

**BIOCHEMICAL AND MOLECULAR CHARACTERIZATION  
OF BEAGLE DOG CYP4A**

RICHARD ALAN GRAHAM

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Approved by

Advisor: Edward L. LeCluyse, Ph.D.

Committee Chair: Dhiren R. Thakker, Ph.D.

Reader: Robert Blouin, Ph.D.

Reader: Mary Paine, Ph.D.

Reader: Bryan Goodwin, Ph.D.

Reader: Ivin S. Silver, Ph.D.

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## LIST OF ABBREVIATIONS

20-HETE	20-hydroxyeicosatetraenoic acid
ACOX	acyl coenzyme A oxidase
AhR	aryl hydrocarbon receptor
BNF	beta-naphthoflavone
BP	base pairs
BROD	7-benzyloxyresorufin O-dealkylation
CAR	constitutive androstane receptor
CLOF	clofibric acid
CPT1A	carnitine palmitoyl transferase-1A
CYP	cytochrome P450
DBD	DNA binding domain
DMSO	dimethylsulfoxide
DR	direct repeat
EROD	7-ethoxyresorufin O-dealkylation
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HMG CoA synthase	3-hydroxy-3-methylglutaryl-CoA synthase
HPLC	high performance liquid chromatography
INH	isoniazid
LBD	ligand binding domain
MGB	minor groove binder
MRM	multiple reaction monitoring

MS	mass spectrometry
PB	phenobarbital
PCN	pregnenolone 16a-carbonitrile
PCR	polymerase chain reaction
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator response element
PXR	pregnane-x-receptor
RIF	rifampin
RXR	9-cis-retinoic acid receptor

## **CHAPTER 1**

### **INTRODUCTION**

## **DRUG METABOLIZING ENZYME INDUCTION**

Induction of drug metabolizing enzymes is an adaptive defense mechanism that serves to protect the cell from accumulating toxicants (endogenous and exogenous) by enabling some xenobiotics to accelerate their own biotransformation (auto-induction) or the biotransformation and elimination of other drugs. Often, xenobiotics can enter the cell with relative ease, due to their high degree of lipophilicity. The first step in detoxification by the process of enzyme induction is generally to increase the expression of Phase I enzymes, for example cytochrome P450 enzymes (CYP), an enzyme system that sets the stage for elimination of xenobiotics, via oxidatative, peroxidative and reductive metabolism. In general, Phase I metabolism introduces a polar functional group onto the non-polar target molecule. Following biotransformation of xenobiotics by CYP, Phase II enzymes (glucuronidation, sulfation, glutathione conjugation, N-acetylation, or conjugation with amino acids) often function to conjugate the product to a more hydrophilic substance thereby increasing the likelihood of excretion from the organism. Recently, a phase III component of biotransformation has been evoked which comprises transport proteins that regulate the access of xenobiotics and drugs to the drug metabolizing enzymes inside the cell. While proteins from the Phase I, Phase II and Phase III class have been shown to be inducible, the magnitude of CYP (Phase I) enzyme induction is by far the greatest.

CYP induction occurs via nuclear receptor mediated transcriptional activation, with few exceptions (e.g., CYP2E1 and CYP2C11, which are induced by mRNA and/or protein stabilization and pulsatile growth hormone secretion in male rats, respectively). Nuclear receptors are ligand activated transcription factors that include receptors for steroid hormones, thyroid hormone, active metabolites of vitamins A and D and orphan nuclear

receptors (receptors for which an endogenous ligand has not been identified) (Whitfield et al., 1999). These receptors form a heterodimeric complex with other receptors and act in the nucleus to mediate gene transcription in response to an inducer or ligand. Specifically, pregnane-X-receptor (PXR), constitutive androstane receptor (CAR), and peroxisome proliferator activated receptor alpha (PPAR  $\alpha$ ) form heterodimers with retinoid-X receptor (RXR) while the aryl hydrocarbon receptor (AhR) binds with AhR nuclear translocator protein (Arnt). CYP1A, CYP2B, CYP3A and CYP4A gene transcription has been shown to be mediated via AhR, CAR, PXR and PPAR  $\alpha$  respectively, in response to enzyme inducers. Cross-regulation of CYP2B and CYP3A genes has been suggested to occur, as these genes respond to both CAR- and PXR-agonists, such as PB and RIF (Moore et al., 2000; Xie et al., 2000).

Activation of nuclear receptors triggered by ligand binding causes a series of events, including recruitment of transcription factors and binding of the nuclear receptor complex to DNA response and/or enhancer elements located within the promoter region proximal to the target gene. These common response elements have been shown to contain the half-site AGGTCA and can be configured into a variety of motifs, including a direct repeat (DR), inverted repeat (IR) or everted repeat (ER) of the half site (Mangelsdorf and Evans, 1995). A further degree of specificity for hormone responses is gained by the number of nucleotides spaced between the two half-sites. Association of ligand-activated nuclear receptor and other transcription factors with the target response element, results in an increase in gene transcription, causing enzyme induction.

A number of P450 enzymes in human and/or rodent liver microsomes are inducible, including various members of the CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, CYP3A, and



CYP4A subfamilies (Parkinson, 2001). To date, nine P450 genes have been sequenced in the dog (compared with up to 60 sequenced genes in the rat) including CYP1A1, CYP1A2, CYP2B11, CYP2C21, CYP2C41, CYP2D15, CYP2E1, CYP3A12, and CYP3A26.

However, for a species that is widely used by the pharmaceutical industry to assess the safety of drugs under development, relatively little information is available in the literature on the inducibility of P450 enzymes in the dog. Several studies have been performed in which a particular P450 cDNA has been cloned and the sequence compared with related enzymes in other species (Ciaccio et al., 1991; Fraser et al., 1997; Graves et al., 1990; Lankford et al., 2000; Ohta et al., 1989; Roussel et al., 1998; Sakamoto et al., 1995). However, much remains to be elucidated about the structure, function, and regulation of dog P450 enzymes.

Two CYP1A enzymes, P450 D2 and P450 D3, have been purified from the liver of polychlorinated biphenyl-treated female beagle dogs (Ohta et al., 1989). Catalytic and structural properties of both proteins were shown to be similar to rat CYP1A2, although P450 D3 exhibits spectral properties similar to those of rat CYP1A1. It is not known whether either of these proteins is constitutively expressed in dog liver (Ohta et al., 1989).

Dog liver microsomes contain CYP2B11 (also called PBD-2), a constitutively expressed and phenobarbital-inducible enzyme with high metabolic activity toward 2,4,5,2',4',5'-hexachlorobiphenyl (Duignan et al., 1988). Like the rat CYP2B1, dog CYP2B11 (PDB-2) catalyzes both the 16 $\alpha$ - and 16 $\beta$ -hydroxylation of testosterone. Another testosterone 16 $\beta$ -hydroxylase has been purified from dog liver and identified as a member of the CYP2C subfamily (Uchida et al., 1990). Whereas rat CYP2B1 catalyzes the 16 $\alpha$ - and 16 $\beta$ -hydroxylation of testosterone at roughly equal rates, dog CYP2B11 preferentially catalyzes the 16 $\alpha$ -hydroxylation of testosterone at 13 to 15 times the rate of testosterone

16  $\alpha$ -hydroxylation (Ohmori et al., 1993). Another CYP2 protein, CYP2D15, has been cloned by Sakamoto et al. (Sakamoto et al., 1995). Recently, CYP2E1 cDNA has been cloned from beagle dogs followed by characterization and expression of the encoded protein (Lankford et al., 2000). Interestingly, the amino acid sequence of dog CYP2E1 exhibits 77% identity to the human ortholog, which is slightly higher than the identity to the rodent or rabbit sequence (75–76%). Characterization of the expressed CYP2E1 protein indicated that dog CYP2E1 has a lower affinity for chlorzoxazone than does human CYP2E (Lankford et al., 2000).

Dog liver is thought to express multiple forms of CYP3A, as has been shown in rat and human. PBD-1, a CYP3A enzyme, was purified from phenobarbital-treated dog liver but also appears to be expressed constitutively (Ciaccio and Halpert, 1989). Molecular and immunochemical analyses indicate the presence of at least one other CYP3A enzyme in dog liver (Ciaccio et al., 1991; Ciaccio and Halpert, 1989). In contrast to rats, there are no marked sex differences in CYP3A activity in dog liver microsomes. Like the corresponding rat enzyme, the dog CYP3A enzyme, CYP3A12 catalyzes the 6  $\alpha$ -hydroxylation of testosterone (Ciaccio and Halpert, 1989). In addition, CYP3A12 catalyzes the 16  $\alpha$ -hydroxylation of testosterone, which is also catalyzed in part by CYP2B11. Recently, a cDNA encoding a protein exhibiting 95.6% amino acid identity with CYP3A12 was isolated from phenobarbital-induced dogs (Fraser et al., 1997). This enzyme, called CYP3A26, is not as prominent as CYP3A12 in hydroxylating steroids.

A CYP4A protein has yet to be identified in dog liver (Adas et al., 1999). DUT-1, purified from liver microsomes of untreated male beagle dogs, catalyzes the 12-

hydroxylation of lauric acid, but the N-terminal sequence of this protein is different from any other P450 characterized to date (Shiraga et al., 1994).

Induction of drug metabolizing enzymes is known to occur across species; however studies on regulation of this process have primarily focused on rodent and human CYPs. There is a species-specific induction profile for the CYP3A family. For example, pregnenolone 16 $\alpha$ -carbonitrile (PCN), a strong inducer of CYP3A in the rat, fails to induce CYP3A6 in the rabbit. Rifampin, a strong inducer of CYP3A4 in the human and CYP3A6 in the rabbit, is not a CYP3A inducer in the rat (Wrighton et al., 1985). Studies to determine the molecular basis of species differences in CYP3A inducibility showed that the differences were a result of an endogenous cellular transcription factor rather than gene structure (Barwick et al., 1996). In these studies, rat, rabbit or human CYP3A response elements were transfected into primary cultures of rat or rabbit hepatocytes treated with dexamethasone, PCN or rifampin. Interestingly, the activation of the CYP3A response element reporter gene acquired the induction characteristics of the cell type into which it was transfected. In other words, rifampin was able to induce the rat CYP3A23 when the rat response element was expressed in primary cultures of rabbit hepatocytes.

A few years after the implication of an endogenous transcription factor to explain CYP3A species differences, the orphan nuclear receptor PXR was discovered and was subsequently shown to play a key role mediating CYP3A gene expression by xenobiotics. Cloning and characterization of PXR from human, rabbit, rat and mouse have shown that there is >95% sequence identity in the DNA binding domains (DBD), whereas the ligand binding domains (LBD) share only 75-80% amino acid identity and display different activation profiles in response to xenobiotics (Zhang et al., 1999; Jones et al., 2000). These

studies suggested that sequence differences in the LBD and not the DBD, appear to serve as the molecular basis dictating the species differences in CYP3A induction (LeCluyse, 2001).

In addition to CYP3A, species differences also exist for other CYPs. CYP1A induction has been shown to be species specific. Omeprazole is known to induce the expression of human CYP1A1/2 through transcriptional activation via the aryl hydrocarbon (Ah) receptor. Studies have shown that CYP1A induction by omeprazole is species specific (Lu and Li, 2001; Shih et al., 1999). For example, treatment of cultured rabbit and human hepatocytes shows increased mRNA expression of CYP1A while treatment of rat hepatocytes yields no change in CYP1A expression.

An important species difference has been identified for induction of CYP4A. Follow up studies of patients who have undergone fibrate drug therapy (Frick et al., 1987), as well as experiments with cultured human hepatocytes, have suggested that humans do not display the same CYP4A inductive effect as do rats and mice (Blaauboer et al., 1990; Richert et al., 2003; Raucy et al., 2004). Similarly, the guinea pig (Tugwood et al., 1998) appears to be refractory to the inductive effects peroxisome proliferators, compounds that markedly induce mouse, rat and rabbit CYP4A.

## **PEROXISOME PROLIFERATION**

Along with enzyme induction, a large number of chemically diverse compounds have been shown to cause hepatic hyperplasia and proliferation of the endoplasmic reticulum and peroxisomes in rodents (Figure 1.1). Such compounds also cause liver tumors in rodents following chronic administration (Reddy and Rao, 1977; Reddy and Qureshi, 1979; Fitzgerald et al., 1981). On the other hand, several non-rodent species (including dog) have been reported to be much less sensitive to peroxisome proliferation (Foxworthy et al., 1990).

Peroxisomes are major sites of oxygen utilization within the cell. They are surrounded by a single membrane and do not contain DNA or ribosomes. Peroxisomes are thought to acquire their protein by selective import from the cytosol. Peroxisomes resemble the endoplasmic reticulum in the sense that they are self-replicating, membrane enclosed organelles that exist without a genome of their own (Alberts B, 2002). Peroxisomes are found in all eukaryotic cells and contain such oxidative enzymes as catalase and urate oxidase at high concentrations. Peroxisomes contain one or more enzymes that use molecular oxygen to remove hydrogen from substrates resulting in the formation of hydrogen peroxide ( $H_2O_2$ ). One major function of reactions by peroxisomes is the metabolism of fatty acids.  $\alpha$ -oxidation results in the alkyl chains of fatty acids being shortened sequentially by two carbon atoms, generating acetyl CoA. Acetyl CoA is exported from the peroxisomes to the cytosol for use in biosynthetic reactions. In mammals,  $\alpha$ -oxidation occurs in both mitochondria and peroxisomes (Alberts B, 2002).

Synthetic peroxisome proliferators are compounds that consist of a broad spectrum of chemicals with little structural similarity other than the presence of an aromatic ring, carboxylic acid and aliphatic chain. Fibrates have been shown to cause peroxisome proliferation, for example the hypolipidemic drug clofibric acid, prescribed for prevention of coronary heart disease in the United States and Europe. Other examples of peroxisome proliferators are certain phthalate ester plasticizers, herbicides and some chlorinated hydrocarbons (Lalwani et al., 1983).

Compounds that cause peroxisome proliferation exert their effects on the liver and other tissues. Peroxisome proliferation results in an increased potential for oxidative DNA damage and decreased gap junctional intercellular communication due to the abundance of

peroxidative enzymes (Bruckner, 2001). Such agents activate the nuclear receptor protein peroxisome proliferator activated receptor alpha (PPAR  $\alpha$ ) which then stimulates the synthesis of several peroxisomal and lipid metabolizing enzymes (e.g., CYP4A), thereby increasing the number and size of peroxisomes in the liver and other susceptible tissues (Lash and Parker, 2001). Following short-term treatment of rats and mice with peroxisome proliferators there is a pleiotropic response which appears to mainly affect the liver. Hepatocytes subsequently display a marked proliferation of peroxisomes and also the smooth endoplasmic reticulum (Latruffe, 1993). In addition to the increase in peroxisomes there is a transcriptional increase in the fatty acid  $\beta$ -oxidation enzymes, including acyl coenzyme A oxidase (ACOX), bifunctional enzymes (BIEN), and 3-ketoacyl-CoA thiolase (thiolase), in the peroxisomal matrix, the CYP4A enzymes found in the endoplasmic reticulum and the cytosolic liver fatty acid-binding protein (L-FABP) (Besnard et al., 1993; Kaikaus et al., 1993; Vanden Heuvel et al., 1993).

In contrast to their carcinogenic effects in rodents, peroxisome proliferators have not been shown to be carcinogenic in other animal species. For example, clofibrate administration to marmosets for 6.5 years (up to 263 mg/kg body weight) did not cause an increase in tumor incidence (Tucker MJ, 1995). Epidemiological studies on the carcinogenic effects of two hypolipidemic drugs administered to humans were suggestive but inconclusive (Bentley et al., 1993). It has been noted, however, that the epidemiological studies were fairly small and therefore may not be sufficiently robust to detect slight increases in human liver carcinogenesis.

A full length cDNA encoding a functional PPAR  $\alpha$  has been cloned from guinea pig and human (Sher et al., 1993; Mukherjee et al., 1994; Bell et al., 1998; Tugwood et al.,

1998). In reporter gene assays using the peroxisome proliferator response element (PPRE) from the rat ACOX promoter, human PPAR $\alpha$ , guinea pig PPAR $\alpha$  and mouse PPAR $\alpha$  display comparable activity (Tugwood et al., 1998). However, the livers of humans and guinea pigs contain approximately an order of magnitude lower PPAR $\alpha$  in comparison to rats and mice suggesting that absolute differences in nuclear receptor levels within the cell play a significant role in the observed species differences in PPAR $\alpha$  activation (Kliwer et al., 1994; Tugwood et al., 1998). Additionally, it has been suggested that active PPAR $\alpha$  in humans may be depleted due to expression of alternatively spliced PPAR $\alpha$  mRNA lacking exon 6 yielding a truncated, inactive PPAR $\alpha$  protein (Tugwood et al., 1996; Palmer et al., 1998).

Compelling evidence for the role of PPAR $\alpha$  in peroxisome proliferation was put forth in a study with transgenic mice lacking PPAR $\alpha$  (Lee et al., 1995; Peters et al., 1997). The targeted mutation resulted in a mutant mouse PPAR $\alpha$  allele that does not produce a functional PPAR $\alpha$  protein. The knockout mice were viable, fertile, and healthy and lacked any observable gross defects, thus implying that mouse PPAR $\alpha$  is not essential in embryonic development. Mice lacking PPAR $\alpha$  do not undergo hepatic peroxisome proliferation, liver enlargement, tumourigenesis, or induction of  $\alpha$ -oxidation enzymes (ACOX, BIEN and thiolase),  $\beta$ -oxidation enzymes (CYP4A1 and CYP4A3) or the cytosolic L-FABP, in response to the peroxisome proliferators clofibrate or Wyeth-14,643 (Wy-14,643). Thus, these data strongly implicate the involvement of PPAR $\alpha$  as a common regulatory mechanism

for controlling the transcriptional activation of the corresponding genes involved in lipid metabolism.

The coordinate induction of lipid-metabolizing enzymes and peroxisome proliferation is an adaptive response resulting in maintenance of homeostasis of cellular lipids, thereby implicating the role of peroxisome proliferation in the regulation of fatty acid metabolism (Lee et al., 1995). A better understanding of the mechanisms regarding the species specific differences in target gene response and ultimately, peroxisome proliferation, could potentially better predict pleiotropic effects brought on by drugs.

## **BIOLOGICAL ROLE OF CYP4A**

CYP4A enzymes are fatty acid hydroxylases that are expressed in most mammalian tissues. These enzymes are selective for the  $\omega$ -hydroxylation of saturated and unsaturated fatty acids (Hardwick et al., 1987; Kimura et al., 1989b; Stromstedt et al., 1990; Wang et al., 1996; Helvig et al., 1998; Nguyen et al., 1999; Hoch et al., 2000), however CYP4A lacks known roles in drug metabolism (Cowart et al., 2002). Metabolism of fatty acids is an important cellular function to facilitate the degradation and elimination of fatty acids that are present at levels in excess of the tissues need for energy production and lipid synthesis (Johnson et al., 2002). Following the  $\omega$ -hydroxylation of long chain fatty acids by CYP4A, oxidation of the alcohol by cytosolic dehydrogenases occurs, leading to the formation of dicarboxylic acids, which are consequently more efficiently oxidized by peroxisomal  $\alpha$ -oxidation than by mitochondrial  $\beta$ -oxidation (Reddy and Mannaerts, 1994). Energy conservation is achieved since  $\alpha$ -oxidation in peroxisomes does not go to completion and the first step in the process is not linked to ATP production. Also, medium chain fatty acids



released during peroxisomal  $\alpha$ -oxidation can be  $\alpha$ -hydroxylated. Following formation of medium and short chain dicarboxylic acids, these compounds can be readily excreted in urine (Johnson et al., 2002).

Arachidonic acid can be metabolized by the classical cyclooxygenase and lipoxygenase pathways to well-characterized metabolites and their respective cardioprotective end products such as prostacyclin (PGI<sub>2</sub>) and 12-hydroxyeicosatetraenoic acid (12-HETE). Major substrates of CYP4A are fatty acids but recent studies have shown that CYP4A can also metabolize other non-fatty acid substrates (Okita and Okita, 2001). CYP4A11 has been shown to partially regulate cell and/or organ physiology by conversion of arachidonic acid to the vasoactive and natriuretic eicosanoid 20-hydroxyeicosatetraenoic acid (20-HETE). Other metabolites of arachidonic acid are the four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET). The EETs are potent dilators of blood vessels resulting from activation of calcium activated potassium channels. 20-HETE, on the other hand, is a potent vasoconstrictor that activates L-type calcium channels which leads to vasoconstriction of vascular smooth muscle (Gross et al., 2005).

Studies have shown that the metabolism of arachidonic acid to 20-HETE displays biphasic kinetics and that both CYP4F2 and CYP4A11 catalyze this reaction in human kidney (Lasker et al., 2000) and human liver (Powell et al., 1998). Recently, animal studies have shown that inhibition of 20-HETE formation by treatment with selective CYP  $\alpha$ -hydroxylase inhibitors can (1) reduce the initial fall in cerebral blood flow after subarachnoid hemorrhage in the rat (Kehl et al., 2002) and (2) reduce myocardial infarct size following ischemia-reperfusion injury in dogs (Nithipatikom et al., 2004). A recent review article suggests that the inhibition of selected CYPs as an approach to the treatment of myocardial

infarction should be further developed (Doggrell, 2004). Due to the emerging importance of CYP4A in the regulation of key physiologic functions, it will be important to elucidate the molecular mechanisms that control the expression of this enzyme.

## **CYP4A REGULATION**

Numerous compounds or physiological conditions have been shown to regulate CYP4A expression in the liver and/or kidney as reviewed previously (Desvergne and Wahli, 1999). Some CYP4A isoforms have been shown to be an indicator of exposure to compounds that are classified as peroxisome proliferators. In rodents, CYP4A enzymes are induced by fibrates and other peroxisome proliferators in liver and kidney. Interestingly, the CYP4A induction response observed in rodents following treatment with peroxisome proliferators is not observed in guinea pig, dog, monkey or human.

### **Molecular Basis for Species Difference in CYP4A Induction**

The mechanism underlying the species specific lack of PPAR mediated target gene response has been studied by a number of investigators; however there is still little evidence to explain the species difference in CYP4A induction. Studies have shown that (1) while the DNA binding domain is highly conserved across species there are amino acid differences in the PPAR LBD. (2) PPAR levels in cultured dog, guinea pig and human hepatocytes are an order of magnitude lower than in rodents. (3) Whereas the rat ACOX (peroxisome proliferator-responsive gene) promoter contains an active PPRE, the human ACOX promoter lacks activity. By analogy, it is plausible that the human (or other non-responsive species) CYP4A gene lacks a functional response element. Another attractive hypothesis to explain the CYP4A species difference is that non-responsive species may lack an activator or have an

active repressor of PPAR $\alpha$  function. Finally, recent studies have identified phosphorylation as a regulator of PPAR $\alpha$ ; however, species differences have not been evaluated regarding this post translational modification (Rochette-Egly, 2003).

### **PPAR Structure**

Three PPAR isotypes exist, PPAR $\alpha$  (NR1C1), PPAR $\beta$  (NR1C2; also called PPAR $\delta$ ) and PPAR $\gamma$  (NR1C3). This subfamily of nuclear receptors is involved in lipid homeostasis, regulation of body weight and food intake, control of inflammation and wound healing (Desvergne and Wahli, 1999; Escher and Wahli, 2000; Michalik et al., 2004). PPAR $\alpha$  is expressed in cells with high catabolic rates of fatty acids and high peroxisome-dependent activities (hepatocytes, cardiomyocytes, proximal tubules of kidney cortex, intestinal mucosa and brown adipocytes). PPAR $\beta$  is ubiquitously expressed with a high concentration in skeletal muscle where it plays an important role in control of fatty acid oxidation (Grimaldi, 2005). Three PPAR $\gamma$  transcripts exist, resulting from alternate promoter usage and splicing: PPAR $\gamma$  1, 2 and 3. PPAR $\gamma$  1 is ubiquitously expressed with predominant expression in adipose tissues and macrophages. PPAR $\gamma$  2 is highly expressed in white adipose tissue where it plays an important role in adipocyte differentiation. PPAR $\gamma$  3 is primarily expressed in the large intestine (Diradourian et al., 2005).

PPARs share a common structure consisting of four major domains, namely, A/B, C, D and E/F (Figure 1.2) (Desvergne and Wahli, 1999). The A/B domain (proximal to the N-terminus) contains a ligand-independent transcriptional activating function (AF-1). The C domain or DNA binding domain consists of two zinc finger-like motifs that can recognize a specific DNA sequence, the PPRE. The D domain is a hinge region that can impact the DNA

binding ability of the receptor. This region is also involved in cofactor interaction. The E/F domain (ligand-binding domain) exhibits a strong ligand-dependent transcriptional activating function (AF-2) and is also involved in the dimerization with the binding partner nuclear receptor, 9-cis retinoic acid receptor (RXR) (Grimaldi, 2005).

PPAR $\alpha$  has been cloned from mouse (Isseemann and Green, 1990), frog (Dreyer et al., 1992), rat (Gottlicher et al., 1992), guinea pig (Bell et al., 1998), chicken (Takada et al., 2000), rhesus monkey (Winegar et al., 2001), human (Sher et al., 1993) and most recently, beagle dog (Nagasawa et al., 2004). Amino acid sequence analysis of the PPAR $\alpha$  LBD indicates a high degree of conservation across species (~90%). While such a high degree of amino acid conservation might suggest similar ligand binding characteristics, it should be noted that a single amino acid change in PPAR $\alpha$  produces PPAR $\alpha$  character in response to agonists (Takada et al., 2000). Furthermore, species differences exist in response to several PPAR $\alpha$  ligands, for example, GW9578, KRP-297, GI262570, L-796449, an effect that might be mediated by PPAR $\alpha$  affinity (Willson et al., 2000). Interestingly, the LBD of dog PPAR $\alpha$  was shown to be 97.0 and 92.4% identical to human and rat PPAR $\alpha$ , respectively (Nagasawa et al., 2004). Further studies revealed that a novel PPAR $\alpha$  agonist, KCL-1999000269 (KCL), transactivated PPAR $\alpha$ , modulated PPAR $\alpha$  target gene expression in hepatocytes, and decreased plasma triglyceride levels in a species dependent manner. These results implicate the importance of the PPAR $\alpha$  LBD in response to PPAR $\alpha$  ligand.

## **Expression Levels of PPAR**

Several literature reports suggest that PPAR levels are an order of magnitude lower in human hepatocyte cultures and human derived cell lines than are levels of the nuclear receptor in rodent hepatocyte cultures and cell lines. Guinea pig, another non-CYP4A inducible species, was reported to have relatively low concentrations of hepatic PPAR (Bell et al., 1998). Interestingly, PPAR levels in cultured dog and human hepatocytes were shown to be expressed at comparable levels to one another but an order of magnitude lower than rat PPAR (Nagasawa et al., 2004). It is intriguing to consider three species that are not responsive to CYP4A inducers express PPAR at markedly reduced levels, relative to species in which CYP4A is highly inducible. In fact, this straightforward correlation has been proposed to explain species differences in PPAR target gene induction and peroxisome proliferation (Palmer et al., 1998); however, it is likely to be an oversimplification since non-CYP4A inducible species are clearly able to undergo some PPAR target gene responses. For example, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG CoA synthase) and carnitine palmitoyl transferase-1A (CPT1A) have been shown to be markedly upregulated following treatment of human hepatocytes with Wy-14,643, suggesting that other cellular factors dictate the species specific CYP4A induction response (Ammerschlaeger et al., 2004).

## **Peroxisome Proliferator Response Elements**

Peroxisome proliferators activate PPAR which forms a heterodimeric complex with RXR (Evans, 1988) and subsequently binds to DNA at the PPRE in the promoter region of

genes associated with the proliferative response, for example, ACOX, rat CYP4A1 and rabbit CYP4A6. The PPREs are comprised of an imperfect direct repeat of a consensus sequence (AGGTCA) separated by one nucleotide (DR-1 element) (Kliewer et al., 1992) that is essential for PPAR binding.

In rabbit, two imperfect DR-1 motifs were identified approximately 677bp upstream of the CYP4A6 gene, and these could be bound by PPAR. Further study demonstrated the importance of the 5'-DR-1 flanking sequences in promoting binding of PPAR/RXR heterodimers and subsequent activation of CYP4A6 gene transcription (Palmer et al., 1995). Subsequently, studies of the DNA elements involved in regulation of rat CYP4A1 identified a PPRE approximately 4,300bp upstream of the gene (Aldridge et al., 1995). Interestingly, a second element was located 35 nucleotides further upstream; however, this element failed to bind PPAR/RXR heterodimers and was unresponsive to Wy-14,643 in gene transfection assays, suggesting that the more distal element was not active. Later work showed that the nucleotides flanking the 5'-region of the DR-1 were essential for PPAR binding, consistent with the rabbit CYP4A6 PPRE (Juge-Aubry et al., 1997). Notably, no PPRE has been identified upstream of the guinea pig or human CYP4A gene (or any other non-responsive species, for that matter), suggesting a strong role for the PPRE in mediating species differences in CYP4A induction and susceptibility to peroxisome proliferation.

### **Post-translational Modification of PPAR**

Recent insights into regulation of PPAR have demonstrated that phosphorylation of this nuclear receptor protein is a key signaling step for several events affecting target gene transcription. Phosphorylation of nuclear receptors occurs on all three major domains, the N-

terminal activation function (AF-1), the ligand-binding and the DNA-binding domains.

PPARs are phosphorylated by multiple kinases, including mitogen-activated protein kinase (MAPK), calcium-dependent protein kinase (PKC), cAMP-dependent protein kinase (PKA) and AMP-activated protein kinase (AMPK), reviewed previously (Rochette-Egly, 2003).

Several years ago, it was demonstrated, for the first time, that rat PPAR $\alpha$  can exist as a phosphoprotein in vivo and the phosphorylation can be mediated by insulin (Shalev et al., 1996). This important finding provided a new direction for the study of PPAR $\alpha$  regulation. Recent work in this area has shown that phosphorylation of nuclear receptors can enhance transcriptional activation by enhancing the recruitment and interaction with transcription factors and coactivators and by stabilization of PPAR $\alpha$  binding to DNA. For example, using hepatocytes isolated from PPAR $\alpha$  knockout mice, it was demonstrated that cotreatment with the PPAR $\alpha$  ligand Wy-14,643 and the PKA activator, cholera toxin, resulted in a synergistic activation of ACOX gene expression (Lazennec et al., 2000). In addition to phosphorylation of nuclear receptors, phosphorylation of coactivators (e.g., SRC-1 (Rowan et al., 2000), TIF2 (Lopez et al., 2001), PCC-1 (Knutti et al., 2001), p/CIP (Font de Mora and Brown, 2000) and p300/CBP (Yuan and Gambia, 2000; Vo and Goodman, 2001)) and corepressors (e.g., SMRT (Hong and Privalsky, 2000)) can also control enhancement of transcriptional activation. These results suggested that nuclear receptor phosphorylation might enhance the activity of other PPAR $\alpha$  target genes. In contrast to activation, nuclear receptor phosphorylation can lead to dissociation from DNA response elements, thereby decreasing ligand affinity. Phosphorylation can also lead to degradation of NUCLEAR RECEPTOR protein.

