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Polycyclic aromatic hydrocarbons and amiodarone pharmacokinetics

By

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To my Mother, Magda Hassan

Abstract

In the treatment of arrhythmias, amiodarone is a primary therapeutic agent. Cytochromes P450 (CYP) 1A1 and 1A2 facilitate biotransformation of amiodarone to the biologically active desethylamiodarone. Side effects have been reported during therapy and some are correlated with increased desethylamiodarone levels. Exposure to polycyclic aromatic hydrocarbons (PAH) like β-naphthoflavone induces CYP1A1 and CYP1A2 and therefore can increase desethylamiodarone levels. Desethylamiodarone, however, was reported to inactivate human CYP1A1 and therefore can conceivably inhibit its CYP1A1-mediated formation.

Our primary objective was to investigate the effect of β-naphthoflavone on amiodarone disposition. Since rats were used, CYP isoenzymes involved in desethylamiodarone formation in human were compared to their rat counterparts. The effect of ketoconazole on desethylamiodarone formation, the inactivating potential of desethylamiodarone on CYP1A1 and the mechanism of β-naphthoflavone-amiodarone interaction were assessed.

Human CYP1A1 and rat CYP2D1 had the highest intrinsic clearance (Cl_{int}) for desethylamiodarone. Human and rat CYP1A2 had the lowest Cl_{int}. Ketoconazole (18.8 μM) inhibited all isoforms except for rat

CYP1A2; it potently inhibited human CYP1A1 and CYP3A4 and rat CYP2D2 and CYP1A1.

After a single amiodarone dose was administered to control and β-naphthoflavone pretreated rats, the plasma area under concentration-time curve (AUC) of desethylamiodarone increased. With multiple doses, amiodarone $AUC_{(0\text{-}24h)}$ decreased in β-naphthoflavone plasma (30%), lung (35%), liver (48%), kidney (52%), heart (34%), and intestine (43%). Desethylamiodarone $AUC_{(0\text{-}24h)}$ increased in β-naphthoflavone plasma (36%), lung (56%), liver (101%), kidney (65%), and heart (73%).

Desethylamiodarone caused no inactivation of CYP1A1 when preincubations were diluted and nicotinamide adenine dinucleotide phosphate (NADPH) was added in the probe incubation samples. Evidence for reversible mixed-competitive inhibition was apparent. Addition and/or replenishment of NADPH were important factors in maintaining control activity.

B-naphthoflavone increased desethylamiodarone formation only in lung and kidney microsomes. Desethylamiodarone formation in liver, intestine and heart microsomes was not altered. Body-weight-normalized liver mass was significantly increased (27%) by β-naphthoflavone.

In conclusion, human CYP1A1 was more efficient in forming desethylamiodarone than rat isoenzyme. Exposure to PAH increased desethylamiodarone levels *in vivo*. Increased desethylamiodarone levels

were partly caused by CYP1A1 induction, and by increased liver mass. Desethylamiodarone did not inactivate CYP1A1 activity.

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Abbreviations

Amiodarone	AM
Desethylamiodarone	DEA
β-naphthoflavone	BNF
Corn oil	CO
Cytochrome P450	CYP
Ketoconazole	KTZ
Mechanism-based inhibitors	MBI
Intrinsic clearance	Cl_{int}
Maximum rate of metabolite formation	V_{max}
Michaelis-Menten constant	K_m
Area under concentration-time curve	AUC
Polycyclic aromatic hydrocarbons	PAH
3-methylcholantherene	3-MC
Multidrug-resistance gene	MDR
P-glycoprotein	P-gp
2,3,7,8-tetrachlorodibenzo-p-dioxin	TCDD
Aryl hydrocarbon receptor	Ahr
Multidrug resistance associated	MRP

proteins	
7-ethoxyresorufin O-deethylase	EROD
Uridine diphosphate glucuronosyltransferase	UGT
Volume of distribution	V_d
Clearance	CL
Elimination half-life	$t_{1/2}$
Maximum tissue or plasma concentration	C_{max}
Time at which C_{max} is obtained	t_{max}
Inhibitory constant	Ki
Nicotinamide adenine dinucleotide phosphate	NADPH
Competitive inhibition constant	K_{ic}
Uncompetitive inhibition constant	K_{iu}

Chapter 1

1.0 Introduction

1.1 Polycyclic aromatic hydrocarbons; definition and sources

Polycyclic aromatic hydrocarbons (PAH) are common environmental contaminants. They are characterized by having two or more merged aromatic rings that are made up of carbon and hydrogen atoms. They are formed by incomplete combustion of organic material from natural or industrial sources. Because of their significant risk to human health, they are discussed extensively in specialized monographs by the International Agency for Research on Cancer, a division of the World Health Organization that is devoted to conducting research on causes, mechanisms and strategies of preventing and controlling human cancer.

PAH can be released into the environment via natural or anthropogenic sources (Baek *et al.*, 1991). Natural emissions can result from forest fires, volcanoes, and coal. From the latter source, however, PAH are considered to be tightly bound to the coal structure and cannot leach out spontaneously (Ipcs, 1998d). Anthropogenic release can happen either unintentionally during coal processing and petroleum production or intentionally through commercial production of compounds such as naphthalene and anthracene which act as moth repellents (Ipcs, 1998d).

Generally the background levels of these compounds are maintained within the range of 0.1-100 ng/m^3 (Simo *et al.*, 1991; Ipcs, 1998b). Human exposure to PAH, in a given population or amongst different populations, is variable (Yang *et al.*, 2003; Huang *et al.*, 2006). Possible reasons for this variability lie in differences in the degree of air pollution, tobacco consumption, ingestion of contaminated food and drinking water, use of PAH-containing products, and work environments especially those involving production of high levels of PAH (Ipcs, 1998a; Huang *et al.*, 2006).

Once in the body, PAH is quickly absorbed, with bioavailability of orally ingested PAH ranging from 20 to 50% (Directorate and Opinions, 2002). Inside the body, PAH distributes to various tissues, with notable accumulation in adipose tissues (Obana *et al.*, 1981; Directorate and Opinions, 2002). An alteration in physiological and biochemical function may ensue, which may be important clinically with possible modulation of the pharmacokinetics and pharmacodynamics of such drugs as theophylline, irinotecan and olanzapine (Horai *et al.*, 1983; Callaghan *et al.*, 1999; van der Bol *et al.*, 2007).

Perhaps the most significant source of PAH exposure is via inhalation of tobacco smoke (Yach, 2005). Tobacco smoke is composed of two phases. The volatile phase constitutes about 95% by weight of cigarette smoke and is made up of a mixture of gases such as carbon monoxide, carbon dioxide and volatile hydrocarbons such as benzene

(Darby *et al.*, 1984; Zevin and Benowitz, 1999). The remaining 5% is composed of particulate alkaloids (mainly nicotine), water and tar (Zevin and Benowitz, 1999). PAH and other carcinogenic aromatic amines are present in the tar component of cigarettes (Zevin and Benowitz, 1999). Additionally, it is known that cooking over charcoal can coat meat with a layer of PAH (Heller *et al.*, 1989). Therefore, the ingestion of smoked and barbecued meats represents a potential source of exposure to PAH (Heller *et al.*, 1989; Ipcs, 1998c). Effects of both of these sources on drug pharmacokinetics have been extensively studied and reviewed.

Most of PAH-related alterations in drug pharmacokinetics have been attributed to its enzyme-inducing properties. However recent developments have pointed out a wide range of PAH-induced effects that can potentially alter drug pharmacokinetics and pharmacodynamics. To our knowledge, there is no specific review of their wide range of effects and how they may lead to pharmacokinetic and pharmacodynamic consequences. In this introduction, mechanisms of PAH-induced alterations in drug pharmacokinetics will be discussed along with examples. Because the most studied source of exposure is through cigarette smoke, several examples will be discussed in relation to this source of exposure. However, whenever possible alterations in drug pharmacokinetics by other rare sources such as exposure to fumes resulting from coke ovens and ingestion of char-grilled meat are discussed.

1.2 PAH absorption and levels in human tissues

PAH compounds are always present in the form of complex mixtures including compounds such as phenanthrene, anthracene, chrysene and bezo[a]pyrene. Levels of PAH have been examined in cigarette smoke from several brands of cigarettes (Ding *et al.*, 2006). Total levels of PAH, represented by the sum of low and high molecular weight PAH, in mainstream smoke (smoke inhaled by smoker) range from 800-2673 ng/cigarette (Ding *et al.*, 2006). Average concentration of individual PAH components in mainstream smoke ranged from 0.08-74.8 ng/cigarette, with levels being 10 times higher in sidestream smoke (smoke released from the end of a lit cigarette) (Darby *et al.*, 1984; Grimmer *et al.*, 1987; Ipcs, 1998c). It is worth noting that levels of PAH in mainstream smoke increase with higher puff volumes and number of puffs taken by smokers, therefore affecting intake by individuals (Kozlowski and O'Connor, 2002). In one study, levels of twelve compounds were measured. Out of these, phenanthrene, fluoranthene and pyrene were the most abundant (Grimmer *et al.*, 1987; Ipcs, 1998c).

While PAH inhaled from mainstream smoke gets deposited in the lungs and upper respiratory tract, a fraction enters the body through the gastrointestinal tract when the inhaled PAH-bound particles are cleared back up the airways and subsequently swallowed (Chen *et al.*, 1989). Respiratory fractional deposition of mainstream smoke particles (defined as total amount deposited/total amount inhaled) can range from 22-75%

with average values of 47% (Hinds *et al.*, 1983; Chen *et al.*, 1989). In rats it was reported that about 16-31% of inhaled smoke entered the body via gastrointestinal absorption with about 20% being deposited in the upper respiratory tract (Chen *et al.*, 1989). Human respiratory intake was previously calculated using PAH concentrations in mainstream smoke and total fractional deposition. For example, in healthy male smokers the intake of bezo[a]pyrene from smoking 20 cigarettes per day was estimated to range from 150 to 750 ng/d assuming a deposition rate of 75% for particulate matter (Scherer *et al.*, 1990).

In non-smokers, diet constitutes the most significant source of PAH, with levels of benzo[a]pyrene intake ranging between 2 to 500 ng/d (Directorate and Opinions, 2002; Ramesh *et al.*, 2004). The levels vary markedly depending on the degree of contamination in water, soil and atmosphere and method employed in food processing (Ramesh *et al.*, 2004). The most abundant PAH in diet are pyrene and fluoranthene, accounting for about half of the total ingested PAH (Phillips, 1999; Ramesh *et al.*, 2004).

The work environment can potentially be the most significant source of PAH exposure. Miners and asphalt workers are exposed to PAH levels ranging from 24 to 737 ng/m^3 and 127 to 2973 ng/m^3, respectively (Seidel *et al.*, 2002; Campo *et al.*, 2006). The significance of these values is underlined when considering that on average, adult humans inhale 15 m^3 of air per day (Ipcs, 1998c).

Although these levels seem quite significant, when PAH were actually measured in human tissues the levels were generally low. The measured concentrations in normal subjects were in the range of fractions to a few nanograms per gram of wet or dry tissue weight for each individual PAH (Obana *et al.*, 1981; Goldman *et al.*, 2001; Directorate and Opinions, 2002; Neal *et al.*, 2008). For example, concentrations of nine PAH were measured in human liver and adipose tissues (Obana *et al.*, 1981). In both tissues pyrene was the most abundant with an average concentration of 0.38 and 1.1 ng/g in liver and fat, respectively (Obana *et al.*, 1981; Directorate and Opinions, 2002). Anthracene was the second most abundant PAH in these tissues at average levels of 0.17 and 0.26 ng/g in liver and fat, respectively (Obana *et al.*, 1981; Directorate and Opinions, 2002). Furthermore, levels of eleven PAH were also measured in human lung samples (Goldman *et al.*, 2001). Of these, fluoranthene was the most abundant with average concentration of 0.093 ng/g. Pyrene and benzo[a]pyrene were the second most abundant PAH components with average concentrations of 0.069 and 0.051 ng/g, respectively (Goldman *et al.*, 2001).

Despite these low levels, as mentioned above, several factors can affect PAH levels in the body. For example, cigarette smoking significantly increases PAH levels in human lung. Total PAH concentrations in human lung were found to be significantly higher (~2-fold) in smokers than non-smokers (Goldman *et al.*, 2001). Components increasing in response to

cigarette smoke were benzo(k)fluoranthene, indeno(1,2,3)pyrene, benzo(g,h,i)perylene and benzo(a)pyrene, with folds of increase ranging between 1.43 and 2.63 (Goldman *et al.*, 2001). Some reports have assessed exposure to PAH using established simple assays of common PAH hydroxyl metabolites such as 1-hydroxypyrene in urine. Using this assay, it was consistently shown that levels of 1-hydroxypyrene increase significantly (~ 2-fold) in urine samples from smokers compared to non smokers (Jongeneelen, 1997; Jacob *et al.*, 1999; Kawamoto *et al.*, 2007).

Generally, PAH exposure can be assessed using non-specific (for whole PAH group) or agent-specific biomarkers (Jongeneelen, 1997). Agents that are commonly monitored in the latter approach are pyrene, phenantherene and benzo[a]pyrene (Jongeneelen, 1997; Jacob *et al.*, 1999). The first approach monitors general biological changes that are known to be caused by almost all PAH (Jongeneelen, 1997). Examples of non-specific assays include measurement of aromatic DNA adducts, urinary thioethers, urinary mutagenicity and stepwise and/or simultaneous assays of different PAH metabolites in urine (Chung *et al.*; Jongeneelen, 1997). Most of these assays, however, have limited sensitivity and high variability (Jongeneelen, 1997; Feng *et al.*, 2006).

As mentioned previously, indicators of a single PAH ingredient can also be used to assess the overall exposure to PAH. For example, assays of agents such as benzo[a]pyrene, pyrene and phenanthrene and/or their respective metabolites in body fluid and/or tissue samples have been

used. Protein- or DNA-adducts to benzo[a]pyrene are also useful in that regard, particularly when cancer risk assessment is of concern (Jongeneelen, 1997; Goldman *et al.*, 2001). However, a clear correlation of PAH-exposure and benzo[a]pyrene adducts to hemoglobin, DNA or protein was not consistently observed (Jongeneelen, 1997; Scherer *et al.*, 2000). Monitoring levels of pyrene and phenanthrene, and/or their metabolites, was consistently shown to be useful. Unlike benzo[a]pyrene and its metabolite (3-hydroxy benzo[a]pyrene), pyrene and phenantherene are dominant components in PAH mixtures and usually present at higher levels (Jongeneelen, 1997).

As a marker, 1-hydroxypyrene is extensively used in the assessment of human exposure to PAH. Ease of detection using HPLC separation and fluorescence detection, sensitivity and low cost make this technique suitable for routine assessment of exposure (Jongeneelen, 1997). More importantly, its concentration in urine samples correlates with PAH intake. However the relationship is not linear. For example, an increase in PAH intake of 100- to 250-fold leads to a 4- to 12-fold increase in the urinary concentration of 1-hydroxypyrene (Jongeneelen, 1994). Other reports showed that the square root or natural logarithmic transformations of urine concentrations of 1-hydroxypyrene significantly correlate with pyrene and benzo[a]pyrene concentrations in air samples (r=0.978 and 0.959 respectively) (Zhao *et al.*, 1990; Ny *et al.*, 1993; Shepperd *et al.*, 2009). Furthermore, levels of pyrene in air samples

correlated with total PAH, using natural logarithmic transformed data, in a work environment, with correlation coefficients ranging from 0.8-0.9 in some reports (Ny *et al.*, 1993; Mannschreck *et al.*, 1996; Jacob *et al.*, 1999).

1.3 Alteration of factors dictating drug disposition

Although they are considered as potent inducers of the phase I metabolizing enzymes cytochrome P450 (CYP) 1A1, 1B1 and 1A2, studies have shown that PAH can result in a wide range of effects. Therefore, several mechanisms could contribute to PAH-mediated alteration of drug pharmacokinetics and pharmacodynamics. This can potentially involve enzyme induction, alterations of blood components, liver size, liver blood flow and modulation of uptake or efflux transporters. Changes produced by PAH in each of these processes will be discussed in the following section. In our discussion, attempts to compare changes in each of these processes in rat, as a commonly used animal model, and human will be made so that changes that are relevant to humans can be pointed out.

1.3.1 Transporter proteins

Several investigators have studied the effect of PAH on different efflux and influx transporters. However, the results of these reports vary greatly, especially between animal models and humans.

1.3.1.1 Rat

Earlier reports studied the effect of single intraperitoneal dose (10 µg) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on multidrug-resistance (MDR1), a family of genes that encodes for P-glycoprotein (P-gp) efflux transporters; gene expression was studied in livers collected (72 h after TCDD administration) from adult male Fischer rats (Burt and Thorgeirsson, 1988). In the same study the role of aryl hydrocarbon receptor (Ahr), a transcription factor involved in transcriptional regulation of CYP1A induction, was investigated using mice with (C57BL/6) or without (DBA/2) fully functioning Ahr receptors. While TCDD successfully induced MDR expression in rats and C57BL/6 mice, DBA/2 mice failed to show any induction; thus potential implication of Ahr in MDR induction was considered (Burt and Thorgeirsson, 1988). However, later reports showed that TCDD-related induction of MDR was only observed when large doses, 200-fold higher than the maximum dose required to cause maximum induction of CYP1A, were used and therefore involvement of Ahr in MDR induction was later ruled out (Gant *et al.*, 1991; Fardel *et al.*, 1996).

It was thought, however, that PAH-mediated MDR induction is mediated by a mechanism that has cross-substrate specificity with Ahr (Fardel *et al.*, 1996). Consistent with this hypothesis, other PAH were also capable of inducing the MDR gene. For example, exposure to 5-50 µM 3-methylcholantherene (3-MC) for 48 h caused dose-dependent induction of MDR1 gene expression, P-glycoprotein (P-gp) protein and activity in non-

parenchymal rat liver epithelial cell line (SDVI), with enhanced gene expression being also reported for 25 µM benzo(a)pyrene (Fardel *et al.*, 1996). However, MDR2 and MDR3 gene expression was not detectable in the SDVI cell line in the presence and absence of 3-MC (Fardel *et al.*, 1996). In rat primary hepatocytes, when 50 µM 3-MC was incubated for 36 or 72 h, no significant alteration in P-gp protein expression and activity was observed (Chieli *et al.*, 1994). However, when 3-MC was coincubated with rhodamine-123, a specific P-gp substrate, evidence for competitive inhibition of P-gp was apparent (Chieli *et al.*, 1994). It was also found that untreated primary hepatocytes spontaneously over-express functional P-gp with time in culture (Chieli *et al.*, 1994).

Evidence also exists showing that rat organic anion transporting polypeptide 2 (Oatp2), the basolateral uptake transporter involved in uptake of cardiac glycosides into liver, was inhibited (20%) by 3-MC (Rausch-Derra *et al.*, 2001). Oatp1 protein expression, however, was unaffected in 3-MC (20 mg/kg/day for 4 days) treated rats (Rausch-Derra *et al.*, 2001). In terms of their effect on Oatp2 activity, ouabain uptake in hepatocytes from 3-MC treated rats was not changed, indicating that any inhibitory effect of 3-MC on Oatp2 protein levels is likely of minor or no relevance (Eaton and Klaassen, 1979) .

Multidrug resistance associated proteins (MRP) are canalicular or basolateral efflux transporters. Gene expression of several of these isoforms was induced in mice liver [MRP2 (~126%), MRP3 (~267%),

MRP5 (~589%), MRP6 (~255%) and MRP7 (~185%)] in response to treatment with different PAH such as TCDD (40 µg/kg) and β-naphthoflavone (BNF) (200 mg/kg) (Maher *et al.*, 2005). MRP1 gene expression, however, was not affected by any of these treatments (Maher *et al.*, 2005).

1.3.1.2 Human

In humans there are two MDR isoforms, MDR1 and MDR2. MDR1 is highly expressed in liver and is responsible for transport of xenobiotics (Schuetz *et al.*, 1995). Benzo[a]pyrene and chrysene increased MDR1 gene expression in human epithelial colorectal adenocarcinoma (Caco-2) cells in a dose-dependent (1-10 µM) manner (Lampen *et al.*, 2004). When 3-MC (1 or 2 µg/ml) and TCDD (1 nM) were incubated with human hepatocytes, however, MDR was induced only in 62% and 55% of the samples treated with 3-MC and TCDD, respectively, with CYP1A1 and CYP1A2 gene expression being induced in all samples (Schuetz *et al.*, 1995). The rest of the samples showed either no change or decreased expression in response to treatments (Schuetz *et al.*, 1995; Runge *et al.*, 2000).

Involvement of Ahr in regulation of the MDR gene was excluded due to lack of induction of MDR in a large proportion of the samples and the observation of almost equivalent potencies in MDR-induction mediated by 3-MC and TCDD although the second had 30,000 times greater affinity

for Ahr (Schuetz *et al.*, 1995). Possible association of low basal and inducible CYP1A1 mRNA levels with high basal and inducible MDR levels, respectively in response to PAH was observed (Schuetz *et al.*, 1995).

A recent study showed that BNF (10 µM) potently inhibited MDR1 gene expression (52%) (Nwankwo, 2007). An inhibitory effect of BNF on P-gp activity was assumed because BNF treatment enhanced the cytotoxic effect of adriamycin in an adriamycin resistant mammary adenocarcinoma cell line although drug cellular uptake was not measured (Nwankwo, 2007). Variable PAH-mediated MDR-regulation was explained based on linked regulation of MDR1 and CYP1A. For example, in the presence of weak inducers, it is likely that there would be induction of MDR genes, leading to a cooperative elimination of the xenobiotic (Nwankwo, 2007). In the case of potent inducers, down regulation of MDR would be expected (Nwankwo, 2007).

P-gp gene expression and functional activity, indicated by cellular accumulation of daunorubicin, in mononuclear cells and granulocytes in peripheral blood was significantly induced by inhalation of industrial solvents (Hegewisch-Becker *et al.*, 1993). Although these solvents contain a large amount of aliphatic and chlorinated hydrocarbons, their complex composition and presence of other materials made it difficult to relate the increase in P-gp expression to a certain group of compounds, considering that in the same study, cigarette smoke had no effect on P-gp expression (Hegewisch-Becker *et al.*, 1993).

Furthermore, PAH-enriched diet had no effect on P-gp gene and protein expression in mucosal biopsy samples (Fontana *et al.*, 1999). However, higher mean values of MDR1 mRNA were observed in non-neoplastic bronchial specimens in smokers compared to non-smokers, with differences not being statistically significant (Lechapt-Zalcman *et al.*, 1997). Furthermore, some reports are available showing that cigarette smoke induces P-gp expression in lung cancer patients (Volm *et al.*, 1991).

The effect of PAH on the expression of another efflux transporter, MRP, was also investigated in humans or human-derived cell lines. There are nine MRP isoforms identified in humans, with MRP2 being mainly localized to the canalicular membrane and involved in excretion of xenobiotics into bile (Maher *et al.*, 2005). In a human liver cell line (HEPG2), treatment with 5 and 10 µM BNF significantly increased (2-fold) levels of MRP3 mRNA, a basolateral export pump (Hitzl *et al.*, 2003). In contrast, liver samples derived from smokers showed no change in MRP3 gene expression (Hitzl *et al.*, 2003). MRP3 protein levels, however, were numerically higher (43%) in smokers than non-smokers, with the difference being nonsignificant (Hitzl *et al.*, 2003).

Overall it seems that effects of PAH on transporter proteins are highly variable and unclear. Where some reports have shown significant induction of MDR and MRP expression, inhibitory effects and lack of change were also observed. To date there is no clear explanation for

these variable effects. However PAH-related effects on transporter proteins seem to happen at relatively high concentrations. Whether these changes are relevant to humans exposed to common PAH sources such as diet and cigarette smoke seem to be unlikely.

1.3.2 Blood components

Some alterations in serum proteins in response to cigarette smoking were previously reported. Perhaps one of the most important effects on plasma proteins is the change in α_1-acid glycoprotein levels (an acute phase reaction protein that binds principally to basic drugs). It was shown earlier that in healthy human smokers, plasma concentrations of α_1-acid glycoprotein were 34% higher than in non-smokers (Benedek *et al.*, 1984). The same study showed a non-significant trend of lower albumin levels in smokers (decrease of 6%) (Benedek *et al.*, 1984).

Although levels of α_1-acid glycoprotein increased in smokers, the unbound fraction of propranolol, which binds mainly to lipoproteins and α_1-acid glycoprotein, showed a non-significant trend of reduction (20%) in smokers (Benedek *et al.*, 1984). Unbound fractions of albumin bound drugs such as phenytoin, sulfisoxazole and diazepam also were not affected by smoking (Benedek *et al.*, 1984; Ruiz-Cabello and Erill, 1986).

The mechanism of increase in α_1-acid glycoprotein in smokers is not known; however studies in animal models have shown that PAH could affect its level. For example, intraperitoneal injection of 100 µmol/kg 3-MC

to male Sprague-Dawley rats led to increased (~5-fold) liver gene expression of acute phase genes such as α_1-acid glycoprotein (Kondraganti *et al.*, 2005). This indicates that elevation of α_1-acid glycoprotein in plasma of smokers is likely caused by the PAH component of cigarette smoke.

Studies have also shown that cigarette smoking is associated with disturbances in lipoprotein levels and composition. For example, high density lipoprotein cholesterol, cholesterol ester and plasma lipoprotein A-1 were lower in smokers than non-smokers, with changes being reversible after smoking cessation (Dullaart *et al.*, 1994; Richard *et al.*, 1997). These changes were likely imparted by elevation of cholesteryl ester transfer protein and phospholipid transfer protein activities in smokers (Dullaart *et al.*, 1994).

PAH also caused more drastic changes in lipoprotein levels in animal models such as White Carneau Pigeons. Plasma cholesterol and low density lipoprotein levels were increased after 3 to 6 month of weekly intrapectoral injections of different PAH such as benzo[a]pyrene, 3-MC and 7,12-dimethylbenz[a,h]anthracene (Revis *et al.*, 1984). In contrast to what was observed in smokers, levels of high density lipoprotein cholesterol were not significantly altered (Revis *et al.*, 1984). Consequently the potential involvement of PAH in cigarette-smoke related disturbances in lipoprotein levels is not likely. The authors observed that

the tested PAH, however, tended to associate with lipoproteins (Revis *et al.*, 1984).

