Genetic Variants of Flavin-containing Monooxygenases: Consequences for Drug Metabolism

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Statement

I, Asvi Francois confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The metabolism of the anti-tubercular drug, thiacetazone (TAZ) by human FMOs *in vitro* and the disposition of TAZ *in vivo* in mice were studied. Reverse phase chromatography confirmed TAZ to be a substrate for human FMO1, FMO2.1 and FMO3 with the formation of TAZ-sulphinic acid and TAZ-carbodiimide via a TAZ- sulphenic acid intermediate. The products are the same as those formed by the *Mycobacterium tuberculosis* enzyme EtaA, the enzyme responsible for TAZ activation. Kinetic studies found FMO2.1 to be significantly more efficient at TAZ oxygenation than EtaA, FMO1 and FMO3. Asians and Europeans do not express functional FMO2 in their lungs as a result of a premature stop codon. However about 28% of African individuals lack this mutation. The products of FMO2 are expected to be toxic to mammalian cells; therefore individuals expressing FMO2 in their lungs may be at higher risk of FMO-dependent TAZ bioactivation.

Protein variants of FMO3 were analysed for their ability to catalyse TAZ oxygenation. Kinetic studies showed that the L360P variant displayed a significantly higher catalytic activity towards TAZ than the wild type protein. The K158/G308 protein was inactive towards TAZ, whereas K158 or G308 variants oxygenated TAZ. These findings may reflect the underlying mechanism of TAZ-dependent liver toxicity reported in patients taking TAZ as part of treatment for TB.

Mouse liver and lung microsome experiments indicated that both FMOs and cytochromes P450 (CYPs) metabolise TAZ *in vitro*. FMO contribution was higher in the lung than the liver. Kinetic studies using microsomes from *Fmo1* knockout mice show FMO1 to be the predominant contributor to TAZ oxygenation *in vitro*.

Metabolism of TAZ in liver and lungs of mice *in vivo* was not observed, however TAZ, TAZ-sulphenic acid, TAZ-sulphinic acid and TAZ-carbodiimide were identified in kidney.

For my Mother and Lord Vishnu...

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[E]	Enzyme Concentration
A	Absorbance
Abs	Absorbance
AG	Arabinogalactan
ANTU	Alpha-Naphthylthiourea
ASW	African Ancestry in Southwest USA
BLAST	Basic Local Alignment Search
Bp	Base Pairs
BSA	Bovine Serum Albumin
BZD	Benzydamine
C57BL/6	C57 Black 6 Mouse Strain
cDNA	Complimentary DNA
CMAS	Cyclopropane Mycolic Acid Synthase
CRP	NADPH-Dependent Cytochrome P450 Reductase
CYP	Cytochrome P450
D	Aspartic Acid
DNA	Deoxyribonucleic Acid
D-PBS	Dulbelcos's Phosphate Buffered Saline
DTNB	5,5-Dithio-bis(2-nitrobenzoic acid)
DTT	Dithiotreitol
E	Glutamic Acid
ε	Molar Extinction Co-effiecient
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EMB	Ethambutol
ETA	Ethionamide
EtaA	Ethionamide-Activating Enzyme
ETA-AMIDE	2-Ethyl-4-amidopyridine
ETA-NITRILE	2-Ethyl-4-cyanopyridine
ETA-SO	Ethionamide *S*-oxide
FAD	Flavin Adenine Dinucleotide
FAD-OOH	Hydroperoxyflavin
Fig.	Figure
FMN	Flavin Adenine Mononucleotide
FMO	Flavin Containing Monooxygenase
G	Glycine
g.	Gene Cooridinate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Gln	Glutamine
GSH	Glutathione
H	Histidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HIV	Human Immunodefiency Virus

HNF1	Hepatocyte Nuclear Factor 1
HPLC	High Performace Liquid Chromatography
Hr	Hour(s)
I	Isoleucine
INH	Isoniazid
K	Lysine
*K*cat	Turnover Number
K_M	Michaelis Constant
KO	Knockout
L	Leucine
LAM	Lipoarabinomannan
LC-MS	Liquid Chromatography-Mass Spectroscopy
Leu	Leucine
LKW	Luhya in Webuye, Kenya
M	Methionie
M. bovis	*Mycobacterium bovis*
M. smegmatis	*Mycobacterium smegmatis*
M.tuberculosis	*Mycobacterium tuberculosis*
m/z	Mass-to-charge ratio
MA	Mycolic Acid
MAME	Mycolic Acid Methyl Esters
MDR-TB	Multi-drug resistant TB
Min	Minute(s)
MKK	Maasai in Kinyawa, Kenya
MMLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
MS	Mass Spectroscopy
N.D	Not Detected
n.d	Not determined
NADPH	β-Nicotinamide Adenine Dinucleotide Phosphate (Reduced)
P	Probability
P	Proline
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
Pro	Proline
PTU	Phenylthiourea
PZA	Pyrazinamide
Q	Glutamine
qRT-PCR	Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction
RIF	Rifampicin
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

S.D.	Standard Deviation
S.E	Standard Error
SAM	*S*-adenosyl methionine
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sec	Second(s)
Sf9	*Spodoptera frugiperda*
SM	Streptomycin
SNP	Single Nucleotide Polymorphism
TAZ	Thiacetazone
TB	Tuberculosis
TBE	Tris/Borate/EDTA Solution
TBS	Tris Buffered Saline
TEMED	*N*,*N*,*N'*,*N'*-Tetramethylethylenediamine
TMA	Trimethylamine
TMAU	Trimethylaminuria
TNB	2-nitro-5-thiobenzoate
Tricine	N-(Tri(hydroxymethyl)methyl)glycine
Tris	Tris[hydroxymethyl]aminomethane
UK	United Kingdom of Great Britain and Northern Island
USA	United States of America
UV	Ultraviolet
*V*max	Maximal Velocity
WHO	World Health Organisation
X-MDR TB	Extensively Drug Resistant TB
YRI	Yoruba in Ibadan, Nigeria

A word about human and mouse gene nomenclature.

A human gene is written in italics and capital letters e.g. *FMO*, whereas the human protein is written in capitals e.g. FMO. In mice the protein is also written in capitals e.g. FMO, but the gene is written in italics with the first letter uppercase and the following letters in lowercase e.g. *Fmo*.

Chapter 1: Introduction

1.1 Xenobiotic Metabolism

Xenobiotic metabolism refers to the biochemical pathways involved in the processing of an exogenous substance. The principle site of xenobiotic metabolism is the liver, but biochemical transformations, to a greater or lesser extent occur throughout the body and depend on the site of xenobiotic exposure. Xenobiotic metabolism can be considered in two phases. Phase I metabolism is the stage at which polar groups are either introduced or exposed in the xenobiotic. This step is achieved by oxidation catalysed by Cytochrome P450 Monooxygenases (CYPs), Flavin Containing Monooxygenases (FMOs), Alcohol and Aldehyde Dehydrogenases, Monamine Oxidase or Peroxidases or hydrolysis by Esterases, Amidases or Epoxide Hydrolase. Phase II metabolism refers to the detoxification of modified products of Phase I metabolism by conjugation to water-soluble, non reactive chemical moieties to facilitate their excretion from the body. This step is catalysed mainly by Transferases e.g. Glutathione *S*-Transferase, Sulfotransferases or UDP Glucuronyl Transferase.

After the CYPs, the FMOs are the largest group of enzymes involved in the Phase I metabolism of drugs and other xenobiotics and provide the focus for this investigation.

1.2 FMOs

FMOs (EC 1.14.12.8) are β-Nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)-dependant enzymes that catalyse the *N*- and *S*-oxidation of a wide range of compounds. They contain flavin dinucleotide (FAD) as a prosthetic group and are dependant on molecular oxygen for enzyme catalysis.

1.2.1 Discovery of FMOs

In 1960, a study described how rat liver microsomes contained enzymes that catalysed the oxygen- and NADPH-dependent oxidation of a number of azo dyes (Miller *et al.* 1960). The localisation (membrane bound), co-factor requirement (FAD), and activity of this enzyme (oxidation) towards xenobiotics was similar to that observed for CYPs which had recently been discovered at the time. Studies with purified pig liver microsomes provided evidence that this enzyme was not a CYP. It was immunologically distinct from the microsomal NADPH-dependent NADPH cytochrome P450 reductase and unlike this enzyme, which contains FAD and flavin mononucleotide (FMN), FAD was the only prosthetic group found in the pig liver oxidase (Masters *et al.* 1971). Further studies with purified pig liver microsomes, confirmed the dependence of this oxidase enzyme on FAD but not FMN (Pettit *et al.* 1964). At the time, most of the characterisation of the pig liver enzyme had been done using *N*, *N*- dimethylaniline as a model substrate and so 'amine oxidase' and 'dimethylaniline monooxygenase' were apt names for this enzyme. The enzyme was initially classified as a mixed function-amine *N*-oxidase because it consumes two atoms of oxygen per substrate where one atom is transferred to the nitrogen of the amine moiety and the other is reduced to form one molecule of water. Further studies with a panel of xenobiotics revealed that the enzyme was capable of reacting with a remarkably broad and rather promiscuous range of compounds with no common chemical feature (described in section 1.2.3). The name mixed function amine oxidase was now too restrictive in describing the reactions catalysed by this enzyme. The enzyme was given the trivial name flavin containing monooxygenase which is usually abbreviated to 'FMO'.

1.2.2 FMO Enzyme Mechanism

The mechanism of FMO enzyme catalysis was determined by extensive kinetic and spectral studies by Poulsen and Zeigler in the 1970's (Poulsen *et al.* 1979).

A sequential cycle is observed in FMO enzyme catalysis with the first step being the reduction of the prosthetic FAD by NADPH (Fig 1.1). The reduced form ($FADH_2$) can rapidly react with molecular oxygen to generate a hydroperoxyflavin (FAD-OOH) radical. In a mechanism that distinguishes FMOs from all other monooxygenases, the formation of this radical does not require binding of the oxygenatable substrate to the active site of the enzyme, in fact the hydroperoxyflavin formation occurs *before* the interaction of substrate and FMO (Poulsen *et al.* 1979). The enzyme-bound C4a-hydroperoxyflavin is stable from minutes to hours at 4˚C and the radical is presumed to be in this state in a cell. Thus, FMOs have been described to be in a 'cocked gun' state, ready to attack any substrate bearing a soft nucleophile moiety, which has gained access to the active site bearing the hydroperoxyflavin radical. In the presence of a suitable substrate (see next section) FMO transfers one atom of molecular oxygen (from the FAD-OOH) to oxygenate the substrate, while the other is reduced to form water. The oxygenated substrate is released immediately and the next and rate-limiting step is the release of water. The last step in the cycle is the release of $NADP^+$ and then the regeneration of FAD-OOH starts again. It is important to note that substrate binding has no effect on the velocity of product formation; that is decided by the rate-limiting step (the release of water), despite this, the enzyme displays Michaelis-Menten kinetics (Poulsen *et al.* 1979).

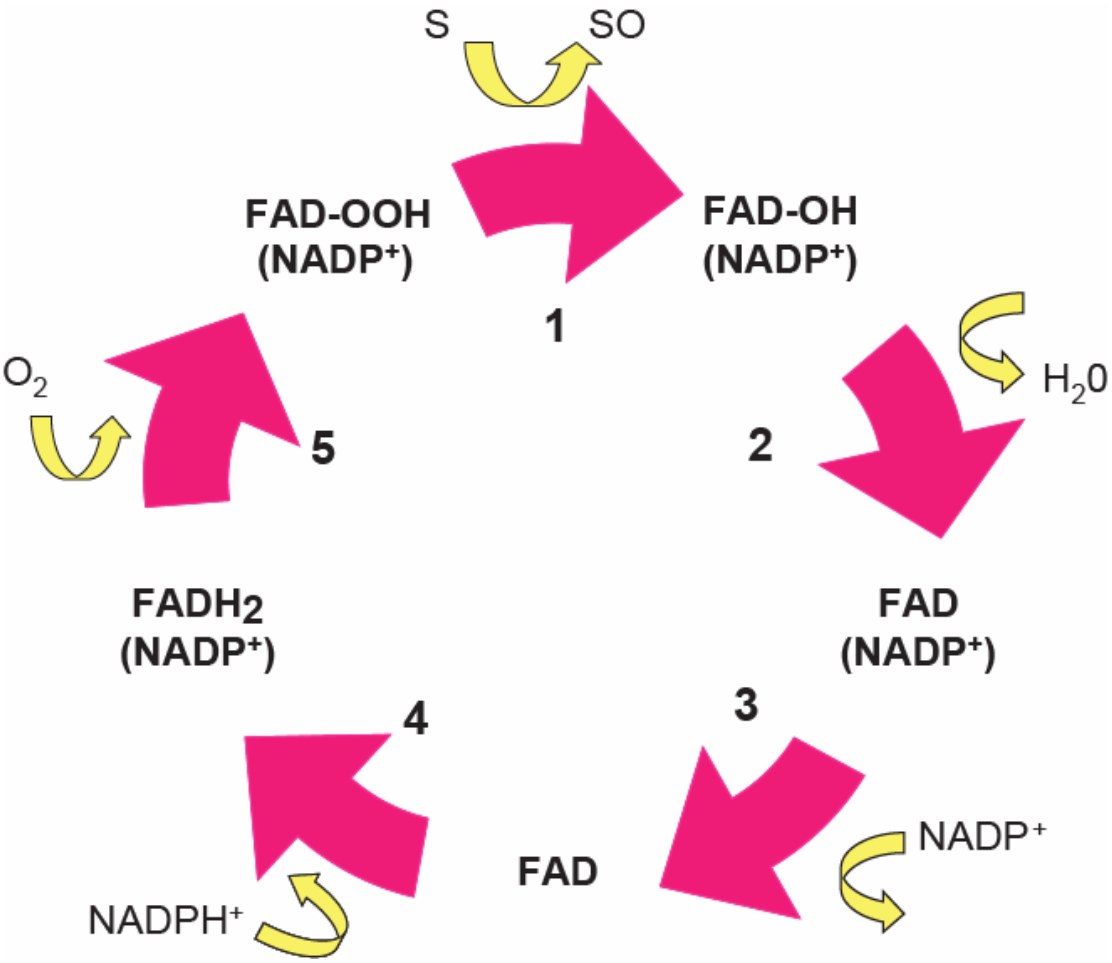


Figure 1.1: Major Steps in the Catalytic Cycle of FMO.

