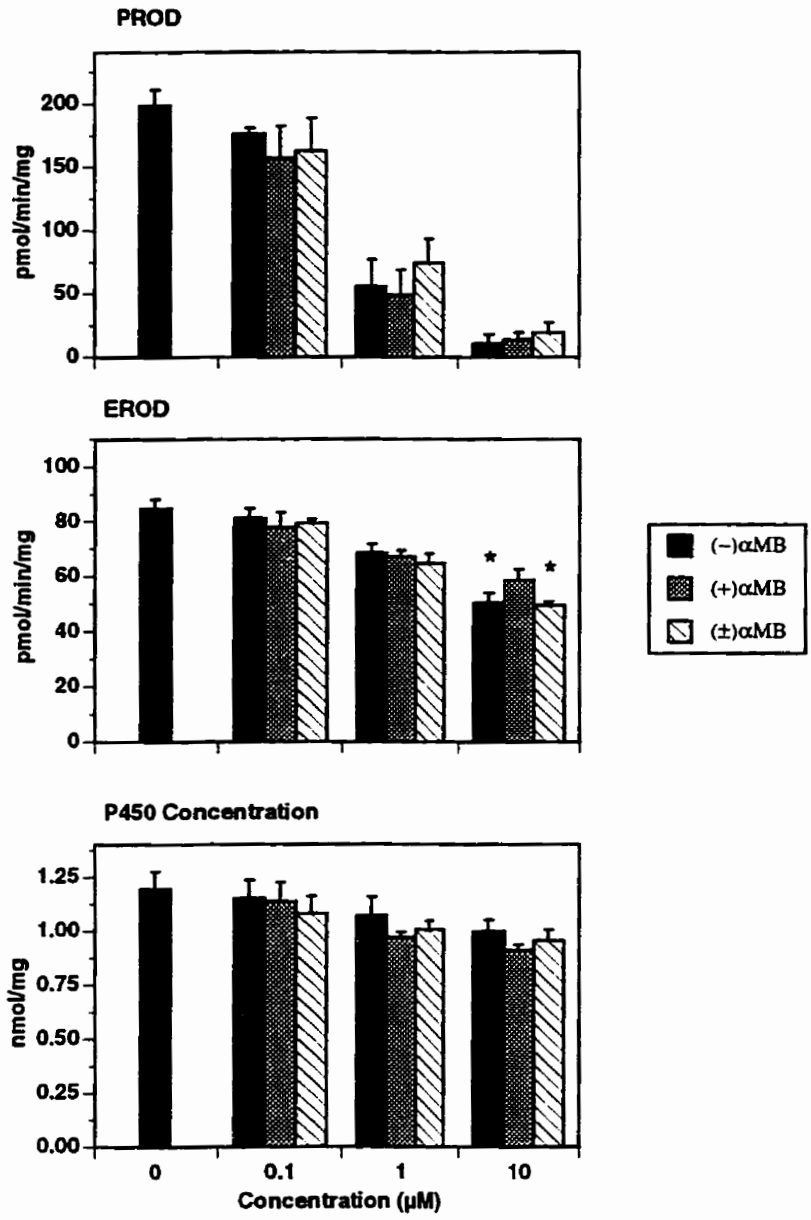


Figure 4.6
Maximum extent of MB inactivation of hepatic PROD and EROD activity and CYP content by individual αMB enantiomers or racemate. Guinea pig hepatic microsomes were incubated for 30 min in the presence of NADPH and (-)αMB, (+)αMB or (±)αMB at the indicated concentrations. Subsequently, the inhibited microsomes were washed by sedimentation and resuspension and were assayed for PROD, EROD and CYP content as described in Materials and Methods. Each bar represents the mean ± SD of experiments performed in duplicate using microsomes prepared from 4 individual livers. * significantly different from (+)αMB, $p<0.05$.

higher Km value for αMB than BBT (Chapter 2 and Sinal and Bend, 1995). While the effects of different sized α-substituents have been documented, the effect of stereochemistry at this chiral center position has not. To this end, individual enantiomers of αMB were prepared and identified using established chromatographic and spectroscopic methods. Based upon these data and information obtained from the literature, a tentative assignment of absolute configuration was also obtained for each of the enantiomers. A kinetic study of these compounds was undertaken in order to compare the enantiomers in terms of inactivation rates and isozyme selectivity. Previous studies of these ABT derivatives with respect to inactivation kinetics have helped to rationalize the CYP isozyme selectivity of this class of compounds as well as the relative potency and selectivity of αMB *vs.* BBT. For example, inactivation of rabbit CYP2B4 and CYP2B5 is much more rapid for αMB (k_{inact} = 0.68 and 0.55 min^{-1}, respectively) compared with BBT (0.29 and 0.18 min^{-1}), consistent with the greater isozyme selectivity of the former (Grimm *et al.*, 1995). Similarly, inactivation of guinea pig pulmonary CYP2B-dependent PROD activity by 25 nM αMB is much more rapid ($t_{1/2}$ = 0.9 min) than for equimolar BBT ($t_{1/2}$ = 32 min) (Chapter 3 and Sinal and Bend, 1996). The maximal rate constant for inactivation (k_{inact}) reflects both the rate of conversion of inhibitor to reactive intermediate(s) and the partition ratio, a measure of the efficiency of generating reactive intermediate(s) as opposed to stable products (Halpert, 1995; Rando, 1984). Thus, k_{inact} provides a measure of isozyme selectivity as well as a means of comparing the inactivation of a specific enzyme activity, under identical reaction conditions, by different compounds.

This study demonstrates a stereoselective difference for MB inactivation of CYP2B by enantiomers of αMB. The shorter $t_{1/2}$ values at all of the concentrations studied, and the larger k_{inact} value, clearly indicate that (-)αMB is a more rapid inactivator of CYP2B-dependent PROD when compared with (+)αMB. In contrast, there were no differences between the inactivation of CYP1A-dependent EROD activity by enantiomers of αMB. Consistent with previous data (Chapter 3 and Sinal and Bend, 1996), the faster rate of

PROD *vs.* EROD inactivation indicates that the selectivity of αMB for CYP2B(s) is due, at least in part, to a higher rate of inactivation. The much larger degree of competitive inhibition of PROD *vs.* EROD activity, as determined by incubation with the inhibitors in the absence of NADPH, also indicates that selectivity for CYP2B is due, in large part, to a greater binding affinity, in spite of the similar K_I values determined for MB inactivation. It is important to note that the PROD and EROD assays were performed after a 100-fold dilution of the inhibition incubation, and hence, the actual concentration of the inhibitors in the assay mixture was approximately 1% of that shown in the figures and tables. Thus, the actual affinity of αMB for binding to CYP2B and CYP1A isozymes is somewhat under represented. Surprisingly, (+)αMB caused a greater degree of competitive inhibition at the three highest concentrations used, but a similar K_I value for PROD inactivation, when compared with (-)αMB. This indicates that while affinity is important for metabolic activation of αMB, oxidation is most likely the rate-limiting step for MB inactivation.

The experiments involving 30 min incubations of the inhibitors with guinea pig hepatic microsomes and NADPH were performed to determine the maximum extent of inactivation by αMB enantiomers. While these data further demonstrate the selectivity of αMB for CYP2B *vs.* CYP1A inactivation, there were no differences between the enantiomers. The similarity of PROD and EROD inactivation, as well as loss of spectral CYP, indicates that 30 min is sufficient time to overcome the difference in initial inactivation rates exhibited by the two αMB enantiomers. Furthermore, these data indicate that while significant differences in the kinetic parameters for CYP2B inactivation by αMB do exist, it seems unlikely that any differences exist for the reaction mechanism. Taken together, these data indicate that the stereochemical orientation of the α-substituent of αMB has significant effects on binding affinity and oxidation rates, but does not affect the reaction mechanism that ultimately results in MB inactivation of CYP.

All of the *N*-aralkylated derivatives of ABT were designed to have molecular features that structurally mimic benzphetamine, a substrate for CYP2B-dependent *N*-

demethylation (Serabjit-Singh *et al.*, 1983). Common structural features include an *N*-benzyl group, an aromatic region of similar size, an amino group in the desired region of oxidation, and an α-carbon for substitution. Consistent with the stereoselectivity for MB inactivation of CYP2B(s) by αMB demonstrated in this study, benzphetamine also exhibits stereoselectivity for CYP2B-dependent metabolism. For example, the V_{max} for amphetamine formation via α-carbon oxidation is 3-fold greater for (+)benzphetamine than for (-)benzphetamine, while the K_m values are similar (Beckett and Gibson, 1978). Given that a difference was found for rates of inactivation for αMB enantiomers, but not the K_I value, α-carbon oxidation may be an important determinant of the rate of CYP2B inactivation.

Studies with radiolabeled BBT have shown that this ABT derivative is metabolized by guinea pig hepatic microsomes (Woodcroft *et al.*, 1997) or purified rat CYP2B1 (Kent *et al.*, 1997a) to benzotriazole, benzaldehyde, ABT and a previously unidentified metabolite designated as #27 (Fig. 4.7), which is generated at a relatively high rate (12 nmol/nmol CYP/min). Recently, #27 has been putatively identified as a dimeric product of BBT oxidation (Kent *et al.*, 1997b). Interestingly, this is the sole metabolite generated by a mutant form of rat CYP2B1, which contains a Gly478 to Ala substitution, and is not inactivated by BBT (Kent *et al.*, 1997b). Benzotriazole, benzaldehyde and ABT are believed to result from oxidation at the 1-amino nitrogen of BBT (Woodcroft *et al.*, 1997), while the BBT dimer product is thought to arise from α-carbon oxidation (Kent *et al.*, 1997b). Molecular modeling studies, in combination with experimental observations, indicate that mutation of Gly478 to Ala results in steric hindrance that favours oxidation of BBT at the α-carbon, rather than the 1-amino nitrogen (Kent *et al.*, 1997b). Taken together, these data indicate that 1-amino nitrogen oxidation results in generation of a reactive intermediate(s) capable of inhibiting rat CYP2B1 in a MB manner, while α-carbon oxidation results in metabolism of BBT to a non-inhibitor dimer. By analogy, the stereoselectivity of guinea pig CYP2B inactivation shown in this study may indicate that,

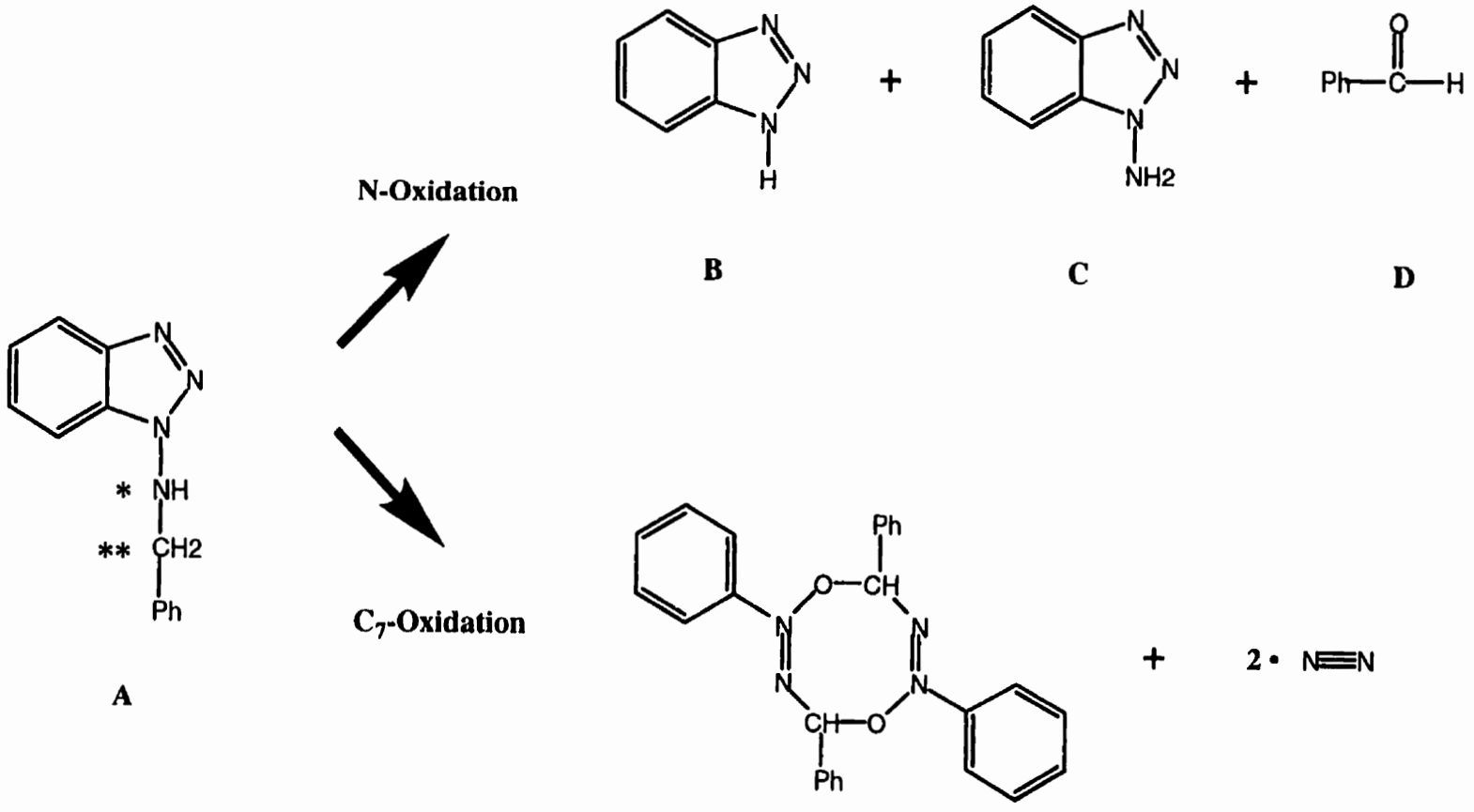


Figure 4.7
Simplified scheme outlining the potential outcomes of BBT metabolism initiated at the amino-nitrogen (*) or C_7-carbon (**). Parent compound and metabolites shown are: (A) BBT, (B) benzotriazole, (C) 1-aminobenzotriazole, (D) benzaldehyde, (E) BBT dimer and, (F) molecular nitrogen. Ph is used to differentiate the benzene ring of the *N*-benzyl group from the benzene ring of the benzotriazole moiety of BBT.

compared with (-)αMB, (+)αMB is oriented in the CYP active site in a manner more favourable to α-carbon oxidation, leading to more frequent metabolism to non-inhibitory products, such as the dimer observed for BBT. This difference in the apparent partition ratio is consistent both with the faster rate of inactivation of guinea pig hepatic CYP2B isozymes by (-)αMB, compared with (+)αMB, and the equivalent extent of inactivation after 30 min. Presumably, guinea pig hepatic CYP1A isozymes do not catalyze α-carbon oxidation of αMB, or are not sensitive to the stereochemistry of the α-substituent.

In conclusion, MB inactivation of CYP2B-dependent PROD activity in hepatic microsomes prepared from PB-treated guinea pigs, exhibits stereoselectivity for (-)αMB with respect to inactivation rate. In contrast, (+)αMB has a higher binding affinity for CYP2B based upon the amount of competitive inhibition of PROD activity, although this was not reflected by a difference of K_I values. MB inactivation of CYP1A-dependent EROD activity by αMB is stereoselective neither with respect to inactivation rate nor binding affinity. After extended incubations in the presence of inhibitor and NADPH, stereoselectivity for maximum PROD or EROD inactivation, or CYP loss, was absent. We conclude that MB inactivation of guinea pig hepatic CYP2B, but not CYP1A isozymes by αMB, occurs in a stereoselective manner, most likely as a result of a difference in the balance between metabolic activation and deactivation for each αMB enantiomer.

Chapter 5

Differential *in vivo* Effects of α-Naphthoflavone and β-Naphthoflavone on CYP1A1 and CYP2E1 in Rat Liver, Lung, Heart and Kidney[4]

5.1 Objectives

Tissue selective patterns in the spectrum and inducibility of CYPs can be an important determinant of the toxicity experienced by any particular organ upon exposure to xenobiotics. CYP1A1 metabolizes a variety of PAHs to mutagenic metabolites (Shimada *et al.*, 1992). This isoform is constitutively expressed at very low levels in most tissues, but is induced dramatically in response to a number of its substrates, including 3MC or BaP (Denison and Whitlock, 1995). Induction of CYP1A1 occurs primarily at the level of gene transcription and is mediated by the AHR, a ligand activated transcription factor (Rowlands and Gustafsson, 1997). CYP2E1-dependent metabolism has been implicated in the toxicity of a wide variety of low molecular weight chemicals including benzene, alcohol and the widely used drug acetaminophen (Lee *et al.*, 1996b; Valentine *et al.*, 1996; Wu and Cederbaum, 1996). In contrast to CYP1A1, CYP2E1 is expressed constitutively in a number of tissues and is induced at the pre- and post-translational level depending upon the identity of the inducer, as well as the dosage and duration of treatment (Hu *et al.*, 1995).

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4 A version of this chapter has been submitted for publication.

Sinal CJ, Webb CD and Bend JR (1998) Differential in vivo effects of α-naphthoflavone and β-naphthoflavone on CYP1A1 and CYP2E1 in rat liver, lung, heart and kidney. Currently under review for publication in *J. Biochem. Mol. Toxicol.*

In addition to CYP1A1 induction, AHR agonists cause a diversity of toxic effects, the most common of which are immunosuppression, tumour promotion, a number of tissue specific histopathological changes and a loss of body weight (Pohjanvirta and Tuomisto, 1994). A number of studies have clearly demonstrated that starvation and altered nutritional status can increase CYP2E1 expression, presumably due to an accumulation of ketone bodies resulting from altered fatty acid and glucose metabolism (Leakey *et al.*, 1989; Lieber, 1997; Manjgaladze *et al.*, 1993; Soh *et al.*, 1996). Thus, it is possible that the pathobiological changes associated with AHR agonists could cause alterations in the expression of CYP2E1, in addition to their direct effects on CYP1A1 expression. The goal of the present study was to test the hypothesis that pathobiological changes caused by exposure to AHR agonists, can alter the expression of CYP genes not under direct transcriptional control of the AHR. To this end, changes in rat CYP1A1 and CYP2E1 mRNA and catalytic activity in response to *in vivo* treatment with the AHR agonist βNF, and the comparatively weak AHR agonist α-naphthoflavone (αNF), were investigated. A comparison of the differential effects of these treatments on liver, lung, kidney and heart CYP are also presented.

5.2 Materials and Methods

5.2.1 Materials

NADPH, sarkosyl, *p*-nitrophenol, diethyldithiocarbamate, αNF and βNF were purchased from Sigma Chemical Co. (St. Louis, MO); Resorufin, 7-ethoxyresorufin and 7-pentoxyresorufin from Molecular Probes Inc. (Eugene, OR.); [α-^{32}P]dCTP (3000 Ci/mmol) from ICN Biomedicals Canada (Montreal, PQ); Prime-a-gene random primer DNA labeling kit from Promega (Madison, WI); *Taq* DNA polymerase and Superscript II RNase H$^-$ reverse transcriptase from Gibco-BRL Canada (Burlington, ON); all restriction enzymes from Pharmacia Canada (Baie d'Urfe, PQ), and; Hybond-N nylon filters from

Amersham Canada (Oakville, ON). PCR primers were synthesized and purified through HPLC by General Synthesis and Diagnostics (Toronto, ON). αMB was synthesized and purified as previously described (Mathews and Bend, 1986; Woodcroft *et al.*, 1990). All other chemicals (reagent grade or better) were from BDH, Toronto, Canada.

5.2.2 Animal Treatment and Preparation of Microsomes

Male Sprague Dawley rats (250-300g) were treated intraperitoneally with 80 mg/kg βNF or αNF (dissolved in corn oil) or corn oil alone for 4 days and sacrificed 24 hr following the last injection by asphyxiation with CO_2. All animals were allowed free access to food and water throughout the treatment period. Liver, lung, kidney and heart microsomes were prepared by differential centrifugation as previously described (Bend *et al.*, 1972). Microsomal protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Microsomes were stored at -80°C until use.

5.2.3 RNA Extraction and Analysis

Total cell RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1987). For the preparation of slot blots, 20 µg of RNA were denatured by heating to 70°C for 15 min in a solution of 50% deionized formamide, 7% formaldehyde and 1X SSC buffer (0.15 M NaCl, 0.015 M sodium citrate). The samples were then cooled quickly on ice, mixed with 2 volumes of 20X SSC and applied to Hybond-N nylon filters using a BioRad (Mississauga, ON) Bio-Dot SF vacuum filtration manifold. After rinsing the wells with 6X SSC (2 x 500 µL), the RNA was fixed to the filters by baking at 80°C for 2 hr. Pre-hybridization of the filters was carried out in a solution containing 6X SSPE (0.9 M NaCl, 0.06 M $NaH_2PO_4 \cdot H_2O$, 0.006 M Na_2EDTA), 50% deionized formamide, 5X Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS) and 100 µg/mL

sheared salmon sperm DNA for 4 hr at 42°C. Hybridization with the random primer ^{32}P-labeled cDNA probes was carried out in the same solution, without Denhardt's reagent, for 16-24 hr at 42°C. The filters were then washed twice at room temperature using a solution of 2X SSPE/0.5 % SDS for 15 min. This was followed by a 30 min wash using 0.1X SSPE/0.5% SDS at 42°C and a final 30 min wash using the same solution at 65°C for 30 min. The washed filters were sealed in plastic wrap and exposed to Kodak X-OMAT AR film in the presence of an intensifier screen at -80°C. After exposure, the cDNA probe was stripped from the filters by washing in 0.1X SSPE/0.5% SDS at 100°C prior to subsequent probe hybridizations. Autoradiographs were scanned using an Hewlett-Packard desktop scanner and analyzed using NIH Image analysis software.

The 1406 bp cDNA probe for rat CYP1A1 mRNA was obtained by EcoRI/PstI digestion of plasmid pA8 (Hines *et al.*, 1985) which was generously provided by Dr. Ronald Hines (Wayne State University). The 883 bp cDNA probe for rat HO-1 mRNA was prepared as described previously (Sinal and Bend, 1997). A cDNA probe for rat CYP2E1 mRNA was prepared by reverse transcription coupled to PCR amplification using the primers F2E1-3 5'-AAAAGAATTCGCACATGGCGGTTCTTGGCATCA-3' (forward) and R2E1-1 5'-AAAAGGTACCAGGTCTCATGAACGGGGAATGAC-3' (reverse). These primers corresponded to nucleotides 8-31 and 1477-1500, respectively, of the published (Song *et al.*, 1986) cDNA sequence and include a clonable EcoRI or KpnI restriction site (underlined). Briefly, 1 μg of total RNA obtained from rat liver was used as a template for first strand cDNA synthesis using reverse transcriptase according to the manufacturer's (Gibco-BRL) specifications and by using R2E1-1 as the primer. Then, 10% of the cDNA product was added to a standard PCR mixture according to the manufacturer's instructions (Gibco-BRL) using F2E1-3 and R2E1-1 as specific primers. PCR amplification was performed using a Perkin-Elmer Cetus (model 480; Norwalk CT) DNA thermal cycler using the following temperature profile: initial denaturation for 5 min at 95°C; 35 cycles of 45 sec at 95°C (denaturation), 45 sec at 60°C (annealing), 1.5 min at

72°C (extension); and a final extension of 6 min at 72°C. Subsequently, the 1513 bp cDNA product was purified from the PCR reaction by agarose gel (1%) electrophoresis in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM Na_2EDTA, pH 7.2) using a Prep-A-Gene nucleic acid purification kit (Bio-Rad Canada, Mississauga, ON).

All probes were ^{32}P-labeled by the random primer method according to the manufacturer's (Promega) instructions and were used in the following order: CYP2E1, CYP1A1, HO-1 and glyceraldehyde 6-phosphate dehydrogenase (GAPDH).

5.2.4 Enzyme Assays

For assays of EROD activity, incubations contained microsomal protein (liver, 0.1 mg; lung and kidney, 0.25 mg; heart, 0.5 mg) and 1.3 μM 7-ethoxyresorufin (in Me_2SO) made up to a final volume of 975 μL with 0.1 M potassium phosphate buffer (pH 7.8) containing 1 mM EDTA. The samples were allowed to preincubate for 5 min in a shaking water bath maintained at 37°C. Reactions were started by the addition of 25 μL NADPH (final concentration = 1 mM) and were allowed to incubate, with shaking, for 6 min prior to quenching with 2 volumes of ice-cold methanol. Precipitated protein was removed by centrifugation (Sorvall GLC-1, M rotor) for 5 min at 3000 RPM . The fluorescent product, resorufin, was measured at an excitation wavelength of 535 nm and an emission wavelength of 585 nm using a Perkin-Elmer fluorescence spectrophotometer (model LS-5B). The rate of EROD activity was calculated based on a standard curve of fluorescence *vs.* resorufin concentration. Lung microsomal PROD activity was determined identically to EROD activity with 0.25 mg protein and a final substrate concentration of 5 μM.

For assays of PNP activity, incubations contained microsomal protein (1 mg) and 200 μM *p*-nitrophenol made up to a final volume of 975 μL with 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$. The samples were allowed to preincubate for 5 min in a shaking water bath maintained at 37°C. Reactions were started by the addition of 25 μL NADPH (final concentration = 1 mM) and were allowed to incubate, with shaking, for 20

min prior to adding 0.25 mL ice-cold 0.6 N perchloric acid and placing the tubes on ice. Precipitated protein was removed by centrifugation (Sorvall GLC-1, M rotor) for 5 min at 3000 RPM . After mixing the resultant supernatant with 100 μL 10 M NaOH, the amount of 4-nitrocatechol formed was measured by its absorbance at λ = 546 nm using a Beckman DU-65 spectrophotometer. The rate of PNP activity was calculated based on an extinction coefficient of ε=10.28 $mM^{-1} \cdot cm^{-1}$ (Reinke and Moyer, 1985). All assays were verified to proceed linearly with respect to time and protein concentration.

5.2.5 Inhibition Incubations

Incubation mixtures contained 5 mg/mL of pulmonary microsomal protein and 1-100 μM inhibitor in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. The inhibitors, diethyldithiocarbamate (DEC) or αMB were dissolved in methanol, added to the incubation vessel and the methanol was removed under a gentle stream of nitrogen prior to the addition of the other reaction components. Methanol alone was added to control incubations. Reactions were started by the addition of NADPH (1 mM final) and were maintained at 37°C in a shaking water bath for 30 min. Washing of the microsomes to remove excess inhibitor was accomplished by dilution of the incubations (25-fold) with ice-cold incubation buffer, sedimentation at 100 000 x g for 60 min at 4°C (Beckman XL-90 ultracentrifuge; 50Ti rotor), and resuspension of the microsomal pellet in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. Subsequently, the microsomes were centrifuged at 412 160 x g for 15 min at 4°C (Beckman TL-100 ultracentrifuge; TLA 100.3 rotor) followed by resuspension in the same buffer. After determination of protein concentration, these washed microsomes were assayed for PROD, EROD and PNP activities as described above.

5.2.6 Statistics

Data were analyzed by one-way ANOVA, followed by the Tukey-Kramer multiple comparisons test using SuperANOVA v1.11 for Macintosh software (Abacus Concepts, Berkeley, CA). A critical significance level of $p<0.05$ was chosen for all comparisons. Regression analysis was performed using StatView v4.5 for Macintosh software (Abacus Concepts, Berkeley, CA).

5.3 Results

5.3.1 Effect of αNF and βNF on Body and Organ Mass

Nutritional status is known to influence the expression of a number of CYPs, most notably CYP2E1 (Leakey *et al.*, 1989; Yang *et al.*, 1992). Furthermore, AHR agonists such as βNF have a number of toxicological effects, including a profound wasting syndrome (Pohjanvirta and Tuomisto, 1994). As an indicator of the role that these factors may have played in the modulation of CYP after acute administration of αNF or βNF, the total body mass of the animals was monitored throughout this study. The animals were treated with corn oil, αNF or βNF once daily, from days 1 through 4, with sacrifice occurring on day 5. As shown in Fig. 5.1, the animals that received corn oil or αNF experienced a small (5%) weight gain over the course of treatment. In contrast, animals treated with βNF exhibited a significant decline in body mass that amounted to 12% by the time of sacrifice (Day 5). The total wet mass of the liver, lung, kidney and heart from the animals used in this study were also measured at the time of sacrifice. With the exception of the liver, all of the organs obtained from βNF treated animals had a lower mass when compared to organs isolated from corn oil or αNF treated animals (Fig. 5.2). The difference between the masses of the lungs, kidneys and heart (13%, 10% and 12%, respectively) from βNF treated animals compared to corn oil treated animals was consistent with the loss of total body mass, however, statistical significance was not achieved in this

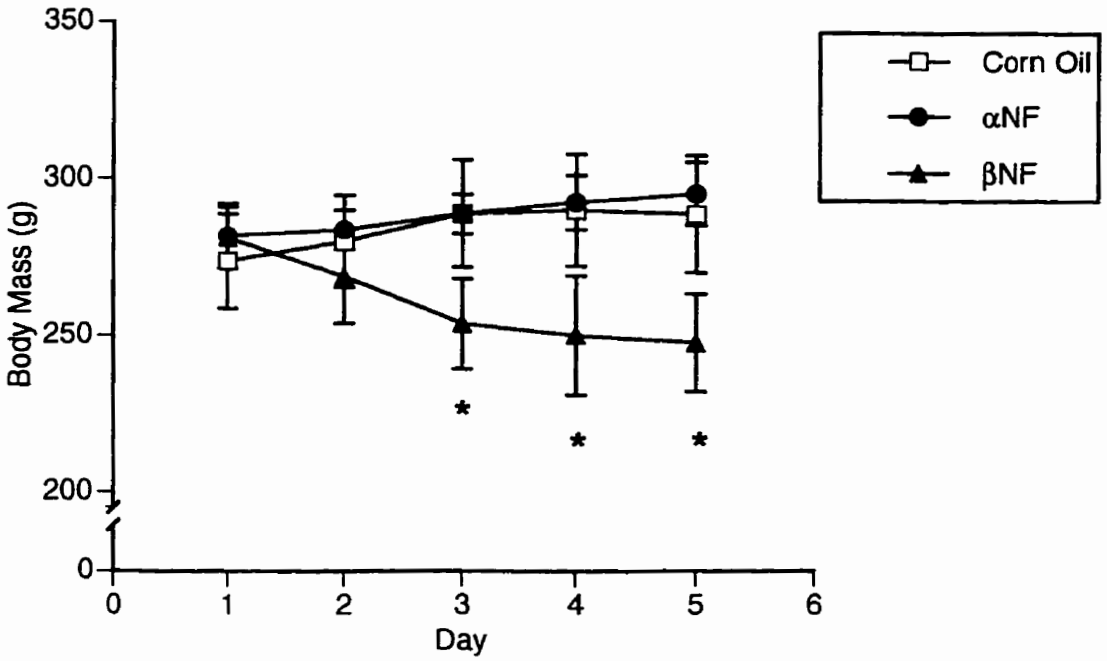

Figure 5.1
Total body mass of rats treated with corn oil, αNF or βNF. Values shown are the mean ± SD (N=4). * significantly different from corn oil or αNF treated animals ($p<0.05$).