It was shown earlier that cigarette smoke increases hematocrit, blood and plasma viscosity and total plasma protein concentrations in otherwise healthy smokers (Levenson *et al.*, 1987). Reasons for these changes and whether they are caused by the PAH component of cigarette smoke are poorly defined. However, drug distribution within components of blood can be altered in smokers. For example, changes in the erythrocyte-plasma partition ratio of the anticancer drug, irinotecan, were observed in smokers (Dumez *et al.*, 2005). Lower partition ratios were observed in smokers, with more drug being detected in erythrocytes of nonsmokers compared with smokers (Dumez *et al.*, 2005). Cigarette smoke was thought to decrease irinotecan affinity to erythrocytes (Dumez *et al.*, 2005). The lower amount of drug incorporated in erythrocytes of smokers was thought to be responsible for poorer response seen in smokers because erythrocytes were thought to be a key factor in drug transport to tissues, importantly to the site of action. The importance of erythrocytes in the drug effect was attributed to their occupation of a relatively large volume of blood, their large numbers and their relatively long life span (Dumez *et al.*, 2005).

1.3.3 Metabolizing enzymes

Although PAH are generally considered as potent inducers of CYP1A isoenzymes, they also induce other drug metabolizing enzymes.Their effects on different phase Ⅰ and phase Ⅱ metabolizing enzymes in human and rat will be discussed in the following section.

1.3.3.1 Human

1.3.3.1.1 Phase Ⅰ metabolizing enzymes

Duodenal biopsies from healthy humans fed char-grilled meat for 7 days or from cigarette smokers showed marked induction in CYP1A1 mRNA, protein and activity (Buchthal *et al.*, 1995; Fontana *et al.*, 1999). In colon, however, protein and CYP1A1-mediated 7-ethoxyresorufin O-deethylase (EROD) activity remained below the lowest limit of quantitation after char-grilled diet (Fontana *et al.*, 1999). Char-grilled meat diet also induced hepatic CYP1A2 activity (89%), as indicated by the [^{13}C-3-methyl] caffeine breath test (Fontana *et al.*, 1999).

In smokers, liver biopsy samples showed a 3.5-fold induction of CYP1A2 protein, 4.3-fold increase in phenacetin O-deethylation and 3.2-fold increase in EROD compared to non-smokers with a strong correlation being observed between the amount of CYP1A2 protein and activity (Pelkonen *et al.*, 1986; Sesardic *et al.*, 1988). There was no correlation however between plasma levels of cotinine (a major metabolite of nicotine) and EROD activities in human liver biopsy samples (Pelkonen *et al.*,

1986). Studies on primary human hepatocytes also displayed a significant degree of induction (5.7-fold) upon exposure to benzo[a]pyrene (Vakharia *et al.*, 2001). Additionally, although CYP1B1 gene expression was higher in liver samples from smokers, CYP1B1 protein was undetectable in smokers and non-smokers (Chang *et al.*, 2003).

In line with these findings, earlier studies showed that ingestion of charcoal grilled steak enhances antipyrine clearance and phenacetin oral clearances by 20 and 500%, respectively (Heller *et al.*, 1989). This effect was mainly attributed to induction of CYP1A2 which contributes partially or extensively to the metabolism of antipyrine and phenacetin, respectively (Slusher *et al.*, 1987; Xiaodong *et al.*, 1994).

Consistent with findings obtained from liver and duodenal samples of smokers, CYP1A1 activity was significantly induced (3-fold) in cells collected from broncho-alveolar lavage (BAL) of lungs of smokers compared to non-smokers, with increased CYP1A1 and CYP1B1 gene expression being observed (Thum *et al.*, 2006). Increased CYP1A1-related activity, aryl hydrocarbon hydroxylase (14-fold), was also shown in microsomes from lungs of smokers compared to non-smokers (Nakajima *et al.*, 1995).

Other phase I metabolizing enzymes such as CYP3A4/5 (protein or activity) in intestine and liver were not affected by ingestion of a char-grilled diet (Fontana *et al.*, 1999). Furthermore, microsomal epoxide

hydrolase activity in lung was not affected by cigarette smoking (Nakajima *et al.*, 1995).

1.3.3.1.2 Phase ll metabolizing enzymes

Duodenal uridine diphosphate glucuronosyltransferase (UGT) activity was not altered in smokers compared to non-smokers (Buchthal *et al.*, 1995). Although not changed in duodenal samples, the activity of hepatic bilirubin UGT in smokers was thought to increase because cigarette-smoking men showed significantly lower (15.4%) serum bilirubin concentrations than men who never smoked (Van Hoydonck *et al.*, 2001). However, bilirubin as an antioxidant, might have been consumed in scavenging free radicals produced by cigarette smoke, therefore contributing to lower levels of bilirubin in smokers (Van Hoydonck *et al.*, 2001). Nevertheless, the induction of UGT in response to exposure to cigarette smoke in mice and induction of 3,6-dihydroxychrysene diglucuronide formation in duodenal biopsies from smokers compared to non-smokers support the presence of some inducing effects of smoke on UGT activity (Buchthal *et al.*, 1995; Van Hoydonck *et al.*, 2001).

Additionally, treatment of human hepatocytes with TCDD caused induction of glutathione S-transferase activity in some hepatocyte samples (2 to 3 out of 6 samples) (Schrenk *et al.*, 1995). Absence of induction in the rest of the samples suggested the presence of polymorphism in the inducibility of glutathione S-transferase. In contrast, in lung cytosol

fractions obtained from smokers glutathione-S-transferase protein and activity (indicated by 1-chloro-2,4-dinitrobenzene) were not altered (Nakajima *et al.*, 1995).

1.3.3.2 Rat

1.3.3.2.1 Phase I metabolizing enzymes

Treatment with BNF or Aroclor 1254 (a mixture of polychlorinated biphenyl compounds) caused a dramatic ~73-78% reduction in liver content of CYP2C11, an isoenzyme which constitutes >50% of the total CYP content in male rats. At the same time, CYP1A1 and CYP1A2 content were strongly increased by both compounds (Guengerich *et al.*, 1982; Sinal *et al.*, 1999). The down regulation of CYP2C11 in response to PAH was later confirmed and explained based on the contribution of the Ahr in the down-regulation of the isoenzyme (Safa *et al.*, 1997). In rat lung, CYP1A1 protein expression was constitutively minimal whereas the level of CYP1A2 was barely detectable; 3-MC treatment preferentially induced CYP1A1 with no increase in CYP1A2 levels being observed (Keith *et al.*, 1987). Similarly, CYP1A1 was induced in the endothelium of arteries, arterioles, veins and venules in response to 3-MC (Keith *et al.*, 1987).

In rats exposed to cigarette smoke (1 or 6 h per day for 2 or 8 weeks), although liver CYP1A1, 1A2 and CYP2B1/2 related enzymatic activities were increased (Kushinsky and Louis, 1976; Wardlaw *et al.*, 1998), the response of other tissues was different. For example, in lung,

CYP1A1 and CYP2B1/2 enzymatic activities were significantly induced and reduced, respectively (Wardlaw *et al.*, 1998). Consistently, CYP2B1-mediated activity was increased and decreased in rat liver and lung, respectively after BNF treatment (Sinal *et al.*, 1999). PAH-mediated effects on CYP2B1 were attributed to a non-Ahr dependent pathway since a previous study showed that CYP2B1 was down-regulated in both Ahr-responsive and non-responsive mouse strains (Sinal *et al.*, 1999).

In nasal and olfactory microsomes discrepencies between protein expression and enzymatic activities of CYP1A1, 1A2 and 2B1/2 were observed. For example, protein expression of CYP1A1 was induced in olfactory and nasal respiratory microsomes while CYP1A2 and CYP2B1/2 were reported to be either not significantly altered or showing a trend of decline in both tissues, respectively (Wardlaw *et al.*, 1998). Enzymatic activities of CYP1A1, 1A2 and CYP2B1/2, however, were each inhibited in olfactory microsomes, with similar inhibitory effect being observed on CYP2B1/2 in nasal respiratory microsomes (Wardlaw *et al.*, 1998). Consistently, intraperitoneal injections of benzo[a]pyrene, arochlor 1254 and 3-MC did not increase the aryl hydrocarbon hydroxylase activity in nasal tissue whereas TCDD resulted in increased activity of the same reaction (Bond, 1983).

Gene expression of CYP2E1, which is regulated by fasting or starvation, was increased in liver and extra-hepatic tissues after intraperitoneal injection of BNF (80 mg/kg/day for 4 days). Its activity,

however, was only increased in kidney microsomes (Sinal *et al.*, 1999). Changes in CYP2E1 expression and activity were believed to have occurred through BNF-induced pathobiological changes rather than Ahr agonism since BNF-treated rats showed marked weight loss. Other phase Ⅰ metabolising enzymes were also induced by PAH. For example, 3-MC induced microsomal epoxide hydrolase (2.6-fold) activities in rat liver (Kondraganti *et al.*, 2008).

1.3.3.2.2 Phase Ⅱ metabolizing enzymes

Administration of benzo[a]pyrene induced some conjugative pathways such as UGT activity (2.1-6.4-fold) and glutathione S-transferase (1.6-fold) in female Wistar rats (Elovaara *et al.*, 2007). In the same study the effect of molecular size on inducibility of phase Ⅰ and phase Ⅱ enzymes was tested. Similar to benzo[a]pyrene, 3-MC induced UGT activity (4.4-fold) and glutathione S-transferase (1.8-fold) in Sprague–Dawley rats (Kondraganti *et al.*, 2008).

1.3.3.3 Comparison between human and rat

While in rats PAH treatment can induce a wide range of phase Ⅰ and phase Ⅱ enzymes, only induction of CYP1A1 and 1A2 was consistently observed in humans exposed to common PAH sources, with potential evidence of induction in UGT activity. Although humans and rats consistently showed induction of CYP1A1 and CYP1A2 in hepatic and most extra-hepatic organs, some qualitative and quantitative differences in

induction were suggested. For example, upon incubation of 10^{-12} M TCDD with human hepatocytes, induction of human CYP1A2 was more predominant than CYP1A1 whereas in rat at 10^{-10} M TCDD induction of CYP1A1 was more predominant than CYP1A2 at the levels of gene expression and protein (Xu *et al.*, 2000). The article suggested that PAH preferentially induce CYP1A2 in humans while preferential induction of CYP1A1 occurs in rats. In contrast, other studies showed that induction of both isoforms in human hepatocytes is also possible (Meunier *et al.*, 2000; Xu *et al.*, 2000).

Generally rat hepatocytes showed more potent increases in EROD activity in response to TCDD than did human hepatocytes (Schrenk *et al.*, 1995; Xu *et al.*, 2000; Elovaara *et al.*, 2007). Differences in extent of induction could be explained by the fact that TCDD-treated rat hepatocytes contained about 4-5 times more Ahr than human hepatocytes (Xu *et al.*, 2000). However, it was thought that it does not truly explain the lower inducibility in humans since it was shown previously that there was no correlation between intrinsic Ahr levels and enzyme inducibility (Xu *et al.*, 2000).

Indeed, there were large inter-individual differences in the magnitude of induction of CYP1A activity upon exposure of human hepatocytes to PAH or in liver samples of smokers (Sesardic *et al.*, 1988; Schrenk *et al.*, 1995; Meunier *et al.*, 2000; Vakharia *et al.*, 2001). For example, in a relatively large sample population (n=74) the degree of

BNF-mediated induction of EROD ranged from 2 to 37.3-fold (Meunier *et al.*, 2000). Increases of CYP1A1 activity of up to 100-fold in lungs of smokers were also reported, with up to 200-fold variability among smokers. These observations indicate that unlike rats, humans differ in their susceptibility to CYP1A induction. Indeed, a recent study reported that variability associated with basal or induced enzyme activity measured in humans exceeded that usually observed constitutively or inducibly in rat (Elovaara *et al.*, 2007).

In an effort to identify factors that could lead to this variability in CYP1A induction among humans, the influence of number of cigarette consumed per day was investigated. Only 20% of inter-individual differences in EROD was explainable by number of cigarettes smoked/day (Anttila *et al.*, 2001). The presence of polymorphism in the inducibility of CYP1A was also proposed (Schrenk *et al.*, 1995). One of the early recognised polymorphisms in CYP1A1 is the substitution of valine for isoleucine at the catalytic site of the enzyme. It was shown that such substitution resulted in enhancing CYP1A1 inducibility in smokers (Crofts *et al.*, 1994; Zhang *et al.*, 1996). Similarly polymorphic inducibility of CYP1B1 was proposed (Toide *et al.*, 2003). The incidence of subjects with low, intermediate, and high inducibility were 54.2%, 34.7%, and 11.1%, respectively, with a strong significant correlation being seen between CYP1B1 mRNA and concentration of dioxin in the strong inducibility groups (Toide *et al.*, 2003).

1.3.4 Liver mass and blood flow

Liver size or liver volume is an important determinant of the capacity of individuals to clear drugs, especially those which are exclusively eliminated by metabolism (Spoelstra *et al.*, 1986; Johnson *et al.*, 2005; Wynne, 2005; Fanta *et al.*, 2007). It is well known that direct exposure to different PAH can increase the ratio of liver to total body mass in rats (Kushinsky and Louis, 1976). In animal models benzo[a]pyrene and benz[a]anthracene increased the liver mass by 27 and 19%, respectively (Torronen *et al.*, 1981). In a recent study it was shown that PAH increased total cell number (by ~100%), the number of cells undergoing S-phase and cyclin A protein when incubated with rat hepatic oval 441WB-F344 cell lines (Zatloukalova *et al.*, 2007).

Cigarette smoke can also cause significant increases (12%) in rat liver mass, with the percentage of increase being equivalent to that produced by administration of single dose of 30 mg/kg benzo[a]pyrene (Kushinsky and Louis, 1976). In a human-derived cell lines, hepatocellular carcinoma (HepG2), PAH were found to upregulate genes such as ferritin heavy polypeptide 1 (FTH1) and peroxiredoxin 1 (PRDX1) (Castorena-Torres *et al.*, 2008). These genes were thought to affect cell proliferation (Castorena-Torres *et al.*, 2008).

Indeed, some clinical studies in humans have demonstrated that cigarette smoking increases (by 3- to 8-fold) the risk of developing liver

hyperplasia (increased cell proliferation), with the risk increasing with the number of cigarettes consumed (Karhunen and Penttila, 1987; Scalori *et al.*, 2002). In these studies, liver hyperplasia was diagnosed by either direct examination of autopsy samples or by employing ultrasound, biphasic computed tomography or magnetic resonance (Karhunen and Penttila, 1987; Scalori *et al.*, 2002). In the study conducted on autopsy samples, liver mass was significantly larger in males with hyperplastic nodules (31 g/kg) than in males without liver lesions (26 g/kg) (Karhunen and Penttila, 1987).

Although hyperplasia has been reported, an increase in liver volume as a consequence of cigarette smoking has not been demonstrated. There was no significant alteration in liver volume in smokers (1427±299 ml) vs. non-smokers (1293±144 ml) (Spoelstra *et al.*, 1986; Andersen *et al.*, 2000). Liver volume was measured by computed tomography or ultrasonic scanning techniques in both studies. In one study, although smokers showed non-significantly higher liver volume ($p=0.19$ for body weight-corrected liver volume), the small number of subjects studied (17 in total, 8 smokers and 9 non-smokers) might have obscured any effect of smoking (Spoelstra *et al.*, 1986). In the second study, larger samples of smokers (n=24) and non-smokers (n=32) were recruited yet no alteration in liver volume was reported (Andersen *et al.*, 2000).

Although an increase in liver mass in response to cigarette smoke and PAH treatment has been shown in animal models such as rat, extrapolation of this finding to humans should be made cautiously because the available data are in conflict. Nevertheless, laboratory animals tend to be more homogenous in terms of genetics and environmental factors compared to humans. Whether smoking, for example, leads to significant changes in liver mass might require a study involving hundreds rather than tens of subjects.

Hepatic blood flow has also been compared in smokers vs. non-smokers. Most of these studies used indocyanine green, a diagnostic dye, that is highly extracted by liver and therefore its clearance is used as an indicator of hepatic blood flow (Wood *et al.*, 1979). It was found that neither cigarette smoke or PAH altered liver blood flow in human and animal models (Nies *et al.*, 1976; Wood *et al.*, 1979; Huet and Lelorier, 1980; Eldon *et al.*, 1987). A non-significant tendency towards lower hepatic blood flow values in smokers compared to non-smokers was also previously observed (Huet and Lelorier, 1980).

1.4 Drug pharmacokinetics and pharmacodynamics

Alteration in drug pharmacokinetics and pharmacodynamics resulting from exposure to sources of these compounds will be discussed in the following section with examples. Changes in pharmacokinetic parameters of each of these drugs as a result of cigarette smoking or ingestion of char-grilled diet are outlined in table 1.

1.4.1 Antipyrine

Antipyrine, an analgesic and antipyretic therapeutic agent, does not bind to plasma proteins and is metabolized slowly but almost entirely by liver, conferring it a low hepatic extraction ratio (Branch *et al.*, 1974). As a result of enzyme induction, its clearance has been reported to increase in smokers compared to non-smokers after oral and intravenous administration (Scavone *et al.*, 1997). Mean cumulative urinary excretions of antipyrine metabolite, 3-hydroxymethylantipyrine, were higher in cigarette smokers (Eichelbaum *et al.*, 1982). Because these metabolites are formed mainly by CYP1A2, cigarette smoke mediated induction of this isoform is the likely causative mechanism behind the increased clearance (Scavone *et al.*, 1997). Furthermore, antipyrine oral clearance and half-life were increased and decreased, respectively by ingestion of charcoal-grilled meat for a total of eight days (Pantuck *et al.*, 1976; Heller *et al.*, 1989).

Consistent with increased clearance in humans, administration of antipyrine to 3-MC-pretreated rats increased its clearance (138%) and decreased its half-life (53%) (Danhof *et al.*, 1979). A nonsignificant trend of increased intrinsic clearance (Cl_{int}) (54%) to 4-hydroxy-antipyrine was observed when antipyrine metabolism was studied in microsomes prepared from 3-MC treated rats compared to controls (Kahn *et al.*, 1982).

1.4.2 Phenacetin

The pharmacokinetics of phenacetin, once a popular analgesic and antipyretic therapeutic agent with moderate to high extraction ratio in liver (Inaba *et al.*, 1979), were altered by ingestion of charcoal-grilled meat for eight days. Oral clearance of phenacetin, a CYP1A2 substrate (Donato *et al.*), was increased five-fold (Heller *et al.*, 1989). Similarly, when phenacetin was administered orally to current smokers, its oral clearance was increased 2.5-fold with half-life not being altered (Dong *et al.*, 1998).

In PAH-treated animal models, phenacetin metabolism was also increased. For example, phenacetin O-deethylation increased markedly when phenacetin was incubated with microsomes from 3-MC-treated rats (van Bree *et al.*, 1986). Cl_{int} was increased 2.5-fold (van Bree *et al.*, 1986). Clearance of phenacetin also increased (5-fold) after intravenous administration to 3-MC-pretreated rats (Klippert *et al.*, 1983).

A phenacetin metabolite, N-acetyl-p-aminophenol (acetaminophen), which is perhaps the most widely used analgesic in its own right, is cleared mainly through conjugative pathways with glucuronic acid and sulfate moieties. Its clearance was not altered by ingestion of charcoal-grilled beef for five days (Anderson *et al.*, 1983).

1.4.3 Methylxanthines

1.4.3.1 Theophylline

The pharmacokinetics of theophylline, an anti-asthma methylxanthine with low hepatic extraction ratio, were altered by smoking and diet inclusive of charcoal-grilled foods (Kappas *et al.*, 1978; Otero *et al.*, 1996). Its clearance and elimination half-life increased and decreased respectively in smokers compared to non-smokers (Hunt *et al.*, 1976; Horai *et al.*, 1982; Horai *et al.*, 1983). Cigarette smoking patients would likely need larger daily doses, as much as 2000 mg/day, of theophylline to control asthma whereas non-smokers required doses that ranged between 600 to 900 mg/day (Horai *et al.*, 1982). PAH-inducible CYP1A2 was found to be involved in theophylline metabolism (Zhang and Kaminsky, 1995). Similarly, clearance of theophylline was increased in 3-MC pretreated rats (Groen *et al.*, 1992).

Although theophylline clearance is clearly enhanced by exposure to cigarette smoke, exposure to coke oven emissions did not alter theophylline clearance and was assumed to be due to the exposure to other components that have inhibitory effects on drug metabolising enzymes such as heavy metals and carbon monoxide (Haughey and Jusko, 1988).

1.4.3.2 Caffeine

Caffeine is a commonly used social drug present in many beverages (coffee, tea and sodas) and also used clinically in the treatment of neonatal apnea. A cup (150 mL) of coffee or tea is equivalent to a caffeine dose of approximately 70 and 25 mg respectively (Seng *et al.*, 2009). It enhances mental activity by stimulating neurons involved in arousal and by inhibiting neurons involved in sleep (Seng *et al.*, 2009). In smokers, its clearance and half-life were increased and decreased, respectively after administration of 3 or 5 mg/kg oral doses (Seng *et al.*, 2009). To reach plasma levels within the reported beneficial range for alertness, 4.5-9 µg/ml, higher doses of caffeine were required for smokers (476 mg repeated every 6 h) compared with non-smokers (340 mg repeated every 8 h).

Caffeine metabolism, in terms of theobromine, theophylline, paraxanthine and 1,3,7 trimethyluric acid formation, was also induced (4-11-fold over controls) when it was incubated with microsomes from 3-MC pretreated rats (Bonati *et al.*, 1980).

1.4.4 Clomipramine

Plasma trough levels of clomipramine, a tricyclic antidepressant with low to medium hepatic extraction ratio (Lainesse *et al.*, 2006), were significantly reduced (52%) in smokers upon long term treatment (John *et al.*, 1980). Levels of its metabolite, desmethylclomipramine, however, were

not significantly altered (John *et al.*, 1980). Lack of increase in desmethylclomipramine was attributed to possible induction of formation of other ring hydroxylated metabolites or enhanced induction of desmethylclomipramine clearance (John *et al.*, 1980). Tolerability was generally poorer in non-smokers compared to smokers, indicating that higher drug levels in non-smokers might have been associated with increased risks of side effects (John *et al.*, 1980).

Attempts to increase exposure to clomipramine by increasing its dose in heavy smokers fails to achieve higher concentrations and appropriate drug effectiveness. For example, after administring relatively high doses (150-225 mg/day for three weeks) of clomiparmine to a heavy smoker, clomipramine was shown to be ineffective in managing depression due to unusually low drug plasma levels (Conus *et al.*, 1996). Co-treatment with fluvoxamine (100 mg/day), a potent CYP1A2 inhibitor, enhanced response to clomipramine treatment within 4 days. This was accompanied by 4-fold increase in clomipramine plasma levels, thus indicating that decreased drug exposure was the cause of poor response (Conus *et al.*, 1996).

1.4.5 Benzodiazepine derivatives

Studies have shown that benzodiazepine-related side effects such as drowsiness were less frequent in smokers than non-smokers (Norman *et al.*, 1981). For some drugs evidence exists for a pharmacokinetic-based

interaction and resultant decrease in drug exposure caused by enzyme induction. There are other examples, however, where drug disposition was not altered by cigarette smoke. For these drugs, presence of pharmacodynamic-based interactions with the cigarette smoke ingredient nicotine was proposed.

For example, the pharmacokinetics of diazepam, midazolam and lorazepam were compared in smokers and non-smokers (Ochs *et al.*, 1985). Cigarette smoke did not alter the pharmacokinetics of any of these drugs after intravenous administration to healthy volunteers (Ochs *et al.*, 1985). Presence of an interaction at a pharmacokinetic level between cigarette smoking and any of these drugs was therefore excluded as the reason for the notably lower incidence of side effects related to these drugs.

Upon oral administration of clorazepate, it is subjected to rapid complete decarboxylation in the acidic environment in the stomach to form the active anxiolytic metabolite desmethyldiazepam which is also an active metabolite of diazepam and known to be slowly eliminated by liver with an apparently low extraction ratio (Ochs *et al.*, 1986). No clorazepate is detected in plasma (Norman *et al.*, 1981).

Upon oral administration of clorazepate (20 mg) to healthy volunteers, plasma half-life and oral clearance of desmethyldiazepam were decreased (46%) and increased (175%), respectively in smokers

compared to non-smokers (Norman *et al.*, 1981). When the same dose was given intravenously, however, no significant changes in clearance, volume of distribution, serum unbound fraction or elimination half-life were apparent (Donato *et al.*; Ochs *et al.*, 1986). Absolute oral bioavailability of desmethyldiazepam after an oral dose (20 mg) of clorazepate was 1.3 and was significantly different from 1 (Ochs et al., 1982). After oral administration, clorazepate is quickly converted to desmethyldiazepam in the stomach. This process, however, is expected to be slower upon intravenous administration of clorazepate since there is no contact with the acidic medium in the stomach (Ochs et al., 1982). Differences in level of desmethyldiazepam after both routes might have contributed to differences in the findings of both studies.

Olanzepine, a benzodiazepine derivative with antipsychotic properties, possesses a moderately low plasma clearance of 26 L/h (Callaghan *et al.*, 1999). Its plasma levels, oral volume of distribution and oral clearance decreased (34%), increased (234%) and increased (40-206%) in smokers with effects being more potent as consumption of cigarettes increased to ≥5 cigarette/day (Callaghan *et al.*, 1999; Wu *et al.*, 2008; Citrome *et al.*, 2009). Monitoring plasma levels of olanzepine in smokers was thought to be essential because a concentration of 9.3 ng/ml was required for appropriate control of schizophrenia while plasma concentrations of 20.6 ng/ml were associated with increased risk of weight gain (Wu *et al.*, 2008). Non-smoking psychiatric patients receiving

olanzapine showed better control of their symptoms, although smokers received higher dose (33%) than non-smokers (Carrillo *et al.*, 2003). Furthermore, olanzapine-related side effects were more common among non-smokers as a result of higher drug levels than smokers.

CYP1A2 enzyme activity and serum steady state olanzapine concentrations were increased (6-fold) and decreased (5-fold), respectively in smokers compared to non-smokers (Carrillo *et al.*, 2003). Cases of olanzapine related toxicity were reported upon decreasing cigarette consumption, especially for those smoking at least one pack/day. Lowering (30 to 40%) drug dose upon cigarette smoke cessation/decrease was successful in reversing toxicity and bringing levels back to pre-cessation state (Lowe and Ackman, 2010). Since the drug is metabolized by CYP1A2, co-administration with CYP1A2 inhibitors such as fluvoxamine increased its plasma levels (119%) and decreased its clearance by approximately 50%, with plasma levels of the metabolite formed by CYP1A2 pathway, N-desmethyl olanzapine being decreased by 77%, (Callaghan *et al.*, 1999).

1.4.6 Thiothixene

Similar to benzodiazepines, in anti-schizophrenic therapy, smokers generally require significantly higher doses (45-77%) of neuroleptic agents than non-smokers (Ereshefsky *et al.*, 1991; Salokangas *et al.*, 1997). In some cases smoking did not affect plasma levels of these drugs and

pharmacological or pharmacodynamic basis for the interaction was proposed and confirmed. This interaction was based on the fact that nicotine was shown to have a stimulatory effect on dopamine, stimulating its release and inhibiting its degradation, and therefore increasing the dose requirements of antidopaminergic agents (Sagud *et al.*, 2009).