Functioning as a key mediator in fatty acid metabolism and inflammatory response (Blanquart et al., 2003), suggests that PPAR $\alpha$  expression must be under tight control, enabling the receptor to react to acute conditions. This hypothesis led to the discovery that PPAR $\alpha$  is an unstable protein that can be stabilized by ligand activation. Further studies demonstrated that PPAR $\alpha$  is degraded via the ubiquitin proteasome pathway and ligand stabilization results from a decrease in the protein ubiquitination (Blanquart et al., 2002).

### **PPAR $\alpha$ Signal Transduction Pathway**

While much is known about the basic mechanism of PPAR $\alpha$  mediated transcriptional activation, several recent studies have focused on the signal transduction pathway that controls upstream signaling events. It is becoming apparent that protein-PPAR $\alpha$  interactions are a key signaling event that occurs prior to nuclear receptor binding to DNA response element. Several proteins have been shown to regulate PPAR $\alpha$  activity, including the deoxyuridine triphosphatase (dUTPase) (Chu et al., 1996), heat shock protein 90 (hsp90) (Sumanasekera et al., 2003a; Sumanasekera et al., 2003b) and hepatitis X associated protein 2 (XAP2). dUTPase serves as a repressor of rat PPAR $\alpha$  by preventing formation of a PPAR $\alpha$ /RXR $\alpha$  heterodimer. PPAR $\alpha$  is inhibited by association with hsp90 in an isoform specific manner, whereas XAP2 is an inhibitor of all three PPAR subtypes (Sumanasekera et al., 2003b). A recent finding suggests that the ribosomal protein L11 interaction with PPAR $\alpha$  can decrease binding to the PPRE, resulting in an inhibition of PPAR $\alpha$  target gene expression (Gray et al., 2006). While the biological significance of PPAR-protein interactions is still unclear, it is becoming apparent that the well studied model of nuclear receptor mediated transcriptional activation (ligand activation of nuclear receptor followed



by binding specific sequences in the 5' regulatory region of target genes) is an oversimplification of regulatory control (Figure 1.3). Nuclear receptor-protein interactions as an emerging area of research should provide important mechanistic insights into species differences in the regulation of PPAR target genes.

## **RATIONALE AND OVERVIEW OF DISSERTATION PROJECT**

Beagle dog continues to be a commonly used non-rodent species for toxicological, pharmacokinetic and pharmacological studies in the pharmaceutical industry. Results of these studies are critical in establishing the preclinical efficacy and safety of a drug candidate. Moreover, high significance is placed on animal pharmacokinetic data and therefore autoinduction represents a major complication that must be addressed during drug discovery and development. Compared to other animal species, including humans, our knowledge of the function and regulation of canine CYP enzymes remains incomplete. Thus, an aim of the dissertation project is to evaluate the hypothesis that dog CYP enzymes respond to prototypical enzyme inducers in a manner similar to the corresponding human CYP enzymes (Chapter 2). We therefore propose to examine the induction profile of several CYP enzymes in cultured male dog hepatocytes (in vitro) and in microsomes prepared from the livers of male and female dogs that were treated with prototypical CYP inducers (ex vivo). These inducers will include - naphthoflavone, 3-methylcholanthrene, phenobarbital, isoniazid, rifampin and clofibrilic acid. In each case, several marker substrate reactions and Western immunoblotting will be used to assess CYP enzyme induction. In order to use dog hepatocyte cultures as a model to study P450 enzyme induction it is important to understand which dog P450s are inducible by prototypical rodent and human P450 inducers.

Information regarding the regulation of transcriptional CYP enzyme induction has been

lacking. Indeed, to date, full length sequences of dog AhR, CAR or PXR have not been reported. Graham et al. evaluated CYP2B11, CYP2C21, CYP2C41 and CYP3A12 mRNA expression in male and female dog liver (Graham et al., 2003). While each of these CYP mRNA isoforms were detected in dog liver with no sex differences in expression, the authors did not evaluate the mRNA expression of other important CYPs in these studies (e.g., CYP1A1, CYP1A2 and CYP3A26). Furthermore, at this time, studies have not been reported that discern time- and concentration-dependent changes in CYP mRNA and protein expression. Therefore, studies evaluating the time- and concentration-dependent induction of CYP1A, CYP2B and CYP3A using primary cultures of canine hepatocytes treated with BNF, PB and RIF are presented (Chapter 3). Determination of the concentration response profiles for CYP1A, CYP2B and CYP3A induction with BNF, PB and Rif, respectively, will provide insight into the similarities and differences in dog and human gene regulation. In general, the outcome of the study is important, because relatively little is known about the induction of canine cytochrome P450 enzymes. Furthermore, comparison of derived EC<sub>50</sub> values for CYP induction across species should identify similar or disparate modes of enzyme regulation. The proposed research should enhance our understanding of the similarities and differences in dog and human CYP regulation, thereby, resolving any uncertainties concerning the use of cultured dog hepatocytes as a surrogate model to predict human CYP induction.

Historically, CYP4A has been known as a selective fatty acid  $\omega$ -hydroxylase, however more recently, CYP4A has been shown to partially regulate cell and/or organ physiology by conversion of arachidonic acid to the vasoactive and natriuretic metabolite, 20-HETE. Due to the emerging importance of CYP4A in the regulation of key physiologic functions, it will be important to further characterize the factors that regulate this enzyme.

While CYP4A is presumed to be expressed in beagle dog, there has been no report confirming this assumption. Determination of the dog CYP4A cDNA sequence allows for detection dog CYP4A mRNA using real-time PCR. Studies were therefore designed to clone CYP4A from beagle dog liver. Studies to evaluate the inducibility of dog liver CYP4A were initiated in order to evaluate species differences in the nuclear-receptor mediated regulation of this CYP subfamily. Furthermore, studies are outlined to evaluate the tissue-specific mRNA expression of dog CYP4A isoforms (Chapter 4).

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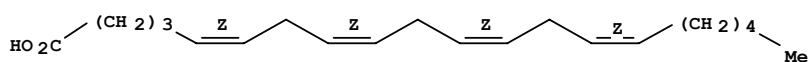
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**Table 1.1** Known Beagle Dog CYP Proteins

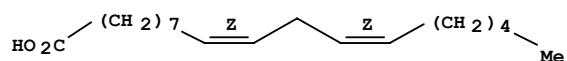
Protein Name	Gene Symbol	Known Inducer <sup>a</sup>	Reference <sup>b</sup>
CYP1A1	P56590	BNF	1,2
CYP1A2	NP_001008720	BNF	1,2
CYP2B11	P24460	PB	2,3
CYP2C21	A37222	Not Tested	
CYP2C41	AAC08738	Not Tested	
CYP2D15	BAA04220	Not Tested	
CYP2E1	AAF88144	Not Inducible	4
CYP3A12	P24463	PB, RIF	2,5
CYP3A26	AAN47145	PB, RIF	2,5

<sup>a</sup>Abbreviations: BNF ( -Naphthoflavone), PB (Phenobarbital), RIF (Rifampin)

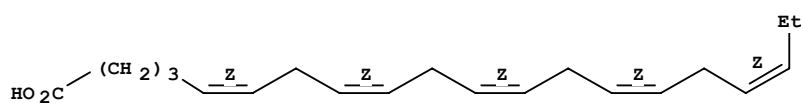
<sup>b</sup>(1) (McKillop et al., 1998); (2) (Nishibe and Hirata, 1993); (3) (Graves et al., 1990); (4) (Lankford et al., 2000b); (5) (Ciaccio and Halpert, 1989).



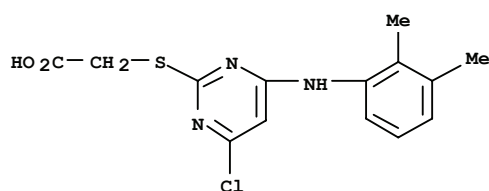
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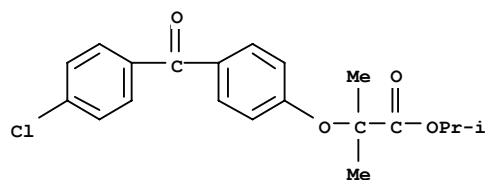
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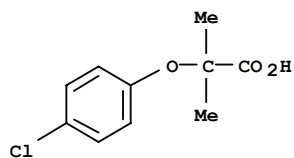
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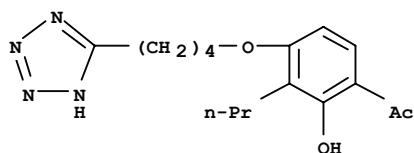
Wy-14,643



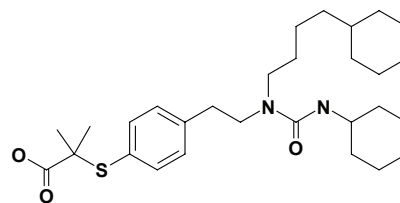
Clofibric Acid



LY-171883



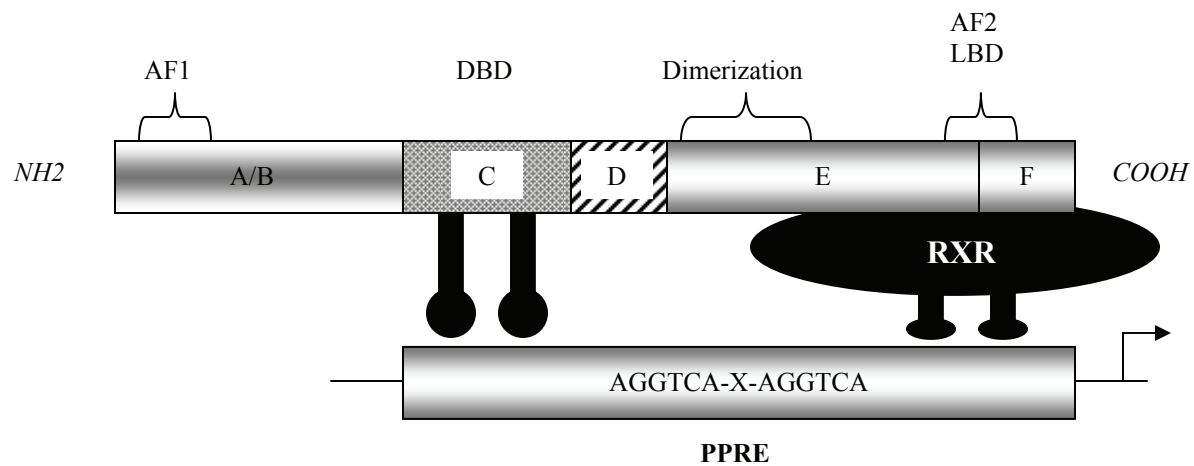
Fenofibrate



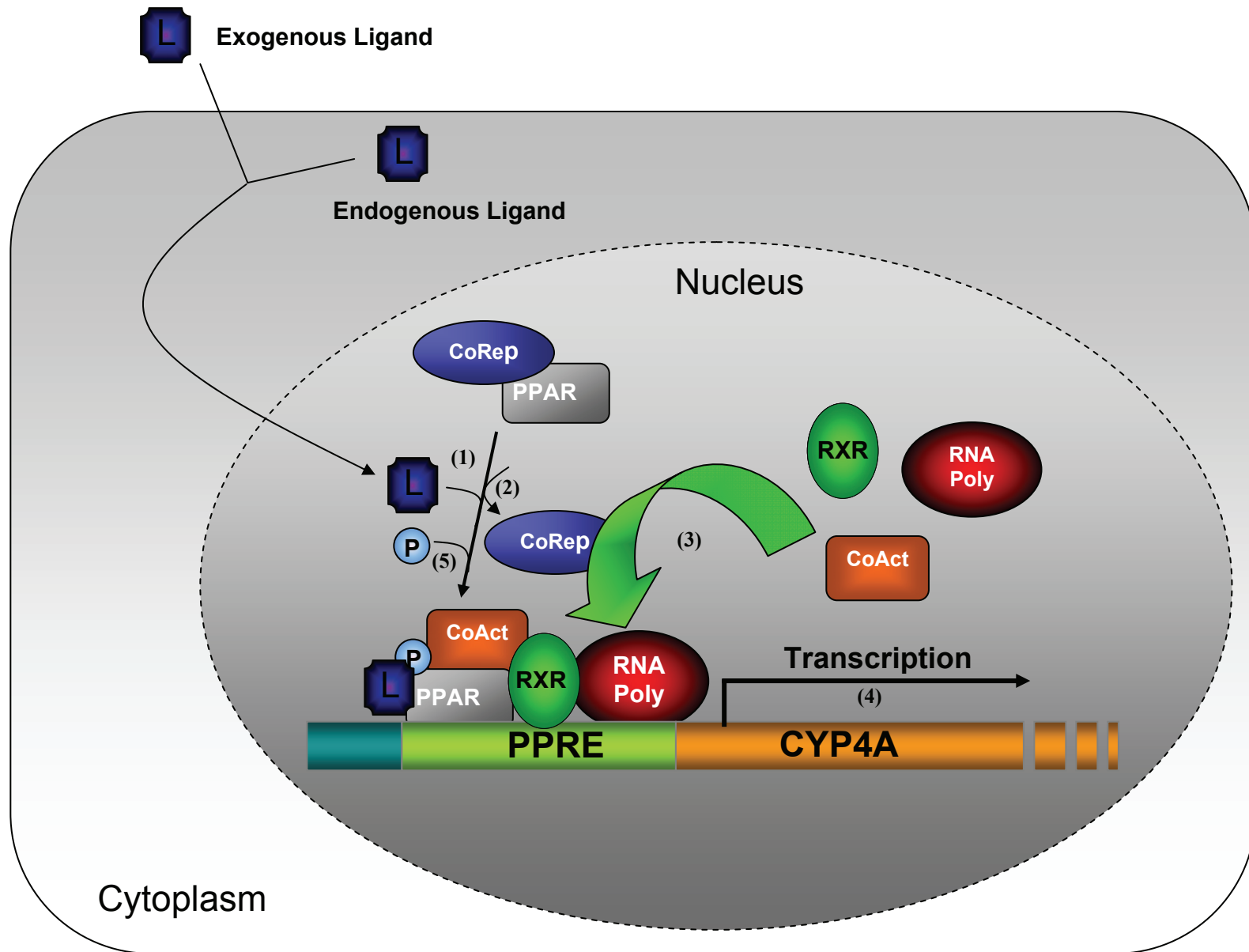
GW7647X

**Figure 1.1** Structures of select PPAR ligands. Arachidonic, linoleic and eicosapentaenoic acid are endogenous ligands, while Wy-14,643, clofibric acid, LY-171883, fenofibrate and GW7647X are synthetic ligands.





**Figure 1.2** Structure of PPAR AF1: activating function-1, DBD: DNA binding domain, AF-2: activating function-2, LBD: ligand binding domain, RXR: 9-cis retinoic acid receptor, PPRE: peroxisome proliferator response element.



**Figure 1.3** Overview of the CYP4A transcriptional activation process controlled by PPAR. Ligand binding (1) causes displacement of constitutive PPAR /corepressor complex (2). Ligand activated PPAR /RXR heterodimer binds to the PPRE upstream of the CYP4A gene causing recruitment of additional coactivators (CoAct) and transcription factors (3), including RNA polymerase (RNA Poly). Subsequently, an increase in CYP4A RNA transcription is achieved (4). Post-translational modification, including phosphorylation (5), has been shown to stabilize PPAR thereby preventing degradation of the protein and/or activation complex.

## **CHAPTER 2**

### **IN VIVO AND IN VITRO INDUCTION OF CYTOCHROME**

#### **P450 ENZYMES IN BEAGLE DOG**

This chapter is based on the following original publication:

Graham RA, Downey A, Mudra D, Krueger L, Carroll K, Chengelis C, Madan A and Parkinson A (2002) In vivo and in vitro induction of cytochrome P450 enzymes in beagle dogs. *Drug Metab Dispos* **30**:1206-13.

## ABSTRACT

The aim of this study was to determine the in vitro and in vivo effects of several prototypical inducers, namely  $\beta$ -naphthoflavone, 3-methylcholanthrene, phenobarbital, isoniazid, rifampin, and clofibric acid, on the expression of cytochrome P450 (P450) enzymes in beagle dogs. For the in vitro induction study, primary cultures of dog hepatocytes were treated with enzyme inducers for 3 days, after which microsomes were prepared and analyzed for P450 activities. For the in vivo induction study, male and female beagle dogs were treated with enzyme inducers for 4 days (with the exception of phenobarbital, which was given for 14 days), after which the livers were removed and microsomal P450 activities were determined ex vivo. Treatment of male beagle dog hepatocyte cultures ( $n = 3$ ) with  $\beta$ -naphthoflavone or 3-methylchloranthrene resulted in up to a 75-fold increase in microsomal 7-ethoxyresorufin O-dealkylase (CYP1A1/2) activity, whereas in vivo treatment of male and female beagle dogs with  $\beta$ -naphthoflavone followed by ex vivo analysis resulted in up to a 24-fold increase. Phenobarbital caused a 13-fold increase in 7-benzyloxyresorufin O-dealkylase (CYP2B11) activity in vitro and up to a 9.9-fold increase in vivo. Isoniazid had little or no effect on 4-nitrophenol hydroxylase (CYP2E1) activity in vitro. Rifampin caused a 13-fold induction of testosterone 6 $\beta$ -hydroxylase (CYP3A12) activity in vitro and up to a 4.5-fold increase in vivo. Treatment of dogs in vivo or dog hepatocytes in vitro with clofibric acid appeared to have no effect on CYP4A activity as determined by the 12-hydroxylation of lauric acid. In general, the absolute rates (picomoles per minute per milligram of microsomal protein) of P450 reactions catalyzed by microsomes from cultured hepatocytes (i.e., in vitro rates) were considerably lower than those catalyzed by microsomes from dog liver (i.e., ex vivo rates). These results

suggest that beagle dogs have CYP1A, CYP2B, CYP2E, and CYP3A enzymes and that the induction profile resembles the profile observed in humans more than in rats.

## INTRODUCTION

Enzyme induction enables some xenobiotics to accelerate their own biotransformation (auto-induction) or the biotransformation and elimination of other drugs. Drugs and new molecular entities are often screened for their ability to induce cytochrome P450 (P450) and other phase I and phase II enzymes with the aim of predicting or explaining drug-drug interactions in humans, and increases in liver weight and/or proliferation of the endoplasmic reticulum or peroxisomes, pharmacokinetic tolerance, and/or formation of liver and thyroid tumors in rodents. Enzyme induction is often evaluated by an *ex vivo* procedure whereby the xenobiotic is administered to rats and mice (or other laboratory animals) *in vivo*, followed by an evaluation of changes in the levels of liver microsomal P450 and other enzymes *in vitro* (Parkinson, 2001).

Beagle dog is widely used throughout drug discovery and development in the pharmaceutical industry. However, surprisingly little information is available on the regulation of P450 induction and regulation in this species. In other species levels of P450 enzymes are affected by age, gender and hormonal status as well as by exposure to drugs. For example, gender differences are pronounced in some species, such as rat, but not in others, such as monkey and human.

Due to the lack of information on the inducibility of P450 enzymes in the dog, we examined the induction profile of several P450 enzymes in cultured male dog hepatocytes (*in vitro*) and in microsomes prepared from the livers of male and female dogs that were treated with prototypical P450 inducers (*in vivo*). These inducers included  $\beta$ -naphthoflavone, 3-methylcholanthrene, phenobarbital, isoniazid, rifampin, and clofibric acid. In each case,

several marker substrate reactions and Western immunoblotting were used to assess P450 enzyme induction.



## **MATERIALS AND METHODS**

### **Chemicals and Reagents**

Insulin, Dulbecco's modified Eagle's medium, GlutaMAX-1 (dipeptide L-alanyl-L-glutamine 200 mM supplied in 0.85% NaCl), modified Chee's medium, minimal essential medium nonessential amino acids, and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Matrigel and ITS<sup>+</sup> (insulin, transferrin, selenium) were purchased from Collaborative Biomedical Products (Bedford, MA). Collagenase (type I) was purchased from Worthington Biochemicals (Freehold, NJ). Vitrogen 100 was purchased from Celtrix (Santa Clara, CA). Androstenedione, L-arginine, bovine serum albumin, clofibrilic acid, dexamethasone, DMSO, fetal bovine serum, EGTA, D-(+)-glucose, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, L-glutamine, 11 $\beta$ -hydroxytestosterone, lauric acid, NADP, 1 $\beta$ -naphthoflavone, 4-nitrophenol, 4-nitrocatechol, Percoll, phenobarbital, testosterone, thymidine, and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid protein assay reagents were purchased as a kit from Pierce Chemical Co. (Rockford, IL). NuPage gels and related electrophoresis reagents were purchased from Novex (San Diego, CA). Polyvinylidene difluoride membranes were purchased from Bio-Rad (Hercules, CA). BCIP/NBT phosphatase substrate was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). 7-Ethoxyresorufin, 7-benzyloxyresorufin, and resorufin were purchased from Molecular Probes Inc. (Junction City, OR). [<sup>14</sup>C]Lauric acid (58 Ci/mol) was purchased from ICN Radiochemicals (Irvine, CA). 6 $\beta$ -Hydroxytestosterone and 16 $\beta$ -hydroxytestosterone were purchased from Steraloids, Inc. (Wilton, NH). Solvents were purchased either from Fisher Scientific (Pittsburgh, PA) or Aldrich Chemical Co. (Milwaukee, WI).

## **Hepatocyte Isolation and Culture**

Three male beagle dogs (Covance Research Products, Inc., Cumberland, VA) were euthanized, and the livers were perfused by a modification of the previously described two-step collagenase digestion method (Seglen, 1976; Seglen et al., 1980; Quistorff et al., 1989; LeCluyse et al., 1996, Madan et al., 1999). Hepatocytes were maintained in culture for 3 days before treatment with P450 inducers. On day 4, the medium was aspirated and replaced with 3 ml of supplemented modified Chee's medium containing vehicle or the inducer. Cultures were treated daily for 3 consecutive days with either vehicle (0.1% DMSO or 0.1% saline), -naphthoflavone (33  $\mu$ M), 3-methylcholanthrene (10  $\mu$ M), phenobarbital (250  $\mu$ M), isoniazid (100  $\mu$ M), rifampin (50  $\mu$ M), or clofibrilic acid (100  $\mu$ M). (Prototypical inducers were dissolved in DMSO, except isoniazid, which was dissolved in saline.) At the end of the treatment period, the hepatocytes were harvested, and microsomes were prepared as described previously (Madan et al., 1999). The microsomal samples were stored at -80°C for later analysis of P450 activities. The protein concentration in the microsomal samples was determined with a BCA Protein Assay Kit, according to Technical Bulletin 23225X from Pierce Chemical Co. (Smith et al., 1985; Wiechelman et al., 1988).

## **Treatment of Dogs in vivo and Preparation of Microsomes**

Male and female beagle dogs (7–18 months old; Ridgman Farms, Mt. Horeb, WI) were treated by subcutaneous injection with corn oil vehicle (two males and two females), saline vehicle (two males and two females), 10 mg/kg/day -naphthoflavone (two males and one female), 10 mg/kg/day rifampin (two males and two females), 10 mg/kg/day clofibrilic acid (two males and one female), or phenobarbital (two males and two females). Animals treated with phenobarbital were treated for 14 consecutive days with dosing escalations from 10

mg/kg/day (days 0 and 1) to 20 mg/kg/day (days 2 through 5) to 30 mg/kg/day (days 6 through 13). Dogs treated with vehicle or enzyme inducers (other than phenobarbital) were treated for 4 consecutive days. After completion of the dosing regimen, the dogs were euthanized by intravenous injection with sodium pentobarbital (5 ml per dog) followed by excision of the livers, which were subsequently perfused with chilled saline. After perfusion, the livers were snap-frozen in liquid nitrogen and stored at -70°C. Microsomes were prepared as described previously (Pearce et al., 1996). This experimental design was reviewed and approved by the Institutional Animal Care and Use Committee of WIL Research Laboratories, Inc. (Ashland, OH).

### **Enzyme Assays**

The O-dealkylation of 7-ethoxyresorufin and 7-benzyloxyresorufin, the 6 $\beta$ -, 16 $\beta$ -, and 16 $\alpha$ -hydroxylation of testosterone, the 4-hydroxylation of nitrophenol, and the 12-hydroxylation of lauric acid were determined by methods described previously (Burke and Mayer, 1974; Wood et al., 1983; Koop, 1986; Sonderfan et al., 1987; Romano et al., 1988; Sonderfan and Parkinson, 1988; Giera and Van Lier, 1991; Tierney et al., 1992; Burke et al., 1994; Pearce et al., 1996). The incubation conditions for each of the assays are given in Table 2.1.

### **Western Immunoblotting**

Microsomal samples were analyzed by Western immunoblotting to determine levels of immunoreactive CYP1A, 2B, 3A, and 4A. Microsomes were subjected to SDS-polyacrylamide gel electrophoresis, based on the method originally described by Laemmli (1970). Briefly, microsomes were mixed in a 1:1 ratio with NuPage sample dilution buffer (pH = 8.5) containing 1.09 M glycerol, 141 mM Tris-base, 106 mM Tris-HCl, 73 mM SDS,

0.51 mM EDTA, 0.22 mM Serva Blue G250, and 0.175 mM phenol red and heated at 100°C for 2 to 5 min. The denatured proteins (up to 10 µg per lane, as specified in figure legends) were subjected to electrophoresis on precast 4 to 12% NuPage bis-Tris gels (pH 6.4 gels; constant voltage of 200 V; electrophoresis time ~55 min) (Novex). Proteins were transferred electrophoretically to polyvinylidene difluoride membranes and subjected to immunoblotting, based on the method by Towbin et al. (1979), with a Blot Module from Novex. Membranes were incubated in blocking buffer containing 10% (w/v) Carnation nonfat dry milk and 0.05% (v/v) Tween 20 in Tris-buffered saline (10 mM Tris-HCl and 150 mM NaCl, pH = 7.4) and then probed with polyclonal antibodies raised against purified rat liver microsomal CYP1A1, CYP2B1, CYP3A1 (Parkinson and Gemzik, 1991), or CYP4A (Affinity Bioreagents, Golden, CO) at final concentrations ranging from 0.25 µg/ml to 10 µg/ml. The secondary antibody was affinity-purified goat-anti-rabbit IgG (H + L) conjugated with alkaline phosphatase from Kirkegaard and Perry Laboratories, which was diluted in blocking buffer to a final concentration of 0.25 µg/ml. Membranes were washed three times with Tris-buffered saline, and the proteins were visualized by incubation with BCIP/NBT phosphatase substrate.

### **Statistical analysis**

For analysis of microsomes from the in vitro cultures, data are mean ± standard deviation of three preparations. An asterisk (\*) indicates a statistically significant ( $p < 0.05$ ) difference from control as determined by a one-way repeated measures analysis of variance test followed by a Dunnett's post hoc test. For ex vivo analysis of microsomes, data are duplicate measurements of a pool of microsomes from two dog livers (with the exception of the -

naphthoflavone and clofibric acid females, in which case, only a single dog liver was available).

## **RESULTS AND DISCUSSION**

There are several reports on the cloning and sequencing of cDNAs encoding dog P450 enzymes, and on the characterization of the catalytic activity of dog P450 enzymes (Gee et al., 1984; Duignan et al., 1987; Ciaccio and Halpert, 1989; Ciaccio et al., 1991; Graves et al., 1990; Nicolas et al., 1995; Sakamoto et al., 1995; Ekins et al., 1996; Fraser et al., 1997; Nishibe et al., 1998; Roussel et al., 1998; Adas et al., 1999; Lankford et al., 2000; Hewitt et al., 2001). Several studies have shown that a select compound can cause induction of one or more P450 enzymes in dog (McKillop and Pickup, 1991; Robertson et al., 1995; Nishibe and Hirata, 1995; McKillop et al., 1998; Mae et al., 1998). However, little information is available on the profile of P450 enzymes induced by prototypical P450 enzyme inducers, namely, those that cause CYP1A, CYP2B, CYP2E, CYP3A, or CYP4A induction in other mammalian species (McKillop 1985; Nishibe and Hirata, 1993; Nishibe and Hirata, 1995; Nishibe et al., 1998). Along with enzyme induction, a large number of chemically diverse compounds have been shown to cause hepatic hyperplasia and proliferation of the endoplasmic reticulum and peroxisomes in rodents. Such compounds also cause formation of liver tumors in rodents after chronic administration (Reddy and Rao, 1977; Reddy and Qureshi, 1979; Fitzgerald et al., 1981). On the other hand, nonrodent species, including dog, have been reported to be much less sensitive to peroxisome proliferation (Foxworthy et al., 1990).

### **Evaluation of Dog Hepatocytes**

Viability of the final preparation of hepatocytes (after Percoll gradient centrifugation) was greater than 70% for each of the three preparations of dog hepatocytes. Dog hepatocytes attached to collagen-coated culture dishes. After 6 days in culture, representative culture

dishes seeded with freshly isolated hepatocytes were photographed under light microscopy (Figure 2.1). Hepatocytes exhibited morphology traits consistent with normal cells: the cells were cuboidal and contained granular cytoplasm with one or two centrally located nuclei (Figure 2.1). Interestingly, the cellular morphology of the cultured dog hepatocytes closely resembled that of cultured human hepatocytes but not rat hepatocytes (LeCluyse et al., 1999, 2000). Unlike rat hepatocyte cultures, dog hepatocyte cultures tended to be completely confluent, covering nearly 100% of the culture dish. Even though the collagen substratum and Matrigel overlay caused cells to spread and flatten to a certain degree, the hepatocytes retained a high degree of three-dimensional architecture.

#### **Induction of cytochrome P450 content in dogs in vivo**

The effects of treating male and female dogs in vivo with  $\beta$ -naphthoflavone, phenobarbital, rifampin, or clofibric acid on total P450 content are shown in Table 2.2. Treatment with  $\beta$ -naphthoflavone resulted in up to a 1.5-fold increase in P450 content, whereas treatment with phenobarbital or rifampin resulted in up to 2.0- and 3.1-fold increases, respectively. On the other hand, treatment with clofibric acid resulted in up to a 32% decrease in P450 content. The determination of total P450 content requires relatively large amounts of microsomal protein (approximately 1 mg per assay); therefore, this assay was not performed with microsomal samples from cultured hepatocytes.

#### **Induction of EROD (CYP1A1/2) Activity**

Although EROD activity has not been shown to be specific for dog CYP1A as it has for the rat, there is indirect evidence suggesting that dog CYP1A catalyzes EROD. McKillop (1985) reported that EROD activity increases 3- to 5-fold in dogs treated with the CYP1A inducer  $\beta$ -naphthoflavone but not with the CYP2B inducer phenobarbital. CYP1A1 and

CYP1A2 mRNA are expressed at low levels in the liver of untreated dogs, but the levels increase after treatment with the polychlorinated biphenyl mixture, Kaneclor KC-500 (Uchida et al., 1990). Treatment of cultured dog hepatocytes with  $\beta$ -naphthoflavone has been shown to increase EROD activity by 25-fold (Nishibe and Hirata, 1993).