Substrate binding is not required for the cycle to begin. In the first step, the xenobiotic substrate **S** is oxidised to **SO** by the enzyme-bound 4a-hydroperoxyflavin (FAD-OOH). **SO** is released immediately and the release of water in step 2 is rate-limiting. The subsequent steps 3-5 simply regenerate the FAD-OOH form of the enzyme. Adapted from Ziegler 2002.

1.2.3 Substrate Specificity

In general, any compound bearing a soft nucleophile that can gain access to the enzyme-bound C4a-hydroperoxyflavin site is a potential substrate for an FMO. The unique mechanism of FMOs is theorised to be the basis of the broad range of compounds that are substrates for this enzyme. Substrates include hydrazines (Prough *et al.* 1981), phosphines (Smyser *et al.* 1985), iodine boron containing compounds (Jones *et al.* 1986), sulphides (Hamman *et al.* 2000), selenium bearing compounds (Ziegler *et al.* 1992) and an array of amines (Ziegler 2002). There is evidence of a number of endogenous compounds being FMO substrates including methionine (Duescher *et al.* 1994), cysteamine, cysteine- and homocysteine-*S*-conjugates (Ziegler 1993).

Structure-activity studies with purified and microsomal pig liver FMO indicate that the overall size and shape of a nucleophilic substrate are major factors limiting access to the FMO-bound hydroperoxyflavin radical.

1.2.4 Reactions Catalysed by FMOs: Detoxification *vs* Bioactivation

Detoxification refers to the removal of toxic metabolites by the process of oxidation or conjugation, i.e. it is a form of xenobiotic metabolism. Conversely bioactivation is the process of metabolism in which a generated metabolite is more toxic or reactive than the parent compound. FMOs generally catalyse the former reaction that results in the formation of metabolites with reduced pharmacological and toxicological properties. For instance, the neurotoxicant, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is readily *N*-oxygenated by FMO to the *N*-oxide, a non-toxic and easily excretable metabolite (Weisman *et al.* 1985; Cashman *et al.* 1986). As with every rule however, there are exceptions. In the case of certain sulphur-containing compounds such as thiocarbamides, FMOs efficiently catalyse their bioactivation.

Thiourea and *N,N*-mono or disubstituted thioureas are amongst the best substrates for FMOs (Poulsen *et al.* 1979; Henderson *et al.* 2004a; Onderwater *et al.* 2004; Onderwater *et al.* 2006). Initial work by Poulsen and Zeigler using phenylthiourea (Poulsen *et al.* 1979), demonstrated that FMOs catalyse a two-step, sequential oxidation of thiocarbamides to generate the sulphinic acid via a sulphenic acid intermediate (Fig 1.2). The sulphenic acids of thiocarbamide compounds are strong electrophiles that are highly susceptible to reduction by thiol-containing molecules, such as glutathione (GSH). The net effect of the non-enzymatic reduction of sulphenic acid by GSH results in the formation of oxidised GSH (GSH disulphide, GSSG) and regeneration of the parent thiocarbamide (Fig. 1.3A). In the presence of glutathione reductase, a futile cycle is established whereby the formation of sulphenic acid by the action of FMO is concomitantly linked to the reaction of glutathione reductase to regenerate GSH in its reduced form (Fig. 1.3A and B). Both enzymatic pathways are powered by the oxidation of NADPH to $NADP^+$.

The glutathione status within a cell (i.e. the disulfide:thiol ratio), if disturbed, can affect the activity of numerous enzymes and thus can alter normal cellular function (reviewed elsewhere by Kosower *et al.* 1978). An increased ratio of GSSG:GSH is an indication of oxidative stress and triggers cellular pathways to correct it (reviewed by Kosower *et al.* 1978). Studies with rat hepatocytes have determined two mechanisms by which the liver may attempt to correct this redox imbalance. When the amount of GSSG exceeds that of GSH in a cell, reductases catalyse the reduction of the oxidised form at the expense of NADPH oxidation, but when the rate of GSH oxidation exceeds that of GSSG reduction, excess GSSG is excreted in bile. Livers of rats perfused with thiourea or phenylthiourea led to the efflux of GSSG (but not GSH) in the bile of such animals confirming this hypothesis (Krieter *et al.* 1984). *N*-benzylimidazole a potent CYP inhibitor, had no effect on the

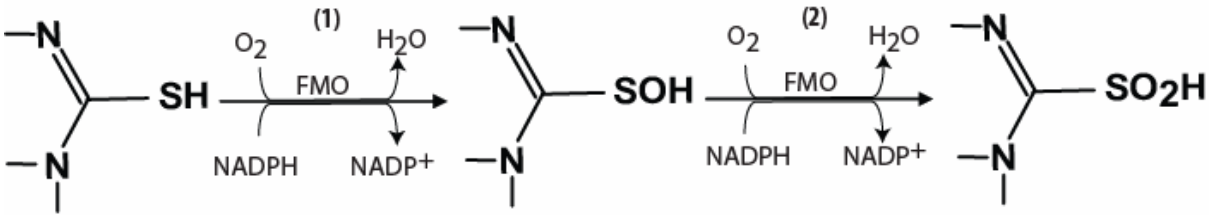


Figure 1.2: Oxidation of Thiourea by FMO. A sequential two-step oxidation of thiourea catalysed by FMO generates (1) formamadine sulphenic acid and (2) formamadine sulphinic acid. Adapted from Poulsen *et al.* 1979.

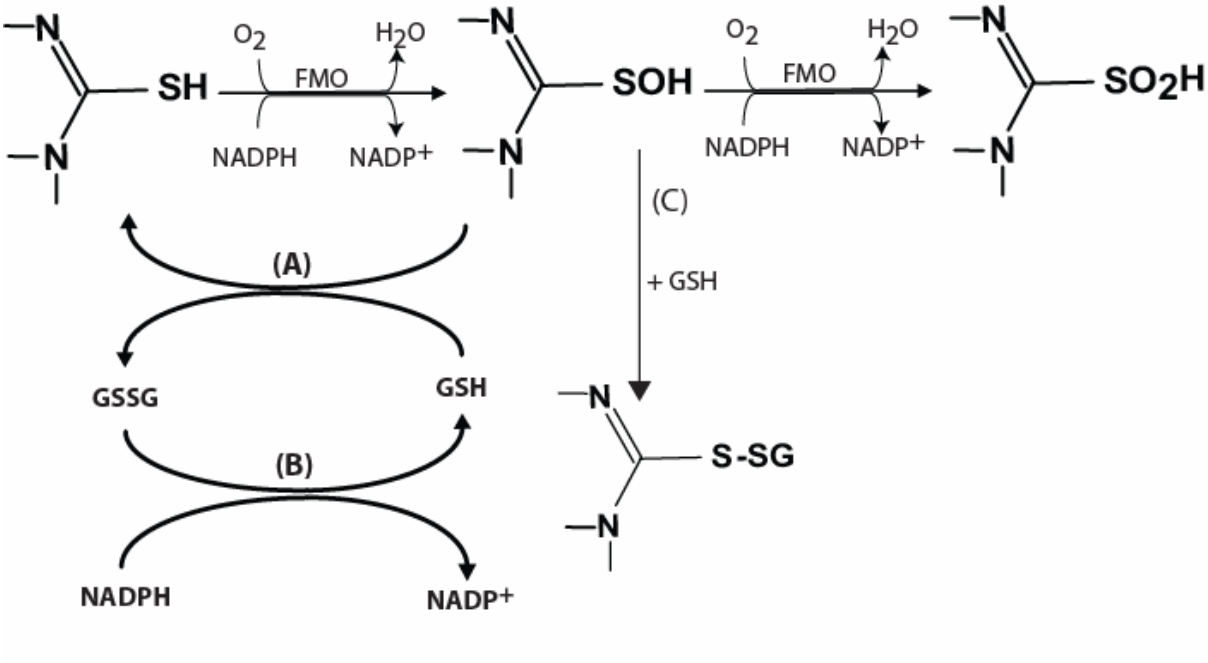


Figure 1.3: Interaction of Glutathione (GSH) with FMO-generated metabolites of Thiocarbamide S-oxygenation.

(A) Non-enzymatic reduction of formamidine sulphenic acid to the parent thiocarbamide by GSH and the formation of GSH disulfide. (B) Enzymatic regeneration of GSH by the oxidation of NADPH. (C) Non-enzymatic reaction of formamadine sulphenic acid and GSH.

amount of GSSG excreted in bile and thus indicated that this monooxygenase was not involved in the bioactivation reaction. The contribution of hydroperoxidase and glutathione peroxidase in the bioactivation process was not determined to be significant as selenium-deficient mice excreted similar amounts of GSSG in bile when compared to mice supplemented with selenium (Krieter *et al.* 1984). Lack of GSSG efflux in bile after administration of mice with the corresponding nitrogen analogue of thiourea and phenylthiourea (i.e. urea and phenylurea) was consistent with a model in which a reactive sulphur-containing derivative of the thiocarbamide was responsible for the toxicity. Given the substrate specificity of FMO and the formation of sulphenic acid species by this enzyme led to a FMO-dependent pathway being implicated in the bioactivation of thiourea and phenylthiourea in the liver of rats (Krieter *et al.* 1984).

In addition to the exhaustion of GSH in the redox cycling of sulphenic acids, depletion of NADPH as a result of the uncontrolled oxidation reactions may lead to the depletion of glycogen. NADPH is largely maintained from the dehydrogenation of glucose-6-phosphate, a process that occurs as part of the pentose phosphate pathway. Since glucose-6-phosphate is derived from glycogenolysis, the need to regenerate NADPH from $NADP^+$ results in the depletion of glycogen (Krieter *et al.* 1984). In the liver, thiocarbamide bioactivation and GSH:GSSG imbalance may directly contribute to glycogen depletion as glycogenolysis is also stimulated by elevated GSSG levels (Sies *et al.* 1978). This has been defined as the basis of glycogen depletion observed in liver of rats perfused with thiocarbamides (Krieter *et al.* 1984).

The reactive SO^- moiety of sulphenic acids can attack the thiol group of GSH directly, generating a glutathione-sulphenate adduct and thus further depleting the concentration of GSH in a cell (Giri *et al.* 1970; Krieter *et al.* 1984) (Fig. 1.3C).

Other than the thiol-group in glutathione, sulphenic acid metabolites can react with thiol-containing molecules such as cysteine residues in proteins thus perturbing protein function (Decker *et al.* 1992).

The sulphenic acid metabolite of thiourea is implicated in forming DNA adducts (Ziegler-Skylakakis *et al.* 1998). Furthermore the sulphinic acid of thiourea has been demonstrated to initiate DNA repair synthesis, form DNA adducts and cause the formation of micronuclei in cultured rat hepatocytes (Ziegler-Skylakakis *et al.* 1998). A micronuclei test is used to assess DNA damage induced by a compound. Micronuclei form when a chromosome or fragment of a chromosome fails to incorporate into daughter nuclei at the time of cell division.

1.3 Human FMOs

In humans, five *FMO* genes are known to encode protein (*FMO1-5*) (Phillips *et al.* 1995). *FMOs* 1, 2 3 and 4 are found in a cluster with *FMO6,* a putative pseudogene, at 1q23-4 (Shephard *et al.* 1993; Hernandez *et al.* 2004). *FMO5* is located further away from this cluster at 1q21 (McCombie *et al.* 1996). A second cluster on chromosome 1 contains five *FMO* pseudogenes, *FMO7P-FMO11P*, which are located within a cluster ~4Mb to the centromeric side of the functional *FMO* gene cluster (Hernandez *et al.* 2004).

FMOs 1-5 exhibit 50-59% amino acid identity across mammalian species including humans (Lawton *et al.* 1994). A primary sequence alignment of the five functionally active human FMOs is given in Figure 1.4 and illustrates the highly conserved motifs identified in all mammalian FMOs. Two GXGXXG motifs, known to be important in the binding of the

Figure 1.4: A primary sequence alignment of the five functional FMOs in humans. The primary sequence of FMOs 1-5 are aligned. An asterix denotes conserved identical residues, a colon indicates conserved similar residues, and a dot indicates highly similar residues. The 'GXGXXG' FAD and NADPH binding moieties are boxed in red along with the FMO characteristic 'FATGY' motif boxed in blue. Primary sequences were aligned using the ClustalW2 tool available at http://www.ebi.ac.uk/Tools/clustalw2/index.html.

Adenine dinuceotide phosphate (ADP) moiety in FAD and NADPH are present at positions (residues 9-14 and 191-196, respectively) within human FMOs (Fig. 1.4) and other examined mammalian FMOs. The FAD-binding site is contained within a highly conserved motif that predicts a βαβ secondary structure, a Rossman fold, known to be important in binding dinucleotides (Wierenga *et al.* 1986). In addition to this motif, an FMO characteristic motif 'FATGY' has been identified and is present at identical positions within the human FMOs (Fig.1.4) and is thought to play a role in *N*-oxidation.

1.3.1 FMO1

FMO1, purified from porcine liver microsomes, was the first FMO to be isolated from porcine liver microsomes and was named as a mixed function amine oxidase (Ziegler *et al.* 1971) (see section 1.2.1). A cDNA corresponding to the human FMO1 protein was identified in 1991 (Dolphin *et al.* 1991) and was formally named as FMO1 in 1994 (Lawton *et al.* 1994). The human gene encodes a polypeptide of 532 amino acid residues that has a molecular weight of 60,603 Daltons.

FMO1 is highly expressed in the liver of most adult mammals such as pig, rabbit, rat, dog and mouse (Gasser *et al.* 1990; Lawton *et al.* 1990; Cherrington *et al.* 1998; Lattard *et al.* 2002; Stevens *et al.* 2003) but the gene is not expressed in the liver of adult human. Neither the mRNA (Phillips *et al.* 1995; Dolphin *et al.* 1996) nor protein (Yeung *et al.* 2000; Koukouritaki *et al.* 2002) of FMO1 is detected in the adult liver. The lack of *FMO1* expression in the adult human liver is thought to be due to the insertion of several long interspersed nuclear elements (LINEs), just upstream of the proximal promoter P0 (Shephard *et al.* 2007), which influence the expression in the adult, but not in the foetal liver. FMO1 is expressed in foetal liver of humans (Dolphin *et al.* 1991; Phillips *et al.* 1995; Dolphin *et al.*

1996; Yeung *et al.* 2000; Koukouritaki *et al.* 2002), but its expression is switched off in a mechanism linked tightly to birth but not gestational age (Koukouritaki *et al.* 2002). The repression is likely to be due to increased methylation, around the time of birth, of the proximal promoter P0 due to the close presence of LINE elements (Shephard *et al.* 2007)

FMO1 expression is abundant in the adult human kidney, and the gene is also, to a lesser extent, expressed in the small intestine (Yeung *et al.* 2000), stomach and endocrine organs such as the thymus, testis, pancreas, adrenal cortex and thyroid (Hernandez *et al.* 2004). FMO1 is also detected in the foetal kidney, but unlike in the liver, expression in this tissue increases after birth (Dolphin *et al.* 1991; Phillips *et al.* 1995; Dolphin *et al.* 1996; Yeung *et al.* 2000; Krause *et al.* 2003). The continued expression of the gene in adult tissues such as the kidney is due to the use of tissue-specific, alternative, downstream promoters (Shephard *et al.* 2007).