An example where drug pharmacokinetics was altered in smokers is thiothixene. Its oral clearance was increased in smokers compared to non-smokers (Ereshefsky *et al.*, 1991). Monitoring of drug levels in smokers was thought to be important since it was shown that adequate clinical response was associated with plasma levels between 2-15 ng/ml (Ereshefsky *et al.*, 1991).

1.4.7 Hydrocodone

Upon long term treatment with hydrocodone, a narcotic analgesic therapeutic agent, smokers' serum levels were 78% less than those of non-smokers (Ackerman and Ahmad, 2007). They also required significantly larger (59%) doses of hydrocodone to control their pain, with their final pain score being still significantly greater than non-smokers (Ackerman and Ahmad, 2007). The decrease in analgesic response in smokers was attributed to stimulant action of nicotine, decreased pain tolerance normally associated with smoking and decreased serum analgesic levels (Ackerman and Ahmad, 2007).

1.4.8 Propranolol

Propranolol, an antihypertensive agent with moderately high extraction ratio (Suzuki *et al.*, 1980), is metabolized via three main pathways, namely glucuronidation, side-chain oxidation and ring oxidation (Walle *et al.*, 1987). Plasma levels decreased (38%) after oral administration of an 80 mg tablet of propranolol in healthy smoking volunteers whereas half-life remained unchanged. The increased oral clearance was accompanied by increased clearance through glucuronidation (55%) and side chain oxidation (122%) with ring oxidation pathway remaining unchanged (Walle *et al.*, 1987). The contribution of CYP1A1/2 to propranolol side-chain oxidation was confirmed in animal models through either the inhibitory effect of alpha-naphthoflavone or the inductive effect of 3-MC (Walle *et al.*, 1987). Increased glucuronidation of propranolol in smokers is one of the few drug examples showing increased glucuronidation activity in smokers.

1.4.9 Irinotecan

Irinotecan, an anticancer drug with low hepatic extraction ratio (Farabos *et al.*, 2001), is metabolized by carboxylestrases to yield an active metabolite (SN-38) which is subjected to glucuronidation, forming SN-38G (van der Bol *et al.*, 2007). Although the drug is not metabolized by CYP1A isoforms, its $AUC_{0\text{-}100h}$ and clearance significantly decreased (15.3%) and increased (18%), respectively in smokers (van der Bol *et al.*,

2007). Levels of its metabolite, SN-38, were also decreased (39%) in smokers (van der Bol *et al.*, 2007).

It was noted that decreased levels of irinotecan and SN-38 were associated with decreased drug effectiveness (van der Bol *et al.*, 2007). The mechanism involved in this interaction is not confirmed. However, the authors attributed the increased clearance to an inducing effect of cigarette smoke on either CYP3A4/5 or carboxylestrases which was accompanied by increased glucuronidation. Indeed, although levels of SN-38G were not altered in smokers, the relative extent of glucuronidation, defined as molar $AUC_{0\ to\ 100h}$ ratio of SN-38G to SN-38 was significantly higher in smokers (van der Bol *et al.*, 2007).

1.4.10 Erlotinib

Erlotinib, used in treatment of certain types of lung cancers, is metabolized mainly by CYP3A4 and to a relatively smaller extent by CYP1A2 and CYP1A1 to two O-demethylated active metabolites among other metabolites (Hamilton *et al.*, 2006). After erlotinib (150 mg) was administered orally to healthy cigarette smoking volunteers, it's Cmax, $AUC_{0-\infty}$ decreased by 35% and 64%, respectively. Cmax and $AUC_{0-\infty}$ of its metabolites also decreased by 39% and 55%, respectively (Hamilton *et al.*, 2006).

Doubling the erlotinib dose (300 mg) in smokers achieved similar plasma levels of erlotinib and metabolites as those achieved in non-

smokers after administering 150 mg dose (Hamilton *et al.*, 2006). The survival time for patients who were current or former smokers was significantly lower than that of patients who had never smoked, with drug related side effects being less common in smokers (Hughes *et al.*, 2009). The maximum tolerated dose of erlotinib in smokers was twice (300 mg/day) that of non-smokers (150 mg/day) with plasma steady state trough levels, at these dose levels, in smokers being equivalent to those of non-smokers (1.22 µg/ml vs. 1.24 µg/ml, respectively) (Hughes *et al.*, 2009).

In support of the hypothesis that smoking decreases drug exposure and therefore drug benefit, the study showed evidence of improved clinical response in smokers treated with higher (250-300 mg/day) doses of erlotinib than those treated with lower dose (150 mg/day). The survival time of smokers receiving erlotinib doses (250 or 300 mg) was 9.56 months while in those receiving 150 mg was 5.45 months (Hughes *et al.*, 2009).

1.4.11 Quinine

Quinine is an anti-malarial drug with low hepatic extraction ratio (Roy *et al.*, 2002); its half-life and oral clearance decreased and increased respectively in otherwise healthy smokers after single oral dose of quinine sulphate, with the unbound fraction being unaltered (Wanwimolruk *et al.*, 1993). However, the clearance of unbound quinine was increased by 50%

(Wanwimolruk *et al.*, 1993). The dose excreted as unchanged quinine in the urine (0-48h) decreased by 30% in smokers (Wanwimolruk *et al.*, 1993), with renal clearance of drug being similar in both groups.

Quinine is metabolized mainly by CYP3A4 with little or no contribution of human CYP1A1 or 1A2 (Wanwimolruk *et al.*, 1993; Zhang *et al.*, 1997). Although its pharmacokinetics were altered in otherwise healthy smokers, no significant differences in its pharmacokinetics or its effectiveness were noted upon repeated oral administration in cigarette smoking malaria patients (Pukrittayakamee *et al.*, 2002). It was observed that in patients with malaria, quinine clearance is significantly reduced compared with healthy volunteers (Pukrittayakamee *et al.*, 2002). The decrease in quinine clearance in patients was attributed to an inhibitory effect of malaria on CYP isoenzymes (Pukrittayakamee *et al.*, 2002).

Table 1: Changes in pharmacokinetic parameters, clearance (CL), oral clearance (CL/F), volume of distribution (V_d), oral volume of distribution (V_d/F) and half-life ($t_{1/2}$) for different drugs as a result of cigarette smoking or ingestion of char-grilled diet.

Drug	Pharmacokinetics parameters					
	CL	CL/F	V_d	V_d/F	$t_{1/2}$	Reference
Antipyrine 1. Cigarette smoke 2. Charcoal-grilled meat Heller 1989	Increased (63%) -	Increased (59-69%) Increased (20%)	No change -	No change -	Decreased (17-42%) Decreased (20%)	(Wood *et al.*, 1979; Eichelbaum *et al.*, 1982; Spoelstra *et al.*, 1986; Heller *et al.*, 1989; Scavone *et al.*, 1997)
Phenactin 1. Cigarette smoke 2. Charcoal-grilled meat Heller 1989	-	2.5-fold Increased (5-fold)	-	-	-	(Dong *et al.*, 1998) (Pantuck *et al.*, 1976; Heller *et al.*, 1989)
Theophylline	Increased (18-45%)	Increased (125%)	No change	Increased (32%)	Decreased (14-27%)	(Hunt *et al.*, 1976; Horai *et al.*, 1982; Horai *et al.*, 1983; Haughey and Jusko, 1988)
Desmethyldiazepam	-	Increased (175%)	-	No change	Decreased (46%)	(Norman *et al.*, 1981)
Olanzapine	-	Increased (40-206%)	-	Increased (234%)	No change	(Callaghan *et al.*, 1999; Wu *et al.*, 2008)
Irinotecan	Increased (18%)	-	-	-	Not mentioned	(van der Bol *et al.*, 2007)
Quinine	-	Increased (77%)/No change[a]	-	-	Decreased (38%)/No change[a]	(Wanwimolruk *et al.*, 1993; Pukrittayakamee *et al.*, 2002)
Thiothixene	-	Increased (36%)	-	-	-	(Ereshefsky *et al.*, 1991)
Erlotinib	-	Increased (144%)	-	-	Decreased (57%)	(Hamilton *et al.*, 2006)
Caffeine	-	Increased (55%)	-	No change	Decreased (12%)	(Seng *et al.*, 2009)
Propranolol	-	Increased (77%)	-	-	No change	(Walle *et al.*, 1987)

1.5 Amiodarone

1.5.1 Pharmacokinetics

Amiodarone (AM) is a commonly prescribed class III anti-arrhythmic drug that is used in the treatment of life threatening ventricular and supraventricular arrhythmias (Echt *et al.*, 1991; 1992; Naccarelli *et al.*, 2000). In humans AM has relatively low blood clearance, long terminal elimination half-life and large volume of distribution (Holt *et al.*, 1983). Similarly, in rats, AM has a large volume of distribution yet its clearance and half-life are relatively greater and shorter, respectively than those observed in humans (Weir and Ueda, 1986; Shayeganpour *et al.*, 2005; Shayeganpour *et al.*, 2008). Its hepatic extraction ratio in human and rat were reported to be low (0.13) and moderate (0.49), respectively (Holt *et al.*, 1983; Shayeganpour *et al.*, 2005). AM is extensively bound to plasma proteins (>90%) in both species (Lalloz *et al.*, 1984).

Elimination of AM occurs mainly via oxidative metabolic pathways in liver, with renal elimination being negligible in human and rat (Holt *et al.*, 1983; Weir and Ueda, 1986). Hepatic biotransformation results in several metabolic products, with the CYP super-family being implicated. These products include mono-N-desethylamiodarone (DEA), di-N-desethylamiodarone and de-iodinated desethylamiodarone, each of which is present in blood and/or plasma of humans and animal models (Latini *et al.*, 1984; Young and Mehendale, 1986; Fabre *et al.*, 1993). Of these, DEA

is especially important due to its pharmacological and toxicological activities and the presence of high circulating plasma and tissue levels in humans, which often exceed the concentrations of the parent drug itself (Brien *et al.*, 1987; Kannan *et al.*, 1989; Stark *et al.*, 1991; Massey *et al.*, 1995; Meng *et al.*, 2001).

Several CYP isoenzymes have been implicated in the biotransformation of AM to DEA in human and rat (Fabre *et al.*, 1993; Ohyama *et al.*, 2000a; Shayeganpour *et al.*, 2006a). These reports have demonstrated the involvement of CYP3A4, 1A1, 1A2, 2D6 and 2C8 in DEA formation in human whereas CYP3A1 and 1A1 were involved in DEA formation in rats (Fabre *et al.*, 1993; Ohyama *et al.*, 2000a; Shayeganpour *et al.*, 2006a).

1.5.2 Pharmacology and toxicology

Although AM is known to prolong repolarization and refractoriness of atrial and ventricular muscles, a property of class III anti-arrhythmic agents, studies have shown that it can also act as a sodium channel blocker (inactivated), beta-blocker and calcium channel blocker (Mason, 1987; Stark *et al.*, 1991). Similarly DEA was found to possess similar anti-arrhythmic properties and was thought to contribute to the anti-arrhythmic effect of AM (Stark *et al.*, 1991). AM also causes a wide range of side effects that involve organs such as liver, lung and thyroid (Beddows *et al.*, 1989; Pollak, 1999; Bargout *et al.*, 2000; Waldhauser *et al.*, 2006). For

most AM-induced side effects, DEA was shown to be more toxic than AM (Beddows *et al.*, 1989; Pollak, 1999; Waldhauser *et al.*, 2006).

Evidnece for a correlation between AM or DEA concentration and antiarrhythmic effect or side effects is present. For example, significant correlations between AM antiarrhythmic effects, measured as percentage change in electrocardiographic parameters, and plasma and/or myocardial drug concentrations were observed (Connolly *et al.*, 1984; Debbas *et al.*, 1984). Similar to AM, strong correlations were observed between DEA plasma and/or myocardial concentrations and electrocardiographic parameters (Nattel, 1986; Kharidia and Eddington, 1996). Furthermore, recent studies have shown that some of AM-induced toxicological effects correlated with AM and DEA concentrations in the affected organs, one of these notably being the lungs in which pulmonary fibrosis may ensue (Camus *et al.*, 2004). Data are available showing an association between pulmonary toxicity and higher DEA:AM serum concentration ratios in patients receiving AM (Pollak, 1999).

Chapter 2

2.0 Rationale

A significant contribution of DEA to some of the side effects of AM was previously reported (Beddows *et al.*, 1989; Bargout *et al.*, 2000; Waldhauser *et al.*, 2006). Indeed several side effects, especially pulmonary toxicity, were associated with higher DEA levels, and more potent toxic effects have been consistently shown for DEA than for AM (Beddows *et al.*, 1989; Bargout *et al.*, 2000). Studies have shown that CYP1A1 and CYP1A2 are involved in AM metabolism in humans and rats (Ohyama *et al.*, 2000a; Shayeganpour *et al.*, 2006a). There is also literature showing that CYP1A2 and 1A1 are significantly expressed in hepatic (~ 10% of total CYP content) and extra-hepatic tissues such as lung (Shimada *et al.*, 1996). As previously discussed, both of these isoenzymes were consistently shown to be inducible in smokers.

Given these facts, it is surprising that little or no study has been directed to the effect of PAH on AM pharmacokinetics and pharmacodynamics. Based on involvement of CYP1A1 and 1A2 in AM metabolism, potential alteration of AM pharmacokinetics and enhanced DEA production in smokers is expected. Furthermore, cigarette smoke might increase the risk of developing AM-related side effects through increased DEA production. Indeed, a recent study in hamsters showed

that AM-treated hamsters that are exposed to cigarette smoke developed more pronounced lung inflammation than those treated with AM or smoke alone (Bhavsar *et al.*, 2007). However, potential enhancement of pulmonary DEA production and its involvement in aggrevating lung inflammation in cigarette smoke-exposed hamsters was not investigated. Additionally, data on the comparative DEA-forming activities of both human CYP1A1 and 1A2 with their rat counterparts are not available.

In an earlier study the effect of (±)-ketoconazole (KTZ) on DEA formation was explored (Shayeganpour *et al.*, 2006a). KTZ, a selective CYP3A inhibitor, non-selectively inhibited DEA formation by rat CYP1A1. The importance of this observation lay in that KTZ is often used in drug interaction studies as a presumed selective inhibitor of CYP3A4 (Wrighton and Ring, 1994). It was also used to establish the role of human CYP3A4 in AM metabolism in human liver microsomes (Fabre *et al.*, 1993; Ohyama *et al.*, 2000a).

Furthermore, combined medicinal use of KTZ and AM can lead to a significant interaction *in vivo*, which is of clinical importance given that concentrations of AM are subject to therapeutic drug monitoring (Pollak, 1999). In humans interaction of AM with azole antifungal agents is well recognized, and could possibly lead to serious clinical side effects such as Torsades de pointes secondary to overly prolonged QT interval (Kounas *et al.*, 2005). It has been assumed that this interaction is due to inhibition of CYP3A isoenzymes by KTZ (Kounas *et al.*, 2005). Given that KTZ was

shown to inhibit AM metabolism by CYP1A1, this cannot be said with certainty.

Furthermore, it has been reported that DEA is a strong inactivator, or mechanism-based inhibitor (MBI), of human CYP1A1 with inhibitor concentration causing 50%-maximal inactivation being 1 μM (Ohyama *et al.*, 2000b). This claim raises some question as to the relevance of CYP1A1 induction for AM disposition and CYP1A1-mediated DEA formation in humans as DEA might inhibit its own CYP1A1-mediated formation. In reviewing the earlier report, we have found that methodological factors could have caused a skewed view of whether DEA is an MBI.

Two designs are typically utilized for MBI assessment (Silverman, 1995a). Although each incorporates a preincubation stage in which the enzyme is exposed to the suspected MBI, they differ in the second stage in which the MBI-exposed enzyme is exposed to a CYP-specific probe substrate. Using one approach, an aliquot of the preincubated CYP-MBI is diluted 50- to 100-fold before being exposed to the CYP-specific probe (Silverman, 1995a). In the second approach the probe substrate is directly added to the preincubated CYP-MBI without dilution (Silverman, 1995a). In both cases saturating concentrations of the CYP probe is used (Silverman, 1995a). The former protocol is most commonly employed and is considered superior because the dilution step minimizes potential

reversible inhibition caused by the suspected MBI (Silverman, 1995a; Yang *et al.*, 2005; Ghanbari *et al.*, 2006).

Indeed, reviews of the experimental protocols used to evaluate MBI found significant variability in the dilution factor which could potentially bias estimates of inactivation parameters (Yang *et al.*, 2005; Ghanbari *et al.*, 2006). To minimize bias, use of maximum-fold of dilution of preincubates (50- to 100-fold), with short preincubation (up to 10 min) and incubation (3 min) times have been recommended (Yang *et al.*, 2005; Ghanbari *et al.*, 2006).

In reviewing the report of DEA as CYP1A1 MBI (Ohyama *et al.*, 2000b), it was apparent that the joint DEA-CYP1A1 preincubation was not diluted before remaining enzyme activity was assessed. Hence, the possibility of reversible inhibition during the incubation phase cannot be excluded.

Chapter 3

3.0 Hypotheses and Objectives

Our studies were done with these hypotheses in mind:

1) DEA forming activity of human and rat CYP isoenzymes are equal.

2) KTZ will inhibit DEA formation by CYP3A especially when used at low concentrations at " Window of Selectivity".

3) Exposure to PAH will enhance AM clearance and increase DEA levels in vivo.

4) Lack of dilution step confounded the findings of a previous assessment of the DEA inactivating effect (Ohyama *et al.*, 2000b) of human CYP1A1.

5) Formation of DEA by PAH-treated microsomes is greater than by controls.

BNF was used in our studies as a representative PAH. Unlike other PAH, BNF (a potent inducer of CYP1A1 and CYP1A2) was thought to be non-carcinogenic and had little toxicity compared to other PAH which makes it attractive in studies focussed on drug related toxicities (McKillop and Case, 1991; Adedoyin *et al.*, 1993). Furthermore, it is rapidly cleared, with blood clearance (130 ml/min/kg) being approximately equal to hepatic blood flow in rats (Adedoyin *et al.*, 1993). It is worth noting that after 100 mg/kg BNF was administered intraperitoneally or orally, BNF was

undetectable which was attributed to its high volume of distribution and clearance (Adedoyin *et al.*, 1993). After BNF was administered intravenously (10 mg/kg), the average terminal-half life was 43 min in unanesthetized rats (Adedoyin *et al.*, 1993). Rapid elimination of BNF might also be a desirable property to minimize potential interactions between the drug and inducer. Therefore changes in pharmacokinetics of the drug are likely caused by BNF-mediated induction of drug metabolizing enzymes and not due to BNF itself.

To allow us to test our hypotheses, our objectives included the following:

- To compare DEA formation by CYP isoenzymes known to contribute to DEA formation in humans to their rat counterparts.
- To examine the Inhibition of DEA formation by KTZ.
- To explore the effect of BNF on AM pharmacokinetics. This included the effect of BNF induction on AM tissue distribution.
- To investigate the effect of DEA on human recombinant CYP1A1 activity and assessing of inactivation potential.
- To examine the role of CYP1A1 induction in DEA formation and the effect of AM treatment on CYP1A1 and CYP1A2 gene expression and activity.

Chapter 4

4.0 Experimental

4.1 Materials

Amiodarone HCl, ethopropazine HCl, nicotinamide adenine dinucleotide phosphate tetrasodium (NADPH), β-naphthoflavone (BNF) and 7-ethoxyresorufin (7-ER) were obtained from Sigma (St Louis, MO, USA). Resorufin was purchased from ICN Biochemicals Canada (Toronto, ON). Desethylamiodarone (DEA) was obtained as a kind gift from Wyeth-Ayerst Research (Princeton, NJ). Isoflurane BP was purchased from Benson Medical Industries (Ontario, Canada). Heparin sodium for injection (1000 U/mL) was obtained from Leo Pharma, Inc. (Thornhill, Ontario, Canada). AM HCl as a sterile injectable solution (50 mg/mL) was purchased from Sandoz (Sandoz Canada, Boucherville,Quebec, Canada). Sodium chloride for injection was from Hospira Healthcare Corporation (Montreal, Quebec, Canada). Methanol, acetonitrile, hexane (all HPLC grade), triethylamine. and sulfuric acid were purchased from EM Scientific (Gibbstawn, NJ). Potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, potassium chloride, magnesium chloride hexahydrate, sucrose, and calcium chloride dihydrate (all analytical grade) were obtained from BDH (Toronto, ON, Canada). Supersomes expressing rat CYP1A1, 1A2, 2C6, 2C11, 2D1 and 2D2 and human CYP1A1, 1A2, 2D6 and 3A4 with supplementation of CYP reductase were purchased

from BD Gentest (Woburn, MA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR Green SuperMix, and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA). Real-time PCR primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) according to previously published sequences. Stock solutions of amiodarone (13.2 mg/mL) or KTZ (1.24 mg/mL) were made in methanol.

4.2 Methods

4.2.1 Studies aimed at comparing DEA formation by CYP isoenzymes known to contribute to DEA formation in humans to their rat counterparts

The formation kinetics of DEA were characterized when AM was exposed to human CYP1A1, 1A2, 3A4 and 2D6 and rat CYP1A1, 1A2, 2C6, 2C11, 2D1, 2D2, and 3A1 isoforms. Each 0.5 mL incubate contained 20 pmol/mL of CYP isoform and 5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer (pH=7.4). To incubations, AM HCl was added to provide for nominal concentrations of 0.5, 2, 5, 10, 20, 40, 80, 100 or 155 µM. For this, AM HCl was dissolved in methanol such that a total methanol concentration of 0.8% was present in each incubation mixture. Oxidative reactions were initiated with the addition of 1 mM NADPH after a 5 min pre-equilibration period. All incubations were performed at 37ºC in a shaking water bath (50 rpm) for 30 min. The

reaction was stopped by adding 1.5 mL ice-cold acetonitrile. Under these conditions of time and protein concentration the increase in DEA formation rates were linear based on preliminary time- and CYP content-linearity studies. Samples were kept at -20º C until assayed for AM and DEA.

4.2.2 Inhibition of DEA formation by KTZ

To study the inhibitory effect of KTZ on AM metabolism, incubations were constituted as above, except that methanol concentrations were 1.6% in AM or AM+KTZ incubates. Increasing the methanol concentration to 1.6% from 0.8% had no effect on the formation rates of DEA by any of the recombinant microsomal preparations. In support of this observation, it was shown previously that methanol concentrations of up to 3% did not affect the inhibitory potency of KTZ on midazolam 1-hydroxylation activity by human liver microsomes (Iwase *et al.*, 2006).

The inhibitory effect of KTZ was examined using two approaches. A single concentration percentage of inhibition was carried out using 18.8 µM of KTZ and 34.5 µM of AM in the presence of the same rat and human CYP used above. For each CYP the determinations were done in triplicate for AM and AM+KTZ incubations. These high concentrations of AM and KTZ were chosen to mimic hepatic levels *in vivo* (Brass *et al.*, 1982; Brien *et al.*, 1987; von Moltke *et al.*, 1996). The inhibitory constant (ki) of inhibition of DEA formation by KTZ was also assessed using the recombinant CYP isoenzymes with lower concentrations of drug and

inhibitor. For this purpose nominal KTZ concentrations were 0, 0.025, 0.04, 0.05, 0.1, 0.16, 0.5, 1, 2.5 or 5 µM with AM concentrations of 0.5, 1, 2, 4, 8 or 20 µM. Incubations were performed in replicates of 2-3 on each day for each incubate concentration of AM and KTZ.

For rat CYP3A1, the percent of disappearances of AM and DEA in the presence and absence of KTZ were compared. 1 µM of AM and DEA were incubated with CYP3A1 and with various concentrations of KTZ (0, 50, 500, 5000 nM) in incubation mixtures as described above. Incubations containing all reaction components except for CYP3A1 were used as controls to provide the initial concentrations of AM and DEA. Remaining concentrations of AM and DEA were determined. Additionally, in the AM-spiked samples the difference between the initial amount of AM and the molar sum of AM+DEA measured was determined. This was assumed to represent other metabolites of AM.

4.2.3 Effect of BNF on AM pharmacokinetics

All experimental protocols involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Sprague-Dawley rats (Charles River, Montreal, Quebec, Canada) were used in all studies involving animals. The average±SD values of their body weights were 288 ± 69 g. All the rats were housed in temperature-controlled rooms with 12 h light/d. The animals were fed a

standard rodent's chow containing 4.5% fat (Lab Diet1 5001, PMI Nutrition LLC, Brentwood, Missouri, USA).

Rats were allocated into two groups. The control group (n=6), received 2 mL/kg/d of corn oil (CO), and the BNF-treated group (n=8), received 80 mg/kg/d of BNF dissolved in CO, through intra-peritoneal injection. Four daily doses of BNF or vehicle were administered. After the 4^{th} dose of BNF, the right jugular veins of all rats were catheterized with Micro-Renathane tubing (Braintree Scientific, Braintree, MA, USA) under isoflurane anesthesia. The cannula was filled with 100 U/mL heparin in 0.9% saline. On the fifth day, the rats were injected with a single dose (25 mg/kg) of AM HCl over ~60 s via the jugular vein cannula, immediately followed by injection of approximately 1 mL of sterile normal saline solution. Thereafter, blood samples (0.15–0.3 mL) were collected from the cannula at approximately 0.08, 0.33, 0.67, 1, 2, 3, 4, 6, 8, 10, 24, and 48 h after dosing. At the time of first sample withdrawal, the first 0.2 mL volume of blood was discarded as previously described (Shayeganpour *et al.*, 2005). To maintain patency of the cannula, heparin in normal saline (100 U/mL) was used to flush each cannula after collection of blood samples. Blood samples were centrifuged at 2000 g for 3 min. The plasma was transferred to new polyethylene tubes and stored at – 20° C until assayed for AM and DEA.

4.2.4 Effect of BNF on AM tissue distribution

Rats were allocated into two groups which received BNF (80 mg/kg/d, for 4 d) or CO (2 mL/kg/d, for 4 d). On the third day of dosing, the right jugular veins of all rats were catheterized in the same manner as described previously. On the same day, the rats received 25 mg/kg/d of AM i.v. through the jugular vein cannula. AM i.v. dosing solution was prepared by dilution of AM HCl solution for injection in normal saline (12.5 mg/mL). AM i.v. dosing was continued for 4 d. After the 4^{th} dose of AM, the rats were exsanguinated by cardiac puncture under isoflurane anesthesia at the approximate times 0.083, 1, 3, 6, 12 and 24 h (n=4-6 for each group, at each time point). Thereafter, blood, intestine, liver, kidneys, heart, and lung tissues were collected. The blood samples were immediately centrifuged at 2000 g for 10 min. The resulting plasma was transferred to new tubes and stored with the rest of the tissues at -20º C.