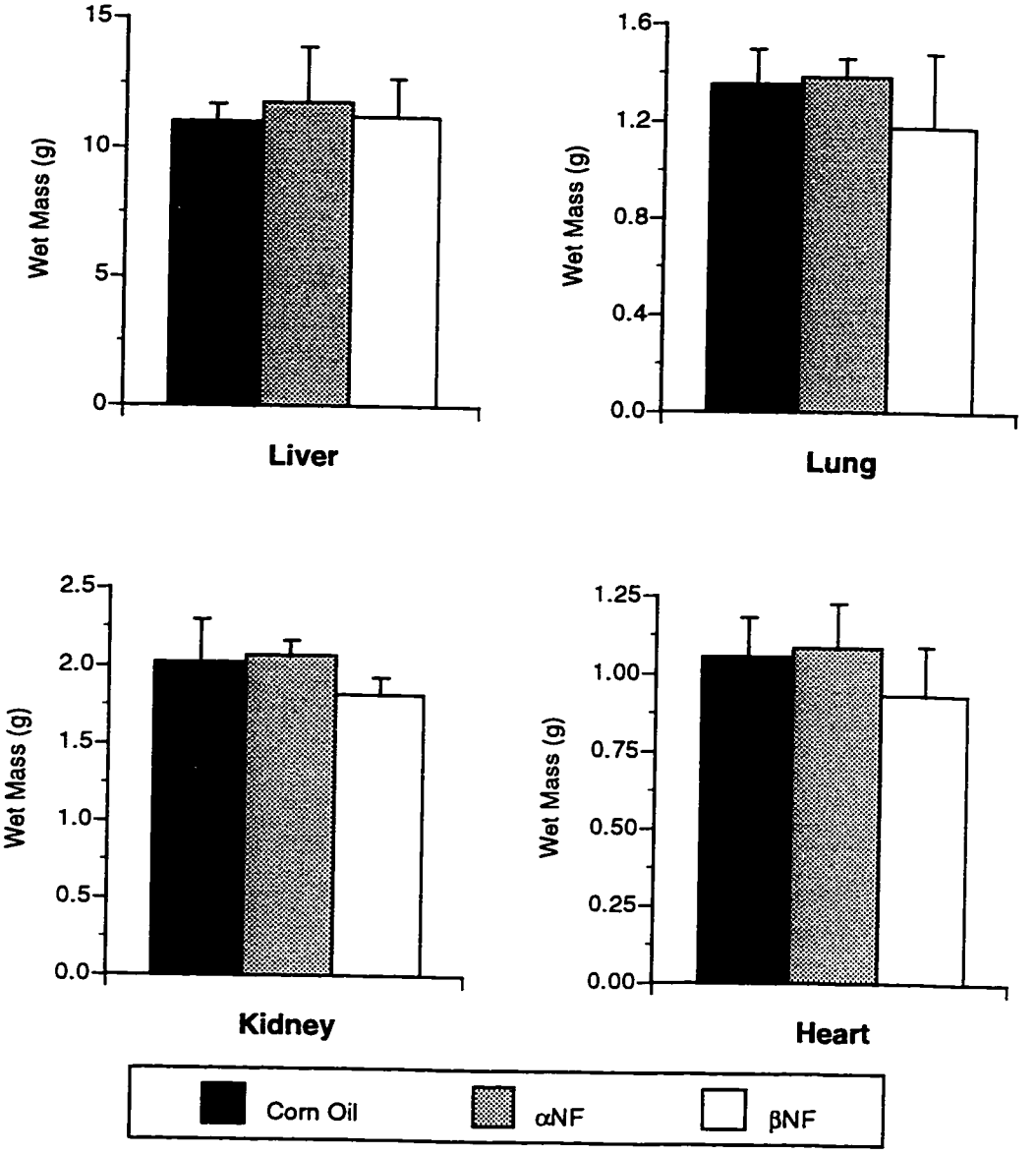

Figure 5.2
Total wet mass at sacrifice of organs harvested from corn oil, αNF or βNF treated rats. Values shown are the mean ± SD (N=4).

case. Also similar to total body mass, the organs harvested from αNF treated animals did not appear to have different masses from those obtained from corn oil treated animals.

5.3.2 Effect of αNF and βNF on CYP1A1 and CYP2E1 mRNA

In order to determine the effect of αNF and βNF treatment on CYP1A1 and CYP2E1, total RNA was prepared from liver, lung, kidney and heart, and analyzed using slot blots. To account for the possibility that treatment with αNF or βNF could induce oxidative stress, a condition which may have modulatory effects on CYP2E1 expression (Tindberg and Ingelman-Sundberg, 1989), the mRNA for HO-1 was also monitored. The expression of this gene is induced by oxidative stress, but is not influenced by AHR ligands (Plewka and Bienioszek, 1994). The steady-state levels of CYP1A1 mRNA were typically very low in all of the organs isolated from corn oil treated animals, but were increased dramatically in response to βNF treatment (Fig. 5.3). Treatment with αNF, an AHR antagonist with some agonist properties, caused relatively minor increases of CYP1A1 mRNA levels in liver, kidney and heart when compared to βNF. However, in lung αNF increased CYP1A1 mRNA levels to approximately 60% of those seen with βNF treatment.

The steady-state levels of CYP2E1 mRNA were readily detected in the liver of corn oil treated animals, with lesser amounts present in kidney, followed by lung and heart (Fig. 5.3). While αNF treatment caused comparatively small changes in the levels of CYP2E1 mRNA in all of the organs studied, βNF appeared to have more substantial effects. For example, while liver CYP2E1 mRNA was marginally greater in βNF *vs.* corn oil treated (1.4-fold), more substantial elevations were observed for lung and kidney (2.0- and 2.5-fold, respectively), with the greatest increase associated with heart (5.5-fold). Neither αNF nor βNF appeared to have any effect on the steady-state levels of HO-1 mRNA in any of the organs studied, when compared with corn oil treated animals.

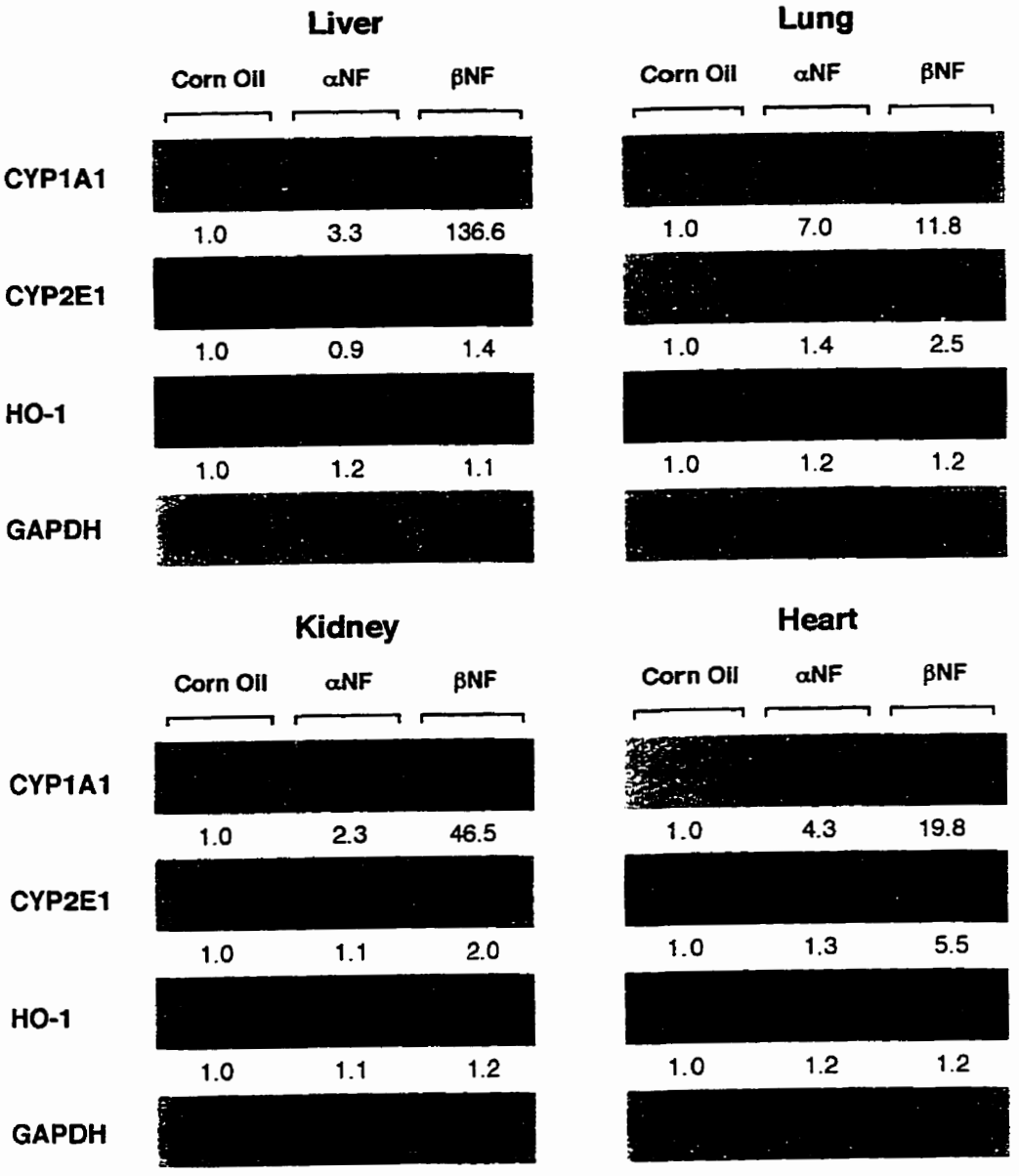

Figure 5.3
Representative slot blots of total RNA isolated from liver, lung, kidney or heart of corn oil, αNF or βNF treated rats. Numbers shown represent the fold change of densitometric signal (corrected for GAPDH) relative to that obtained for corn oil treated animals. Values given are the average obtained from the RNA isolated from four individual animals.

5.3.3 Effect of αNF and βNF on Cytochrome P450-Dependent Catalytic Activity

In order to determine the effect of αNF and βNF on CYP-dependent catalytic activity, EROD was used as a selective marker for CYP1A1 (Burke *et al.*, 1985), while PNP is considered to be a reaction principally catalyzed by CYP2E1 (Mishin *et al.*, 1996). As expected, and consistent with the changes observed for CYP1A1 mRNA, βNF treatment caused a substantial increase of EROD activity in rat liver, lung, kidney and heart (Fig. 5.4). Also in agreement with the mRNA data, αNF caused comparatively small changes in liver and kidney EROD activity. The exception to this was the lung where the EROD activity of pulmonary microsomes prepared from αNF treated rats was approximately 60% of that associated with βNF treatment. A similar, but smaller effect was also observed for the heart where the EROD activity of microsomes from αNF treated rats was approximately 35% that for βNF.

In agreement with observed changes of CYP2E1 mRNA, PNP activity was slightly greater (1.4-fold) with microsomes prepared from the livers of βNF treated animals when compared with corn oil controls (Fig. 5.5). Similarly, kidney microsomal PNP activity was significantly greater (3.1-fold) with βNF treatment, compared with corn oil. Treatment of the animals with αNF was apparently without effect on liver or kidney PNP activity. Although the largest change in CYP2E1 mRNA levels caused by βNF treatment was associated with the heart, PNP was not detectable in heart microsomes prepared from corn oil, αNF or βNF treated rats. In contrast to the other tissues, αNF or βNF treatment caused significant decreases (72% and 27% of corn oil, respectively) of lung microsomal PNP activity.

A comparison of the steady-state levels of lung CYP2E1 mRNA with PNP activity revealed an obvious discrepancy: although CYP2E1 mRNA levels were greater in the lungs of βNF *vs.* corn oil treated animals, PNP activity was substantially less. These data suggest that CYP2E1 may not be the predominant enzyme catalyzing PNP activity in rat

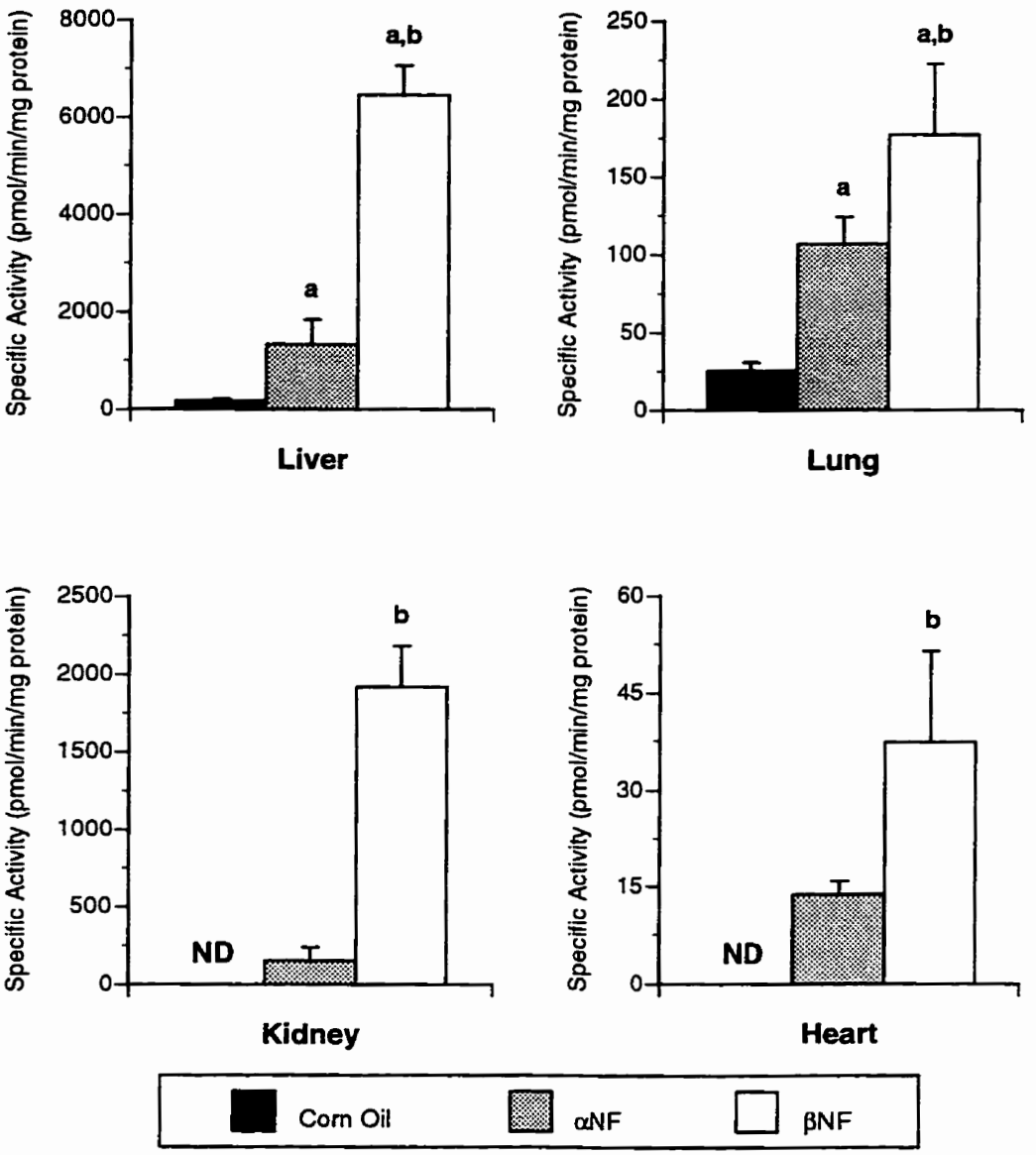

Figure 5.4
EROD activity of liver, lung, kidney or heart microsomes prepared from corn oil, αNF or βNF treated rats. Values shown are the mean ± SD (N=4). **a**, significantly different from corn oil treated animals ($p<0.05$); **b**, significantly different from corn oil or αNF treated animals ($p<0.05$); **ND**, not detected (<1 pmol/min/mg microsomal protein).

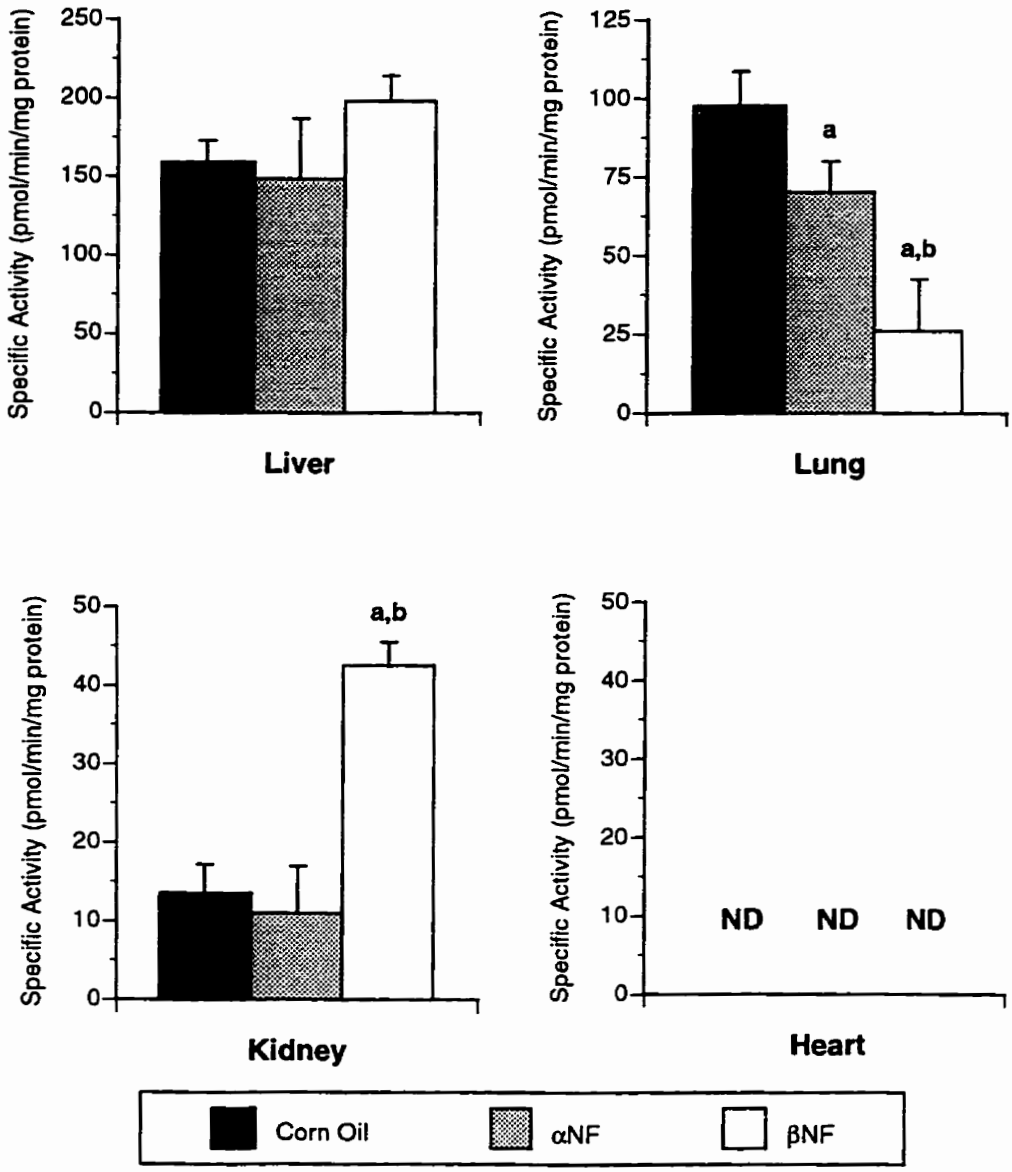

Figure 5.5
PNP activity of liver, lung, kidney or heart microsomes prepared from corn oil, αNF or βNF treated rats. Values shown are the mean ± SD (N=4). **a**, significantly different from corn oil treated animals ($p<0.05$); **b**, significantly different from corn oil or αNF treated animals ($p<0.05$); **ND**, not detected (<1 pmol/min/mg microsomal protein).

lung. A significant negative correlation ($R = -0.838$, $p<0.0007$) was found between lung EROD and PNP activities (Fig. 5.6A), raising the possibility that the depression of PNP activity was related to the ability of αNF or βNF to act an AHR agonist in rat lung. CYP2B1 is a major isozyme expressed in rat lung (Keith *et al.*, 1987; Omiecinski *et al.*, 1990a), and thus was considered a reasonable candidate to account for PNP metabolism in lung microsomes. Similar to PNP activity, lung microsomal CYP2B1-dependent PROD activity was decreased by αNF or βNF (48% and 17% of corn oil, respectively) treatment (Fig. 5.6B). A significant ($R = 0.887$, $p = 0.0001$) positive correlation was also found between the PNP and PROD activities of lung microsomes prepared from the individual animals treated with corn oil, αNF or βNF (Fig. 5.6C), suggesting that CYP2B1 is a major contributor to lung microsomal PNP activity.

To investigate this possibility, we performed additional studies using the MB inactivators αMB and DEC. The former is a well characterized CYP2B-selective inhibitor in lung (Knickle *et al.*, 1994; Mathews and Bend, 1993; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990), while the latter has been reported as selective for CYP2E1 (Guengerich *et al.*, 1991). As shown in Fig. 5.7, incubation of rat lung microsomes from corn oil treated rats with αMB caused a greater dose-dependent loss of PROD activity compared to PNP activity. For example, 1 μM αMB caused approximately 55% loss of PROD activity, compared with 10% for PNP. Similarly, at the highest concentration of αMB used a 90% loss of PROD compared with 50% for PNP activity was observed. EROD activity was also inactivated by αMB at higher concentrations, with approximately 75% loss occurring at 100 μM. When DEC was used as the inhibitor, the losses of PROD and PNP activity were very similar at all of the concentrations studied, with the exception of 100 μM where a significantly greater loss of PNP compared with PROD activity was found. For the most part, EROD activity was not substantially affected by DEC, with a 35% loss occurring only at 100 μM.

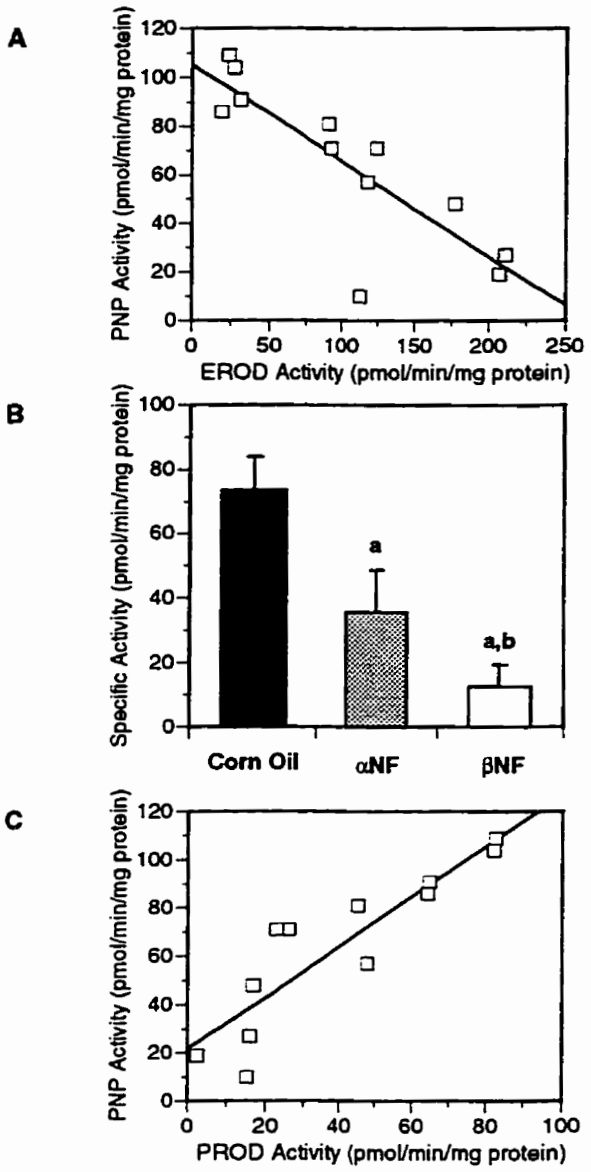

Figure 5.6

A. Correlation of EROD and PNP activities of lung microsomes prepared from corn oil, αNF or βNF treated rats. Each data point represents the results obtained for an individual animal. R = -0.838, p = 0.0007 for regression analysis.

B. PROD activity of lung microsomes prepared from corn oil, αNF or βNF treated rats. Values shown are the mean ± SD (N=4). **a**, significantly different from corn oil treated animals ($p<0.05$); **b**, significantly different from corn oil or αNF treated animals ($p<0.05$).

C. Correlation of PROD and PNP activities of lung microsomes prepared from corn oil, αNF or βNF treated rats. Each data point represents the results obtained for an individual animal. R = 0.887, p = 0.0001 for regression analysis.

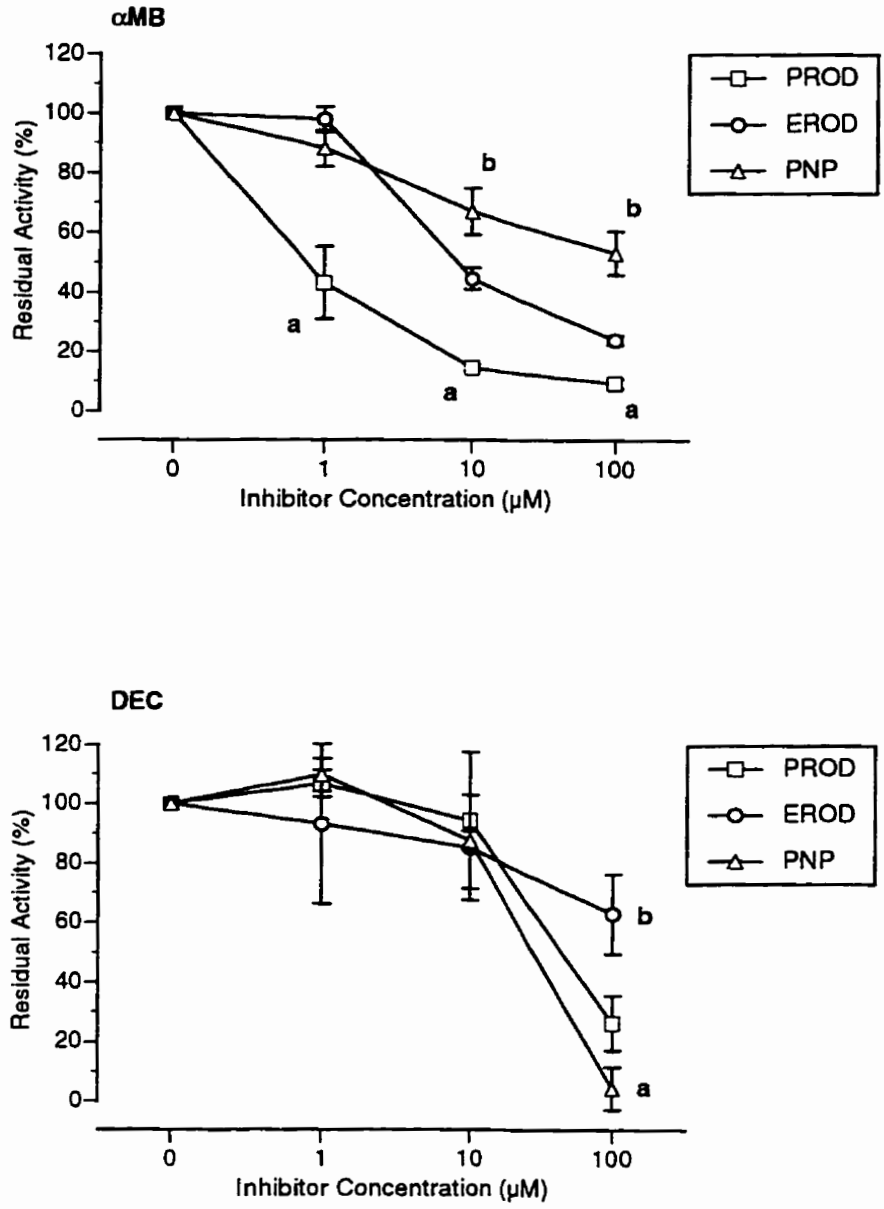

Figure 5.7
Dose-dependent inhibition of pulmonary CYP-dependent activity by αMB or DEC in microsomes prepared from corn oil treated rats. Control (100%) values for PROD, EROD and PNP activities were 37.9 ± 5.4, 5.0 ± 0.2 and 99.9 ± 14.8 pmol/min/mg microsomal protein, respectively. Values are mean ± SD (N=3). **a**, significantly different from PNP and EROD ($p<0.05$); **b**, significantly different from PROD and EROD ($p<0.05$).