Levels of expression of FMO1 in the kidney (47 ± 9 pmol/mg microsomal protein) (Yeung *et al.* 2000) are not much lower than that observed in the liver for the major hepatic CYP isoform, CYP3A4 (96 ± 51 pmol/mg microsomal protein) (Shimada *et al.* 1994). In kidney, the amount of FMO1 is greater than that of total CYP expression and thus is likely to be an important contributor to renal xenobiotic metabolism.

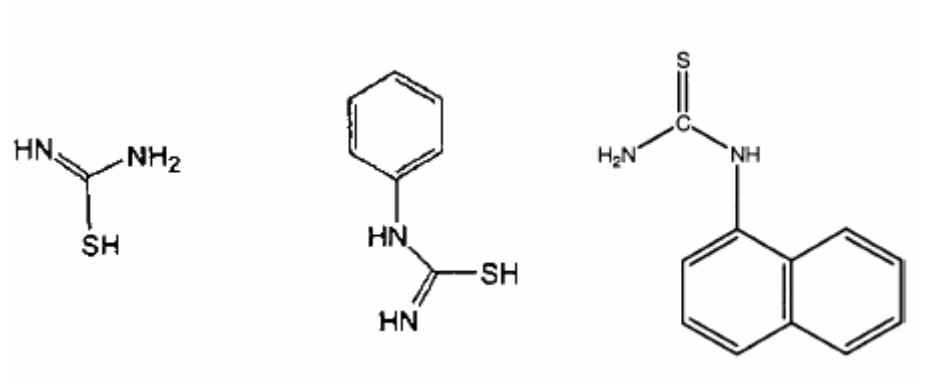
FMO1, among all the FMO isoforms has the broadest substrate specificity. This is an attribute of the size and dimension of the access channel through which a substrate gains entry to the active site. The access channel in human FMO1 is estimated to be 5 Å in diameter and the active site containing FAD-OOH is at least 5 Å below the surface of the channel (Kim *et al.* 2000). This is the theorised basis by which FMO1 can oxygenate substrates as small as thiourea with the same efficiency as it metabolises large and bulky compounds such as tricyclic tertiary amines (Fig. 1.5). A wide range of xenobiotics and foreign compounds are among its substrates including therapeutic drugs, pesticides and

endogenous compounds. A list of all known substrates of human FMO1 that are of therapeutic importance has been listed in Tables 1.1 and 1.2.

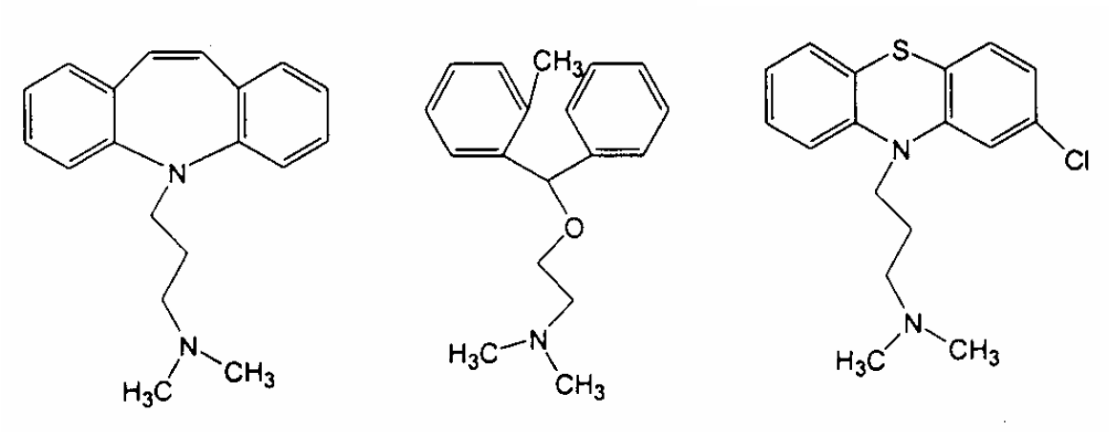
1.3.1.1 Inter-Individual Differences in Expression of Human FMO1: Consequences for Drug Metabolism

Human *FMO1* is the most conserved gene amongst the *FMOs* within the gene cluster located on 1q23-4. In a recent study, four non-synonymous single nucleotide polymorphisms (SNPs) were identified in the protein coding region of *FMO1* in African-American subjects (Furnes *et al.* 2003). The genetic substitutions (and subsequent amino acid substitution) were observed to be g.9614C>G (**H97Q**), g.23970A>G (**I303V**), g.23971T>C (**I303T**) and g.27362C>T (**R502X**) and were estimated to occur at a relatively low frequency of 1-2% in this population (Furnes *et al.* 2003). Functional studies with heterologously expressed protein variants indicated a moderate but distinct substrate-specific difference towards typical FMO1 substrates including the hyperthyroidism drug, methimazole, the anti-depressant drug imipramine and the pesticide agent fenthion. An interesting finding was that the truncated variant, R502X was capable of oxidising imipramine and fenthion but was inactive towards methimazole.

Recently, studies have reported inter-individual differences in levels of protein expression of human FMO1 in the kidney (Yeung *et al.* 2000; Koukouritaki *et al.* 2002; Krause *et al.* 2003). As FMOs are generally not inducible by environmental factors, it is plausible to assign such inter-individual differences to genetic variability within promoter and regulatory sequences within the gene that ultimately lead to different amount of protein being produced. A single nucleotide substitution, g.-9,536C>A (*FMO1*6)* was identified in the upstream region of exon 0 that happens to be within a Yin Yang 1 (YY1) binding site sequence (Hines *et al.* 2003). The YY1 site also overlaps binding sequences of other



Thiourea Phenylthiourea Alpha-naphthylthiourea



Imipramine Orphenadrine Chlorpromazine

Figure 1.5: Selected substrates of human FMO1. Chemical structures of xenobiotics are given to illustrate the diversity in size and dimension of substrates oxygenated by human FMO1. Phenylthiourea and alpha-naphthylthiourea are thiocarbamides where as Imipramine and Chlorpromazine are tricyclic tertiary amines. Orphenadrine is a methylated derivative of diphenylhydramine. (Figure adapted from (Kim *et al.* 2000).

Table 1.1 Nitrogen-containing drugs oxygenated by Human FMO1

Substrate	Type of Drug or health condition	Product	Reference
Benzydamine	non-steroidal anti-inflammatory agent (rheumatism)	*N*-oxide	(Lang *et al.* 2000; Rettie *et al.* 2000)
Chlorpromazine	Dopamine D2 antagonist (antipsychotic)	-	(Kim *et al.* 2000)
Deprenyl	Monoamine oxidase type B inhibitor (Parkinsons Disease)	hydroxylamine	(Szoko *et al.* 2004)
Imipramine	5HT/noradrenaline uptake inhibitor (anti-depressant)	*N*-oxide	(Kim *et al.* 2000)
Itopride	dopamine D2 antagonist (gastroprokinetic agent)	*N*-oxide	(Mushiroda *et al.* 2000)
Methamphetamine	Psychostimulant	Hydroxylamine	(Szoko *et al.* 2004)
N- deacetyl ketoconazole*	anti-fungal agent	*N*-hydroxy	(Rodriguez *et al.* 2000)
Olopatadine	anti-histamine agent	*N*-oxide	(Kajita *et al.* 2002)
Orphenadrine	Anti-cholinergic (Parkinson's Disease)	-	(Kim *et al.* 2000)
SNI-2011	muscarinic receptor agonist (Sjogren's Syndrome)	*N*-oxide	(Washio *et al.* 2001)
Tamoxifen	estrogen-receptor modulator (Breast Cancer Therapy)	*N*-oxide	(Parte *et al.* 2005)
Xanomeline	muscarinic receptor agonist (Alzheimer's disease)	*N*-oxide	(Ring *et al.* 1999)

*Major metabolite of anti-fungal agent ketoconazole.

- product not identified

In many cases, FMO1 is not the only enzyme involved in the metabolism of the drug *in vivo*.

This table appears in the review by Phillips, Francois and Shephard (Phillips 2007) which is bound into the back of this thesis.

Table 1.2: Suphur-containing drugs oxygenated by Human FMO1

Substrate	Type of Drug or health condition	Product	Reference
Ethionamide	Antibiotic (tuberculosis)	*S*-oxide/sulphinic acid	(Henderson *et al.* 2008)
Methimazole	Thyroperoxidase inhibitor (hyperthyroidism)	*S*-oxide	(Furnes *et al.* 2004)
S – methyl esonarimod*	cytokine production inhibitor (rheumatism)	*S*-oxide	(Ohmi *et al.* 2003)
Tazorotenic acid**	Retinoic acid receptor modulator (acne/psoriasis)	*S*-oxide	(Attar *et al.* 2003)
Thiacetazone	Antibiotic (tuberculosis)	Sulphinic acid/carbodiimide	(Qian *et al.* 2006)

*active metabolite of parent compound esonarimod.

** active metabolite of parent compound tazarotene.

In some cases FMO1 is not the only enzyme involved in the metabolism of the drug *in vivo*.

This table appears in the review by Phillips, Francois and Shephard (Phillips 2007) which is bound into the back of this thesis.

transcription factors, mainly Oct1 and HNF1 (Hines *et al.* 2003). YY1 is a transcription factor which is known to negatively regulate *FMO1* expression in rabbit (Luo *et al.* 2001). Transcriptional reporter assays with human *FMO1* gene constructs however suggest YY1 to be a positive regulator of FMO1 expression, but only in the presence of other yet to be identified upstream regulators. In humans therefore, it is suggested that the disruption of the consensus YY1 binding site as a result of the g.-9,536C>A mutation results in the downregulation of transcription of *FMO1*. This finding has been postulated as the basis of inter-individual difference in *FMO1* expression levels observed in adult kidney (Hines *et al.* 2003). Analysis of the frequency of the variant allele, *FMO1*6* was not found to be statistically different among African Americans or Northern European-Americans but was found to be prevalent in Hispanic Americans at a significantly higher frequency of 26.9% (Hines *et al.* 2003). A significantly higher amount of FMO1 in kidney obtained from African individuals than Caucasians has been reported. However, it is noteworthy that the comparison made in this study may not be appropriate as kidney biopsies were used for African sample analysis while samples from cadavers were analysed for all Caucasians (Krause *et al.* 2003).

The genetic variations observed in *FMO1* may have consequences for the ability of individuals to metabolise therapeutic drugs and other xenobiotics that are substrates for this enzyme. For example, *Fmo1* (-/-) mice administered with the anti-depressant drug imipramine, display exaggerated pharmacological behavioural responses including tremors and body spasms and increased amounts of parent drug in the plasma and kidney (Hernandez *et al.* 2009). Conversely, higher levels of FMO1 in the kidney may be associated with increased renal toxicity as a result of bioactivation of xenobiotics containing sulphur moieties.

1.3.2 FMO2

In 1984, direct evidence of the existence of multiple forms of FMO was obtained when an enzyme from rabbit lung was purified. The 'lung' FMO was distinctly different from the 'liver' FMO (now known as FMO1) in that it had restricted substrate specificity related to steric properties, greater thermal stability and displayed a higher pH optimum for enzyme activity (Williams *et al.* 1984; Williams *et al.* 1985; Nagata *et al.* 1990; Williams *et al.* 1990; Venkatesh *et al.* 1992). This enzyme is now know as FMO2.

FMO2 is highly expressed in the lung of mammals including rabbits (Williams *et al.* 1984; Tynes *et al.* 1985; Williams *et al.* 1985; Lawton *et al.* 1990; Williams *et al.* 1990), guinea pig (Nikbakht *et al.* 1992), mouse (Karoly *et al.* 2001), and rhesus macaque (Yueh *et al.* 1997). FMO2 expression in rabbit has been estimated to represent 10% of total lung protein (Williams *et al.* 1984; Tynes *et al.* 1985). The mRNA of FMO2 is the most abundant amongst the isoforms in human lung (Zhang *et al.* 2006) but efforts to isolate and purify the protein from this tissue proved unsuccessful (Whetstine *et al.* 2000).