4.2.5 Effect of DEA on human recombinant CYP1A1 activity and assessing of inactivation potential

4.2.5.1 Test for DEA as a CYP1A1 substrate

In the presence of 0.5 ml of 0.5 M phosphate buffer supplemented with 5 mM $MgCl_2 \cdot 6H_2O$ and 1 mM NADPH, 0.8 µM of DEA was incubated for 0, 5, 10, and 20 min with CYP1A1 (20 pmol/ml) in quadruplicate. For this and all incubations outlined below, incubations involving CYP were performed in a shaking water bath at 37°C. The reaction was terminated

at the end of the predefined times by adding 1.5 ml ice-cold acetonitrile. Samples were then frozen at -20°C until assayed for remaining DEA.

4.2.5.2 Self inhibition of DEA formation

In the media described above, 128 µM AM was incubated with 40 pmol/ml CYP1A1 in the presence and absence of 8.3 µM DEA. These AM and DEA concentrations were selected based on those observed in human liver *in vivo* (Brien et al., 1987). After 30 min 100 µl was transferred to tubes containing 300 µl ice-cold acetonitrile. Tubes containing the added amount of DEA without CYP1A1 were assayed for the initial amount of DEA ($DEA_{initial}$). The expected total amount (DEA_{exp}) in the AM+DEA containing tubes was estimated as $DEA_{exp}= DEA_{initial}+DEA_{formed}$, with DEA_{formed} being the amount assayed in the AM-only incubations. The values of the DEA_{exp} were then compared with the measured amount of DEA in the incubations containing AM+DEA.

4.2.5.3 Effect of DEA on EROD

5 µM DEA was coincubated (n=5) with 10 pmol/ml CYP1A1 and 1.5 µM 7-ethoxyresorufin (7-ER) in the presence of 0.1 M potassium phosphate buffer (pH=7.8) containing 1 mM ethylene diamine tetraacetic acid disodium (EDTA). The final volume was 1 ml with concentrations of organic solvent in the tubes containing 0 and 5 µM DEA being 1%. After 5-min, 1 mM NADPH was added to initiate EROD and 3 min after that, 2 ml ice-cold methanol was added to stop the reaction. Concentrations used for

methanol and DMSO were 0.5 and 0.07-0.5 %, respectively. It is worth mentioning that earlier reports showed that methanol and DMSO can be used in concentration of 1 and 0.1%, respectively, without affecting CYP3A activity (Iwase *et al.*, 2006). In our lab, we have observed that increasing concentration of DMSO from 0.07 to 0.5% does not affect EROD activity.

4.2.5.4 Kinetic analysis of the effect of DEA on EROD

In the presence of 10 pmol/ml of CYP1A1 and the same incubation media described above, various concentrations of DEA (0, 0.25, 0.5, 1, 2.5, and 5 µM) were incubated with different concentrations of 7-ER (0.05, 0.2, 0.75, and 1.5 µM; in duplicate for each concentration). After a 5-min equilibration period, 1mM NADPH was added to start the reaction. The reaction was then stopped after 3 min with 2 ml ice-cold methanol.

4.2.5.5 Inactivation experiments

Two protocols were used. Methanolic DEA solutions were added to the tubes and dried in vacuo to remove the solvent. The dried residues were reconstituted with CYP1A1-containing buffer. Preliminary experiments using aliquots of CYP1A1-containing buffer confirmed that DEA was soluble in the buffer.

4.2.5.5.1 Protocol A

Here dilution of the preincubation mixture was adopted, to minimize the possibility of inhibition of EROD by DEA. DEA (0, 0.5, 1, 2 or 5 µM; n=3-5 per incubation) was added to 0.1 M potassium phosphate (pH=7.8) containing 1 mM EDTA and 0.4 nmol/ml of CYP1A1. NADPH (1 mM) was added to start the preincubation phase which lasted for 0, 2, 5, 10, 20 and 30 min. Afterwards, 20 µl was transferred to a fresh 980 µl volume of buffer + 0.3 µM 7-ER. 1 mM NADPH was added to the EROD incubation tubes 1 min before transfer of the preincubation aliquot; 3 min later 2 ml of ice-cold methanol was added.

4.2.5.5.2 Protocol B

This was the basic procedure as described by Ohyama et al. (2000) where EROD was directly assessed in the preincubation media without dilution (Ohyama *et al.*, 2000b). Into the EDTA-containing buffer described above was added NADPH (0.25 mM) and 0 or 2 µM DEA (n=6 each; total volume 100 µl). Preincubation times were 0, 10, 20, or 30 min, after which 2 µM of 7-ER was directly added to initiate EROD. After 15 min, 2 ml of ice-cold methanol were added to stop the reaction. In a modification of the procedure, in the 30 min preincubation, extra NADPH (0.25 mM) was added to the buffer at the time of addition of 7-ER.

4.2.5.5.3 Experimental impact of NADPH in the pre- and post-incubation phases

DEA (0, 0.5, 1 or 2 µM) was added to the CYP1A1-containing preincubation buffer as used in Protocol A, but without NADPH. After 0, 2, 5, or 10 min (n=3), 20 µl was transferred to the incubation media containing 7-ER and NADPH. Three min later 2 ml ice-cold methanol was added. The same experiment was also repeated with NADPH and DEA present in the preincubation but absent from the incubation phase.

At the end of the EROD incubation experiments in section ***4.2.5.3*** to ***4.2.5.5.3***, protein was precipitated by centrifugation for 5 min at 2500×*g*. Thereafter, the fluorescent metabolite, resorufin, was measured as described previously (Gharavi and El-Kadi, 2005).

4.2.6 Role of CYP1A1 induction in DEA formation and effect of AM treatment on CYP1A1/2 gene expression and activity

4.2.6.1 Animal treatment

Male Sprague–Dawley rats (Charles River, Quebec, Canada) were used in the study. The average±SD values for their body weights were 302±55 g. At the beginning of the study, there were no significant differences in the body weights of rats assigned to different treatment groups. As mentioned previously all the rats were housed in temperature controlled rooms with 12 h light per day. The animals were fed a standard rodent chow containing 4.5% fat (Lab Diet1 5001, PMI nutrition LLC,

Brentwood, USA). Free access to food and water was permitted during experimentation.

The rats were allocated into two groups. The control group (n=9) received 2 ml/kg/day of corn oil (CO) ip for 4 days. The BNF treated group (n=12) received 80 mg/kg/day of BNF ip for 4 days. The 80 mg dose of BNF was dissolved in 2 ml CO so that the control and the treated groups received the same amounts of vehicle on a weight basis. One day after the last dose, the rats were anesthetized using isoflurane, and their liver, intestine, kidney, lung and heart tissues were excised. The collected tissues were directly frozen in liquid nitrogen and kept at – 80 ºC.

In studying the effect of AM treatment on basal and BNF mediated induction of CYP1A isoforms, rats were administered CO, BNF, CO+AM or BNF+AM (n=4-10 per group). On the day before the study the right jugular veins of all rats were catheterized with polyethylene tubing (Becton Dickinson, Sparks, MD, USA) under isoflurane anesthesia. On the first day of the study CO, BNF, CO+AM and BNF+AM treatments were started. BNF and CO doses and routes of administration were similar to those mentioned previously. AM was administered intravenously (25 mg/kg/day). All treatments were continued for four days. One day after the last dose, the rats were killed by cervical dislocation under isoflurane anesthesia, and their liver, intestine, kidney, lung and heart tissues were excised, directly frozen in liquid nitrogen and kept at – 80 ºC.

4.2.6.2 RNA Extraction and cDNA Synthesis

Total RNA was isolated from the frozen tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. First strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions.

4.2.6.3 Quantification by Real-Time PCR

Quantitative analysis of specific mRNA expression was performed by real-time PCR by subjecting the resulting cDNA to PCR amplification in 96-well optical reaction plates using the ABI Prism 7500 System (Applied Biosystems). The 25 µl reaction mix contained 0.1 µl of 10 µM forward primer, 0.1 µl of 10 µM reverse primer, 12.5 µl of SYBR Green Universal Mastermix, 11.05 µl of nuclease-free water and 1.25 µl of cDNA sample. The primers used in the current study were similar to those used in a previously published study (Zordoky and El-Kadi, 2007). Forward primer sequences were CTGGTTCTGGATACCCAGCTG, GTCACCTCAGGGAATGCTGTG and GGCCAAGGTCATCCATGA for CYP1A1, CYP1A2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Backward primer sequences were CCTAGGGTTGGTTACCAGG, GTTGACAATCTTCTCCTGAGG and TCAGTGTAGCCCAGGATG for CYP1A1, CYP1A2 and GAPDH,

respectively. No-template controls were incorporated to test for the contamination of any assay reagents. The thermocycling conditions were initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

4.2.6.4 Real-Time PCR Data Analysis

The real-time PCR data were analyzed using the relative gene expression (i.e., ΔΔ CT) method, as described in Applied Biosystems User Bulletin No. 2 and explained by Livak and Schmittgen (Livak and Schmittgen, 2001). The data are presented as the fold change in gene expression normalized to the endogenous reference gene (GAPDH) and relative to a calibrator. CO was used as the calibrator to measure the change in gene expression by different treatments.

4.2.6.5 Preparation of microsomes and CYP1A activity measurement

Tissue-specific microsomes were prepared using a previously published method (Barakat et al., 2001). Briefly, all tissues were washed in ice-cold KCl (1.15%, w/v), cut into pieces and homogenized separately in ice-cold sucrose solution (5 g of tissue in 25 ml of 0.25 M sucrose). Tissue homogenates were centrifuged at 600 × g for 8 min. The supernatant was then centrifuged at 12000 × g for 10 min. Thereafter, supernatants resulting from the previous step were mixed with 8 mM

$CaCl_2$ and centrifuged at 27,000 × g for 15 min. The consequent pellets were suspended in 0.15 M KCl and recentrifuged at 27000 × g for 15 min. Final pellets were re-suspended in cold sucrose (25 mM) and stored at -80°C. The microsomal protein concentration was measured using the Lowry method (Lowry et al., 1951).

CYP1A activity in the microsomes was assessed by measuring EROD activity (Sinal et al., 1999). In 0.1 M potassium phosphate buffer (pH=7.8), 7-ER (1.5 µM) was incubated with the microsomal protein (0.25 and 0.5 mg/ml for hepatic and extra-hepatic microsomes, respectively). The final volume was 1 ml. The samples were allowed to pre-equilibrate for 5 min in a shaking water bath at 37°C. The reaction was initiated by the addition of 0.8 mM NADPH and was allowed to incubate for 3 or 5 min for hepatic and extra-hepatic microsomes, respectively. The reaction was then stopped by adding 2 ml ice-cold methanol. The precipitated protein was removed by centrifugation for 5 min at 120 × g. Thereafter, the fluorescent product, resorufin, was measured in the supernatant at excitation wavelength of 545 nm and emission wavelength of 575 nm.

4.2.6.6 DEA formation by hepatic and extra-hepatic microsomes

The incubation mixtures contained 1 mg/ml microsomal proteins, 0.1-180 µM of AM HCl, 1 mM NADPH, and 5 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer (pH =7.4). The final volume ranged between 0.2 to 0.5 ml. The oxidative reactions

were started by the addition of 1 mM NADPH after a 5-min pre-equilibration period. The incubation times were 10, 60, 10, 30 and 3 min for liver, lung, intestine, kidney and heart microsomes, respectively. At these times the rates of DEA were found to be linear in preliminary time linearity studies. At the end of the incubation time, the reaction was stopped by adding ice-cold acetonitrile (three times the original final volume).

Additionally, DEA metabolism by rat recombinant CYP1A1 isoenzyme was investigated. In the presence of the previously described phosphate buffer, DEA (0.15 µM) was incubated with 40 pmol/ml CYP1A1 (n=3). The final volume was 0.5 ml. The reaction was started by adding 1 mM NADPH and 100 µl samples were taken at 0, 30, 60 and 120 min. The aliquots were instantly added to tubes containing 300 µl ice-cold acetonitrile to stop the reaction.

4.2.7 AM and DEA assay

For incubations involving recombinant enzyme systems (supersomes) and microsomal incubations of liver, heart and intestine, an HPLC method was used for analysis of AM and DEA (Jun and Brocks, 2001; Shayeganpour *et al.*, 2006a). The assay had a validated lower limit of quantitation of 35 ng/ml for both AM and DEA based on 100 µL of rat plasma (Jun and Brocks, 2001). Briefly, 30 µL internal standard (ethopropazine HCl 50 µg/mL,) was added to each tube containing 0.5 mL

microsomal incubation mixture and 1.5 mL of acetonitrile. Samples were vortex mixed for 30 s and centrifuged for 2 min at 2500 g to facilitate removal of protein. Thereafter, 7 mL of hexane was added and the mixture was vortex mixed for 45 s, then centrifuged for 3 min. The final organic layer was transferred in to new tubes, evaporated to dryness, reconstituted with mobile phase and injected into the HPLC apparatus. This assay had reported intraday and interday coefficients of variation of less than 20 % (Jun and Brocks, 2001). The reported intraday accuracy ranged from 84.8 % to 114% whereas, the interday accuracy ranged from 97.4 % to 103 % (Jun and Brocks, 2001).

In biological rat plasma and tissues samples and in microsomal incubations involving lung and kidney, another validated liquid chromatography and mass spectrometry method was used for the simultaneous assay of AM and DEA in plasma and tissues (Shayeganpour *et al.*, 2007). This assay method uses the same basic extraction procedure as described previously. However, the mobile phase was pumped as a linear gradient from methanol: formic acid 0.2%, 40:60 to 90:10 v/v over 12 min, then back to the original 40:60 v/v composition over 3 min. A constant flow rate of 0.2 mL/min was used throughout. The column was heated to 45°C during the chromatographic run. This assay had a validated lower limit of quantitation of 2.5 ng/mL for both AM and DEA based on 100 µL of rat plasma (Shayeganpour *et al.*, 2007). The

intraday and interday coefficients of variation were equal or less than 18%, and mean error was <12%.

4.2.8 Data analysis

All compiled data were expressed as means±SD unless otherwise indicated. To determine the kinetics of DEA formation by the various microsomal preparations, Vmax and km were determined by fitting the simple Michaelis-Menten model to the DEA formation rate vs. AM concentration data using non-linear regression analysis (Shayeganpour *et al.*, 2006b). To judge the goodness of fit, Aikake Information Criteron, sum of least squares analysis and correlation coefficients were used. The Vmax and km were thus determined. The intrinsic clearance (Cl_{int}) for AM metabolism to DEA was calculated by determining the quotient of Vmax to km.

In the inhibition studies which used large concentrations of AM and KTZ, the effect of KTZ on DEA formation was expressed as percentage of inhibition. The ki values were determined from the incubations with lower concentrations of AM and KTZ using a two step process (Bourrie *et al.*, 1996). After assay of the samples, the formation data for DEA were subjected to Lineweaver-Burke analysis. Linear regression analysis of the 1/DEA formation velocity to 1/AM concentrations yielded slopes that represented the km to Vmax ratios for each KTZ concentration. Following this, secondary plots of km/Vmax vs. KTZ concentration were constructed.

These plots were also subjected to linear regression analysis, with the x-axis intercept representing the ki values.

For the pharmacokinetic study, non-compartmental methods were used to calculate pharmacokinetic parameters. The elimination rate constant (λ_z) was estimated by subjecting the plasma concentrations in the terminal phase to linear regression analysis. The terminal elimination phase half-life ($t_{1/2}$) was calculated by dividing 0.693 by λ_z. The concentration at time 0 h after i.v. dosing was estimated by back extrapolation to time zero using the first three measured log-transformed concentrations (5 min, 20 min and 40 min) after dosing. Area under plasma concentration vs time curve ($AUC_{0-\infty}$) was calculated using the combined log-linear trapezoidal rule from time 0 h post dose to the time of the last measured concentration, plus the quotient of the last measured concentration divided by λz. C_{max} and t_{max} values were estimated by visual examination of concentration vs time profiles. Clearance (CL) was calculated as CL= Dose/$AUC_{0-\infty}$, and steady-state volume of distribution (Vd_{ss}) was calculated as Vd_{ss}= CL×AUMC/AUC, where AUMC is area under the first moment plasma concentration vs time curve, from time of dosing to infinity. The accumulation factor (R) was estimated as R= $(AUC_{0-T})_{n=4} \div (AUC_{0-T})_{n=1}$, where n is the number of doses. In the tissue distribution study, AUC_{0-24} for plasma or tissue concentrations were determined for AM and DEA from the time of dosing to the time of the last measured concentration (24 h).

In assessing the mechanism of inhibition of DEA on EROD, the equations describing competitive, non-competitive, uncompetitive and mixed type of inhibitions were fitted to the data as described previously (Cortes *et al.*, 2001; Venkatakrishnan *et al.*, 2003). The optimal mechanism of inhibition and the values of the inhibition constants were then determined from the model that yielded the least residual sum of squares and Akaike Information Criterion (Akaike, 1974).

To determine the kinetic constants for DEA formation by liver and kidney microsomal preparations, Michaelis-Menten models for single and two enzymes were fitted to DEA formation rates using non-linear regression analysis (Shayeganpour *et al.*, 2006a). The total sum of squares and Akiake information criteria were used to judge the goodness of fit. In liver and kidney microsomes, a single enzyme model was found appropriate and the intrinsic clearance (Cl_{int}) for DEA formation was calculated by determining the quotient of V_{max} to k_M. This model uses the following equation (Venkatakrishnan et al., 2003):

$$\boldsymbol{v} = \frac{\boldsymbol{V_{max}} \times [\boldsymbol{AM}]^{\boldsymbol{n}}}{[\boldsymbol{k_m}]^{\boldsymbol{n}} + [\boldsymbol{AM}]^{\boldsymbol{n}}}$$

Where v is the rate of DEA formation, V_{max} is the maximal rate of DEA formation, k_M is the affinity constant, [AM] is AM concentration and n is the shape factor required to fit sigmoidal shapes. When n=1, the model is reduced to the simple Michaelis-Menten equation. Since linear increases in DEA formation rates versus the tested range of AM

concentrations were mostly seen in lung, intestine and heart microsomes, linear regression was used for analysis of DEA formation rate vs. AM concentration data. The goodness of the fitted linear regression data was judged by the r^2 value. Cl_{int} was determined from the slope of the regression line through the concentration versus v relationships.

An *in vitro* half-life ($t_{1/2}$) method was used to assess DEA metabolism by rat CYP1A1 (Obach, 1999; Venkatakrishnan et al., 2003). The first order rate constant (k) for DEA depletion was determined from the slope of the linear regression line of the plot of log percentage DEA remaining vs incubation time. The amount of DEA measured at 0 min was set as 100%. $t_{1/2}$ and Cl_{int} were then calculated from the following equations, respectively

$$\boldsymbol{t_{1/2} = \frac{0.693}{k}}$$

$$\boldsymbol{Cl_{int} = \frac{0.693}{T_{1/2} \times [M]}}$$

Where [M] is the concentration of the microsomal protein in the incubation medium.

4.2.9 Statistical analysis

One-way analysis of variance, Duncan's multiple range post hoc test and Student's unpaired *t* tests were used as appropriate to assess the significance of differences between groups. Where the ranked data did not

conform to normality, Kruskal-Wallis one way analysis of variance on ranks and Dunn's test were used. Microsoft Excel (Microsoft, Redmond, WA), SPSS version 12 (SPSS Inc., Chicago, IL) or SigmaPlot 11.0 (Systat software, Inc. Chicago, IL) were used in statistical analysis of data. The level of significance was set at $p < 0.05$.

In the tissue distribution study, where destructive sampling was used, comparison of total $AUC_{0\text{-}24h}$ in CO- and BNF-treated rats was done according to Bailer's method (Bailer, 1988). Briefly, the SD of partial AUC was estimated to assess significance of differences. In this test, α was 0.05, the critical value of Z (Z_{crit}) for the 2–sided test after Bonferroni adjustment was 2.24, and the observed value for Z (Z_{obs}) was calculated as previously described (Bailer, 1988).

Chapter 5

5.0 Results

5.1 DEA formation by CYP isoenzymes known to contribute to DEA formation in humans: comparison to their rat counterparts

There were notable differences between the isoenzymes in the speed at which the formation of DEA occurred (Figure 1). For most CYP, the formation data were well fitted to the simple Michaelis-Menten model which permitted calculation of km, Vmax and Cl_{int} (Table 2). Only rat CYP1A2 did not conform to the model (Figure 2). It showed a linear increase in DEA formation rates within the range of concentrations tested. Cl_{int} for CYP1A2 (Table 2) was approximated by the slope of DEA formation rate velocity plots (Figure 2). In rat, CYP2D1 was by far the most prolific metabolizing enzyme, possessing the highest average Vmax and Cl_{int} (Table 2, Figure 1). In contrast CYP1A2 displayed the least Cl_{int} for formation of DEA. Although rat CYP1A1, 2C6 and 2C11 seemed to possess higher affinity for AM, significant differences among the isoenzymes were not apparent (Table 2). Kinetic analysis of DEA formation by the human CYP revealed that CYP1A1 had the highest DEA forming efficiency as indicated by its Cl_{int}. Furthermore, both CYP1A1 and 3A4 had significantly higher affinity for AM than the rest of the tested human isoenzymes, as indicated by their low km values (Table 2, Figure 1). The tested rat isoforms were ranked for Cl_{int} in the following descending order; CYP2D1 > 2C11 > [2C6 = 2D2 = 3A1 = 1A1 = 1A2]. In

contrast, human isoforms were ranked as follows; CYP1A1 > 3A4 > [2D6 = 1A2].

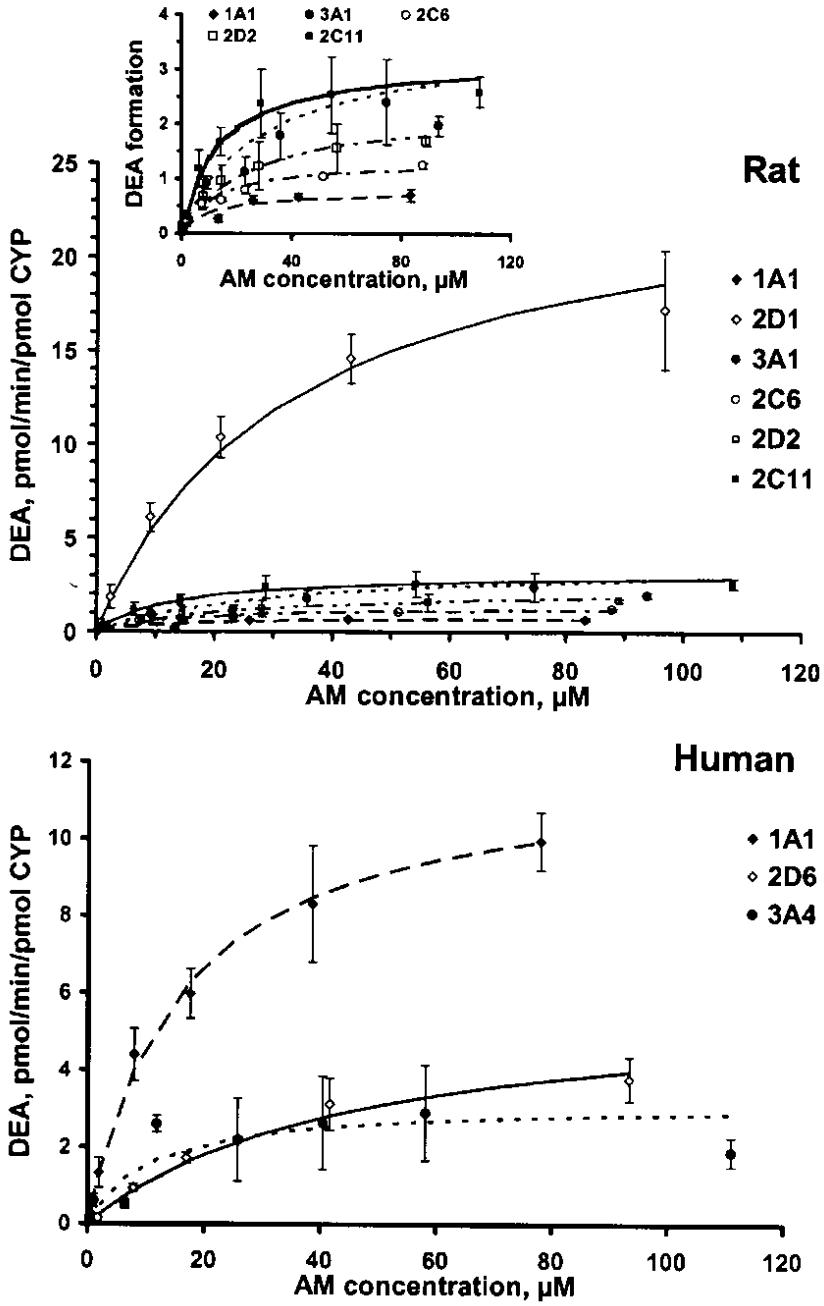

Figure 1: Desethylamiodarone (DEA) formation rates (mean±SD, n=3) vs. Amiodarone (AM) concentrations by rat (top panel) and human CYP (lower panel). The Michaelis Menten model was fitted to the data (lines). Inset in upper panel shows lower velocity rat CYP.