5.4 Discussion

The data presented in this study demonstrate the differential effects of αNF and βNF on the expression CYP1A1- and CYP2E1-dependent catalytic activity and mRNA, in a tissue selective manner. While βNF dramatically increased the steady-state level of CYP1A1 mRNA and EROD activity in all of the tissues studied, the magnitude of CYP1A1 induction differed among the tissues studied. For example, while αNF caused only small increases of CYP1A1 in liver and kidney, lung CYP1A1 mRNA and EROD activity was increased to 60% of that observed for βNF treatment (Figs. 5.3, 5.4). A similar, but smaller effect was also observed for heart CYP1A1 mRNA and EROD activity. Prior studies have demonstrated the ability of αNF to inhibit CYP1A1 gene expression induced by AHR agonists through competitive interactions for binding with the AHR (Merchant *et al.*, 1992; Merchant and Safe, 1995). In addition, the ability of αNF to act as a weak AHR agonist and CYP1A1 inducer in cell culture has been documented (Santostefano *et al.*, 1993), although the concentrations required to increase CYP1A1 mRNA were quite high (10 μM). CYP1A1, CYP3A and UDP-glucuronosyltransferases, all of which have greater activities in liver compared with lung or heart, have been implicated in the metabolism of αNF and other related flavonoids (Andries *et al.*, 1990; Ebner and Burchell, 1993; Green *et al.*, 1994; Lee *et al.*, 1994). Thus, the efficacy of αNF for CYP1A1 induction in lung, and to a lesser extent in heart, may have been due to a higher effective concentration of the compound in these tissues as a result of deficient metabolism relative to the liver.

Treatment with βNF also resulted in an increase of CYP2E1 mRNA in all of the tissues studied, with the most pronounced effects occurring in heart, followed by kidney, lung and liver (Fig. 5.3). Similarly, liver and kidney CYP2E1-dependent PNP activity was also increased in microsomes prepared from βNF *vs.* corn oil treated animals. While substantial increases of EROD activity occurred in all of the tissues in response to βNF treatment, the increases of PNP activity were comparatively small. In addition, no PNP

activity was detected in heart microsomes prepared from corn oil, αNF or βNF treated rats, while in lung these treatments clearly decreased PNP activity. Thus, it is unlikely that CYP1A1 induction was responsible for the increased rates of PNP activity in any of the tissues studied. CYP2E1 expression can be influenced at the transcriptional, post-transcriptional and post-translational (Ronis *et al.*, 1996) levels, however none of these mechanisms have been reported to involve AHR-dependent signalling pathways. Thus, it is unlikely that the increases of CYP2E1 mRNA and catalytic activity observed here resulted from a direct transcriptional mechanism similar to that characterized for CYP1A1 (Rowlands and Gustafsson, 1997). Instead, it is more likely that CYP2E1 induction was secondary to the pathobiological changes, and in particular, the substantial weight loss associated with the AHR-mediated toxicity caused by βNF treatment. The animals in this study that were treated with βNF clearly experienced a significant decrease in total body mass, while animals treated with the much weaker AHR agonist, αNF, or corn oil, did not (Fig. 5.1). Similarly, decreases of a similar magnitude were also found for the organ masses of lung, kidney and heart, although statistical significance was not achieved in this case (Fig. 5.2). The lack of effect of αNF compared with βNF on total body and organ masses most likely reflects the weak AHR agonism and/or increased metabolism of this compound compared with βNF. Changes in nutritional status and fasting can increase CYP2E1 catalytic activity through both pre-translational mechanisms, as opposed to low dose, acute ethanol treatment, which appears to increase CYP2E1 catalytic activity principally through protein stabilization (Hu *et al.*, 1995). In the present study, the increase of PNP activity observed for kidney microsomes prepared from βNF treated rats was accompanied by an increase in the steady-state level of CYP2E1 mRNA. Furthermore, increases of CYP2E1 mRNA were also observed to differing degrees in all of the tissues obtained from βNF treated rats. In contrast, αNF treatment caused comparatively little effect on CYP2E1 mRNA levels in any of the tissues studied. Taken together, these data indicate that the increases of CYP2E1 mRNA and catalytic activity

associated with βNF treatment did not result from any direct actions of this compound on CYP2E1 expression, but rather occurred secondary to changes in nutritional status associated with AHR-mediated toxicity. Induction of oxidative stress, which may influence CYP2E1 expression (Tindberg and Ingelman-Sundberg, 1989), did not appear to be a contributing influence, as only minor changes in HO-1, a sensitive marker of oxidative stress, were observed in response to βNF treatment.

Similar to the other tissues studied, βNF treatment caused an elevation of CYP2E1 mRNA in rat lung, while αNF had a comparatively minor effect (Fig. 5.3). However, no increase of PNP activity was found, despite that PNP activity was readily detected with microsomes prepared from corn oil treated rats (Fig. 5.5). Instead, substantial decreases of lung microsomal PNP activity were associated with βNF or αNF treatment. This apparent disparity of effects on CYP2E1 mRNA and catalytic activity led us to consider the possibility that CYP isozyme(s) other than CYP2E1 may be primarily responsible for PNP activity in rat lung. Consistent with this, kinetic analysis has indicated the presence of at least two enzymes with distinct Km values that contribute to PNP activity in sheep lung microsomes (Arinc and Aydogmus, 1990). A substantial decrease of lung microsomal CYP2B1-dependent PROD activity was observed in the present study for animals treated with αNF or βNF (Fig. 5.6B). While smaller decreases of rat lung CYP2B1 catalytic activity have been reported elsewhere after βNF treatment (Rabovsky and Judy, 1987), direct comparison with our data is difficult due to the shorter duration of treatment employed in the previous study. Administration of Aroclor 1260, a mixture of PCBs, was previously shown to cause decreased rabbit lung CYP2B4 protein and catalytic activity, while liver and lung CYP1A1 was induced substantially (Serabjit-Singh *et al.*, 1983). Removal of the co-planar isomers and dibenzofurans from Aroclor 1260 did not affect the loss of lung CYP2B4, however induction of CYP1A1 was decreased substantially. Similarly, the selective down-regulation of mouse lung CYP2B10 in response to Aroclor 1254 has also been reported concomitant with induction of hepatic CYP1A1, CYP1A2 and

CYP2B, as well as renal and pulmonary CYP1A1 (Beebe *et al.*, 1995). Since a similar depression of lung CYP2B10 was observed in the previous study using both AHR-responsive (C57) and -non responsive (DBA) mouse strains, a lack of AHR involvement was indicated. While the negative correlation between EROD and PNP activities observed in this study suggests a relationship between AHR agonism and CYP2B1 expression in rat lung, it does not directly demonstrate a causal relationship.

A significant positive correlation was found between lung microsomal PNP and PROD activities in this study suggesting that CYP2B1 makes a major contribution to PNP activity in rat lung. Consistent with this, previous studies have demonstrated the ability of phenobarbital to increase hepatic PNP activity in rat (El Dieb *et al.*, 1993; Reinke and Moyer, 1985). While our studies with the CYP2B-selective MB inactivator αMB are consistent with a contribution by this isoform to lung microsomal PNP activity, the quantitative importance may be limited. For example, at a concentration of 100 μM, αMB inhibited 90% of lung microsomal PROD activity, while only approximately 50% of total PNP activity was lost. These data suggest that the contribution of CYP2B isozyme(s) to PNP activity in lung microsomes from untreated rats may be limited to 50%, assuming no concomitant inhibition of CYP2E1 by αMB. Interestingly, DEC caused similar losses of PROD and EROD activity of rat lung microsomes at all but the highest inhibitor concentration. It is likely that this reflects cross inhibition of CYP2B and CYP2E1 isozymes by DEC (Chang *et al.*, 1994), as well as the contribution of both of these isozymes to rat lung microsomal PNP activity. Thus, while these data do not definitively establish that lung PNP activity is attributable in large part to CYP2B1, they do demonstrate that CYP isoform(s) other than CYP2E1 make significant contributions to PNP activity in this tissue.

The magnitude of the increase of hepatic CYP2E1-dependent catalytic activity and mRNA observed in the present study is consistent with changes reported previously for rat hepatic PNP activity after βNF treatment (Reinke and Moyer, 1985). Similarly, increases

of rat intestinal CYP2E1 protein in response to treatment with the AHR agonists βNF or 3-methylcholanthrene have been detected using immunohistochemical methods (Matsuda *et al.*, 1995). While the toxic effects of AHR agonists with respect to the modulation of xenobiotic metabolizing enzymes have been extensively studied, our work indicates that CYP2E1 induction secondary to AHR mediated toxicity must also be considered. CYP2E1 has been implicated in the bioactivation of a variety of low molecular weight carcinogens such as benzene (Valentine *et al.*, 1996), as well as in the toxic effects of commonly consumed drugs such as acetaminophen and alcohol (Lee *et al.*, 1996b; Wu and Cederbaum, 1996). Furthermore, evidence exists indicating that reactive oxygen species are formed as a result of "leaky" CYP2E1-dependent metabolism in the presence and absence of substrates (Ingelman-Sundberg and Johansson, 1984; Persson *et al.*, 1990). Given the real possibility of exposure to AHR agonists, such as polyaromatic hydrocarbons contained within cigarette smoke, combined with other factors that can affect CYP2E1 such as nutritional status, concurrent exposure to substances bioactivated to toxic metabolites by CYP2E1 raises the possibility of toxicological interactions. The tissue selectivity of these effects, particularly in the case of the kidney where the largest changes of CYP2E1-dependent PNP activity were observed, suggests the possibility of organ specific effects. Finally these data raise a cautionary note for the interpretation of *in vivo* toxicology data derived from studies in which βNF is used as a selective CYP1A inducer. While this is essentially true in liver, a significant increase of kidney PNP activity and decrease of lung PNP and PROD activities were also observed. Given that changes, not only in the activity, but also the spectrum of CYP isozymes present in a tissue can cause profound changes in the metabolism of xenobiotics, the assumption that βNF is a highly selective inducer of CYP1A isozymes in all tissues is not valid. This is of particular significance to extrahepatic tissues where comparatively low levels of most xenobiotic metabolizing enzymes are expressed.

Chapter 6

Liver Transplantation Induces CYP1A1-Dependent Monooxygenase Activity in Rat Lung and Kidney[5]

6.1 Objectives

Although liver transplantation has been the subject of intensive study (Bussuttil *et al.*, 1994), comparatively little is known about the effects of the procedure on CYP-dependent metabolism. This deficiency of information is of particular significance with respect to the battery of drugs commonly administered to patients following liver transplantation (Zetterman, 1994). The characteristically small therapeutic indices and high toxicity of many immunosuppressive drugs require a thorough understanding of potential changes in drug metabolism following liver transplantation to design safe and effective drug therapies for this group of patients. Furthermore, due to the high incidence of multiple organ failure and renal toxicity associated with liver transplantation and immunosuppressive therapy (Bismuth *et al.*, 1993), the effect of this procedure on extrahepatic CYP activity is also of importance. The experiments presented in Chapter 5 have demonstrated that pathobiological stress associated with AHR-mediated toxicity has differing effects on CYP catalytic activity and steady-state mRNA levels, depending upon the isozyme and/or tissue studied. The objective of this study was to test the hypothesis

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5 A version of this chapter has been published.

Sinal CJ, Zhu L-F, Zhong R, Cherian MG and Bend JR (1995) Liver transplantation induces cytochrome P450 1A1 dependent monooxygenase activity in rat lung and kidney. *Can. J. Physiol. Pharmacol.* **73**: 146-152.

that differential isozyme- and organ-selective effects on CYP catalytic activity also occur during pathobiological states not associated with xenobiotic administration. To this end, orthotopic liver transplantation was used as a medically relevant model to assess hepatic and extrahepatic microsomal CYP-dependent metabolism during pathobiological stress in the rat.

6.2 Materials and Methods

6.2.1 Chemicals

NADPH and hemin were purchased from Sigma Chemical Co. (St. Louis, MO); 7-ethoxyresorufin, 7-pentoxyresorufin and resorufin from Molecular Probes Inc. (Eugene, OR); and all other chemicals (reagent grade or better) from BDH Inc. (Toronto, ON).

6.2.2 Animal Treatments

Male rats of inbred Lewis strain (250-300 g), obtained from Charles River (Montreal, PQ) were housed in individual plastic cages under controlled temperature conditions and 12 hr light and dark cycles. Rats were given water and standard rat chow ad libitum.

6.2.3 Liver Transplantation

Rat chow was withheld and the rats were given 5% glucose and 0.9% saline ad libitum 24 hr prior to surgery. Prior to, and during surgery, rats were anesthetized by inhalation of halothane (1.5% in air). Orthotopic, nonarterialized liver grafts were performed as previously described (Kamada and Calne, 1983). Briefly, the hepatic artery was ligated and divided and the graft was perfused *in situ* with 10 mL of heparinized lactate Ringer's solution at 4°C through a fine cannula inserted in the portal vein. The graft was removed and preserved in lactated Ringer's solution at 4°C. The livers of the recipient rats

were removed and the suprahepatic inferior vena cava was anastomosed end-to-end to the suprahepatic inferior vena cava of the donor liver with 7/0 prolen sutures. Similarly, the portal vein and infrahepatic inferior vena cava were also anastomosed using the cuff technique. The common bile ducts were then anastomosed with a stent. The rats were injected with 10 mg/kg morphine 1 hr after the operation and given food and water ad libitum. The rats were active and showed no sign of discomfort 12 hr after surgery. They were given penicillin G, 6 u/100g/day i.m. for 7 days. This experimental protocol was reviewed and approved by the animal use committee at the University of Western Ontario.

6.2.4 Preparation of Microsomes

Livers removed from the recipient animals, and lungs and kidneys from the donor animals, served as controls (donor) for the assays of CYP monooxygenase activity. Recipient animals were killed 1, 3, 7 or 21 days after transplantation by bleeding from the abdominal aorta under halothane anesthesia. Livers, lungs and kidneys from these animals served as experimental (recipient) samples. All tissues were rapidly removed, rinsed in 0.1 M potassium phosphate buffer (pH 7.4) containing 150 mM KCl, and were flash frozen in liquid nitrogen. Liver, lung and kidney microsomes were prepared by differential centrifugation as previously described (Bend *et al.*, 1972). Microsomal protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Microsomal CYP content was determined from the dithionite difference spectrum of carbon monoxide-saturated microsomes using a Beckman DU-65 spectrophotometer with ε=100 $mM^{-1} \cdot cm^{-1}$ (Estabrook *et al.*, 1972).

6.2.5 Assays of Microsomal Monooxygenase Activity

Rates of PROD and EROD activity were determined based on the methods of Burke and Mayer (1974) and Lubet *et al.* (1985) respectively. The measurements were performed in fluorimeter cuvettes maintained at 37°C with stirring in a Perkin-Elmer fluorescence

spectrophotometer (model LS-5B). Incubations contained 0.3 mg (hepatic PROD and EROD; pulmonary PROD) or 0.6 mg (pulmonary EROD; renal PROD and EROD) of microsomal protein and were made up to a volume of 1.42 mL with 0.1 M potassium phosphate buffer (pH 7.8). After 5 min of temperature equilibration, 5 μL 7-pentoxyresorufin or 7-ethoxyresorufin (both in Me_2SO; final concentration of 4 and 1μM, respectively) were added and a baseline fluorescence scan was recorded (excitation = 525 nm; emission = 585 nm). Reactions were initiated by the addition of 75 μL of NADPH dissolved in incubation buffer to give a final concentration of 1 mM. The reaction was allowed to proceed for 10 min and the rate of formation of resorufin was calculated by comparison to known amounts (5 pmol) of resorufin added in 5 μL Me_2SO to the incubations. All reactions were verified to proceed linearly at the substrate and protein concentrations chosen.

6.2.6 Assay of Microsomal Heme Oxygenase Activity

The rate of microsomal HO activity was determined based on the method of Trakshel *et al.* (1986). Briefly, incubations contained 1 mg/mL microsomal protein, 1.5 mg/mL 105 000 x g supernatant from liver homogenate as a source of biliverdin reductase and 25 μM hemin (dissolved in 0.1 M NaOH) made up to a final volume of 2 mL with buffer (0.1 M potassium phosphate , pH 7.4). These incubation volumes were divided into equal 1 mL aliquots (sample and reference) and were incubated at 37°C for 5 min. Reactions were started by adding NADPH (in buffer; 400 μM final) to the sample or an equivalent volume of buffer to the reference. After incubation for 8 min at 37°C, with shaking, the sample volume was scanned spectrophotometrically (Beckman DU-8) from 450 to 550 nm using the reference volume as a blank. The amount of bilirubin formed was quantitated from the absorbance change at 470 nm relative to 530 nm using an extinction coefficient of 40 $mM^{-1} \cdot cm^{-2}$ (Trakshel *et al.*, 1986).

6.2.7 Statistics

Statistical analysis was performed on the raw experimental data against the corresponding control data using the unpaired, two-tailed Student's t-test and StatView v4.51 for Macintosh software (Abacus Concepts, Berkeley, CA).

6.3 Results

6.3.1 Hepatic Cytochrome P450

PROD and EROD activities were used as selective markers for CYP2B- and CYP1A1-dependent monooxygenase activity, respectively (Burke *et al.*, 1985; Lubet *et al.*, 1985; Nerurkar *et al.*, 1993). In general, both of these activities were significantly decreased at all time points in hepatic microsomes from recipient *vs.* donor animals (Fig. 6.1). Similarly, hepatic microsomal CYP content was significantly decreased. In contrast, hepatic microsomal HO activity was markedly elevated at all time points in the recipient *vs.* donor animals. For example, HO activity for the recipients was maximal 7 days after transplantation, whereas hepatic CYP concentration, PROD and EROD activities were lowest at this time point.

Of interest, the decrease and recovery of hepatic CYP content and CYP-dependent oxidation appeared to occur in a biphasic manner. An initial decrease was observed after 1 day for transplant *vs.* control animals. Following this, a small but consistent recovery of CYP content and PROD and EROD activities was observed after 3 days. Subsequently, these values were lowest in recipient *vs.* donor animals 7 days after transplantation. Finally, a modest recovery of total CYP and EROD activity occurred after 21 days, with a more substantial recovery of hepatic PROD activity apparent. This pattern of alternating decreases and increases was also observed in a reciprocal manner for hepatic HO activity.

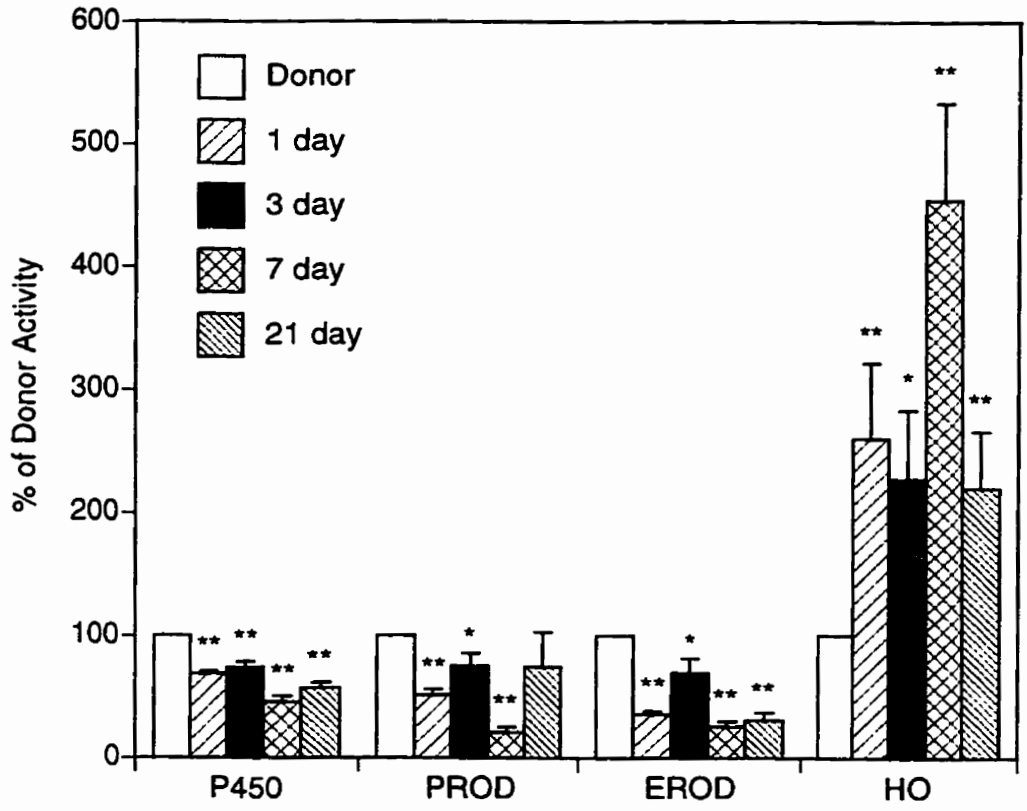

Figure 6.1
The effect of liver transplantation on hepatic microsomal CYP content, PROD, EROD and HO activities. Samples represent the means of data obtained from at least 5 individual livers (N=3 for 3 day group). Donor values for 1, 3, 7 and 21 day groups respectively, were 0.79 ± 0.07, 0.88 ± 0.06, 0.68 ± 0.04 and 0.70 ± 0.14 nmol/mg/protein for [CYP]; 10.9 ± 1.5, 11.5 ± 1.0, 9.7 ± 2.2 and 10.1 ± 2.5 pmol/min/mg protein for PROD; 126 ± 20, 80. ± 6, 139 ± 11 and 94 ± 18 pmol/min/mg protein for EROD; and, 38.2 ± 9.0, 50.6 ± 12.5, 26.6 ± 9.0 and 37.8 ± 17.9 pmol/min/mg protein for HO.
*$p < 0.05$, **$p < 0.01$, significantly different compared to donor group.

6.3.2 Pulmonary Cytochrome P450

Consistent with the data obtained for liver, a significant depression of both pulmonary PROD and EROD activities was found in recipient *vs.* donor animals 1 day after liver transplantation (Fig. 6.2). However, in contrast to the hepatic results, this decrease occurred without a concomitant elevation of pulmonary HO activity. Similar to the pattern observed for hepatic CYP, pulmonary PROD and EROD activities recovered substantially by 3 days after transplantation, however, a subsequent depression of the pulmonary activities was not observed at 7 or 21 days. CYP2B-dependent PROD activity in recipient animals was slightly elevated at 3 days, but was not significantly different from the donor animals at 7 or 21 days. In sharp contrast, CYP1A1-dependent EROD activity of lung microsomes from recipient animals was increased substantially 7 and 21 days (4.2- and 7.6-fold vs donor values), respectively, after transplantation. However, no changes in pulmonary HO activity were observed 3, 7 or 21 days after liver transplantation.

6.3.3 Renal Cytochrome P450

Similar to the hepatic and pulmonary data, a slight decrease of CYP-dependent PROD and CYP1A1-dependent EROD activity was apparent 1 day after transplantation for renal microsomes from recipient *vs.* donor animals (Fig. 6.3). However, these decreases were not significant. Furthermore, no significant change in PROD activity relative to the donor animals was noted after 3, 7 or 21 days. In contrast, renal EROD activity was increased by 7 and 21 days after transplantation. This increase was not as great as the pulmonary response, but was still substantial (approximately 2-fold of the donor animals after 7 and 21 days). Although an unexpected decrease of renal microsomal HO activity was observed in the recipient animals at 1 day, no significant changes were evident 3, 7 or 21 days after transplantation.

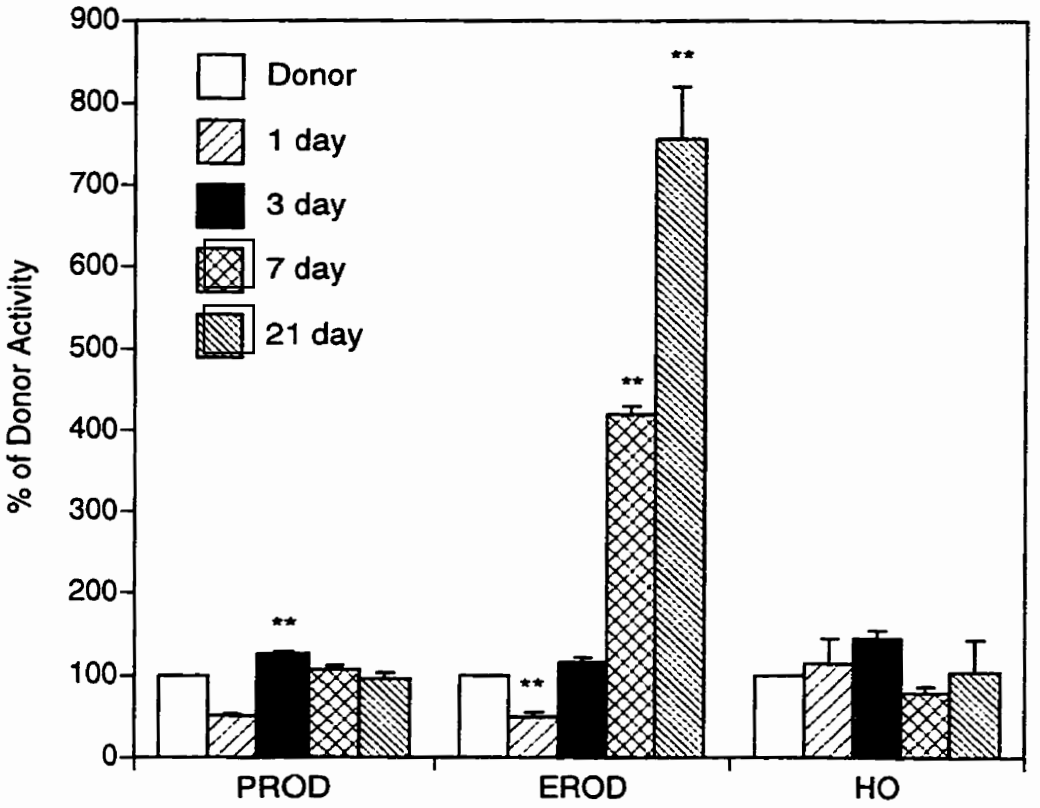

Figure 6.2
The effect of liver transplantation on pulmonary microsomal CYP-dependent PROD, EROD and HO activities. Samples represent data obtained from three individual experiments using pools of lung from 3-6 animals. Donor values (pmol/min/mg protein) for 1,3,7 and 21 day groups respectively, were 33.1 ± 2.2, 39.2 ± 1.7, 37.6 ± 0.6 and 34.2 ± 1.2 for PROD; 1.80 ± 0.11, 1.09 ± 0.27, 1.47± 0.24 and 1.05 ± 0.10 for EROD; and 21.5 ± 3.6, 38.3 ± 10.5, 32.2 ± 7.5 and 21.5 ± 5.6 for HO.
**$p < 0.01$, significantly compared to corresponding donor group.