The effects of treating dog hepatocyte cultures ( $n = 3$ ) with  $\beta$ -naphthoflavone and 3-methylcholanthrene, phenobarbital, isoniazid, rifampin, or clofibric acid on EROD activity are shown in Figure 2.2A. Treatment of cultured hepatocytes with  $\beta$ -naphthoflavone or 3-methylcholanthrene resulted in a 75-fold induction of EROD (CYP1A1/2) activity, whereas the other inducers had little or no effect (Figure 2.2A). Both the absolute rates (expressed as picomoles per minute per milligram of microsomal protein) and fold induction of CYP1A1/2 by  $\beta$ -naphthoflavone were reproducible ( $<25\%$  relative standard deviation) among three preparations of cultured dog hepatocytes. The effects of treating male or female beagle dogs in vivo with  $\beta$ -naphthoflavone, phenobarbital, rifampin, or clofibric acid, followed by ex vivo analysis of the liver microsomal samples, are shown in Figure 2.2B. Treatment of dogs with  $\beta$ -naphthoflavone resulted in up to a 24-fold increase in EROD activity. Western immunoblotting confirmed that treatment of male or female dogs in vivo or in vitro with  $\beta$ -naphthoflavone (or 3-methylcholanthrene; in vitro only), but not phenobarbital, rifampin or clofibric acid, caused a marked increase in immunoreactive CYP1A1/2 (Figure 2.2C).

There are two notable differences between the CYP1A induction in vitro and ex vivo. First, EROD activity (picomoles per minute per milligram of microsomal protein) in vitro was approximately one-tenth of that observed ex vivo. Second, Western immunoblotting revealed two immunoreactive proteins (tentatively identified as CYP1A1 and CYP1A2) in



microsomal samples from dogs treated with  $\beta$ -naphthoflavone in vivo, whereas the same treatment in vitro appeared to induce a single immunoreactive protein (Figure 2.2C). The exact identity of these immunoreactive proteins is not known; however, since the lower band appeared in untreated male and female dogs, it would seem, by analogy with most other species (with the exception of guinea pig), that the lower band is CYP1A2 (Thomas et al., 1984). By a similar comparison, it appears that only CYP1A1 induction was detected in vitro.

### **Induction of BROD (CYP2B11) Activity**

7-Benzyloxyresorufin O-dealkylation has been shown to be a specific substrate for dog CYP2B11 (Klekotka and Halpert, 1995). Like the corresponding rat enzyme, dog CYP2B11 (PDB-2) catalyzes both the 16 $\alpha$ - and 16 $\beta$ -hydroxylation of testosterone; however, dog CYP2B11 preferentially catalyzes the 16 $\alpha$ -hydroxylation of testosterone at 13 to 15 times the rate of testosterone 16 $\beta$ -hydroxylation (Coulter et al., 1993; Ohmori et al., 1993).

Treatment of cultured dog hepatocytes with phenobarbital resulted in a 13-fold induction of BROD (CYP2B11) activity, whereas the other inducers tested had little or no effect (Figure 2.3A). Both the absolute rates (expressed as picomoles per minute per microgram of microsomal protein) and fold induction of BROD by phenobarbital were reproducible (<25% relative standard deviation) among three preparations of cultured dog hepatocytes.

Treatment of dogs with phenobarbital resulted in up to a 9.9-fold increase in BROD activity (Figure 2.3B). Western immunoblotting confirmed that treatment of male or female dogs with phenobarbital, but not  $\beta$ -naphthoflavone, rifampin, or clofibric acid, caused a marked increase in immunoreactive CYP2B11 (Figure 2.3C). Induction of CYP2B11 in cultured hepatocytes was also measured by testosterone 16 $\alpha$ -hydroxylase activity (Duignan et al.,

1987). The pattern of induction of testosterone 16  $\alpha$ -hydroxylase activity (Table 2.2) was similar to that shown in Figure 2.3A and B, for BROD activity.

It should be noted that the “induced” level of CYP2B11 (in vitro) on the Western immunoblot appeared to be the same as the “control” level in microsomes prepared from untreated male beagle dog, an observation that is consistent with the BROD results (Figure 2.3A and B). These data suggest a marked difference in the degree to which CYP2B11 is expressed in vitro versus ex vivo.

### **Induction of 4-Nitrophenol Hydroxylase (CYP2E1) Activity**

Lankford et al. (2000) have isolated and characterized a full-length CYP2E1 cDNA from a beagle liver cDNA library. The deduced amino acid sequence shares 77% identity to rat, rabbit, and human CYP2E1. In rodents, CYP2E1 catalyzes the hydroxylation of 4-nitrophenol (Koop, 1986). Treatment of cultured hepatocytes with the prototypical enzyme inducers had little or no effect on 4-nitrophenol hydroxylase activity (Figure 2.4). Induction of CYP2E1 was also measured by chlorzoxazone 6-hydroxylase activity, a measure of human CYP2E1 activity. In agreement with the data shown in Figure 2.4 for 4-nitrophenol hydroxylation, treatment with prototypical P450 inducers had little or no effect on chlorzoxazone 6-hydroxylase activity (data not shown). Western immunoblotting for detection of the CYP2E1 isozyme was not performed. Consistent with our results, Jayyosi et al. (1996) reported that treatment of dogs with isoniazid had only a slight effect on chlorzoxazone 6-hydroxylase activity and immunoreactive CYP2E1 levels.

### **Induction of Testosterone 6 $\alpha$ -Hydroxylase (CYP3A12) Activity**

Treatment of cultured hepatocytes with phenobarbital or rifampin resulted in a 7.3- and 13-fold induction of testosterone 6  $\alpha$ -hydroxylase (CYP3A12) activity, respectively, whereas

the other inducers examined had little or no effect (Figure 2.5A). Western immunoblotting confirmed that treatment of cultured hepatocytes with phenobarbital and rifampin, but not - naphthoflavone or 3-methylcholanthrene, isoniazid, or clofibrilic acid, caused a marked increase in immunoreactive CYP3A12 (Figure 2.5C). It should be noted that the induced levels of CYP3A12 on the Western immunoblot indicated a level of protein expression comparable with that observed in microsomes prepared from untreated male beagle dog, an observation that is consistent with the testosterone 6 -hydroxylase results (Figure 2.5A and B). These data also suggest a marked difference in the degree to which CYP3A12 is expressed in vitro versus ex vivo.

Treatment of dogs with phenobarbital or rifampin resulted in up to a 2.6- and 4.5-fold induction of testosterone 6 -hydroxylase (CYP3A12) activity, respectively, whereas the other inducers examined had little or no effect (Figure 2.5B). Western immunoblotting confirmed that treatment of male or female dogs with phenobarbital or rifampin, but not - naphthoflavone or clofibrilic acid, caused a marked increase in immunoreactive CYP3A12 (Figure 2.5C). As noted previously for CYP1A and CYP2B, the CYP3A12 activity in vitro was substantially lower (~1/5) than that observed ex vivo. However, in the case of CYP3A12, the fold induction was greater in vitro, which is attributable to the higher control CYP3A12 activity ex vivo. These results are in agreement with a study that reported a 5-fold induction of testosterone 6 -hydroxylation in dog hepatocytes followed by treatment with 30  $\mu$ M rifampin (Nishibe and Hirata, 1995).

#### **Induction of Lauric Acid 12-Hydroxylase (CYP4A) Activity**

Treatment of freshly isolated hepatocytes with clofibrilic acid had little or no effect on lauric acid 12-hydroxylase activity (CYP4A); however, phenobarbital and rifampin appeared

to increase this activity by 2-fold (Figure 2.6A). Treatment of dogs in vivo with the prototypical inducers examined had little or no effect on lauric acid 12-hydroxylase activity (Figure 2.6B). The lack of CYP4A induction by clofibric acid was confirmed by Western immunoblotting (Figure 2.6C); however, the 2-fold increase in activity by phenobarbital and rifampin was not associated with a 2-fold increase in CYP4A levels. As in other cases, the rate of lauric acid 12-hydroxylation was substantially lower in vitro compared with that observed ex vivo.

Treatment of rats with the peroxisome proliferator, clofibric acid, results in induction of lauric acid 12-hydroxylase activity, a marker of CYP4A1–3. In contrast to the rat, human CYP4A does not appear to be an inducible enzyme (Butterworth et al., 1989). DUT-1, purified from liver microsomes of untreated male beagle dogs, catalyzes the 12-hydroxylation of lauric acid, but the NH<sub>2</sub>-terminal sequence of this protein is different from that of any other P450 characterized to date (Shiraga et al., 1994). Treatment of dog hepatocytes with clofibric acid and subcutaneous injection of male and female beagle dogs with clofibric acid had no effect on lauric acid 12-hydroxylase activity. Consistent with this finding, clofibric acid did not induce immunoreactive CYP4A either in vitro or in vivo. Since DUT-1 does not bear amino acid sequence homology with CYP4A proteins (Shiraga et al., 1994), it is possible that DUT-1 (or another enzyme that catalyzes lauric acid 12-hydroxylation) is inducible by rifampin and phenobarbital. Assuming dogs do have a CYP4A enzyme(s) that catalyzes the 12-hydroxylation of lauric acid, the current data suggest that its lack of inducibility is similar to that of human CYP4A. Additionally, similar to humans, dogs appear to be less sensitive to peroxisome proliferation, a phenomenon that is associated with CYP4A induction in rodent species only (Foxworthy et al., 1990).

## CONCLUSION

In the present study, we attempted to evaluate the effects of prototypical P450 enzyme inducers on the in vivo and in vitro expression of P450 enzymes in dogs.  $\beta$ -Naphthoflavone or 3-methylchloranthrene resulted in a large increase in microsomal 7-ethoxyresorufin O-dealkylase (CYP1A1/2) activity in vitro and in vivo. Phenobarbital caused up to a 13-fold increase in 7-benzoyloxyresorufin O-dealkylase (CYP2B1) activity in vitro and in vivo. Isoniazid had little or no effect on 4-nitrophenol hydroxylase activity in vitro. Rifampin caused up to a 13-fold induction of testosterone 6 $\beta$ -hydroxylase (CYP3A12) activity in vitro and in vivo. Treatment of dogs in vivo or dog hepatocytes in vitro with clofibrate appeared to have no effect on CYP4A activity as determined by the 12-hydroxylation of lauric acid. In general, the absolute rates (picomoles per minute per milligram of microsomal protein) of P450 reactions catalyzed by microsomes from cultured hepatocytes (i.e., in vitro rates) were considerably lower than those catalyzed by microsomes from dog liver (i.e., ex vivo rates). These results suggest that beagle dogs have CYP1A, CYP2B, CYP2E, and CYP3A enzymes and that the induction profile resembles the profile observed in humans more than in rats.

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**Table 2.1** Incubation conditions for microsomal P450 assays

Assay	Incubation Conditions		
	[Protein] (mg/mL)	Incubation Time (min)	[Substrate] ( M)
In Vitro			
P450 content	ND	ND	ND
EROD	0.05	20	10
BROD	0.05	20	10
4-Nitrophenol hydroxylation	0.10	60	100
Testosterone oxidation	0.05	32	250
Lauric acid hydroxylation	0.10	40	100
Ex vivo			
P450 content	1.0	N/A	N/A
EROD	0.10	10	10
BROD	0.10	10	10
4-Nitrophenol Hydroxylation	ND	ND	ND
Testosterone Oxidation	0.20	8	250
Lauric Acid Hydroxylation	0.40	10	100

ND, Not determined; N/A, Not applicable ; CYP, Cytochrome P450; EROD, 7-Ethoxyresorufin O-dealkylation; BROD, 7-Benzyloxyresorufin O-dealkylation.

**Table 2.2** The effect of treating dog hepatocytes or subcutaneous injection of dogs with prototypical CYP inducers on CYP content and testosterone 16  $\beta$ -hydroxylation (CYP2B) activity.

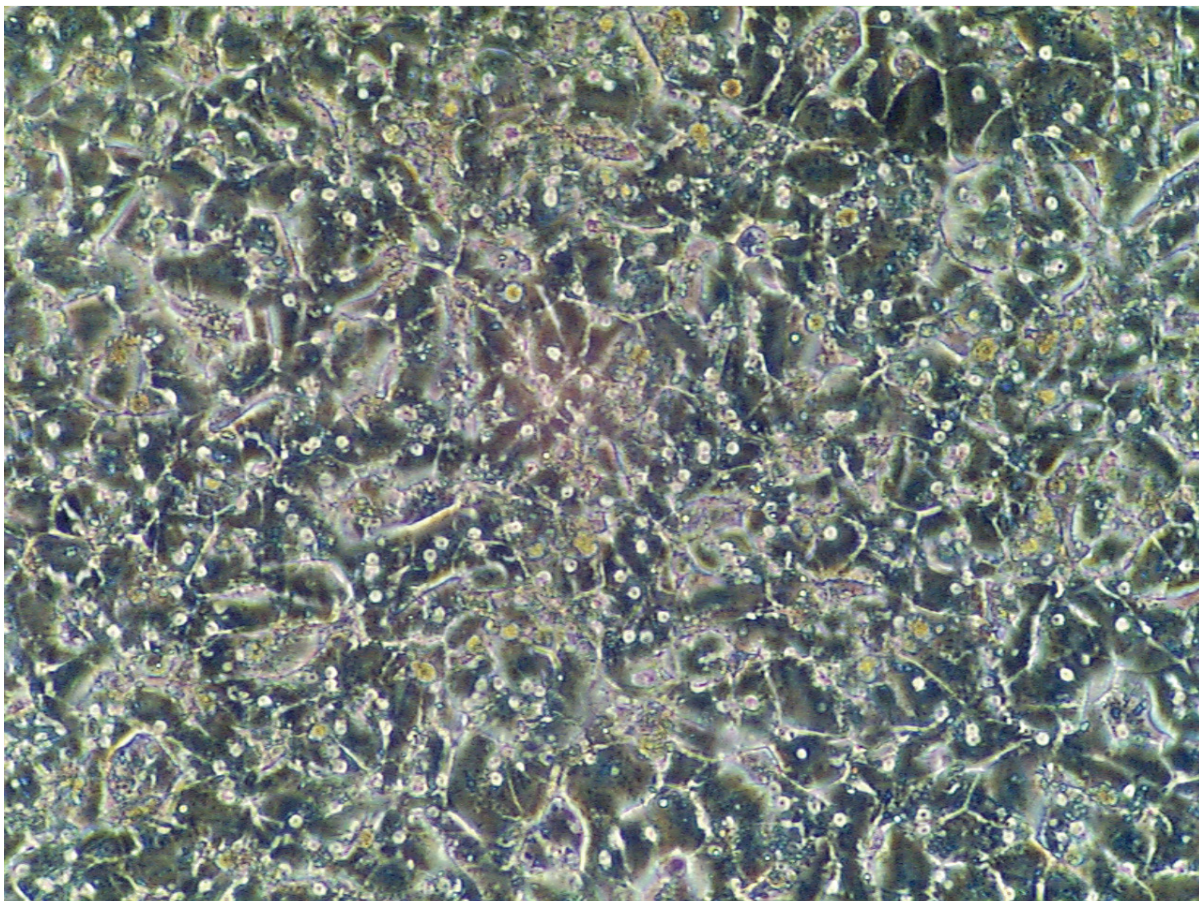
Treatment	CYP Content <sup>a</sup> (nmol/mg protein)	Testosterone 16 $\beta$ -hydroxylation <sup>a</sup> (pmol/mg protein/min)
In vitro <sup>b</sup>		
0.1% DMSO	ND	BLQ
Saline	ND	BLQ
-Naphthoflavone	ND	BLQ
3-methylchloranthrene	ND	BLQ
Phenobarbital	ND	105 $\pm$ 36
Isoniazid	ND	BLQ
Rifampin	ND	11.4 $\pm$ 4.0
Clofibric acid	ND	BLQ
Male Ex vivo <sup>c</sup>		
Corn oil	0.430	577
Saline	0.376	530
-Naphthoflavone	0.585	196
Phenobarbital	1.30	2710
Rifampin	0.865	687
Clofibric acid	0.259	363
Female Ex vivo <sup>c</sup>		
Corn oil	0.383	753
Saline	0.435	495
-Naphthoflavone	0.593	226
Phenobarbital	1.20	3390
Rifampin	0.650	605
Clofibric acid	0.259	344

ND, Not determined; BLQ, Below limit of quantification.

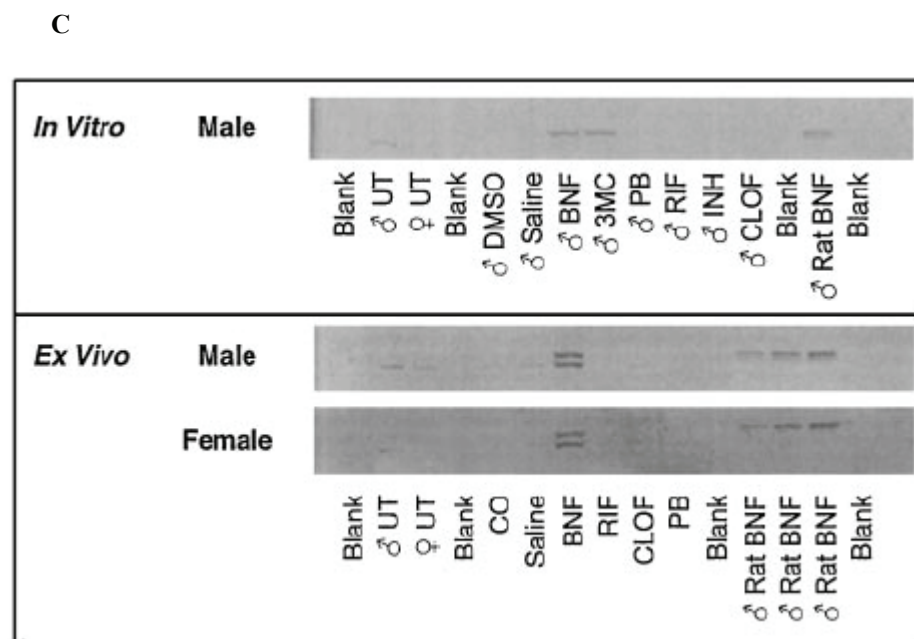
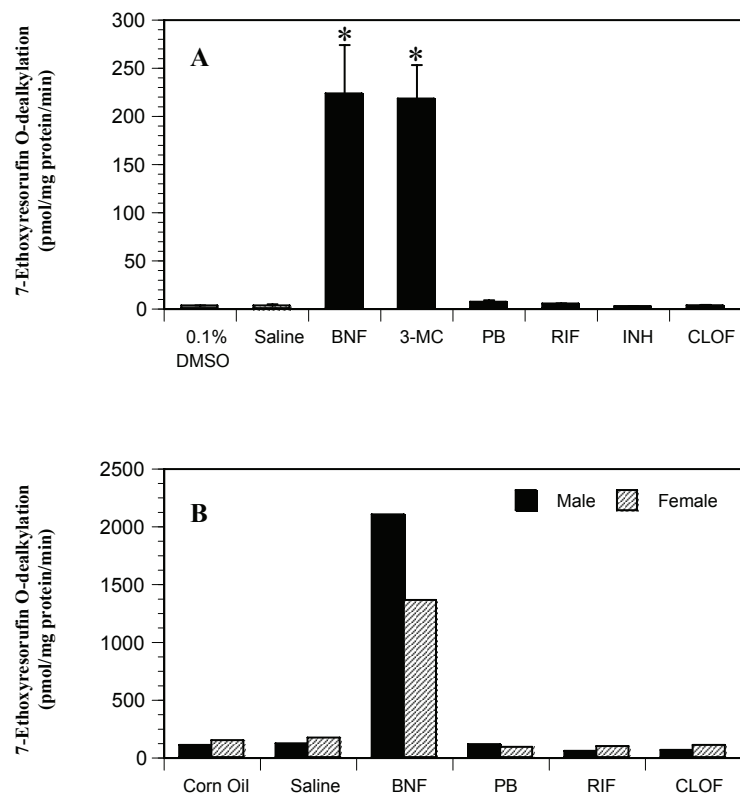
<sup>a</sup>Cytochrome P450 content and testosterone 16  $\beta$ -hydroxylation were determined as described under Materials and Methods. The units for activity data are pmol/min/mg microsomal protein, and those for CYP content are pmol/mg microsomal protein.

<sup>b</sup>Data are mean  $\pm$  standard deviation of three preparations of dog hepatocytes, each analyzed in duplicate.

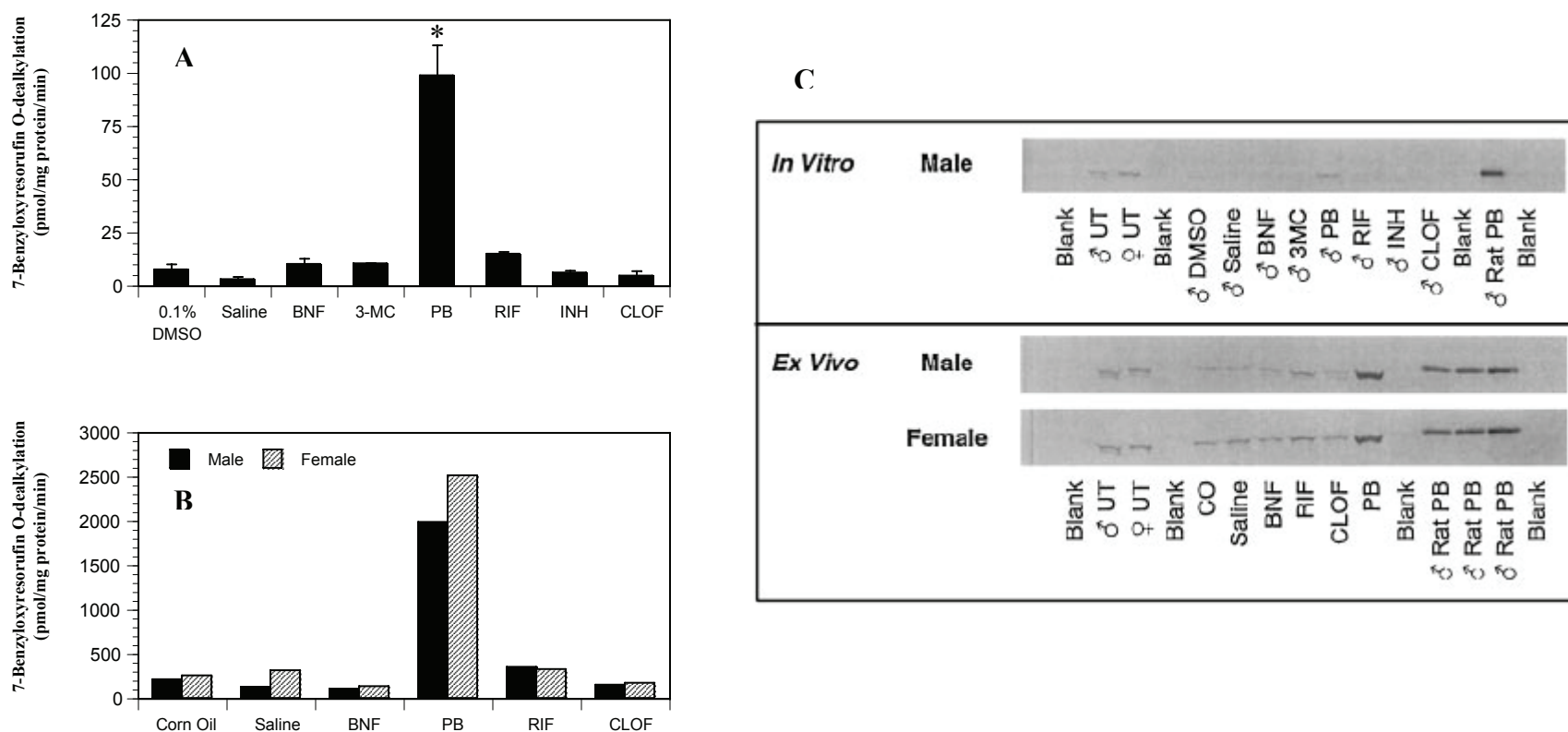
<sup>c</sup>Data are duplicate measurements of a pool of microsomes from two dog livers (with the exception of the -naphthoflavone and clofibric acid females, in which case, only a single dog liver was available).



**Figure 2.1** Photomicrograph of primary cultures of dog hepatocytes treated with 0.1% DMSO (vehicle control). Freshly isolated dog hepatocytes were isolated and cultured for six days as described in Materials and Methods. On day six, the cultures were photographed under a phase contrast microscope. Magnification factor = 300.

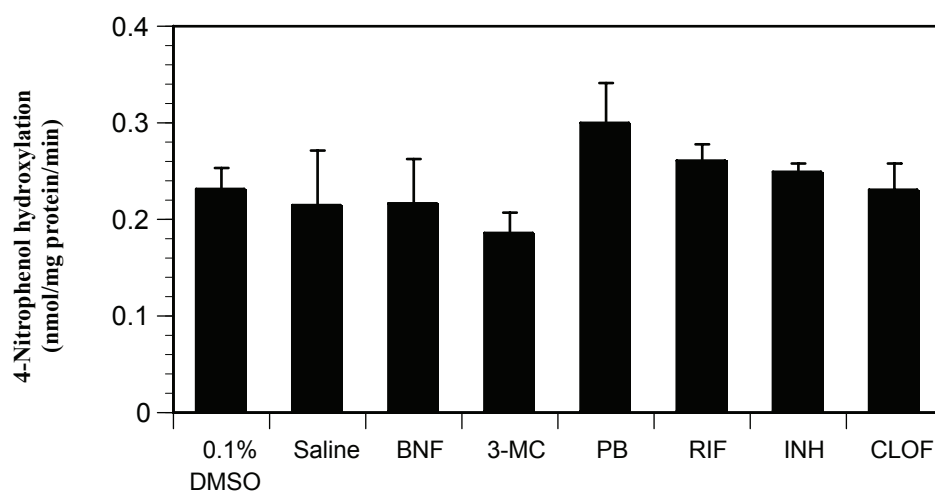


**Figure 2.2** The effect of treating dog hepatocytes or subcutaneous injection of dogs with prototypical CYP inducers on 7-ethoxyresorufin O-dealkylase (CYP1A) activity. Freshly isolated dog hepatocytes were isolated and cultured for up to six days, and Beagle dogs were treated by subcutaneous injection as described in Materials and Methods. (A) In vitro cultures treated with prototypical inducers. (B) Male and female Beagle dogs were treated with prototypical inducers. (C) Microsomes were analyzed by Western immunoblotting. Gels were loaded with 10  $\mu$ g/lane (except for Rat BNF samples, which were 0.05, 0.10 and 0.20  $\mu$ g/lane for ex vivo samples and 0.1  $\mu$ g/lane for in vitro samples). Samples of rat liver microsomes prepared from rats treated with one of four prototypical inducers were also analyzed as reference standards. Asterisk (\*) indicates  $p < 0.05$  as described in Materials and Methods. UT, untreated; DMSO, dimethylsulfoxide; BNF, -naphthoflavone; 3MC, 3-methylcholanthrene; PB, phenobarbital; RIF, rifampin; INH, isoniazid; CLOF, clofibrilic acid; CO, corn oil.



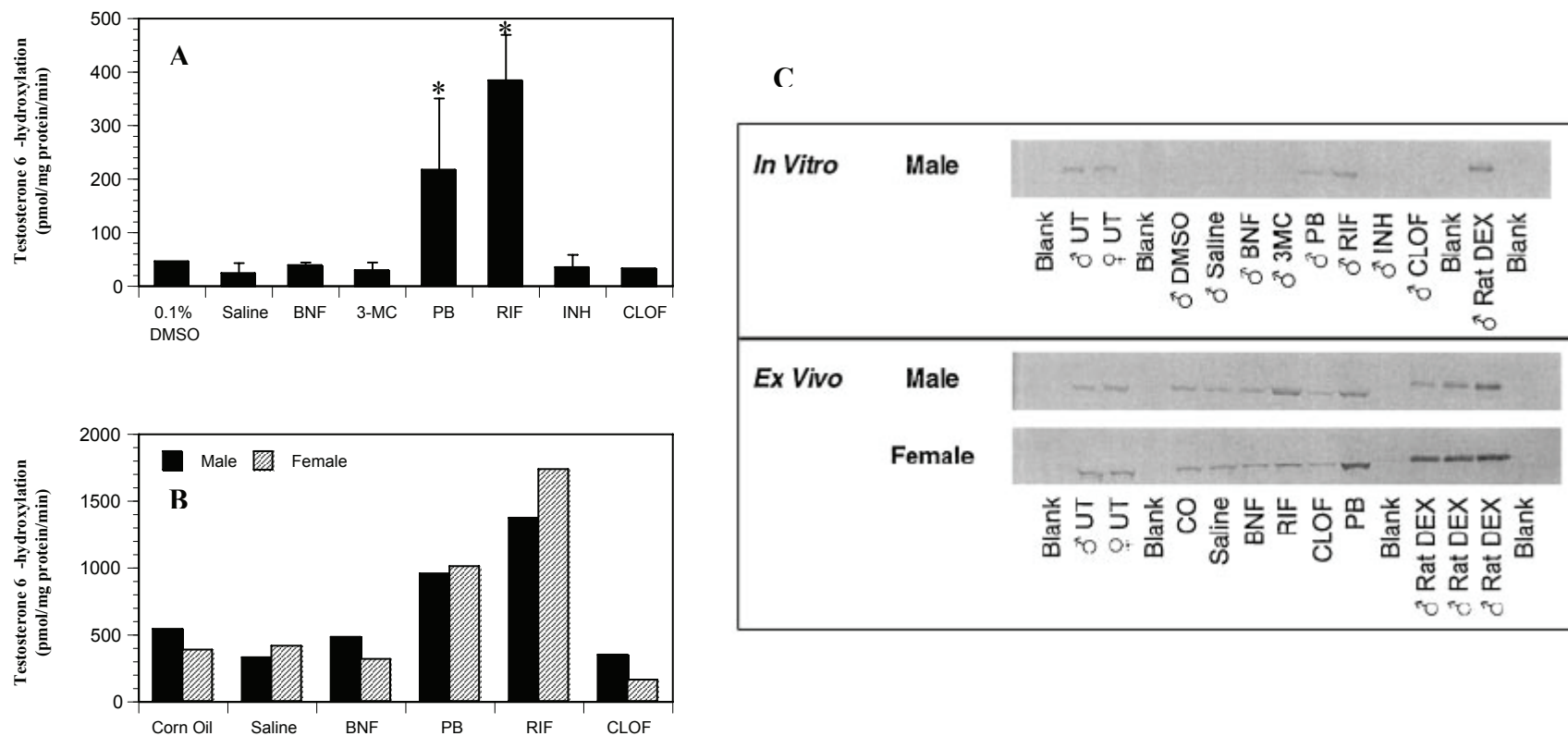
**Figure 2.3** The effect of treating dog hepatocytes or subcutaneous injection of dogs with prototypical CYP inducers on 7-benzoyloxyresorufin O-dealkylase (CYP2B) activity. Freshly isolated dog hepatocytes were isolated and cultured for up to six days, and Beagle dogs were treated by subcutaneous injection as described in Materials and Methods. (A) In vitro cultures treated with prototypical inducers. (B) Male and female Beagle dogs were treated with prototypical inducers. (C) Microsomes were analyzed by Western immunoblotting. Gels were loaded with 10  $\mu$ g/lane (except for Rat PB samples, which were 0.05, 0.10 and 0.20  $\mu$ g/lane for ex vivo samples and 0.1  $\mu$ g/lane for in vitro samples). Samples of rat liver microsomes prepared from rats treated with one of four prototypical inducers were also analyzed as reference standards. Asterisk (\*) indicates  $p < 0.05$  as described in Materials and Methods.