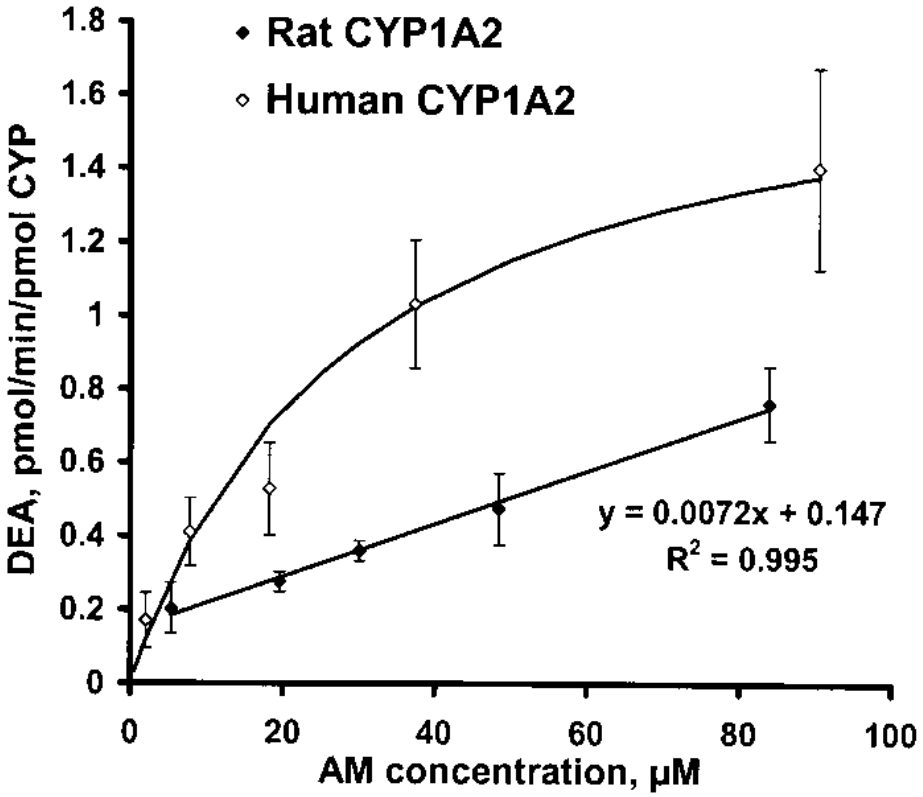

Figure 2: Desethylamiodarone (DEA) formation rates from amiodarone (AM) (means±SD, n=3) by rat CYP1A2, and human CYP1A2. The simple Michaelis-Menten model was fitted to the data for human CYP1A2 (line). Linear regression was used to get the slope of the line and estimate of Cl_{int} for rat CYP1A2.

Table 2: Desethylamiodarone (DEA) kinetic (means±SD, n=3) and inhibitory constants of V_{max}, k_m, Cl_{int} and ki in the presence of recombinant rat and human CYP isoenzymes.

CYP	Vmax, pmol/min/pmol CYP	km, µM	Cl_{int}, µL/min/pmol CYP	ki, nM
Rat				
1A1	0.770 ± 0.0546	10.7 ± 3.03	0.0753 ± 0.0162	379
1A2	ND	ND	0.00757 ± 0.000446	ND
2C6	1.34 ± 0.124	12.2 ± 2.29	0.111 ± 0.0130	1715
2C11	3.20 ± 0.662	13.3 ± 9.05	0.308 ± 0.150	2646
2D1	25.04 ± 7.031	33.6 ± 16.5	0.802 ± 0.173	4000
2D2	2.32 ± 0.460	25.6 ± 8.51	0.0970 ± 0.0333	89.3
3A1	3.57 ± 1.24	28.6 ± 13.5	0.130 ± 0.0216	ND
Human				
1A1	11.9 ± 1.58	15.9 ± 3.33	0.760 ± 0.0578	51.7
1A2	1.82 ± 0.550	28.9 ± 15.2	0.0685 ± 0.0186	4777
2D6	5.89 ± 1.29	45.5 ± 13.3	0.131 ± 0.0105	ND
3A4	3.15 ± 1.65	10.5 ± 3.73	0.304 ± 0.109	70.2
ND: Not determinable				

5.2 Inhibition of DEA formation by KTZ

In incubations containing high concentrations of drug and inhibitor, KTZ led to significant reductions in DEA formation rates by all of the rat isoenzymes except for CYP1A2 (Figure 3). The extent of inhibition was 86%, 76%, 58%, 88%, 55%, and 53% for CYP2D1, CYP2D2, CYP2C6, CYP2C11, CYP3A1 and CYP1A1, respectively (Figure 3). Interestingly, CYP3A1 seemed to be one of the least affected rat isoforms. Similar to rat, for the tested human isoforms, high concentrations of KTZ resulted in significant reductions in DEA formation rates by all of the tested enzymes (Figure 3). The human CYP most inhibited by KTZ were CYP1A1 and CYP3A4 (92.6% and 91.3% respectively). Human CYP2D6 (71.4 %) and CYP1A2 (50.9%) were somewhat less inhibited by KTZ.

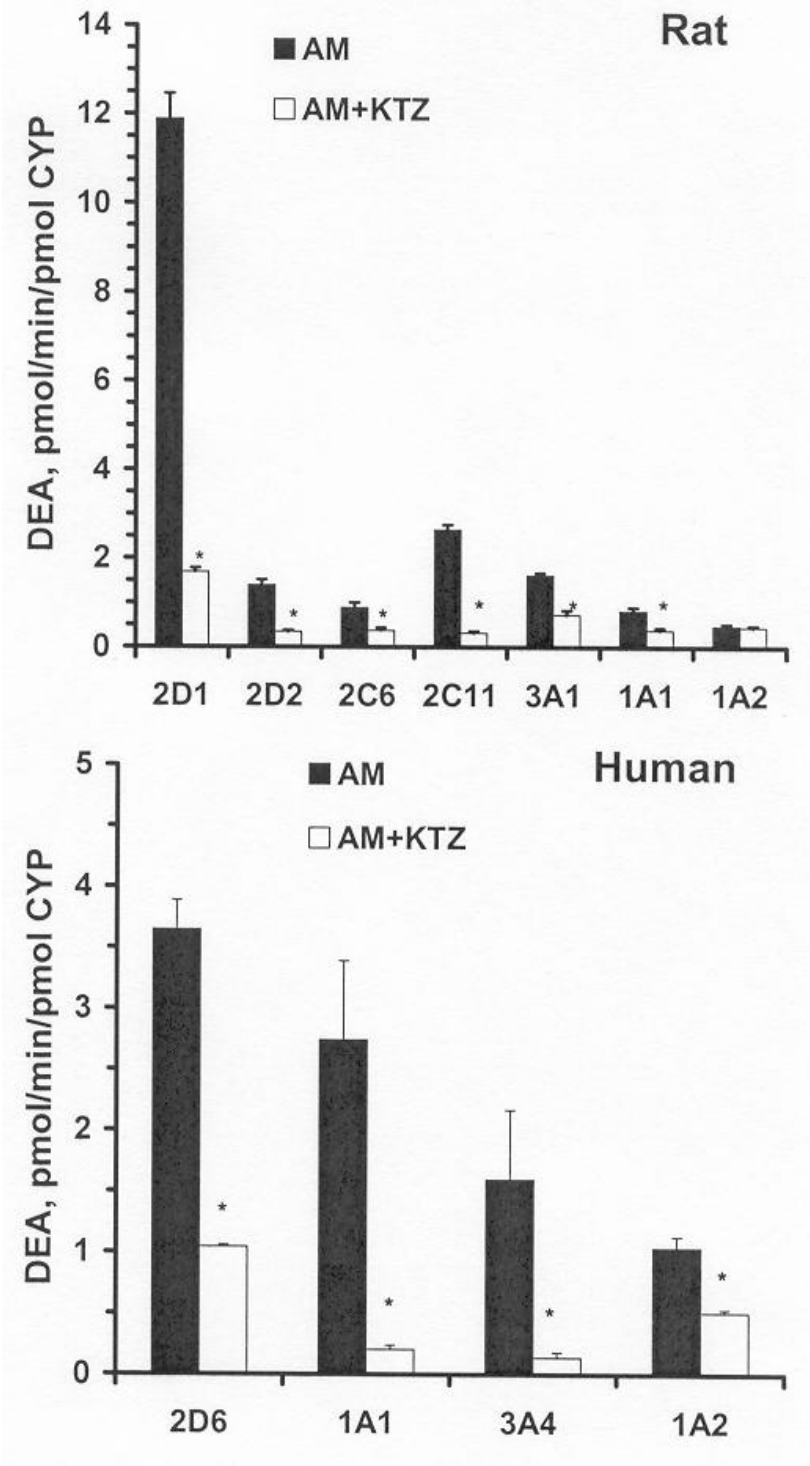

Figure 3: Desethylamiodarone (DEA) formation rates from amiodarone (AM) (mean±SD, n=3) in the presence and absence of 18.8 µM KTZ with 34.5 µM AM by rat CYP2D1, 2D2, 2C6, 2C11, 3A1, 1A1 and 1A2 and human CYP2D6, 1A1, 3A4, and 1A2. *Denotes a significant difference from respective controls.

Very good to excellent regression coefficients were obtained for most of the Lineweaver-Burke transformations (Figures 4 and 5). A concentration-dependent inhibition of DEA formation rate was observed in incubations containing rat CYP1A1, 2C6, 2C11, 2D2 and 2D1 and human CYP1A1, 1A2 and 3A4 (Figures 4 and 5). A concentration-dependent decrease in affinity (increase km) was observed for human CYP1A1 and 1A2 and rat CYP2C6 and 2D2, which is consistent with a competitive-type of inhibition. On the other hand, a concentration-dependent decrease in Vmax was observed for human CYP3A4 and rat CYP1A1, which is consistent with a noncompetitive-type of inhibition. A mixed-type of inhibition, characterized by an increase as well as a decrease in km and Vmax values, respectively, was observed for rat CYP2C11 and 2D1.

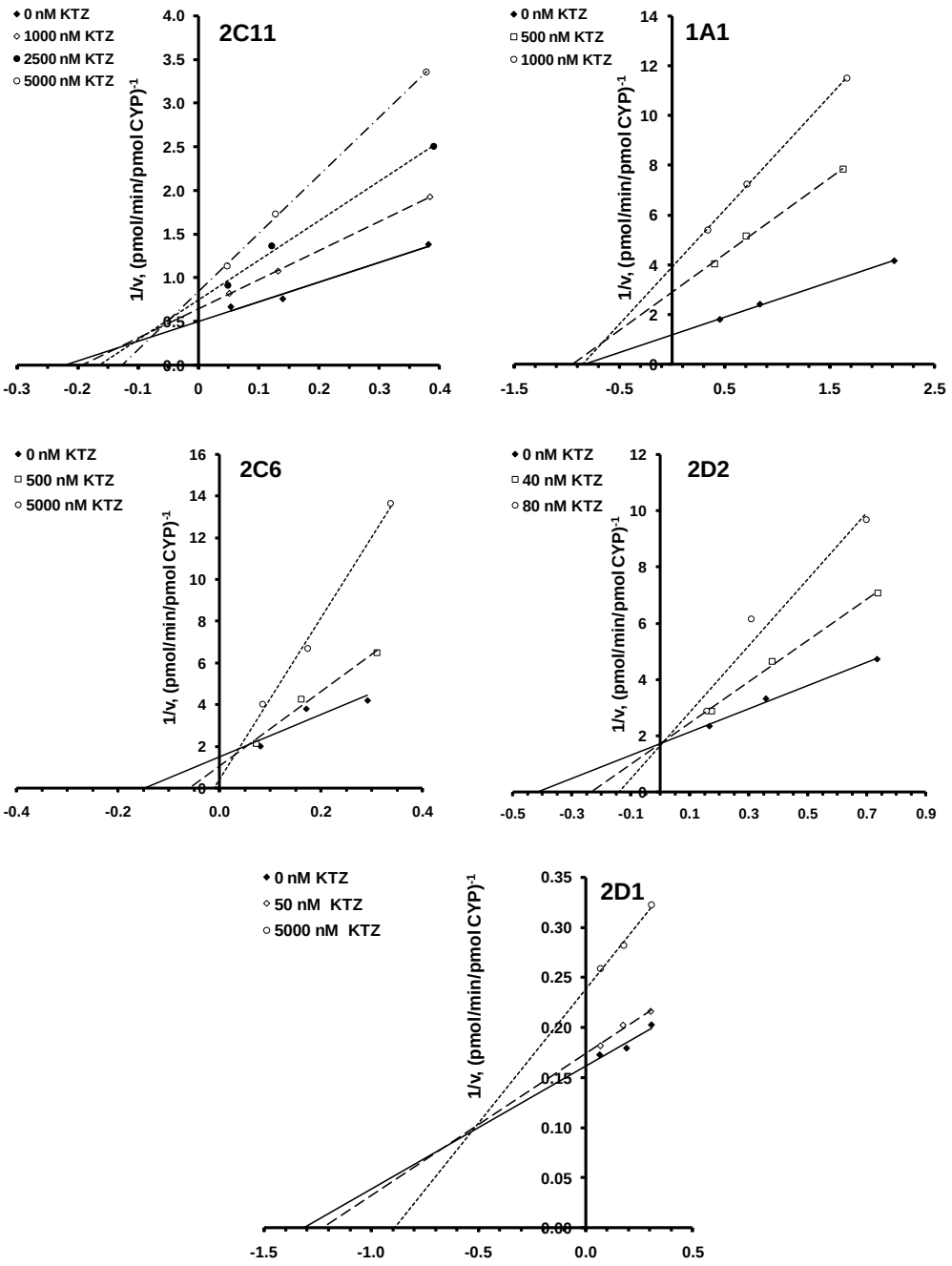

Figure 4: Lineweaver-Burke plots for rat CYP1A1, 2C6, 2C11, 2D1, and 2D2.

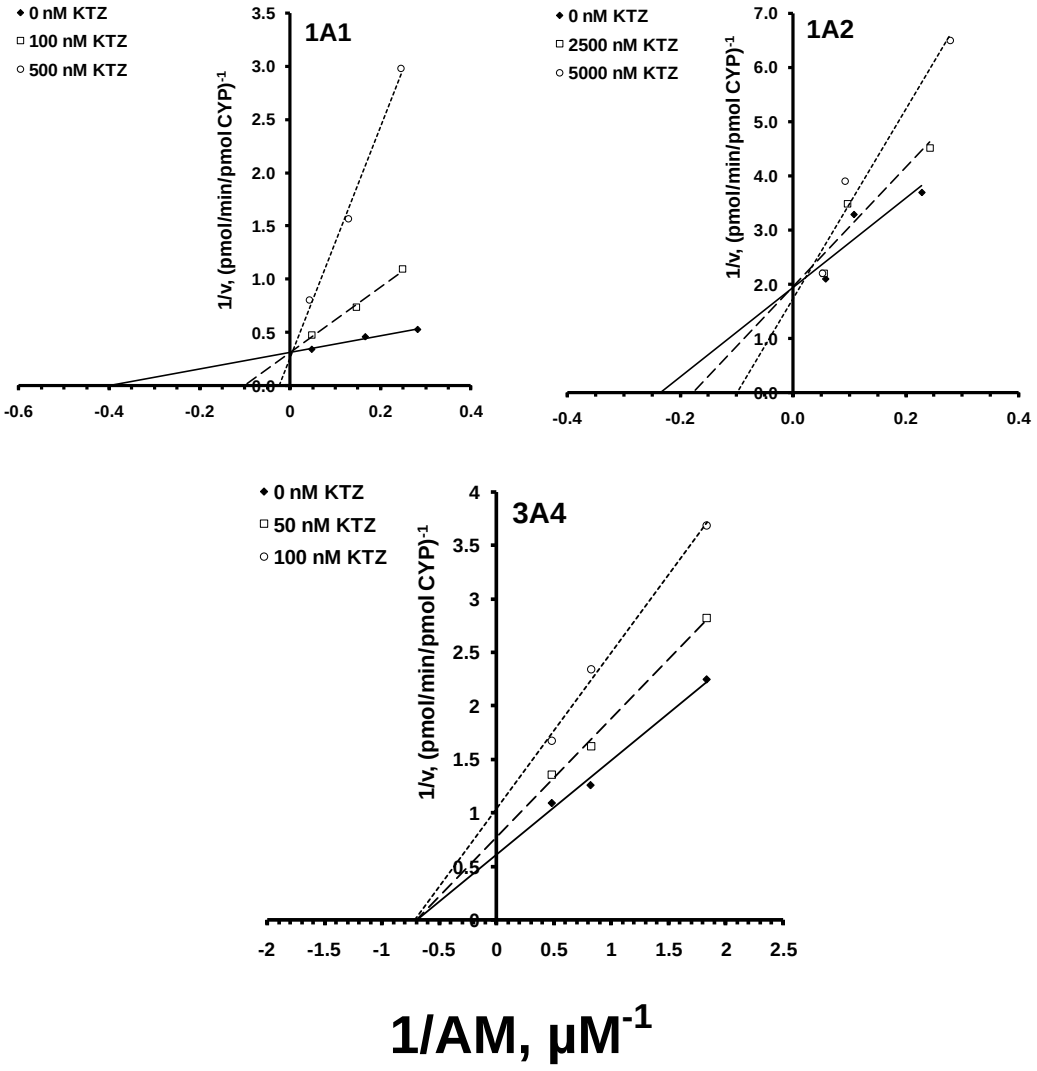

Figure 5: Lineweaver-Burke plots for human CYP1A1, 1A2and 3A4.

In the secondary plots used to determine ki (Figure 6), means±SD of r^2 of slopes for different KTZ concentrations were 0.97±0.029, 0.97±0.048, 0.99±0.014, 0.90±0.10 and 0.95±0.060 for rat 2D2, 1A1, 2C11, 2D1 and 2C6, respectively. For human isoenzymes, r^2 values of Lineweaver-Burke plots were 0.95±0.091, 0.82±0.12 and 0.98±0.020 for 1A1, 1A2 and 3A4, respectively. The determined ki values were noticeably lowest for rat CYP1A1 and 2D2, and for human 1A1 and 3A4 isoforms (Figure 6, Table 1). The inhibition was much weaker for rat CYP2C6, 2C11 and 2D1 and human 1A2. Although high concentrations of KTZ caused inhibition of DEA formation by human 2D6 (Figure 3), at lower concentrations there was no apparent decrease in DEA formation.

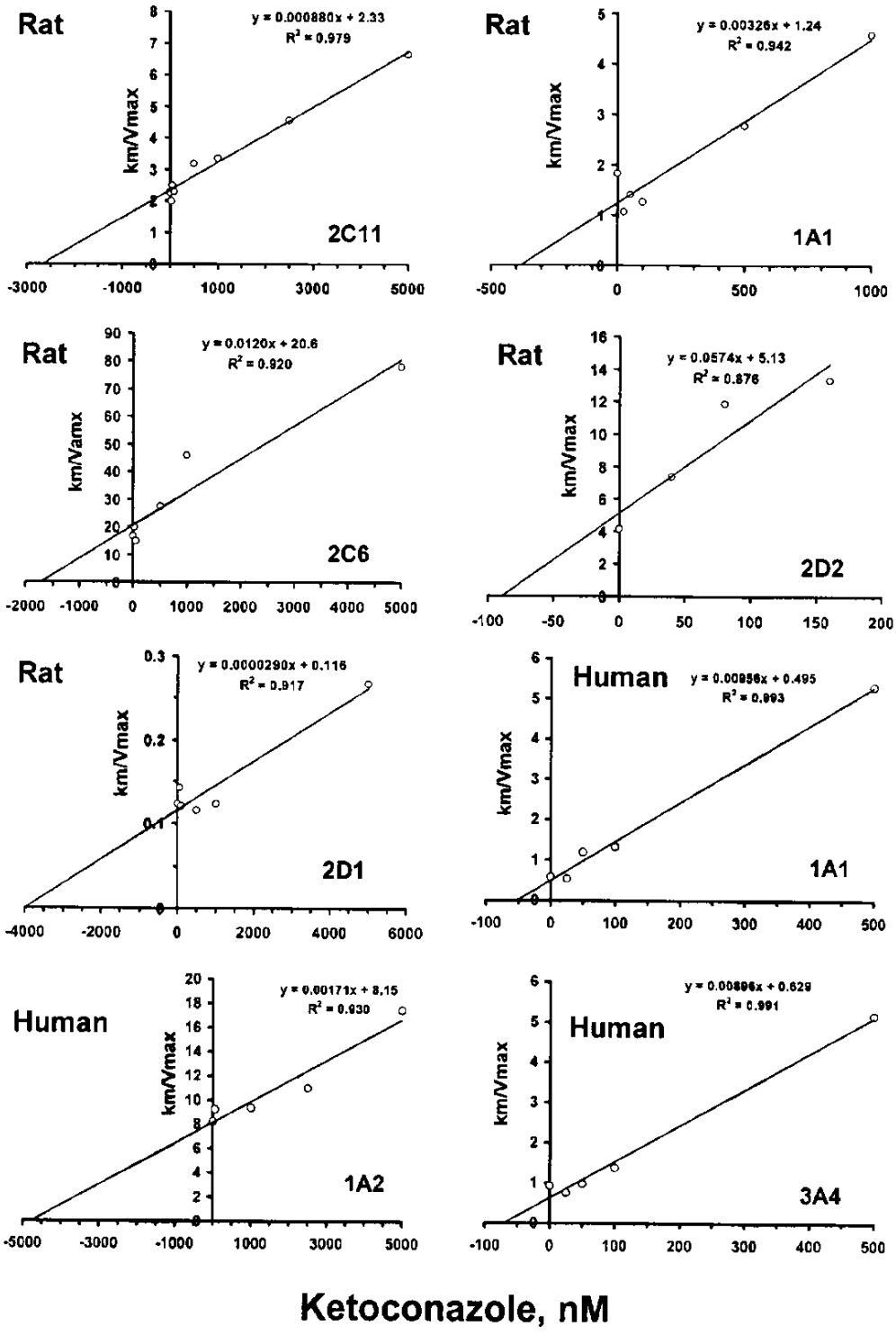

Figure 6: Secondary plots of k_m/V_{max} (min/pmol CYP/mL) vs. KTZ concentration for A.) rat CYP1A1, 2C6, 2C11, 2D1, and 2D2 and B.) human CYP1A1, 1A2, 3A4. The ki values are provided in Table 2.

A paradoxical increase in DEA formation rates was observed when low concentrations of KTZ were incubated with AM in CYP3A1 incubations (Figure 7). To better explore this unexpected finding, similar concentrations of AM and DEA were spiked with CYP3A1 and the decrease in concentrations of both analytes, and concentrations of DEA formed from AM, were assessed. It was noted that when directly spiked with CYP3A1, mean DEA concentrations decreased minimally and non-significantly. In contrast, the concentrations of AM were observed to significantly decrease by up to 60% or more. Increasing KTZ caused the reductions in AM concentrations to be attenuated (Figure 7). This corresponded with a reduction in the DEA formed from AM at concentrations of 5000 nM of KTZ. It was also noted that upon incubation with CYP3A1, there was approximately 28% of the initial AM unaccounted for.

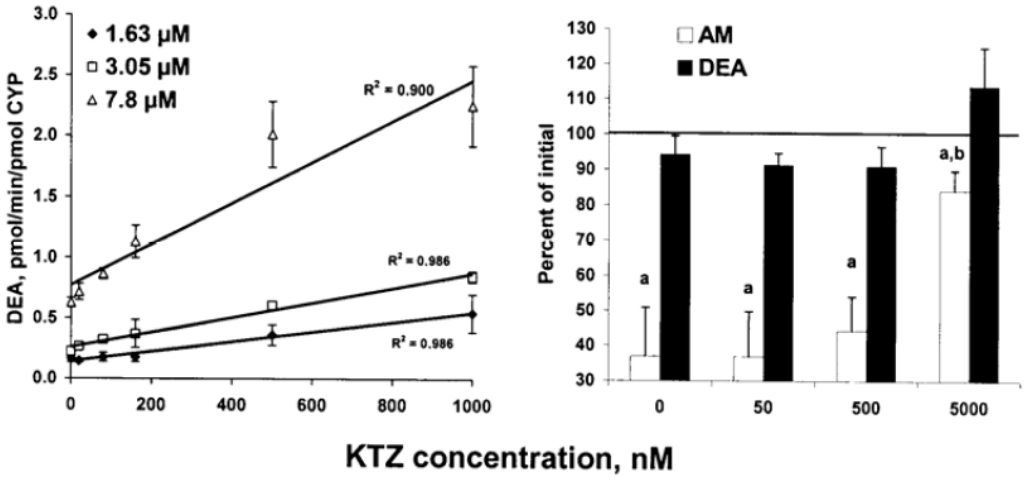

Figure 7: Formation of desethylamiodarone (DEA) by CYP3A1. Left panel, DEA formation (mean±SD) when AM (1.63, 3.05 and 7.8 µM) was present with and without KTZ. Right panel, percent of AM or DEA remaining when 1 µM of each was incubated with CYP3A1 in the presence and and absence of varying concentrations of KTZ. [a] denotes significant difference from amount originally added. [b] denotes significant difference from the microsomal incubation without KTZ.

5.3 Effect of BNF on AM pharmacokinetics

Plasma concentrations of AM resulting after administration of a single i.v. dose of AM HCl declined in a manner consistent with multicompartmental pharmacokinetics in both groups (Figure 8). Furthermore, in terms of AUC there was no significant difference between the plasma AM concentrations measured in BNF- and CO-treated rats (Table 3). Additionally, no significant differences were noticed in other AM pharmacokinetic parameters such as $t_{1/2}$, CL, Vd_{ss} and Vd_{β} measured in BNF- and CO-treated rats (Table 3). In contrast to AM, for DEA the CYP1A1-induced animals had significant 2.9-, 3.2-, 3.4-fold increase in the $AUC_{0\text{-}24h}$, $AUC_{0\text{-}48h}$ and $AUC_{0\text{-}\infty}$, respectively (Figure 8, Table 3). Additionally, there were no significant differences between the C_{max}, t_{max}, and $t_{1/2}$ of the DEA metabolite in BNF- and CO-treated rats.

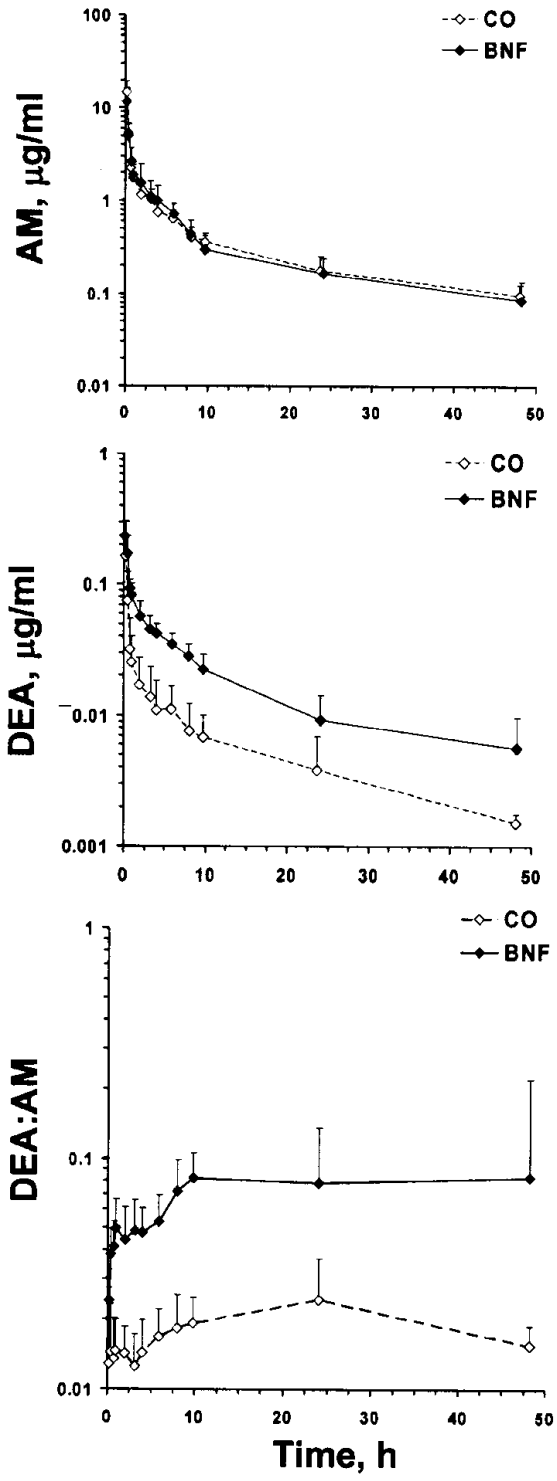

Figure 8: First and second panels represent plasma concentration versus time curves of amiodarone (AM) and desethylamiodarone (DEA) (means±SD) following intravenous administration of 25 mg/kg of AM HCl to corn oil- or β-naphthoflavone-treated rats. The third panel represent concentration ratios of DEA/AM in corn oil- or β-naphthoflavone-treated rats.