Genotypic analysis revealed that the *FMO2* gene, in humans has ethnic-specific variation in its base sequence (Dolphin *et al.* 1998; Whetstine *et al.* 2000). All Europeans and Asians genotyped to date possess the *FMO2**2 allele in which a C>T substitution at position 1414 bp (in the cDNA) replaces a glutamine amino acid residue at position 472 with a premature stop codon in the polypeptide (Q472X) (Fig. 1.6) (Dolphin *et al.* 1998). This position corresponds to g.23238C>T (Q472X) of the gene. A proportion of individuals of African descent however, possess an *FMO2* gene that encodes a full-length protein (Dolphin *et al.* 1998). This allele is called *FMO2*1*. The truncated protein, FMO2.2, when compared with FMO2 from rabbit, guinea pig and rhesus macaque lacks 64 amino residues from its carboxy terminus. Analysis of the product of heterologously expressed FMO2.2 cDNA revealed that the truncated protein is catalytically inactive (Dolphin *et al.* 1998). In the

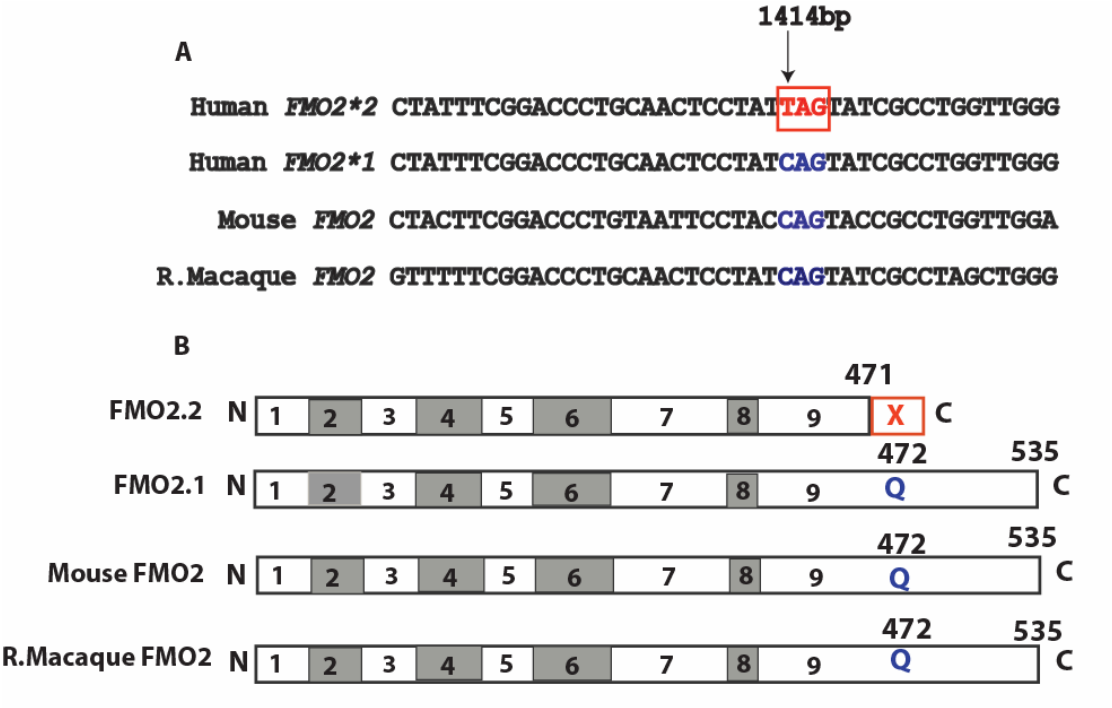


Figure 1.6: The Nonsense Mutation in Human FMO2 is absent in Other Mammalian Species: (A) The codon at position 1414 bp (relative to the A of the ATG translation initiation codon) in the ancestral gene of human *FMO2*1* and mouse and rhesus macaque *FMO2* is given in blue. The corresponding codon in human *FMO2*2* is boxed in red and the position of the nucleotide base substitution is marked with an arrow. **(B)** Horizontal bars represent the polypeptide chains encoded by genes given in part (A). N and C denote the amino- and carboxy- termini respectively. Alternative grey and white boxes indicate regions encoded by exons 1-9.

human lung, the truncated protein is thought to be rapidly degraded because of incorrect folding (Dolphin 1998) and this probably explains why attempts to purify FMO2 from human lung had been unsuccessful (Whetstine *et al.* 2000). In contrast, when the full-length FMO2.1 protein is expressed in a heterologous system it is catalytically active (Dolphin *et al.* 1998).

A recent study in which 1800 people, of recent African descent from sub-Saharan Africa were genotyped, up to 50% of individuals were found to possess at least one copy of the ancestral allele *FMO2*1* (Veeramah *et al.* 2008). Individuals with one or two *FMO2*1* alleles are thus expected to express the full-length and catalytically functional protein (FMO2.1) in the lung (Dolphin *et al.* 1998; Krueger *et al.* 2002; Veeramah *et al.* 2008) (Fig. 1.6). The ancestral allele occurs at relatively low frequency in North Africa and its distribution in sub-Saharan Africa differs significantly within this region (Fig. 1.7). The *FMO2*1* allele has also been observed in 5-7 % of Hispanics of African decent (Whetstine *et al.* 2000).

The nonsense mutation g.23238C>T (Q472X) is not present in non-human primates such as the chimpanzee (*Pan troglodytes*) and the gorilla (*Gorilla gorilla*) and must have therefore arisen in the human lineage some time after the divergence of the *Homo* and *Pan* clades took place some 6 million years ago (Brunet *et al.* 2002).

The full length human FMO2 protein contains 535 amino acid residues and has a molecular weight of 60, 920 Daltons. Analysis of microsomes prepared from a lung sample obtained from a single individual heterozygous for the *FMO2*1* allele detected the expression of FMO2.1 protein in this tissue. The microsomal sample was capable of *S*-oxygenating methimazole with a similar catalytic efficiency as that observed for heterologously expressed human FMO2.1 (Dolphin *et al.* 1998; Krueger *et al.* 2002).

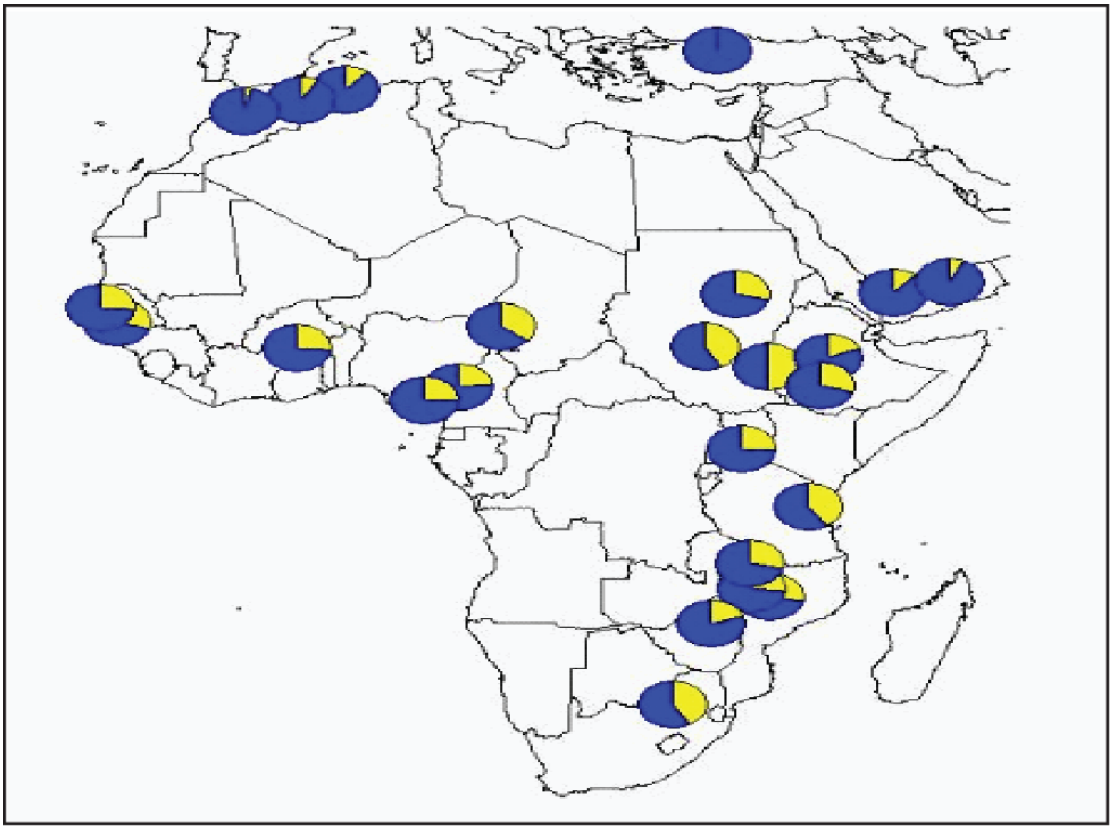


Figure 1.7: The Frequency and Distribution of the Human *FMO2*1* allele in Africa: Pie charts illustrating the prevalence of the human *FMO2*1* allele in different parts of Africa. Yellow = frequency of individuals possessing at least one allele encoding human *FMO2*1*, blue = individuals homozygous for the human *FMO2*2* allele. Figure taken from Veeramah *et al.* 2008.

1.3.2.1 Structure-Function Studies: The Restricted Substrate Specificity of FMO2

Limited structure-function studies have been carried out with human FMO2. A kinetic study using a panel of phenothiazine derivatives indicated that the oxygenatable moiety of a compound bearing large or bulky groups, needed to be a certain distance away from them to gain access to the active site (Fig. 1.8) (Krueger *et al.* 2005). For instance, FMO2.1 was not active towards a phenothiazine derivative with a 3-carbon side chain but was active when the side chain increased in length from 3 to 5 or 8 alkyl groups (Fig. 1.8). A similar study with rabbit FMO2 and porcine FMO1 demonstrated how the latter efficiently catalysed the oxidation of phenothiazine derivatives with a 3-carbon side chain whereas FMO2 could not (Nagata *et al.* 1990). Using a panel of thioureas of various sizes, Nagata et al. demonstrated that unlike porcine FMO1, rabbit FMO2 could not catalyse the oxygenation of 1,3-diphenylthiourea, the largest thiourea tested in this investigation (Nagata *et al.* 1990). These findings predicted that in addition to the access channel of FMO2 being narrower than that of FMO1, the catalytic centre of FMO2 was considerably deeper within the enzyme than FMO1 (Nagata *et al.* 1990; Kim *et al.* 2000).

1.3.2.2 Inter-individual Differences in human FMO2 expression: Consequences for Xenobiotic Metabolism

The principle inter-individual difference in FMO2 expression in humans is the expression of functionally active (FMO2.1) or inactive (FMO2.2) protein in the lung. As detailed in the preceding section, the majority of the world's population does not produce FMO2 in their lungs, but a significant proportion of sub-Saharan Africans do. The amount of FMO2.1 expressed in the lung of individuals possessing the ancestral gene has been estimated to be around 8.8 pmol/mg microsomal protein which is comparable if not greater than most CYPs expressed in this tissue (Krueger *et al.* 2002; Henderson *et al.* 2004b).

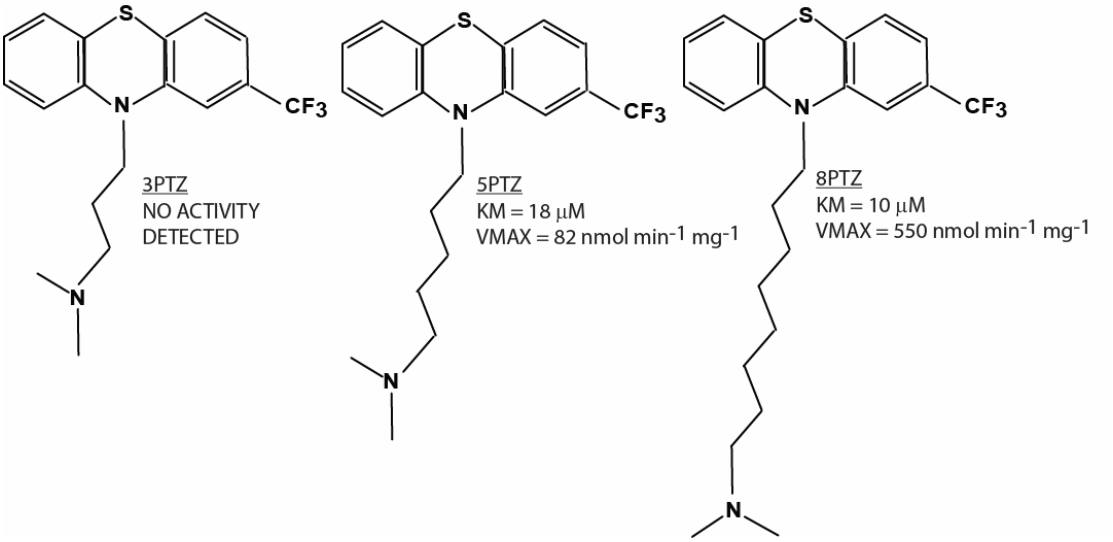


Figure 1.8: Human FMO2.1 substrate specificity towards phenothiazines. Activity of FMO2.1 towards phenothiazine with tertiary amine substituents of differing lengths. 3PTZ, 5PTZ and 8PTZ represent phenothiazine derivaties with 3, 5 or 8 carbon alkyl side chains respectively. Adapted and revised from Krueger *et al.* 2005.

It is therefore plausible to assume that individuals expressing FMO2.1 will have altered metabolism of drugs and xenobiotics in the lungs. This raises an important question of whether such individuals are more or less predisposed to FMO-dependent toxicity of xenobiotics, whether the activity of this enzyme will affect the efficacy of a drug or whether these individuals may be at a genetic advantage with regard to protection from exogenous toxicants.

To date, limited studies have investigated the contribution of FMO2.1 in drug metabolism, probably because of the apparent lack of this isoform in human lung. The recent discovery that a significant proportion of individuals carry the *FMO2*1* allele has however generated interest in the toxicological consequences of xenobiotic metabolism by this enzyme.

Heterologously expressed human FMO2.1 is capable of catalysing the *S*-oxygenation of a wide range of thiourea-containing compounds including phenylthiourea, alpha-naphthylthiourea and ethylenethiourea (Henderson *et al.* 2004a). Thiourea containing compounds are used extensively in industry and to a certain extent are used in household products. Thiourea is an industrial chemical with a number of uses, it is used extensively in the manufacture of flame retardant resins and is the main component of liquids used to clean items made of silver. Recently, the interest in thiourea-containing drugs has risen since the development of Trovirdine, an *N*-substituted thiocarbamide as a potent HIV-1 non-nucleoside, reverse transcriptase inhibitor (Cantrell *et al.* 1996; Onderwater *et al.* 2004). Alpha-naphthylthiourea (ANTU) is used extensively as a rodenticide. The product of the FMO-mediated oxygenation of thioureas, the sulphinic acid metabolite, is generated via a sulphenic acid intermediate that is more toxic than the parent compound. As detailed in section 1.2.4, the intermediate metabolite is capable of redox cycling in the presence of glutathione and glutathione reductase which is hypothesised to result in oxidative stress.

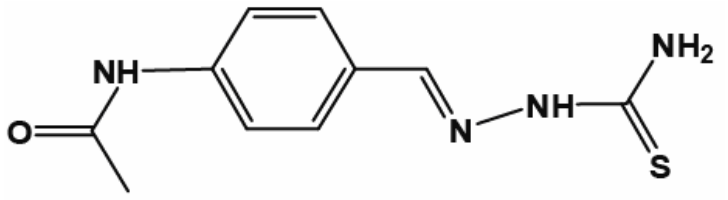
Other than reacting with glutathione, the sulphenic acid metabolite can react with sulphhydrl groups including cysteine residues of other proteins. This is the mechanism by which the FMO-dependent *S*-oxygenation of phenylthiourea (PTU) was shown to inhibit the activity of CYP1A1 (Onderwater *et al.* 1999) in rat hepatocytes. In rats, ANTU is toxic to the lungs causing severe pulmonary edema and vascular injury which has been demonstrated to be FMO-dependent (Boyd *et al.* 1976; Lee *et al.* 1980; Scott 1990). Since ANTU is sold in a powdered form, inhalation of ANTU particles is likely to be toxic to individuals expressing FMO2.1 in the lungs.