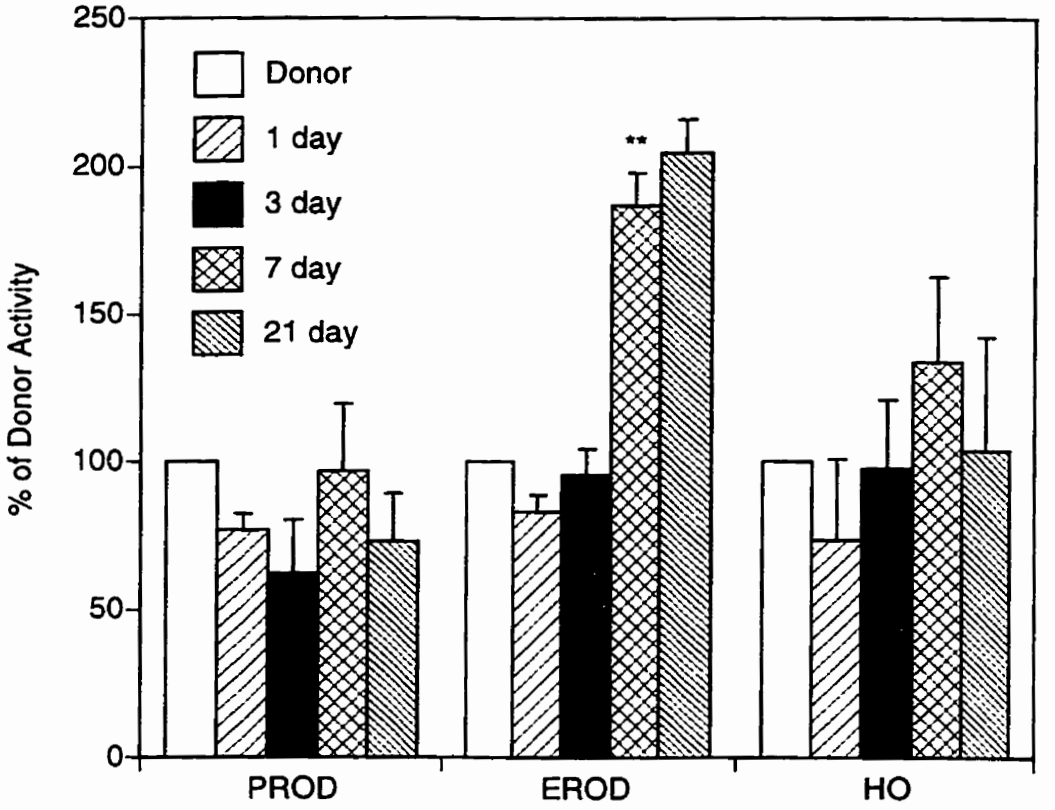

Figure 6.3
The effect of liver transplantation on renal microsomal CYP-dependent PROD, EROD and HO activities. Samples represent data obtained from three individual experiments using pools of kidney from 3-6 animals. Donor values (pmol/min/mg protein) for 1,3,7 and 21 day groups respectively, were 30.74 ± 0.12, 0.66 ± 0.18, 0.96 ± 0.12 and 0.74 ± 0.06 for PROD; 0.59 ± 0.02, 0.91 ± 0.06, 1.01 ± 0.08 and 0.62 ± 0.09 for EROD; and 19.7 ± 5.7, 27.2 ± 5.5, 38.4 ± 5.1 and 25.8 ± 7.2 for HO.
*$p < 0.05$, **$p < 0.01$, significantly different compared to donor group.

6.4 Discussion

Depression of hepatic CYP-dependent drug metabolism during inflammatory or immunostimulatory conditions is well known (Azri and Renton, 1991; Renton and Knickle, 1990). This effect can be mimicked by the direct administration of some cytokines including IL-1 (Barker *et al.*, 1992), IL-6 (Fukuda and Sassa, 1994), TNFα (Abdel-Razzak *et al.*, 1993b) and interferon (Cribb *et al.*, 1994) as well as by bacterial endotoxin (Morgan, 1993a). Therefore, it was not surprising to observe a decrease in hepatic microsomal CYP concentration and its associated monooxygenase activities after liver transplantation in the rat. Reperfusion injury involving free radicals, Kupffer cell activation and leukocyte adhesion is known to occur in recipients of liver transplants (Lemasters and Thurman, 1997; Marzi *et al.*, 1993; Takei *et al.*, 1991). Thus, a contributing factor to depression of hepatic CYP monooxygenase function observed in this study is probably the release of cytokines by Kupffer cells, activated blood cells and injured hepatocytes.

Cytokines generally down-regulate CYP at the transcriptional level (Abdel-Razzak *et al.*, 1993b) in an isozyme-selective manner. Recent evidence (Fukuda and Sassa, 1994) indicates that at least one cytokine, IL-6, may indirectly down-regulate hepatic CYP1A1 transcription by a decrease in the level of free heme as a result of microsomal HO induction. In addition to being necessary for CYP synthesis, free heme is also a positive regulator of CYP1A1 gene transcription in rat liver (Bhat and Padmanaban, 1988a; 1988b). Consistent with this, in our study, the level of hepatic HO activity was clearly inversely related to total microsomal CYP concentration as well as CYP monooxygenase activity. The direct degradation of CYP isozymes due to heme removal by HO, as suggested by Kutty *et al.* (1988), might have also been a contributing factor.

Finally, due to the substantial production of reactive oxygen species in the livers of transplant recipients (Connor *et al.*, 1992) it is also possible that peroxidative inactivation

of CYP isozymes (Karuzina and Archakov, 1994) occurred. Impaired cellular metabolism and activation of CYP-specific intracellular degradation mechanisms (Eliasson *et al.*, 1992) might also contribute.

In contrast to the hepatic data, pulmonary microsomal CYP1A1 displayed a time-dependent increase in activity up to 21 days after liver transplantation. Hepatic Kupffer cells represent the largest fixed macrophage population in the body. Therefore, the capacity of this cell mass for the release of cytokines is expected to be large, and to have a substantial systemic influence. Furthermore, the pulmonary circulation represents the first major vascular bed into which substances released by the liver will be delivered. Thus, effects similar to those seen in the liver might be expected. The high incidence of cytokine-related pulmonary vascular and respiratory problems experienced by liver transplant recipients strongly suggests this occurs (Afessa *et al.*, 1993; Colletti *et al.*, 1990). Consistent with this concept, we observed an initial decrease in pulmonary CYP-dependent PROD and EROD activity 1 day after transplantation. However, the subsequent recovery of pulmonary CYP and selective induction of CYP1A1-dependent EROD activity suggest that additional mechanisms are active. It is possible that the level of inflammatory mediators released from the liver decreases over time such that their efficacy becomes limited to the immediate site(s) of injury. Additionally, secondary sites of inflammation in the lung may have a greater influence on pulmonary CYP monooxygenases than substances released from the primary site of injury. At least one cytokine, IL-2, has been shown to induce CYP1A1 catalytic activity in rat liver (Kurokohchi *et al.*, 1993). Therefore, a further possibility is the alteration of the spectrum of inflammatory mediators released from the liver and/or secondary sites of inflammation over time.

The delayed increase of pulmonary CYP1A1-dependent activity might also suggest an indirect effect, possibly involving perturbation of a physiological substrate. For example, the congenitally jaundiced Gunn rat exhibits a marked endogenous activation of hepatic CYP1A1 and CYP1A2 (Kapitulnik and Gonzalez, 1993). Bilirubin may represent

an endogenous substrate for CYP1A1 (De Matteis *et al.*, 1991b). Therefore, endogenous activation of CYP1A1 in the Gunn rat may be related to the hyperbilirubinemia caused by UDP-GT deficiency. Consistent with this, it is possible that hyperbilirubinemia produced by impaired hepatic function and HO induction in the recipient rats of this study contributed to the increase in pulmonary microsomal CYP1A1-dependent EROD activity. Furthermore, the lack of pulmonary HO induction might allow for exposure to elevated plasma levels of liver-derived bilirubin in the absence of any HO-mediated limitation of free heme in lung cells. Increase of EROD activity was probably not observed in our transplanted livers due to the greater influence of cytokine-mediated CYP depletion, HO-mediated heme degradation and oxidative damage. Work from our laboratory has shown that administration of a single dose of sodium arsenite to male Sprague-Dawley rats results in a tissue- and isozyme-selective increase in pulmonary CYP1A1-dependent catalytic activity, while hepatic CYP-dependent activity is markedly decreased (Albores *et al.*, 1995). The similar effects of pathobiological stresses produced by arsenite administration or liver transplantation suggest that analogous mechanisms contribute to the modulation of CYP activity in both models.

The pattern of CYP-monooxygenase modulation seen in recipient renal microsomes was similar to that of lung. Thus, it is likely that the mechanisms responsible for the pulmonary effects are applicable to the kidney. The lower magnitude of the increase in renal CYP1A1-dependent EROD activity (compared to lung) may reflect a lower target concentration of inflammatory mediator(s) and/or reduced responsiveness of this tissue. Studies of drug metabolism in the kidney after liver transplantation are important due to the high incidence of acute renal toxicity during immunosuppression therapy (Porayko *et al.*, 1993; 1994). The data presented in this study indicate that the elevated plasma level of drugs such as cyclosporin A and FK-506 in liver transplant recipients is due, at least partially, to depressed hepatic CYP-monooxygenase activity. Specifically, hepatic CYP3A-dependent activity, which is known to be involved in the metabolism of many of

these drugs, is also decreased after liver transplantation in the human (Azoulay *et al.*, 1993). Furthermore, the risk of acute renal toxicity is most severe in the first week after liver transplantation (Porayko *et al.*, 1993; 1994). In our study, CYP-dependent oxidation in recipient renal microsomes was depressed 1 day after transplantation. However, by 7 days after transplantation, at least one CYP-dependent activity was increased. Thus, while hepatic metabolism is maximally decreased 1 week after transplantation, it is possible that the increase of extrahepatic CYP1A1-dependent metabolism may provide partial compensation and confer a protective effect against the toxicity of any immunosuppressive drugs oxidatively metabolized by this isoform. This is not very important for cyclosporin A or FK-506 for their major pathways of oxidation are catalyzed by CYP3A4 and CYP3A2 in humans and rats, respectively (Isogai *et al.*, 1993; Karanam *et al.*, 1994).

In conclusion, liver transplantation in the Lewis rat results in a significant depression of hepatic cytochrome P450 monooxygenase activity up to 21 days after surgery. Simultaneously, CYP1A1-dependent oxidation in pulmonary and renal microsomes is substantially increased, whereas CYP2B-dependent metabolism in lung is essentially unchanged. These results are consistent with other work in our laboratory demonstrating a similar tissue- and isozyme-selective increase of pulmonary CYP1A1 catalytic activity after a single dose of sodium arsenite. The tissue specific alterations observed in this study are most likely the result of a variety of inflammatory responses involving cytokines and possible impairment of the metabolism of endogenous substrates of CYP isozymes. The data presented in this study suggest the existence of generalized stress responses to inflammation, both locally and secondary to the primary insult. The results of this study demonstrate that changes in hepatic and extrahepatic CYP-dependent metabolism do occur as a result of liver transplantation. In lung and kidney these are related to isozyme-selective up-regulation. Through the use of this and other model systems, such as arsenite administration, the role of CYP isozyme modulation in the generalized stress responses of cells and tissues can be clarified.

Chapter 7

Modulation of Liver, Lung and Kidney Cytochrome P450 After Acute Sodium Arsenite Administration in the Rat

7.1 Objectives

Arsenite (As^{+3}) is a naturally occurring metalloid that has been associated with an increased risk of cancer in certain highly exposed populations (Chen *et al.*, 1992a). Induction of various hepatic chemical detoxication enzymes, such as NADPH:quinone oxidoreductase (Falkner *et al.*, 1993a) and metallothionein (Albores *et al.*, 1992b), as well as heat shock proteins (Bauman *et al.*, 1993) and HO (Keyse and Tyrrell, 1989), occurs rapidly following As^{+3} treatment. Acute As^{+3} treatment also decreases hepatic microsomal CYP content and catalytic activity (Albores *et al.*, 1992a). However, comparatively little is known regarding the effects of this metalloid on extrahepatic CYP. This is of considerable interest with respect to the pivotal role of these enzymes in chemical carcinogenesis (Guengerich, 1991; Shimada *et al.*, 1992) and the fact that extrahepatic organs, such as the lung, are known to be sites of As^{+3}-mediated carcinogenesis (Hertz-Picciotto and Smith, 1993).

Previously, As^{+3} was shown to potentiate the induction of guinea pig pulmonary CYP1A1-dependent EROD activity by βNF, while causing substantial decreases of CYP-dependent metabolism and increases of HO-dependent metabolism in the liver (Falkner *et al.*, 1993b). Subsequently it was reported that As^{+3}, in the absence of known CYP inducers, selectively increases CYP1A1-dependent EROD activity in rat lung (Albores *et*

al., 1995). Published data (Sinal *et al.*, 1995) and the experiments presented in Chapter 6 demonstrate that orthotopic liver transplantation in the rat causes a similar isozyme-selective increase of CYP1A1-dependent EROD activity in the lung and kidney, while severely compromising CYP-dependent metabolism and inducing HO in the liver. The objective of this study was to test the hypothesis that acute administration of As^{+3} causes a selective increase of CYP1A1 mRNA and catalytic activity by a mechanism similar to that observed for other pathobiological states such as orthotopic liver transplantation. To this end, the effect of this treatment on the CYP content, catalytic activity and mRNA levels in rat liver, lung and kidney was assessed over a period of ten days. The catalytic activity and mRNA levels of HO, a sensitive marker of oxidative stress, were determined over the same time period. To investigate the possibility that perturbations in the levels of circulating heme and its metabolites may contribute to the extrahepatic modulation of CYP observed during conditions of pathobiological stress, plasma bilirubin levels were also monitored.

7.2 Materials and Methods

7.2.1 Materials

Sodium arsenite, NADPH, hemin, bilirubin, PB and *n*-lauroylsarcosine were purchased from Sigma Chemical Co. (St. Louis, MO); 7-pentoxyresorufin, 7-ethoxyresorufin and resorufin were from Molecular Probes, Inc. (Eugene, OR); [α-^{32}P]dCTP (>3000 Ci/mmol) from ICN Biomedicals Canada (Montreal, PQ); Prime-a-Gene random primer DNA labelling kit from Promega (Madison, WI); Hybond-N nylon filters from Amersham Canada Ltd. (Oakville, ON); all restriction enzymes from Pharmacia Canada Inc. (Baie d'Urfe, PQ); *Taq* DNA polymerase and Superscript II RNase H$^-$ reverse transcriptase were from GIBCO BRL Canada (Burlington, ON); PCR primers were synthesized and purified by high performance liquid chromatography by General Synthesis

and Diagnostics (Toronto, ON); all other chemicals (reagent grade or better) were purchased from BDH Inc. (Toronto, ON)

7.2.2 Animal Treatment and Preparation of Microsomes

Male Sprague-Dawley rats (250-300 g) (Charles River, St. Constant, PQ) were injected s.c. with sodium arsenite in saline at 75 μmol/kg. Control animals were injected with saline alone. Standard rat chow and water were provided ad libitum. The animals were killed by CO_2 asphyxiation at the specified intervals after injection. This experimental protocol was reviewed and approved by the animal use committee at the University of Western Ontario.

Lung, kidney and liver microsomes were prepared by differential centrifugation of homogenized tissue as previously described (Bend *et al.*, 1972). Microsomal protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. Hepatic microsomal CYP content was determined from the carbon monoxide difference absorption spectrum of dithionite reduced microsomes with $\varepsilon = 91\ mM^{-1} \cdot cm^{-1}$.

7.2.3 Assays of Microsomal Monooxygenase Activity

Rates of PROD and EROD activity were determined essentially as previously described (Albores *et al.*, 1995) based on the methods of Lubet *et al.* (1985) and Burke and Mayer (1974), respectively. Briefly, all measurements were performed in fluorimeter cuvettes maintained at 37°C with stirring in a Perkin-Elmer LS-5B fluorescence spectrophotometer. Incubations contained 0.3 mg (liver and lung) or 0.75 mg (kidney) of microsomal protein in 0.1 M potassium phosphate buffer (pH 7.8). After temperature equilibration, 7-pentoxyresorufin or 7-ethoxyresorufin (both in Me_2SO) were added to final concentrations of 4 and 1 μM, respectively. Reactions were started by the addition of NADPH to a final concentration of 1 mM. Changes in fluorescence (excitation = 525 nm;

emission = 585 nm) were monitored for 10 min, and the rate of formation of resorufin was calculated by comparison with known amounts (5 pmol) of resorufin added to the incubations. Product formation was verified to be linear with time and protein concentration under these reaction conditions.

7.2.4 Assay of Microsomal Heme Oxygenase

The rate of microsomal HO activity was determined essentially as described previously (Sinal *et al.*, 1995) based on the method of Trakshel *et al.* (1986). Briefly, incubations contained 1 mg/mL microsomal protein, 1.5 mg/mL 105 000 x g supernatant protein from control liver homogenate as a source of biliverdin reductase, and 25 μM hemin. Incubations were performed at 37°C using 0.1 M potassium phosphate buffer (pH 7.4) and were initiated by adding NADPH to a final concentration of 400 μM. The amount of bilirubin formed was quantitated from the absorbance change at 470 nm relative to 530 nm, using an extinction coefficient = 40 $mM^{-1} \cdot cm^{-1}$. Product formation was verified to be linear with time and protein concentration under these reaction conditions.

7.2.5 cDNA Probes

The 1406 bp cDNA probe for rat CYP1A1 mRNA was obtained by EcoRI/PstI digestion of plasmid pA8 (Hines *et al.*, 1985) which was generously provided by Dr. Ronald Hines (Wayne State University). The 484 bp cDNA probe for rat CYP2B1/2 mRNA was obtained by HindIII/NcoI digestion of plasmid pB7 (Affolter *et al.*, 1986) which was generously provided by Dr. Alan Anderson (Laval University). A cDNA probe for rat HO-1 mRNA was prepared by reverse transcription coupled to polymerase chain reaction amplification (RT-PCR) using the primers FHO1 5'-GAGGTGCACATCCGTGCAGAGA-3' (forward) and RHO1 5'-GCAGTCATGGTCAGTCAACATGG-3' (reverse). Briefly, 1 μg of total RNA from the liver of a sodium arsenite-treated rat was used as a template for first strand cDNA synthesis

using reverse transcriptase according to the manufacturer's (BRL) specifications and by using RHO1 as the primer. Following this, 10% of the cDNA products was added to a standard PCR mixture according to the manufacturer's instructions (BRL) using FHO1 and RHO1 as specific primers. PCR amplification was performed with a Perkin Elmer DNA thermal cycler (model 480) using the following temperature profile: initial denaturation for 5 min at 95°C; 35 cycles of 45 sec at 95°C (denaturation), 45 sec at 60°C (annealing) and 1.5 min at 72°C (extension); and a final extension of 6 min at 72°C. Subsequently, the 1132 bp cDNA product, corresponding to nucleotides +67 to +1196 of the published sequence (Shibahara *et al.*, 1985), was purified from the PCR reaction by agarose (1%) gel electrophoresis. Prior to labelling, the HO-1 cDNA was digested with EcoRI/HindIII to yield an 883 bp product corresponding to nucleotides +87 to +970 of rat HO-1 mRNA. This fragment was purified from a 1% agarose gel for use as a specific probe for rat HO-1 mRNA. All probes were ^{32}P-labeled by the random primer method according to the manufacturer's (Promega) instructions.

7.2.6 Preparation and Analysis of Total RNA

Total cell RNA was isolated by the acid/guanidinium thiocyanate/phenol/chloroform extraction method of Chomczynski and Sacchi (1987). Northern blot analysis of total RNA was performed as described elsewhere (Sambrook *et al.*, 1989). Briefly, aliquots of RNA were separated in a denaturing (2.2 M formaldehyde) agarose (1.2%) gel and transferred to Hybond-N nylon filters. The RNA was fixed to the filters by baking at 80°C for 2 hr. Pre-hybridization of the filters was carried out in a solution containing 6 x SSC (0.9 M NaCl, .09 M sodium citrate), 50% deionized formamide, 5 x Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin), 0.5% SDS and 100 µg/mL sheared salmon sperm DNA for 4 hours at 42°C. Hybridization with the ^{32}P-labeled cDNA probes was carried out in the same solution, minus Denhardt's reagent, for 16-24 hr at 42°C. The filters were then washed twice at room temperature in 2 x SSC,

0.5% SDS for 15 min. This was followed by a 30 min wash in 0.1 x SSC, 0.5% SDS at 42°C and a final 30 min wash in 0.1 x SSC, 0.5% SDS at 65°C for 30 min. The washed filters were sealed in plastic wrap and exposed to Kodak X-OMAT AR film in the presence of an intensifier screen at -80°C. Hybridization signals were quantitated by densitometry and are expressed relative to the signals obtained for GAPDH mRNA.

7.2.6 Assay of Plasma Bilirubin

Whole blood was collected from saline and sodium arsenite treated rats under phenobarbital anesthesia (200 mg/kg) by cardiac puncture using a heparinized butterfly cannula apparatus. Blood was collected into heparinized tubes and immediately centrifuged at 10 000 x g for 10 min at 4°C. The plasma supernatant was aliquoted to fresh tubes and stored at -80°C until analysis (< 1 week later). Colourimetric determination of total plasma bilirubin was performed after coupling with diazotized sulfanilic acid using a commercially available assay kit (Total Bilirubin Kit, Sigma Chemical Co., St. Louis MO). The concentration of total bilirubin in plasma was quantitated by comparison with known amounts of bilirubin standard prepared in a 1 mg/mL solution of bovine serum albumin. All of the procedures described above were performed under low light conditions to minimize photooxidation of bilirubin.

7.2.8 Statistics

All experimental data were analyzed by the unpaired, Student's *t*-test for statistically significant differences between the arsenite-treated and corresponding saline control groups using StatView v4.51 for Macintosh software (Abacus Concepts, Berkeley, CA).

7.3 Results

7.3.1 Hepatic Cytochrome P450 Content

Treatment of rats with a single dose (75 μmol/kg, s.c.) of sodium arsenite resulted in a significant decrease (66%) in total hepatic CYP concentration after 1 day when compared with saline-treated controls (Fig. 7.1). Following this, a time-dependent recovery of CYP occurred, with no significant differences apparent between control and treated animals from 2 days onward.

7.3.2 Modulation of Microsomal Cytochrome P450 and Heme Oxygenase Activities

PROD activity is primarily catalyzed by CYP2B isozymes in rat liver and lung, while EROD activity is mainly due to CYP1A1/1A2 isozymes in rat liver and CYP1A1 in rat lung and kidney (Burke *et al.*, 1985). Thus, these activities are useful markers for differential effects of acute sodium arsenite administration on CYP-dependent catalytic activity. Consistent with the total CYP content data, significant decreases of hepatic PROD and EROD activity, compared with saline-treated controls, were observed 1 day after a single dose of sodium arsenite (Fig. 7.2). Similarly, renal EROD activity was also significantly decreased after 1 day. There were also indications of decreased renal PROD and lung PROD and EROD activity, however these changes were not significant.

Liver and kidney PROD activity fully recovered 2 days after arsenite treatment and remained equivalent to control levels up to 10 days afterward. A similar recovery was seen for lung microsomes, however an apparent increase in PROD activity occurred 7 and 10 days after As^{+3} treatment. This difference was significant only after 10 days when the PROD activity was approximately 125% of control.

Similar to PROD, EROD activity displayed substantial, but differing degrees of recovery from 2 days onward. For example, 2 days after treatment, liver EROD activity of

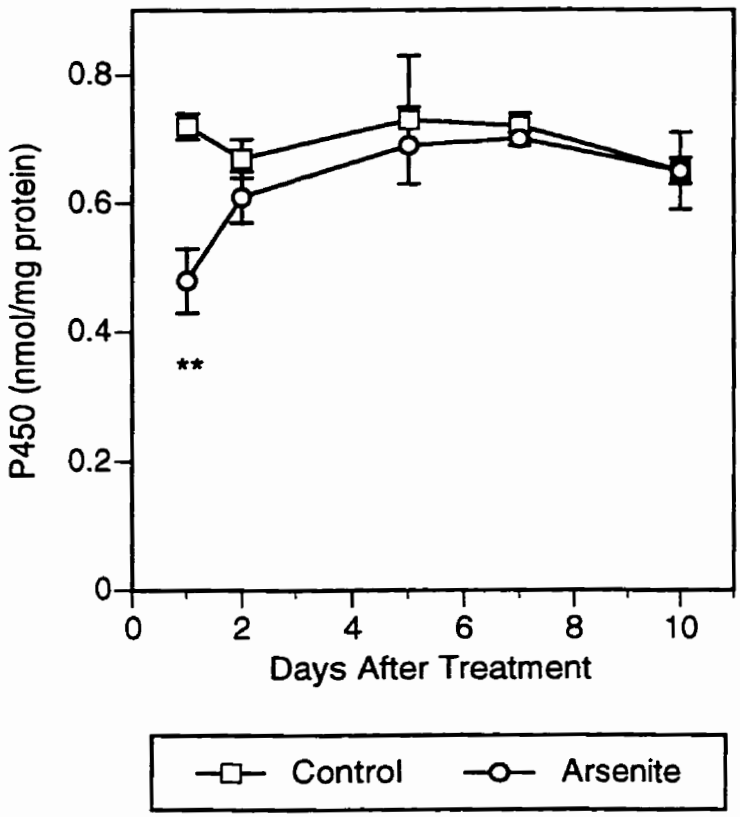

Figure 7.1
Time course for changes in liver microsomal CYP content of rats 1, 2, 5, 7 or 10 days after treatment with saline (control) or sodium arsenite. Each data point represents the mean ± standard deviation of duplicate experiments performed with the livers of three individual animals. ** $p<0.01$ compared with the respective saline control group.

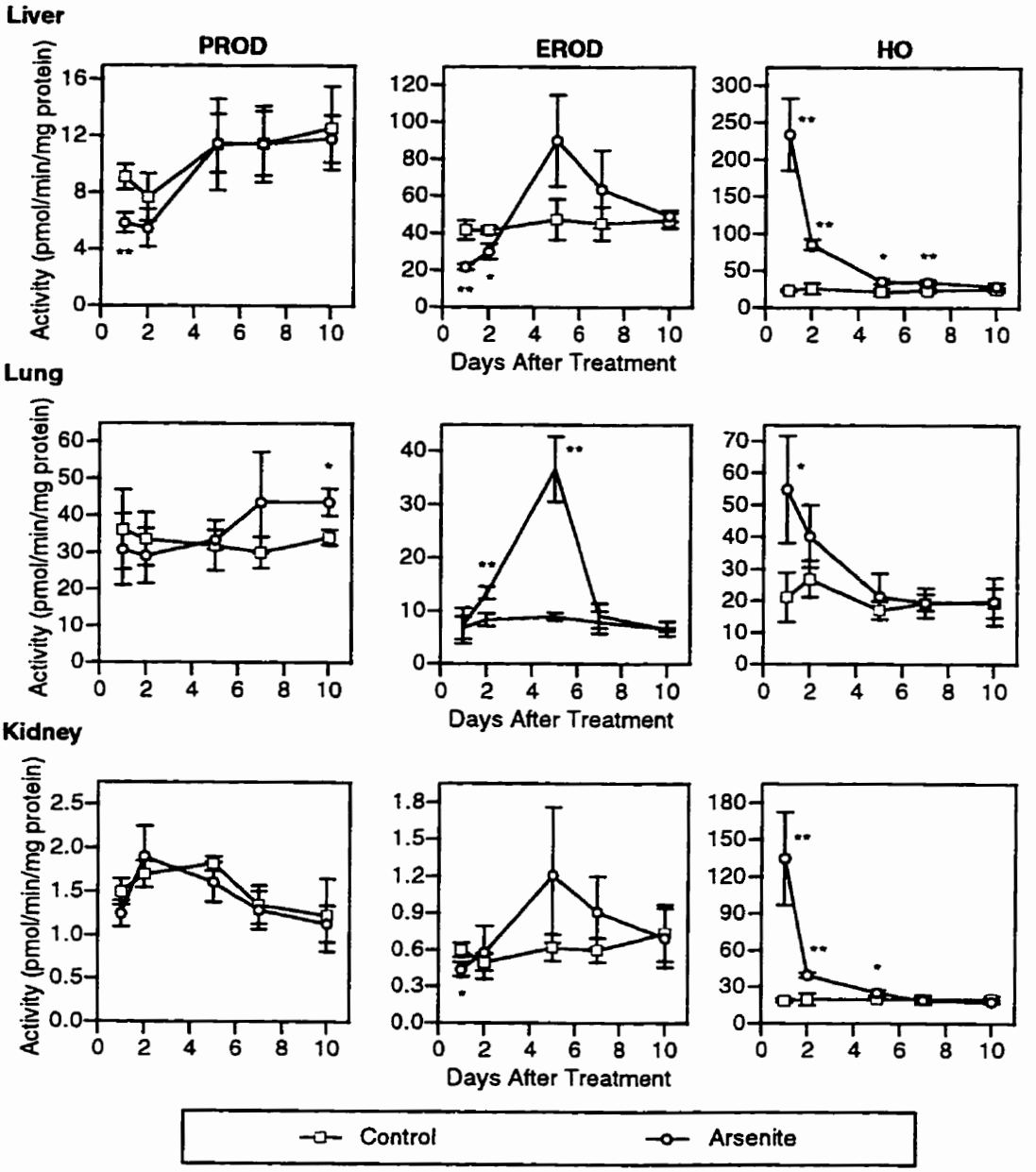

Figure 7.2
Time course for changes in liver, lung and kidney microsomal PROD, EROD and HO activities of rats 1, 2, 5, 7 or 10 days after treatment with saline (control) or sodium arsenite (As^{+3}). Each data point represents the mean ± standard deviation of duplicate experiments performed with the tissues of three individual animals. * $p<0.05$ and ** $p<0.01$ compared with the respective saline control group.

microsomes from sodium arsenite-treated animals remained significantly lower than that of saline-treated controls (Fig. 7.2). On the other hand, kidney EROD was not different from control and lung EROD was significantly increased to approximately 160% of control values. However, a marked increase of microsomal EROD activity occurred uniformly for all tissues 5 days after As^{+3} treatment. Although liver and kidney EROD activities were increased to 190% and 200% of control values, respectively, only the increase in lung EROD activity (400%) was found to be statistically significant. Following this, a decline to control EROD values was observed for all tissues by 10 days after As^{+3} treatment.