Table 3: Means±SD (range in parentheses) of pharmacokinetics of amiodarone (AM) after the administration of a single iv bolus dose of AM HCl 25 mg/kg. Asterisks represent significant differences between corn oil- and the β-naphthoflavone-treated rats.

	Control		**BNF**	
	AM	**DEA**	**AM**	**DEA**
AUC_{0-24h}	17.0 ± 3.26	0.244 ± 0.146	17.0 ± 7.30	0.701 ± 0.135*
(μg·h/L)	(13.5-22.0)	(0.172-0.540)	(10.8-32.3)	(0.525-0.961)
$AUC_{0-\infty}$	22.4 ± 4.22	0.316 ± 0.180	28.5 ± 21.5	1.07 ± 0.388*
(μg·h/L)	(18.1-29.4)	(0.197-0.679)	(13.3-78.2)	(0.605-1.77)
C_{max}	-	0.164 ± 0.133	-	0.253 ± 0.0819
(μg/mL)		(0.0796-0.426)		(0.172-0.379)
t_{max}[a]	-	0.150	-	0.150
(h)		(0.100-0.317)		(0.100-0.200)
$t_{1/2}$	18.6 ± 2.89	17.3 ± 6.11	17.9 ± 3.92	17.1 ± 10.8
(h)	(14.6-21.9)	(9.02-27.0)	(11.6-22.7)	(6.04-42.1)
CL	1.14 ± 0.199	-	1.30 ± 0.433	-
(L/kg/h)	(0.850-1.29)		(0.692-1.88)	
Vd_{ss}	18.8 ± 6.38	-	20.1 ± 7.06	-
(L/kg)	(7.18-26.6)		(11.1-32.6)	
Vd_{β}	30.8 ± 7.58	-	33.7 ± 10.4	-
(L/kg)	(21.4-43.7)		(18.5-47.8)	

[a] indicate median values

5.4 Effect of BNF on AM tissue distribution

Generally, AM concentrations were higher than those of DEA in plasma and tissues of both CO- and BNF-treated rats. Consistent with earlier reports, plasma concentrations of both AM and DEA were the lowest compared to the rest of the tissues, whereas lung showed the highest levels of both AM and DEA. High concentrations of AM in CO- and BNF-treated tissues were observed as quickly as 5 min after dosing, after which exponential decline in the measured concentrations was observed (Figure 9).

The mean C_{max} values for AM were 265, 131, 68.9, 90.4, and 27.5 µg/mL or g in control lung, liver, kidney, heart, and intestine samples, respectively. In BNF-treated tissues, the mean C_{max} values for AM were 251, 100, 54.3, 103, and 26.6 µg/mL or g in lung, liver, kidney, heart, and intestine samples, respectively. Additionally, in control rats the C_{max} values for DEA occurred at about 5 min in plasma and liver, 1 h in kidney and heart, and 3 h in lung and intestine samples (Figure 9). The observed C_{max} values for DEA were 0.200, 31.5, 7.29, 4.07, 3.25, and 5.02 µg/mL or g in control plasma, lung, liver, kidney, heart, and intestine samples, respectively. Furthermore, the maximum DEA concentrations were observed at about 1 h in all of the tested BNF-treated plasma and tissue samples, with estimated C_{max} values of 0.217, 42.9, 22.9, 14.3, 8.03, and

9.78 µg/mL or g in BNF-treated plasma, lung, liver, kidney, heart, and intestine samples, respectively.

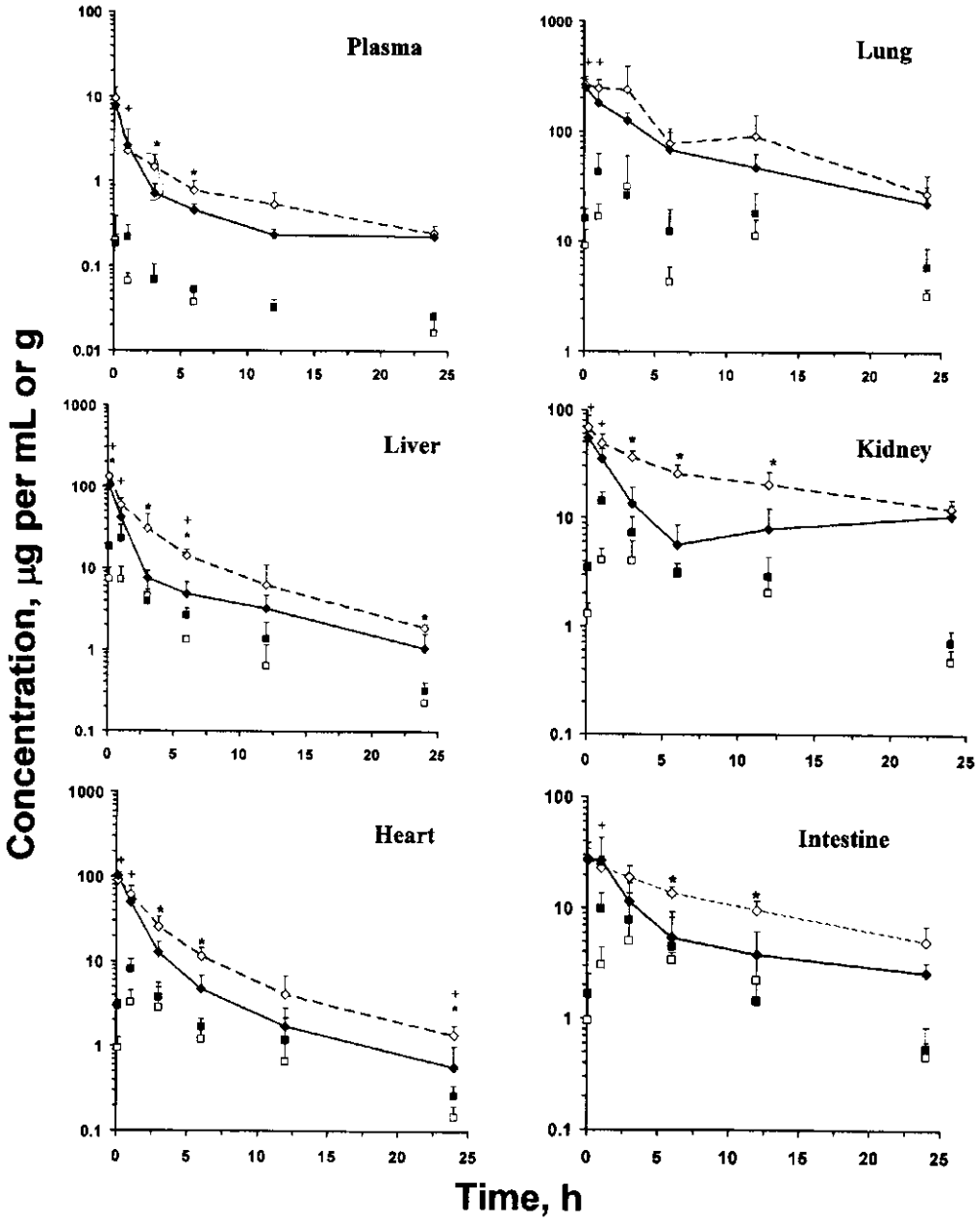

Figure 9: Concentrations (mean±SD) of amiodarone (AM) (CO ◊ , BNF ◆) and desethylamiodarone (DEA) (CO □ , BNF ■) versus time curves in plasma, lung, liver, kidney, heart and intestine following four intravenous doses of 25 mg/kg/d of AM HCL to corn oil- or β-naphthoflavone-treated Sprague-Dawley rats.* and + indicate significant differences between corn oil- and β-naphthoflavone-treated rats at that time point ($p<0.05$).

Unlike the case for the single dose study, BNF treatment resulted in a general decrease in the AM $AUC_{0\text{-}24h}$ after repeated doses. The decrease in AM $AUC_{0\text{-}24h}$ was significant in plasma and tissues of BNF- compared to CO-treated rats (Table 4, Figure 9). The percentage reductions were 30%, 35%, 48%, 52%, 34%, and 43% in BNF-treated plasma, lung, liver, kidney, heart and intestine, respectively. For DEA, BNF treatment caused an overall increase in the concentrations of DEA. The increase in DEA $AUC_{0\text{-}24h}$ was statistically significant in BNF-treated plasma, lung, liver, kidney, and heart samples. The percent of increases observed in these tissues were 36%, 56%, 101%, 65%, and 73%, respectively (Table 4, Figure 9). Although a trend of higher DEA levels was apparent in BNF-treated intestine (27%), a statistically significant difference was not observed (Table 4, Figure 9). Furthermore, the DEA/AM ratios of the $AUC_{0\text{–}24h}$ were higher in the BNF plasma, lung, liver, kidney, and heart by 1.9-, 2.5-, 3.8-, 3.4-, and 2.7-fold, respectively (Table 4, Figure 10). In a less intensive assessment, we tested the effect of BNF treatment on AM and DEA uptake in the brain and spleen at a single time point (6 h) after AM administration. The levels of DEA were significantly higher in BNF-treated brain (0.22 µg/g) and spleen (4 µg/g) compared to the control brain (0.15 µg/g) and spleen (2.8 µg/g). Levels of AM, on the other hand, were significantly lower in BNF-treated spleen (12.2 µg/g) compared to the control (25.3 µg/g). Unlike spleen, levels of AM were not

significantly altered in BNF-treated brain compared to the CO-treated ones.

The mean accumulation factors after multiple doses of AM were estimated to be 1.27 and 0.895 in the CO- and BNF-treated rats, respectively. On the other hand, the accumulation factors estimated for DEA were higher (3.8 and 1.8, respectively) than that of AM.

Table 4: Tissue uptake of amiodarone (AM) and desethylamiodarone (DEA) in rats given iv bolus dose of AM HCl 25 mg/kg/d, for 4 d. The means±SD of AUC0-24h (µg.h/g) is shown. *represents significant differences between the corn oil- and the β-naphthoflavone-treated rats.

	Control			BNF			BNF:Control	
	AM	DEA	DEA/AM	AM	DEA	DEA/AM	AM	DEA
Plasma	21.7±1.52	0.934±0.0775	0.043	15.3± 1.12*	1.27 ± 0.0663*	0.083	0.705	1.36
Lung	2430±281	249±35.7	0.10	1570 ± 125*	388 ± 48.8*	0.25	0.646	1.56
Liver	362±28.6	38.9±5.92	0.11	187 ± 13.8*	78.1 ± 8.33*	0.42	0.517	2.00
Kidney	566±33.1	51.6±3.93	0.091	273 ± 23.8*	85.0 ± 8.17*	0.31	0.482	1.64
Heart	296±19.6	24.5±3.25	0.083	196 ± 10.8*	42.4 ± 5.26*	0.22	0.662	1.73
Gut	273±13.8	55.7±7.70	0.20	156 ± 18.6*	70.8 ± 11.8	0.45	0.571	1.27

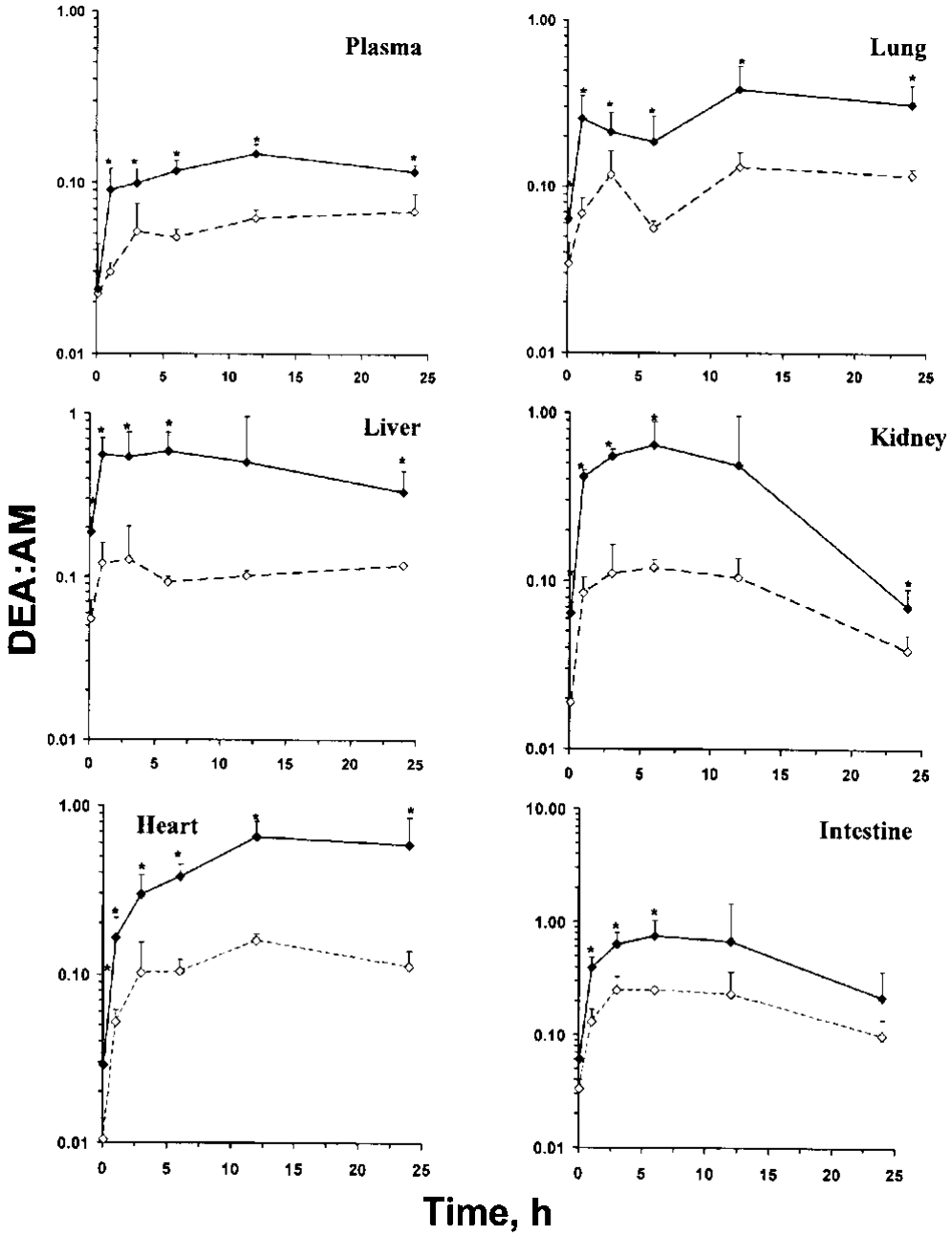

Figure 10: The ratio of desethylamiodarone (DEA):amiodarone (AM) (means±SD) versus time curves measured in plasma, lung, liver, kidney, heart and intestine following the intravenous administration of four doses of 25 mg/kg/d of AM HCl to corn oil- or β-naphthoflavone-treated Sprague-Dawley rats. Open and closed symbols indicate corn oil- and β-naphthoflavone-treated rats, respectively. * indicate significant difference ($p<0.05$).

5.5 Effect of DEA on human recombinant CYP1A1 activity and assessing of inactivation potential

5.5.1 DEA metabolism and CYP1A1 inhibition

With coincubation of DEA and CYP1A1, no significant reduction in DEA was observed at any of the predetermined time points (Figure 11). Although not a substrate for CYP1A1, DEA still caused a significant reduction of 38% in EROD when coincubated with 7-ER. The resorufin formation rates were 24±2.3 and 15±1.9 pmol/min/pmol CYP in 0 and 5 µM DEA-containing incubations, respectively. This was consistent with the finding that with coincubation of preformed DEA and AM, the measured DEA was significantly 19% lower than expected based on incubation of AM alone (Table 5).

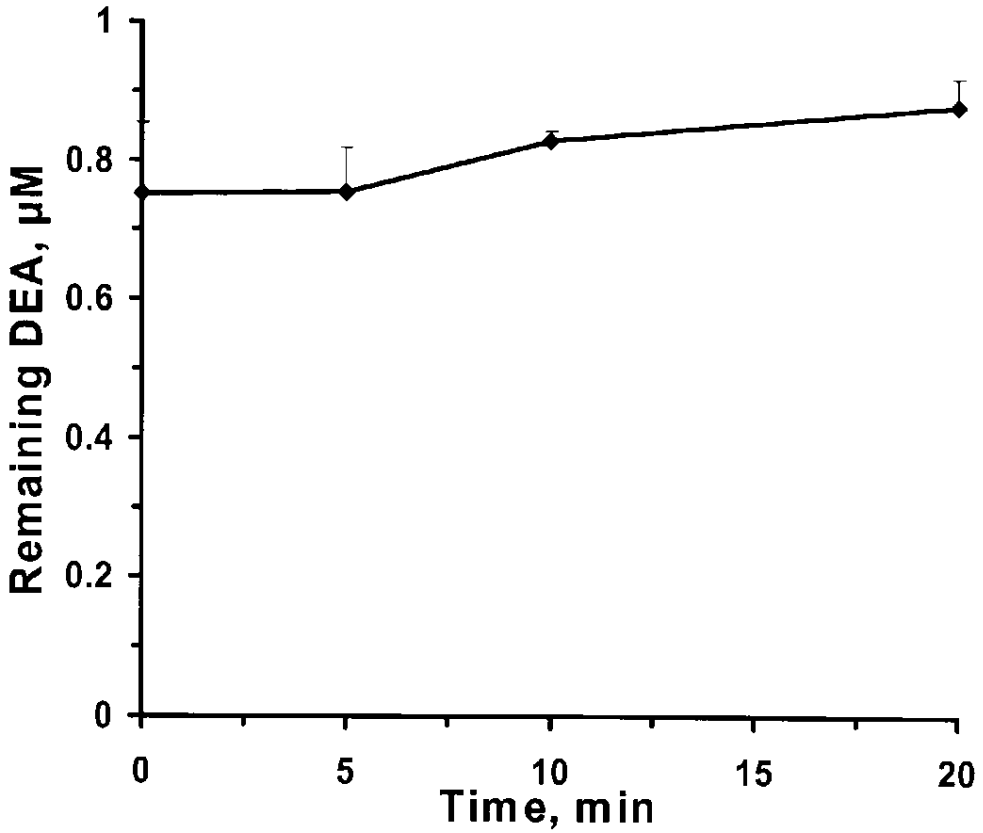

Figure 11: Concentration of desethylamiodarone (DEA) remaining vs. incubation times. DEA (0.8 µM) was incubated with 20 pmol/ml human CYP1A1 and NADPH for 0, 5, 10, and 20 min.

Table 4: The self-inhibiting effect of DEA on CYP1A1-mediated biotransformation of AM to DEA.

Incubations	Amount of DEA (μM)
$DEA_{Initial}$ (in DEA-only incubations)	8.34 ± 0.28
DEA_{Formed} (in AM-only incubations)	6.95 ± 0.14
$Total_{Assayed}$ (in AM+DEA incubations)	12.4 ± 0.42
$Total_{Exp}$ ($DEA_{Initial}$ + DEA_{Formed})	15.3 ± 0.28*

128 μM AM was incubated with 40 pmol/ml CYP1A1 in the presence and absence of 8.3 μM DEA. Tubes containing the added amount of DEA without CYP1A1 were assayed for the initial amount of DEA ($DEA_{initial}$). The expected total amount of DEA ($Total_{exp}$) in the AM+DEA containing tubes was estimated as ($Total_{exp}$) = $DEA_{initial}$+DEA_{formed}, with DEA_{formed} being the amount assayed in the AM-only incubations. * denotes significant difference between the expected amount of DEA ($Total_{Exp}$) and actual measured amount in AM+DEA incubations ($Total_{Assayed}$).

In assessing the mechanism of inhibition, observed data were best fitted to the following equation:

$$v = \frac{V \times a}{k_m \cdot \left[1 + \frac{[I]}{K_{ic}}\right] + a \cdot \left[1 + \frac{[I]}{K_{iu}}\right]}$$

in which V is the limiting rate, a is the substrate concentration (7-ER), k_m is the Michaelis constant, [*I*] is the concentration of the inhibitor (DEA), K_{ic} is the competitive inhibition constant and K_{iu} is the uncompetitive inhibition constant. In competitive inhibition [*I*]/K_{iu} is negligible, in uncompetitive inhibition [*I*]/K_{ic} is negligible, and in pure non-competitive inhibition the two inhibition constants are equal, K_{ic}= K_{iu} (Cortes *et al.*, 2001). The data were plotted in two different ways, 1/v vs [*I*] (Dixon plot) and 7-ER/v vs [*I*] plots

(Figure 12). The different lines represent resorufin formation rates at various substrate (7-ER) concentrations. These lines meet in a point which is below the abscissa axis in the 7-ER/v vs [*I*] plot (Figure 12, upper panel) and above the axis in the plot of 1/v vs [*I*] (Figure 12, lower panel). These plots indicate that the mechanism of inhibition is mixed with a predominantly competitive component as described previously (Cortes *et al.*, 2001). The competitive (k_{ic}) and the uncompetitive inhibition constants (k_{iu}) were determined to be 2.1 µM and 9.6 µM, respectively.

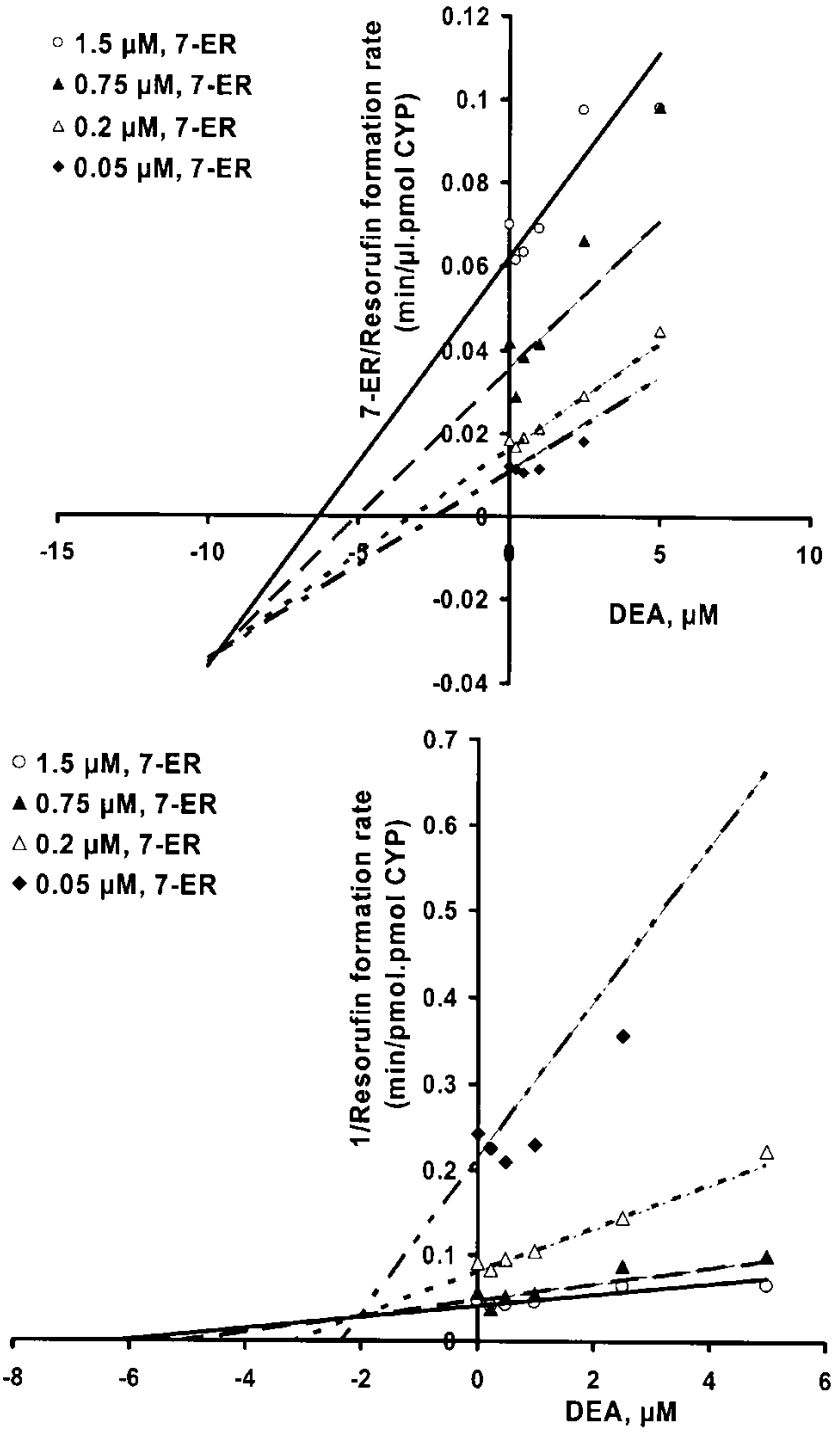

Figure 12: The inhibition of human CYP1A1 by desethylamiodarone (DEA). Upper Panel, 7-ER/Resorufin formation rate against DEA concentrations plot of the data. Lower Panel, Dixon plot (1/Resorufin formation rate against DEA concentrations) of the inhibition data is presented. The results of the mixed-competitive model fitted data in both graphs are represented by the lines.

5.5.2 Assessment of Inactivation

In Protocol A, the remaining EROD was not significantly altered by DEA (Figure 13A). Using Protocol B, however, EROD was only discernable in the 0 and 2 μM DEA-containing tubes at the 0 min preincubation time (Table 6), where EROD activity was significantly lower with 2 μM DEA (33%). When extra NADPH was added concurrently with 7-ER, there was some recovery of EROD (Table 6).