Recent studies have demonstrated the activity of human FMO2.1 towards pesticides such as phorate and disulfoton (Henderson *et al.* 2004b). Phorate and disulfoton are thioether-containing organophosphate insecticides used extensively in agriculture. They are broad-spectrum insecticides used to protect crops such as corn, potatoes, cotton and grain, including wheat. Exposure to these compounds occurs both in occupational settings and to the public in general. In the occupational setting, the primary route of exposure is inhalation and dermal, whereas in the general population it is inhalation, diet and dermal (Brokopp *et al.* 1981). As inhalation is a major route of exposure, extensive pulmonary metabolism of such compounds is expected. The role of CYPs in organophosphate metabolism is well documented. As with other organophosphates, CYPs can catalyse the desulphuration to yield the corresponding oxon, a toxic metabolite and potent inhibitor of acetylcholinesterase (Kulkarni 1984). CYPs can also catalyse the formation of the *S*-oxide and further oxidise this metabolite to generate a sulphone (Kulkarni *et al.* 1984). In addition to the toxic oxon, the sulphone metabolite of organophosphate pesticides is also a acetylcholinesterase inhibitor (Levi *et al.* 1988). Human FMO2.1-dependent sulphoxidation of phorate and disulfuton generates the *S*-oxide, a typical detoxification product and does not catalyse the second oxidation reaction to the sulphone (Henderson *et al.* 2004b). With the higher FMO:P450

ratio in the lungs and the high catalytic activity displayed by FMO2.1 towards these compounds, plus the lack of production of toxic oxons and sulphone metabolites (generated by CYP oxidation), suggests that individuals expressing FMO2.1 in the lung are protected from bioactivated species of such exogenous compounds. Conversely one may argue that the extensive formation of the *S*-oxide by FMO2.1 in the lungs may provide a source for the CYPs to catalyse the secondary oxidation reaction of this metabolite to give more of the toxic sulphone species.

With respect to altered drug metabolism in the lung, anti-tubercular drugs are of particular concern. Thiacetazone and Ethionamide are prodrugs that require metabolic activation by *Mycobacterium tuberculosis* to exert an anti-microbial effect (Baulard *et al.* 2000; DeBarber *et al.* 2000; Vannelli *et al.* 2002; Qian *et al.* 2006; Dover *et al.* 2007). A detailed description of these drugs and their activation will be given in section 1.4.2. Thiacetazone and Ethionamide are thiocarbamide-containing compounds and thus are expected to be FMO substrates. Human FMO1 and FMO3 are capable of catalysing the *S*-oxygenation of thiacetazone to form the sulphinic acid and carbodiimide derivatives of this drug (Qian *et al.* 2006). Although an intermediate was not identified, a sulphenic acid metabolite is postulated to be the precursor of the observed products. *In vitro* studies have demonstrated that the metabolites of this reaction lead to the depletion of glutathione and thus it represents a bioactivation. Whether or not FMO2.1 can catalyse this reaction is still to be determined and provides the focus of experiments described in this thesis. Given that tuberculosis is a major problem in a world region where the frequency of the *FMO2*1* allele is considerably high, raises the question of whether individuals given this drug as part of chemotherapy will be at a greater risk of drug toxicity. It also poses the question of whether the 'use' of thiacetazone by FMO2.1 in the lung may affect drug efficacy. It is tempting to assume that thiacetazone will be a substrate for FMO2.1 as FMO1 and FMO3 are capable of

A



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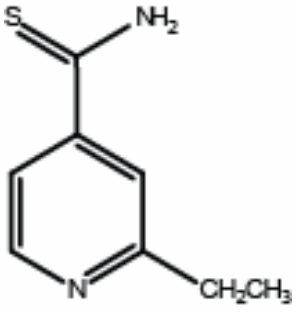


Figure 1.8A: Chemical Structures of Second-line Antibiotics: (A) Thiacetazone and (B) Ethionamide.

its *S*-oxygenation, but caution must be exercised when extrapolating substrate specificities among FMO isoforms. As detailed in the preceding section, FMO2 has the most restricted substrate specificity when compared to both FMO1 and FMO3. Marked differences in substrate oxygenation between isoforms is a result of the dimension of the channel that gives access to the active site of the enzyme and has been described in section 1.3.2.1.

1.3.3 FMO3

A cDNA for human FMO3 was identified in 1992 (Lomri *et al.* 1992) and it encoded a polypeptide of 532 amino acid residues with a molecular weight of 60, 047 Daltons.

FMO3 is the major FMO isoform present in the adult human liver (Phillips *et al.* 1995; Dolphin *et al.* 1996). FMO3 mRNA has also been detected in the lung, kidney, adrenal medulla and cortex, pancreas, thyroid, gut and brain (Hernandez *et al.* 2004). Low levels of FMO3 are detected in embryonic liver cells but not in the foetus. Currently, the mechanisms and factors required for the expression of FMO3 in the embryo, silencing through foetal development and reactivation after birth are not known. After birth the onset of FMO3 expression in the liver occurs in three distinct phases between 3 weeks and 10 months of age, 10 months and 11 years and between 11 and 18 years of age (Koukouritaki *et al.* 2002). Unlike the regulation of human *FMO1* in the foetal liver which is tightly linked to birth but irrespective of gestational age, the onset of *FMO3* gene expression after birth is less defined. Birth is necessary but not sufficient for expression of FMO3 in the adult liver and some individuals show no expression of this protein up until 10 months of age (Koukouritaki *et al.* 2002).

In terms of substrate specificity, FMO3 is an intermediate between FMO1 and FMO2. It can metabolise substrates which are excluded from the active site of FMO2 (such as secondary amines, (Cashman *et al.* 1999)) but cannot catalyse the oxidation of bulkier

compounds such as imipramine which are good substrates for FMO1 (Kim *et al.* 2000). Given the relatively broad substrate specificity of this enzyme and its expression in the liver, FMO3 is considered the most important FMO isoform in hepatic drug metabolism in humans. Compounds of therapeutic importance metabolised by FMO3 are given in Tables 1.3 and 1.4.

Unlike other FMO isoforms, an endogenous role for FMO3 in humans is established. The bacterial break-down product of dietary choline in the gut is trimethylamine (TMA), a compound with an extremely strong, fish-like odour. In the liver, FMO3 catalyses the *N*-oxygenation of TMA to its *N*-oxide, a metabolite with no detectable offensive odour. TMA *N*-oxide is excreted from the body via the urine and sweat. Impairment of this detoxification reaction results in the rare but serious condition of Trimethylaminuria, which will be described below.

1.3.3.1 Genetic Variants of human *FMO3*

Human *FMO3* is the most polymorphic among the *FMO* genes with more than 40 SNPs identified across the entire length of this gene (reviewed elsewhere by Phillips 2007 and Phillips *et al.* 2008). These variations, many in the form of single nucleotide substitutions, may have consequences for the ability of individuals to metabolise therapeutic drugs and other xenobiotics that are substrates for this enzyme.

1.3.3.2 FMO3 Variants and Trimethylaminurea

Some of the documented SNPs identified in *FMO3* are rare (occurring at a frequency of <1% in a population) and result in the total loss of FMO3 catalytic activity. A list of these mutations has been given elsewhere (for a review see Phillips 2007). Such polymorphisms

Table 1.3: Nitrogen-containing drugs oxygenated by Human FMO3

Substrate	Type of Drug Or Health Condition	Products	Reference
Amphetamine	dopamine transporter ligand (antipsychotic)	hydroxylamine	(Cashman *et al.* 1999; Szoko *et al.* 2004)
Benzydamine	non-steroidal anti-inflammatory (rheumatism)	*N*-oxide	(Lang *et al.* 2000; Stormer 2000)
Clozapine	antipsychotic agent	*N*-oxide	(Tugnait *et al.* 1999)
5,6-Dimethylxanthenone 4-acetic acid (DMXAA)	antitumour agent	Hydroxylamine	(Zhou *et al.* 2002)
Deprenyl	monoamine oxidase type B inhibitor (Parkinson's Disease)	Hydroxylamine	(Szoko *et al.* 2004)
Itopride	Dopamine D2 antagonist (gastroprokinetic agent)	*N*-oxide	(Mushiroda *et al.* 2000)
K11777	Cysteine protease inhibitor (Agent against *Trypanosoma Cruzi*)	*N*-oxide	(Jacobsen *et al.* 2000)
Methamphetamine	psychostimulant	Hydroxylamine	(Cashman *et al.* 1999; Szoko *et al.* 2004)
N-deacetyl ketokanozole*	anti-fungal agent	*N*-hydroxy/nitrone	(Rodriguez *et al.* 2000)

Table 1.3: Nitrogen-containing drugs oxygenated by Human FMO3 (*continued*)

Substrate	Type of Drug Or Health Condition	Products	Reference
Nicotine	Stimulant	*Trans N*-oxide	(Park *et al.* 1993)
Olapatadine	anti-histamine agent	*N*-oxide	(Kajita *et al.* 2002)
Pyrazolacridine	Anti-tumour agent	*N*- oxide	(Reid *et al.* 2004)
Ranitidine	Anti-histamine (stomach ulcers/Zollinger Ellison syndrome)	*N*-oxide	(Chung *et al.* 2000)
S16020 (Olavicine Derivative)	anti-tumour agent	*N*- oxide	(Pichard-Garcia *et al.* 2004)
Tamoxifen	estrogen-receptor modulator (Breast Cancer Therapy)	*N*- oxide	(Mani *et al.* 1993)
Xanomeline	muscarinic receptor agonist (Alzheimer's disease)	*N*- oxide	(Ring *et al.* 1999)

*Major metabolite of anti-fungal agent ketoconazole.

In some cases FMO3 is not the only enzyme involved in the metabolism of the drug *in vivo*. **This table appears in the review by Phillips, Francois and Shephard (Phillips 2007) which is bound into the back of this thesis.**

Table 1.4: Suphur-containing drugs oxygenated by Human FMO3

Substrate	Type of Drug Or Health Condition	Products	Reference
Ethionamide	antibiotic (tuberculosis)	*S*-oxide/sulphinic acid	(Henderson *et al.* 2008)
MK-0767 methyl sulfide	peroxisome proliferator receptor activator (diabetic)	*S*-oxide	(Karanam *et al.* 2004)
Ranitidine	Anti-histamine (stomach ulcers/Zollinger Ellison syndrome)	*S*-oxide	(Chung *et al.* 2000)
S - methyl esonarimod *	cytokine production inhibitor (rheumatism)	*S*-oxide	(Ohmi *et al.* 2003)
Sulindac Sulphide**	Non-steroidal anti-inflammatory agent (Colorectal Cancer)	*S*-oxide	(Hamman *et al.* 2000)
Tazarotenic Acid***	Retinoic acid receptor modulator (acne/psoriasis)	*S*-oxide	(Attar *et al.* 2003)
Thiacetazone	antibiotic (tuberculosis)	sulphinic acid /carbodiimide	(Qian *et al.* 2006)

*active metabolite of parent compound esonarimod.

** active metabolite of parent compound sulindac.

*** active metabolite of parent compound tazarotene.

In some cases FMO3 is not the only enzyme involved in the metabolism of the drug *in vivo*.

This table appears in the review by Phillips, Francois and Shephard (Phillips 2007) which is bound into the back of this thesis.

are causative of Trimethylaminuria (TMAU) or 'Fish-odour syndrome', a condition in which individuals have a body odour similar to that of rotten fish. In the absence of functional FMO3 in the liver, trimethylamine derived from the diet or from the reduction of dietary precursors such as choline, accumulates within the body and is then excreted in its raw, odorous form in the bodily fluids. The smell of rotten fish thus emanates from a patient's urine, sweat and breath. Affected individuals have >40% unmetabolised TMA in their urine compared to 0-9% in unaffected individuals (Cashman *et al.* 2003). More than the physiological effects, the psychological and social consequences of this condition are important. Patients affected by this condition generally have trouble integrating into society because they are often perceived by others as being unhygienic. In some cases individuals are known to have taken their own lives as a means of escape from their condition (Todd 1979).

The first described clinical report of TMAU was in 1970 (Humbert *et al.* 1970) but it is not the first TMAU-like reference on record. Shakespeare's The Tempest describes a character, Caliban of which was written:''What have we here? A man or a fish? Dead or alive? A fish: he smells like a fish; a very ancient and fish-like smell'' (Man Burrows 1975). The earliest reference is thought to be in a Hindu folklore, contained in the Indian epic, Mahabharata dating back to nearly 1400 BC, Satyavata, a young women condemned to solitary life as a ferry women was cast from society because she smelt of ''rotten fish'' (New York Times 2005).

TMAU patients display altered metabolism of therapeutic drugs that are substrates for FMO3. For instance, the non-steroidal anti-inflammatory drug Benzydamine (BZD) is metabolised to its *N*-oxide by FMO3 in the liver and a measure of the BZD *N*-oxide: BZD ratio in the serum and urine is considered as an index for FMO3 activity *in vivo* (Stormer 2000). In a recent study, TMAU patients were administered BZD and monitored for the

amount of metabolised and unmetabolised drug in their urine and serum (Mayatepek *et al.* 2004). The ratio of BZD *N*-oxide: BZD displayed by TMAU patients was considerably lower from that observed for control subjects. This study was the first to demonstrate the effect of FMO3-defiency on drug metabolism. The study did not however, determine the clinical implications of the altered metabolism of BDZ.

1.3.3.3 Inter-individual Differences and the Impact on Drug Metabolism

During the course of study on the genetic causes of TMAU, numerous SNPs were identified in *FMO3* which affected the catalytic activity of the enzyme but were not causative of TMAU (Table 1.5). The polymorphisms are relatively common (ranging from 2-50% depending on the population sampled) and thus are of particular interest as they may alter the metabolism of therapeutic drugs in the general population. For instance, an individual with a polymorphism in *FMO3* that causes a reduction in oxidation activity (e.g. D132H or E158K) may be considered a poor or ‘‘slow’’ metaboliser of FMO3 substrates and therefore may have heightened risk of overdose. Conversely an individual possessing a variant FMO3 with increased catalytic activity (e.g. L360P) may be considered as a ‘‘rapid’’ metaboliser of a drug. This may lead to lower efficacy of a drug metabolised by FMO3 and ineffective dose prescription due to more rapid drug clearance.