UT, untreated; DMSO, dimethylsulfoxide; BNF, 6-benzyl-5-naphthoflavone; 3MC, 3-methylcholanthrene; PB, phenobarbital; RIF, rifampin; INH, isoniazid; CLOF, clofibric acid and CO, corn oil.



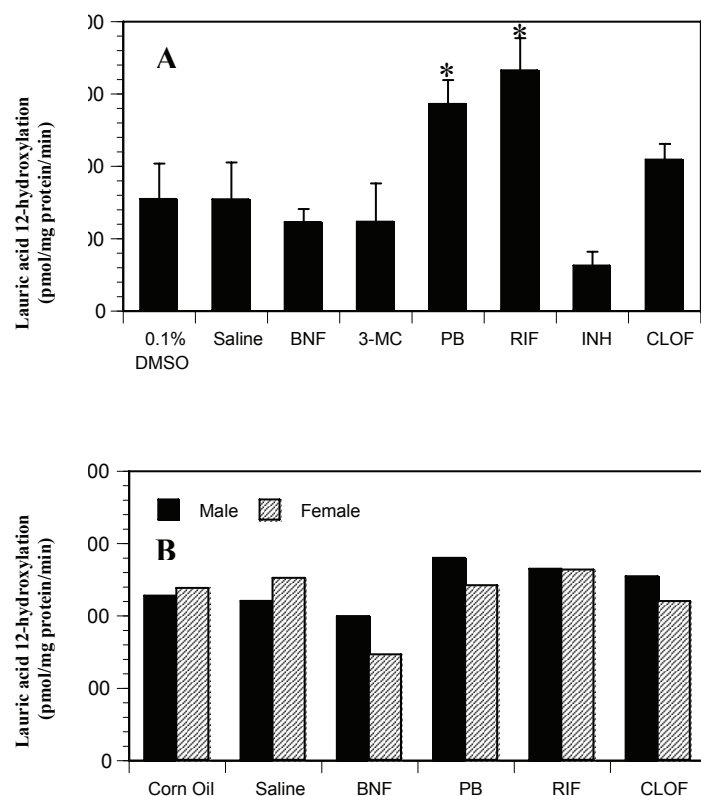
**Figure 2.4** The effect of treating dog hepatocytes with prototypical CYP inducers on 4-nitrophenol hydroxylase (CYP2E) activity. Freshly isolated dog hepatocytes were isolated and cultured for up to six days as described in Materials and Methods. Cultures were treated with prototypical P450 enzyme inducers and analyzed for CYP2E activity as described in Materials and Methods. UT, untreated; DMSO, dimethylsulfoxide; BNF, -naphthoflavone; 3MC, 3-methylcholanthrene; PB, phenobarbital; RIF, rifampin; INH, isoniazid; CLOF, clofibric acid and CO, corn oil.



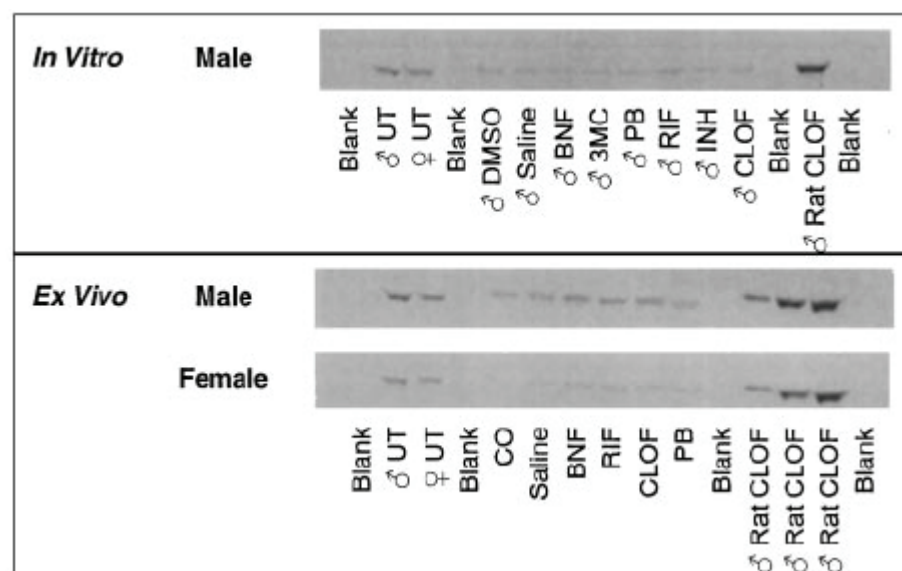


**Figure 2.5** The effect of treating dog hepatocytes or subcutaneous injection of dogs with prototypical CYP inducers on testosterone 6-hydroxylase (CYP3A) activity. Freshly isolated dog hepatocytes were isolated and cultured for up to six days, and Beagle dogs were treated by subcutaneous injection as described in Materials and Methods. (A) In vitro cultures treated with prototypical inducers. (B) Male and female Beagle dogs were treated with prototypical inducers. (C) Microsomes were analyzed by Western immunoblotting. Gels were loaded with 10  $\mu$ g/lane (except for Rat DEX samples, which were 0.05, 0.10 and 0.20  $\mu$ g/lane for ex vivo samples and 0.1  $\mu$ g/lane for in vitro samples). Samples of rat liver microsomes prepared from rats treated with one of four prototypical inducers were also analyzed as reference standards. Asterisk (\*) indicates  $p < 0.05$  as described in Materials and Methods. UT, untreated; DMSO, dimethylsulfoxide; BNF,  $\beta$ -naphthoflavone; 3MC, 3-methylcholanthrene; PB, phenobarbital; RIF, rifampin; INH, isoniazid; CLOF, clofibrac acid; CO, corn oil and DEX, dexamethasone.





C



**Figure 2.6** The effect of treating dog hepatocytes or subcutaneous injection of dogs with prototypical CYP inducers on lauric acid 12-hydroxylase (CYP4A) activity. Freshly isolated dog hepatocytes were isolated and cultured for up to six days, and Beagle dogs were treated by subcutaneous injection as described in Materials and Methods. (A) In vitro cultures treated with prototypical inducers. (B) Male and female Beagle dogs were treated with prototypical inducers. (C) Microsomes were analyzed by Western immunoblotting. Gels were loaded with 10  $\mu$ g/lane (except for Rat CLOF samples, which were 0.05, 0.10 and 0.20  $\mu$ g/lane for ex vivo samples and 0.1  $\mu$ g/lane for in vitro samples). Samples of rat liver microsomes prepared from rats treated with one of four prototypical inducers were also analyzed as reference standards. Asterisk (\*) indicates  $p < 0.05$  as described in Materials and Methods. UT, untreated; DMSO, dimethylsulfoxide; BNF,  $\beta$ -naphthoflavone; 3MC, 3-methylcholanthrene; PB, phenobarbital; RIF, rifampin; INH, isoniazid; CLOF, clofibric acid and CO, corn oil.

## **CHAPTER 3**

### **TEMPORAL KINETICS AND CONCENTRATION-RESPONSE RELATIONSHIPS FOR INDUCTION OF CYP1A, CYP2B AND CYP3A IN PRIMARY CULTURES OF BEAGLE DOG HEPATOCYTES**

This chapter is based on the following original publication:

Graham RA, Tyler LO, Krol WL, Silver IS, Webster LO, Clark P, Chen L, Banks T and LeCluyse El (2006) Temporal Kinetics and Concentration-Response Relationships for Induction of CYP1A, CYP2B and CYP3A in Primary Cultures of Beagle Dog Hepatocytes. *J. Biochemical and Molecular Toxicology* **20(2)**: 69-78.

## ABSTRACT

Compared to other species, little information is available on the xenobiotic-induced regulation of cytochrome P450 enzymes in the beagle dog. Dogs are widely used in the pharmaceutical industry for many study types, including those that will impact decisions on compound progression. The purpose of this study was 1) to determine the temporal kinetics of drug-induced changes in canine CYP1A, CYP2B, and CYP3A mRNA and enzymatic activity, and 2) to characterize concentration-response relationships for CYP1A2, 2B11 and 3A12 using primary cultures of canine hepatocytes treated with  $\beta$ -naphthoflavone (BNF), Phenobarbital (PB) and Rifampin (RIF), respectively. CYP1A1 and CYP1A2 mRNA exhibited maximal expression (12,700-fold and 206-fold, respectively) after 36 hr of treatment with BNF. PB treatment, but not RIF treatment, caused maximal induction of CYP2B11 mRNA (149-fold) after 48 hr of treatment. CYP3A12 and CYP3A26 mRNA levels were increased maximally after 72 hr of treatment with PB and RIF (CYP3A12, 35-fold and 18-fold, CYP3A26, 72-fold and 22-fold with PB and RIF treatment, respectively). Concentration-response relationships for BNF induced 7-ethoxyresorufin *O*-dealkylation (EROD) ( $EC_{50}=7.8 \pm 4.2 \mu\text{M}$ ), PB induced 7-benzyloxyresorufin *O*-dealkylation (BROD) ( $EC_{50}=123 \pm 30 \mu\text{M}$ ) and PB and RIF induced testosterone 6 $\beta$ -hydroxylation ( $EC_{50}=132 \pm 28 \mu\text{M}$  and  $EC_{50}=0.98 \pm 0.16 \mu\text{M}$ ) resembled the relationship for human CYP induction compared to that of rodent. Interestingly, RIF had no effect on CYP2B11 expression, which represents a species difference overlooked in previous investigations. Overall, the induction of dog CYP1A, CYP2B and CYP3A exhibits characteristics that are intermediate to those of rodent and human.

## INTRODUCTION

Beagle dog is a model species that is widely used in the pharmaceutical industry drug discovery and development. Canine in vitro systems (e.g., cDNA expressed enzymes, microsomes, S9 and suspension and/or cultures of hepatocytes) are used to determine intrinsic clearance, to assess CYP induction and to identify metabolic pathways for new chemical entities. Moreover, dogs are widely used for in vivo analysis of drug metabolism and disposition, in addition to their routine use in safety assessment studies. Considering the degree to which the beagle dog is utilized throughout the drug development process, relatively little is known about the function and regulation of drug metabolizing enzymes in this species.

To date, nine CYP genes have been sequenced in the dog including CYP1A1 and CYP1A2 (Uchida et al., 1990a), CYP2B11 (Graves et al., 1990), CYP2C21 (Uchida et al., 1990a), CYP2C41 (Blaisdell et al., 1998a), CYP2D15 (Sakamoto K. et al., 1995), CYP2E1 (Lankford et al., 2000a), CYP3A12 (Ciaccio et al., 1991), and CYP3A26 (Fraser et al., 1997a). Dog CYP1A1/2, CYP2B11 and CYP3A12 have been shown to be inducible by the prototypical human CYP inducers BNF, PB and RIF (Nishibe et al., 1998; Korytko et al., 1999; Graham et al., 2002). It has been established that species differences in CYP induction exist. For example, pregnenolone 16 $\alpha$ -carbonitrile (PCN) is an efficacious inducer of rodent but not human CYP3A, whereas RIF is an efficacious inducer of human but not rodent CYP3A. Differences in the PXR ligand binding domain have been implicated as the basis for species differences in CYP3A induction (LeCluyse, 2001). However, for the known beagle dog genes little more than cloning, expression and characterization of a few CYP

cDNAs has been completed. To date, full length sequences of dog AhR, CAR or PXR have not been reported.

Previously we reported the in vitro and ex vivo induction of CYP in the dog using prototypical human CYP inducers (Graham et al., 2002). We found that beagle dog has CYP1A, CYP2B, and CYP3A and the in vitro and ex vivo induction responses elicited by the prototypical human CYP inducers BNF, PB and RIF (at a single concentration) were more similar to the response reported for human than rat. The present study expands on our previous studies by determining the temporal kinetics and concentration-response relationship for the induction of CYP1A, CYP2B and CYP3A mRNA and enzyme activity following treatment of cultured dog hepatocytes with the prototypical inducers BNF, PB and RIF. The purpose of this work is to provide further insight into the similarities and differences in dog and human CYP regulation.

## **MATERIALS AND METHODS**

### **Chemicals and Reagents**

Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Cambrex Bioscience (Walkersville, MD). Insulin, GlutaMAX-1 (dipeptide L-alanyl-L-glutamine 200 mM supplied in 0.85% NaCl), penicillin-streptomycin, SuperScript II, Reverse Transcriptase and TRIzol<sup>®</sup> Reagent were purchased from Invitrogen (Carlsbad, CA). Biocoat<sup>®</sup> plates, Matrigel and ITS<sup>+</sup> (insulin, transferrin, selenium) were purchased from BD Biosciences (Bedford, MA). Collagenase (type I), cortexolone, dexamethasone, DMSO, fetal bovine serum, EGTA, D-(+)-glucose, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, L-glutamine, NADP, 7-naphthoflavone, Percoll<sup>®</sup>, phenobarbital, 7-Ethoxyresorufin, 7-benzyloxyresorufin, resorufin, testosterone, thymidine, and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid protein assay reagents were purchased as a kit from Pierce Chemical Co. (Rockford, IL). 6-Hydroxytestosterone was purchased from Steraloids, Inc. (Wilton, NH). Genomic DNA and solvents were purchased from EMD Biosciences (Darmstadt, Germany). RNeasy<sup>®</sup> 96 RNA extraction kit was purchased from Qiagen (Valencia, CA). TaqMan<sup>®</sup> primers and probes were purchased from Biosource International (Camarillo CA). qPCR MasterMix Plus<sup>®</sup> was purchased from Eurogentec (SanDiego, CA). DNase was purchased from Ambion Inc. (Austin, TX). Ribogreen RNA quantitation kit was purchased from Molecular Probes (Eugene, OR).

### **Hepatocyte Isolation and Culture**

Male beagle dogs (GSK, RTP North Carolina) were euthanized and the livers were perfused by a modification of the previously described collagenase digestion method (Seglen,

1976; Seglen et al., 1980; LeCluyse et al., 1996; Madan et al., 1999). Hepatocytes were maintained in culture for 48 hr before treatment with CYP inducers. After the adaptation period, medium was replaced with 3 mL of Dulbecco's modified Eagle's medium (DMEM) containing vehicle or the inducer. For temporal kinetic studies hepatocytes (n=1 dog) were cultured in 60-mm Biocoat<sup>®</sup> dishes and treated daily for up to 72 hr with vehicle (0.1% DMSO), BNF (50  $\mu$ M), PB (1000  $\mu$ M) or RIF (10  $\mu$ M). At the end of the treatment period, a single 60-mm Biocoat dish per treatment group was harvested at t=0, 1.5, 3, 6, 12, 18, 24, 36, 48 and 72 hr for mRNA or microsomal protein analysis. Samples at each timepoint were tested in duplicate. For concentration response studies, hepatocyte cultures (n=3 dogs) were treated daily for 3 consecutive days with vehicle (0.1% DMSO), BNF (0.1-100  $\mu$ M), PB (10-2000  $\mu$ M), or Rif (0.1-50  $\mu$ M). At the end of the treatment period, hepatocytes were harvested, and microsomes were prepared from three 60-mm Biocoat<sup>®</sup> dishes per treatment group at t=72 hr, as described previously (Madan et al., 1999). The microsomal samples were stored at -70°C for later analysis of CYP activities. The protein concentration in microsomal samples was determined with a BCA Protein Assay Kit, according to Technical Bulletin 23225X from Pierce Chemical Co. (Smith et al., 1985). Samples were tested in duplicate in all enzymatic activity assays.

### **Enzymatic assays**

Microsomal samples were analyzed to determine EROD (CYP1A1/2), BROD (CYP2B11) and testosterone 6 $\beta$ -hydroxylase (CYP3A12) activities with modifications of previously described methods (Burke and Mayer, 1974; Sonderfan et al., 1987; Klekotka and Halpert, 1995). Studies were conducted to demonstrate that metabolite formation was proportional to incubation time and microsomal protein concentration (data not shown).

Incubations were performed in duplicate in 96-deepwell plates on an Eppendorf Shaker (37°C/900 rpm) by a Tecan Genesis 150 automated liquid handling instrument. Incubation mixtures (total volume = 250 µL) consisted of 25 µL dog liver microsomes (0.2 mg/mL final concentration), 200 µL substrate mix [potassium phosphate buffer (100 mM), EDTA (1.2 mM), MgCl<sub>2</sub> (3.2 mM) and 7-ethoxyresorufin (10 µM), 7-benzyloxyresorufin (10 µM) or testosterone (250 µM)]. Reactions were started with 25 µL of an NADPH-generating system (glucose-6-phosphate [5 mM], glucose-6-phosphate dehydrogenase [1 unit/mL] and NADP [1 mM]). After 20 minutes, samples containing 7-ethoxy- or 7-benzyloxyresorufin were quenched with 250 µL chilled acetone. After 30 minutes, samples containing testosterone were quenched with 500 µL chilled acetonitrile. Samples were centrifuged for 15 minutes at 3400 rpm (~2500 g) and transferred to a 96-well plate for analysis by fluorescence (535 excitation/595 emission) (EROD and BROD) or a 96-deepwell plate for LC/MS/MS analysis (testosterone 6β-hydroxylation). Eight-point standard curves containing resorufin (0 to 1250 pmol) or 6β-hydroxytestosterone (0 to 5000 pmol) were treated in the same manner as experimental samples. All samples fell within the range of the standard curve for each assay.

### **EC<sub>50</sub> Modeling**

Concentration-effect profiles were generated for induction of CYP1A, CYP2B and CYP3A by BNF, PB, and RIF. Catalytic activities were converted to percentage of maximum effect (E(%)) according to equation 1 where  $E_{\text{obs}}$  is the observed activity,  $E_0$  is the baseline activity, and  $E_{\text{max}}$  is the maximum activity achieved (Faucette et al., 2004). The relationship between E(%) for enzyme activity and inducer concentration (C) was fit with a standard Hill equation where 100% was substituted for  $E_{\text{max}}$  by nonlinear least-squares



regression (WinNonlin, Version 3.1) to recover estimates of the  $EC_{50}$  and sigmoidicity factor ( $\gamma$ ) for each concentration-effect profile (equation 2).

$$(1) \quad E(\%) = \left( \frac{E_{obs} - E_0}{E_{max} - E_0} \right) * 100\% \quad (2) \quad E(\%) = \left( \frac{100 * C^\gamma}{EC_{50}^\gamma + C^\gamma} \right)$$

### LC/MS/MS Analysis

The supernatant from the acetonitrile precipitation of incubations was analyzed for the presence of 6 $\beta$ -hydroxytestosterone and cortexolone (IS) by a LC-MS/MS method. Chromatographic separations were carried out using a Cohesive Technologies TX-2 HTLC system. A 10  $\mu$ l aliquot of incubate was injected onto a 50 x 0.5 mm I.D., 60  $\mu$ m Cyclone column (Cohesive Technologies). Samples were cleaned for 30 s at 2.0 ml/min with 9 mM acetic acid. Samples were then back-flushed off the cleanup column onto the 50 x 2.0 mm I.D., 3.0  $\mu$ m YMC ODS-AQ analytical column (Waters). The analytes were separated with a linear gradient, with mobile phase changing from 5% to 35% acetonitrile in 3.0 min and then rising linearly to 95% acetonitrile in 1.0 min. Total run time was 9.7 min. Mass spectrometric analyses were performed using an Applied Biosystems API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray interface operated in the positive ionization mode. The elution of 6 $\beta$ -hydroxytestosterone and cortexolone was monitored by multiple reaction monitoring (MRM) for the transition of precursor-to-product ions at  $m/z$  305.1  $\rightarrow$  269.1 and 347.0  $\rightarrow$  269.3, respectively.

## **RNA analysis**

Total RNA was extracted from hepatocytes by column extraction using a Qiagen RNeasy<sup>®</sup> 96 RNA extraction kit (Qiagen, Valencia, CA). Following extraction, samples were DNase treated and quantified using a Ribogreen<sup>®</sup> RNA quantitation kit (Molecular Probes, Eugene, OR), and cDNA was synthesized using Superscript II<sup>™</sup> RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA). The resultant cDNA template was used to quantify the number of copies of mRNA for selected CYP genes using an ABI 7900 Sequence Detection System. TaqMan primer and probe sequences are shown in Table 3.1. Serially diluted dog genomic DNA was used as a standard for determining the relative copy number of each isoform. Resulting copy numbers were normalized to the total RNA concentration, and the fold change of treated samples compared to vehicle control treated samples was calculated. DNase-treated samples that were not reverse transcribed were also included to ensure complete removal of any contaminating genomic DNA.

## **RESULTS**

### **Dog Hepatocyte Culture Morphology**

Male beagle dog hepatocytes were cultured for 48 hr following isolation with a once daily change of medium (Figure 3.1). Trypan blue viability of the final suspension of hepatocytes was ~90% for all hepatocyte preparations. Three hours following plating of hepatocytes (Matrigel<sup>®</sup> overlay performed), approximately 75% confluency was observed, consisting of mostly small, round hepatocytes (Figure 3.1A). At 6 hr after plating, some of the cells appeared to spread and take on a cuboidal shape (Figure 3.1B), continuing progressively for ~48 hr at which point the monolayer was 100% confluent (Figure 3.1F). After the initial 48 hr adaptation period, hepatocyte cultures were treated with solvent control (0.1% DMSO) or prototypical inducers for an additional 72 hr (Figure 3.1G-I). Up to 72 hr after the initiation of treatment, cell morphology did not appear to change to an appreciable extent.

### **Temporal Kinetics for Induction of CYP1A, CYP2B and 3A mRNA and Enzyme Activities**

Following 48 hr of acclimation to the culture conditions, dog hepatocytes were treated for 72 hr with 0.1% DMSO, 50  $\mu$ M BNF, 1 mM PB or 10  $\mu$ M RIF. Under control conditions CYP mRNA levels increased over the initial 6 hrs after 0.1% DMSO treatment followed by a decline between 6 and 12 hr (most dramatic for CYP2B11) after which levels increased slightly through 72 hr (Figure 3.2A). The induction of CYP mRNA in dog hepatocyte cultures over time following treatment with BNF, PB or RIF is shown in Figure 3.2 (B-F). CYP1A1 and CYP1A2 mRNA increased (12,700-fold and 206-fold, respectively) over 36 hr following treatment with BNF. There was a decrease in CYP1A1 and CYP1A2

mRNA expression between 18 and 24 hr after treatment followed by a sharp spike between 24 and 36 hr. CYP1A1 and CYP1A2 mRNA levels were maximally induced after 36 hrs of treatment and decreased between 36 and 72 hr. Microsomal EROD activity remained unchanged over the initial 12 hr after BNF treatment and increased between 12 and 72 hr (Figure 3.2B and C). Maximal expression of CYP1A activity was observed after 72 hrs of BNF treatment.

PB treatment increased CYP2B11 mRNA levels (up to 149-fold) over the initial 48 hr of treatment followed by a decline in mRNA expression between 48 and 72 hr. Microsomal BROD activity remained unchanged up to 12 hr after PB treatment and increased between 12 and 72 hr. By contrast, CYP2B11 mRNA and BROD activity were unchanged over the 72 hr treatment period with RIF (Figure 3.2D).

CYP3A12 and CYP3A26 mRNA levels increased maximally over 72 hr of treatment with PB and RIF (CYP3A12, 35-fold and 18-fold; CYP3A26, 72-fold and 22-fold, respectively). CYP3A12 and CYP3A26 mRNA levels appeared to level off between 24 and 36 hr following treatment with PB or RIF followed by a sharp increase between 36 and 48 hr. After 72 hr of PB treatment, CYP3A26 mRNA expression was 2-fold greater than CYP3A12 expression, whereas RIF treatment increased CYP3A12 and CYP3A26 expression to the same extent. An increase in microsomal testosterone 6 $\beta$ -hydroxylation was observed as early as 3 hr after treatment with PB and RIF and continued to increase over the 72 treatment period (Figure 3.2E and F). Enzyme induction kinetics resembled CYP3A12 (and incidentally, CYP3A26) mRNA kinetics following treatment with PB and RIF (Figure 3.2E). The rate of testosterone 6 $\beta$ -hydroxylation was approximately 2-fold higher with PB treatment than with RIF treatment.

## Concentration-Response Relationship for CYP1A1/2, CYP2B11 and CYP3A12

### Induction

Microsomal CYP1A1/2, CYP2B11 and CYP3A12 specific activities were determined from dog hepatocyte cultures (n=3) following 72 hr treatment with BNF, PB or RIF via EROD, BROD and testosterone 6 $\beta$ -hydroxylation. As shown in Figure 3.3A, CYP1A control activities (0.1% DMSO) were similar for the three separate preparations of dog hepatocytes; however BNF-induced EROD activities varied (up to 6.5-fold at  $E_{max}$ ) between animals (dog 2>3>1). However, a concentration-dependent response was observed between 0.1 and 100  $\mu$ M for all three hepatocyte preparations. An  $EC_{50}$  of 7.8  $\pm$  4.2  $\mu$ M was calculated from the converted data (equation 1) for BNF induction of CYP1A.

Similar to CYP1A activities, CYP2B11 control activities were in good agreement across hepatocyte preparations; however, PB-induced CYP2B11 activities were variable (up to 2.2-fold at  $E_{max}$ ) (Figure 3.4A). The rank order for rate of BROD following PB treatment was dog 2>3>1. The  $EC_{50}$  for PB induction of CYP2B11 was estimated to be 123  $\pm$  30  $\mu$ M (Figure 3.4B). In contrast to PB treatment, RIF treatment failed to induce CYP2B11 activity to an appreciable extent, and no  $EC_{50}$  value was obtainable (Figure 3.4A inset).

The rank order of the hepatocyte preparations for PB and RIF induction of CYP3A was different than observed for CYP1A induction by BNF and CYP2B induction by PB (dog 2>1>3, Figure 3.5A and 5C, respectively). The  $EC_{50}$  for PB induction of CYP3A12 was 132  $\pm$  28  $\mu$ M (Figure 3.5B). The  $EC_{50}$  for RIF induction of CYP3A12 was 0.98  $\pm$  0.16  $\mu$ M (Figure 3.5D). Treatment of dog hepatocytes with either PB or RIF resulted in an increase in microsomal testosterone 6 $\beta$ -hydroxylation, however PB treatment resulted in a larger increase ( $E_{max}$ ) than RIF treatment.

## **Experimental Design Limitations**

The concentration range of enzyme inducers was chosen based on concentration response profiles in other species reported in the literature. Following evaluation of concentration response data from the first preparation of dog hepatocytes, the concentration range of PB and RIF were adjusted to include additional concentrations near or below the apparent EC<sub>50</sub>. Therefore 10 µM PB was only used for dogs 2 and 3 cultures and 25 and 50 µM PB only for dog 3 cultures. Hepatocytes from dogs 2 and 3, but not dog 1 were treated with 0.1 and 0.5 µM RIF.

## DISCUSSION

Beagle dogs (and associated pre-clinical models) are widely used in the pharmaceutical industry during drug discovery and development. While much is known about the regulation of drug metabolizing enzymes in other model species (e.g., rat, mouse) little information is available on the regulation of dog drug metabolizing enzymes. The purpose of the present study was two-fold: (1) to examine the temporal kinetics of CYP1A1, CYP1A2, CYP2B11, CYP3A12 and CYP3A26 mRNA induction and enzyme activity in cultured dog hepatocytes and (2) to determine a concentration-relationship of dog microsomal CYP1A1/2, CYP2B11 and CYP3A12 enzyme induction following treatment of hepatocyte cultures with BNF, PB and RIF.

Previously we described the morphology of cultured dog hepatocytes as resembling human more than rat. This observation was based on the tendency of dog hepatocytes to slowly form confluent monolayers over the course of a 5-day culture period (Graham et al., 2002). In the present study we show a representative time course to attain hepatocyte monolayer confluency in cultured dog hepatocytes (Figure 3.1). While bile canaliculi seem to be apparent as early as 24 hr after plating, this observation has not been tested. Few descriptions of dog hepatocyte morphology exist in the literature. Foxworthy et al. noted that dog hepatocytes are smaller in size (thereby requiring higher cell seeding density) than rat or monkey and that dog hepatocytes required 20% FCS for optimal attachment in culture (Foxworthy et al., 1990). In contrast to these observations, we found that a similar seeding density to rat or human hepatocytes and addition of 5% FBS to the seeding media was adequate for culture of dog hepatocytes. These differences are likely related to differences in perfusion buffers and isolation techniques between the two laboratories.

It is generally considered appropriate to evaluate CYP induction after 48-72 hr of drug treatment in cultured hepatocytes from rat and human liver; however detailed investigations into the temporal kinetics of mRNA expression and enzyme activity have not been reported previously for cultured dog hepatocytes. Likewise, several reports exist that show prototypical human CYP inducers also induce in the dog, however, concentration response relationships for these effects have not been compared. For example, PB induces both rat and human CYP2B enzymes, although the concentration response profile in the two species are dramatically different, suggesting differences in the regulation of rat and human CYP2B expression (LeCluyse et al., 1999; Faucette et al., 2004).

In our studies of CYP mRNA induction kinetics, several key observations were made during the time course of measurement. Between 1.5 and 6 hr after treatment of dog hepatocyte cultures with solvent control (0.1% DMSO), there was an increase in CYP1A1, CYP1A2, CYP2B11, CYP3A12 and CYP3A26 mRNA levels, followed by a marked decrease between 6 and 12 hr. From 12-72 hr after 0.1% DMSO treatment, an apparent steady state was achieved for all isozymes, although there was a slight trend toward increasing mRNA expression (most notably for CYP3A12 and CYP3A26). This effect might be attributed to the presence of 100 nM dexamethasone in the culture medium, since dexamethasone has been shown to induce human CYP3A4, albeit at higher concentrations, e.g., 2  $\mu$ M and above (McCune et al., 2000). Following BNF treatment, there was a decrease in CYP1A1 and CYP1A2 mRNA expression between 18 and 24 hr, followed by a marked increase after the second BNF treatment at 24 hr (Figure 3.2 B and C). The decrease in CYP1A mRNA before the second BNF treatment might be attributed to the metabolism of BNF in the hepatocyte culture. It has been reported that the terminal half-life for BNF



disposition in the rat is 40 min (Adedoyin et al., 1993). In addition, these authors report that BNF clearance in the rat was time dependent, presumably due to induction CYP1A enzymes. Interestingly, CYP1A enzyme induction first occurs at 18 hr (4-fold), the time point at which CYP1A mRNA levels begin to decline. Following treatment with BNF or PB, increases in CYP1A1, CYP1A2 and CYP2B11 mRNA were apparent after only 1.5-3 hr, whereas increases in CYP1A and CYP2B enzyme activity were not apparent until 12 hr after treatment (Figure 3.2B-D). In contrast, following treatment with PB or RIF, an increase in CYP3A enzyme activity was apparent after only 3 hr, an effect that might be attributed to isozyme differences in protein synthesis rates (Figure 3.2E and F). Further investigation would be required to fully characterize these differences in induction kinetics. Maximal induction of CYP1A1, CYP1A2, CYP2B11, CYP3A12 and CYP3A26 occur approximately 48 hr after inducer treatment, suggesting that 48 hr is an appropriate endpoint to evaluate mRNA induction. On the other hand, enzyme activity continued to increase 72 hr after inducer treatment for all isozymes evaluated, indicating that an endpoint of 72 hr (or longer) would be appropriate.