In contrast to total CYP, microsomal HO activity was elevated dramatically by 1 day after As^{+3} treatment (Fig. 7.2). The relative magnitude of induction of HO activity for the three tissues studied was liver > kidney > lung. Interestingly, this order of induction was paralleled by the relative order of decreases of CYP content and/or activity observed for these tissues. The amount of time required for the return of HO activity to basal levels in each of the tissues appears to be related to the initial level of induction. For example, liver HO activity was approximately 1000% of control 1 day after As^{+3} treatment and did not return to control levels until 10 days post-treatment. In contrast, lung HO activity was only approximately 160% of control 1 day after As^{+3} treatment, but was not significantly different from control after 2 days. Similar to the decreases in CYP-dependent catalytic activity 1 day after As^{+3} treatment, the magnitude of increase of EROD activity appears to have been inversely related to the level of HO induction in the three tissues studied. Specifically, in the lung, HO activity was increased the least after 1 day, and returned to control values more rapidly than either the liver or kidney. Similarly, EROD activity was increased much more dramatically in the lung than in the other tissues studied.

7.3.3 Modulation of Cytochrome P450 and Heme Oxygenase mRNA

To determine if the modulation of CYP-dependent PROD and EROD activity is mediated, at least in part, at the pre-translational level, we carried out northern blot

hybridization analysis for CYP1A1, CYP2B1/2, HO-1 and GAPDH mRNAs (Fig. 7.3). Hybridization of total lung RNA blots with a cDNA probe for CYP1A1 mRNA showed a time-dependent increase in the accumulation of this message species 1,2 and 5 days after As^{+3}-treatment when compared with saline-treated controls. In contrast, no significant changes in the mRNA levels for CYP2B1/2, HO-1 or the housekeeping gene GAPDH were apparent at any time point. CYP1A1 or CYP2B1/2 transcripts were not detected in blots prepared from liver or kidney RNA 5 days (Fig. 7.3) or at any other time point after saline or As^{+3} treatment (data not shown). Weak signals were obtained for HO-1 mRNA on liver blots prepared from As^{+3}- but not saline-treated rats 1 day after treatment (data not shown). However, this message species was not detected on liver or kidney blots after 5 days (Fig. 7.3) or at any other time point (data not shown).

Analysis of the densitometry signals obtained for lung CYP1A1 and CYP2B1/2, relative to GAPDH mRNA (Fig. 7.4) showed a time-dependent increase in the accumulation of CYP1A1 mRNA which corresponded well with the increases measured for CYP1A1-dependent EROD activity (Fig. 7.2). Significant increases in the level of this transcript were noted 1 (146% of saline control), 2 (196%) and 5 days (437%) after treatment. In contrast, no significant changes in CYP2B1/2 mRNA were noted for saline-*vs.* As^{+3}-treated animals.

7.3.4 Modulation of Plasma Bilirubin Levels

Heme metabolism and elimination of bile pigments are profoundly altered by As^{+3} treatment (Albores *et al.*, 1989). As an indicator of the effect of As^{+3} treatment on heme and bile pigment metabolism of the animals used in this study, total plasma bilirubin was measured. As shown in Fig. 7.5, As^{+3} treatment caused a rapid increase of plasma bilirubin concentration, which was maximal (11-fold) 1 day after treatment, compared with saline treated controls. The highest concentrations of plasma bilirubin corresponded well with the time (1 day) of the greatest HO-1 induction after As^{+3} treatment in all of the tissues

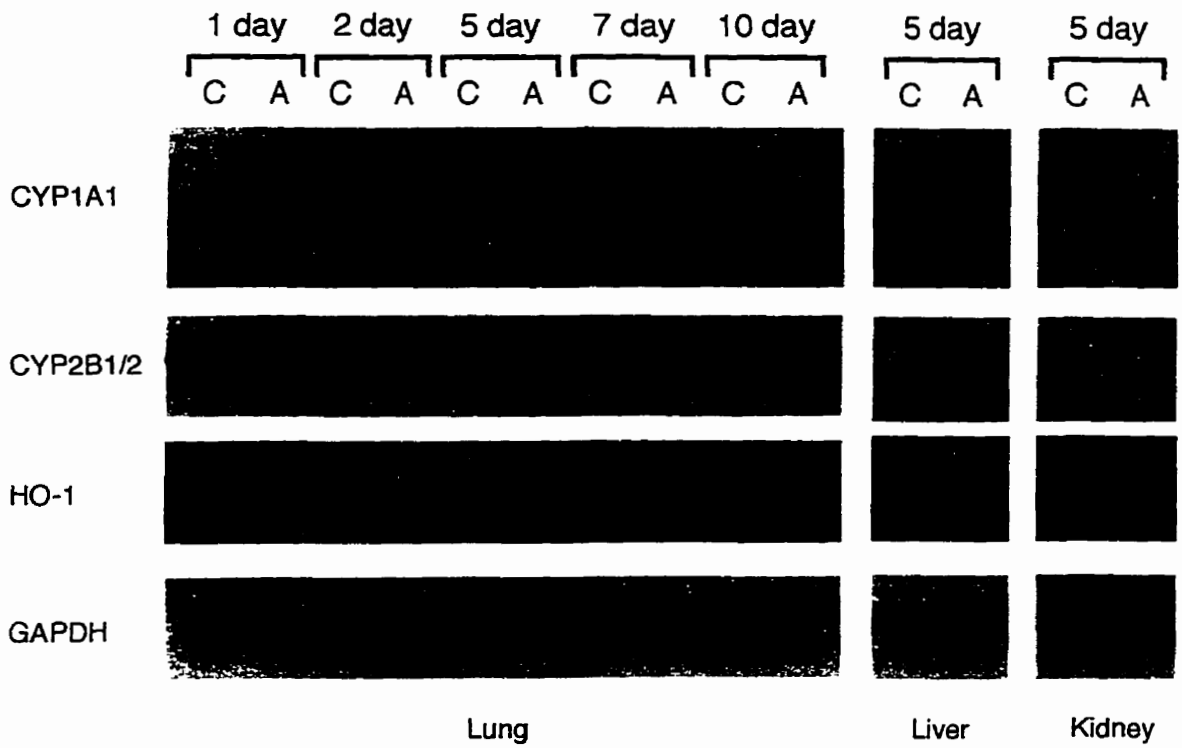

Figure 7.3
Northern blot analysis of total RNA isolated from the liver, lung and kidney of rats 1, 2, 5, 7 or 10 days after treatment with saline (C) or sodium arsenite (A). Total RNA (25 μg) was separated on a 1.2% denaturing (formaldehyde) agarose gel, transferred to nylon membranes and was hybridized with ^{32}P-labeled cDNA probes specific for CYP1A1, CYP2B1/2, HO-1 and GAPDH mRNAs.

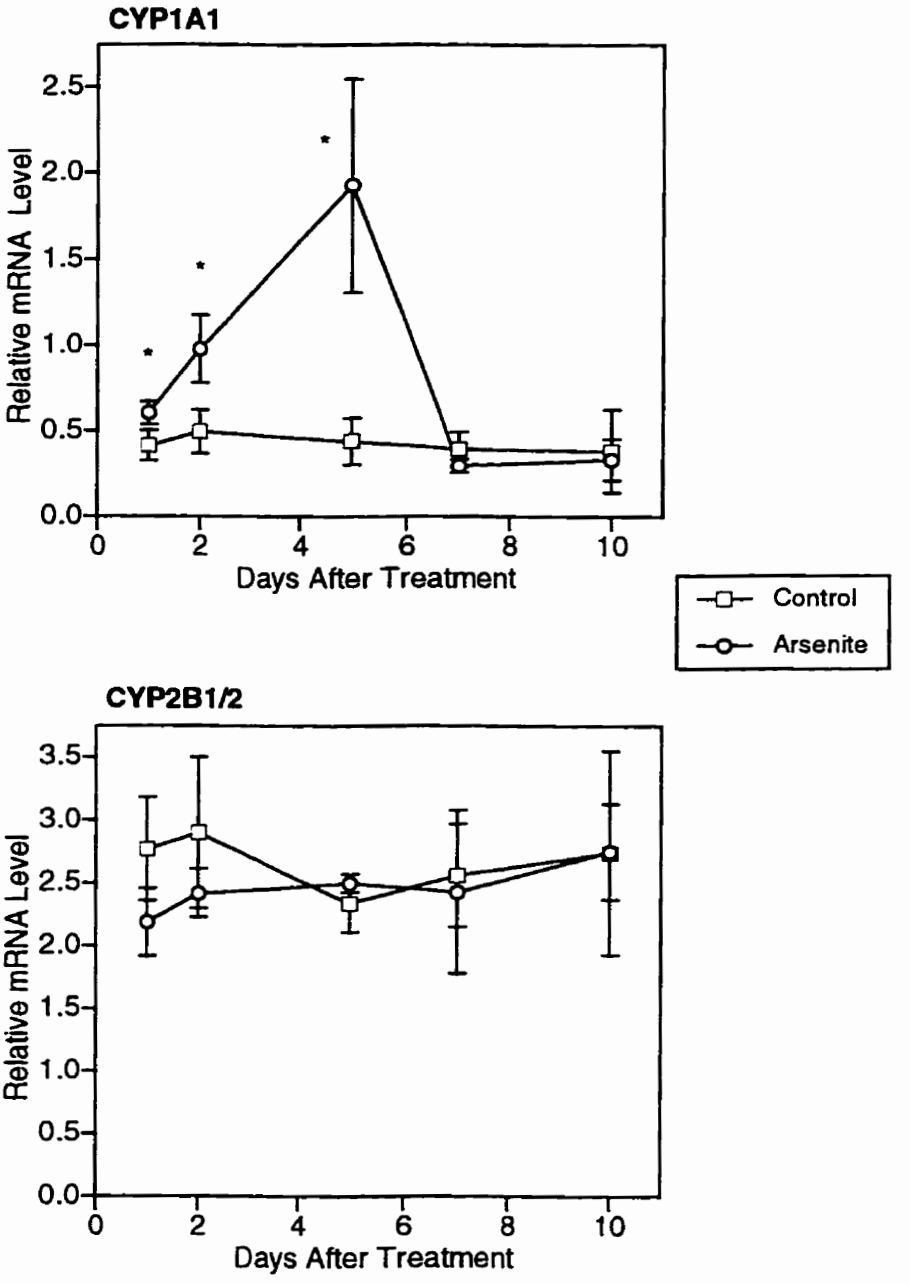

Figure 7.4
Time course for changes in lung CYP1A1 and CYP2B1/2 mRNAs of rats 1, 2, 5, 7 or 10 days after treatment with saline (control) or sodium arsenite (As^{+3}). The mRNAs were quantitated by densitometry and are expressed relative to the signal intensities obtained for GAPDH mRNA. Each data point represents the mean ± standard deviation of experiments performed with the lungs of three individual animals. * $p<0.05$ compared with the respective saline control group.

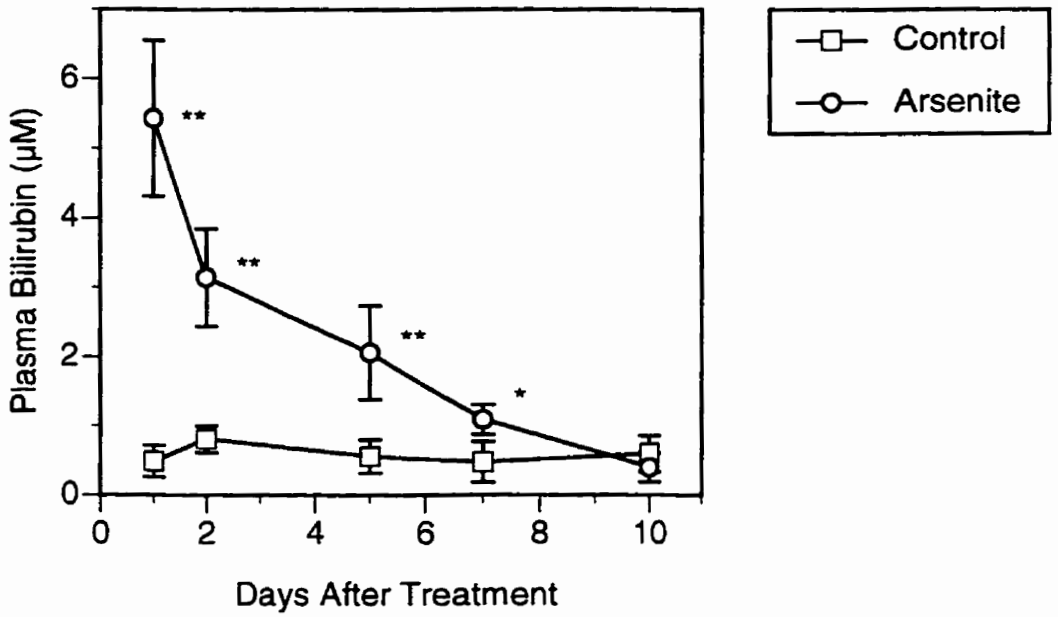

Figure 7.5
Time course for changes in serum bilirubin level of rats 1, 2, 5, 7 or 10 days after treatment with saline (control) or sodium arsenite (As^{+3}). Each data point represents the mean ± standard deviation of experiments performed with the serum of three individual animals. * $p<0.05$ and ** $p<0.01$ compared with the respective saline control group.

studied (Fig. 7.2). Furthermore, a temporal correspondence with maximum depression of hepatic CYP content, PROD and EROD activity in response to As^{+3} treatment was also observed (Figs. 7.1, 7.2). Consistent with the modulation of hepatic HO-1 activity, total plasma bilirubin remained significantly elevated in As^{+3}- *vs.* saline-treated rats up to 10 days after treatment.

7.4 Discussion

We have reported increased rat pulmonary CYP1A1-dependent EROD activity after acute sodium arsenite treatment in the rat (Albores *et al.*, 1995). To our knowledge, the findings presented in that study demonstrated, for the first time, a selective increase of the catalytic activity of a CYP isozyme after As^{+3} treatment. We report here that this phenomenon is mediated, at least in part, at the pre-translational level, suggesting the possibility of transcription induction. Acute As^{+3} administration is known to affect the expression of a number of intracellular proteins. For example, it is a potent inducer of heat shock and other stress-related proteins such as HO-1, as demonstrated in this and other studies (Keyse and Tyrrell, 1989). The data presented in this study indicate that a similar influence is exerted upon the expression of CYP1A1.

Previous work has shown that acute administration of sodium arsenite decreases the hepatic content of microsomal CYP (Albores *et al.*, 1992a). This effect has also been demonstrated in this study, however it is apparent that this depression is transient in nature. By extending our study to 10 days after treatment, we have shown that recovery of CYP content and/or catalytic activity is rapid and isozyme selective. Furthermore, an apparent selective increase of liver EROD activity was found after 5 days, although it was not quite significant ($p = 0.053$). In contrast to the selective increase of hepatic EROD activity, the decreases of CYP-dependent oxidation did not appear to be selective for EROD vs. PROD activity.

The role of HO in the modulation of CYP catalytic activity and mRNA after As^{+3} administration is not readily apparent from our study. One thing that is clear however, is that CYP-dependent catalytic activity is almost always maximal or minimal when tissue HO activity is minimal or maximal, respectively. Whether this relationship is causal or coincidental is not clear from our data. Two possibilities are that HO contributed to the modulation of CYP observed in this study, or alternatively, that HO merely served as a good marker for the level of stress experienced by a particular tissue. While ample evidence exists for the latter view (Applegate *et al.*, 1991; Tacchini *et al.*, 1995), demonstrations of the former are lacking. The possibility of direct degradation of CYP isozymes by HO has been suggested by Kutty *et al.* (1988), however a requirement for CYP denaturation and subsequent heme release was also suggested by these data. Furthermore, recent evidence (Fukuda and Sassa, 1994) indicates that the cytokine IL-6 may indirectly down regulate hepatic *CYP1A1* gene transcription. This is thought to be mediated by a decrease in cellular free heme, a proposed positive regulator of *CYP1A1* gene transcription (Bhat and Padmanaban, 1988a; 1988b), as a result of microsomal HO induction.

If HO activity is considered solely as an indicator of the level of stress experienced by a tissue at a particular point in time, it becomes clear that the effect of As^{+3} on CYP is time-dependent. For example, 1 or 2 days after treatment, the level of oxidative stress experienced by the liver and kidney is relatively high as indicated by microsomal HO activity. Concomitant with this, total CYP and/or CYP catalytic activity were decreased, indicating the domination of negative regulatory influences. Subsequent to this, HO activity decreases, presumably indicating a further decrease in oxidative stress. After 5 days, it appears that positive regulatory influences, selective for CYP1A1, are dominant although transient in nature.

The delayed increase of CYP1A1 mRNA and catalytic activity in the lung suggest that As^{+3} did not act directly to exert this effect. Instead, it is more likely that As^{+3}

treatment initiates a series of physiological and cellular responses that ultimately contribute to CYP1A1 induction. Furthermore, no measurable increase of CYP1A1 mRNA and/or catalytic activity was observed in any of the tissues studied while HO was elevated. Therefore, oxidative stress induced by sodium arsenite is probably not the direct cause for this effect. In addition, the selectivity of the induction (CYP1A1 vs. CYP2B1/2) argues against a generalized rebound or over-compensatory cellular response to the initial decrease. Some cytokines have been shown to regulate CYP in an isozyme-selective manner (Abdel-Razzak *et al.*, 1994; Clark *et al.*, 1995; Morgan, 1993a). While most cytokines down-regulate CYP, at least one cytokine, IL-2, may induce CYP1A1 catalytic activity in rat liver (Kurokohchi *et al.*, 1993). Thus, it is possible that some circulating cytokine, or combination of inflammatory mediators, produced at specific times as a result of As^{+3}-induced oxidative stress, is capable of exerting a selective positive modulating effect on CYP1A1.

The delayed increase of CYP1A1 activity in all of the tissues studied also suggests perturbation of a physiological substrate. Acute sodium arsenite administration results in profound alterations of heme metabolism and elevations of the biliary excretion of bilirubin (Albores *et al.*, 1989). Consistent with this, we have demonstrated that acute sodium arsenite treatment also causes large increases of plasma total bilirubin in the rat. The congenitally jaundiced Gunn rat exhibits marked endogenous activation of hepatic *CYP1A1* and *CYP1A2* gene expression (Kapitulnik and Gonzalez, 1993), as well as severe hyperbilirubinemia due to impaired elimination resulting from a mutation in the bilirubin UDP-GT gene (Iyanagi *et al.*, 1989). Administration of the *CYP1A* inducer TCDD to Gunn rats significantly lowers plasma bilirubin levels (Kapitulnik and Ostrow, 1978), whereas PB, an inducer of other CYP isoforms, does not (Cohen *et al.*, 1985). A TCDD-inducible bilirubin oxidase activity, that is inhibited by an antibody to CYP1A1/CYP1A2, has been described (De Matteis *et al.*, 1991a). Taken together, these data indicate that bilirubin may serve as a substrate for CYP1A1 or CYP1A2 in a metabolic pathway for

elimination separate from bilirubin UDP-GT. Substrate-mediated regulation is common for many CYP genes, particularly for CYP1A1 (Denison and Whitlock, 1995). Thus, it is conceivable that elevated circulating bilirubin levels in As^{+3} treated rats may have contributed to the increase in pulmonary CYP1A1 mRNA and catalytic activity observed in this study.

Orthotopic liver transplantation, a procedure that results in profound pathobiological stress and increased plasma bilirubin levels (Forster *et al.*, 1993), is also associated with increased extrahepatic CYP1A1-dependent EROD activity (Chapter 6 and Sinal *et al.*, 1995). Consistent with this, the increase of EROD activity and CYP1A1 mRNA after acute sodium arsenite administration in this study was most prominent extrahepatically, particularly in lung. The largest increases of HO activity in response to As^{+3} treatment were found in liver, and the least, in lung (Fig. 7.2). Furthermore, the highest HO activities occurred at the same time as the largest decreases of hepatic microsomal CYP content, and CYP-dependent PROD and EROD activities. This suggests that the elevated levels of plasma bilirubin are derived, at least in part, from metabolism of CYP heme. Thus, it is possible that the relative selectivity for CYP1A1 induction in lung may have been a consequence of exposure to and accumulation of bilirubin produced hepatically, combined with a lack of substantial HO induction in the lung. The latter factor is of particular importance as HO induction in lung would be expected to limit the quantity of free heme available for incorporation into CYP apoprotein and would be indicative of oxidative stress, a condition that opposes CYP1A1 induction (Barker *et al.*, 1994). Also of note, the lung receives the total cardiac output and is exposed to all blood-borne constituents. Thus, if the increases of CYP1A1 mRNA and catalytic activity occurred in response to a mediators(s) present within the circulation, it is not surprising that the greatest magnitude of response was observed in the lung.

Induction of CYP1A1 expression by PAHs such as 3MC and BaP is mediated by the AHR, a ligand-activated transcription factor (Denison and Whitlock, 1995). In order to

activate transcription, the ligand-bound AHR must form a heterodimer with the ARNT protein (Okey *et al.*, 1994). Similarly, the transcription activator, hypoxia inducible factor-1 (HIF-1) is also a heterodimer consisting of HIF-1α, a protein closely related to the AHR, and HIF-1β, recently identified as ARNT (Wang *et al.*, 1995). Synthesis of HIF-1α and HIF-1β (ARNT), and the DNA-binding activity of HIF-1 was also shown to be tightly regulated by cellular O_2 tension. Thus, modulation of ARNT, and its companion proteins during or after As^{+3}-induced oxidative stress may have a role in the changes of CYP1A1 mRNA accumulation observed in this study.

Based upon the changes observed for pulmonary CYP1A1 after acute As^{+3}-treatment, it is possible that the ability of this tissue to oxidize exogenous and endogenous substrates and to modulate toxic responses may be altered. Indeed, the increased accumulation of CYP1A1 mRNA, concomitant with increased CYP1A1-dependent catalytic activity, indicates that meaningful pharmacological, toxicological and physiological effects are possible. In particular, CYP1A1 can bioactivate a number of precarcinogens to mutagenic species (McManus *et al.*, 1990) and upregulation of CYP1A1 expression is a known risk factor for lung cancer (Gonzalez, 1997; Ioannides and Parke, 1994). It is important to realize however, that the cell specific nature of many pulmonary toxicants is an important consideration in studies of this type. For example, the lung is a heterogeneous mixture of multiple cell types with CYP1A1 preferentially expressed and induced in Clara, alveolar Type II and endothelial cells (Anttila *et al.*, 1992; Overby *et al.*, 1992). Thus, it is possible that modulation of the CYP monooxygenase system as measured in whole lungs may underestimate the changes typical of select cell types.

In conclusion, rat pulmonary CYP1A1 catalytic activity and mRNA are increased maximally 5 days after acute sodium arsenite treatment. This response exhibits both isozyme selectivity (CYP1A1 vs CYP2B1/2) and tissue selectivity (lung vs liver and kidney), although smaller increases were also noted in liver and kidney. The results of this study indicate that CYP1A1 catalytic activity and mRNA are responsive, both directly and

indirectly, to conditions of oxidative stress. This is consistent with our previous finding that rat lung and kidney CYP1A1-dependent EROD activity is selectively increased following orthotopic liver transplantation (Chapter 6 and Sinal *et al.*, 1995). Taken together, these data suggest a common regulatory mechanism for the modulation of CYP1A1 activity and mRNA during and/or after pathobiological stress.

Chapter 8

Aryl Hydrocarbon Receptor-Dependent Induction of *Cyp1a1* by Bilirubin in Mouse Hepatoma Hepa 1c1c7 Cells[6]

8.1 Objectives

While substrate metabolism and transcriptional regulation of Cyp1a1 by xenobiotics have been extensively studied, analogous roles for endogenous compounds have not been described. Published reports (Albores *et al.*, 1995; Sinal *et al.*, 1995) and the data presented in Chapters 6 and 7 of this thesis have demonstrated that pathobiological states caused by orthotopic liver transplantation or acute sodium As^{+3} treatment are associated with increased CYP1A1 mRNA and catalytic activity in lung and kidney. Concomitant with this, substantial depression of CYP-dependent and increase of HO-dependent catalytic activities also occurs. The elevated levels of plasma bilirubin associated with liver transplantation (Forster *et al.*, 1993), As^{+3} exposure (Chapter 7) and congenital defects in the bilirubin UDP-GT gene (Iyanagi *et al.*, 1989) indicate that this product of HO-mediated heme catabolism may be a mediator of the increased level of CYP1A1 expression associated with these conditions as reported in Chapters 6 and 7 of this thesis and in published accounts (Albores *et al.*, 1995; Kapitulnik and Gonzalez, 1993; Kapitulnik *et al.*, 1987; Sinal *et al.*, 1995).

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6 A version of this chapter has been published.

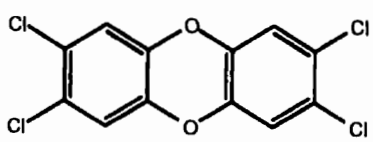
Sinal CJ and Bend JR (1997) Aryl hydrocarbon receptor-dependent induction of Cyp1a1 by bilirubin in mouse hepatoma Hepa 1c1c7 cells. *Mol. Pharmacol.* **52**: 590-599.

The objective of this study was to test the hypothesis that heme and its immediate catabolic metabolites, biliverdin and bilirubin, can directly cause increased expression and catalytic activity of CYP1A1 in a cell culture model. To this end, we examined the effect of treatment with exogenous hemin, biliverdin and bilirubin (Fig. 8.1) on Cyp1a1 mRNA and enzyme activity in mouse hepatoma Hepa 1c1c7 cells. The involvement of the AHR in this process was also investigated using AHR- and ARNT-deficient, mutant Hepa 1c1c7 cells, a DRE/luciferase reporter gene and gel retardation assays. We provide here direct evidence for AHR-dependent regulation of *Cyp1a1* gene expression and enzyme activity by bilirubin, an endogenous metabolite of heme.

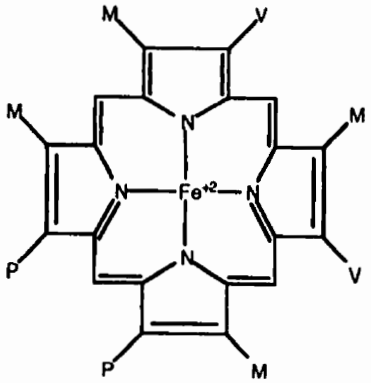
8.2 Materials and Methods

8.2.1 Materials

Hemin IX chloride, bilirubin IX, and biliverdin IX dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO.); meso-bilirubin and bilirubin diglucuronide from Porphyrin Products (Logan, UT); actinomycin D and cycloheximide from Fluka Chemika-BioChemika (Buchs, Switzerland); dioctylamine from Aldrich Chemical (Milwaukee, WI); 7-ethoxyresorufin and resorufin were from Molecular Probes, Inc. (Eugene, OR.); penicillin, streptomycin, amphotericin B, [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (7000 Ci/mmol) from ICN Biomedicals Canada (Montreal, PQ); Prime-a-Gene random primer DNA labelling kit from Promega (Madison, WI); Hybond-N nylon filters from Amersham Canada Ltd. (Oakville, ON); all restriction enzymes from Pharmacia Canada Inc. (Baie d'Urfe, PQ.); *Taq* DNA polymerase, Superscript II RNase H$^-$ reverse transcriptase, fetal calf serum, improved minimal essential media (IMEM) and gentamycin sulfate were from GIBCO BRL Canada (Burlington, ON); TCDD was a generous gift from Dr. Tim Zacharewski (Univ. of Western Ontario); poly(dIdC) was obtained from Boehringer Mannheim (Montreal, PQ); PCR primers were synthesized and purified by high

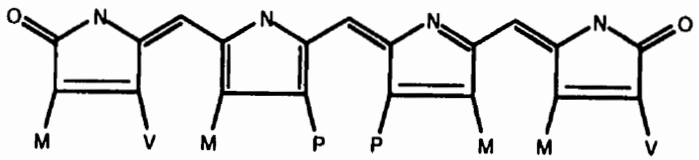


TCDD

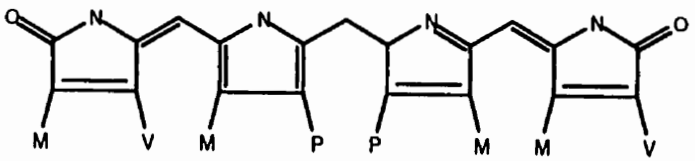


Hemin

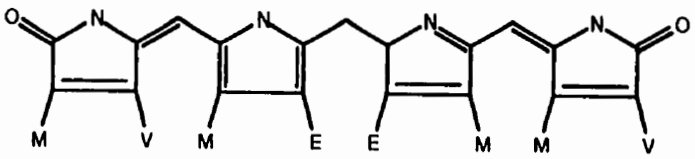
M	=	CH_3
E	=	CH_2CH_3
G	=	$(CH_2)_2COOC(CH_2O)_4COOH$
P	=	$(CH_2)_2COOH$
V	=	$CHCH_2$



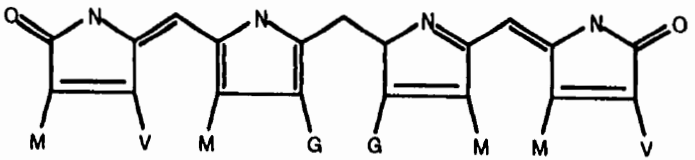
Biliverdin



Bilirubin



meso-Bilirubin



Bilirubin Diglucuronide

Figure 8.1
Structures of the test compounds used in this study.

performance liquid chromatography by General Synthesis and Diagnostics (Toronto, ON); and all other chemicals (reagent grade or better) were purchased from BDH Inc. (Toronto, ON).

8.2.2 Cell Culture and Treatments

The mouse hepatoma Hepa 1c1c7 cell lines (wild type, C4 and C12; generously provided by Dr. O. Hankinson, UCLA) were maintained in a standard medium consisting of IMEM supplemented with 10% fetal bovine serum, 20 μM L-glutamine, 50 μg/mL gentamycin sulphate, 100 IU/mL penicillin, 10 μg/mL streptomycin and 25 ng/mL amphotericin B. Cells were grown in 75 cm^2 tissue culture flasks at 37°C in a 4% CO_2 humidified environment.