The E158K amino acid variant of FMO3 is the product of a G>A substitution at position g.15167 in exon 4. g.15167 (E158K) is by far the most common polymorphism occurring at a frequency of 45-50% in African Americans, 42% in Caucasian Americans and in 19% of Asians (Dolphin *et al.* 1997; Park *et al.* 2002; Koukouritaki *et al.* 2005; Hao *et al.* 2006; Hao *et al.* 2007; Koukouritaki *et al.* 2007). Individuals homozygous for this polymorphism display similar TMA: TMA *N*-oxide ratio to individuals with the reference

Table 1.5: Polymorphic Variants of *FMO3* not causative for TMAU

Variant	exon	Amino Acid Change	Functional Consequence	Reference
g.72G>T	2	E24D	Limited	(Koukouritaki *et al.* 2005)
g.11177C>A	3	V58I	Reduced	(Kubota *et al.* 2002)
g.15089G>C	4	N61K*	reduced or abolished	(Koukouritaki *et al.* 2005)
g.15167G>A	4	N61S	*S*-oxygenation only	(Dolphin *et al.* 2000)
g.15475G>T	5	D132H	substrate-dependant decrease	(Furnes *et al.* 2003; Lattard *et al.* 2003)
g.15550C>T	5	E158K	moderate, substrate-dependent decrease	(Brunelle *et al.* 1997)
g.18281G>A	6	G180V	no effect	(Dolphin *et al.* 1997)
g.18290A>G	6	R205C	moderate decrease	(Fujieda *et al.* 2003)
g.21350T>C	7	V257M	no effect or limited substrate-dependent decrease	(Treacy *et al.* 1998)
g.21443A>G	7	M260	n.d.	(Shimizu *et al.* 2007)
g.21599T>C	7	V277A	n.d.	(Cashman 2002)
g.21604G>C	7	E308G	moderate substrate-dependent decrease	(Treacy *et al.* 1998; Lattard *et al.* 2003)
g.23613G>T	8	L360P	increased activity	(Furnes *et al.* 2003; Lattard *et al.* 2003)
g.24642G>A	9	E362Q	n.d.	(Cashman 2002)
g.24691G>C	9	K416N	Limited	(Koukouritaki *et al.* 2005)
g.24642G>A	9	I486M	n.d.	(Cashman 2002)
g.24691G>C	9	G503R	n.d.	(Furnes *et al.* 2003)

*Likely to be causative for TMAU.
n.d. – Not determined. Coordinates are based on assigning the 'A' of the ATG translation initiation codon as +1
This table appears in the review by Phillips, Francois and Shephard (Phillips 2007) which is bound into the back of this thesis.

genotype (Zschocke *et al.* 1999). The finding is indicative of this amino acid variant having a similar catalytic efficiency to the wild type protein.

g.21443A>G (E308G) is a relatively common polymorphism occurring at a frequency of 18% in Asians, 20% in Caucasian-Americans and in 5% of African-Americans (Treacy *et al.* 1998; Park *et al.* 2002; Koukouritaki *et al.* 2005; Hao *et al.* 2006; Hao *et al.* 2007; Koukouritaki *et al.* 2007). Linkage analysis confirmed g.15167 (E158K) and g.21443A>G (E308G) polymorphisms to be linked in 4% of individuals from a German population (Zschocke *et al.* 2000). In this study, individuals homozygous for both variants *in cis* (on the same allele) were reported to display a mild TMAU phenotype, as confirmed from TMA: TMA *N*-oxide ratios. Interestingly, individuals who had one E158K or E308G variant allele and one E158K variant allele showed little difference between urinary metabolic ratio of TMA: TMA *N* -oxide (0.0084) and individuals with the reference genotype (0.0056). This data suggested, for the first time, that the occurrence of E158K and E308G in *cis* has more effect on catalytic function than a genotype of E158K or E308G and E158K alone (Zschocke *et al.* 2000). Studies with African-American subjects suggested that a haplotype containing E158K and E308G in *cis* occurred at a frequency of around 5% (Koukouritaki *et al.* 2007).

The *in vivo* consequence of the polymorphism g.21599T>C (L360P) is still to be determined, but it is the only polymorphic variant to result in an enzyme with higher catalytic activity (Furnes *et al.* 2003; Lattard *et al.* 2003). The polymorphism has been estimated to occur at a frequency of around 2% in African-Americans and genotypic analysis to date infers the variant to be specific to this ethnic population.

Inter-individual differences of 10- to 20-fold in the amount of hepatic FMO3 protein have been reported (Overby *et al.* 1997; Koukouritaki *et al.* 2002). FMOs are generally considered not to be inducible by environmental factors and therefore such variation must be due to genetic factors. SNPs discovered in the 5'-flanking region of the *FMO3* gene are

implicated in the observed inter-individual differences in levels of FMO3 protein expression in the liver. *In vitro* analysis of such SNPs illustrates their effect on *FMO3* transcription, in some cases effectively silencing it, and in others increasing it as much as 8-fold (Koukouritaki *et al.* 2002).

1.3.4 FMO4

FMO4 is 558 amino acid residues long. Other members of the FMO family, with the exception of FMO2.2, contain between 532 and 535 residues (Phillips *et al.* 1995). Sequence comparisons reveal that the additional residues in FMO4 are contained in a single block located at the C-terminus of the polypeptide (Dolphin *et al.* 1992). It has been suggested that the additional amino acid residues may have arisen as a result of a single point mutation in the termination codon of the ancestral *FMO4* gene.

The mRNA of FMO4 is expressed constitutively at low levels in the liver, kidney and lung of the adult human (Dolphin *et al.* 1992; Dolphin *et al.* 2000; Hernandez *et al.* 2004; Zhang *et al.* 2006). The protein encoded by this gene is still to be detected in human tissue.

Evidence of FMO4 contribution to xenobiotic metabolism is limited. In fact, very little is known of this FMO in general. The probable reason is the difficulty encountered in expressing this FMO isoform in a heterologous system (Itagaki *et al.* 1996).

1.3.5 FMO5

A cDNA encoding human FMO5 was isolated in 1995 (Overby *et al.* 1995; Phillips *et al.* 1995) and consists of a polypeptide of 533 amino acid residues and of molecular weight 60, 255 Dalton. FMO5 is expressed in many foetal and adult tissues, but in humans its main site of expression is adult liver although it is not expressed in the same amounts as FMO3 (Janmohamed *et al.* 2001; Hernandez *et al.* 2004; Zhang *et al.* 2006).

FMO5 is regarded as the ‘‘black sheep’’ of the FMO family with respect to gene expression, gene regulation and substrate specificity. Despite this, it is classified as an FMO because of its sequence similarity to other members of this family.

The *FMO5* gene is not part of the *FMO* gene cluster on human chromosome 1q24.3 but is located ~26Mb closer to the centromere at 1q21.1 (Hernandez *et al.* 2004). *FMO5* is thought to be the ancestral *FMO* gene (Hernandez *et al.* 2004). Currently, FMO5 is the only human FMO demonstrated to be under hormonal regulation (Miller *et al.* 1997).

FMO5 shows little or no activity towards classic FMO substrates such as methimazole (FMO1, FMO2 and FMO3), imipramine (FMO1) and thioureas (FMO1, FMO2 and FMO3) (Overby *et al.* 1995; Henderson *et al.* 2004a; Onderwater *et al.* 2004; Hernandez *et al.* 2009) but efficiently catalyses the *N*-oxygenation of short chain aliphatic primary amines like *n*-octylamine, that are not utilized by other human FMOs. FMO5 is capable of oxidising thioethers with proximal carboxy groups, like the drug Esonarimod, a reaction not catalysed by FMO1 or FMO3 (Ohmi *et al.* 2003).

Sequencing studies performed on human *FMO5* from individuals of African-American descent suggest it is the most conserved isoform of the FMO family (Furnes *et al.* 2003).

Despite its expression in the adult human liver, FMO5 is not thought to play a significant role in drug metabolism as a result of its limited substrate specificity. FMO5 may, however be important in an endogenous metabolic pathway, yet to be identified.

Table 1.6: Summary of Tissue-specific FMO expression in Man

Human FMO	Major site of expression	Reference
FMO1	Kidney	Koukouritaki *et al.* 2002
FMO2	Lung*	Krueger *et al.* 2002
FMO3	Liver	Phillips *et al.* 1995, Koukouritaki *et al.* 2002
FMO4	Not determined	-
FMO5	Liver	Janmohammed *et al.* 2001

*Absent in the majority of the human population, see section 1.3.2 for details.

1.4 Tuberculosis

Tuberculosis (TB) is an infectious disease caused by the opportunistic bacterium, *Mycobacterium tuberculosis*. TB is a severe infection of the lung that can disseminate to other parts of the body such as the lymph nodes, the spine and the central nervous system. The symptoms of pulmonary TB include persistent coughing with blood appearing in sputum, chest pains, weight loss, intermittent fever and night sweats.

1.4.1 Transmission and Pathogenesis

Transmission of the disease occurs when an infected individual expels *Mycobacteria* in the form of aerosols in the air when they cough, sneeze, spit or laugh. The bacteria once inhaled in the lungs of an uninfected person lodge in the distal airways of the alveoli and are engulfed by alveolar macrophages. At this stage the pathogen has one of several fates. In the majority (>90%) of cases, *Mycobacterium* 'hides' within macrophages and evades the primary innate immune response by interfering with intracellular signalling pathways in the macrophage by mechanisms that are currently poorly understood. The pathogen in this state, is non-replicating and initiates a latent infection. This sub-clinical, asymptomatic infection can last for years and in the majority of cases never re-activates. Re-activation or 'post-primary' infection occurs in 5-10 % of individuals and its onset is accelerated in an immunocompromised host. In the remainder of l0% of cases, *Mycobacterium* starts to replicate and the onset of a primary infection develops.

1.4.2 TB Incidence, Prevalence and Mortality

One third of the world's population is infected with TB. The World Health Organisation (WHO) Report 2009 estimated 9.2 million new cases of TB worldwide in 2007, of which 31% were in Africa (WHO 2009). Among the 9.2 million cases, 0.5 million were caused by

multi-drug resistant strains of *Mycobacterium tuberculosis* (MDR-TB) and 5 % of the incident cases were estimated to be HIV-positive. There was an estimated 13.7 million prevalent cases worldwide in 2007 and this estimate is slightly higher than that reported for the previous year (13.4 million in 2006). The number of deaths caused by TB in HIV-negative patients was estimated to be 1.32 million in 2007 and there was an additional 0.48 million TB deaths in HIV-positive cases. In absolute terms, the total number of TB cases is on the rise and the WHO statistics highlight that TB is still a major global health problem. If controls are not imposed, an estimated 1 billion people will be infected by 2020, over 125 million will get sick and more than 30 million will die from this disease (Ghiladi *et al.* 2005).

1.4.3 Genus *Mycobacterium* and Disease

Mycobacterium is a genus of Actinobacteria given its own family name, *Mycobacteriacea*. Several species belonging to this genus are pathogenic and known to cause serious disease in mammals. *Mycobacterium tuberculosis* is the primary causative agent of TB in humans. Other pathogenic species of *Mycobacteria* have been isolated in man; albeit rare they include *Mycobacterium Microti*, *Mycobacterium africanum* and *Mycobacterium canettii* (Miltgen *et al.* 2002; Cadmus *et al.* 2009; Frank *et al.* 2009).

1.4.3.1 *Mycobacterium tuberculosis* (*M.tuberculosis*)

M. tuberculosis is an aerobic, non-motile, polytrophic organism that grows in straight or curved rod-like structures (bacillus). The bacterium has a remarkably slow rate of growth with a doubling time of around 20-30 hours (cf. *E.coli* which can divide every 20 mins under optimum conditions). *M. tuberculosis* is classified as Gram-positive bacteria as it has one phospholipid outer membrane. Despite this feature, the bacteria stains weakly upon Gram-staining and in some cases will not stain at all. The inability to retain Gram stain is because

of the presence of a thick, lipid-rich waxy layer that surrounds the cell wall thus preventing the stain to encounter this barrier. Instead, an acid-fast or Ziehl-Neelsen stain can be employed to identify *M. tuberculosis* in bacterial cultures.

The cell wall of *M. tuberculosis* and species belonging to the *Mycobacterium* genus in general have a unique and complex cell wall structure that is considered one of the attributes of making this organism one of the most successful pathogens of mankind. The cell wall consists of an inner and outer layer that surrounds the plasma membrane of the organism. The outer layer compartment consists of lipids and proteins. Linked to the lipid moiety are polysaccharides including lipoarabinomannan (LAMs), lipomannan and sulpholipids. This layer is the soluble component of the cell wall and contains receptors that interact with the host immune system. The inner layer consists of peptidoglycans (PG), arabinogalactan (AG) and mycolic acids (MA) covalently linked to form a complex known as the MA-AG-PG complex that extends from the plasma membrane towards the outer layer, starting with PG and ending with MA. This complex is insoluble and impermeable and is referred to as the essential core of the cell wall. This component is the target of several antibiotics and will be described later.