We have shown that the concentration response relationship for CYP1A induction by BNF is similar across species. For example the  $EC_{50}$  for induction of rat CYP1A by BNF was reported to be 1.5  $\mu$ M (Madan et al., 1999), whereas we determined the  $EC_{50}$  for induction of dog CYP1A by BNF to be 7.8  $\mu$ M. Furthermore, using cultured human hepatocytes we determined the  $EC_{50}$  for induction of CYP1A by BNF to be 4.2  $\mu$ M (data not shown), similar to the  $EC_{50}$  value of 4.0  $\mu$ M reported by Smith et. al (Smith et al., 2005). These results suggest that regulation of CYP1A might be better conserved across species compared to that of CYP2B and CYP3A.

Treatment of dog hepatocytes with PB resulted in a marked induction of dog CYP2B11 mRNA expression and microsomal BROD activity. The concentration response relationship for PB induction of dog CYP2B11 was similar to induction of human CYP2B6 by PB (Faucette et al., 2004) but an order of magnitude different than induction of rat CYP2B1/2 by PB (LeCluyse et al., 1999). Interestingly, up to 25  $\mu$ M RIF treatment, which causes marked induction of CYP2B6 in primary human hepatocytes, did not cause an increase in dog CYP2B11 mRNA expression or enzymatic activity. This is the first report highlighting a major species difference in CYP2B induction between dog and human, although lack of CYP2B11 induction by RIF has been observed by other investigators. Nishibe et. al., reported that treatment of dogs with RIF resulted in a slight increase ( $\sim$ 1.4-fold) in microsomal testosterone 16  $\alpha$ -hydroxylation (a marker of CYP2B11 and CYP2C21), an effect that is substantially lower than what has been shown in human hepatocytes treated with RIF (Nishibe et al., 1998). In our previous report on induction of dog CYP by prototypical inducers, we also observed a lack of CYP2B11 induction (in vitro and ex vivo) by RIF as measured by BROD and Western blot analysis (Graham et al., 2002). This species difference suggests that there are fundamental differences in the regulation of CYP2B11 by activators of the nuclear receptor PXR (e.g., RIF) as compared to activators of CAR (e.g., PB). As a result, our studies indicate that dog hepatocytes may not be a surrogate to predict human CYP2B induction by PXR activators.

The concentration response relationships for CYP3A12 induction by PB was similar to that observed for induction of CYP3A4 by PB (Faucette et al., 2004). While the rat CYP3A concentration response relationship by PB is somewhat higher compared to human, (rat  $EC_{50}$ =500  $\mu$ M, human  $EC_{50}$ =142  $\mu$ M), the dog  $EC_{50}$  was determined to be nearly identical to human (132  $\mu$ M). By contrast, Table 3.2 shows that the  $EC_{50}$  values for CYP3A induction

by PXR activators RIF and DEX are similar; however, it's difficult to make a concentration response comparison across species since RIF does not induce rat CYP3A to an appreciable extent. However, we have shown that dog CYP3A12 does respond to RIF and that the concentration response relationship is similar to human.

It should be noted that while PB and RIF both induced dog CYP3A mRNA expression and enzymatic activity the magnitude of the maximum response was greater in each case (n=3) with PB treatment. Interestingly, we have shown previously that RIF is more efficacious than PB at inducing dog CYP3A12 in ex vivo studies (Graham et al., 2002). Previous reports would suggest that PB and RIF treatment cause approximately equivalent maximal induction of CYP3A4 in cultures of human hepatocytes (Raucy, 2003). For example, Hariparsad et. al. treated 3 separate cultures of human hepatocytes with 10  $\mu$ M RIF or 2 mM PB and found that in 1 of the 3 cultures CYP3A4 activity was approximately equal. In a second culture, the PB response was about 20% less than the RIF response and in the third culture the RIF response was about 20% less than the PB response (Hariparsad et al., 2004). Taken together these observations suggest that there are several possibilities for the larger increase in CYP3A induction by PB over RIF in dog hepatocyte cultures. First, there could be a difference in nuclear receptor binding to and activation of response elements controlling dog and human CYP3A induction. Second, as RIF has been shown to be a substrate for OATP-C (SLC1B1) and OATP8 (SLC1B3) there could be differences in the affinity for the corresponding dog uptake transporters, or the expression of the transporter could vary in cultured dog and human hepatocytes (Vavricka et al., 2002). Finally, differences could be attributed to differences in metabolism of PB and RIF in cultures of dog and human hepatocytes. Each of these possibilities requires further investigation.

In summary, induction of dog CYP1A, CYP2B and CYP3A by prototypical inducers seems to be more similar to human than rodent. However, important species differences do exist, e.g., lack of CYP2B11 induction by RIF. Results of this study suggest that caution should be exercised when using primary cultures of dog hepatocytes to predict human CYP induction.

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**Table 3.1** Sequences of primers and probes used in the TaqMan assay. Each probe was synthesized with 5'-FAM and 3'-TAMRA. GAPDH was used to monitor the integrity of the mRNA and the efficiency of the cDNA synthesis. Data were not normalized to GAPDH.

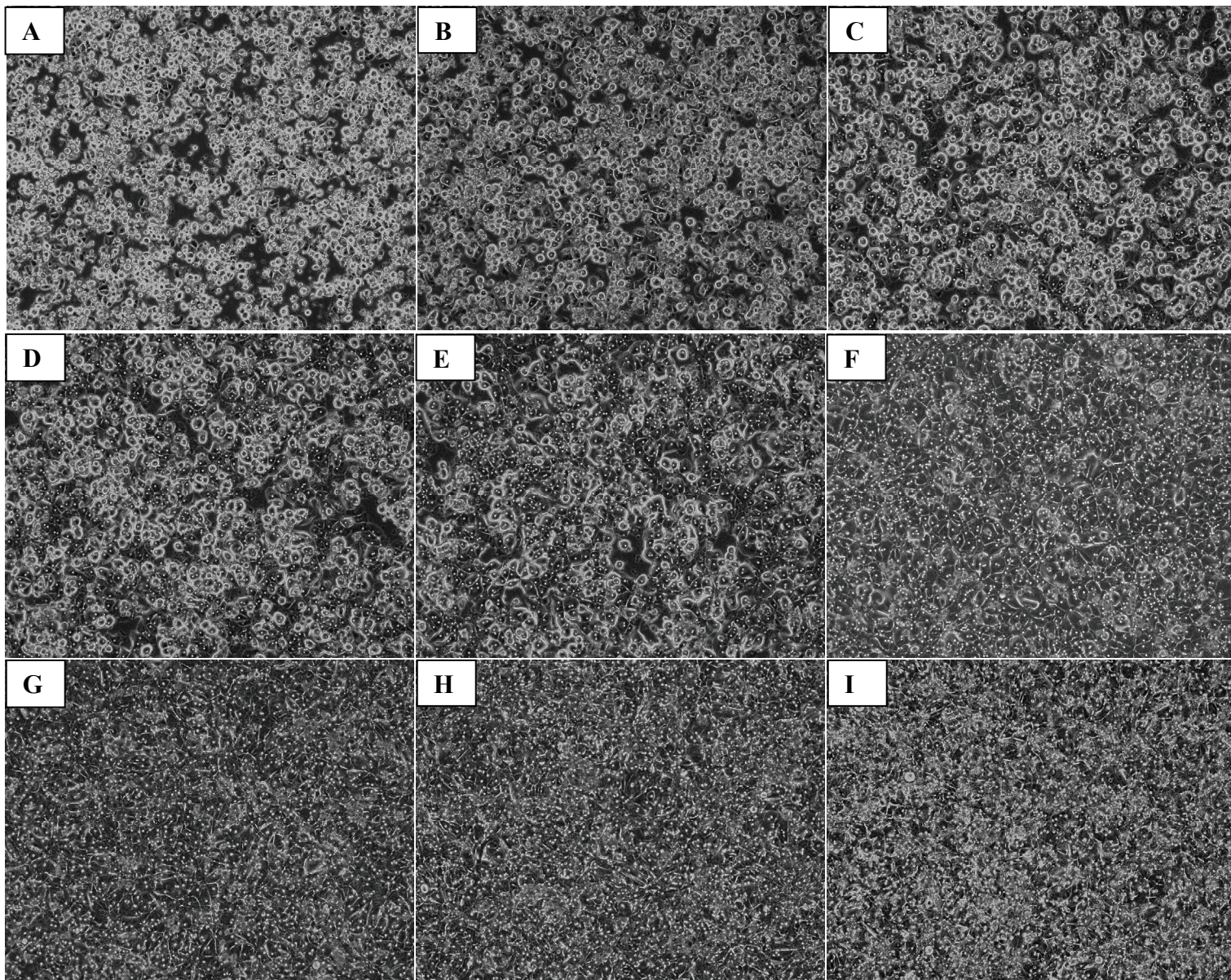
mRNA	Forward Primer	Probe	Reverse Primer	Accession Number
CYP1A1	CCCTCTGGCAGACTAAATTCCA	AGGATGGCCAGAAAGAGAAAGACCTCCA	GAAGTGCATCGGTGAGACCAT	(AACN010067442 & AACN010089968)
CYP1A2	CGGCCAGAGCGATTCT	ACTGCAGATGGCACCGCCATCA	AGCATCACCTTCTCACTCAAGGT	(AACN010103563- AACN010517076)
CYP2B11	CTACAGTGATTGTGTTTTACAGACGAGTA	CAAAGGCCAGGCACTGGCATCA	TCCACTCCCAGCCATATGATC	NM_001006652 and M92447
CYP3A12	GGACTTCCTTTTGTCTTCAAGAAA	CTATCCCAGAACACCAGAGACCTCAATTCCT	CCTACATGAGTGAAACCACATAATCAA	X54915
CYP3A26	AACACAGATCTCCCTGAGAATAAATACTCG	AGGGATTATTCAACCTGAAAAACCCGTTGTTT	CACTCCCATCTCTTGGCTCAA	AF547269
GAPDH	CGACATCAAGAAGGTAGTGAAGCA	AGTGGGTGTCCTGTTGAAGTCA	TCAAAGGCATCCTGGGCTACACTGAGG	AB004358



**Table 3.2** EC<sub>50</sub> species comparisons. Rat and human EC<sub>50</sub> values derived from cultured hepatocytes were obtained from the literature <sup>A</sup>(Madan et al., 1999), <sup>B</sup>(Courtois et al., 2002), <sup>C</sup>Graham, unpublished, <sup>D</sup>(Faucette et al., 2004).

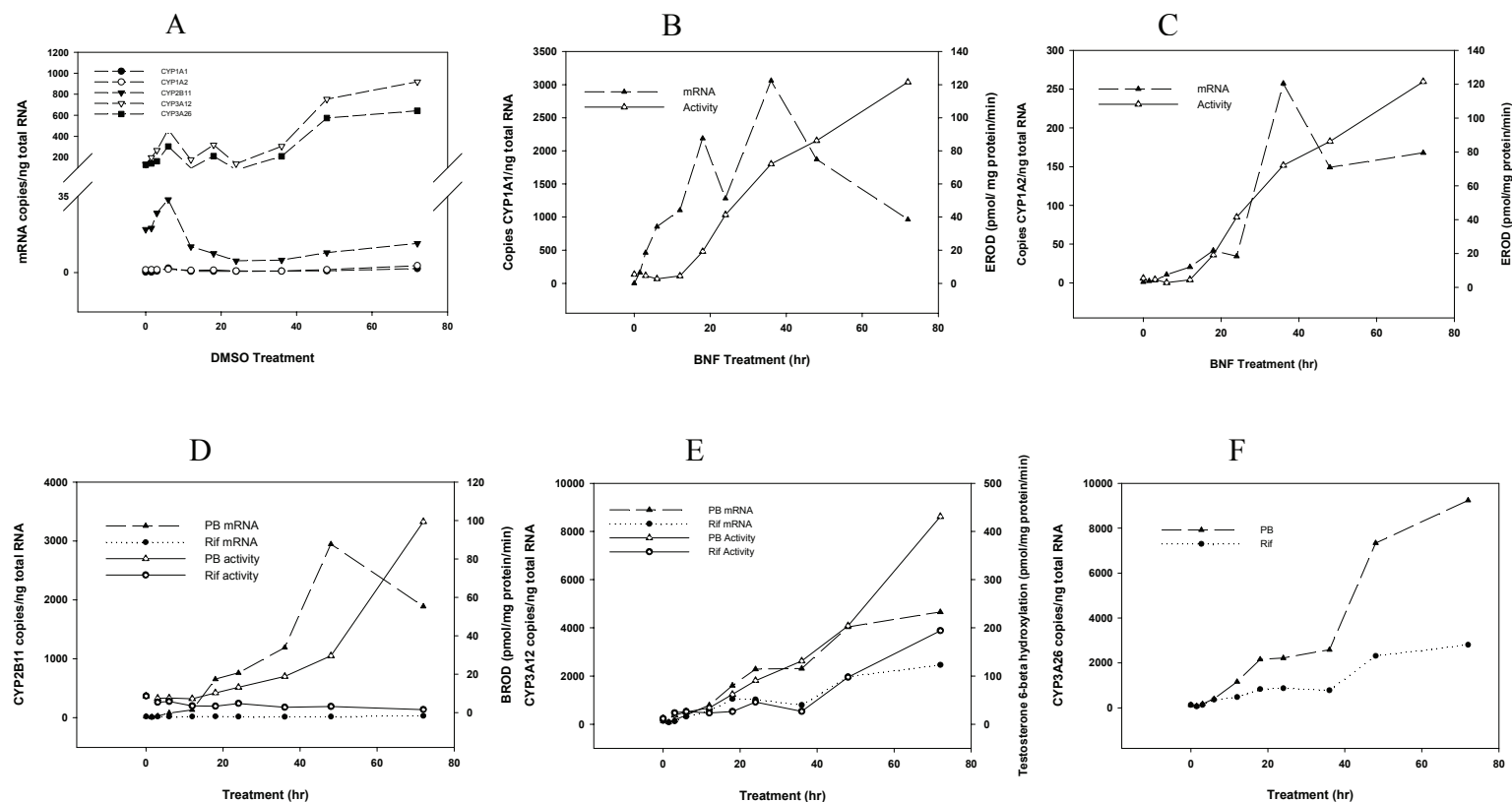
CYP	Marker Activity	Inducer	Rat EC50	Dog EC50	Human EC50
1A	EROD	BNF	1.5 µM <sup>A</sup>	7.8 µM	4.2 µM <sup>C</sup>
2B	PROD/BROD/Bupropion-hydroxylation	PB	10 µM <sup>A</sup>	123 µM	238 µM <sup>D</sup>
3A	Testosterone 6 -hydroxylation	PB	500 µM <sup>B</sup>	132 µM	142 µM <sup>D</sup>
		Dex/Rif	1.3 µM <sup>A</sup>	0.98 µM	0.37 µM <sup>D</sup>



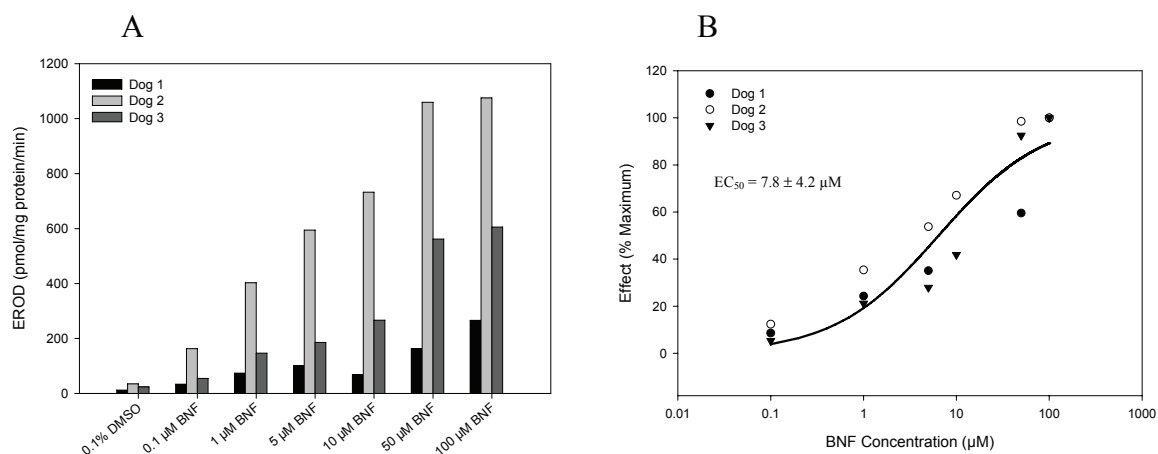




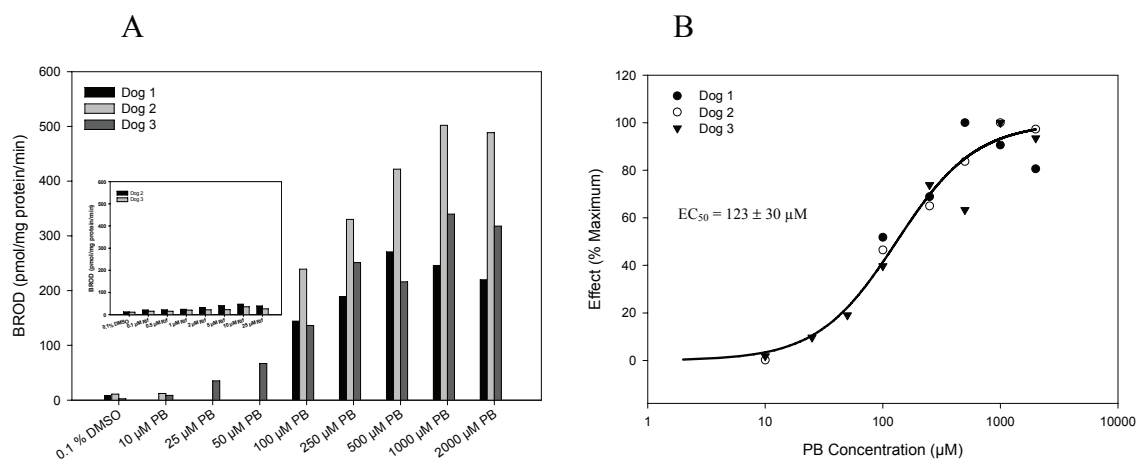
**Figure 3.1** Photomicrograph of cultured dog hepatocytes. Dog hepatocytes were plated and photographed at t=3 hr (A), t=6 hr (B), t=12 hr (C), t=18 hr (D) t=24 hr (E), and t=48 hr (F). Following 48 hr of culture, cells were treated with inducer for an additional 72 hr. (G) 0.1% DMSO treatment, t=24 hr post dosing, (H) 0.1% DMSO treatment, t=48 hr post dosing, (I) 0.1% DMSO treatment, t=72 hr post dosing.



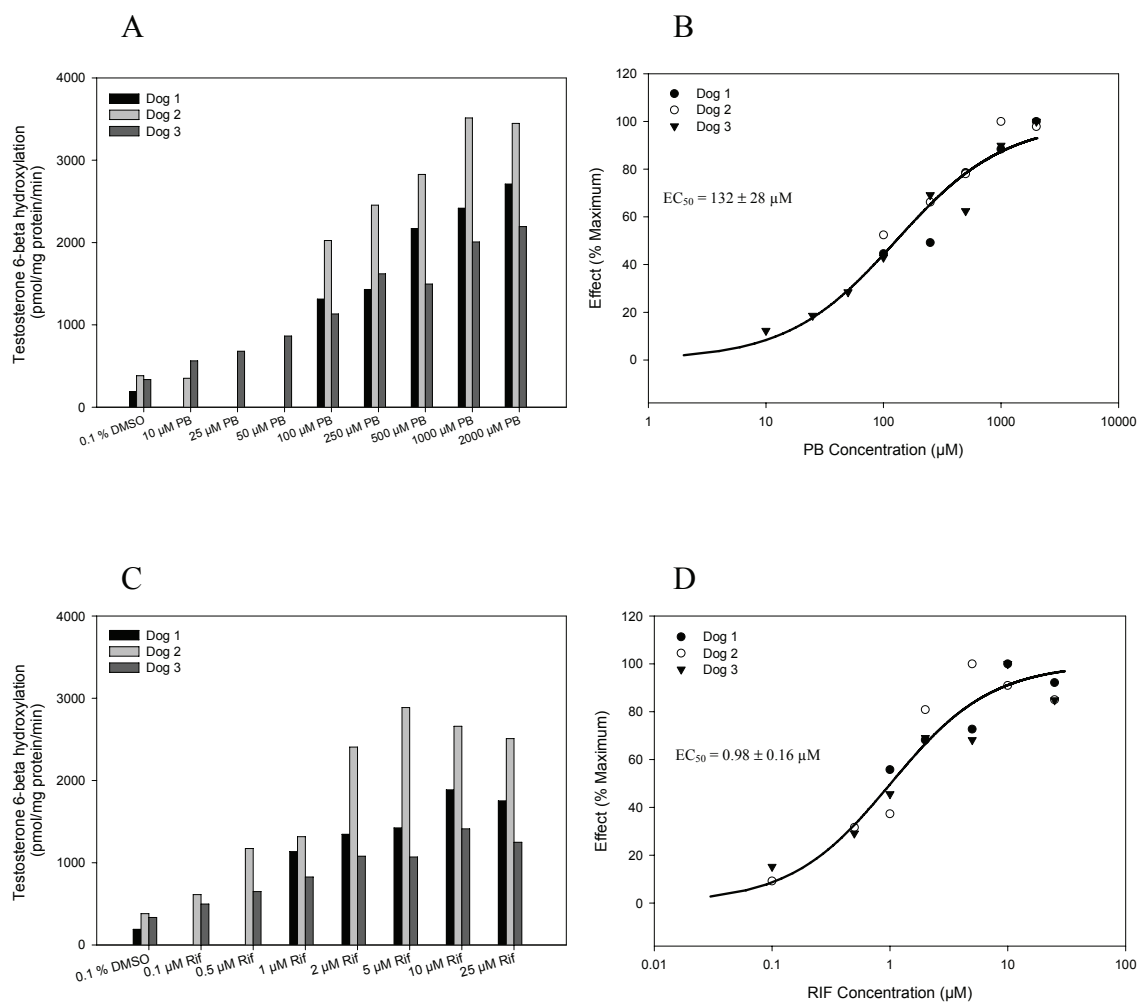
**Figure 3.2** Temporal kinetics of mRNA induction and enzymatic activity. After 2 days of adaptation, hepatocytes were harvested 0, 1.5, 3, 6, 12, 18, 24, 36, 48 and 72 hr after treatment for mRNA expression and 0, 3, 6, 12, 18, 24, 36, 48 and 72 after treatment for evaluation of enzyme activity. (A) CYP1A1, CYP1A2, CYP2B11, CYP3A12 and CYP3A26 mRNA expression over time following treatment with 0.1% DMSO. (B) Time dependent CYP1A1 mRNA expression and enzymatic activity with 50  $\mu$ M BNF treatment. (C) Time dependent CYP1A2 mRNA expression and enzymatic activity with 50  $\mu$ M BNF treatment. (D) Time dependent CYP2B11 mRNA expression and enzymatic activity with 1mM PB or 20  $\mu$ M RIF treatment. (E) Time dependent CYP3A12 mRNA expression and enzymatic activity with 1mM PB or 20  $\mu$ M RIF treatment. (F) Time dependent CYP3A26 mRNA expression with 1mM PB or 20  $\mu$ M RIF treatment.



**Figure 3.3** Concentration response relationship for induction of beagle dog microsomal EROD activity following treatment of cultured hepatocytes with 50  $\mu$ M BNF for 72 hr. (A) 7-ethoxyresorufin O-dealkylation (CYP1A1/2) (B) Normalized concentration response relationship for CYP1A induction by BNF.



**Figure 3.4** Concentration response relationship for induction of beagle dog microsomal BROD activity following treatment of cultured hepatocytes with 1mM PB or 20  $\mu M$  RIF (inset) for 72 hr. (A) 7-benzyloxyresorufin O-dealkylation (CYP2B11) (B) Normalized concentration response relationship for CYP2B induction by PB.



**Figure 3.5** Concentration response relationship for induction of beagle dog microsomal testosterone 6 -hydroxylation following treatment of cultured hepatocytes with 1 mM PB or 20 μM RIF for 72 hr. (A and C) testosterone 6 -hydroxylation (CYP3A12) (B and D) Normalized concentration response relationship for CYP3A induction by PB and RIF.

## **CHAPTER 4**

### **CLONING, TISSUE EXPRESSION AND REGULATION OF BEAGLE DOG CYP4A GENES**

This chapter has been submitted as part of a manuscript to Toxicological Sciences and is formatted in the style of that journal.



## ABSTRACT

In addition to its function as a fatty acid hydroxylase the peroxisome proliferator-activated receptor (PPAR) target gene, CYP4A, has been shown to be important in conversion of arachidonic acid to the potent vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE), suggesting a role for this enzyme in mediating vascular tone. In the present study the cDNA sequence of beagle dog CYP4A37, CYP4A38 and CYP4A39 from liver was determined. Open reading frame analysis predicted that CYP4A37, CYP4A38 and CYP4A39 are each comprised of 510 amino acids with ~90% sequence homology, and ~71% and ~78% homology to rat CYP4A1 and human CYP4A11, respectively. PCR analysis revealed that the three dog CYP4A isoforms are expressed in kidney>liver>>lung>>intestine>skeletal muscle>heart. Treatment of primary dog hepatocytes with the PPAR agonists GW7647X and clofibric acid resulted in an increase in CYP4A37, CYP4A38 and CYP4A39 mRNA expression (up to 4-fold), whereas HMG-CoA synthase mRNA expression was increased to a greater extent (up to 10-fold). These results suggest that dog CYP4A37, CYP4A38 and CYP4A39 are expressed in a tissue dependent manner and that beagle dog CYP4A is not highly inducible by PPAR agonists, similar to the human CYP4A11 gene.

## INTRODUCTION

Fatty acids are vital nutrients for the growth and development of all organisms. In addition to providing substrates for membrane synthesis and energy metabolism they are also components of multiple signaling cascades and directly regulate gene transcription through their activities on transcription factors such as peroxisome proliferator-activated receptor (PPAR ). CYP4A enzymes are fatty acid hydroxylases that are expressed in most mammalian tissues and highly expressed in kidney, liver and lung. These enzymes are selective for the  $\omega$ -hydroxylation of saturated and unsaturated fatty acids (Hardwick et al., 1987; Kimura et al., 1989b; Stromstedt et al., 1990; Wang et al., 1996; Helvig et al., 1998; Nguyen et al., 1999; Hoch and Ortiz De Montellano, 2001), however, CYP4A lacks known roles in drug metabolism (Coward et al., 2002; Johnson et al., 2002). Although fatty acids represent the primary substrates for CYP4A enzymes it has also been shown that these enzymes can metabolize non-fatty acid substrates such as the potent vasodilator 20-hydroxyeicosatetraenoic acid (20-HETE) (Harder et al., 1995; Okita and Okita, 2001). It was recently shown that inhibition of CYP  $\omega$ -hydroxylase results in a reduction in infarct size in a dog model for cardiac injury (Nithipatikom et al., 2004; Nithipatikom et al., 2006) suggesting that CYP4A has a detrimental role in enhancing myocardial injury in canine heart.

Across species, CYP4A is primarily expressed in liver and kidney, with lower expression levels in other tissues. CYP4A1, CYP4A2 and CYP4A3 mRNAs are constitutively expressed in rat liver (Kimura et al., 1989b). In the male rat kidney, CYP4A1 and CYP4A3 mRNAs are expressed at low levels, whereas CYP4A2 mRNA levels were found to be similar to those of maximally induced CYP4A2 mRNA levels in the liver (Kimura et al., 1989b). CYP4A protein was not detected by immunoblot analysis in rat small

intestine (Patel et al., 1998) and was relatively low in rat brain, heart and lung (Zhang et al., 2002). Expression of CYP4A11 mRNA in human tissues has been reported using real time PCR. CYP4A11 was highly expressed in liver and kidney (liver to kidney ratio 2.4:1), whereas expression was relatively low (2-3 orders of magnitude less than liver) in small intestine, adrenal gland, lung, brain, prostate, testis, uterus and placenta (Nishimura et al., 2003).

For many years it has been recognized that the transcriptional activity of the genes encoding various CYP4A subfamily members is dramatically increased by numerous structurally diverse xenochemicals, collectively referred to as peroxisome proliferators (PPs) (Okita and Okita, 2001). In mouse (Bell et al., 1993), rat (Kimura et al., 1989b) and hamster (Choudhury et al., 2000) liver and kidney, CYP4A enzymes are induced by various PPAR activators including the fibrate class of lipid-lowering drugs. It has been shown that in rat liver, CYP4A proteins represent 1-4% of total CYP, but increase to 16-30% following clofibrate administration (Sharma et al., 1989). On the other hand, the CYP4A induction response is not observed in guinea pig (Bell et al., 1993) or in cultured human hepatocytes (Raucy et al., 2004) following treatment with PPs. For example, while treatment of cultured rat hepatocytes with clofibrate elicited a large induction (14-fold) exposure of primary human hepatocytes to the same drug resulted in a modest induction of CYP4A11 mRNA (~2-fold) (Madan et al., 1999; Raucy et al., 2004).

Beagle dog is a model species that is widely used in the pharmaceutical industry during the drug development process. Previously we reported that clofibric acid administration to beagle dogs failed to induce dog liver CYP4A protein or microsomal lauric acid 12-hydroxylation, suggesting that CYP4A is not inducible in this species (Graham et al.,

2002). Results of our previous studies suggested that dog CYP4A might be regulated in a manner similar to CYP4A from other non-responsive species, such as human CYP4A11.

CYP4A enzymes have gained attention recently because they metabolize arachidonic acid to 20-HETE. In fact, it has been suggested that targeting of CYPs in the heart and coronary circulation could be of therapeutic utility in coronary artery disease based in part on results from a dog model of cardiac injury (Doggrell, 2005). Given the importance of various canine models in drug discovery efforts, it is important to characterize dog CYP4A isoform expression and regulation, yet little is known about the sequence homology and regulation of the individual dog CYP4A genes. In the present study, we report on the cDNA cloning, tissue expression and regulation of dog CYP4A37, CYP4A38 and CYP4A39.