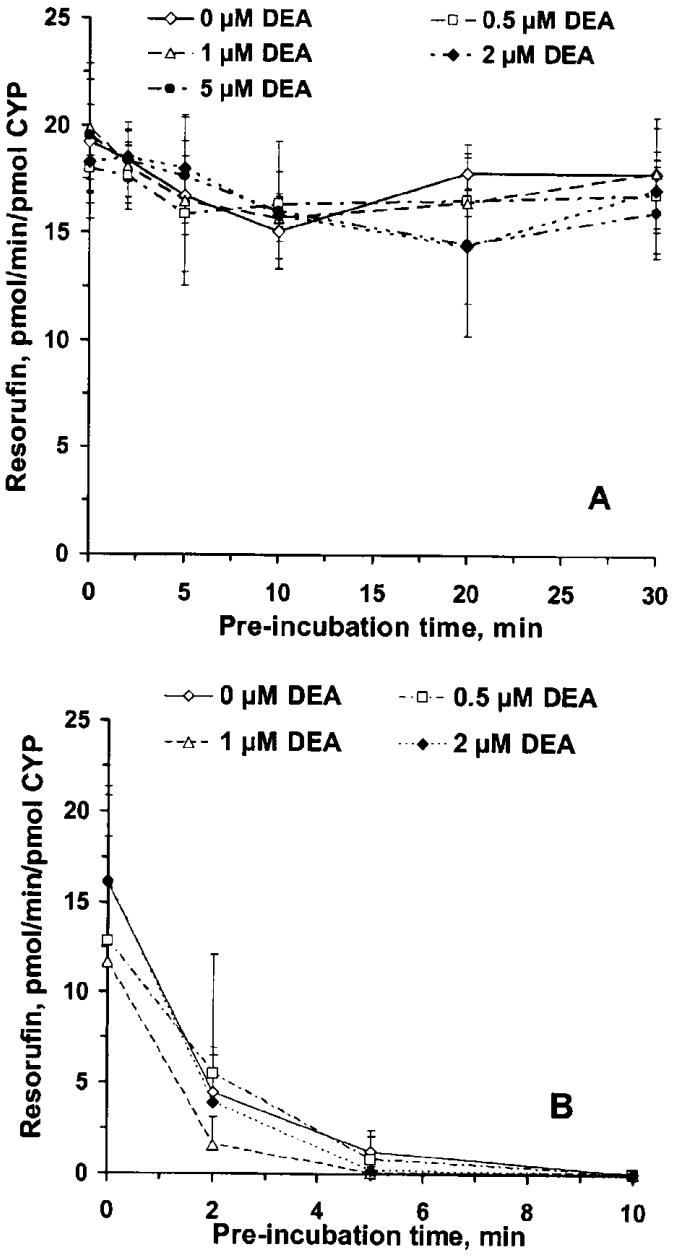

Figure 13: Results from inactivation experiments. Each data point represents the mean±SD of 3 to 5 replicates. A.) Data obtained using Protocol A where dilution of preincubation in 7-ER-containing media was performed. No significant differences were detected as a function of DEA concentration. B.) Inactivation data obtained where NADPH was not added to the buffer containing the 7-ER-containing media, only in the aliquot transferred from the preincubation buffer (see ”4.2.5.5.3 Experimental impact of NADPH in the pre- and post-incubation phases” in Methods). For each DEA concentration, resorufin formation rates at 0 min were significantly ($p<0.05$) higher than at the corresponding later time points. There were, however, no differences in EROD at each discrete preincubation time, between DEA concentrations of 0, 0.5, 1 and 2 µM.

Table 5: Inactivation experiments conducted using Protocol B. Resorufin formation rates (mean±SD) are shown in the presence of 0 and 2 µM desethylamiodarone (DEA).

Preincubation time, min	Resorufin formation rates, pmol/min/pmol CYP	
	0 µM DEA	2 µM DEA
0	3.06±0.28 (2.76-3.49)	2.06±0.23* (1.76-2.39)
10	ND	ND
20	ND	ND
30	ND	ND
30[a]	0.83±0.35 (0.43-1.06)	0.92±0.25 (0.62-1.06)

ND, not detected.

* Significant differences between 0 and 2 µM DEA (unpaired Student's t-test, $p<0.05$).

[a] Modification where 0.25 mM NADPH was added at the time of 7-ER addition.

5.5.3 Experimental impact of NADPH in the pre- and post-incubation phases

In tubes where NADPH was absent in the preincubation phase with or without DEA, there was no significant change in the activity with longer preincubation times. Thus DEA had no direct effect on CYP1A1 activity in the absence of NADPH. When NADPH was present in the preincubation but not supplemented in the incubation phase, EROD was still measurable. However, as time increased there were progressive decreases in EROD in both DEA-free and DEA-containing samples (Figure 13B). Specifically, preincubations of 2, 5 and 10 min produced decreases in activity of 72.0, 92.0 and 99.5%, respectively in the DEA-free samples. This was consistent with Protocol B where EROD was totally absent in the DEA-free tubes at preincubation times of 10 min or longer (Table 6). Furthermore, the CYP1A1 activity in the presence of DEA was not significantly different from that in DEA-free incubations (Figure 13B).

5.6 Role of CYP1A1 induction in DEA formation and effect of AM treatment on CYP1A1/2 gene expression and activity

5.6.1 CYP1A1 and CYP1A2 gene expression in CO-, CO+AM-, BNF-, BNF+AM-treated rats

BNF treatment was associated with significant increases in liver mass compared to the CO treated rats. Liver weights were 17±4.1 g and 13±2.8 g in BNF- and CO-treated rats, respectively. When the liver weight was normalized to body weight measured at the time of collection, the ratios were 0.040±0.0036 and 0.051±0.0050 in the control and BNF-treated rats, respectively ($p<0.05$). The rest of the collected organs showed no significant change in response to BNF treatment.

In terms of the expression of CYP1A1 relative to CYP1A2 in each of the tested organs, CYP1A2 gene expression was significantly higher than CYP1A1 in livers from rats of each group. For example, the levels of CYP1A1 relative to CYP1A2 were 0.00192±0.000569, 0.00561±0.00645, 0.633±0.255 and 0.720±0.195 in CO-, CO+AM-, BNF- and BNF+AM-treated livers, respectively. On the other hand, in each of the examined extra-hepatic tissues, CYP1A1 gene expression was significantly higher than that of CYP1A2. Levels of CYP1A1 mRNA relative to CYP1A2 mRNA ranged from 10 to 422 and 65 to 9639 in different extra-hepatic tissues from control- and BNF-treated groups, respectively.

BNF treatment caused increases in CYP1A2 gene expression in the liver and intestine ($p<0.05$; Table 7). In contrast, CYP1A2 gene

expression was unaffected by BNF treatment in lung, kidney and heart tissues. For CYP1A1, significant induction of gene expression was noticed in all of the tested tissues (Table 7). Comparison of the fold of induction among the tissues revealed that the highest fold of increase in CYP1A1 gene expression occurred in the liver > [heart and kidney] > [lung and intestine]. Treatment with AM did not significantly alter CYP1A1 or 1A2 basal gene expression in the control rats. However, when co-administered with BNF, CYP1A1 gene expression was higher in liver, lung and kidney (Table 7).

Table 7: Fold change in CYP1A1 and CYP1A2 mRNA expression (mean±SD) in β-naphthoflavone (BNF)-, corn oil (CO)-, amiodarone+β-naphthoflavone (AM+BNF)- and amiodarone+corn oil (AM+CO)- treated rats

Treatment	CYP1A1				CYP1A2			
	CO	**CO+AM**	**BNF**	**BNF+AM**	**CO**	**CO+AM**	**BNF**	**BNF+AM**
Liver	1.04±0.309 (n=4)	2.14±2.47 (n=4)	2500±1007[a] (n=4)	3716±1076[a,b] (n=10)	1.02±0.230 (n=4)	0.709±0.115 (n=4)	7.34±1.31[a] (n=4)	9.00±2.02[a] (n=8)
Lung	1.03±0.278 (n=4)	2.28±1.43 v(n=4)	42.8±14.0[a] (n=4)	111±50.4[a,b] (n=5)	1.02±0.192 (n=4)	0.955±0.506 (n=4)	0.647±0.115 (n=4)	0.909±0.317 (n=5)
Intestine	1.05±0.337 (n=4)	2.05±0.867 (n=4)	33.5±12.4[a] (n=4)	41.0±24.3[a] (n=5)	1.21±0.796 (n=4)	0.829±0.182 (n=4)	126±122[a] (n=5)	173±144[a] (n=5)
Kidney	1.21±0.671 (n=4)	2.24±0.854 (n=4)	479±191[a] (n=4)	637±66.0[a,b] (n=8)	1.42±1.36 (n=4)	7.52±7.92 (n=4)	1.30±0.364 (n=4)	2.72±1.12 (n=8)
Heart	1.05±0.362 (n=4)	1.64±0.745 (n=4)	408±234[a] (n=4)	437±45.6[a] (n=5)	1.13±0.564 (n=4)	1.14±0.520 (n=4)	3.28±3.26 (n=4)	1.23±0.531 (n=5)

[a] Different from CO groups ($p<0.05$). [b] Different from BNF ($p<0.05$). Comparisons were done using one-way analysis of variance followed by Duncan's multiple range post hoc tests.

5.6.2 CYP1A1 and 1A2 activity in CO-, CO+AM-, BNF-, BNF+AM-treated rats

Treatment with BNF increased the EROD in the tested microsomes (Table 8) by averages of 17.5, 17.2, 2.49, 459 and 3.42-fold in liver, lung, intestine, kidney and heart respectively. The median values of folds of EROD induction were 15.7, 16.5, 1.43, 473, 3.96- fold in liver, lung, intestine, kidney and heart, respectively. When ranked statistically (Kruskal-Wallis one way analysis of variance on ranks and Dunn's test), the induction was highest in kidney > [lung, liver] > [intestine, heart]. Co-treatment with AM did not significantly alter pulmonary or hepatic microsomal EROD of CO- or BNF-treated rats. Hepatic EROD were 116±44.1, 127±34.9, 1407±570 and 1937±354 pmol/min/mg protein in CO, CO+AM, BNF and BNF+AM treated liver microsomes, respectively. In lung, resorufin formation rates were 7.86±1.89, 7.04±2.72, 75.4±40.9 and 68.0±36.5 pmol/min/mg protein in CO-, CO+AM-, BNF- and BNF+AM-treated microsomes, respectively.

Table 6: Ethoxyresorufin deethylase activity (mean±SD) in microsomes from vehicle control (CO)- and β-naphthoflavone (BNF)-treated rats.

Organ	Treatment	Resorufin, pmol/min/mg protein
Liver	CO (n=5)	118 ± 40.5
	BNF (n=8)	2073 ± 1056*
Lung	CO (n=5)	4.83 ± 3.00
	BNF (n=8)	83.0 ± 25.2*
Intestine	CO (n=5)	3.45 ± 0.239
	BNF (n=8)	8.61 ± 7.05*
Kidney	CO (n=5)	1.88 ± 0.462
	BNF (n=8)	864 ± 48.5*
Heart	CO (n=4)	6.28 ± 3.24
	BNF (n=7)	21.5 ± 2.69*

* Different from respective CO ($p<0.05$, Student's unpaired *t* test).

5.6.3 DEA formation in microsomes from CO- and BNF-treated rats

DEA was formed from AM at detectable levels by all of the tested microsomes. In terms of Cl_{int}, liver was found to have the highest capability of DEA formation. Interestingly, the heart was found to possess considerable DEA-forming activity at a level significantly higher than the other extra-hepatic organs (Table 9). Statistically, Cl_{int} was highest in liver > heart > [intestine, kidney, lung] microsomes. Additionally, linear increases in DEA formation rates versus the tested range of AM concentrations were mostly seen in lung, intestine and heart microsomes. One- and two-enzyme model equations were also fitted to these data but they were found to be either associated with more error or did not yield any significant improvement over the linear regression analysis. The r^2 values were 0.957±0.0424, 0.993±0.00317, and 0.887±0.0902 in CO-treated lung, intestine, and heart samples, respectively. In BNF-treated lung, intestine and heart microsomes, the r^2 values were 0.973±0.0131, 0.977±0.00914, and 0.905±0.0723, respectively. It was not possible to estimate the V_{max} and k_M values in these organs. However, Cl_{int} was estimated from the slopes of the regression lines obtained by linear regression analysis of the data.

Table 7: Kinetic constants (mean±SD) for microsomal DEA formation in vehicle control (CO)- and β-naphthoflavone (BNF)-treated rats.

Organ	Treatment	Vmax, pmol/min/mg protein	Km, µM	Cl_{int}, µL/min/mg protein
Liver	CO (n=5)	594 ± 419	58.6 ± 21.5	7.67 ± 6.19
	BNF (n=5)	557 ± 201	123 ± 104	4.77 ± 2.88
Lung	CO (n=6)	22.3[a]	218[a]	0.0693±0.0254
	BNF (n=9)	41.1 ± 25.0[b]	372 ± 191[b]	0.110±0.0390*
Intestine	CO (n=4)	ND	ND	0.407 ± 0.0668
	BNF (n=4)	ND	ND	0.394 ± 0.114
Kidney	CO (n=8)	33.6 ± 16.6	134 ± 126	0.276 ± 0.130
	BNF (n=12)	49.6 ± 10.6*	143 ± 121	0.478± 0.0635*
Heart	CO (n=4)	ND	ND	1.19 ± 0.191
	BNF (n=4)	ND	ND	1.05 ± 0.232

ND, not determined

* Different from the respective CO ($p<0.05$). Comparisons were made using student's unpaired *t* test.

[a] n=2 where the plateau phase in DEA formation was reached

[b] n=4 where the plateau phase in DEA formation was reached

Treatment with BNF did not significantly alter the DEA formation rates in the liver, intestine or heart microsomes (Figure 14 and Table 9). Although linear increases in DEA formation rates were mostly observed in lung microsomes, two and four of CO- and BNF-treated lungs, respectively, showed metabolic profiles that were best described by the Michaelis-Menten equation for a one-enzyme system. The V_{max} and k_M values for these rats are presented in Table 9. In lung and kidney microsomes, BNF treatment resulted in significant increases in Cl_{int} (Figure 14 and Table 9). The percentages of increase in the Cl_{int} values were 59% in lung and 73% in kidney microsomes. In kidney, where it was possible to estimate the V_{max} and k_M, BNF treatment resulted in a significant increase of 48% in the V_{max}. However, the k_M values were not significantly affected by BNF.

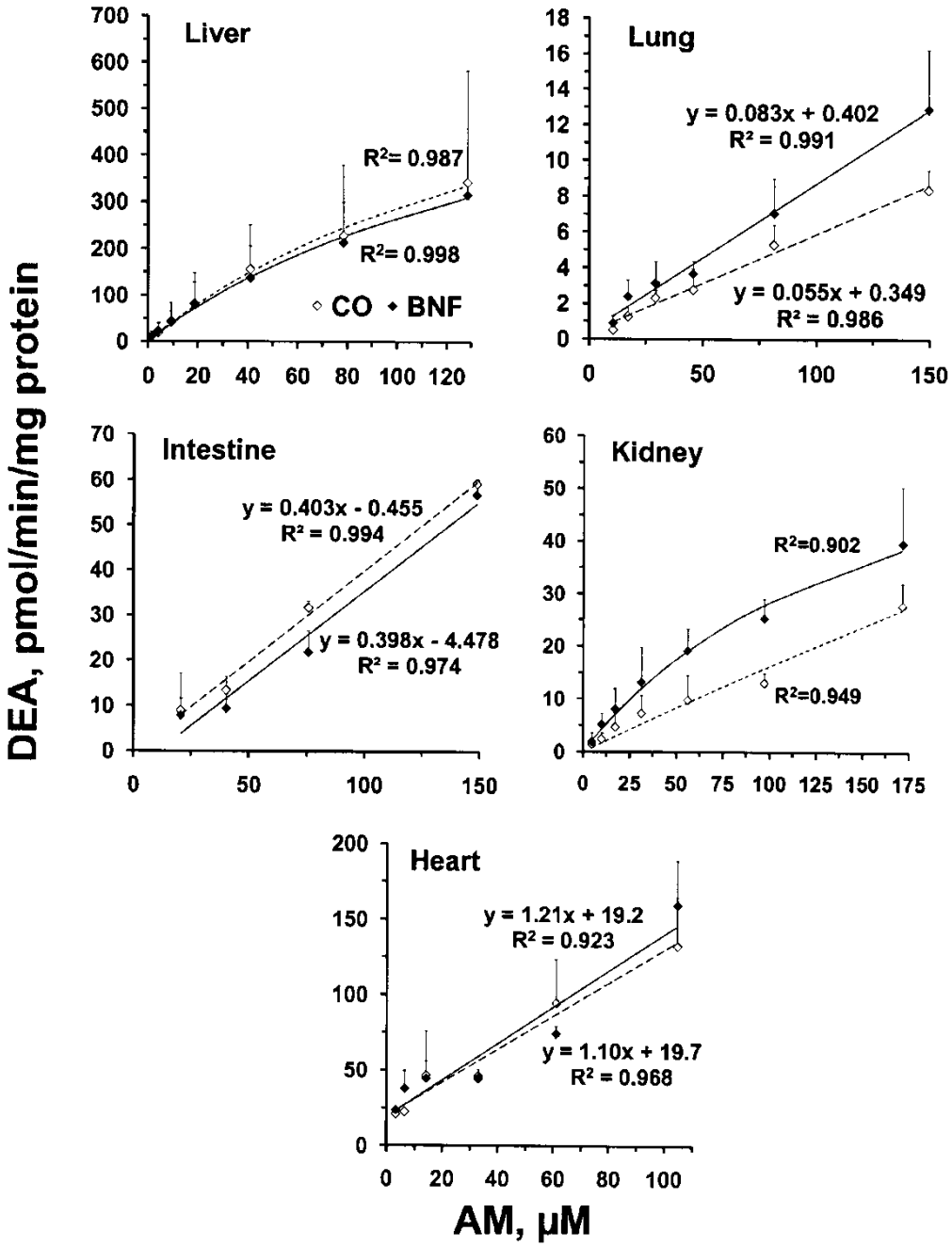

Figure 14: Desethylamiodarone (DEA) formation rates (mean±SD) from amiodarone (AM) in corn oil (CO) and β-naphthoflavone (BNF) treated microsomes. Unfilled markers represent average of data collected from CO-treated liver (n=5), lung (n=6), intestine (n=4), kidney (n=12) and heart (n=4). For relationships where linear regression provided the optimal fit, regression equations and regression coefficient (R^2) are shown. In other cases where the Michaelis Menten equation best fit the data, only the R^2 is shown. Dashed and solid lines represent the fitted data in CO- and BNF-treated microsomes, respectively.

5.6.4 DEA metabolism by rat CYP1A1

When DEA was incubated with rat CYP1A1, significant and progressive reductions in the amount of DEA were observed at all time measures (Figure 15). The *in vitro* determined $t_{1/2}$ and Cl_{int} based on DEA depletion were 57 min and 0.3 µl/min/pmol CYP, respectively.

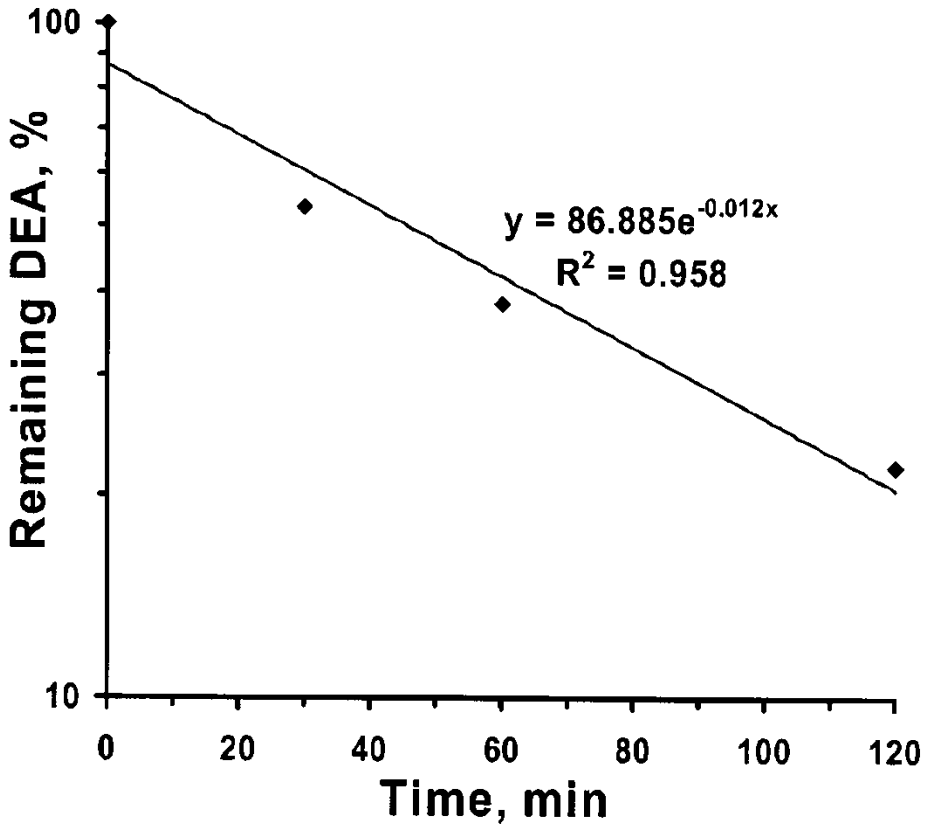

Figure 15: Desethylamiodarone (DEA) remaining (%) vs time after 0.15 µM DEA was incubated with 40 pmol/ml CYP1A1 (n=3/time point).

Chapter 6

6.0 Discussion

Coronary heart disease is considered a prevalent cause of death throughout the world, accounting for 7.2 million deaths (13.2%) worldwide in 2002 (Anonymous, 2007). Cardiac arrhythmia is considered a common form of the cardiovascular diseases, with AM being commonly used as a first-line of treatment (Mason, 1987). Because of its potentially life-threatening side effects, it has been advised that patients should be aware of these side effects through a medication guide that is dispensed with the medication (Anonymous, 2005a). Furthermore, prescribers have been advised to conduct baseline lung, liver and thyroid function tests, an electrocardiogram, serum potassium levels and an ophthalmological examination before, during and even for several months after AM discontinuation (Pollak, 1999; Anonymous, 2005b).

High serum DEA concentrations, specifically DEA:AM ratios of greater than 1.4, are associated with a higher risk of developing AM-induced pulmonary toxicity; for such patients a regular check of the lung diffusion capacity of carbon monoxide is recommended (Pollak, 1999). Furthermore, in liver and thyroid gland DEA was also found to be more toxic than AM (Beddows et al., 1989; Waldhauser et al., 2006). Based on these findings, we postulate that factors leading to increased DEA levels such as exposure to PAH can be potential risk factors for developing AM

side effects. Additionally, the therapeutic range of AM is considered to be 1-2.5 mg/L with arrhythmia increasing in frequency in patients with serum concentrations of less than 1 mg/L (Rotmensch et al., 1984). Therefore, pharmacological activity of AM might be compromised in PAH-exposed patients.

6.1 DEA formation by human and rat CYP isoenzymes and inhibitory effect of KTZ

The metabolism of AM to DEA has been studied in several species including human, rabbit and rat (Young and Mehendale, 1987; Fabre *et al.*, 1993; Trivier *et al.*, 1993; Shayeganpour *et al.*, 2006a). Indeed, this biotransformation has been extensively studied in human and the involvement of CYP3A4, 2C8, 1A2 and 2D6 has already been reported, although the inhibitory effect of KTZ on DEA-formation by these isoenzymes has not been reported (Ohyama *et al.*, 2000a). Furthermore, human CYP1A1-mediated DEA-forming activity of was not deeply investigated probably due to its low constitutive expression (Ohyama *et al.*, 2000a). On the other hand, although the involvement of CYP3A1, 3A2 and 1A1 in AM metabolism in rat has been reported (Shayeganpour *et al.*, 2006a), possible contributions of other CYP isoforms that are abundant in rat liver have not, to date, been well studied.

Consistent with earlier reports, the current results have shown that CYP3A4 possessed the highest activity amongst the constitutively expressed human isoforms. It was also of note, however, that human

CYP1A1 had a high affinity and capacity which translated into a higher Cl_{int} than CYP3A4. Despite this, because significant levels of hepatic CYP1A1 are observed under conditions of induction (Meunier *et al.*, 2000; Xu *et al.*, 2000), CYP3A4 which constitutes ~30% of the total CYP content in human liver is expected to be the major isoform involved in AM metabolism in humans (Shimada *et al.*, 1989). DEA formation by human CYP1A1 might, however, be a consideration in patients with CYP1A1-induced status, which includes patients who are smokers (Pelkonen *et al.*, 1986; Sesardic *et al.*, 1988). In comparison to CYP isoforms tested here, it is known that the mean km, Vmax and Cl_{int} for DEA formation by CYP2C8 are 8.6 μM, 2.3 pmol/min/pmol CYP and 0.261 μL/min/pmol CYP, respectively (Ohyama *et al.*, 2000a). This places the activity of CYP2C8, based on Cl_{int}, below that of both 1A1 and 3A4, respectively.

Of the tested rat isoforms, CYP2D1 showed the highest activity for DEA formation, followed by CYP2C11. The latter isoenzyme is a male-specific isoform comprising up to 54% of the total CYP content in male rat liver and it was earlier known as $CYP450_{UT-A}$ (Guengerich *et al.*, 1982; Wallin *et al.*, 1990). To our knowledge this is the first evidence that rat CYP2D1 and 2C11 are involved in DEA formation. Both isoforms had higher Cl_{int} values than CYP3A1 and CYP1A1 (Table 2), the roles of which in biotransformation of AM to DEA were previously established (Shayeganpour *et al.*, 2006a). In rats, the expressions of genes encoding for CYP2D1, CYP3A1 and CYP2C11 are strong in the liver (Lindell *et al.*,

2003) and, therefore, they could be considered as important contributors to DEA formation in rat.

In comparing human and rat CYP activities, striking differences were found in the comparison of the species-specific homologous enzymes. For example, human CYP1A1, 1A2 and 3A4 had 10-, 9-, 2-fold higher Cl_{int} than the respective rat counterparts (CYP1A1, 1A2, 3A1). Of the rat tested isoforms, only CYP2D1 showed a considerably higher (6.1-fold) Cl_{int} than human CYP2D6. Species-specific differences in isoform activity were also observed by other investigators. For example, the oxidation of benzo[α]pyrene by human CYP1A1 was reported to be less than that of rat CYP1A1 (Guengerich, 1997).