1.4.4 Anti-tuberculosis chemotherapy

There are five main aims of chemotherapy

a. Cure of the patient
b. Prevention of death from active disease or its late effects
c. Avoidance of relapse or re-current disease
d. Prevention of spread of drug-resistant organisms
e. Protection of the community from infection

A group of antibiotics have been dubbed the essential drugs for TB chemotherapy. The drugs are generally prescribed in combination as monotherapy can lead to drug resistance. A two-tier system is established in TB chemotherapy and consists of first-line and second-line antibiotics. First-line antibiotics; isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and streptomycin (SM) are predominantly bactericidal in action. Thiacetazone (TAZ), ethionamide (ETA) and ethambutol (EMB) are second-line antibiotics and are predominantly bacteriostatic. First-line antibiotics are generally more expensive and display higher drug efficacy, second-line drugs on the other hand are cheaper but have higher levels of toxicity and are less effective at treating TB. Among all the current TB drugs, TAZ is the cheapest antibiotic available. The WHO recommends Directly Observed Therapy (DOTs) that consists of the following drug regimen for treatment of active TB infection: 6 months therapy consisting of initial 2 month phase of treatment with four drugs; INH, RIF, PZA and EMB followed by a continuation phase with INH and RIF for another 4 months (WHO 2009). All of these drugs are expensive and are ineffective in treating TB in areas of high incidence of multi-drug resistant TB (MDR-TB). In developing countries, the cost of these drugs is too much to afford and more second-line drugs are substituted for first-line antibiotics. For instance drug regimens in Sudan, Nigeria, Ghana, Senegal and some parts of South America administer the following course of treatment: 4 months intensive chemotherapy with INH, RIF, PZA, EMB followed by 3 months of TAZ and INH (Lawn *et al.* 1999; Mame Thierno *et al.* 2001; Dosumu 2002; Keus *et al.* 2003). Poverty-stricken areas of Africa and certain parts of India however can ill afford this relatively cheap combination and thus resort to a preparation of INH and TAZ (Thiazina) course of treatment which lasts for 18 months, despite it being less effective and causing potentially life-threatening side effects (Nunn *et al.* 1992). The side effects of this drug have been presumed to be from the TAZ component of this preparation (Anonymous 1963; Aquinas 1968; Volek *et al.* 1970; Hussain *et al.* 1973;

Coetzee 1980; Naraqi *et al.* 1980; Short 1980; Jaliluddin *et al.* 1981; Eriki *et al.* 1991; Fegan *et al.* 1991; Kole 1991; Nunn *et al.* 1991; Ipuge *et al.* 1995).

INH is the most potent bactericidal antibiotic amongst the essential drugs and is most effective towards metabolically active and continuously growing bacteria. RIF is a good bactericidal agent towards growing bacteria but is also effective at targeting semi-dormant bacteria. Thus RIF is a good sterilising agent that will kill *Mycobacteria* capable of surviving the action of INH. PZA, like RIF is a good sterilising agent of *Mycobacteria*, especially those that grow in an acidic environment. SM has less bactericidal action but nevertheless is effective.

It is envisaged that tubercle bacilli in lesions within an infected host consist of sub-populations that display differences in metabolic status. These are ‘‘(a) those that are metabolically active, continuously growing bacteria that are killed by the action of INH (but in the case of INH resistance are killed by RIF and SM), (b) semi-dormant bacteria that undergo spurts of metabolism which are killed by RIF, (c) those that are of low metabolic activity and reside in acid pH environment which are killed by PZA and (d) those that are ‘dormant’ which are not killed by any current TB drug’’ (Zhang 2005).

1.4.5 Mechanisms of Drug Action

The action of drugs can be categorised in the following groups, inhibitors of cell wall synthesis (INH, TAZ, ETA, EMB), inhibitors of nucleic acid synthesis (RIF), inhibitors of protein synthesis (SM) and drugs that inhibit or deplete membrane energy (PZA). It is beyond the scope of this report to detail the mechanism of each drug, for a detailed insight into the mode of drug action/activation see review by (Zhang 2005). A brief description will be provided here.

Inhibitors of Cell Wall Synthesis

Inhibitors of cell wall synthesis target components within the lipid rich inner and outer layer that surrounds the cell wall, thereby weakening the cell scaffold that is crucial to bacterium survival. INH is a pro-drug that is activated by *M. tuberculosis* catalase-peroxidase enzyme, KatG ((Zhang *et al.* 1992), Johnson and Schultz 1994) to generate a range of toxic oxygen species. The active species of this activation, the isonicotinic acyl radical, reacts with NAD to form an INH-NAD adduct that inhibits the enzyme InhA (Rozwarski *et al.* 1999). The enoyl-acyl carrier protein reductase, InhA is part of the Fatty Acid Synthase type II (FAS II) complex that is responsible for the synthesis and elongation of mycolic acids. The primary target of INH is thus mycolic acid biosynthesis (Winder *et al.* 1970). Mutations in *KatG* and in *InhA* confer resistance to INH treatment (Banerjee *et al.* 1994).

EMB interferes with the production of arabinogalactan, an important polysaccharide component of the essential core of the cell wall. EMB inhibits the polymerisation of the arabinin moiety of arabinogalactan and LAMs thus weakening the scaffold of the *mycobacterial* cell wall (Zhang 2005). The exact details of EMB action or activation are still poorly understood but aribinosyl transferase, an enzyme involved in arabinogalactan synthesis has been postulated as the target of this drug.

ETA and TAZ also target cell wall biosynthesis and this will be described in detail in section 1.4.6 and 1.4.7 respectively.

Inhibitors of nucleic acid synthesis

RIF is a broad spectrum, rifamycin B derivative that inhibits RNA synthesis in bacteria. RIF inhibits the synthesis of RNA by binding to the β-subunit of bacterial DNA-dependent RNA polymerase (DDRP) thus inhibiting the transcription of RNA and subsequent translation to protein (Zhang 2005). The beta subunit of DDRP is the translational product of the *rpoB*

gene and mutations in a defined 81-bp region of this gene confer RIF resistance (Zhang 2005).

Inhibitors of Protein Synthesis

SM is an aminoglycoside that inhibits translation of bacterial mRNA. It does so by binding to specific components associated with the bacterial ribosome that is composed of a large (50S) and a small (30S) subunit. Studies have identified that SM binds to the 16S rRNA component of 30S and also to the S12 protein that is associated with this complex (Spotts *et al.* 1961). 16S rRNA and S12 are encoded by the *rrs* and *rpsl* genes respectively and mutations in these genes are known to cause resistance to SM action (Garvin *et al.* 1974).

Inhibition and Depletion of Membrane Energy

PZA, a structural analogue of nicotinamide, is a prodrug that requires conversion to its active form pyrazinoic acid (POA) by the pZase/nicotinamidase enzyme (Scorpio *et al.* 1996). The drug has no activity against *Mycobacteria* growing at normal culture conditions near neutral pH, but is effective in killing bacilli at lower pH (Tarshis *et al.* 1953). Acid pH facilitates the formation of uncharged protonated POA that permeates through the membrane easily, this results in the accumulation of POA and the decrease in membrane potential in *M. tuberculosis* (Zhang *et al.* 1999; Zhang *et al.* 2003). Protons flow into the cell and this subsequently causes the collapse of the proton-motive force and affects membrane transport (Zhang *et al.* 2003).

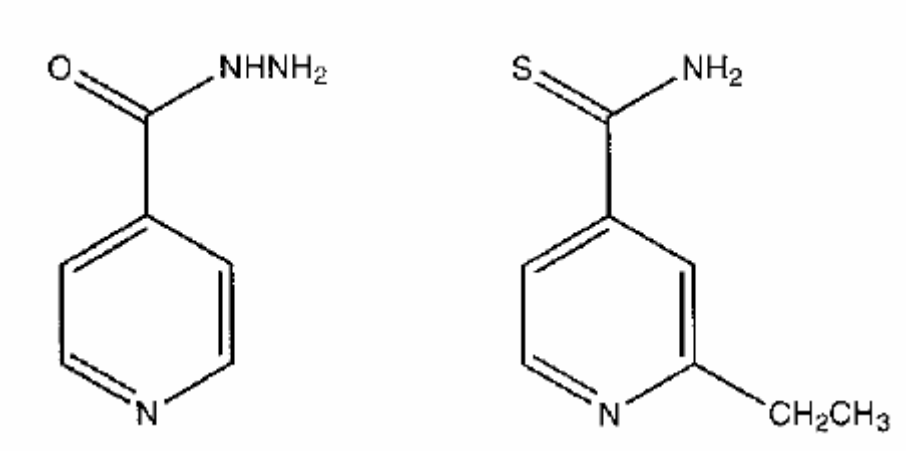
The pZase/nicotinamidase enzyme is encoded by the *pncA* gene in *M. tuberculosis*. Mutations in *pncA* are considered the major mechanism of resistance to this drug (Scorpio *et al.* 1996; Scorpio *et al.* 1997).

1.4.6 Ethionamide

Ethionamide (2-ethylpyradine-4-carbothioamide, ETA) is a second-line antibiotic developed by the Lederle Research Laboratories in 1956 (Liebermann D 1956). The drug has been used in the developing world and in the United States to either treat the *mycobacterium* or reduce the risk of resistance to first-line antibiotics.

ETA is a structural analogue of INH (Fig. 1.9). It was observed that mutations in the promoter and coding region of *Inh*A, which encodes the enzyme that is the cellular target for INH (InhA) conferred resistance to ETA treatment (Banerjee *et al.* 1994). This gave the impression that ETA and INH have common modes of activation, but it was hard to explain why clinical isolates of *M. tuberculosis* that were resistant to INH treatment were still susceptible to ETA and *vice versa*. The lack of cross-resistance suggested that KatG, the catalase peroxidase responsible for INH activation was probably not involved in the conversion of ETA. In 2000, two independent laboratories identified an FAD-containing monooxygenase as the activator of ETA, this enzyme was named Ethionamide-Activating enzyme usually abbreviated to EtaA (also known as EthA) (Baulard *et al.* 2000; DeBarber *et al.* 2000). Clinical isolates containing mutations in the gene encoding EtaA are resistant to ETA action and an activation step for ETA activity is thus necessary for it to exert an anti-tubercular effect. Heterologous expression of the enzyme in *E.coli* confirmed this enzyme to catalyse the oxidation of this drug (Vannelli *et al.* 2002). EtaA will be described in section 1.4.8.

In vitro studies claim that EtaA catalyses the sequential oxidation of ETA to form the *S*-oxide (ETA-SO) and 2-ethyl-4-amidopyridine (ETA-amide) (Vannelli *et al.* 2002). Since the anti-tubercular activity of ETA-amide and ETA-SO is comparable to that exhibited by the parent compound, it is unlikely that these metabolites represent the activated species of this drug. Although not identified, an intermediate precursor of ETA-amide has been



INH ETA

Figure 1.9: Chemical Structures of Isoniazid (INH) and Ethionamide (ETA)

postulated to be the reactive species of ETA oxidation and is predicted to be a sulphinic acid metabolite (Vannelli *et al.* 2002). In an independent study, the *in vivo* metabolism of ETA in *M.tuberculosis* was investigated and in addition to ETA-SO and ETA-amide, the corresponding nitrile, acid and alcohol derivatives of ETA were observed (DeBarber *et al.* 2000). Both studies employed extraction methods prior to MS analyses and some argue that these products may result from spontaneous chemical modification/oxidation rather than an enzymatic reaction as none of these compounds display anti-microbial activity (Hanoulle *et al.* 2006). To establish the product(s) of EtaA catalysed oxidation, one study employed high resolution magic angle spinning-NMR to determine the metabolism and identify the active species of this drug in living *M.smegmatis* (Hanoulle *et al.* 2006). The study investigated the metabolism of ETA in the growth medium and within the cells in real time. The study demonstrated that the concentration of ETA in the growth medium decreased over time and was coupled to the formation of two metabolites. NMR analysis confirmed these as ETA-SO and ETA-alcohol. Analysis of the intracellular distribution of this drug however did not identify ETA, ETA-alcohol or ETA-amide. This suggests that upon activation of ETA, the products are rapidly expelled from the cell. Furthermore, the accumulation of an aromatic ETA derivative was observed within the cell and its formation correlated to the loss of ETA in the growth medium. This metabolite, ETA* is considered to be the active product of EtaA catalysed oxidation, the nature of this species however, is still to be determined but is postulated as the sulphinic acid of ETA (Hanoulle *et al.* 2006). Given that ETA, ETA-SO, and ETA-alcohol were only observed in the growth medium and that ETA* accumulated over time within the cell indicates that ETA activation is coupled to a complex molecular sorting of metabolites and to a selective accumulation of a specific species. (Vannelli *et al.* 2002; Hanoulle *et al.* 2006).

Although the active species of ETA is not identified the mode of action of this drug is established. The cellular target of ETA is the enoyl acyl carrier protein reductase, InhA (Larsen *et al.* 2002). This enzyme is involved in the final step of the Fatty Acid Synthase complex reactions. The actual mechanism by which ETA or its active metabolite inhibit this enzyme is still to be established.

Given that InhA is the cellular target of ETA, it is reasonable to assume that the drug will affect lipid metabolism in *Mycobacteria*. ETA causes the cessation of alpha-, keto and methoxy-mycolate methyl ester synthesis; these lipids are sub-types of mycolic acids that are essential components of the *Mycobacterium* cell wall. Thus impairment in their biogenesis is likely to weaken the cell scaffold and make *Mycobacteria* more susceptible to host immune attacks (Dover *et al.* 2007). More details regarding the role of mycolic acids will be provided in the next section.

1.4.7 Thiacetazone

Thiacetazone (*p*-acetylaminobenzaldehyde thiosemicarbazone, TAZ) is a member of the thiosemicarbazone family of drugs that were developed in the late 1940s by Farbenfabriken Bayer in Germany (Behnisch *et al.* 1950). The therapeutic importance of TAZ was investigated by Domagk (Domagk 1950) when the drug was used to treat some 10, 000 patients in Germany as a single agent in treatment for TB (Mertens *et al.* 1950). As was the norm of the time, these trials were uncontrolled and details referring to drug efficacy, tolerance and safety were not documented but two American observers concluded that TAZ appeared to have ‘‘anti-tuberculous activity of the same order of magnitude as *p*-aminosalicyclic acid; and a potential toxicity somewhat like the arsenics used to treat syphilis’’ (Hinshaw *et al.* 1950; Nunn *et al.* 1993). The drug has not been used in the United States or in Europe (outside Germany) because of the discovery of INH and SM, drugs that

are far more efficient at treating TB (Nunn *et al.* 1993). However the combination of high costs of obtaining first-line antibiotics and the long duration of drug regimens means that poorer countries can ill afford them. This has led to the use of TAZ as either monotherapy or in conjunction with other drugs in areas of Africa, India and South America (Narang ; Anonymous 1963; Fox *et al.* 1974; Neill *et al.* 1990; Nunn *et al.* 1992; Nunn *et al.* 1993; Lawn *et al.* 1999; Mame Thierno *et al.* 2001).