## **MATERIALS AND METHODS**

### **Chemicals and Reagents**

Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Cambrex Bioscience (Walkersville, MD). Insulin, GlutaMAX<sup>®</sup> (dipeptide L-alanyl-L-glutamine 200 mM supplied in 0.85% NaCl), penicillin-streptomycin, NuPage<sup>®</sup> gels, polyvinylidene difluoride membranes and related electrophoresis reagents were purchased from Invitrogen (Carlsbad, CA). ECL<sup>™</sup> Western Blotting Analysis System was purchased from Amersham Biosciences (Buckinghamshire, England). Biocoat<sup>®</sup> plates, Matrigel and ITS<sup>+</sup> (insulin, transferrin, selenium) were purchased from BD Biosciences (Bedford, MA). Collagenase (type I), dexamethasone, DMSO, EGTA, D-(+)-glucose, L-glutamine, Percoll<sup>®</sup>, thymidine, and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid protein assay reagents were purchased as a kit from Pierce Chemical Co. (Rockford, IL). Oligonucleotides for cloning and sequencing were purchased from Sigma-Genosys (St. Louis, MO). TaqMan<sup>®</sup> primers and probes and PCR master mix were purchased from Applied Biosystems (Foster City, CA). Proteinase K and DNase were purchased from Qiagen Inc. (Valencia, CA). Clofibrate was obtained from ICN Biomedicals (Aurora, OH) and GW7647X was prepared in-house as previously described (Brown et al., 2001).

### **PPAR Transfection Assay**

#### **Plasmids**

PCR primers containing KpnI and BamHI restriction sites were used to amplify PPAR ligand binding domain (LBD) fragments from full-length human and dog clones. The LBD fragments were ligated into the multiple cloning site of pFA-CMV (Stratagene, La

Jolla, CA). The resulting constructs (pFA-CMV-GAL4-hPPAR LBD and pFA-CMV-GAL4-dogPPAR LBD) each carried a fusion of the LBD with the yeast-derived GAL4 DNA-binding domain under the control of the CMV immediate early promoter. The reporter construct UAS-tk-Luc carries a single 17 bp (5'-CGGAGTACTGTCCTCCG-3') upstream activating sequence (UAS), the thymidine kinase (tk) minimal promoter, and the firefly luciferase gene.

### **Cell-based luciferase assay**

African Green Monkey kidney cell line CV-1 (ATCC CCL-70) was maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 2mM glutamine, and 1% penicillin/streptomycin (pen/strep). In preparation for luciferase assays, CV-1 cells were grown in charcoal-stripped cell medium containing DMEM/F-12 medium supplemented with 5% or 3% dextran-treated/charcoal-stripped (CS) fetal bovine serum, 2mM glutamine, with or without 1% pen/strep, as described below. CS fetal bovine serum was purchased from Hyclone; all other cell culture reagents were from Gibco.

The luciferase protocol is a multi-day procedure. On day 1, confluent cells in maintenance medium were subcultured 1:10 into T-175 cm<sup>2</sup> flasks containing 50 mL of 3% CS medium with pen/strep. These flasks were allowed to incubate at 5% CO<sub>2</sub> and 37°C for 72 hours.

Cells were harvested by trypsinization and then transfected using FuGENE (Roche, Basel, Switzerland) according to the manufacturer's specifications. Briefly, each transfection contained 0.27 (PPAR ) or 0.55 (PPAR and PPAR ) g pFA\_CMV\_GAL4\_PPAR\_LBD plasmid (human or dog), 10.9 g UAS-tk-Luc, and 24 g pBluescript (carrier DNA). Plasmid DNA was mixed with FuGENE in OptiMEM-1 (Invitrogen, Carlsbad, CA) medium

and incubated for 30 min at room temperature. During this incubation, cells were harvested into 3% CS medium without pen-strep and dispensed at 14 million cells per T-175 cm<sup>2</sup> flask. Transfection mixes were added to the flasks and incubated overnight at 5% CO<sub>2</sub> and 37°C.

Transfected cells were added to 384-well plates containing pharmacological agents. Stock solutions of test compounds were reconstituted in DMSO at a concentration of 1 mM. For 11-point dose-response experiments, the compounds were 3-fold serially diluted in DMSO and then transferred to 384-well assay plates (NUNC, catalog #164564) at 0.5

L/well using a Beckman FX. DMSO and agonist control compound GI249820X (1 mM) were each transferred at 0.5 L/well to columns 23 and 24, respectively, of the 384-well plates. Transfected cells were harvested in 5% CS medium with pen/strep and dispensed at 10,000 cells/well (50 L) onto the prepared 384-well compound plates using a Titertek Multidrop. Following overnight incubation at 5% CO<sub>2</sub> and 37° C, Steady-Glo reagent (Promega, Madison, WI) was added to the assay plates using a Multidrop. Plates were incubated for 10 min to ensure complete cell lysis and read in a ViewLux (PerkinElmer, Wellesley, MA).

### **cDNA Cloning**

Approximately 100 mg of frozen male beagle dog liver was ground to a fine powder using a mortar and pestle under liquid nitrogen. Total RNA was isolated using the Qiagen RNeasy<sup>®</sup> Mini kit (Qiagen Inc., Valencia, CA). RT-PCR was carried out in two steps using the Advantage<sup>™</sup> RT-for PCR Kit followed by the BD Advantage<sup>™</sup> 2 PCR Kit (BD Biosciences, Palo Alto, CA). To determine whether a portion of the putative dog CYP4A cDNA could be amplified by PCR, oligonucleotides were designed based on the published human CYP4A11 cDNA sequence (Accession L04751) cloned from human

kidney. Primer CYP4A-F1 (5'-CACCATGAGTGTCTCTGTGCTGAGCC-3') was based on sequence approximately 100 bp from the 5'-end of the human CYP4A cDNA sequence while primer CYP4A-R1 (5'-CTCCTGAGACGCAGGTGGATTCCAT-3') was approximately 1600 bp from the 5'-end of the human CYP4A sequence, or about the midpoint of the complete transcript. PCR was conducted using CYP4A-F1 and CYP4A-F2 primer pair described above and after agarose gel electrophoresis, a band of the expected size (~1.5 kb) was obtained. The generated cDNA was gel purified using the QIAquick® gel extraction kit (Qiagen Inc., Valencia, CA). Gel purified cDNA was subcloned using the TOPO TA cloning® kit for sequencing (Invitrogen, Carlsbad, CA). cDNA isolated from subsequent clones using the QIAprep® 96 turbo miniprep kit (Qiagen Inc., Valencia, CA) and submitted for sequencing.

The 5'-end of the dog CYP4A sequence determined using the GeneRacer™ kit from Invitrogen Corporation (Carlsbad, California), whereas determination of the 3'-end of the dog CYP4A sequence was accomplished using the SMART™ RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA). Gene specific primers were designed based on the internal sequencing information obtained from the 1.5 kb region described above.

Sequencing of the 3'-RACE products revealed approximately 800 base pairs of untranslated sequence between the stop codon and the poly-A tail. Sequencing of 5'-RACE products resulted in approximately 40 base pairs of untranslated sequence upstream of the open reading frame. Sequence information from the 5'-RACE and 3'-RACE experiments as well as the open reading frame regions was assembled using Sequeuncher alignment software (version 4.0.5).



## **Hepatocyte Isolation and Culture**

Male beagle dogs (GSK, RTP North Carolina) were euthanized and the livers were perfused by a modification of the previously described collagenase digestion method (Seglen, 1976; LeCluyse et al., 1996; Madan et al., 1999). Dog hepatocytes were cultured as described previously (Graham, 2006, in press). Hepatocytes (n=3 dog livers) were cultured in 6-well Biocoat<sup>®</sup> plates and treated daily for 48hr with vehicle (0.1% DMSO), GW7647X (0.01, 0.1, 1, 5 and 10  $\mu$ M), or clofibrilic acid (1, 10, 100, 500 and 1000  $\mu$ M). At the end of the treatment period, each treatment group was harvested for mRNA analysis. Samples from each treatment group were tested in duplicate in subsequent TaqMan<sup>®</sup> assays.

## **RNA Isolation and cDNA synthesis**

For absolute quantification experiments, total RNA was isolated from dog tissue by column extraction using a Qiagen RNeasy<sup>®</sup> Mini kit (Qiagen Inc., Valencia, CA). RNA isolation from skeletal muscle and heart was accomplished using a proteinase K digestion step as recommended by the manufacturer. Following RNA extraction, samples were DNase treated and quantified using a Ribogreen<sup>®</sup> RNA quantitation kit (Molecular Probes, Eugene, OR), and cDNA was synthesized using a cDNA Archive Kit (Applied Biosystems, Foster City, CA).

For relative quantification experiments, total RNA was isolated from cultured dog hepatocytes using the Automated SV 96 Total RNA Isolation System (Promega, Madison, WI) and quantified using a Ribogreen<sup>®</sup> RNA quantitation kit (Molecular Probes, Eugene, OR), and cDNA was synthesized using a cDNA Archive Kit (Applied Biosystems, Foster City, CA).

## **Microsome Preparation and Western Blot**

Microsomes were isolated from dog hepatocyte cultures followed by Western Blot analysis of CYP4A expression as described previously (Graham et al., 2002). The protein concentration in the microsomal samples was determined with a BCA Protein Assay Kit, according to Technical Bulletin 23225X from Pierce Chemical Co. (Smith et al., 1985) (Wiechelman et al., 1988). For Western blots, 10 µg of microsomal protein was loaded per well. The primary antibody was a human CYP4A anti-peptide antibody (Affinity Bioreagents, Golden, CO) at a dilution of 1:1000. The secondary antibody was a peroxidase labeled anti-rabbit antibody included in the ECL™ Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, England) which was diluted 1:1000 in blocking buffer.

## **TaqMan® Assays**

Custom TaqMan® gene expression assays were purchased from Applied Biosystems (Foster City, CA). Briefly, a probe target site was identified at an exon-exon junction in the gene of interest and submitted to Applied Biosystems Global Assays by Design Department using File Builder software program. Assays were supplied at 20 and consisted of primer (forward and reverse) and minor groove binder (MGM) probe. Primer and probe sequences used in this study are listed in Table 4.1.

Absolute quantification experiments were conducted to quantify the number of copies of mRNA for selected cytochrome target genes using an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Plasmid DNA concentration was determined using a Quanti-iT™ PicoGreen dsDNA Assay Kit (Molecular Probes, Eugene, OR). Five point standard curves were prepared from plasmid DNA for CYP4A37, CYP4A38 and CYP4A39 (30, 300, 3000, 30,000 and 300,000 copies). The standard curve Ct values ranged

from 19 to 35. For test samples, each reaction consisted of 100 ng cDNA, 2.5  $\mu$ L 20X assay mix and 25  $\mu$ L master mix in a total volume of 50  $\mu$ L. Standards and samples were evaluated in triplicate. Thermocycling parameters were 50  $^{\circ}$ C (2 min), 95  $^{\circ}$ C (10 min), followed by 40 cycles of 95  $^{\circ}$ C (15sec), 60  $^{\circ}$ C (1 min).

Relative quantification experiments were conducted to determine relative mRNA expression (treated versus control) of target genes using an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). For test samples, each reaction consisted of 50 ng cDNA, 2.5  $\mu$ L 20X assay mix and 25  $\mu$ L master mix in a total volume of 50  $\mu$ L. Target gene expression was normalized to the housekeeping gene cyclophilin. Samples were evaluated in triplicate. Thermocycling parameters were 50  $^{\circ}$ C (2 min), 95  $^{\circ}$ C (10 min), followed by 40 cycles of 95  $^{\circ}$ C (15sec), 60  $^{\circ}$ C (1 min).

## **LC/MS Analysis**

### **GW7647X**

The media samples and supernatant from scraped cell homogenates were analyzed for the presence of GW7647X by a LC-MS/MS method. Chromatographic separations were carried out using a Cohesive Technologies TX-2 HTLC system equipped with an autosampler, two quaternary pumps, two binary pumps and a valve module. A 10  $\mu$ L aliquot of hepatocyte incubate was injected onto a 50 x 0.5 mm I.D., 60  $\mu$ m Cyclone column (Cohesive Technologies, Franklin, MA). Samples were cleaned for 30 s at 2.0 ml/min with 100% aqueous mobile phase (0.1% formic acid). Samples were then back-flushed off the cleanup column onto the 30 x 2.1 mm I.D., 3.0  $\mu$ m Atlantis<sup>TM</sup> dC18 analytical column (Waters Corporation, Milford, MA) in 90 s at 0.1 ml/min with the 50% organic mobile phase

(50:50 acetonitrile / 0.1% formic acid). The 0.1 ml/min flow was combined with a 0.4 ml/min 95% 2.5 mM ammonium acetate, pH=5.5 / 5% acetonitrile flow from eluting pump and the analytes were re-focused on the analytical column. After the transfer the analytical column was washed with 95% aqueous flow for 15 sec and then the analytes were eluted with a linear gradient from 5% to 95% organic in 45 s at 0.6 ml/min. Total run time was 6.6 min. The temperature of the analytical column was maintained at 40 °C. The flow from analytical column was diverted to waste for the first 2.9 min, to MS detector for the next 1.2 min, and to waste again for the remaining run time. The make-up solvent (50:50 acetonitrile/water) was infused into the MS source at 0.5 ml/min when the column effluent was diverted to waste. The HPLC flow was split 50:50 before entering the source.

Mass spectrometric analyses were performed using an Applied Biosystems API 3000 triple quadrupole mass spectrometer equipped with a TurboIonspray interface operated in the positive ionization mode. Nitrogen was used as both the sheath and drying gas at a pressure of 10 arbitrary units and a flow-rate 7 L/min, respectively. The spray voltage was set at 5.5 kV and the drying gas temperature was set at 425 °C. Declustering potential and focusing potential were optimized at 50 and 140 V, respectively. The collision cell was filled with nitrogen at a pressure of 12 arbitrary units and the collision energy was set at 30 eV. The elution of GW7647X was monitored by multiple reaction monitoring for the transition of precursor-to-product ions at  $m/z$  503.2  $\rightarrow$  378.4.

### **Clofibric Acid**

The media samples and supernatant from scraped cell homogenates were analyzed for the presence of clofibric acid by a LC-MS/MS method. Chromatographic separations were carried out using a Cohesive Technologies TX-2 HTLC system equipped with an

autosampler, two quaternary pumps, two binary pumps and a valve module. A 10  $\mu$ l aliquot of hepatocyte incubate was injected onto a 50 x 0.5 mm I.D., 60  $\mu$ m Cyclone column (Cohesive Technologies). Samples were cleaned for 30 s at 2.0 ml/min with 100% aqueous mobile phase (0.1% formic acid). Samples were then back-flushed off the cleanup column onto the 30 x 2.1 mm I.D., 3.0  $\mu$ m Atlantis dC18 analytical column (Waters) in 90 s at 0.1 ml/min with the 50% organic mobile phase (50:50 acetonitrile / 0.1% formic acid). The 0.1 ml/min flow was combined with a 0.4 ml/min 95% 0.025% formic acid / 5% acetonitrile flow from eluting pump and the analytes were re-focused on the analytical column. After the transfer the analytical column was washed with 95% aqueous flow for 15 sec and then the analytes were eluted with a linear gradient from 5% to 95% organic in 45 s at 0.6 ml/min. Total run time was 6.6 min. The temperature of the analytical column was maintained at 40  $^{\circ}$ C. The flow from analytical column was diverted to waste for the first 2.9 min, to MS detector for the next 1.2 min, and to waste again for the remaining run time. The make-up solvent (50:50 acetonitrile/water) was infused into the MS source at 0.5 ml/min when the column effluent was diverted to waste. The HPLC flow was split 50:50 before entering the source.

Mass spectrometric analyses were performed using an Applied Biosystems API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray interface operated in the negative ionization mode. Nitrogen was used as both the sheath and drying gas at a pressure of 10 arbitrary units and a flow-rate 7 L/min, respectively. The spray voltage was set at -4.5 kV and the drying gas temperature was set at 425  $^{\circ}$ C. Declustering potential and focusing potential were optimized at -40 and -130 V, respectively. The collision cell was filled with

nitrogen at a pressure of 10 arbitrary units and the collision energy was set at -20 eV. The elution of clofibric acid was monitored by multiple reaction monitoring for the transition of precursor-to-product ions at  $m/z$  213.0  $\rightarrow$  127.0.

### **Statistical Analysis**

To evaluate the statistical significance of differences between means, equal variance and normality tests were first conducted to determine whether the data were parametrically distributed. For a parametrically distributed data set, a one-way repeated measures analysis of variance (ANOVA) was carried out to determine whether there were significant differences between the group means. Statistically significant differences from the controls were identified by a Holm-Sidak post hoc test ( $p = 0.05$  or 5% level of significance).

## RESULTS

### Cloning of Dog CYP4A37, CYP4A38 and CYP4A39

Two primers (CYP4A-R1 and CYP4A-R2) were designed from the human CYP4A11 cDNA sequence (Accession number L04751) and used to amplify CYP4A from dog liver by RT-PCR. 5'- and 3'-RACE was utilized to determine the full-length cDNA sequence of dog CYP4A37, CYP4A38 and CYP4A39 (Figure 4.1-4.3). The sequence of each isozyme was verified from multiple independent clones and has been submitted to GenBank with accession numbers DQ138950 (CYP4A37), DQ138951 (CYP4A38), and DQ138952 (CYP4A39). The CYP4A37, CYP4A38 and CYP4A39 cDNA sequences are approximately 2,400-bp long and contain a 43, 53 and 40-nucleotide 5'-UTR, respectively, a 1,515-bp open reading frame, and a 769, 763 and 876-bp 3'-UTR downstream from the TAA terminal codon. Each cDNA encodes a protein of 510 amino acids containing the CYP signature motif (FxxGxxxCxG) (444–453) which includes the invariant enzyme active site cysteine (Nelson et al., 1996). The calculated molecular weight of dog CYP4A37, CYP4A38 and CYP4A39 protein is ~59 kDa.

Alignment of rat CYP4A1, dog CYP4A37, CYP4A38, CYP4A39 and human CYP4A11 amino acid sequences suggest a high degree of homology and conservation amongst isoforms (Figure 4.4). The deduced amino acid sequence of CYP4A37, CYP4A38 and CYP4A39 share  $\geq 90\%$  identity to one another and are approximately 71% and 78% identical to rat CYP4A1 (Kimura et al., 1989a) and human CYP4A11 (Kawashima et al., 2000), respectively (Table 4.2).

### **Tissue Expression of Dog CYP4A Isoforms**

Quantitation of CYP4A mRNA from dog tissues (n=3 dogs) using real time PCR indicated that CYP4A37, CYP4A38 and CYP4A39 are highly expressed in kidney and liver (Figure 4.5A). CYP4A37, CYP4A38 and CYP4A39 mRNA was expressed to a similar extent in the liver, whereas kidney expression of CYP4A37 was higher than CYP4A38 and CYP4A39 (7.8-fold and 2.2-fold, respectively). Expression of dog CYP4A37, CYP4A38 and CYP4A39 mRNA was also notable in lung but was ~3% of liver expression. In contrast, CYP4A expression was relatively low in the intestine, skeletal muscle and heart (Figure 4.5B).

### **Potency of PPAR Agonists**

While clofibric acid is widely used as a tool to study PPAR regulation, its potency is relatively weak against PPAR compared to newer molecules. For example GW7647X was shown to be a potent human PPAR agonist with ~200-fold selectivity over PPAR and PPAR (Brown et al., 2001). To evaluate the effect of GW7647X and clofibric acid on dog PPAR activation (Figure 4.6), a transactivation assay using GAL4-PPAR LBD was performed as described in Materials and Methods. GW7647X was highly potent and efficacious against dog PPAR ( $EC_{50}$ =7.6 nM, maximum response=93% of agonist control), whereas clofibric acid showed little discernable activity against this receptor ( $EC_{50}$ >10  $\mu$ M, maximum response=14% of agonist control) (data not shown). Similar results were obtained for activation of human PPAR using GW7647X ( $EC_{50}$ =6.5 nM, maximum response=90% of agonist control) and clofibric acid ( $EC_{50}$ >10  $\mu$ M, maximum response=13% of agonist



control). Furthermore, we have shown that GW7647X demonstrates approximately 100 and 4000-fold selectivity over PPAR $\alpha$  and PPAR $\gamma$  isoforms, respectively (data not shown)

### **Induction of Dog CYP4A Isoforms**

To investigate whether dog CYP4A isoforms were inducible by PPAR agonists, cultures of dog hepatocytes (n=3) were treated with GW7647X or clofibric acid, followed by real time PCR analysis of mRNA expression. Treatment of dog hepatocytes with GW7647X (0.01-10  $\mu$ M) or clofibric acid (1-1000  $\mu$ M) resulted in a slight (up to 1.9-fold) but statistically significant induction of CYP4A37 and CYP4A38 at the highest concentrations tested (Figure 4.7A and B). Notably, CYP4A39 was induced to the greatest extent (up to 4-fold) compared with CYP4A37 and CYP4A38 and a concentration-dependent increase was observed between 0.1 and 10  $\mu$ M and 10 and 1000  $\mu$ M for GW7647X and clofibric acid, respectively (Figure 4.7C). In contrast to the minor induction of dog CYP4A isoforms, the previously characterized PPAR $\alpha$  target gene HMG CoA synthase was induced by ~9.5- and 6-fold by GW7647X and clofibric acid, respectively (Figure 4.7D) (Nagasawa et al., 2004). Western blot analysis indicated that CYP4A protein expression was consistent with mRNA levels following treatment with GW7647X and clofibric acid (Figure 4.7E).

### **Metabolic Stability of Test Compounds in Cell Culture Studies**

Determination of the metabolic stability of GW7647X and clofibric acid in cell culture studies was conducted to understand the relationship between induction of dog CYP4A isoforms and in vitro metabolism. Furthermore, these studies were conducted to understand whether or not drug had sufficient access to the interior of the hepatocytes or if permeability might be attenuating the induction response. Following a 2-day adaptation period, cultured dog hepatocytes were treated with 1  $\mu$ M GW7647X or 1  $\mu$ M clofibric acid

for up to 72hr (changing media every 24hr). As shown in Figure 4.8, the in vitro  $t_{1/2}$  of GW7647X was determined to be 2.4hr, whereas the  $t_{1/2}$  of clofibric acid was 52hr.

To understand whether the in vitro metabolism of GW7647X was limiting the exposure of compound to the hepatocytes, medium containing GW7647X was replaced at 8-hr intervals for a total of 48hr (multidose experiment). Notably, there appeared to be a slight increase in CYP4A37, CYP4A38 and CYP4A39 induction compared to the standard treatment protocol; however, the control levels in the multidose experiment were lower than in the standard treatment giving rise to an apparent increase in CYP4A expression (Figure 4.9).

## DISCUSSION

Beagle dogs (and associated models) are widely used in the pharmaceutical industry during drug discovery and development. While much is known about the regulation of CYP enzymes in other model species (e.g., rat, mouse) little information is available on the regulation of dog CYP enzymes. In the present study the cDNA sequence of beagle dog CYP4A37, CYP4A38 and CYP4A39 from liver was determined and the relative tissue expression examined. In addition, CYP4A induction was measured in cultured dog hepatocytes following treatment with the PPAR agonists, GW7647X and clofibrilic acid.

The deduced amino acid sequence of CYP4A37, CYP4A38 and CYP4A39 share  $\geq 90\%$  identity to one another and are approximately 71% and 78% identical to rat CYP4A1 and human CYP4A11, respectively. The beagle dog liver CYP4A37, CYP4A38 and CYP4A39 mRNA sequences were compared to the boxer genome using NCBI dog genome resources BLAST tool. The high degree of identity between CYP4A37, CYP4A38 and CYP4A39 mRNA and separate boxer CYP4A genes suggested that each beagle CYP4A mRNA produces a single protein (i.e., separate isozymes). There are only a few exceptions to this rule where differential splicing of the transcript results in an enzyme with a new catalytic activity (Nelson et al., 1996).

Sequence analysis suggests that the original mammal CYP4 gene cluster was composed of CYP4X, CYP4A and CYP4B. This set likely derived from the fish CYP4T gene, with the 4B sequence being most like the 4T sequence. It is likely that two serial gene duplications occurred: 4B giving rise to 4A and 4A giving rise to 4X. In humans and chimps, 4X gave rise to 4Z, however, 4Z does not seem to exist in any other species. Rodents have expanded on the basic CYP4 set by expanding the 4As. Opossum doubled 4B

and 4X, but still have just one 4A. Dogs have 4X1, 4A36, 4A37, 4A38, 4A39 and 4B1. The CYP4A36 gene has a sequence gap that contains 5 exons (exons 5-9) so it is not complete (David R. Nelson, personal communication).

Dog CYP4A37, CYP4A38 and CYP4A39 each contain the signature sequence of 10 residues including the invariant active site cysteine residue. It has been established that a heme is covalently bound in the CYP4A family via a conserved glutamic acid residue on the I-helix of the protein and amino acid context of this glutamic acid (EGHDTT) is highly conserved (Hoch and Ortiz De Montellano, 2001). As expected, this active site heme binding motif is completely conserved in beagle dog CYP4A37, CYP4A38 and CYP4A39 (positions 321-326). Although the crystal structure of CYP4A has not been determined, Chang and Loew constructed a three-dimensional model of the human CYP4A11 and identified the location of active site residues (Chang and Loew, 1999). Amino acids identified in the active site of CYP4A11 included tyrosine-120, leucine-132, tyrosine-317, phenylalanine-318, glutamate-321, glycine-322, tyrosine-325, and valine-386. Each of these amino acids is completely conserved in beagle dog CYP4A37, CYP4A38 and CYP4A39. Interestingly, while pig CYP4A21 shares 74% homology with human CYP4A11, this enzyme lacks lauric acid hydroxylase activity, presumably due to amino acid differences identified in the active site region (Lundell et al., 2001). Conservation of key amino acids that have been shown to be involved in substrate region-specificity suggests that dog CYP4A37, CYP4A38 and CYP4A39 enzymes might function as fatty acid hydroxylases, however further studies are needed to demonstrate the catalytic properties of dog CYP4A enzymes.

In the present work we show that beagle dog mRNA expression of CYP4A37, CYP4A38 and CYP4A39 was greatest in the liver and kidney, although there was also notable expression in the lung. Expression levels in intestine, skeletal muscle and heart were low. These results are in agreement with CYP4A tissue expression patterns in other species (e.g., rat and human) (Kimura et al., 1989b; Patel et al., 1998; Zhang et al., 2002; Nishimura et al., 2003). Previous studies have described CYP4A isoform expression profiles and they suggest that rat CYP4A1, CYP4A2 and CYP4A3 are all constitutively expressed at similar levels in the liver. Contrary to rat liver CYP4A expression, profiles in the kidney suggest that CYP4A1 and CYP4A3 are expressed at low levels compared to CYP4A2 (Kimura et al., 1989b). In fact, these studies showed that CYP4A2 expression levels in the kidney of untreated male rats were similar to induced CYP4A2 mRNA levels in the liver. Interestingly, like rat, dog has three CYP4A isoforms that likely encode enzymatically active protein. Furthermore, beagle dog CYP4A37 mRNA expression was ~3-fold higher in kidney than in liver, while CYP4A38 and CYP4A39 kidney expression were comparable to liver expression. The relevance of this differential expression pattern in kidney is unclear, however these results suggest that dog CYP4A37 mRNA expression in the kidney might be regulated in a manner similar to rat CYP4A2.

Follow-up studies of patients who have undergone fibrate drug therapy (Frick et al., 1987) and experiments with cultured human hepatocytes (Blaauboer et al., 1990; Richert et al., 2003) have suggested that humans do not display the same CYP4A inductive effect as that observed in rats and mice. Similarly, the guinea pig (Tugwood et al., 1998) and dog (Graham et al., 2002) appear to be refractory to the CYP4A inductive effects of peroxisome proliferators. While it is generally understood that CYP4A induction in the rodent is

mediated by PPAR $\alpha$ , the mechanism for the observed species differences in CYP4A induction is unclear. Several theories for this apparent lack of inducibility have been advanced. Firstly, it has been suggested that species not responsive to peroxisome proliferators lack a functional peroxisome proliferator response element (PPRE) (Lambe et al., 1999; Woodyatt et al., 1999; Hasmall et al., 2000). To date there has not been a functional PPRE identified in the promoter region of the human CYP4A11 gene, whereas rat CYP4A1 and rabbit CYP4A6 promoter regions contain functional elements (Muerhoff et al., 1992; Aldridge et al., 1995). Secondly, levels of PPAR $\alpha$  are an order of magnitude lower in non-responsive species (e.g., guinea pig, and human) in comparison to rodents (Holden and Tugwood, 1999). Recently it was shown that dog PPAR $\alpha$  is expressed at levels similar to human PPAR $\alpha$  in cultured hepatocytes (Nagasawa et al., 2004). A third possibility stems from the observation that there is a two amino acid difference in the PPAR $\alpha$  LBD between rat and human that is critical for the responsiveness to receptor activation (Miyachi and Uchiki, 2003; Uchiki and Miyachi, 2004). Finally, another hypothesis to explain the CYP4A species difference is that non-responsive species may lack an activator or have an active repressor of PPAR $\alpha$  function. While each of these theories have been investigated to some extent, there has not been a definitive study published to date that clearly demonstrates the mechanism for the observed species differences in CYP4A induction. Further studies of CYP4A regulation in dog and other non-responsive species might help to elucidate the mechanism for species dependent induction of the CYP4A family.

The results of the current studies suggest that dog CYP4A37, CYP4A38 and CYP4A39 are expressed in a tissue dependent manner and that beagle dog CYP4A is not highly inducible by PPAR $\alpha$  agonists. Furthermore, we have clearly shown that the CYP4A

induction response in dog hepatocytes is similar to that observed for human CYP4A11, suggesting a similar mode of regulation. However, further work is required to fully assess the species-specific regulation of the CYP4A induction response.

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**Table 4.1** Sequences of primers and MGB probes used in cloning and TaqMan assays.