Hemin, biliverdin and bilirubin were initially dissolved in a small volume of 1 mM NaOH, and made up to the desired final volume with sterile phosphate buffered saline (PBS), pH 7.4. These solutions were prepared fresh for each experiment under low light conditions. Actinomycin D was dissolved in 75% ethanol, cycloheximide in sterile distilled water and TCDD in Me_2SO. For all experiments, control groups of cultured cells for each of the treatments were administered the appropriate solvent. For analysis of mRNA expression levels, approximately 2 x 10^7 cells were added to 100 mm tissue culture dishes in 10 mL of normal culture media. For analysis of EROD levels, approximately 1 x 10^5 cells were added to each well of a 96-well tissue culture plate in 250 μL of normal culture media. Upon 60-80% confluence (1-2 d), appropriate stock solutions of the test chemicals were added directly to the culture media. For experiments involving actinomycin D or cycloheximide, these chemicals were added 1.5 h prior to the addition of the test compounds.

8.2.3 RNA Extraction and Analysis

After incubation with the test compounds for specified time periods, the culture media was aspirated and the cell monolayers were washed twice with ice-cold PBS. Total cell RNA was isolated by the acid/guanidinium thiocyanate/phenol/chloroform extraction method of Chomczynski and Sacchi (1987). For northern blots, aliquots of RNA were separated in a denaturing (2.2 M formaldehyde) agarose (1.1%) gel and transferred to Hybond-N nylon filters. The RNA was fixed to the filters by baking at 80°C for 2 hr. Pre-hybridization of the filters was carried out in a solution containing 6 x SSPE (0.9 M NaCl, 0.06 M $NaH_2PO_4 \cdot H_2O$, 0.006 M Na_2EDTA), 50% deionized formamide, 5 x Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin), 0.5% SDS and 100 µg/mL sheared salmon sperm DNA for 4 hours at 42°C. Hybridization with the ^{32}P-labeled cDNA probes was carried out in the same solution, minus Denhardt's reagent, for 16-24 hr at 42°C. The filters were then washed twice at room temperature in 2 x SSPE, 0.5% SDS for 15 min. This was followed by a 30 min wash in 0.1 x SSPE, 0.5% SDS at 42°C and a final 30 min wash in 0.1 x SSPE, 0.5% SDS at 65°C for 30 min. The washed filters were sealed in plastic wrap and exposed to Kodak X-OMAT AR film in the presence of an intensifier screen at -80°C. After exposure, the filters were stripped by washing in 0.1 x SSPE, 0.5% SDS at 100°C prior to subsequent cDNA hybridizations.

The 539 bp cDNA probe for mouse CYP1A1 mRNA was generously provided by Dr. T. Zacharewski (University of Western Ontario). A cDNA probe for rat HO-1 mRNA was prepared by RT-PCR as previously described (Chapter 7). All probes were ^{32}P-labeled by the random primer method according to the manufacturer's (Promega) instructions.

8.2.4 EROD Assays

EROD assays were performed on intact, living Hepa cells. A 7-ethoxyresorufin resorufin working solution was produced by dilution of stock (0.4 mM in Me_2SO) 7-

ethoxyresorufin resorufin to a final concentration of 2 μM in assay buffer (0.05 M Tris, 0.1 M NaCl, pH 7.8) that had been pre-warmed to 37°C. After aspiration of the media containing the test compounds, the cell monolayers were rinsed with warmed (37°C) PBS. The working solution (75 μL) was then added to each cell well and the plates were placed in a shaking incubator at 37°C. After 5 min, initial fluorescence (excitation = 545 nm, emission = 575 nm; Baxter 96-well fluorimeter) measurements were recorded from each cell well and the plates were replaced in the incubator. After a further 10 min, a final set of fluorescence measurements of the cell wells was performed. The amount of resorufin formed in each well between the initial and final fluorescence measurements was determined by comparison with a standard curve of known concentrations. Under these assay conditions, the formation of resorufin with respect to time was verified to proceed linearly for a minimum of 15 min after the initial fluorescence measurements. The working solution was then aspirated, the cell wells were rinsed twice with PBS and 50 μL of distilled water were added to lyse the cells. After placing the cell plates at -80°C for 30 min, the cell lysates were allowed to thaw, and protein levels were determined by the method of Lowry *et al.* (1951) using a Molecular Devices Emax microplate reader.

8.2.5 Transient Transfection and Luciferase Assays

Wild type Hepa 1c1c7 cells were plated in 6-well multiwell tissue culture plates at approximately 50-60% confluence 6 h prior to transfection in standard growth media. Each well of cells was transfected with 1.5 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) as a control for transfection efficiency and 2.5 μg of the DRE-luciferase reporter plasmid pGudLuc1.1 (provided by Dr. M. Denison, U.C. Davis) using a standard calcium phosphate co-precipitation technique (Sambrook *et al.*, 1989). The media was aspirated, the cells were washed twice with PBS and the media was replaced 24 h after transfection. Following this, test compounds were added directly to the media (0.1% v/v). The cells were harvested 24 h later and assayed for β-galactosidase activity

using a 96-well absorbance plate reader (Molecular Devices Emax) as per standard techniques (Sambrook *et al.*, 1989). Luciferase activity was measured using a 96-well luminometer (Labsystems Luminoskan) using standard methods (Brasier *et al.*, 1989). Luciferase values were corrected for transfection efficiency (β-galactosidase activity) and are expressed as fold-induction relative to solvent treated control cells.

8.2.6 Gel Retardation Assays

For preparation of guinea pig hepatic cytosol, freshly excised livers were homogenized in ice-cold HEGD buffer (25 mM HEPES, 5 mM EDTA, 10% glycerol, pH 7.4) using three passes with a Teflon-glass homogenizer. The resulting homogenate was centrifuged at 9,000 x g for 20 min at 4°C followed by centrifugation of the supernatant at 100 000 x g for 60 min at 4°C. For preparation of Hepa cytosol, wild-type cells were grown to 90% confluence in 100 mm tissue culture dishes, washed twice with PBS, and detached using trypsin (0.5%). Cells were pelleted at 1000 x g, resuspended in HEGD buffer, homogenized (Ten Broeck) and centrifuged at 100 000 x g for 60 min at 4°C. Aliquots of Hepa cell and guinea pig liver cytosol (100 000 x g supernatant) were flash-frozen in liquid nitrogen and stored at -80°C until use.

The following complementary oligonucleotides were synthesized and used in the gel retardation assays: (i) -998 to -973 of the *Cyp1a1* regulatory region (5'-gatctctTCTCACGCaActccgag-3' and 5'-gatcctcggagTtGCGTGAGAaga-3'; henceforth termed DRE), and (ii) -998 to -973 of the *Cyp1a1* regulatory region containing a single nucleotide transversion substitution (underlined) within the core DRE motif (5'-gatctctTCTCGCGCaActccgag-3' and 5'-gatcctcggagTtGCGCGAGAaga-3'; henceforth termed mutant DRE). Oligonucleotides were ^{32}P-labelled at the 5'-end using T4 polynucleotide kinase and [γ-^{32}P]ATP using standard methodology (Sambrook *et al.*, 1989). After adding NaCl to a final concentration of 100 mM, the oligonucleotides were annealed by heating to 70°C and allowing to cool to room temperature. The ^{32}P-labelled

DRE was then purified by loading on a 15% non-denaturing polyacrylamide gel and electrophoresis in 1x TBE buffer (90 mM Tris, 120 mM boric acid, 4 mM EDTA, pH 8.0). The labelled DRE was visualized by a 10 s exposure to Kodak X-OMAT AR film, excised from the gel and eluted into 400 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for 16 h at 37°C.

Assessment of AHR transformation and DNA-binding was performed essentially as previously described (Denison *et al.*, 1988). Briefly, cytosol from Hepa 1c1c7 cells (5 mg/mL) or guinea pig liver (16 mg/mL) was transformed by incubation at 22°C for 2 h with the test compounds dissolved in Me_2SO. Transformed cytosol (5 µL) was then incubated for 15 min at 22°C with HEGD buffer (15 µL) containing KCl (64 mM final concentration for Hepa cytosol, 96 mM for guinea pig) and 375 ng of poly(dIdC). For the competition assays, unlabelled, annealed DRE was also added to this initial incubation. Labelled, annealed oligonucleotides (100 000 CPM, 0.5 ng) were then added, followed by a second incubation for 15 min at 22°C. The samples were loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed in 1x TBE. After drying, the gels were visualized by exposure to Kodak X-OMAT AR film in the presence of an intensifier screen at -80°C.

8.2.7 Heme Oxygenase and Biliverdin Reductase Assays

Wild-type Hepa 1c1c7 cells were grown to 80-90% confluence in 100 mm tissue culture dishes. After treatment with the test compounds for 6 h, the cell monolayers were washed twice with ice-cold PBS, scraped from the tissue culture dishes using a rubber policeman, and pelleted at 1 000 x g for 1 min at 4°C. Cell pellets were resuspended in 100 µL of hypotonic buffer consisting of 10 mM potassium phosphate buffer (pH 7.4) and 50 µM phenylmethylsulfonyl fluoride. After sitting on ice for 10 min, the cell suspensions were flash frozen in liquid N_2 and subsequently allowed to thaw on ice. Two such freeze-thaw cycles resulted in essentially complete cell lysis as assessed by light microscopy. The lysed cell suspension was then centrifuged at 15 000 x g for 1 min at 4°C. The supernatant

from this spin, which contains both HO and biliverdin reductase activity, was used for all enzyme assays. Protein concentrations were determined by the method of Lowry *et al.* (1951).

For determination of biliverdin reductase activity, incubations contained 1.25 mg/mL cell lysate, 25 μM biliverdin and 1 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.4. For the HO assays, incubations contained 5 mg/mL lysate, 25 μM hemin and 1 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.4. Samples were incubated for 15 min (biliverdin reductase) or 60 min (HO) at 37°C. For the HO samples, additional NADPH was added after 20 and 40 min. Reactions were stopped by the addition of 2 volumes of $CHCl_3$. After vigorous mixing, the samples were centrifuged at 10 000 x g for 5 min and the lower $CHCl_3$ phase was removed to a fresh tube. The $CHCl_3$ was evaporated under a gentle stream of N_2 and the resulting residue was subsequently dissolved in 100 μL of methanol.

Bilirubin content of the extracted samples was analyzed by reverse-phase high performance liquid chromatography (HPLC) based on the method of McDonagh *et al.* (1982). Briefly, the HPLC system consisted of a Waters HPLC pump and injector connected to a Beckman-Altex Ultrasphere-IP column (5 μM, C-18, 25 x 0.46 cm). The mobile phase consisted of 0.1 M dioctylamine in methanol/water (96:4, v:v) at a flow rate of 1 mL/min. A Waters tunable absorbance detector was used for continuous monitoring at 460 nM. The retention time for bilirubin was 6.8 min. Quantitation of bilirubin was achieved by comparison to known quantities of bilirubin dissolved in a 1 mg/mL bovine serum albumin solution and extracted identically to the unknown samples. The limit of detection for this system was 50 pmol.

8.2.8 Statistics

Data were analyzed by analysis of variance (ANOVA) followed by Bonnferroni/Dunn test for multiple comparisons using StatView 4.01 software (Abacus Concepts, Berkeley CA).

8.3 Results

8.3.1 Increase of Cyp1a1 After Heme Compound Treatment

The effect of hemin, biliverdin or bilirubin on steady-state Cyp1a1 mRNA levels as assessed by northern blots is shown in Fig. 8.2. All of the compounds caused a dose-dependent increase of Cyp1a1 mRNA levels 3 h after treatment when compared with solvent treated control Hepa 1c1c7 cells. Bilirubin was clearly the most potent and effective with discernible effects evident at a concentration of 1 μM. At a concentration of 100 μM, bilirubin produced the greatest increase, followed by biliverdin and hemin, respectively. As expected, 1 nM TCDD caused a substantial increase of Cyp1a1 mRNA after 3 h, however, at this time point the effect was not as great as with 100 μM bilirubin. Also shown in Fig. 8.2 is the effect of the tested compounds on the steady-state levels of inducible HO-1 isoform mRNA. Only hemin increased HO-1 mRNA levels in a dose dependent manner. For reference purposes, the blots were stripped and re-hybridized with a cDNA probe for GAPDH mRNA as a loading control for all of the northern blots presented in this study.

Fig. 8.3 shows the time course for Cyp1a1 mRNA increases 0, 1, 2, 4, 8 or 12 h after treatment with 100 μM hemin, biliverdin or bilirubin. The onset of Cyp1a1 increase was rapid for all compounds and was readily apparent by 1 h for biliverdin and bilirubin. However, the increase in response to 100 μM hemin was somewhat delayed, with an increase of Cyp1a1 mRNA levels being clearly achieved only after 2 h. By 24 h, the

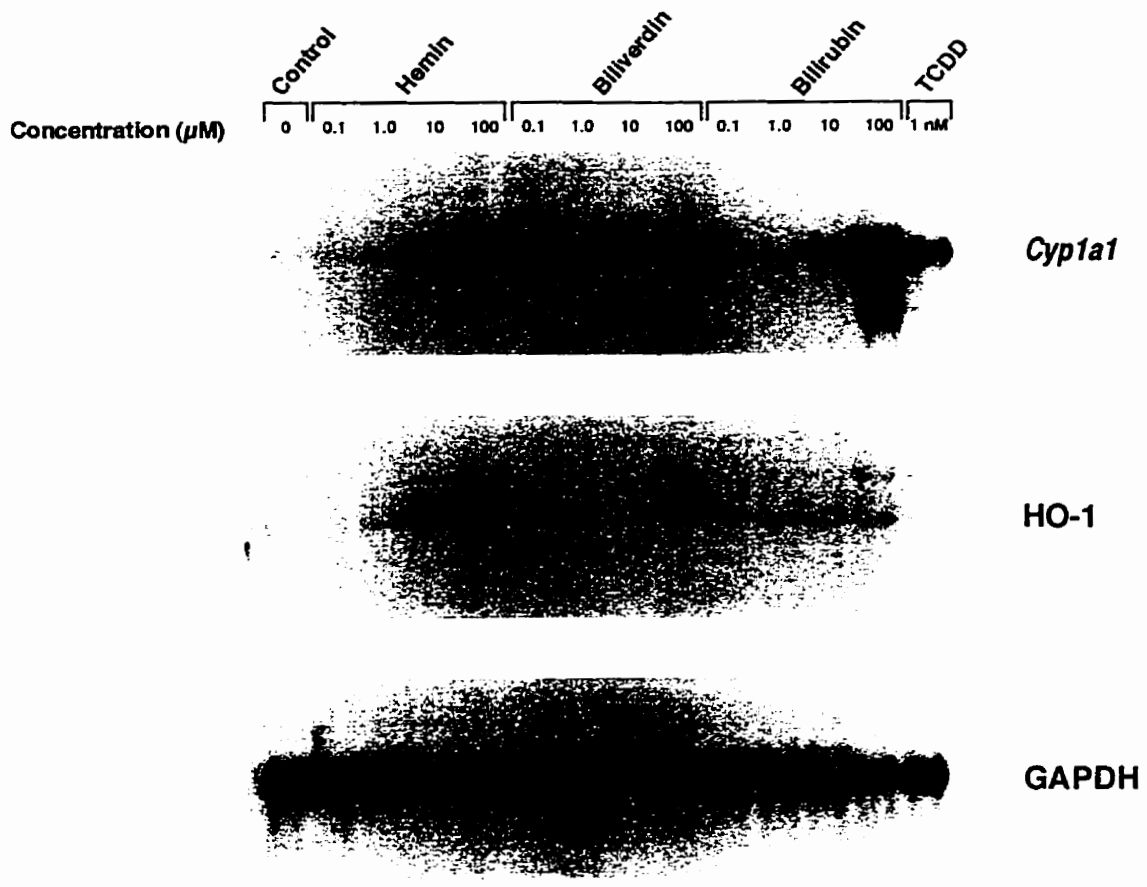


Figure 8.2
Dose-dependent increase of Cyp1a1 mRNA in wild type Hepa 1c1c7 cells 3 h after treatment with hemin, biliverdin or bilirubin as assessed by northern blot analysis. Total RNA (10 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes and hybridized with a ^{32}P-labelled cDNA probe specific for mouse Cyp1a1, HO-1 or GAPDH mRNA. Transcript sizes for Cyp1a1, HO-1 and GAPDH mRNA were approximately 2.9 kb, 1.8 kb and 1.3 kb, respectively. Total RNA isolated from cells 3 h after treatment with 1 nM TCDD was used as a positive control for Cyp1a1 induction.

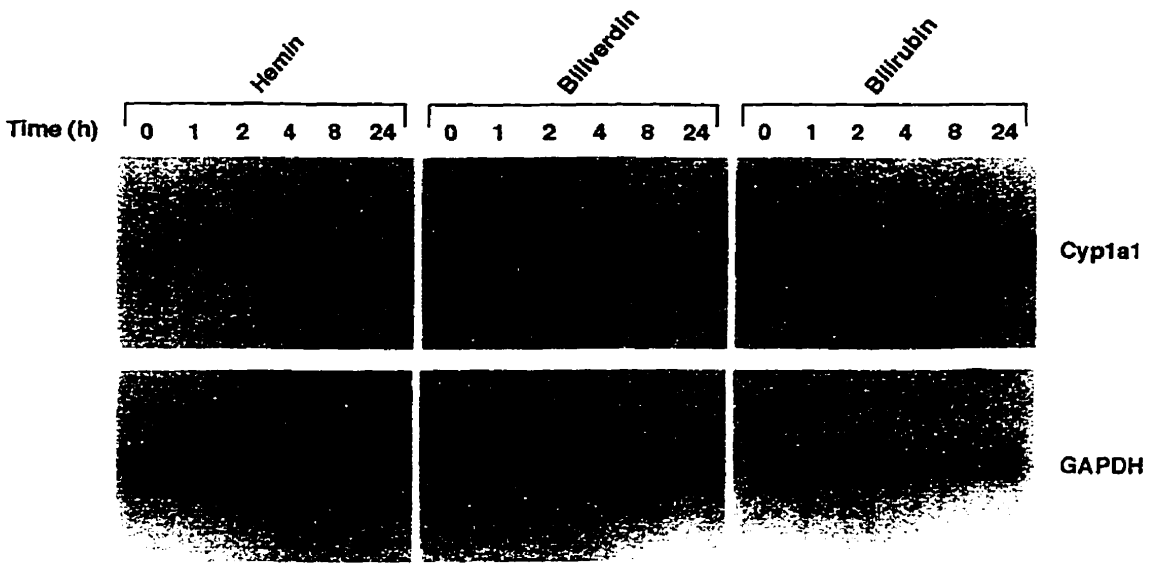


Figure 8.3
Time-dependent increase of Cyp1a1 mRNA in wild type Hepa 1c1c7 cells 0, 1, 2, 4, 8 or 24 h after treatment with 100 μM hemin, biliverdin or bilirubin as assessed by northern blot analysis. Total RNA (10 μg) was separated on a 1.1% formaldehyde denaturing gel, transferred to nylon membranes and hybridized with a ^{32}P-labelled cDNA probe specific for mouse Cyp1a1 or GAPDH mRNA. Transcript sizes for Cyp1a1 and GAPDH mRNA were approximately 2.9 kb and 1.3 kb, respectively.

Cyp1a1 mRNA levels were clearly diminished relative to the maximum for each compound, but remained elevated relative to that seen at 0 h in each case.

To assess the functional implications of exposure to hemin, biliverdin or bilirubin, Cyp1a1-dependent EROD activity was also measured in Hepa 1c1c7 cells (Fig. 8.4). Each of the compounds produced a dose dependent increase of EROD activity 24 h after treatment when compared with solvent exposed control cells. Significant increases of EROD activity were measured at 30 and 100 μM for hemin (2.0- and 5.5-fold, respectively) and at 10, 30 and 100 μM for biliverdin (3.0-, 6.5- and 10.5-fold) and bilirubin (3.0-, 8.0- and 15.0-fold). Furthermore, biliverdin and bilirubin increased EROD activity significantly more than hemin at 10, 30 and 100 μM, while bilirubin produced a significantly greater increase than hemin or biliverdin at 100 μM. The positive control and reference compound TCDD (1 nM) produced an approximately 41-fold increase of EROD activity (Fig. 8.4, inset).

8.3.2 Effect of RNA and Protein Synthesis Inhibitors

To determine if the increase of Cyp1a1 in response to hemin, biliverdin or bilirubin exposure was a result of *de novo* RNA synthesis or a post-transcriptional effect, Hepa 1c1c7 cells were co-treated with the RNA polymerase inhibitor actinomycin D. Treatment with 1 μg/mL actinomycin D 2 h prior to exposure to 100 μM hemin, biliverdin or bilirubin, completely abolished any increase of Cyp1a1 mRNA (Fig. 8.5A). As expected, actinomycin D also inhibited induction by 1 nM TCDD, a finding consistent with an increase in *Cyp1a1* gene transcription by this compound. Actinomycin D also completely blocked the increase of Cyp1a1-dependent EROD activity in response to 100 μM hemin, biliverdin, bilirubin (Fig. 8.5B) or 1 nM TCDD (Fig. 8.5B, inset). Actinomycin D also significantly decreased basal EROD levels in solvent treated Hepa 1c1c7 cells.

We also determined if increased Cyp1a1 mRNA levels due to hemin, biliverdin or bilirubin exposure were dependent upon the action(s) of a highly labile or *de novo*

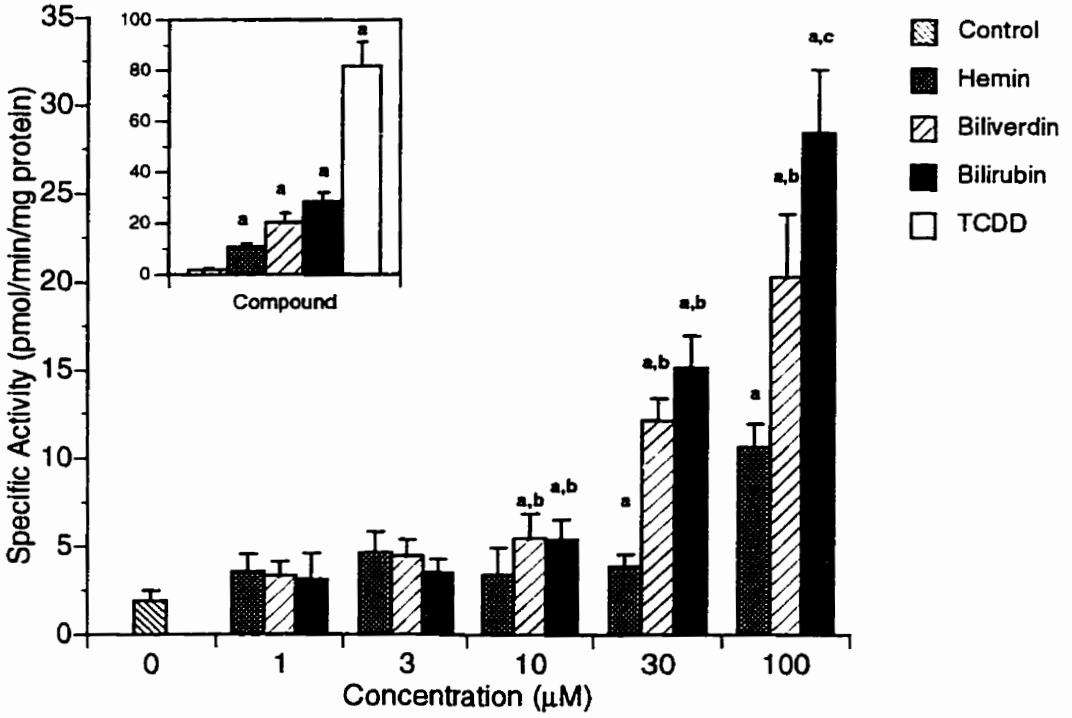


Figure 8.4

Dose-dependent increase of Cyp1a1-dependent EROD activity in wild type Hepa 1c1c7 cells 24 h after treatment with hemin, biliverdin or bilirubin. EROD activity was measured in intact, living cells using a 96-well plate fluorescence assay. Cells treated with 1 nM TCDD were used as a positive control for Cyp1a1 induction (inset). Values shown are the mean ± SD of 12 independent experiments performed in duplicate. **a**, significantly different from 0 μM ($p<0.01$); **b**, significantly different from hemin ($p<0.01$); **c**, significantly different from hemin and biliverdin ($p<0.01$).

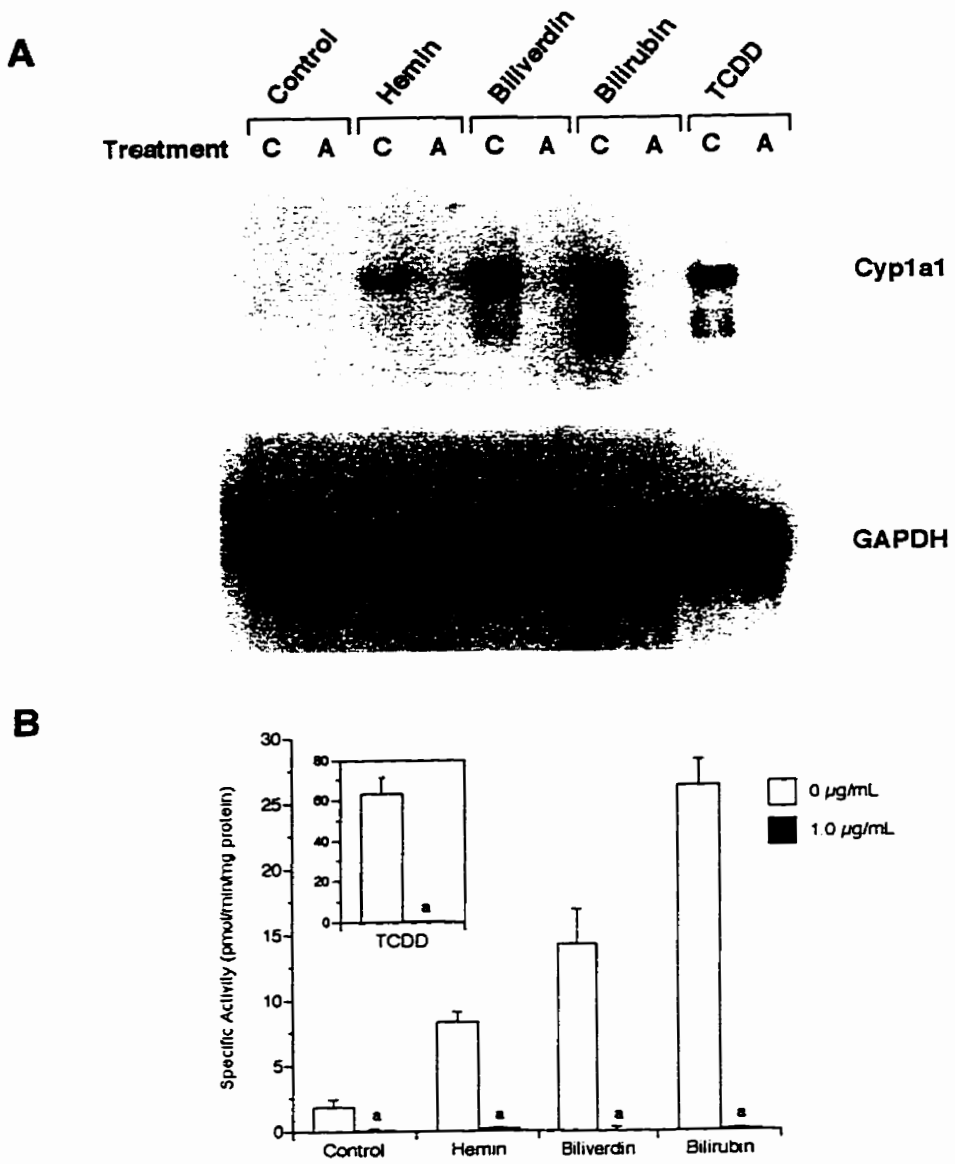


Figure 8.5

(**A**) Inhibition of Cyp1a1 mRNA increase in wild type Hepa 1c1c7 cells by actinomycin D. Cells were treated with 0 (C) or 1 µg/mL actinomycin D (A) 2 h prior to exposure to 100 µM hemin, biliverdin or bilirubin for a subsequent 3 h time period. Treatment with 1 nM TCDD was used as a positive control for Cyp1a1 induction.

(**B**) Inhibition of Cyp1a1-dependent EROD activity increase in wild type Hepa 1c1c7 cells by actinomycin D. EROD activity was measured in intact, living cells 24 h after exposure to 100 µM hemin, biliverdin or bilirubin and co-treatment with 0 or 1 µg/mL actinomycin D. Cells treated with 1 nM TCDD were used as a positive control for Cyp1a1 induction (inset). Values shown are the mean ± SD of 6 independent experiments performed in duplicate. **a**, significantly different from treatment with 0 µg/mL actinomycin D ($p<0.01$).

synthesized protein(s) by treating Hepa 1c1c7 cells with the protein synthesis inhibitor cycloheximide. A 2 h pre-treatment with 1 μg/mL cycloheximide had no discernible effect on the increase of Cyp1a1 mRNA in response to hemin, biliverdin or bilirubin (Fig. 8.6A). This treatment however, did increase Cyp1a1 mRNA levels in vehicle treated and 0.1 nM TCDD treated cells. With respect to EROD activity, cycloheximide completely abolished the increases normally seen in response to hemin, biliverdin or bilirubin treatment (Fig. 8.6B). Furthermore, EROD activity was depressed to values much lower than those observed for vehicle treated control cells indicating a requirement for protein synthesis for increased EROD activity, as well as maintenance of basal levels. Similar effects were observed for TCDD treated Hepa 1c1c7 cells (Fig. 8.6B, inset).