KTZ is commonly used as an *in vitro* selective CYP3A inhibitor in humans and in rats (Ball *et al.*, 1992; Fabre *et al.*, 1993; Lampen *et al.*, 1995; Dilmaghanian *et al.*, 2004; Hasselstrom and Linnet, 2006). As such, KTZ-related *in vivo* drug interactions are commonly presumed to implicate the inhibited drugs as being metabolized by CYP3A4. There are numerous examples in the literature where this presumption is explicit, some of them being presented as late as 2007 (Akram *et al.*, 2007; Ridtitid *et al.*, 2007). Given the previous findings in rat of KTZ-inhibition of CYP1A1 (Shayeganpour *et al.*, 2006a), the current study was also designed to investigate the inhibitory selectivity of KTZ for other human and rat CYP isoforms.

For KTZ use within *in vitro* protocols it was found that although KTZ initially caused a weak accumulation of CYP3A4 protein in human hepatocytes, overall it caused inhibition of protein synthesis and inhibition of CYP3A4 marker activity suggesting a selective inhibitory effect on that isoform (Maurice *et al.*, 1992). Thereafter, it was observed that when chemical inhibitors are used in high concentrations *in vitro* for identification of drug metabolizing enzymes, they tend to lose their selectivity and hence the term “window of selectivity” was introduced. The maximum limit of window of selectivity for KTZ was estimated to be 100-fold its ki value which was 15 nM (Rodrigues, 1994; Bourrie *et al.*, 1996).

At concentrations lying within the KTZ window of selectivity (up to 500 nM), CYP1A1 was potently inhibited by KTZ (Figure 5). The ki value for CYP1A1 inhibition was close to that of CYP3A4 (51.7 nM vs. 70.2 nM, respectively; Table 2). These results came in agreement with another study by Paine et al., 1999 who found that CYP1A1 marker activity was inhibited by KTZ in both human gut and recombinant systems with ki value of 40 nM. The inhibitory effect of KTZ towards human CYP1A2 was very weak compared to CYP1A1 and CYP3A4. Minimal inhibitory effect for KTZ is expected as long as it is used at concentrations below 1.5 µM. However, most drug metabolism studies are conducted with high KTZ concentrations up to 100 µM (Fabre *et al.*, 1993; Ohyama *et al.*, 2000a), a condition that could significantly inhibit CYP1A2 and CYP2D6. For example, DEA formation by CYP2D6 was observed to be inhibited by

approximately 71.4% when KTZ concentrations of 18.8 µM were present (Figure 3).

In earlier reports (Maurice *et al.*, 1992; Bourrie *et al.*, 1996) where KTZ inhibitory selectivity was tested against EROD activity (a marker substrate for CYP1A1), an inhibitory effect was not noticed. The likely cause of this discrepancy is that all of these earlier studies were conducted in human hepatocytes or liver microsomes which in noninduced preparations express little CYP1A1 (Guengerich, 1997). Indeed, the major constitutively expressed isoform of CYP1A is 1A2 in hepatocytes, which was weakly inhibited in our studies (Figure 3).

DEA formation facilitated by rat CYP2D2, 1A1, 2C6, 2C11 and 2D1 were each more sensitive to inhibition by KTZ than CYP3A1 (Figure 5, Table 2), which was inhibited only when a high concentration of KTZ was used (Figure 3). This finding, especially for CYP2C6, is in agreement with other reports which found that KTZ is not a selective inhibitor of CYP3A in rat (Eagling *et al.*, 1998; Kobayashi *et al.*, 2003). As expected, increasing concentrations of KTZ were observed to generally decrease formation of DEA.

A paradoxical increase in DEA formation occurred for CYP3A1 at lower KTZ concentrations ranging from 20 to 1000 nM. Preferential inhibition of metabolism of the formed DEA by the same isoenzyme could have caused an increase in DEA formation in the presence of KTZ (Kozlik

et al., 2001). A second explanation could be KTZ-associated inhibition of formation of other metabolite(s) of AM besides DEA, thus leading to increased amount of AM available to undergo DEA biotransformation. To address these possibilities, we conducted an experiment whereby AM and formed DEA were incubated in the presence of CYP3A1 isoenzyme. No significant metabolism or reduction in the concentration of DEA was observed when directly incubated with CYP3A1 in the absence of KTZ, which rules out the former explanation as being responsible for increased DEA formation in the presence of low concentrations of KTZ and AM. Our analytical method was not designed to measure other metabolites of AM. However, it was observed that the sum of molar-corrected [AM+DEA] formed in the AM-containing incubation was significantly less than the initial molar amount of AM added. This suggests that other metabolites of AM besides DEA are directly formed by CYP3A1, which leaves open the possibility that formation of a non-DEA metabolite is preferentially inhibited by lower concentration of KTZ compared to DEA.

At the *in vivo* level, clinical dosage regimens of AM and KTZ yield hepatic concentrations of both drugs that are in the range of those used in the high concentration *in vitro* experiments. For AM, postmortem and biopsied samples of human liver showed AM concentrations ranging from 7 to 1581 µM (Brien *et al.*, 1987; Ohyama *et al.*, 2000a). Similarly, in rat, high liver AM concentrations (43 and 171 µM) were present after administration of repeated high daily doses of AM (Kannan *et al.*, 1989).

Similar to AM, KTZ liver concentrations were found to be at least 3.2-fold higher than those attained in plasma of rats (Matthew *et al.*, 1993). In human subjects given 600 mg KTZ, mean serum concentrations were reported to be as high as 26.3 µM (Brass *et al.*, 1982). Assuming similar equilibration kinetics between human and rat, intrahepatic concentrations of KTZ would be expected to be significant in humans as well. Hence *in vivo*, KTZ would be expected to significantly inhibit the formation of DEA by each of the tested isoforms except for rat CYP1A2. For *in vivo* experimental results of other drugs besides AM for which KTZ is observed to be an inhibitor, it should be recognized that the involved inhibited isoenzymes may extend beyond those of the CYP3A family (Figure 3).

6.2 Effect of BNF on AM pharmacokinetics and tissue distribution

Our results show that exposure to PAH could significantly influence exposure to AM and formed DEA *in vivo*. After both single and repeated doses of AM administered to BNF-treated rats, plasma concentrations of DEA were significantly increased compared to vehicle-treated rats. The mechanism of increase in DEA levels was assumed to be due to increased DEA formation as a result of induction of CYP1A isoforms. This assumption was supported by the concomitant decrease in AM repeat-dose AUC (Table 4) in all the tissues and plasma samples. However, there was an absence of this trend in plasma after single doses of AM.

There were some differences in the time frame of BNF administration and induction between the single and multiple dose arms, specifically, single dose kinetics were determined the day after the last of four days of dosing with BNF, whereas the tissue distribution study was performed three days after the last dose of BNF was given. However, in an investigation of differences in the degree of induction one and three days after the last dose of BNF, no significant differences were observed (data not shown).

It is worth mentioning that CYP1A2 constitutes about 13% and 2% of the total CYP content in human and rat liver, respectively (Guengerich *et al.*, 1982; Shimada *et al.*, 1994). In contrast, CYP1A1 expression in human and rat liver is negligible (<1%) (Guengerich *et al.*, 1982; Shimada *et al.*, 1994). In extra-hepatic tissues, CYP1A2 is not detectable (de Waziers *et al.*, 1990; Shimada *et al.*, 1996), Whereas, CYP1A1 is significantly expressed in extra-hepatic tissues including lung and intestine (Paine *et al.*, 1999; Sinal *et al.*, 1999). Perhaps importantly for AM, CYP1A1 constitutes up to 35% of the total CYP content in human lung and its activity can be induced (Shimada *et al.*, 1996; Thum *et al.*, 2006). It is also variably expressed in the intestine, with CYP1A marker activity in some individuals actually exceeding that of the liver (Paine *et al.*, 1999).

The tissue distribution study was not designed to reflect true steady-state conditions. Amiodarone was limited to four daily doses to ensure complete dose administration through the implanted cannulae. It

was felt that it would be overly difficult to maintain cannula patency past four days. Although based on the measured terminal phase half-life in plasma after 24-48 h, steady-state might have been assumed to be in effect for AM in the tissue distribution study; a more prolonged half-life is known to be present (~44 h) (Shayeganpour *et al.*, 2005). Based on this, the tissue distribution samples can be assumed to represent values obtained at about 78% of the true estimated steady-state trough values with doses given every 24 h. In the event of a longer undisclosed terminal phase half-life in rat, this value would be lower.

6.3 Effect of DEA on human recombinant CYP1A1 activity and assessing inactivation potential

By definition, MBIs are considered as substrates of the target enzyme that undergo catalytic transformation to products which, prior to their release from the active site, essentially cause an irreversible inactivation of the enzyme (Silverman, 1995b). The data, however, suggested that DEA is not a substrate for human CYP1A1 (Figure 11). In support of this observation Kozlik et al. (2001) found that DEA is mainly metabolized by CYP3A and that the role of CYP1A is not significant (Kozlik *et al.*, 2001). Although not a substrate, DEA still was able to decrease EROD facilitated by CYP1A1 mainly by a competitive mechanism (Figure 12), and was also able to minimally inhibit its own formation (19%) (Table 5).

In replicating the basic procedure (Protocol B) outlined by Ohyama et al. (2000), incubations containing DEA yielded EROD that was only discernable at the 0 min preincubation time (Table 6). In the absence of other data this would suggest a very strong inactivating effect on CYP1A1. However, using the same protocol (Table 6), the same outcome was noted when DEA was absent. When dilution of the preincubate was employed but in the absence of additional NADPH in the EROD-assessment phase and for shorter preincubation time intervals (Figure 13, lower panel), the baseline enzyme activity substantially and progressively decreased with longer preincubation times.

However, when NADPH was added during both the preincubation and EROD measurement phase buffers in Protocol A, EROD was relatively stable and remarkably higher than seen with Protocol B, clearly suggesting the lack of inactivation of CYP1A1 by DEA (Figure 13, upper panel). In line with this observation, when another 0.25 mM NADPH was added following the 30 min preincubation period of Protocol B, enzyme activity was partially recovered (to 27-44% from 0) in both the 0 and 2 μM DEA incubations with no evidence of CYP1A1 inhibition (Table 6). We also noticed in a preliminary time-linearity study that the EROD formation continues to be linear only up to 3 min, which was the incubation time used in our study. In the earlier report (Ohyama *et al.*, 2000b), the EROD incubation was conducted for 15 min which complicates interpretation of the results.

Our report is not the first to show such decreases in activity with preincubations of CYP and NADPH. Enzyme-specific decreases of 65% to 98% in activity with extended preincubations have been encountered in studies which required preincubation in the presence of NADPH or NADPH regenerating systems (Ness *et al.*, 1987; Ogus and Ozer, 1991; Foti and Fisher, 2004; Polasek *et al.*, 2004). Probably, for this reason it has become a standard procedure to replenish NADPH during the incubation phase of the reaction (Ness *et al.*, 1987; Chang *et al.*, 2001; Polasek *et al.*, 2004). This practice allows recovery of most of the activity, as shown here (Table 6 and Figure 13, Upper Panel) (Ness *et al.*, 1987; Chang *et al.*, 2001; Polasek *et al.*, 2004). In light of this intrinsic reduction in activity with prolonged preincubations it becomes apparent that controls (MBI-free) should be preincubated for the same time intervals as the MBI-containing incubations, as previously recommended by Silverman, 1995.

Some investigators have explained this decrease in activity with extended preincubation times based on the decrease in NADPH stability and/or enzyme thermal liability (Ness *et al.*, 1987; Foti and Fisher, 2004). A more sound explanation was offered by Vermilion and Coon (1978) who showed that NADPH-P450 reductase, which is the enzyme responsible for transferring electrons from NADPH to the CYP can actually inhibit the CYP activity (Guengerich, 2008), apparently in an enzyme-specific manner (Ness *et al.*, 1987; Ogus and Ozer, 1991; Foti and Fisher, 2004). This inhibitory effect was thought to result from the production of the

oxidized product ($NADP^+$) which acts as a competitive inhibitor of the reductase, thereby preventing the flow of electrons from NADPH to the CYP enzyme (Vermilion and Coon, 1978; Guengerich, 2008). In support of this assumption is the increase in activity noted after replenishing the NADPH (Table 6). Furthermore, in our study, because EROD was unaffected in samples preincubated without NADPH an intrinsic degradation of CYP1A1 alone could be ruled out.

Evidence of potential involvement of human CYP1A1 in DEA formation is present in the literature and further confirmed by our results (Table 2 and Figure 1) (Ohyama *et al.*, 2000a). Consequently, one might expect that increased CYP1A1 activity would increase the toxicity of AM as a consequence of there being an increased formation of the more toxic metabolite, DEA. In the event of DEA inactivating CY1A1, however, such a concern would be blunted. We have shown that, in contrast to a previous report (Ohyama *et al.*, 2000b), DEA does not appear to inactivate CYP1A1, and hence a safety concern in the use of AM in patients with induced levels of CYP1A1 is valid.

6.4 Role of CYP1A1 induction in DEA formation and effect of AM treatment on CYP1A1 and 1A2 gene expression and activity

Our results show that, at the level of gene expression, BNF resulted in significant increases in CYP1A1 expression in each of the tested tissues. These results are consistent with previous reports which showed that CYP1A1 gene expression was significantly induced in almost

all of the tested tissues by a similar BNF dose (Sinal *et al.*, 1999). CYP1A2 mRNA in contrast, was only induced in liver and intestine. Although BNF is considered as a prototypical inducer of CYP1A1 and 1A2, the rest of the tested tissues did not show significant induction in CYP1A2 gene expression at this dose level. In an earlier study in rat, BNF was administered at 25 mg/kg/d for 2 days (Iba *et al.*, 1999). This dose level failed to upregulate CYP1A2 gene expression to detectable levels in lung and kidney (Iba *et al.*, 1999). In testis-derived cell lines, although CYP1A2 gene expression was constitutively detectable, it was not induced by TCDD or 3-MC, indicating that the induction of this isoform might be tissue-specific in nature (Tsuchiya *et al.*, 2003).

We observed some discordance between the magnitude of increase in CYP1A1 and CYP1A2 mRNA expression and changes in functional activity in some of the tissues (Tables 7 and 8). This was particularly true of the liver, intestine and heart tissues. In lung and kidney, however, folds of induction in CYP1A1 gene expression matched well with those observed at the level of activity, indicating that in these organs induction of CYP1A1 is likely to occur mainly at the gene expression level. Specifically, in kidney, CYP1A1 mRNA increased 479-fold with BNF, whereas EROD was 459-fold higher. Statistically similar magnitudes of increases in mRNA and EROD in lung of 42.8- and 17.2-fold, respectively were also observed.

In studying the effect of BNF on AM pharmacokinetics and tissue distribution (section 6.2 in discussion), our results showed that exposure to a representative PAH (BNF) can markedly alter AM pharmacokinetics (Figure 8 and Table 3). We had also observed significant BNF-related increases in DEA concentrations in plasma, lung, liver, kidney and heart samples after multiple intravenous doses of AM (Figure 9 and 10 and Table 4). In an effort to discern the mechanism responsible for the increase in DEA levels seen *in vivo*, we examined the metabolism of AM by various tissue microsomes. We found that exposure to BNF led to significant increases in DEA-forming activity, but only in lung and kidney microsomes (Figure 14 and Table 9). Furthermore, in lung and kidney, increases noted in EROD are likely mainly due to CYP1A1 induction (as indicated by gene expression analysis, Table 7). Therefore, it appears that increases in DEA levels in these organs are mainly due to CYP1A1 and not 1A2.

For many drugs, liver is the main organ involved in drug metabolism and hence the lack of increase in hepatic DEA formation after BNF might seem paradoxical. It should be noted, however, that DEA can be formed by other CYP isoenzymes which are much more efficient at facilitating the bioconversion (Figure 1 and Table 2) (Ohyama *et al.*, 2000a). In contrast to 7-ER which is metabolized mainly by CYP1A1, in rats DEA could be formed by several isoforms including CYP3A1, 2D1/2, 2C11, 2C6, 1A1 and 1A2, with CYP1A1 and 1A2 being among the least

efficient isoforms (Shayeganpour *et al.*, 2006a). Several of these isoforms were shown to be co-expressed with CYP1A1 or 1A2 in liver, intestine, heart, lung and kidney (Debri *et al.*, 1995; Thum and Borlak, 2000; Schaaf *et al.*, 2001; Yan and Caldwell, 2001; Mitschke *et al.*, 2008). These CYP isoenzymes also contribute to a much higher relative composition of hepatic CYP than does CYP1A1 and 1A2. The overall increase in DEA will hence be affected by the relative contribution of these isoforms to DEA formation compared to CYP1A1 and 1A2 isoforms within each organ, and as such induction of CYP1A may not be expected to significantly increase the overall DEA-forming activity by liver microsomes. On the other hand, on a relative basis CYP1A1 constitutes a larger mass of CYP in the lung and kidney than liver, which explains the increase in DEA formation in these tissues in response to CYP1A1 induction (Shimada *et al.*, 1996; Schaaf *et al.*, 2001; Korashy *et al.*, 2004).

Although DEA formation was only increased in lung and kidney after BNF pretreatment, the increases observed *in vivo* were much higher than can be explained by these relatively (compared to liver) modest increases. Although the metabolic efficiency of liver microsomes was not significantly altered by BNF in a microsomal system, it was observed that body-weight-normalized liver mass was increased (27%, $p<0.05$). This increase in liver mass, even with unchanged microsomal protein normalized metabolic activity, could have resulted in an overall increase in hepatic DEA formation *in vivo*. This effect will not be evident in the *in vitro*

microsomal incubations since protein concentration is kept constant in control and BNF-treated microsome incubations.

As discussed previously, exposure to PAH can result in a wide range of effects. Although an inhibitor of P-gp (Katoh *et al.*, 2001), we could not find literature evidence of AM being a substrate for P-gp or MRP. DEA on the other hand was shown not to be a substrate for P-gp (Seki *et al.*, 2008). Furthermore, since AM is bound to lipoproteins, displacement of AM from these proteins is likely. However changes in lipoprotein levels were seen after chronic treatment (3-6 month) with PAH (Revis *et al.*, 1984). Based on this information changes in efflux transporters or plasma protein binding of AM are likely to be irrelevant and do not fully explain our findings. On the other hand, changes in expression of drug metabolizing enzymes and increase of liver mass are potential mechanisms by which increases noted in DEA formation either *in vitro* or *in vivo* can be explained.

BNF is known, however, to induce CYP1B1, CYP2B1 and some phase Ⅱ metabolizing enzymes. However, of the phase Ⅰ metabolizing enzymes, only CYP1A1 and 1A2 are considered as relevant to DEA metabolite formation based on the minimal contribution of CYP1B1 and 2B1 to its formation from AM that was seen previously (Ohyama *et al.*, 2000a; Shayeganpour *et al.*, 2006a). To our knowledge there is no literature evidence of a contribution of PAH-inducible phase Ⅱ metabolizing enzymes to AM metabolism. Therefore the potential role of BNF-inducible

phase Ⅱ metabolizing enzymes in enhancing AM clearance in BNF-treated rats can not be ruled out.

CO, the vehicle for BNF, was chosen based on previous reports (Sinal *et al.*, 1999). Corn oil does not alter the activity of some CYP isoforms such as CYP2C11 and CYP3A (Brunner and Bai, 2000) and it yielded pharmacokinetic parameters in CO rats (Table 2) that were closely matched by the same parameters in CO-free rats (Shayeganpour *et al.*, 2005).

Another factor to consider in evaluating the relatively low microsomal formation of DEA is the possibility of sequential metabolism of DEA by CYP1A. We tested metabolism of DEA by rat CYP1A1 and found that it was capable of metabolizing the preformed metabolite. In this case, one might underestimate the formation of DEA in the presence of microsomes from BNF-treated rats because as DEA is formed by induced CYP1A1 its clearance would be likewise increased by CYP1A1, possibly resulting in little or no net increase in microsomal formation being realized. Our data show that DEA was not a substrate for human CYP1A1 (Figure 11) and therefore we did not expect DEA to be metabolized by rat CYP1A1. In contrast to our expectations, when DEA was incubated with rat CYP1A1, decreases in DEA were observed with increasing length of exposure to the enzyme (Figure 15).

Although DEA was depleted quickly and efficiently by rat CYP1A1 *in vitro,* this decrease in DEA is expected to be attenuated in the presence of AM *in vitro* in microsome incubations or *in vivo* due to the competitive effect of AM which is normally present at higher concentrations than DEA (Figure 9). Besides, when DEA-forming activity by CYP1A1 was compared in human and rat isoforms, it was noted that human CYP1A1 had a 10-fold higher intrinsic clearance than the rat counterpart (Figure 1 and Table 2). Additionally, unlike rats, DEA was not metabolized by human CYP1A1 (Figure 11 and 15). Therefore, the induction of CYP1A1 by PAH, as observed in rat, would be expected to cause an even greater increase in DEA exposure in humans.

A separate study was also conducted in rats to investigate the effect of AM treatment, and consequently exposure to the formed DEA, on CYP1A gene expression and activity. Our results showed that the combination of BNF+AM treatment did not decrease, but rather increased, CYP1A1 gene expression compared to that of BNF alone in liver, lung and kidney (Table 7). However, when tested at the activity level (EROD), taking BNF+AM-treated rat lung and liver micrsomes as examples, there were no significant increases in activity in control- and BNF-treated microsomes, indicating that AM-related increases in CYP1A1 gene expression are probably insignificant. These results show that exposure to high concentrations of AM or DEA *in vivo* does not inactivate CYP1A1, which is consistent with our results with human CYP1A1 (Figure 13A).

The metabolism of AM to DEA by rat lung and kidney microsomes after induction with BNF has been reported to be small (detectable but not measurable). Based on the results, it was believed that metabolism of AM by both organs was not important (Rafeiro et al., 1990). It is important to recognize that the assay used cited a lower limit of quantitation (LLQ) that is much higher (1.5 nmol/ml, or 968 ng/ml) than many existing assays for DEA. For example, two assays developed in our laboratory cited LLQ of 33 and 2.5 ng/ml (29 to 387 times more sensitive), respectively based on 100 μl of specimen (Jun and Brocks, 2001; Shayeganpour et al., 2007). Thus, limitation in assay sensitivity likely obscured the true impact of CYP1A1 induction on lung and kidney metabolism of AM.

Chapter 7

7.0 Conclusions

Of the AM-induced side effects, pulmonary toxicity is considered the most serious and has the highest mortality (Mason, 1987). Recently, some studies were conducted to identify factors that could increase the risk of pulmonary toxicity and hence aid in developing guidelines for its monitoring in AM-treated patients. For example, patient age, duration of AM therapy, preexisting lung injury, patient body mass index and increased DEA/AM serum concentration ratio were identified as probable risk factors (Pollak, 1999; Ashrafian and Davey, 2001; Okayasu *et al.*, 2006; Ernawati *et al.*, 2008). With this in mind, it may be relevant that the DEA/AM ratio was increased in the BNF-induced rat lungs (Table 4; Figures 9 and 10).

In vitro metabolism of AM was species-dependent. In rat, besides CYP3A1 and 1A1, whose roles in DEA formation were already established, our results showed that CYP2D1 and 2C11 are important facilitative enzymes in formation of DEA in rats. For humans, CYP1A1 and CYP3A4 were most efficient at converting AM to DEA. In the presence of high concentrations such as those expected in liver after *in vivo* administration, KTZ was observed to be a generalized inhibitor of DEA formation. At lower concentrations, KTZ was found to be most potent in

inhibition of human CYP1A1 and CYP3A4. In rats, KTZ inhibitory potency was highest for CYP2D2 and CYP1A1.

Exposure to PAH components can result in an overall increase in the DEA concentrations combined with a decrease in AM levels in the plasma, lung, liver, kidney, and intestine after multiple doses of AM. In patients exposed to environmental sources of PAH (including those exposed to tobacco smoke) such an event could conceivably be of clinical significance considering the pharmacological and toxicological properties of AM and its DEA metabolite.

We also found that DEA was not a substrate for human CYP1A1. However, DEA could inhibit EROD in a mixed competitive fashion. It minimally inhibited its CYP1A1-mediated formation (19%). Importantly, DEA was not an MBI for CYP1A1.The previous report suggesting that of DEA was an inactivator of CYP1A1 may have been confounded by depletion of NADPH during the preincubation phase.

The mechanism of increase in DEA levels seen *in vivo* was partly imparted by increased DEA formation through CYP1A1 induction in lung and kidney. An increase in body weight-normalized liver size provides a potential mechanism for explaining the differences in the extent of increase in DEA levels noted *in vivo* compared to that observed *in vitro*. Additionally, increases noted in DEA levels are possibly attenuated by the increase in DEA clearance, especially in the rat.

AM administration did not alter CYP1A1 basal or BNF-induced activity, thus further confirming a lack of inactivating potential for DEA on CYP1A1. Interestingly, heart possessed a considerable DEA forming activity that was higher than the rest of the tested extra-hepatic tissues. To date, one of the most serious side effects of AM therapy is pulmonary toxicity, which has been associated with high exposure of DEA in some patients. The increase in pulmonary-CYP1A1 mediated formation of DEA from AM was less than that noted for the increase in CYP1A1 expression. Nevertheless, due to the high concentrations and long residence time of AM in lung, the increase in pulmonary-derived DEA could increase the risk of pulmonary-related AM toxicity. Further studies are needed to confirm this assumption.

Chapter 8

8.0 Future directions

Pointing out factors that can lead to altered AM disposition is important for a proper control of arrhythmias and avoiding drug-related side effects. Based on the results observed in the rat and differences in its DEA-forming CYP1A isoenzyme activities compared to humans, studies aimed at investigating DEA and AM levels in PAH-exposed and non-exposed humans, such as smokers and non-smokers, or human-derived models, such as human hepatoma cell line, will be important.

Furthermore, studies focusing on possible pharmacological and toxicological changes in susceptible tissues imparted by CYP1A induction would help provide context to the possible clinical significance of induction to AM therapy. Clinical studies on efficacy of AM and dose requirement in smokers and non-smokers are important. Comparison of the degree of toxicity using biopsy samples from organs susceptible to AM and DEA toxicity from smokers and non-smokers will be useful in shedding light on relevance of PAH exposure to AM therapy. Human cell lines of organs such as liver or lung could also be used.

Increased liver mass and its effect on drug metabolism in humans is particularly interesting since it could lead to clinically significant changes in drug pharmacokinetics. Whether smoking or exposure to PAH in

general can directly increase liver mass in human should also be addressed. Although some reports have showed significant association between the two phenomena, it does not prove the presence of a cause-effect relationship.

Although it was not our main concern, our studies did show that heart can efficiently metabolize AM. The intrinsic clearance for DEA formation in the heart was higher than that of other tested extra-hepatic tissues including the intestine. The significance of this finding lies in the fact that heart is the site of action for AM and variation in anti-arrhythmic control might be better explained by heart metabolic status and levels of the drug within the heart.

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