Primer	Sequence (5'-3')	Orientation	Purpose	Target
CYP4A-F1	5'-CACCATGAGTGTCTCTGTGCTGAGCC-3'	Forward	PCR Cloning	37, 38 and 39
CYP4A-R1	5'-CTCCTGAGACGCAGGTGGATTCCAT-3'	Reverse	PCR Cloning	37, 38 and 39
CYP4A-R2	5'-CCAGGGCGCCAAAGCTAAGG-3'	Reverse	5'-RACE	37, 38 and 39
d4A-gsp1(f)	5'-GGACAGAGAAATACCCCTGTGCCA-3'	Forward	sequencing /3'-RACE	37 and 38
d4A-gsp2(r)	5'-ATCAGTGAAGGTGATGGGCTTGCT-3'	Reverse	sequencing	37, 38 and 39
d4A-gsp3(f)	5'-ATGGAGAATGGGAAGGGCTTGTCTGA-3'	Forward	sequencing	37, 38 and 39
d4A-gsp4(r)	5'-TGGTGTCCAAGGTCATCAAGGAGA-3'	Reverse	Sequencing/5'-RACE	37, 38 and 39
d4A-gsp5(f)	5'-AAACCCTACGTGAGACTCATGGCT-3'	Forward	sequencing /3'-RACE	37, 38 and 39
d4A-gsp6(f)	5'-ATGCCCTTCACCACAACCCAAATG-3'	Forward	sequencing	39
d4A-gsp7(f)	5'-TTGCCATGAACGAGTTGAAGGTGG-3'	Forward	sequencing	37, 38 and 39
d4A-gsp9(f)	5'TGTTCCTTGCTGCCTACTTGTCT-3'	Forward	sequencing	37 and 38
d4A-gsp11(f)	5'-TGCCAACTTACTCCAGTCTCAGTG-3'	Forward	sequencing	39
d4A-grp1&2 (r)	5'-AAGTGGACCAAAGGTGGACTGACA-3'	Reverse	sequencing	37 and 38
d4A-grp 3 (r)	5'-TGGGTACACAGGTAACACACAGGA-3'	Reverse	sequencing	39
GROUP1-AF	ACCCAGACTACATGAAGATGATCCT	Forward	TaqMan	37
GROUP1-AR	AGGAGCAAACCATAACCCTATCCA	Reverse	TaqMan	37
GROUP1-AM1	CTGGTGGTTCTACACATT	Probe	TaqMan	37
GROUP2-AF	GGCACAGGCAAGAGTTCCA	Forward	TaqMan	38
GROUP2-AR	GGCGCAAGGGTATTTCTCTGT	Reverse	TaqMan	38
GROUP2-AM2	CAGTCCCCGACCCTTT	Probe	TaqMan	38
GROUP3-AF	GGCACAAGCGGGAGTTG	Forward	TaqMan	39
GROUP3-AR	TGGGAAGTCCTCTATCCATTTCAGT	Reverse	TaqMan	39
GROUP3-AM2	CAGCTCGTCTCCTTTTT	Probe	TaqMan	39
PPIA.4sF	TGCACCGCCAAGACTGA	Forward	TaqMan	Cyclophilin
PPIA.4sR	GACCTTGCCAAAGACCACATG	Reverse	TaqMan	Cyclophilin
PPIA.4sM1	CTTGCCGTCCAACCAC	Probe	TaqMan	Cyclophilin
HMGCoA-F	GCCAACTGGGTGGAATCCA	Forward	TaqMan	HMG CoA synthase
HMGCoA-R	GATACACTGCAATGTCTCCACAGA	Reverse	TaqMan	HMG CoA synthase
HMGCoA-M1	CCTGGGATGGTCGCTATG	Probe	TaqMan	HMG CoA synthase

**Table 4.2** Amino acid sequence identity of select CYP4A proteins.

	<b>CYP4A1</b>	<b>CYP4A37</b>	<b>CYP4A38</b>	<b>CYP4A39</b>	<b>CYP4A11</b>
<b>CYP4A1</b>					
<b>CYP4A37</b>	71.7				
<b>CYP4A38</b>	72.7	93.5			
<b>CYP4A39</b>	71.1	90.4	90.6		
<b>CYP4A11</b>	77.2	78.2	78.8	78.6	

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-43                               agcaacactcagggagagtcagtgaccagcagccccctgaacc
1  ATGAGTGTCTCTGTACTGAGCCTCACCAGACCCCTGGACACTGTGTCTGGGCTCCTGCAGCTGGCCTCCCTGCTA
   M S V S V L S L T R P L D T V S G L L Q L A S L L
76  GGCTGTGCTCTGTCTGCTCAAGGCAGCACAACTCTACCAGCGCAGGCAGTGGCTGCTGAAAGCTGTCCAGCAG
   G L A L L L L K A A Q L Y Q R R Q W L L K A V Q Q
151 TTCCCGTCCCCCTCCTTCCCACTGGTTCTTCGGGCACAAAGCAGGAGTTCCAAACGGGTGAGGAGCTGCAAGTGT
   F P S P P S H W F F G H K Q E F Q T G Q E L Q V F
226 CAGAAATGGACAGAGAAATACCTTGTGCCAGTCCTCGTTGGCTATGGGGAAGCAGAGTTAGCCTCGTAGTCTAT
   Q K W T E K Y P C A S P R W L W G S R V S L V V Y
301 GACCCAGACTACATGAAGATGATCCTGGGGCGATCAGACCCAAAAGCTGGTGGTTCTACACATTTCATCACTCCC
   D P D Y M K M I L G R S D P K A G G S Y T F I T P
376 TGGATAGGGTATGGTTTGTCTCTGCTGCATGGAGAGGCATGGTTCCAGCACCGGAGGATGCTGACCCAGCCTTC
   W I G Y G L L L L H G E A W F Q H R R M L T P A F
451 CACTATGACATCCTGAAGCCTTACGTGAGACTCATGGCTGACTCTGTCCAAGTGATGCTGGACAAGTGGGAGGAA
   H Y D I L K P Y V R L M A A D S V Q V M L D K W E E
526 CTCCTCAGGAGTGTGCTCTAGAGATCTTTGAAACACGTCTCCTTGATGACCTTGGACACCATTCAGAGTGT
   L L T Q N S H L E I F E H V S L M T L D T I M K C
601 GCCTTCAGCTACCAGGGCAACCACAGGCAGGAGGAACTCCAGGCTACATTCAGGCCATTCGAGATATGAAC
   A F T Q G N H Q A D R N S Q A Y I Q A I R D M N
676 AACCTGTGTTTGGCCGGGTGAGGAATGTTTCTACAGAAAAGACATCTTCTACGGGCTGACCTCTGAGGCGCGC
   N L V F A R V R N V F Y Q K D I F Y G L T S E G R
751 AGGAACCAACAAGGCCCGCCAGATTGCCCATCAACACACAGACCGAGTGATCAAGCTGAGGAAGGCACAGCTACAG
   R N H K A R Q I A H Q H T D R V I K L R K A Q L Q
826 GACGAGGAGGAGCTGGAGAAGTCCAGGAACAAGAGGCACTTGGACTTCTGGACATCCTCTTTGCCAAAATG
   D E G E L E K V R N K R H L D F L D I L L F A K M
901 GAGAATGGGAAGGGCTTGTCTGACAAGGACCTCCGTGCAGAAGTGGACACATTCATGTTTGAGGGGATGACACC
   E N G K G L S D K D L R A E V D T F M F E G H D T
976 ACAGCGAGTGGCATCTCCTGGATCCTCTATGCTCTGGCCACACACCCGAGCATCAGCAGAGATGCCGGGAGGAG
   T A S G I S W I L Y A L A T H P E H Q Q R C R E E
1051 ATCCAGAGCCTTCTGGGAGATGGTGCCTCCATCACCTGGGAGCACCTGGACAGATGCCCTACACCACCATGTGC
   I Q S L L G D G A S I T W E H L D Q M P Y T T M C
1126 ATCAAGGAGGATGCTGCACTCTATCCACAGTTCAGGTGTTGGCAGAGAGCTCAGCAAGCCCATCACCTTCACT
   I K E A L R L Y P P V P G V G R E L S K P I T F T
1201 GATGGACGCTCCTTGCCCAAAGGATTCTTAGTCTCGTCTCTCTTTATGCTCTTACCACAACCCGAATGTGTGG
   D G R S L P K G F L V S L S F Y A L H H N P N V W
1276 CCAGACCCAGAGTGTTCGACCCTTCCCGCTTTCAGCAGATGCCTCTCGACACAGCCATGCTTTCCTGCCCTTC
   P N P E V F D P S R F A P D A S R H S H A F L P F
1351 TCAGGAGGATCAAGGAAGTGCATTGGGCAGCACTTTGCCATGAACGAGTTGAAGGTGGCGGTGGCCCTGACCCGTG
   S G G S R N C I G Q H F A M N E L K V A V A L T T L
1426 CTCGCTTTGAGTGGCACCAGATCCCTTCAAGATCCCTGTTCCAACTCCAAGAATTGTATTGATGATCAAGAAT
   L R F E L A P D P F K I P V P T P R I V L M S K N
1501 GGGATCCACCTGCATCTCAGGAAGCTCTCTAAcccttggtgggacaaggatgagtgccgagggcctcctgcctc
   G I H L H L R K L L *
1576 cctttctgtctctcagtcagtcctcctgtctgtctcacttctcgtctccctctgcccacctgcc
1651 agccttctcgtctcctcctcctcctgttctgtcacttgagattgtgcttctgtgtctcacccttttccaagattc
1726 cttctgtttgtctgttaccatctcctaccgaggtgtcactctgactgttgatgcaaattgtgattcttgcct
1801 gtcccttcacgctgtctgtgtttccctgtcatgtgttctgtcctacttctgtctgtcctataatgtttctct
1876 tgctgctttaccagttacacaaacttagttccttcaaagagcacaatgggtaactctgtgttttaggagatca
1951 gaaatccaaaatgagtcactgagtgcaatgaaggcatgtagatgctctaagggaatgtcttctcttgcct
2026 tttctgccttctcagaatccactcttagggctatcaatgaggggactaagcatcctcatttatcatggaaataca
2101 cttttcttgccttcaactgagtcagatgttaaccataactatcaaatttttagcgtaatatctagactgtttaa
2176 ttaaacaagggtaactccagccaatccaagtgcacccgaaaccaagacatgtcagtcacgcttgggtctacttgg
2251 cattcacacattcccttacaccatacttcgtcctaactaagtaaaataaaagtcagtccttaccacaaaaaaaaa
2326 aaaaaaaaaaaaaaaaaa (a) n

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**Figure 4.1** Full-length cDNA and deduced amino acid sequence of beagle dog liver CYP4A37. In the cDNA sequence, lowercase letters represent the 5' and 3' untranslated regions, and uppercase letters represent the coding region. The predicted amino acid sequence is shown beneath the coding sequence. The nucleotide sequence of beagle dog CYP4A37 mRNA has been submitted to the GenBank nucleotide database with accession number DQ138950.



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-53          agaggattttcagcaacagtcagggagagtcagcaaacactggccctgaacc
1  ATGAGTGTCTCTGTACTGAGCCTCACCAGACCCCTGGACACTGTGTCTGGGCTCCTGCAGCTGGCCTCCCTGCTA
   M S V S L L S L T R P L D T V S G F L Q V A S L L
76  GGCCTGGCTCTGTGTGCTCAAGGCAGCACAGTTCTACTGGCACAGGAGTGGCTGCTCAAAGCTATTCAGCAG
   G L A L L L L K A A Q F Y W H R Q W L L K A I Q Q
151 TTCCCGTCCCCCTCCTTCCCACTGGTTCTTCGGGCACAGGCAAGAGTTCCAAAAGGGTCGGGAGCTGCAAGAGCTG
   F P S P P S H W F F G H R Q E F Q K G R E L Q E L
226 AAGAAAAGGACAGAGAAATACCTTGCGCCAGTCCTCGTTGGCTATGGGGAAGCAGAGTTAGCCTCGTAGTCTAT
   K K R T E K Y P C A S P R W L W G S R V S L V V Y
301 GACCCAGACTACATGAGGATGATCCTGGGGCAGTCAGACCCAAAATCTGATGGCTCCTACAGACTCATGGCTCCC
   D P D Y M R M I L G R S D P K S D G S Y R L M A P
376 TGGATAGGGTATGGTTTGTCTCTGCTGAATGGAGAGATATGGTTCCAGCACCGGCGGATGCTGACCCAGCCTTC
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   H Y D I L K P Y V R L M A D S V Q V M L D K W E T
526 CTCCTCAGGAGTGGAGTATGAGATCTTGAACACGTCCTTGGATGACCTTGGACACCATTTGAAGTGT
   L L T Q N S H L E I F E H V S L M T L D T I M K C
601 GCCTTCAGCTACCAGGGCAACCAGGCAGGACAGGAACTCCAGGCCTACATTCAGGCCATTCGAGATCTGAAC
   A F P D Y M R M I L G R S D P K S D G S Y R L M A P
676 AACCTGTGTTTGGCCCGGTGAGGAACGTTTCTACACAGAAAGACATCTTCTACGGGCTGACCTCTGAGGCGGC
   N L V F A R V R N V F Y Q K D I F Y G L T S E G R
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   R N H K A C Q L A H E H T D R V I K L R K A Q L Q
826 GACGAGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGTGGAGT
   D E G E L E K I R N K R H L D F L D I L L F A K M
901 GAGAACGGGAGGGGCTTGTCTGACAAGACCTGCGTGCAGAAAGTGGACACATTCATGTTTGAGGGGATGACACC
   E N G R G L S D K D L R A E V D T F M F E G H D T
976 ACAGCGAGTGGATCCTCCTGATCCTCTGATCCTGATCCTGATCCTGATCCTGATCCTGATCCTGATCCTGATC
   T A S G I S W I L Y A L A T H P E H Q Q R C R E E
1051 ATCCAGAGCCTTCTGGGGGATGGTGTCTCCATCACATGGGAGCACCTGGACAGATGCCCTACACCACCATGTGC
   I Q S L L G D G A S I T W E H L D Q M P Y T T M C
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1501 GGGATCCACCTGCATCTCAGGAAGCTCCTCTAAcccttggtgggacaaggatgagccctgagggcctcctgcctt
   G I H L H L R K L L *
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2251 acattcccttacatcatacttgcctcctaactaagtaaaataaagtcatgcttctacaaaaaa
2326 aaaaaaaaaaaaaa (a) n