8.3.3 Aryl Hydrocarbon Receptor-Dependent Induction of Cyp1a1

The availability of mutant Hepa 1c1c7 cell lines that lack an AHR-dependent Cyp1a1 induction mechanism (Hankinson *et al.*, 1991) permits testing for the involvement of this signal transduction pathway in the actions of a compound. Fig. 8.7 shows the lack of Cyp1a1 mRNA increase in ARNT-deficient Hepa 1c1c7 C4 (C4) and AHR-deficient Hepa 1c1c7 C12 (C12) cells 3 h after treatment with 100 μM hemin, biliverdin or bilirubin or the positive control compound, 1 nM TCDD. Consistent with these data, no EROD activity was detected after treatment of C4 or C12 cells with these compounds, solvent (Fig. 8.8) or 1 nM TCDD (Fig. 8.8, inset). In contrast significant increases of Cyp1a1 mRNA and EROD activity were found upon treatment of wild type Hepa 1c1c7 cells with each of the test compounds.

To obtain further evidence for involvement of an AHR-dependent mechanism, wild type Hepa 1c1c7 cells were transiently transfected with the DRE-luciferase reporter construct plasmid pGudLuc1.1 (Garrison *et al.*, 1996). This construct contains a portion of the 5'-regulatory region of the Cyp1a1 gene (-1301 to -819) that encompasses 4 copies of the DRE enhancer sequence located upstream of a structural gene coding for the enzyme

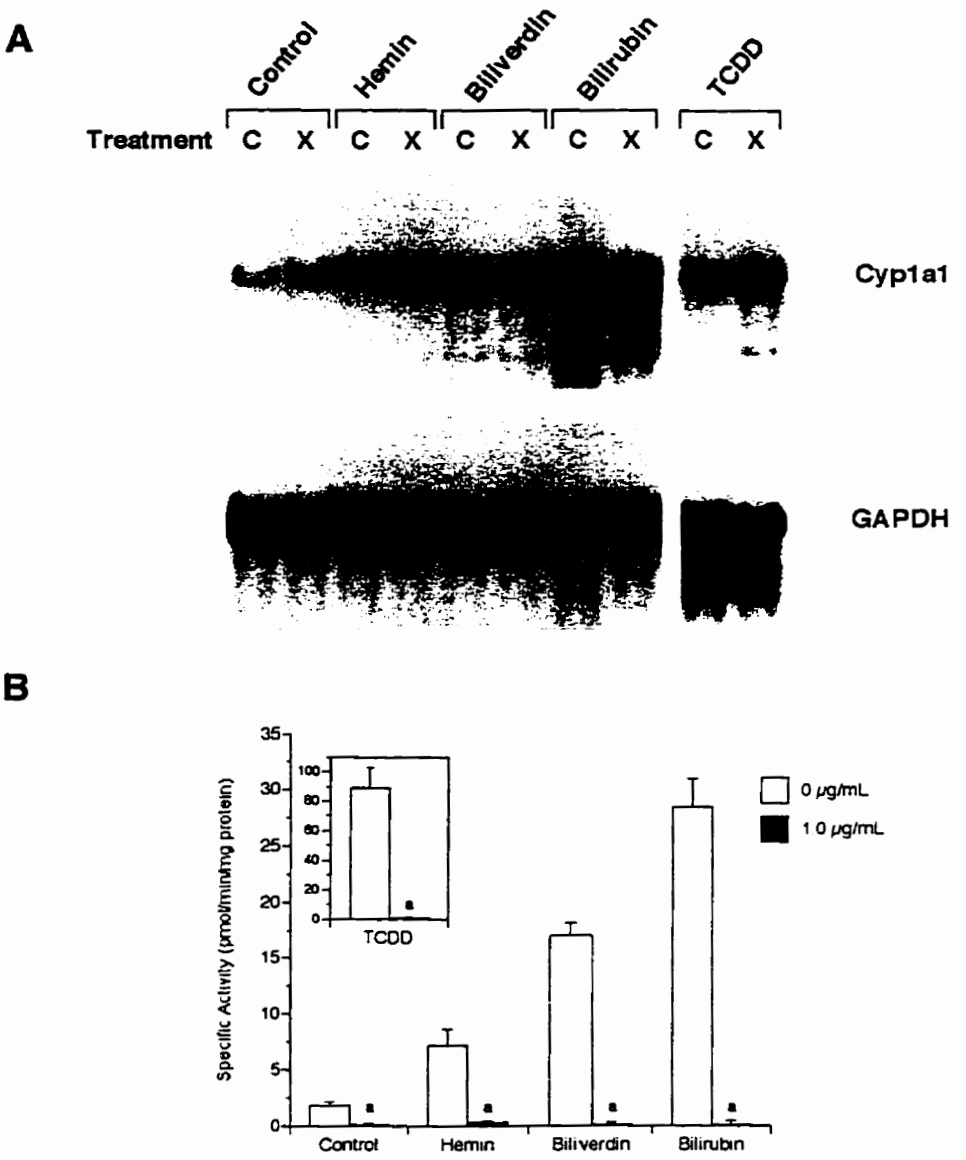


Figure 8.6

(**A**) Effect of cycloheximide on Cyp1a1 mRNA increase in wild type Hepa 1c1c7 cells. Cells were treated with 0 (C) or 1 µg/mL cycloheximide (X) 2 h prior to exposure to 100 µM hemin, biliverdin or bilirubin for a subsequent 3 h time period. Treatment with 1 nM TCDD was used as a positive control for Cyp1a1 induction.

(**B**) Inhibition of Cyp1a1-dependent EROD activity increase in wild type Hepa 1c1c7 cells by cycloheximide. EROD activity was measured in intact, living cells 24 h after exposure to 100 µM hemin, biliverdin or bilirubin and co-treatment with 0 or 1 µg/mL cycloheximide. Cells treated with 1 nM TCDD were used as a positive control for Cyp1a1 induction (inset). Values shown are the mean ± SD of 6 independent experiments performed in duplicate. **a**, significantly different from treatment with 0 µg/mL cycloheximide ($p<0.01$).

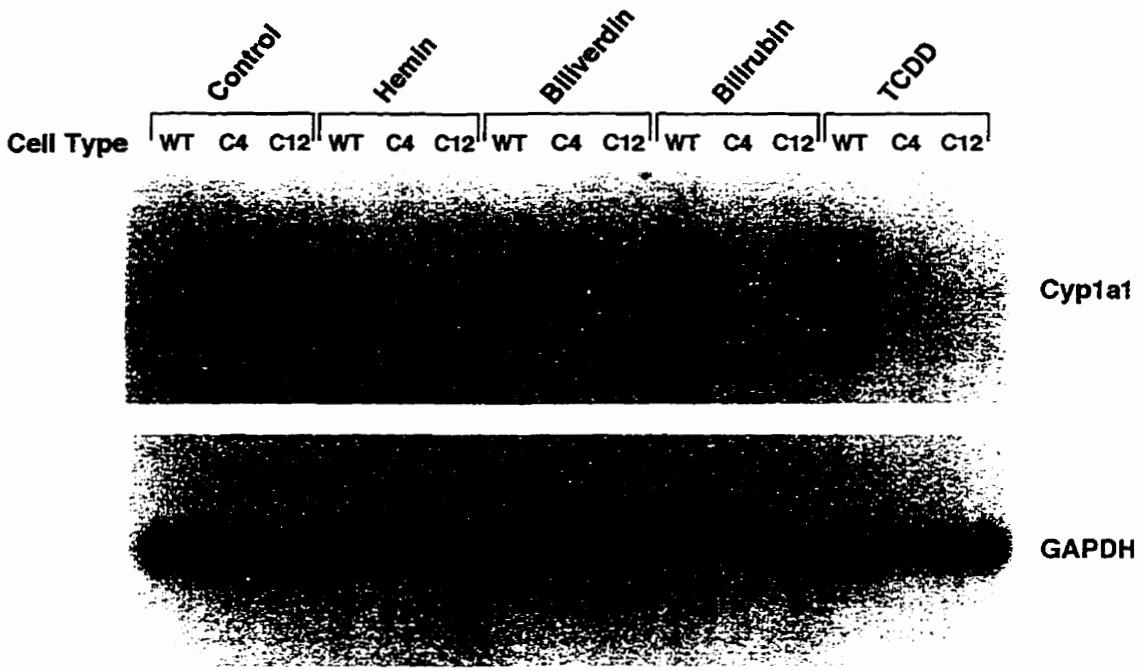


Figure 8.7
Lack of Cyp1a1 mRNA increase in mutant Hepa 1c1c7 cells. Wild type (WT), ARNT-deficient (C4) or AHR-deficient (C12) Hepa 1c1c7 cells were exposed to 100 μM hemin, biliverdin or bilirubin for a 3 h time period. Total RNA isolated from cells 3 h after treatment with 1 nM TCDD was used as a positive control for Cyp1a1 induction.

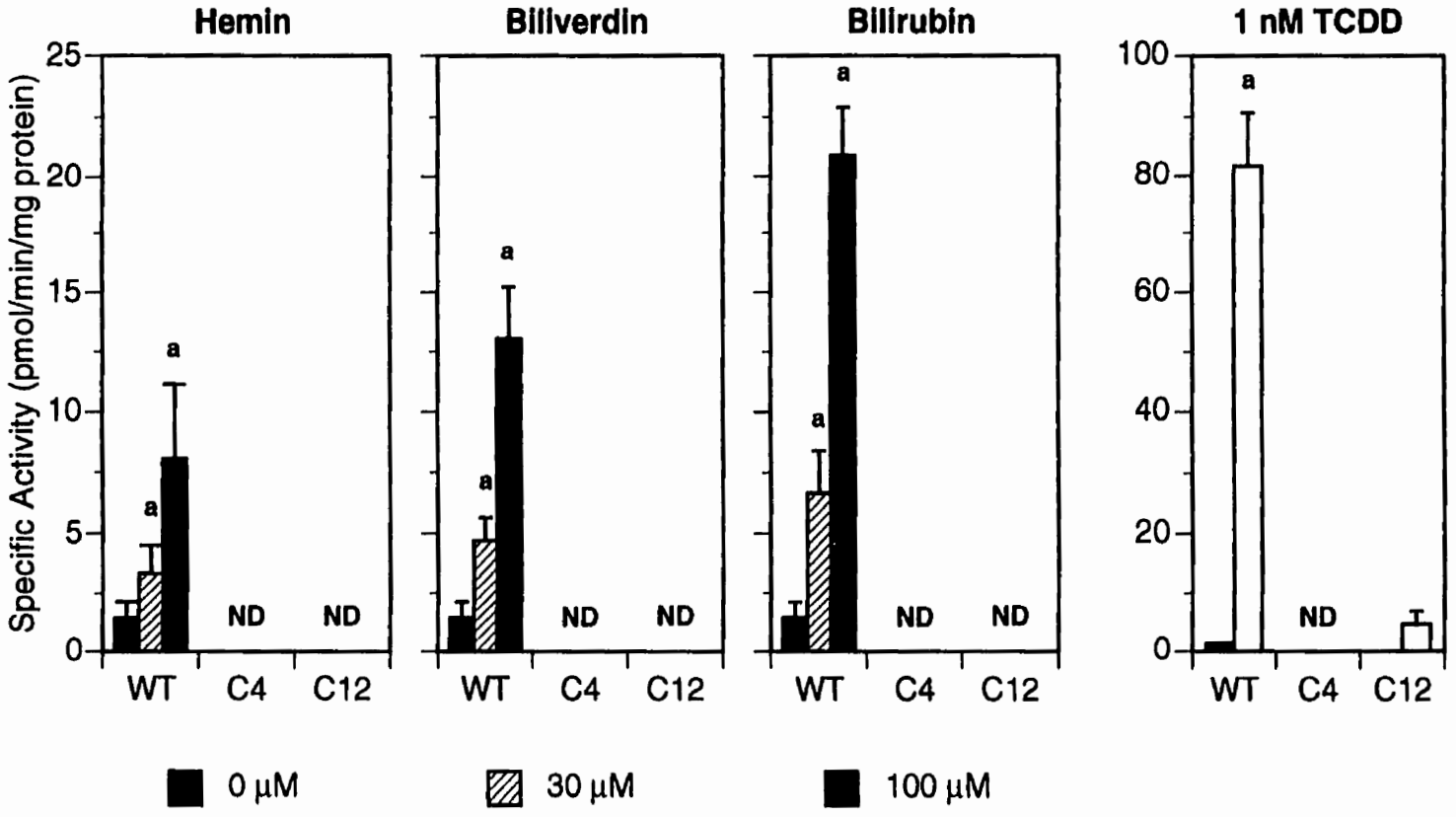


Figure 8.8

Lack of increase of Cyp1a1-dependent EROD activity in mutant Hepa 1c1c7 cells 24 h after treatment with hemin, biliverdin or bilirubin. EROD activity was measured in intact, living wild type (WT), ARNT-deficient (C4) or AHR-deficient (C12) cells using a 96-well plate fluorescence assay. Cells treated with 1 nM TCDD were used as a positive control for Cyp1a1 induction. Values shown are the mean ± SD of 6 independent experiments performed in duplicate. **a**, significantly different from 0 μM ($p<0.01$); **ND**, not detected (< 1 pmol/min/mg protein).

luciferase. Thus, AHR-dependent inducible expression is conferred upon the luciferase gene by the presence of the DRE enhancer sequences. The advantage of this bioassay is that luciferase induction can only occur through the AHR. Therefore other mechanisms such as mRNA stability (Pasco *et al.*, 1988) that might influence EROD activity are excluded. Treatment of transiently transfected cells with hemin, biliverdin or bilirubin significantly increased luciferase activity in a dose-dependent manner when compared with solvent treated control cells (Fig. 8.9). Specifically, induction by 10 or 100 μM biliverdin (3.0- and 11.5-fold, respectively) or bilirubin (4.0- and 16.5-fold) was significantly greater than that observed for equimolar hemin (1.5- and 2.5-fold). Furthermore, 100 μM bilirubin increased luciferase activity significantly more than observed for equimolar biliverdin or hemin. As expected, treatment with 1 nM TCDD produced the greatest induction (52-fold) of all tested compounds (Fig. 8.9, inset). With respect to 10 and 100 μM biliverdin and bilirubin, the magnitude of increase of luciferase activity was similar to that measured for EROD activity. However, the magnitude of luciferase induction (2.5-fold) for 100 μM hemin was approximately half of that seen for EROD induction (5.5-fold).

Ligand-dependent transformation of the AHR is required for AHR-mediated transcriptional activation. We performed gel retardation assays to determine if hemin, biliverdin or bilirubin were capable of inducing the *in vitro* formation of AHR/ARNT heterodimers that are capable of specific binding to ^{32}P-labelled double-stranded oligonucleotides containing a normal DRE sequence. After incubation of these test compounds with Hepa 1c1c7 cell cytosol *in vitro*, only bilirubin induced the formation of a heterodimer:^{32}P-DRE complex that co-migrated with that induced by TCDD (Fig. 8.10A). In order to obtain evidence that the apparent absence of Hepa AHR transformation by hemin and bilirubin was not due to a lack of sensitivity, gel retardation assays were also performed with guinea pig hepatic cytosol, a preparation that provided a greater magnitude of response in our experiments. Similar to Hepa cells, only bilirubin caused the formation

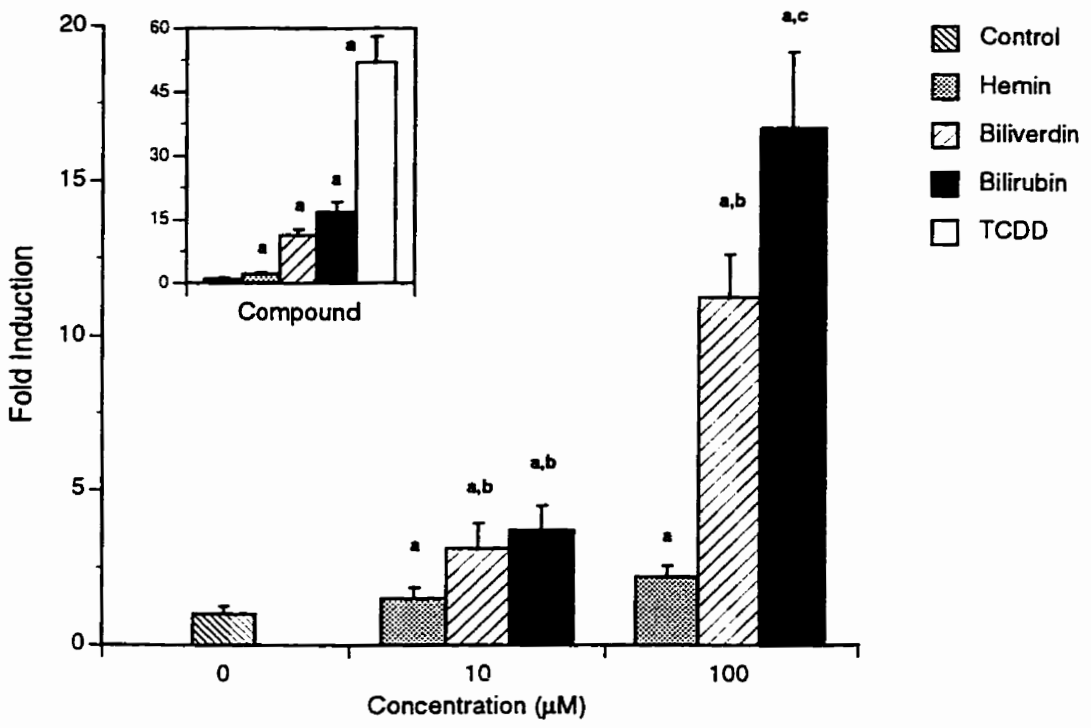


Figure 8.9

Dose-dependent increase of DRE/luciferase reporter construct (pGudLuc1.1) activity in wild type Hepa 1c1c7 cells 24 h after treatment with hemin, biliverdin or bilirubin. Wild type Hepa 1c1c7 cells were transiently transfected with pGudLuc1.1 and pCH110 as described under Materials and Methods. Cells treated with 1 nM TCDD were used as a positive control for luciferase induction (inset). Fold-induction represents the luciferase activity, corrected for transfection efficiency (β-galactosidase activity from pCH110), relative to that measured in vehicle (0 μM) treated cells. Values shown are the mean ± SD of 6 independent experiments performed in duplicate. **a**, significantly different from 0 μM ($p<0.01$); **b**, significantly different from hemin ($p<0.01$); **c**, significantly different from hemin and biliverdin ($p<0.01$).

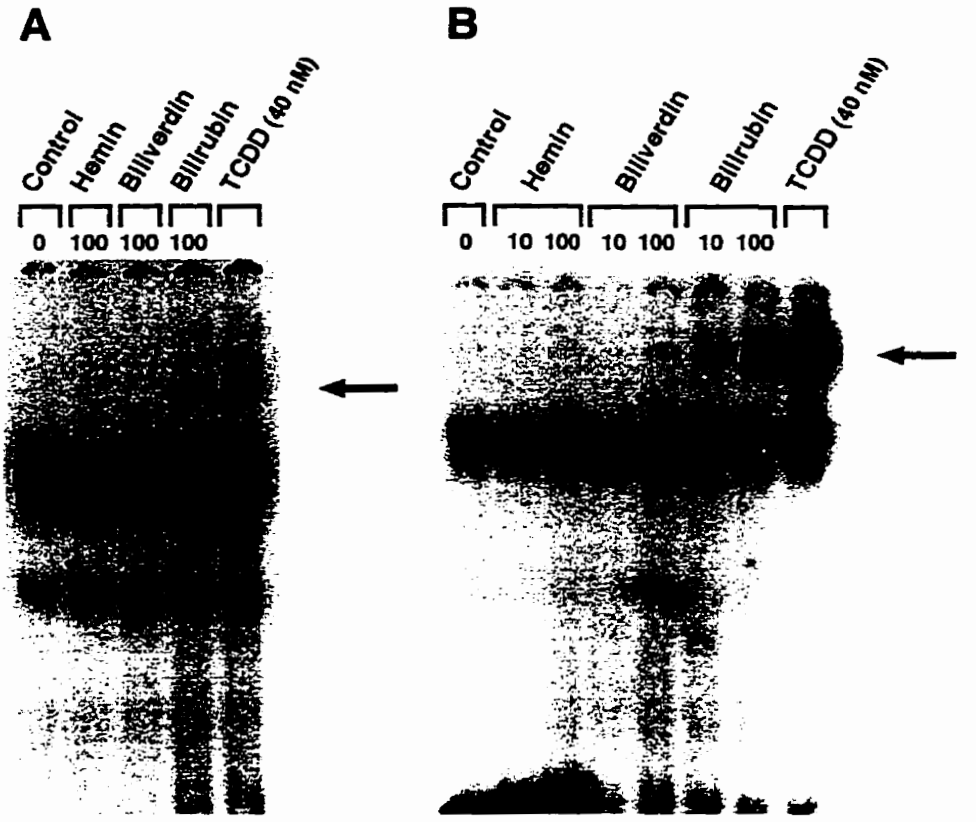


Figure 8.10
Formation of DRE-binding complexes from (A) Hepa 1c1c7 cell or (B) guinea pig hepatic cytosol after *in vitro* transformation with bilirubin. Untreated (Control), hemin, biliverdin or bilirubin treated cytosol was mixed with a ^{32}P-labelled DRE oligonucleotide and the formation of protein-DNA complexes was analyzed by non-denaturing PAGE. Cytosol treated in vitro with 40 nM TCDD was used as a positive control for AHR transformation. The numbers represent the concentration (μM) of test compound and the arrows denote the positions of the ligand-inducible protein-DNA complexes.

of a heterodimer:^{32}P-DRE complex that co-migrated with that induced by TCDD (Fig. 8.10B). Formation of this complex was dose-dependent, occurred in the same concentration range as observed for Cyp1a1 mRNA induction by bilirubin, and was maximal at a concentration of 100 μM (the highest concentration tested). The specificity of this interaction was verified by the ability of an excess of unlabelled DRE oligonucleotide to compete away the bilirubin-induced binding of the transformed AHR to a ^{32}P-labelled DRE in a concentration-dependent manner (Fig. 8.11). The sequence specificity of this interaction was investigated further in experiments with a mutant DRE oligonucleotide containing a single nucleotide transversion within the core of the DRE consensus sequence (GCGTG → GCGCG). Mutations in this core sequence dramatically decrease AHR binding (Shen and Whitlock, 1992; Yao and Denison, 1992). Addition of an excess of this unlabelled mutant oligonucleotide failed to compete away the signal representing the protein:DNA complex at concentrations identical to that used for unlabelled wild type DRE.

8.3.4 Structural Selectivity of Cyp1a1 Induction

Our experiments with hemin, and in particular with biliverdin which has a very similar structure to bilirubin, demonstrate the structural specificity of these compounds for AHR transformation and Cyp1a1 induction. To investigate the structure-activity relationship further, we performed experiments with meso-bilirubin, in which the propionic acid groups are substituted with ethyl groups (Fig. 8.1). Experiments were also carried out using bilirubin diglucuronide, the predominant form of circulating bilirubin in normal mammals (Chowdhury *et al.*, 1994), in which glucuronic acid is attached to the propionic acid groups via an ester linkage (Fig. 8.1). Consistent with our other experiments, treatment of wild type Hepa 1c1c7 cells with 100 μM bilirubin caused a substantial increase of Cyp1a1-dependent EROD activity and steady-state Cyp1a1 mRNA levels (Fig. 8.12). In contrast, treatment with 100 μM meso-bilirubin resulted in a comparatively minor increase of Cyp1a1 mRNA and no significant changes of EROD activity. Similarly, 100

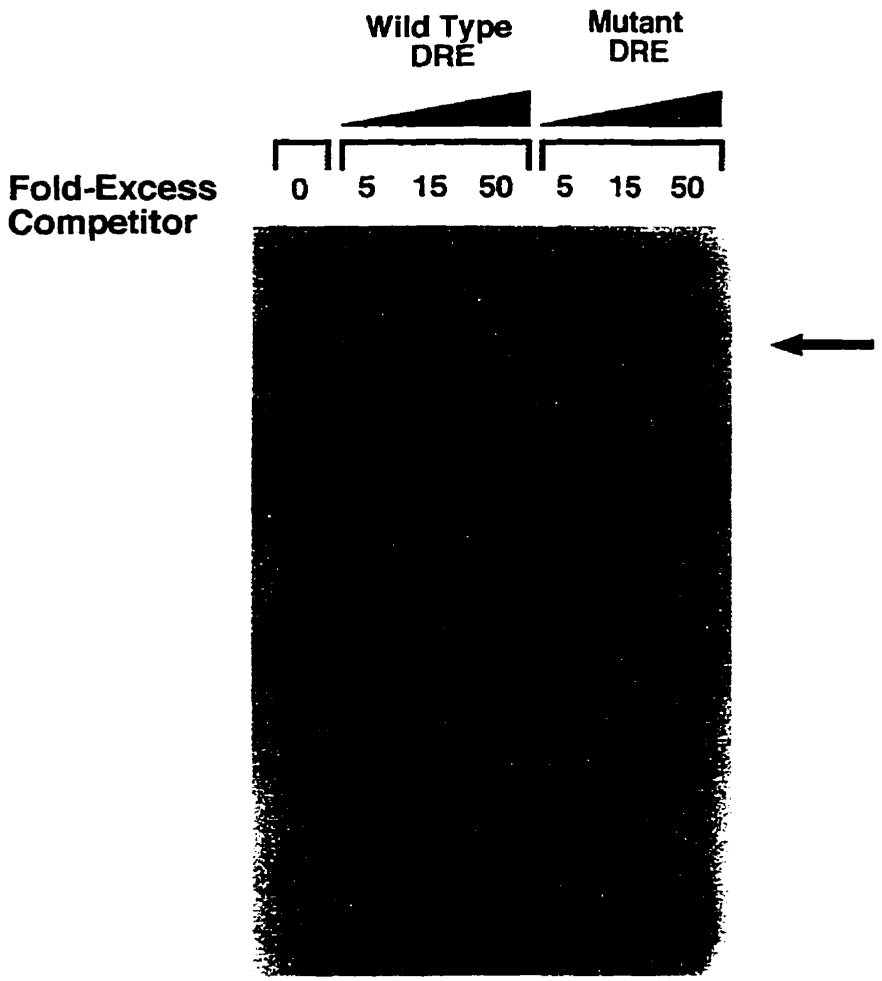


Figure 8.11
DNA sequence specificity for the binding of transformed bilirubin-AHR DRE-binding complexes. After 2 h treatment with 100 μM bilirubin, transformed hepatic cytosol was incubated a further 15 min with 5-, 15- or 50-fold excess of unlabelled wild type or mutant DRE oligonucleotide. Following this, the samples were mixed with a ^{32}P-labelled wild type DRE oligonucleotide and the formation of protein-DNA complexes was analyzed by non-denaturing PAGE. The arrow denotes the position of the ligand-inducible protein-DNA complex.

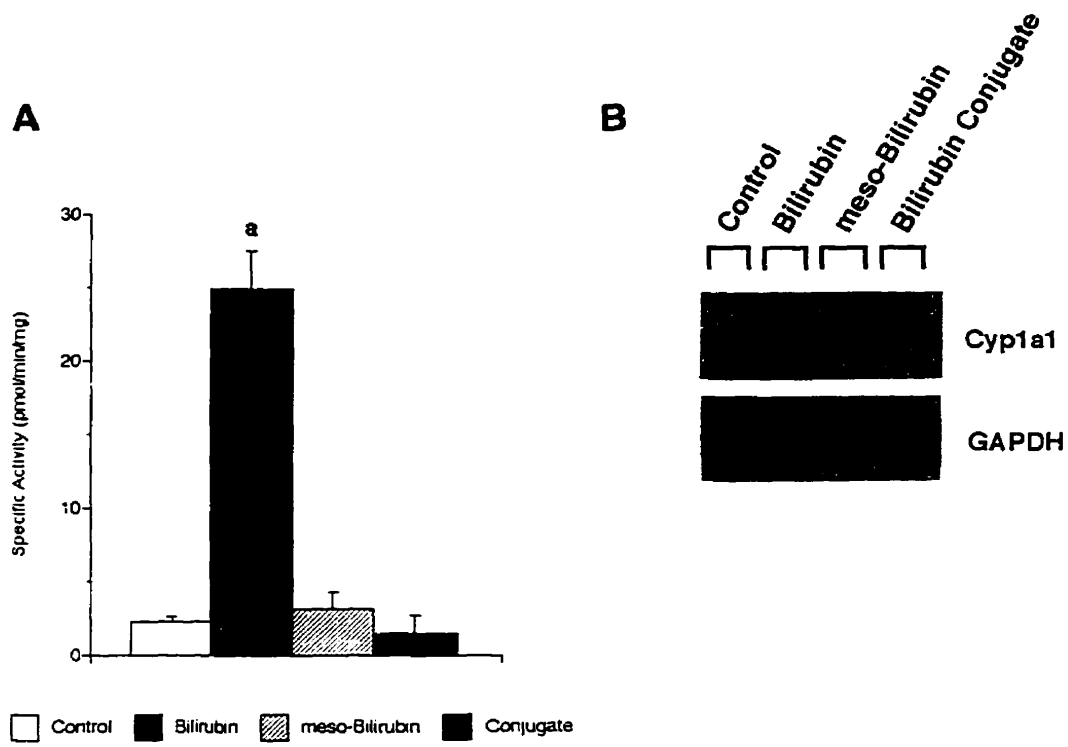


Figure 8.12
Effect of 100 μM bilirubin, meso-bilirubin or bilirubin diglucuronide on Cyp1a1 (A) EROD activity or (B) mRNA level in wild type Hepa 1c1c7 cells. EROD activity and Cyp1a1 mRNA levels were assessed 24 and 3 h, respectively, after treatment with the test compounds. EROD values shown are the mean ± SD of 6 independent experiments performed in duplicate. **a**, significantly different from control, meso-bilirubin and bilirubin diglucuronide ($p<0.01$).