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**Figure 4.2** Full-length cDNA and deduced amino acid sequence of beagle dog liver CYP4A38. In the cDNA sequence, lowercase letters represent the 5' and 3' untranslated regions, and uppercase letters represent the coding region. The predicted amino acid sequence is shown beneath the coding sequence. The nucleotide sequence of beagle dog CYP4A38 mRNA has been submitted to the GenBank nucleotide database with accession number DQ138951.

```

-40          aacactcagggagagtcagtgaccagcagccccctgaacc
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   G L A L L L L K A A Q L Y Q R R Q W L L K A V Q Q
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   F P S P P S H W L F G H K R E L Q K G D E L Q L L
226 CTGAAATGGATAGAGGACTTCCCATGTGCATTTTCTCGCTGGATATGGGGCACAGAGGACAGATCATGATCTAT
   L K W I E D F P C A F S R W I W G T E A E I M I Y
301 GACCCCTGACTACATGAAACTGATCCTGGGGCGATCTGACCCAAAACAGATGGCTCCTACAAACTCATGGCTCCC
   D P D Y M K L I L G R S D P K P D G S Y K L M A P
376 TGGATAGGGTATGTTTGTCTCTGCTGAATGGAGAGACATGGTCCCAGCACCGGCGGATGCTGACCCGGCCTTC
   W I G Y G L L L L N G E T W S Q H R R M L T P A F
451 CACTATGACATCCTGAAACCTTACGTGAGACTCATGGCTGACTCTGTCCAAGTGATGCTGGACAAGTGGGAGGAA
   H Y D I L K P Y V R L M A D S V Q V M L D K W E E
526 CTCCTCAGTCAGAACTCAGATCTGGAGATCTTGGACATGTCTCCTTGATGACCTTGGACACCATTGGAAGTGT
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601 GCCTTCAGCTACCAGGGCAACCACAGGCAGACAGGAACTCCAGGCCTACATTTCAGGCCATTTCGAGATCTGAAC
   A F P D Y M K L I L G R S D P K P D G S Y K L M A P
676 AACCTGTGTTTGTCCCGGATGAGGAACGTTTTCTACACAGAAAGACATCTTCTACGGGCTGACCTCTGAAGCCGC
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2401 acaaaaaaagttttaatgcataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa (a) n

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**Figure 4.3** Full-length cDNA and deduced amino acid sequence of beagle dog liver CYP4A39. In the cDNA sequence, lowercase letters represent the 5' and 3' untranslated regions, and uppercase letters represent the coding region. The predicted amino acid sequence is shown beneath the coding sequence. The nucleotide sequence of beagle dog CYP4A39 mRNA has been submitted to the GenBank nucleotide database with accession number DQ138952.

CYP4A1: MSVSALSSTRFTGSISGFLQVASVLGLLLLLLVKAVQFYLRQWLLKAFQQFPSPFHWFF :60  
 CYP4A37: MSVSVLSLTRPLDTSVGLLQLASLLGLALLLLKAAQLYQRRQWLLKAVQQFPSPPSHWFF :60  
 CYP4A38: MSVSLLSLTRPLDTSVGLQVASLLGLALLLLKAAQFYWHRQWLLKAIQQFPSPPSHWFF :60  
 CYP4A39: MSVSVLSLTRPLDTSVGLLQLASLLGLALLLLKAAQLYQRRQWLLKAVQQFPSPPSHWLF :60  
 CYP4A11: MSVSVLSPSRLIGDVSGILQAASLLILLLLLIKAVQLYLHRQWLLKALQQFPCPPSHWLF :60

CYP4A1: GHK-QFQGDKELOQIMTCVENFPSAFPRFWGSKAYLIVYDPDYMKVILGRSDPKANGVY :119  
 CYP4A37: GHKQEFQGTQELQVFKWTEKYPACASPRWLWGSRVSLVVYDPDYMKMILGRSDPKAGGSY :120  
 CYP4A38: GHRQEFQKGRELQELKKRTEKYPACASPRWLWGSRVSLVVYDPDYMRLILGRSDPKSDGSY :120  
 CYP4A39: GHKRELQKGDDELQLLLKWIIEFPCAFSRWIWGTEAEIMIYDPDYMKLILGRSDPKPDGSY :120  
 CYP4A11: GHIQELQDDQELQRIQKWVETFPSACPHWLWGKVRVQLYDPDYMKVILGRSDPKSHGSY :120

CYP4A1: RLLAPWIGYGLLLLLNGQPWFQHRRLTPAFHYDILKPYVKNMADSIRLMMLDKWEQLAGQD :179  
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 CYP4A38: RLMAPWIGYGLLLLLNGEIIWFQHRRLTPAFHYDILKPYVRLMADSVQVMLDKWEELLTQN :180  
 CYP4A39: KLMAPWIGYGLLLLLNGETWSQHRRLTPAFHYDILKPYVRLMADSVQVMLDKWEELLSON :180  
 CYP4A11: RFLAPWIGYGLLLLLNGQTFWQHRRLTPAFHYDILKPYVGLMADSVRVMLDKWEELLGQD :180

CYP4A1: SSIEIFQHIISLMTLDTVMKCAFSHNGSVQVDGNYKSYIQAIGNLNDLFHSRVRNIFHQND :239  
 CYP4A37: SHLEIFEHVSLMTLDTIMKCAFSYQGNHQADRNSQAYIQAIRDMNNLVFARVRNVFYQKD :240  
 CYP4A38: SHLEIFEHVSLMTLDTIMKCAFSYQGNHQADRNSQAYIQAIRDLNNLVFARVRNVFYQKD :240  
 CYP4A39: SHLEIFDHVSLMTLDTIMKCAFSYQGNHQADRNSQAYIQAIRDLNNLVFARMNRNVFYQKD :240  
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CYP4A1: TIYNFSSNGHLFNRACQLAHDHTDGVIKLRKDQLQNAAGELEKVKKKRRLDFLDILLLARM :299  
 CYP4A37: IFYGLTSEGRNRHKARQIAHQHTDRVIKLRKAQLQDEGELEKVRNKRHLDFLDILLFAKM :300  
 CYP4A38: IFYGLTSEGRNRHKACQLAHEHTDRVIKLRKAQLQDEGELEKIRNKRHLDFLDILLFAKM :300  
 CYP4A39: IFYGLTSEGRNRHKACQLAQHTDRVIKLRKAQLQDKGELEKVRNKRHLDFLDILLFAKV :300  
 CYP4A11: TIYSLTSAGRWTHRACQLAQHTDQVIQLRKAQLQKEGELEKIKRKRHLDFLDILLLAKM :300

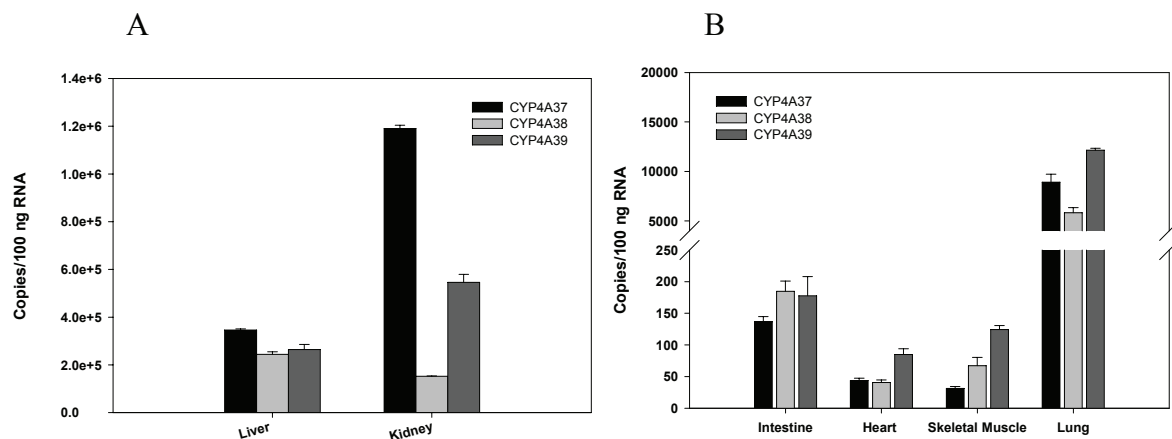
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 CYP4A39: ENGRGLSDKDLRAEVDTFMFEGHDTTASGISWILYALATHPEHQQRCEEIQSLLGDGAS :360  
 CYP4A11: ENGSLSDKDLRAEVDTFMFEGHDTTASGISWILYALATHPKHQERCEEIHSLLGDGAS :360

CYP4A1: ITWDHLDQIPYTTMCIKEALRLYPVPGIVRELSTSVTFPDGRSLPKGIQVTLISIYGLHH :419  
 CYP4A37: ITWEHLDQMPYTTMCIKEALRLYPVPGVGRELSKPITFTDGRSLPKGFLVLSFYALHH :420  
 CYP4A38: ITWEHLDQMPYTTMCIKEALRLYPVPGVGRELSKPITFPDGRSLPRGFLVLLSFYALHH :420  
 CYP4A39: ITWEHLDQMPYTTMCIKEALRLYPVPGVGRELSKPITFPDGRSLPKGFLVLLSFYALHH :420  
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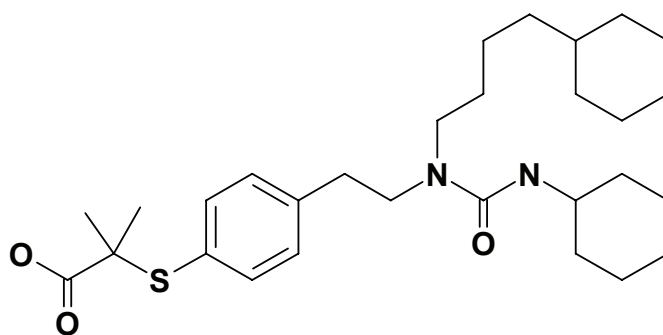
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 CYP4A37: NPNVWPNEVFDPSRFAPDASRHSHAFLPFSGGSRCIGQHFAMNELKVAVALTLLRFEL :480  
 CYP4A38: NPNVWPNEVFDPSRFAPDASRHSHAFLPFSGGSRCIGQHFAMNELKVAVALTLLRFEL :480  
 CYP4A39: NPNVWPNEVFDPSRFAPGVSRHSHAFLPFSGGSRCIGQHFAMNEMKVAVALTLLRFEL :480  
 CYP4A11: NPKVWPNEVFDPSRFAPGSAQHSHAFLPFSGGSRCIGKQFAMNELKVATALTLLRFEL :480

CYP4A1: LPDPTKVPIPLPRLVLKSKNGIYLYLKKLH :509  
 CYP4A37: APDPFKIPVPTPRIVLMSKNGIHLHLRKLH :510  
 CYP4A38: APDPFRIPVPTPRIVLMSKNGIHLHLRKLH :510  
 CYP4A39: APDPFRIPVPTPKIVLMSKNGIHLRLRLH :510  
 CYP4A11: LPDPTRIPITPIARLVLKSKNGIHLRLRLLPNPCEDKDQL :519

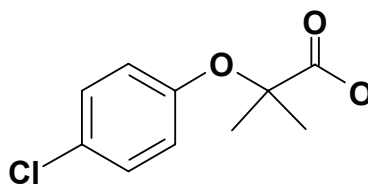
**Figure 4.4** Multiple sequence alignment of the deduced amino acid sequence of dog CYP4A37, CYP4A38 and CYP4A39 with the sequences of rat CYP4A1 (M57718) and human CYP4A11 (L04751). The alignment was performed with the CLUSTAL W program (gap penalty 10). Residues with a black background represent absolutely conserved amino acids.



**Figure 4.5** CYP4A37, CYP4A38 and CYP4A39 tissue expression. CYP4A37, CYP4A38 and CYP4A39 expression in dog liver and kidney (A) and intestine, heart, skeletal muscle and lung (B). Equal amounts of RNA were pooled from n=3 dog tissues and CYP4A37, CYP4A38 and CYP4A39 expression was determined using specific TaqMan assays. Copy numbers of each isoform were determined from standard curves prepared from CYP4A37, CYP4A38 and CYP4A39 plasmid DNA.

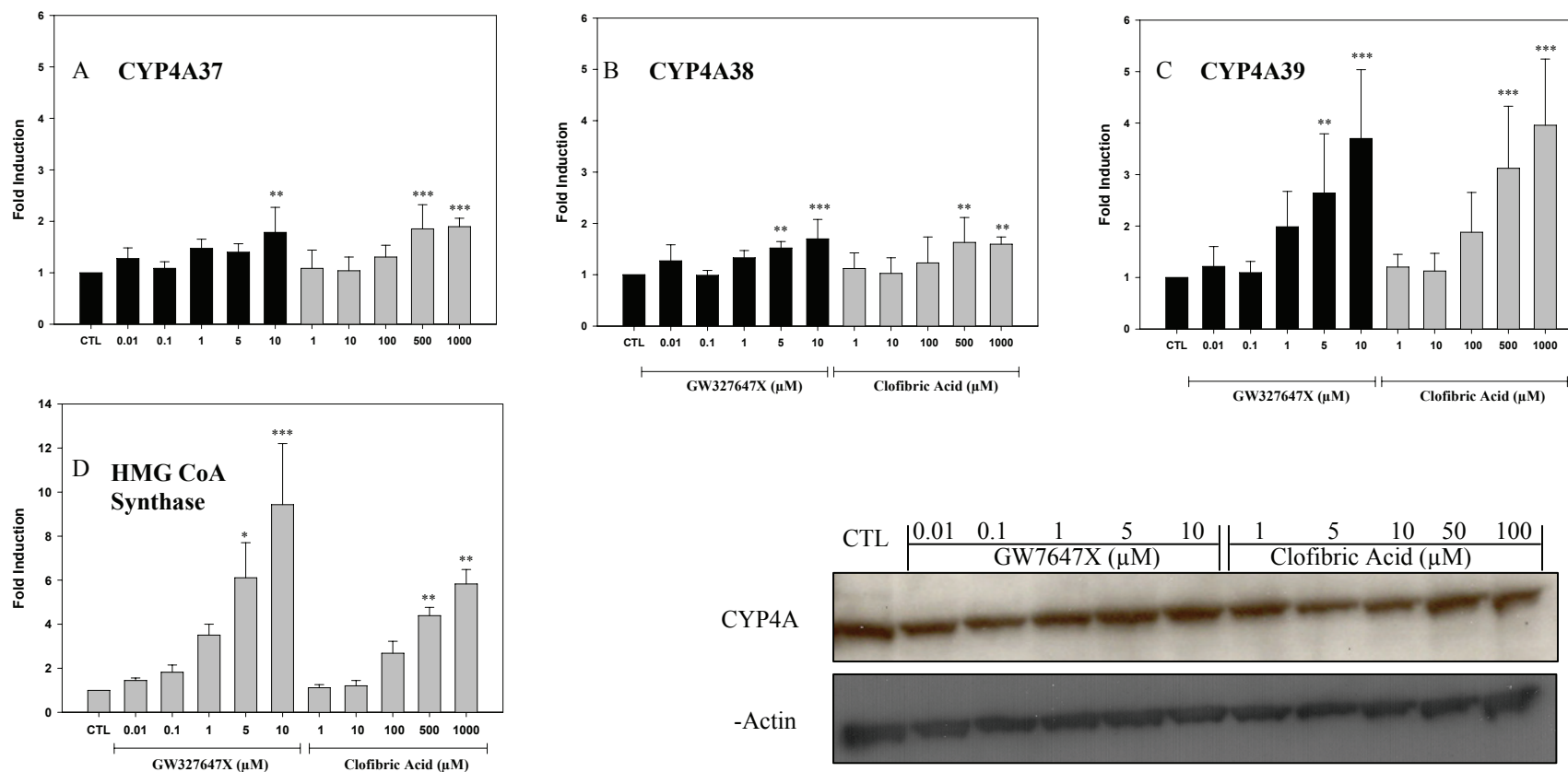


(A) GW7647X

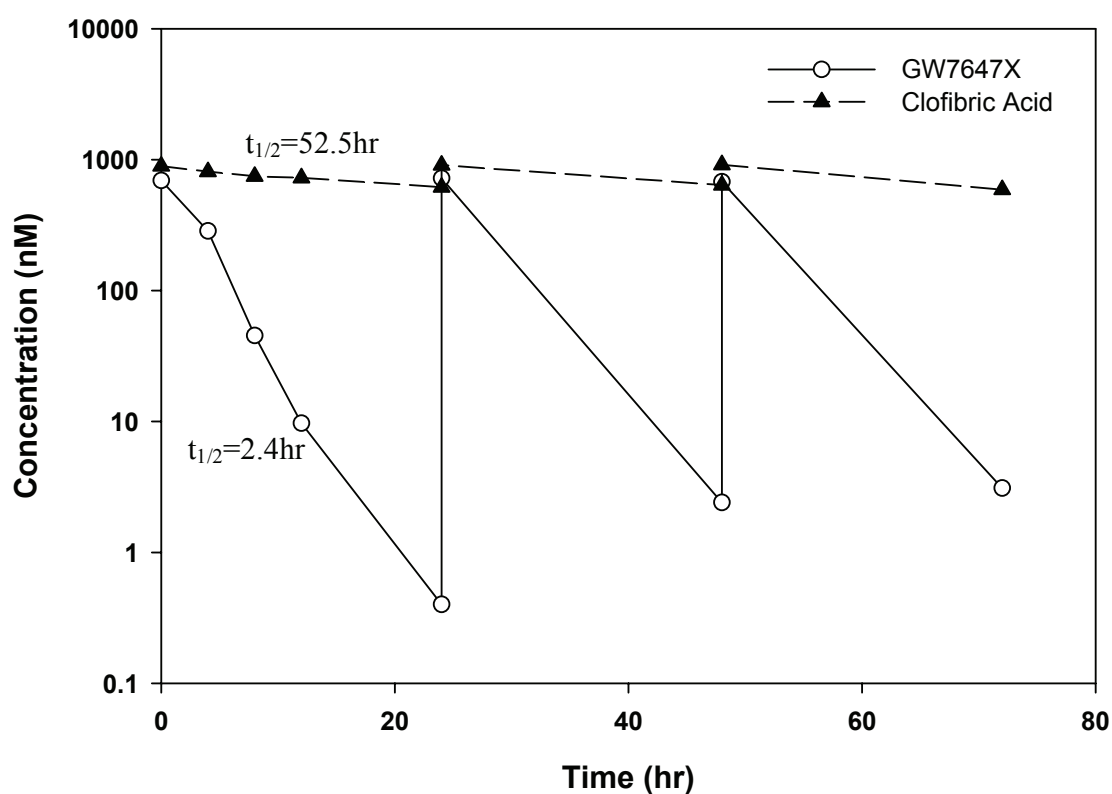


(B) Clofibric Acid

**Figure 4.6** Structure of the PPAR agonist GW7647X (A) and clofibric acid (B).

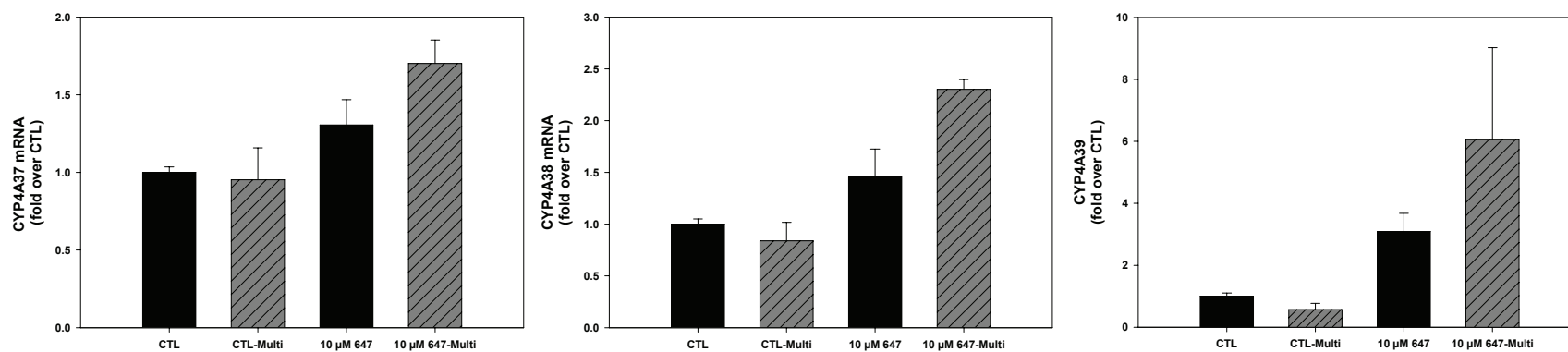


**Figure 4.7** Concentration response relationship for induction of beagle dog RNA following treatment of cultured hepatocytes with GW7647X or clofibric acid for 48hr. RNA expression from dog hepatocyte cultures was determined using specific TaqMan assays. Relative quantification of gene expression was conducted by normalizing target gene expression for each treatment group to cyclophilin. CYP4A37 (A), CYP4A38 (B), CYP4A39 (C), HMG CoA synthase (D). Data are expressed as mean (n=3 hepatocyte cultures)  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001. (E) Representative Western blot of microsomes (10  $\mu$ g/lane) isolated from cultured dog hepatocytes.



**Figure 4.8** Metabolic stability of GW7647X and clofibric acid in cultured dog hepatocytes. Metabolic stability was determined by monitoring the disappearance of compound over time (0, 4, 8, 12, and 24hr). The half-life of GW7647X and clofibric acid was estimated under the assumption that the disappearance followed a first-order linear kinetic process. Data points represent the average of two determinations.





**Figure 4.9** Modulation of CYP4A expression in dog hepatocytes by multiple dosing of GW327647X. Dog hepatocytes were exposed to a single dose of 10 μM GW7647X or 0.1% DMSO, for 48 h (STD), with fresh drug added at 24hr. Parallel cultures received repeat doses of either 10 μM GW327647X or 0.1% DMSO in 8hr intervals for 48hr (MULTI). All cells were collected at 48hr, and CYP4A37, CYP4A38 and CYP4A39 mRNA levels were quantified by real-time PCR (TaqMan) normalized to cyclophilin mRNA, and expressed as fold induction over CTL. Data are expressed as means  $\pm$  S.D. of triplicate measurement.

## **CHAPTER 5**

## **CONCLUSIONS**

Prediction of drug-drug interactions is an important part of drug development. Currently, primary human hepatocytes are the preferred model system to predict the potential for one type of drug-drug interaction, namely enzyme induction. However, the limited availability and cost of primary human hepatocytes represents a major barrier to the utility of this model system in early stage drug discovery. Therefore, identification of a surrogate species to predict human CYP induction would be of considerable value.

Beagle dog is widely used as a model in the pharmaceutical industry, particularly in preclinical toxicology, pharmacology and drug metabolism/pharmacokinetic studies. Previous studies have identified various CYP proteins in the beagle dog (see Chapter 1 for a detailed review); however, little information was available on the inducibility of these enzymes in dog liver by prototypic CYP-inducing agents such as phenobarbital or rifampin. As a result, it has remained unclear how the regulation of dog CYP gene expression, both in vivo and in vitro, relates to that of human.

Consequently, a major aim of the current dissertation work was to investigate the hypothesis that dog liver CYP enzymes are inducible in a manner corresponding to the orthologous human CYP enzymes. To this end, prototypical rat and human CYP inducers were tested for their ability to induce dog CYP in vitro and in vivo (Chapter 2). Treatment of primary cultures of male beagle dog hepatocytes with CYP inducers revealed that beagle dog expressed CYP1A, CYP2B, CYP2E, CYP3A and CYP4A and that the regulation of dog CYP expression seems to be more similar to that observed in human than rat. For example, the well-characterized human CYP3A inducer rifampin also activated dog CYP3A expression in primary canine hepatocytes. Importantly, rifampin is not an inducer of rat CYP3A. This species-specific divergence in CYP3A induction can be explained by the

pharmacology of PXR activation in humans, dogs, and rats. Thus, while rifampin is capable of binding and activating dog and human PXR, it shows no activity on the rodent receptor. A detailed examination of PXR activation performed by Moore et al. (Moore et al., 2002) suggests that several compounds including the environmental pollutant *trans*-nonchlor and the orphan drug SR12813 are capable of strongly activating dog and human PXR but exhibit no activity towards murine PXR. These results, in conjunction with the studies presented in Chapter 2, suggest that primary cultures of dog hepatocytes might represent an alternate model system to predict human CYP induction. An extended comparison of the pharmacology of dog and human PXR activation and induction of CYP3A gene expression should yield valuable insights into the utility of the dog hepatocyte culture system characterized in Chapter 2.

In order to further examine dog CYP gene regulation, the time- and concentration-dependence of CYP induction was examined (Chapter 3). For this purpose, time and concentration-response relationships were derived following treatment of primary dog hepatocyte cultures with prototypical CYP1A, CYP2B and CYP3A inducers, namely - naphthoflavone, phenobarbital and rifampin, respectively. The compounds are well-documented activators of the aryl hydrocarbon receptor and the orphan nuclear receptors CAR and PXR, respectively. It was apparent from these studies that 48 h and 72 h after drug treatment were appropriate time points to monitor both mRNA expression and CYP enzyme activity. Furthermore, while concentration-response relationships for induction of dog CYP1A, CYP2B and CYP3A by these inducers exhibited characteristics closer to human than rat, rifampin did not induce dog CYP2B highlighting a major species difference for the first time. This result was surprising since multiple studies have indicated crosstalk in the

regulation of CYP2B and CYP3A subfamily members by PXR and CAR (Wang and Negishi, 2003). As outlined above, it is unlikely that this difference is due to pharmacology of dog PXR since dog CYP3A was efficaciously induced by rifampin. Since rifampin has previously been shown to be an activator of dog PXR, this observation suggests that the lack of dog CYP2B induction by this compound could be due to structural differences in the regulatory regions of the dog CYP2B gene, although this possibility remains to be investigated. Importantly, the phenobarbital-dependent induction of CYP3A-mediated testosterone 6 $\alpha$ -hydroxylation was observed in dog hepatocytes. Since this activity is most likely mediated by CAR, these data suggest that the complex interplay between CAR and PXR reported on the rodent and human CYP3A promoters is conserved in dogs. Taken together, these data indicate that the primary dog hepatocyte model characterized in Chapter 3 is capable of supporting the aryl-hydrocarbon-, CAR-, and PXR-dependent transactivation of CYP expression observed in vivo (Chapter 2). Moreover, the cross-regulation of CYP3A, but not CYP2B, by CAR and PXR is intact in beagle dog. The significance of this observation is unclear but warrants further investigation using additional chemical tools such as the PXR ligands outlined above.

Although CYP4A isoforms have been isolated and characterized in multiple vertebrates, to date, little is known about the expression and inducibility of dog CYP4A isoforms. The cloning, tissue expression and regulation of beagle CYP4A genes is described in Chapter 4. Sequence analysis revealed that at least three CYP4A isoforms, namely CYP4A37, CYP4A38 and CYP4A39 are expressed in beagle dog. Conservation of key amino acid, particularly in the active site region of the enzyme, suggests that dog CYP4A mRNA encodes active enzyme. Evaluation of tissue expression revealed that dog CYP4A

levels are tissue dependent with relatively high levels found in kidney and liver, with much lower levels in lung, intestine, skeletal muscle and heart. These results are in agreement with CYP4A tissue expression patterns in other species (e.g., rat and human) (Kimura et al., 1989; Nishimura et al., 2003; Patel et al., 1998; Zhang et al., 2002). It was evident following treatment of primary cultures of dog hepatocytes with a potent PPAR $\alpha$  agonist that the CYP4A isoforms were not induced to an appreciable extent. Importantly, expression of a previously characterized PPAR $\alpha$  target gene, namely HMG-CoA Synthase (Nagasawa et al., 2004) was retained, suggesting that the apparent lack of CYP4A responsiveness was not due to a loss of PPAR $\alpha$  activity in this culture system. Treatment of rat and human hepatocytes with clofibric acid results in a strikingly different CYP4A response, whereby rat CYP4A is markedly induced and human CYP4A expression is barely increased (Figure 5.1). Thus, the lack of CYP4A induction observed in human and dog hepatocytes suggests that dog and human CYP4A are regulated in a comparable manner.

For many years it has been understood that treatment of rodents with the hypolipidemic drug clofibrate causes marked hepatomegaly characterized by peroxisome proliferation, hyperplasia, hypertrophy and ultimately hepatocarcinogenesis. Although this phenomenon is known to be mediated by PPAR $\alpha$  (these effects are lost in mice harboring a disrupted PPAR $\alpha$  gene) the precise molecular mechanism responsible for these effects has remained elusive. Importantly, humans are relatively refractory to these effects and clofibrate has been used for decades for treatment of dyslipidemia. Although the reason for this species divergence in response to PPAR $\alpha$  activators is unclear, a number of studies have implicated CYP4A in the pleiotropic response to peroxisome proliferators observed in rodents (Simpson, 1997). Furthermore, CYP4A has been shown to play a role in

maintenance of vascular tone via metabolism of arachidonic acid to the potent vasoconstrictor, 20-HETE. Recently, animal studies have shown that inhibition of 20-HETE formation by treatment with selective CYP  $\omega$ -hydroxylase inhibitors can (1) reduce the initial fall in cerebral blood flow after subarachnoid hemorrhage in the rat (Kehl et al., 2002) and (2) reduce myocardial infarct size following ischemia-reperfusion injury in dogs (Nithipatikom et al., 2004). A recent review article suggests that inhibition of selected CYPs as an approach to the treatment of myocardial infarction should be further developed (Doggrell, 2004).

Several plausible theories have been put forth to explain species differences in peroxisome proliferation, and thus, CYP4A induction. Firstly, studies have shown that while the PPAR DBD is highly conserved across species, there are amino acid differences in the PPAR LBD that might confer species differences, analogous to the mechanism put forth to explain species differences in CYP3A induction (LeCluyse, 2001). A second supposition to explain species differences in target gene induction is the observation that PPAR levels in dog, guinea pig and human hepatocytes are an order of magnitude lower than in rodents (Choudhury et al., 2000; Nagasawa et al., 2004). Thirdly, it is possible that the CYP4A gene of non-inducible species lacks a functional response element, in contrast to rat CYP4A1 and rabbit CYP4A6, for which a functional element has been reported (Aldridge et al., 1995; Palmer et al., 1995). Another hypothesis to explain the CYP4A species difference is that non-responsive species may lack an activator or have an active repressor of PPAR function. Finally, recent studies have identified phosphorylation as a regulator of PPAR; however, species differences have not been evaluated regarding this post translational modification.

Recent cloning and characterization of dog PPAR $\alpha$  revealed the LBD is 97% identical to that of human and 92.4% identical to that of rat (Nagasawa et al., 2004). Nagasawa et al. demonstrated that another PPAR $\alpha$  target gene, HMG CoA synthase, was markedly induced to an equivalent extent in rat and dog hepatocytes following treatment with a potent PPAR $\alpha$  agonist, KPL, whereas treatment with fenofibrate and Wy,14643 induced this target gene in rat, but not in dog hepatocytes. Interestingly, KPL was demonstrated to be more potent against dog than rat PPAR $\alpha$  with EC<sub>50</sub> values of 0.48 and 10.01  $\mu$ M, respectively (Nagasawa et al., 2004). If PPAR $\alpha$  LBD differences were responsible for species differences in target gene response, the rat HMG CoA synthase response would not be expected after treatment with KPL. Furthermore, studies have shown that PPAR $\alpha$  ligands have differential effects on PPAR $\alpha$  target genes in other non-responsive species. For example, while CYP4A induction does not occur to an appreciable extent in human hepatocytes after treatment with PPAR $\alpha$  ligand, the PPAR $\alpha$  target genes CPT1A and HMG CoA synthase are markedly upregulated (Napal et al., 2005) (Lawrence et al., 2001) (Figure 5.2). The most compelling evidence suggesting the PPAR $\alpha$  LBD does not mediate species-specific CYP4A induction was presented in a study utilizing a PPAR $\alpha$  humanized mouse model (expressing human PPAR $\alpha$  but not mouse PPAR $\alpha$  in the liver) (Cheung et al., 2004). Treatment of PPAR $\alpha$  humanized mice with Wy-14,643 and fenofibrate resulted in a robust induction of mouse CYP4A (equivalent to wild type mice), an outcome that would not be expected if ligand affinity was different between mouse and human PPAR $\alpha$ . Taken together, these results suggest that other factors besides PPAR $\alpha$  structure dictate species differences in CYP4A induction.



One theory suggests that species differences in PPAR target gene induction result from differences in hepatic PPAR expression. For example, it has been established that mouse and rat has much higher levels of expression of the receptor in the liver than does human (Palmer et al., 1998). CYP4A is highly inducible in hamster even though hamster PPAR levels are intermediate compared to human and rat, discounting the importance of absolute nuclear receptor levels to elicit a robust CYP4A induction response (Choudhury et al., 2004). A difference in PPAR expression levels is likely to be an oversimplification for species differences in CYP4A induction since non-CYP4A inducible species are clearly able to undergo some PPAR target gene responses. For example, HMG CoA synthase and CPT1A have been shown to be markedly upregulated following treatment of human hepatocytes with Wy14,643, suggesting that other cellular factors dictate the species specific CYP4A induction response (Ammerschlaeger et al., 2004).

In rabbit, two imperfect DR-1 motifs were identified approximately 677bp upstream of the CYP4A6 gene, and these could be bound by PPAR (Palmer et al., 1995). Further work demonstrated that the DR-1 motif is required for the transcriptional activation of the CYP4A6 gene by peroxisome proliferators. Subsequently, characterization of rat CYP4A1 regulation by PPAR revealed a functional PPRE approximately 4,300bp upstream of the gene (Aldridge et al., 1995). Interestingly, a second element was located 35 nucleotides further upstream; however this element failed to bind PPAR/RXR heterodimers and was unresponsive to Wy-14,643 in gene transfection assays, suggesting that the more distal element was not active.

More recently, the gene encoding human CYP4A11 was isolated and its complete genomic sequence determined. Inspection of the genomic sequence revealed there was no PPRE within the 5'-promoter region flanking the CYP4A11 gene (Kawashima et al., 2000). It should be noted however, that these authors only searched ~2300bp upstream of the CYP4A11 transcription start site, presumably due to the lack of any additional sequence information at the time. With the conclusion of the human genome project, detailed sequence information from thousands of genes is readily available. Examination of ~10kb of sequence upstream of CYP4A11 transcription start site revealed the presence of two putative PPRES, one of which was nearly identical in location to the previously identified rat PPRES (Figure 5.3). As part of the work for this dissertation, electromobility shift assays were conducted to determine whether either of these novel response elements could interact with PPAR. Interestingly, neither of the putative CYP4A11 PPRES bound PPAR in these experiments, whereas strong binding was observed with the previously characterized rat CYP4A1 PPRES (Figure 5.4). Notably, a PPRES has not been identified from a species that lacks inducible CYP4A, strongly suggesting a role for the PPRES in mediating species differences in CYP4A induction.

Speculation as to how some species adapted an inducible form of CYP4A, or alternatively, how some species adapted a non-inducible form of CYP4A through evolution, is a fascinating exercise. There are well documented studies proving that mouse, rat, hamster and rabbit CYP4A is highly inducible, whereas guinea pig, monkey and human CYP4A is weakly inducible, at best. Most recently, the work presented in this dissertation clearly shows that dog CYP4A is not inducible, relative to the rodent CYP4A (Chapter 4). Preliminary studies indicate that CYP4A is not inducible by clofibrate in fish or the

American alligator (Emblidge and Delorenzo, 2006; Ertl et al., 1999), suggesting the primitive species did not have an inducible form of this enzyme. It has been suggested that all P450s evolved from a common ancestral P450 gene, likely to have first evolved approximately 3.5 billion years ago (Loomis, 1988).

Throughout its evolutionary history, the CYP superfamily is thought to have undergone repeated rounds of gene and genome duplication. CYP4 is thought to be one of the oldest CYP families, having evolved about 1.25 billion years ago as a way to maintain the membrane integrity of early eukaryotic cells (Nebert and Gonzalez, 1985). Phylogenetic analysis of CYPs supports the notion that a dramatic expansion of several gene families seems to have occurred approximately 400 million years ago. It has also been suggested that CYPs represent one of the most rapidly evolving gene families, presumably due to continuously changing exposure to toxic compounds produced by plants (Danielson, 2002). Most multigene families exist in a continuous state of flux, often times diverging by gene duplication events. After such an event, the resulting gene copy (or copies) will spontaneously mutate subject to pressure of natural selection, as long as one copy maintains its original function. The duplicated copy, free to drift genetically, becomes a pseudogene, and in some cases takes on a novel function to enhance the sustainability of the organism (Danielson, 2002).

Human has two CYP4A isoforms, CYP4A11 and CYP4A22, that appear to have duplicated recently which suggests that the ancestor of rodent and human had a single, likely non-inducible, CYP4A gene (Nelson et al., 2004). It seems possible that rodents (with the exception of guinea pig) attained an inducible CYP4A due to gene duplication events, potentially by expanding upon the PPRE-like sequence identified upstream of the human

CYP4A11 gene as part of work conducted for this dissertation (Figure 5.3). Four CYP4A forms have been identified in rat, namely, CYP4A1, CYP4A2, CYP4A3 and CYP4A8. While CYP4A1 is the most studied of the four rat CYP4A isoforms, sequence analysis indicates that rat CYP4A1 gave rise to CYP4A2 and CYP4A3, all three of which are inducible isoforms. Although not well characterized, rat CYP4A8 has not been shown to be inducible and it differs considerably in sequence from the other rat CYP4A isoforms. It is interesting to theorize that rat CYP4A8 exists as a remnant of a non-inducible ancestral rat CYP4A isoform that may have given rise to the three inducible CYP4A isoforms in this species. While it seems feasible that a gene duplication event gave rise to inducible CYP4A in certain rodent species, it is unclear as to why this event might have occurred.

One of the most perplexing species differences in CYP4A response to peroxisome proliferators is the lack of induction in guinea pig. Guinea pig, like mouse, rat, hamster and rabbit, belongs to the Phylum Chordata, Subphylum Vertebrata, Class Mammalia, Order Rodentia, although the classification of guinea pig as a rodent has been debated (Graur et al., 1991). Since these species are closely related, it seems likely that guinea pig was not susceptible to the same environmental pressure as other rodents that invoked inducible CYP4A. Diet is a logical culprit for evolution of CYP4A species differences; however, on the surface its role is unclear since like mouse, hamster and rabbit, guinea pig is a vegetarian species, whereas rat is omnivorous. However, guinea pig might not have faced the same environmental pressure as other rodents, suggesting that regional dietary differences could have caused evolution of inducible CYP4A in rodents. Interestingly, mouse, rat and hamster seem to have evolved in Asia, whereas guinea pig is indigenous to South America.

Interestingly, guinea pig, like human and other primates are not able to synthesize ascorbic acid and therefore must attain vitamin C from the diet. While this commonality between species that lack a CYP4A induction response is intriguing, it is not likely correlated since dog is able to synthesize ascorbic acid but is not responsive to CYP4A inducers. There are numerous biochemical, environmental, geographical, etc. connections that can be drawn between inducible and non-inducible species; however, there is no clear answer as to why the CYP4A gene family evolved inducible isoforms.

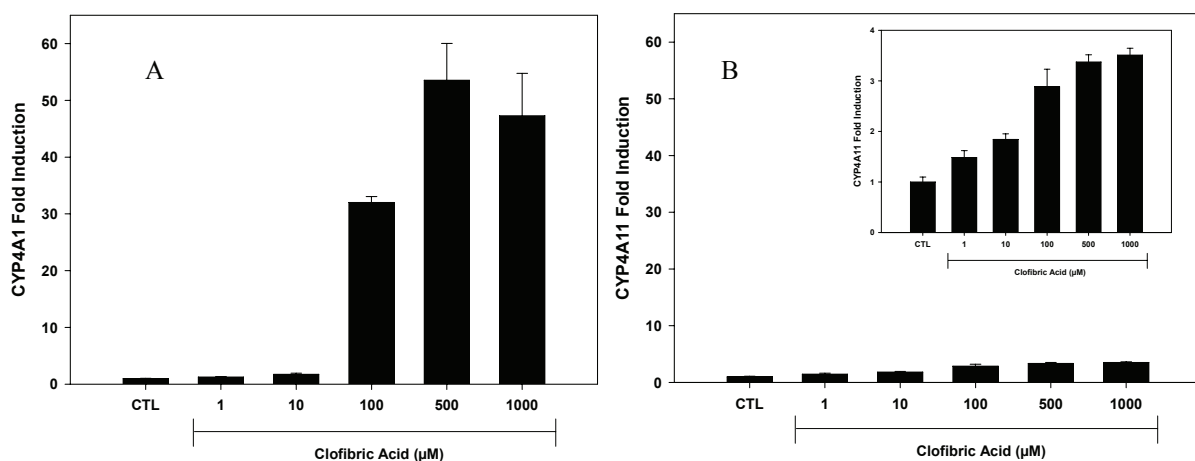
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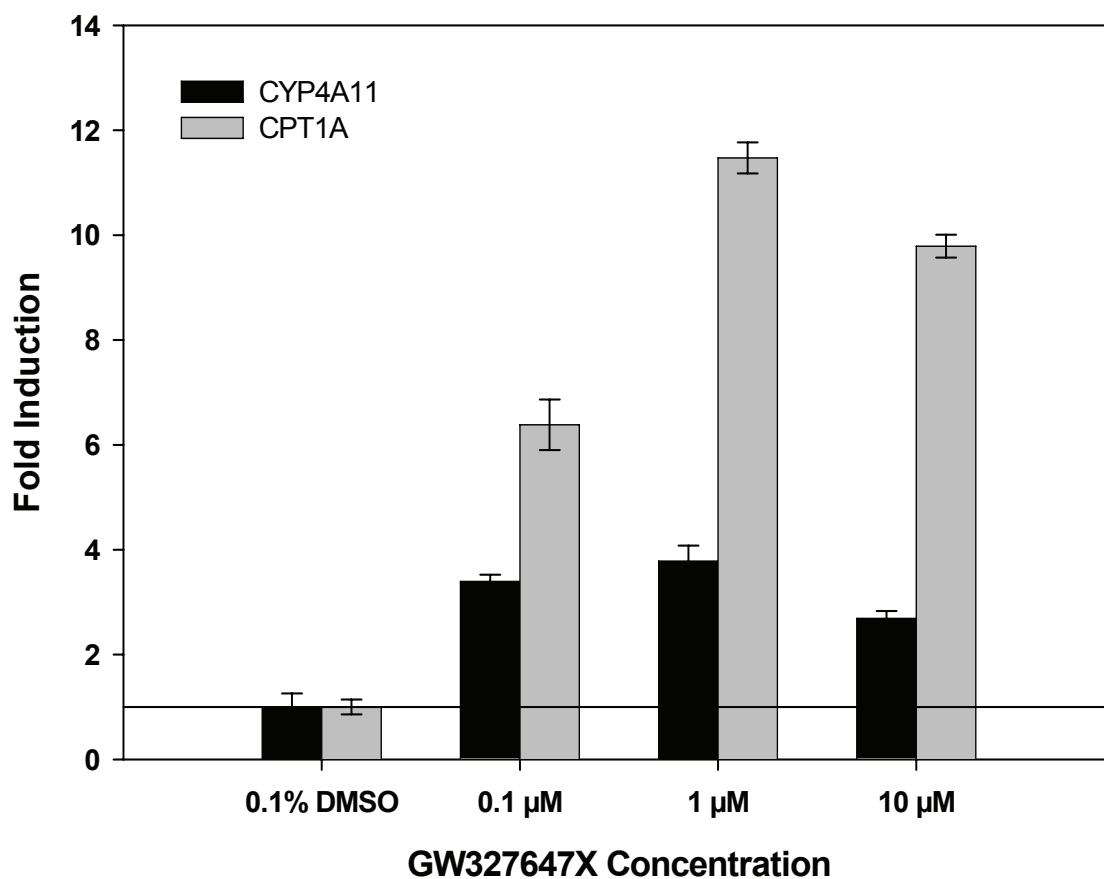
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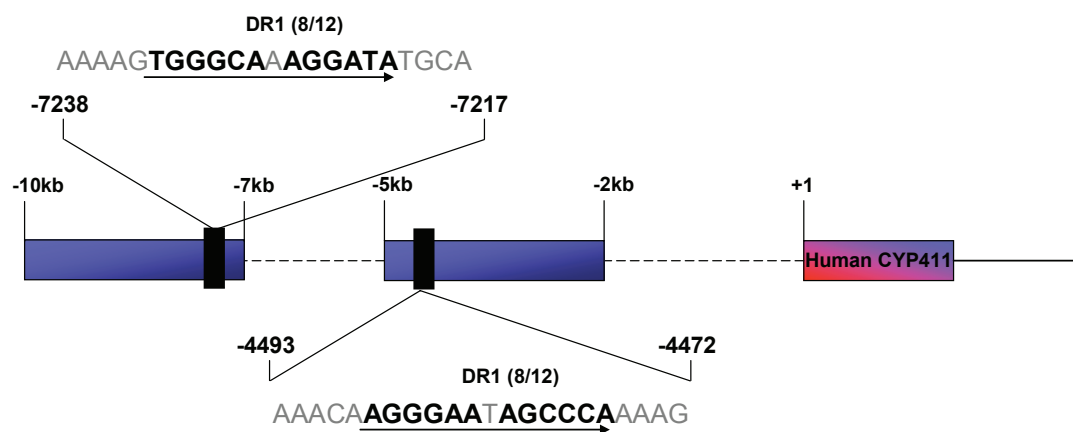




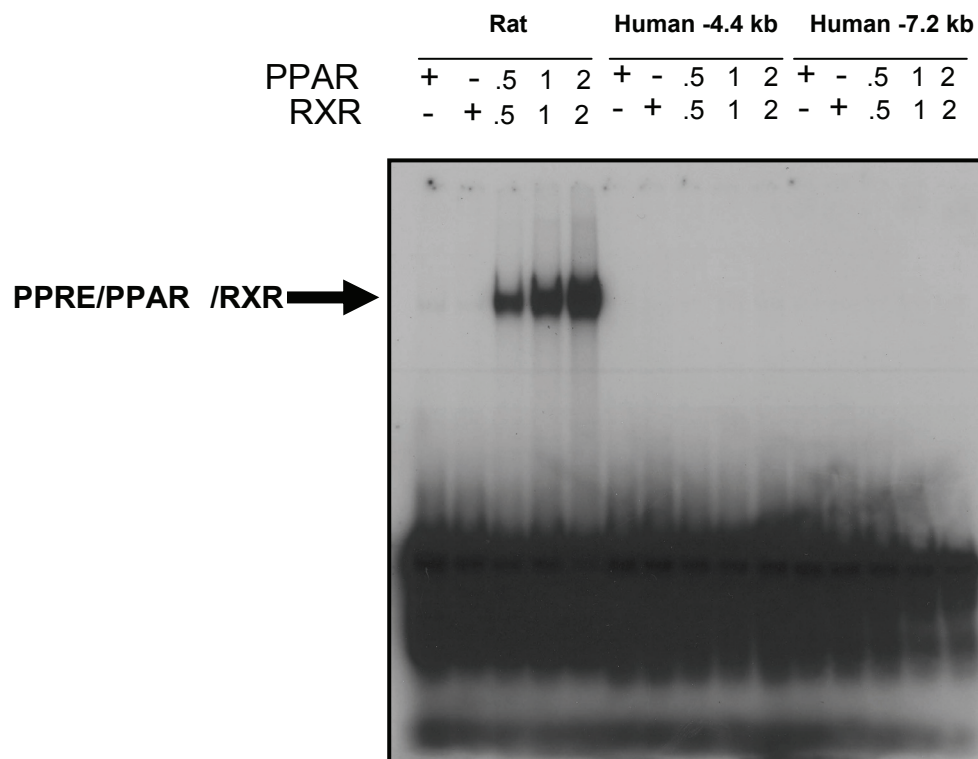
**Figure 5.1** CYP4A induction in cultured rat (A) and human (B) hepatocytes after treatment with clofibric acid. Inset (B): human CYP4A11 induction with different scale highlighting a concentration response relationship for clofibric acid induction of CYP4A. Data are presented as mean  $\pm$  SD of triplicate measurements in a single hepatocyte culture.



**Figure 5.2** Induction of the PPAR target genes CYP4A11 and carnitine palmitoyl acyl-CoA transferase 1 (CPT1A) in cultured human hepatocytes following treatment with PPAR ligand, GW327647X. Horizontal line represents control level (0.1% DMSO) for each target gene. Data are presented as mean  $\pm$  SD of triplicate measurements in a single hepatocyte culture.



**Figure 5.3** Putative PPAR response elements identified from the 5'-flanking region of the human CYP4A11 gene (NM\_000778) using the nuclear receptor binding site algorithm NUBIScan.v2.1.



**Figure 5.4** Electromobility shift analysis was performed using radiolabeled, double-stranded oligonucleotides that correspond to the rat DR1 containing element identified previously or the two putative human DR1 elements at -4.4kb and -7.2 kb upstream of a transcription start site. PPAR and RXR protein was translated in vitro prior to use in binding assays. Numbers above figure indicate volume of each component added in  $\mu$ L. Arrow indicates position of PPAR /RXR/DR-1 oligonucleotide complex.