μM bilirubin diglucuronide was also without effect on Cyp1a1 mRNA levels or EROD activity.

8.3.5 Heme Metabolism by Hepa 1c1c7 Cells

To determine if Hepa 1c1c7 cells are capable of heme metabolism, cell lysates were incubated, in the presence of NADPH, with hemin or biliverdin as a substrate. As shown in Table 8.1, Hepa cells exhibited a constitutive level of HO activity that was induced approximately 4-fold, by 6 h after treatment of the cells with 100 μM hemin. This was consistent with the increase of HO-1 mRNA observed after hemin treatment (Fig. 8.2). Hepa cells also expressed constitutive biliverdin reductase activity that was unaffected by hemin treatment (Table 8.1). However the rate of biliverdin metabolism in Hepa cell lysates was much greater than that for basal or induced HO activity, indicating that the latter represents the rate-limiting step in heme metabolism to bilirubin.

8.4 Discussion

In this study, we have shown that hemin, and the endogenous heme metabolites biliverdin and bilirubin, can modulate the expression of Cyp1a1 in Hepa 1c1c7 cells. Previous studies have indicated that endogenous heme may play a role in regulating transcription of a number of CYP genes. For example, it has been reported that induction of CYP1A1/1A2 and CYP2B1/2B2 mRNAs by 3MC and PB, respectively, is inhibited by the simultaneous administration of heme biosynthesis blockers such as cobalt chloride (Bhat and Padmanaban, 1988a; Dwarki *et al.*, 1987; Rangarajan and Padmanaban, 1989). This inhibition was subsequently overcome by supplementation with low levels of exogenous hemin (Bhat and Padmanaban, 1988a; Dwarki *et al.*, 1987). However, in these studies heme was indicated to be a positive factor for induction of CYP gene transcription by prototypical inducers, not an inducer itself. In this sense our data are not consistent

Table 8.1 - Heme oxygenase and biliverdin reductase activity of Hepa 1c1c7 cells. Cell lysates prepared from control or hemin-treated cells (100 μM for 6 h) were incubated with 2 μM hemin or biliverdin in the presence of 1 mM NADPH for determination of heme oxygenase or biliverdin reductase activity, respectively. Bilirubin formation was quantitated by HPLC as described in Materials and Methods. Values represent the mean ± SD of 3 (heme oxygenase) or 4 (biliverdin reductase) individual experiments.

	Specific Activity (pmol/min/mg protein)	
Cell Treatment	Heme Oxygenase	Biliverdin Reductase
Control	2.5 ± 0.7	68.1 ± 8.4
Hemin (6 h)	10.2 ± 1.0[a]	63.6 ± 7.2

[a] significantly different from control ($p<0.01$).

with these previous studies in that we have demonstrated that hemin can increase Cyp1a1 mRNA and EROD activity in the absence of other inducers. However, this difference might be reconciled, in part, by assigning an indirect or precursor role for hemin in Cyp1a1 induction.

The normal route for endogenous heme degradation consists of an initial NADPH- and P450 reductase-dependent oxidation of heme to biliverdin by microsomal HO (Maines, 1988). Subsequently, biliverdin is converted to bilirubin by the NADPH-dependent cytosolic enzyme, biliverdin reductase (Chowdhury *et al.*, 1994). Our data clearly show that this metabolic pathway is active in Hepa 1c1c7 cells and is induced by treatment with hemin. For several reasons, our data suggest that bilirubin, produced by endogenous metabolism of hemin and biliverdin, is the ultimate inducer of Cyp1a1 mRNA and EROD activity. First, bilirubin is a more potent and effective inducer of Cyp1a1 mRNA and EROD activity in Hepa 1c1c7 cells than hemin or biliverdin. The lower efficacy of hemin, and to a lesser extent, biliverdin, is consistent with their precursor roles in the biochemical pathway for bilirubin biosynthesis, as well as the relative rates of metabolism observed in Hepa 1c1c7 cell lysates. However, it is important to note that our data do not take into account the relative rate or extent of uptake of these compounds into the cells, a parameter that was not measured as part of this study. Second, the relative lag time for the onset of Cyp1a1 mRNA induction after treatment is greater for hemin than for biliverdin or bilirubin, consistent with a temporal requirement for the conversion of hemin to biliverdin by HO, the rate limiting enzyme of heme degradation in Hepa 1c1c7 cells. The induction of HO-1 mRNA by hemin, but not biliverdin or bilirubin, is of potential relevance in this regard. Third, while experiments with the mutant cell lines indicated an AHR-dependent mechanism for Cyp1a1 induction by all of the compounds, only bilirubin caused ligand-dependent conversion of the AHR to its DNA binding form. The gel retardation assays showed that biliverdin or hemin lack this activity. Taken together, these data suggest that

bilirubin, derived from the enzymatic degradation of hemin or biliverdin, is the ultimate inducer of Cyp1a1 mRNA and EROD activity.

The results of the gel retardation assay are direct and convincing evidence for an AHR mechanism for Cyp1a1 induction by bilirubin. Moreover, a number of our other results and comparisons with TCDD also support this conclusion. Specifically, the lack of increase of Cyp1a1 mRNA and EROD activity by bilirubin or TCDD in the ARNT-defective (C4) and AHR-defective (C12) mutant Hepa 1c1c7 cells provides additional proof that an intact AHR signal transduction mechanism is required. Induction of the DRE-luciferase reporter construct pGudLuc1.1 by bilirubin and TCDD in transiently transfected Hepa 1c1c7 cells also strongly implies AHR-dependent transcriptional activation. Inhibition of the Cyp1a1 mRNA increase in cells treated with the RNA synthesis inhibitor actinomycin D demonstrates a requirement for *de novo* mRNA synthesis, consistent with increased gene transcription mediated by the ligand bound AHR complex. Finally, the inability of the protein synthesis inhibitor cycloheximide to abrogate increases of Cyp1a1 mRNA in response to bilirubin or TCDD indicates a lack of requirement for other highly labile transcription factors or *de novo* synthesis of another protein factor(s) required for induction. Surprisingly, cycloheximide did not appear to potentiate the increase of Cyp1a1 mRNA in response to bilirubin as has been reported for TCDD (Lusska *et al.*, 1992). Inhibition of bilirubin induction of EROD activity by cycloheximide indicates that *de novo* Cyp1a1 apoprotein synthesis is required. Taken together, these data suggest that bilirubin induces *Cyp1a1* gene transcription in an AHR-dependent fashion, in a manner highly similar, but not identical to that for TCDD.

The AHR binds a number of environmental contaminants including PAHs, halogenated PAHs, heterocyclic amines and PCBs, all of which have polycyclic lipophilic structures with extensive planar regions (Landers and Bunce, 1991). Studies of the three-dimensional structure of bilirubin indicate a biplanar structure that resembles a half-open book, which is produced by rotation about the central carbon atom that links the two

dipyrrole groups (Bonnett *et al.*, 1976; Tiribelli and Ostrow, 1996). This results in two distinct planar regions that lie essentially perpendicular (θ = 100°) to one another, a conformation which is stabilized by hydrogen bonding of each of the carboxyl groups of the two propionic acid moieties with the oxygens and nitrogens of the opposite dipyrrole (Bonnett *et al.*, 1976; Nogales and Lightner, 1995). This three-dimensional structure, combined with the highly conjugated and lipophilic nature of bilirubin, is consistent with an ability to serve as an AHR ligand. It is unknown what structural implications result from the presence of a double bond at the central carbon which joins the two dipyrrole groups in biliverdin (Fig. 8.1). However, it is possible that this double bond restricts free rotation and bending about the central carbon atom and prevents the adoption of a biplanar structure as predicted for bilirubin. In addition to providing confirmation that bilirubin is an AHR ligand and inducer of AHR-mediated gene transcription, a recent study has indicated that biliverdin may also serve as an AHR ligand (Denison *et al.*, 1998). The reason for the apparent discrepancy between these data and our findings regarding biliverdin are unknown, however methodological considerations such as differing sensitivity of some of the techniques employed (*i.e.* gel shift analysis *vs.* displacement of [^{3}H]TCDD binding to the AHR) may have contributed. Replacement of the propionic acid groups with ethyl groups in meso-bilirubin obviously precludes hydrogen bonding between the two planar regions formed by the dipyrroles, a requirement for stabilization of the biplanar structure of bilirubin (Bonnett *et al.*, 1976). A similar prediction can be made for the attachment of glucuronic acid groups to the propionic acid groups in the bilirubin diglucuronide. Furthermore, the glucuronic acid groups are also expected to increase substantially the physical dimensions and hydrophilicity of the diglucuronide, both of which would reduce cell uptake and subsequent AHR binding. Thus, it is not surprising that neither meso-bilirubin nor bilirubin diglucuronide are effective inducers of Cyp1a1-dependent EROD activity or Cyp1a1 mRNA levels.

A number of other aspects of our data are also worth noting. First, the level of Cyp1a1 mRNA increase 3 h after treatment with 100 μM bilirubin was higher than that observed for 1 nM TCDD. In contrast, induction of EROD and luciferase activities 24 h after treatment with 100 μM bilirubin was usually only about 25% of that observed for 1 nM TCDD. Initial dose-response experiments were carried out to verify that induction of EROD and luciferase reporter gene activity was maximal when Hepa 1c1c7 cells were treated with 1 nM TCDD (data not shown). It is possible that the discrepancy of TCDD-induced Cyp1a1 mRNA and EROD activity, when compared with 100 μM bilirubin, resulted from a relative lack of endogenous metabolism of TCDD. Thus, while initial levels of Cyp1a1 mRNA are greater with bilirubin, TCDD most likely caused a more sustained induction. The possibility of post-transcriptional effects on Cyp1a1 mRNA and/or protein by TCDD can also not be definitively ruled out. Second, it is surprising that bilirubin produced such a large magnitude of Cyp1a1 mRNA increase, but yet did not appear to be nearly as effective as TCDD at transforming the AHR to its DNA binding form. One possible explanation is that additional factors and/or signalling pathways contribute to Cyp1a1 induction by bilirubin, although the lack of Cyp1a1 induction in the mutant Hepa 1c1c7 cell lines argues against this. Furthermore, the similar magnitude of DRE/luciferase reporter gene and EROD induction by 30 or 100 μM bilirubin also suggests that other factors are not involved. Further studies may help to explain this behaviour. Finally, the magnitude of induction of luciferase reporter gene activity (2.5-fold) by 100 μM hemin was clearly lower than that observed for EROD activity (5.5-fold). This suggests that hemin itself may have specific transcriptional or post-transcriptional effects on Cyp1a1, independent of its conversion to the AHR ligand, bilirubin. Possibilities that have been suggested previously include positive regulation of induced transcription elongation (Bhat and Padmanaban, 1988a) and/or post-translational augmentation of CYP enzyme activity by an increase in free heme available for incorporation into CYP apoprotein (Jover *et al.*, 1996).

The normal route for bilirubin excretion involves conjugation to bilirubin mono- and di-glucuronides that are subsequently excreted in the bile (Chowdhury *et al.*, 1994). In humans with Crigler-Najjar syndrome and in the congenitally jaundiced Gunn rat, bilirubin conjugation is absent, owing to a mutation in the bilirubin UDP-GT gene (Iyanagi *et al.*, 1989). This results in an accumulation of unconjugated bilirubin in the plasma. Bilirubin has been reported to have beneficial antioxidant effects (Stocker *et al.*, 1987b), however it is clear that severe neurotoxicity (kernicterus) can result from elevated bilirubin levels (Chowdhury *et al.*, 1994). While UDP-GT mutants exhibit pronounced hyperbilirubinemia, the plasma levels of unconjugated bilirubin remain relatively constant, indicating a balance between formation and elimination. This suggests that alternate forms of bilirubin disposal exist and become important in the absence of conjugation. Administration of the CYP1A1 inducer TCDD to Gunn rats significantly decreases unconjugated bilirubin levels in the plasma (Kapitulnik and Ostrow, 1978), while PB, an inducer of different CYP2B, 2C and 3A isoforms, does not (Cohen *et al.*, 1985). A PAH inducible bilirubin oxidase activity has been identified in hepatic microsomes prepared from Gunn rats or chick embryos. (De Matteis *et al.*, 1989). This reaction requires NADPH and O_2 and is inhibited by an antibody that recognizes both CYP1A1 and CYP1A2 (De Matteis *et al.*, 1991a). Taken together, these data suggest a role for CYP1A1 and/or CYP1A2 as an alternate route for bilirubin degradation. Regulation of CYP genes, especially *CYP1A1*, by their substrates is very common (Denison and Whitlock, 1995). Thus, if bilirubin is in fact a substrate for CYP1A1, it is not surprising that elevated levels of CYP1A1 mRNA and protein are present in the liver of Gunn rats (Kapitulnik and Gonzalez, 1993; Kapitulnik *et al.*, 1987). Furthermore, pathobiological conditions, such as orthotopic liver transplantation (Chapter 6 and Sinal *et al.*, 1995) and acute sodium arsenite exposure (Chapter 7 and Albores *et al.*, 1995), that increase plasma bilirubin levels, are also associated with an isozyme-selective increase of CYP1A1 expression in lung and kidney.

Plasma bilirubin concentrations in normal humans range from 5-20 μM (Chowdhury *et al.*, 1994). A portion of this bilirubin exists as water-soluble conjugates, but the majority is highly bound with serum albumin. We have demonstrated in this study that bilirubin diglucuronide does not induce *Cyp1a1* gene expression. Therefore, under normal physiological conditions it is unlikely that bilirubin influences CYP1A1 expression to any significant degree. However, in the Gunn rat and in humans with Crigler-Najjar syndrome, where bilirubin conjugation and elimination are impaired, plasma levels of bilirubin can reach 50-350 μM and 300-800 μM, respectively (Chowdhury *et al.*, 1994). Bilirubin concentrations at this level clearly do have the potential to substantially affect expression of CYP1A1, and possibly other AHR-regulated genes, as demonstrated by our experiments. It is important to note that our experiments were performed using immortalized cell lines, and as such, extrapolation to the *in vivo* situation must be done with caution. However, the faithful reproduction of hepatic basal and inducible *Cyp1a1* expression by Hepa 1c1c7 cells in combination with the demonstration of bilirubin-inducible AHR transformation using cytosol prepared from guinea pig hepatic tissue, suggests that these data are relevant to regulation of Cyp1a1 by bilirubin *in vivo*.

The data presented here are consistent with previous studies that have demonstrated functional associations between Cyp1a1 and bilirubin disposal, as well as bilirubin and Cyp1a1 regulation. Furthermore, our data are the first to clearly demonstrate that bilirubin, an endogenous metabolite of heme, can directly regulate *Cyp1a1* gene expression and enzymatic activity in an AHR-dependent manner. Also of importance is the demonstration that changes in levels of heme catabolites, and potentially, heme precursors, may have significant effects on Cyp1a1 expression. DRE enhancer sequences and AHR-dependent gene regulation have been identified for other CYP genes such as CYP1A2 (Quattrochi *et al.*, 1994) and CYP1B1 (Tang *et al.*, 1996). Additionally, a number of other enzymes, including some glutathione *S*-transferase and UDP-GT isoforms, as well as NADPH:quinone oxidoreductase, involved in chemical detoxication also exhibit AHR-

dependent induction mechanisms (Falkner *et al.*, 1993a; Nebert *et al.*, 1990; Prestera *et al.*, 1993). Thus it is possible, and indeed likely, that heme metabolism is linked to the regulation of some of these genes.

Chapter 9

Concluding Remarks and Future Experiments

9.1 Concluding Remarks

The research findings presented in this thesis represent novel and significant contributions to the understanding of isozyme- and tissue-selective modulation of CYP by MB inactivation and pathobiological stress. With respect to the *N*-aralkylated derivatives of ABT, MI complexation has been identified as a novel mechanism by which these compounds interact with CYP. Based upon the comprehensive characterization presented in Chapter 2, it was concluded that MI complexation does not contribute to MB inactivation of CYP2B by BBT, αMB or αEB, but is more likely to reflect interactions with other PB-inducible (probably CYP3A) isozymes. The principal evidence supporting this conclusion includes the irreversible inactivation of CYP2B at inhibitor concentrations much lower than that required for MI complexation, attenuation by GSH of MI complexation but not MB inactivation of CYP, the instability of MI complexes after formation and the absence of MI complexation with pulmonary microsomes where CYP2B is expressed at significant levels. The detailed kinetic studies presented in Chapter 3 have also provided new significant information about the MB inactivation of CYP by αMB and BBT. For example, evidence was obtained for a previously undescribed mechanism of CYP inhibition via the conversion of BBT, but not αMB, to a metabolite(s) capable of reversible inhibition of CYP1A2. Furthermore, the tissue selectivity of BBT and αMB for pulmonary *vs.* hepatic CYP2B inactivation could be rationalized in large part by the very rapid and potent inactivation of

this isozyme in guinea pig lung compared with liver microsomes. Similarly, the more rapid inactivation of CYP2B by αMB compared with BBT in guinea pig lung microsomes is also consistent with the greater potency and selectivity of the former. The data presented in Chapter 4 reaffirm the selectivity of αMB for CYP2B *vs.* CYP1A1 inhibition in terms of inactivation kinetics. The demonstration that the chirality of the α-carbon substituent is an important mechanistic determinant of the rate of inactivation is consistent with other studies which have shown that oxidation at this carbon is required for generation of the reactive inhibitory species (Kent *et al.*, 1997a; 1997b; Woodcroft *et al.*, 1997).

The isozyme-selectivity exhibited by the ABT derivatives makes them particularly valuable for studies of CYP2B-dependent metabolism in the lung. For example, αMB and other MB inactivators have been used in a complementary approach employing an inhibitory antibody to conclusively identify CYP2B18 as the sole contributor to the formation of EETs from AA in guinea pig lung (Knickle and Bend, 1994). While inhibitory antibodies exhibit a high degree of selectivity and effectiveness in such *in vitro* studies, MB inactivation by αMB or BBT can be used with intact cell systems and can thus be used for studies involving isolated cells, perfused organs and *in vivo*. For example, αMB inhibits bioactivation of the lung-selective toxicant 3-methylindole by goat lung microsomes *in vitro* (Huijzer *et al.*, 1989). Given previous demonstrations of the selectivity of αMB for pulmonary *vs.* hepatic CYP in vivo (Knickle *et al.*, 1994; Mathews and Bend, 1993), it should be possible to elucidate the contribution of lung CYP-dependent metabolism to pulmonary 3-methylindole toxicity. Furthermore, depending upon the dose required and the associated toxicity, prophylactic treatment with αMB may also prove effective to mitigate the toxic effects of 3-methylindole. The studies presented in this thesis provide important mechanistic information about the ABT derivatives and will aid in the rational design and development of other MB inactivators, some of which may be used for therapeutic purposes, including chemoprevention.

The experiments presented in this thesis also demonstrate that pathobiological stress is associated with isozyme- and tissue-selective modulation of CYP. This most likely results from a combination of the direct effects elicited by exposure to toxic chemicals and the indirect effects of tissue injury/inflammation which ultimately cause perturbations of endogenous metabolism and changes in the levels of endogenous compounds. For example, the data presented in Chapter 5 demonstrate that administration of βNF to rats causes a direct AHR-mediated induction of CYP1A1 in all tissues studied. At the same time, the toxicity associated with this treatment also indirectly caused tissue-selective changes in the expression and catalytic activity of CYP2E1, most likely as a result of a disruption of physiological homeostasis at multiple levels. In Chapter 6, orthotopic liver transplantation was shown to differentially depress hepatic CYP-dependent catalytic activity, and to selectively increase CYP1A1 catalytic activity in rat lung, and to a lesser extent in kidney. A very similar response to acute sodium arsenite treatment was observed in Chapter 7 where hepatic CYP was decreased and pulmonary CYP1A1 mRNA and catalytic activity were selectively increased. It is believed that these findings represent the first reports of pulmonary CYP1A1 induction in response to pathobiological stress caused by liver transplantation or acute sodium arsenite administration. The temporal delay of lung CYP1A1 induction in each case, in the absence of co-treatment with known inducers of this isozyme, indicated that alterations of physiological and cellular homeostasis contributed to this response. Both of these models also share the common feature of profound, and in the case of liver transplantation, sustained hepatic HO induction. This observation, the elevation of plasma bilirubin associated with both of these models as reported in Chapter 7 and elsewhere (Albores *et al.*, 1989; Chowdhury *et al.*, 1994; Forster *et al.*, 1993; Kaplan and Szabo, 1983) and reports of indirect evidence that bilirubin may modulate CYP1A1 expression (Kapitulnik and Gonzalez, 1993; Kapitulnik *et al.*, 1987), led to an examination of the effect of heme and two of its immediate catabolites on CYP1A1 expression in a cell culture system. The data presented in Chapter 8 demonstrate directly that bilirubin induces

CYP1A1 expression and catalytic activity in an AHR-dependent manner. These data represent the first report of an endogenous compound that is both a ligand for the AHR and a mediator of CYP1A1 expression. Furthermore, these results also indicate that bilirubin accumulation may contribute, in large part, to the induction of extrahepatic CYP1A1 during pathobiological states.

The data presented in this thesis represent a major contributionto research in the fields of CYP regulation during pathobiological stress and AHR-dependent gene expression. It is now recognized that CYP1A1 expression and inducibility is an important factor in the etiology of human cancer, particularly in the lung (Gonzalez, 1997; Spivack *et al.*, 1997). The finding that pathobiological states can increase the expression of this isoform in lung may lead to the recognition of additional risk factors for pulmonary carcinogenesis and other outcomes resulting from exposure to toxic chemicals. The identification of bilirubin as an endogenous AHR ligand also has implications in cell biology and physiology. While the data presented in this thesis provide evidence only for the induction of CYP1A1 as a result of increased bilirubin formation during pathobiological states, a role for this heme catabolite in homeostasis is also possible. For example, the results of a recent study (Denison *et al.*, 1998) indicate that bilirubin may induce its own metabolism and conjugation by the PAH-inducible bilirubin UDP-GT isoform and that this may represent an endogenous signalling role for the AHR. Finally, while this homeostatic mechanism is not a sufficient explanation for the advantage obtained by the initial development of the AHR in simple organisms, it may represent an evolutionary pressure that has contributed to the maintenance and adaptation of this receptor to perform essential physiological and developmental functions in higher organisms.

9.2 Future Experiments

Some future areas of study stemming from the research presented in this thesis are outlined below:

- To further determine the utility of *N*-aralkylated derivatives of ABT as *in vivo* diagnostic probes of CYP-dependent metabolism and potential therapeutics through detailed toxicological and time course studies.
- To further examine the metabolic activation and mechanisms of inactivation by *N*-aralkylated derivatives of ABT through identification of the sites of covalent modification of CYP apoprotein and the chemical identity of the adducts.
- To gain further evidence that AHR-dependent mechanisms are responsible for the selective induction of CYP1A1 in response to pathobiological states. This could be addressed most directly by using AHR non-responsive (DBA) and AHR-knockout mouse strains.
- To further characterize the changes in heme metabolism that occur during pathobiological states and to quantitate the accumulation of heme metabolites in plasma and tissues.
- To characterize the regulation by bilirubin of other CYPs (*i.e.* CYP1A2, CYP1B1) and xenobiotic metabolizing enzymes (*i.e.* glutathione *S*-transferase Ya, NADPH:quinone oxidoreductase, bilirubin UDP-GT) known to be induced in an AHR-dependent manner.
- To determine if AHR-mediated mechanisms contribute to the toxicity (*i.e.* kernicterus) associated with elevated plasma levels of bilirubin.
- To characterize the dose-response relationships of bilirubin to CYP1A1 induction *in vivo*. This could be addressed most directly by administration of exogenous bilirubin to animals deficient in bilirubin conjugation due to

genetic defects (*i.e.* Gunn rat) or treatment with bilirubin UDP-GT inhibitors (*i.e.* buprenorphine).

- To examine possible relationships between other pathobiological states (*i.e.* hyperoxia) that have been reported to induce pulmonary CYP1A1. The identification of HIF-1α as a bHLH protein that heterodimerizes with ARNT and activates gene expression indicates that interactions with bilirubin-liganded AHR at the DRE or other DNA response elements may occur.

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IMAGE EVALUATION
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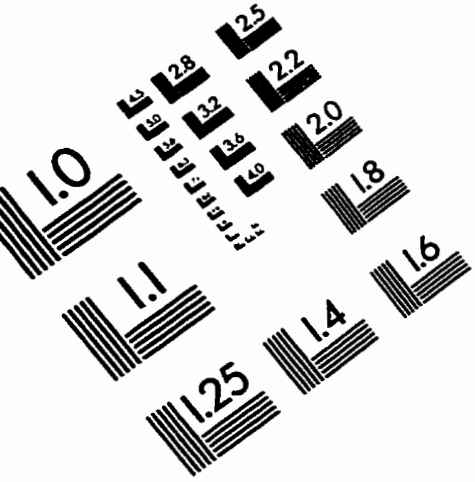

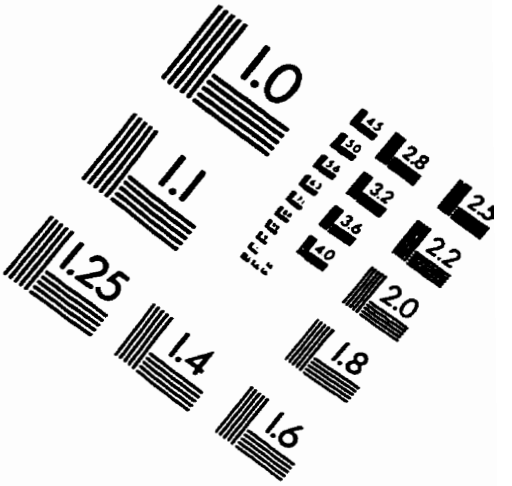

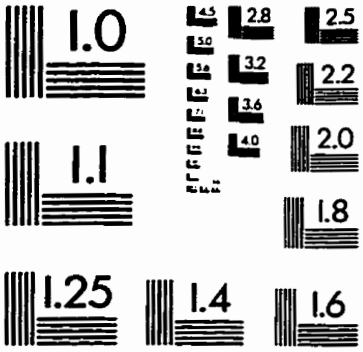

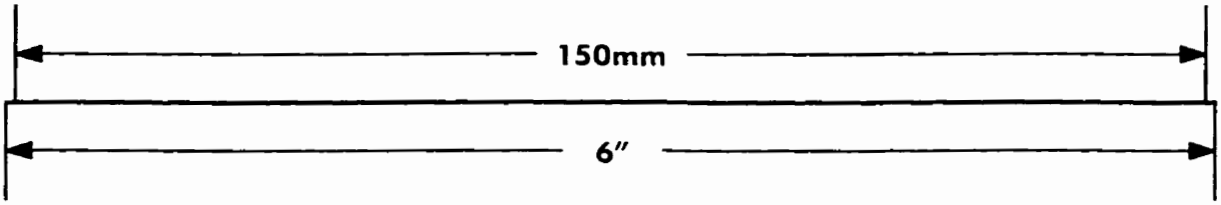

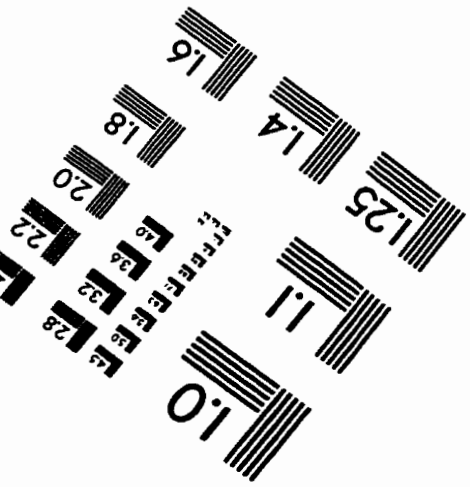

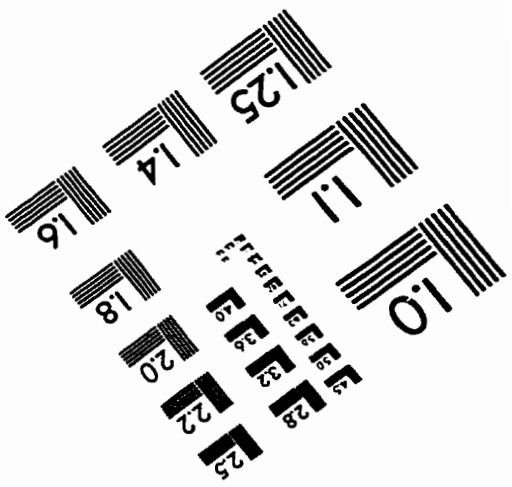

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