The main role of TAZ in current drug regimens is to prevent the emergence of *Mycobacteria* that are resistant to first line drugs such as INH and RIF. Despite TAZ being a bacteriostatic drug with poor sterilising activity (Heifets *et al.* 1990), recent interest in this drug has arisen since it has not been used in the developed world. This means that *M. tuberculosis* strains resistant to drugs prescribed in industrialised countries are likely to be susceptible to TAZ treatment. For example, a recent report described the treatment of an individual suffering from chronic TB infection in Japan whose sputum smeared positive for TB during and after extensive chemotherapy with INH, RIF and PZA (Wada *et al.* 2007). Drug susceptibility tests confirmed the individual to suffer from a multi-drug resistant strain of *M. tuberculosis*. TAZ was included in the regimen as a 'last resort' and after four months of therapy, the individual's sputum smeared negative for the first time since the onset of infection. 3 years after the termination of TAZ therapy in a follow-up, the patient displayed negative sputum smears and no signs of relapse were observed (Wada *et al.* 2007). This finding is valuable as the level of multi-drug resistant *M. tuberculosis*, i.e. strains resistant to INH and RIF are on the rise and pose a major obstacle in the successful treatment of this disease. Furthermore, statistics regarding X-MDR TB, (that is individuals infected with bacterial strains resistant to INH and RIF and at least three of the six second line drugs) confirm its rise in industrialised parts of the world (WHO 2009). A major problem regarding

the progression of TAZ as a mainstay in the developed world however is the level of toxicity observed in regimens containing this drug.

The first controlled clinical trial of TAZ chemotherapy was conducted in East Africa in which 51 patients with active TB infections were administered 150 mg of TAZ daily (Anonymous 1960). During this study, severe skin rashes were observed in about 20% of patients and a single case of Steven's Johnson syndrome was reported. Stevens-Johnson syndrome is a life threatening disease in which cell death causes the epidermis to separate from the dermis and is usually described as 'skin falling off'. Since this study, numerous reports of dermatological reactions including Stevens-Johnson syndrome and Lyell disease, inflicted by TAZ usage have been documented (Harland 1962; Ferguson *et al.* 1971; Hussain *et al.* 1973; Bedi *et al.* 1974; Sahi *et al.* 1974; Purohit *et al.* 1976; Strobel *et al.* 1979; Naraqi *et al.* 1980; Strobel *et al.* 1980; Anonymous 1981; Gupta *et al.* 1983; Mullick *et al.* 1986; Fegan *et al.* 1991; Fegan *et al.* 1991; Nunn *et al.* 1991; Chintu *et al.* 1993; Kelly *et al.* 1994; Ipuge *et al.* 1995; GOTHI 1998 ; Lawn *et al.* 1999; Dieng *et al.* 2001; Mame Thierno *et al.* 2001). Stevens-Johnson syndrome is more common in HIV-positive patients than HIV-negative patients that take TAZ as part of TB treatment (Colebunders 1989; Colebunders *et al.* 1989; Elliott *et al.* 1990; Elliott *et al.* 1996) and work in Uganda specifically implicated TAZ as the responsible drug (Eriki *et al.* 1991). Therefore TAZ is strongly contraindicated in patients that are HIV-positive. Reports of adverse skin reactions have been made in smaller district programmes in India, Kenya, Tanzania and Senegal whereby cutaneous rashes are reported as common (Shane *et al.* 1951; Ipuge *et al.* 1995; GOTHI 1998 ; Mame Thierno *et al.* 2001). A common complaint of patients given this drug is of gastrointestinal disturbances such as nausea and vomiting (Shane *et al.* 1951; Teklu 1976; GOTHI 1998 ; Mame Thierno *et al.* 2001) this is a potential problem with regard to drug compliance as chemotherapy may last for up to 24 months with this drug. Liver function tests performed, such as thymol

turbidity and bromsulphthalein staining on patients prescribed with TAZ, indicate higher incidence of liver toxicity (Hinshaw *et al.* 1950; Shane *et al.* 1951; Anonymous 1963). Jaundice has also been reported in around 4% of individuals in India given TAZ as chemotherapy (Narang ; Sharda *et al.* 1976; Gupta *et al.* 1977). Jaundice was frequently reported in patients on TAZ containing regimens in controlled studies conducted in Africa, Singapore and Hong Kong (Anonymous 1963; Aquinas 1968; Anonymous 1971). These findings are hard to interpret for two reasons; TAZ is usually administered as a preparation with INH (Thiazina) or as multi-drug therapy with other first- and second-line antibiotics and thus it cannot be assumed that TAZ is responsible for inducing hepatotoxicity, secondly disseminated TB infections of the liver may also lead to hepatic dysfunction. To complicate the interpretation further, contradictory findings have been reported about the role of INH in potentiating TAZ-induced toxicity in individuals being given Thiazina or regimens in which the drugs are prescribed together. One case study of 13 British TB patients reported liver toxicity and liver damage in individuals prescribed INH and TAZ but not in those prescribed TAZ as monotherapy (Pines 1964). This would indicate a role of INH in liver toxicity rather than TAZ. Though uncommon, INH-induced hepatotoxicity has been reported and in part has been associated to one's acetylator status (Rubin 1952, Reynolds 1962, Bahrs 1963, Pande 1996, Huang 2002, Possuelo 2008, Bozok Centintas 2008). A study in Zimbabwe however, observed hepatotoxicity in patients given TAZ, INH, PZA and SM but not in drug regimens in which TAZ was omitted, the study also noted that adverse-drug reactions showed no correlation to INH acetylator status (Neill *et al.* 1990). Although a role of INH cannot be ruled out entirely, it is generally accepted that TAZ is the main contributor of liver toxicity and liver damage (Anonymous 1963; Aquinas 1968; Axton 1971; Sharda *et al.* 1976; Gupta *et al.* 1977; Coetzee 1980).

Details regarding the metabolism and potential mechanism of toxicity of TAZ are poorly documented, probably because the development of this drug before the advent of clinical trials and drug licensing and also owing to the lack of TAZ used in the United States. The drug is administered orally in a tablet form and is likely to be well absorbed; hepatic metabolism is thus expected to be important, though bioavailability data is not available. UV-spectral and HPLC analysis of human urine and faecal samples suggest that 15-20% of the drug is excreted unchanged (Ellard *et al.* 1974; Jenner 1983; Jenner *et al.* 1984). Spectral analysis observed *p*-aminobenzaldehyde thiosemicarbazone and *p*-acetylaminobenzoic acid in urine (Ellard *et al.* 1974) but these metabolites were not identified in later work using HPLC (Jenner 1983; Jenner *et al.* 1984). The latter method detected two unidentified metabolites, although in small quantities in urine (Jenner 1983; Jenner *et al.* 1984).

The mode of action of TAZ in killing *M. tuberculosis* has recently been investigated. TAZ, like other TB drugs is a pro-drug that requires metabolic activation by an enzyme specific to *Mycobacteria*. The enzyme necessary (but not sufficient) to activate TAZ is the bacterial FMO, EtaA (Baulard *et al.* 2000; DeBarber *et al.* 2000; Qian *et al.* 2006; Dover *et al.* 2007; Alahari *et al.* 2009). In the presence of NADPH and molecular oxygen, EtaA catalyses the oxidation of TAZ to generate two major metabolites; TAZ-sulphinic acid and TAZ-carbodiimide (Qian *et al.* 2006). Experiments with chemical oxidants such as H_2O_2 and the desulphuration agent cuprous chloride (CuCl), demonstrate that TAZ-sulphinic acid and TAZ-carbodiimide are not products of sequential oxidation, i.e. the carbodiimide is not formed by desulphuration of the sulphinic acid, but instead both products are formed by independent enzymatic reactions of TAZ probably through an unidentified intermediate. Given that sulphinic acids can be generated by the oxidation of sulphenic acids and the fact that EtaA is a monooxygenase, the authors of this study postulated a TAZ-sulphenic acid metabolite as the intermediate precursor for both products (Fig. 1.10) (Qian *et al.* 2006).

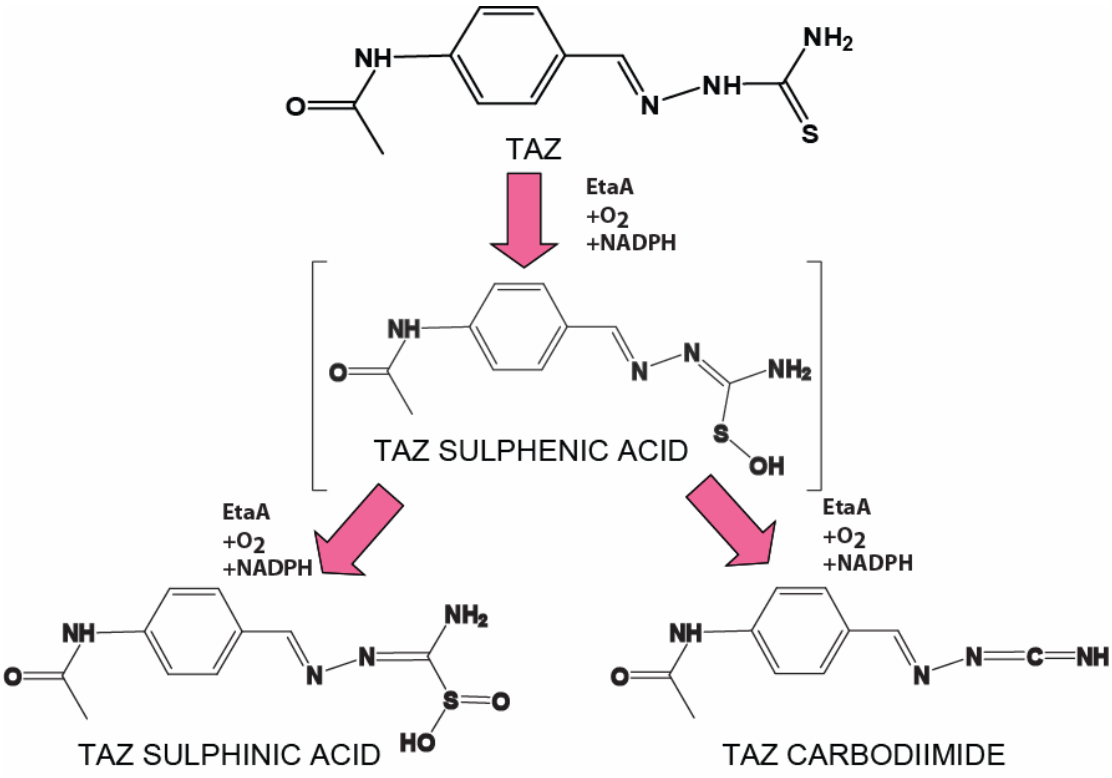


Figure 1.10: EtaA Catalyses the Two-Step Oxidation of TAZ. Chemical structures of oxidative products generated by the oxygen- and NADPH-dependant oxidation of TAZ by EtaA. The structure of the postulated sulphenic acid intermediate is given in brackets (Qian *et al.* 2006).

Recent work has identified stains of *M. bovis* that are resistant to TAZ but carry a functional EtaA (Alahari *et al.* 2009). This implies that in addition to EtaA, other activators of TAZ are present. The *Mycobacterial* methyltransferase Mma4 is necessary in the activation of TAZ, though currently it is unclear how this enzyme interacts with EtaA and whether the enzymes share a common target (Alahari *et al.* 2009). Although the mode of TAZ action has recently been established, the drug metabolite responsible for inhibiting the growth of *M. tuberculosis* is still unknown.

TAZ is an inhibitor of cell wall mycolic acid biogenesis (Alahari *et al.* 2007; Dover *et al.* 2007; Alahari *et al.* 2009). Mycolic acids are mixtures of alpha, methoxy- and keto-mycolic acid methyl esters that are branched, long chain fatty acids representing the key components of the hydrophobic cell wall. These lipids provide several protective features including resistance to oxidative injury, low permeability to antibiotics and the ability to persist within the host (Daffe *et al.* 1998; Dubnau *et al.* 2000; Glickman *et al.* 2000). Synthesis of alpha- and keto-mycolic acid methyl esters occurs through a coordinated pathway involving oxidation, methylation and *cis* or *trans*-cyclopropanation that is catalysed by cyclopropane mycolic acid synthases (CMASs). In a recent study, the effect of TAZ on wild type and TAZ-hypersensitive strains of *M. bovis* were studied to elucidate the mechanism by which this drug impairs cell wall biogenesis (Dover *et al.* 2007). TAZ inhibits the formation of all sub-types of mycolates and results in the accumulation of alpha- and keto-mycolate precursors. These lipids, when analysed by NMR and MS, lack the cyclopropane ring moiety and imply that the cellular target of TAZ may be enzymes that catalyse this step (Alahari *et al.* 2007). The cellular target of TAZ has thus been identified as the *S*-adenosyl methionine (SAM)-dependant methyltransferases, Mma2, Mma4 and PcaA (Alahari *et al.* 2007). The actual mechanism by which TAZ or its reactive metabolite inhibits the function of such enzymes is still unclear. It is noteworthy that although the action of ETA

on *M. bovis* does affect mycolic acid synthesis, it does not lead to the accumulation of unsaturated mycolates, this implies ETA and TAZ inhibit mycolic acid biogenesis by different mechanisms. Furthermore, TAZ does not inhibit the function of enzymes involved in the FAS II synthase pathway i.e. InhA, KasA, KasB, MabA or mtFabH and further suggests that TAZ and ETA although activated by the same enzyme have different cellular targets (Dover *et al.* 2007).

1.4.8 Ethionamide-Activating Enzyme (EtaA): A bacterial FMO

In 2000, two independent studies identified a gene Rv3854c (*EtaA*) in the *M. tuberculosis* genome that encoded a protein responsible for ETA activation (Baulard *et al.* 2000; DeBarber *et al.* 2000). The protein was accordingly named Ethionamide-Activating enzyme and is usually abbreviated to EtaA or EthA.

Characterisation of EtaA revealed that it was 488 amino acid residues in length and was membrane associated (Vannelli *et al.* 2002). UV-spectral analysis of EtaA yielded a spectrum displaying absorbance maxima at 365 nm and 440 nm suggesting the presence of a flavin containing prosthetic group (Vannelli *et al.* 2002). Extraction of the flavin species identified FAD and not FMN as the prosthetic group in this enzyme. The enzyme demonstrated dependence on molecular oxygen and NADPH. EtaA is inactive under anaerobic conditions and NADH can not be substituted for NADPH. The size, prosthetic group, dependence on oxygen and NADPH and enzyme localisation of EtaA are consistent with it being a flavin-containing monooxygenase (Vannelli *et al.* 2002). A primary sequence alignment of EtaA and human FMO1, 2.1 and 3 is given in Figure 1.11 and demonstrates that the bacterial enzyme contains the characteristic 'GXGXXG/A' consensus sequence that is important in binding dinucleotides in eukaryotic FMOs. As described in the preceding section, EtaA catalyses the *S*-oxidation of TAZ

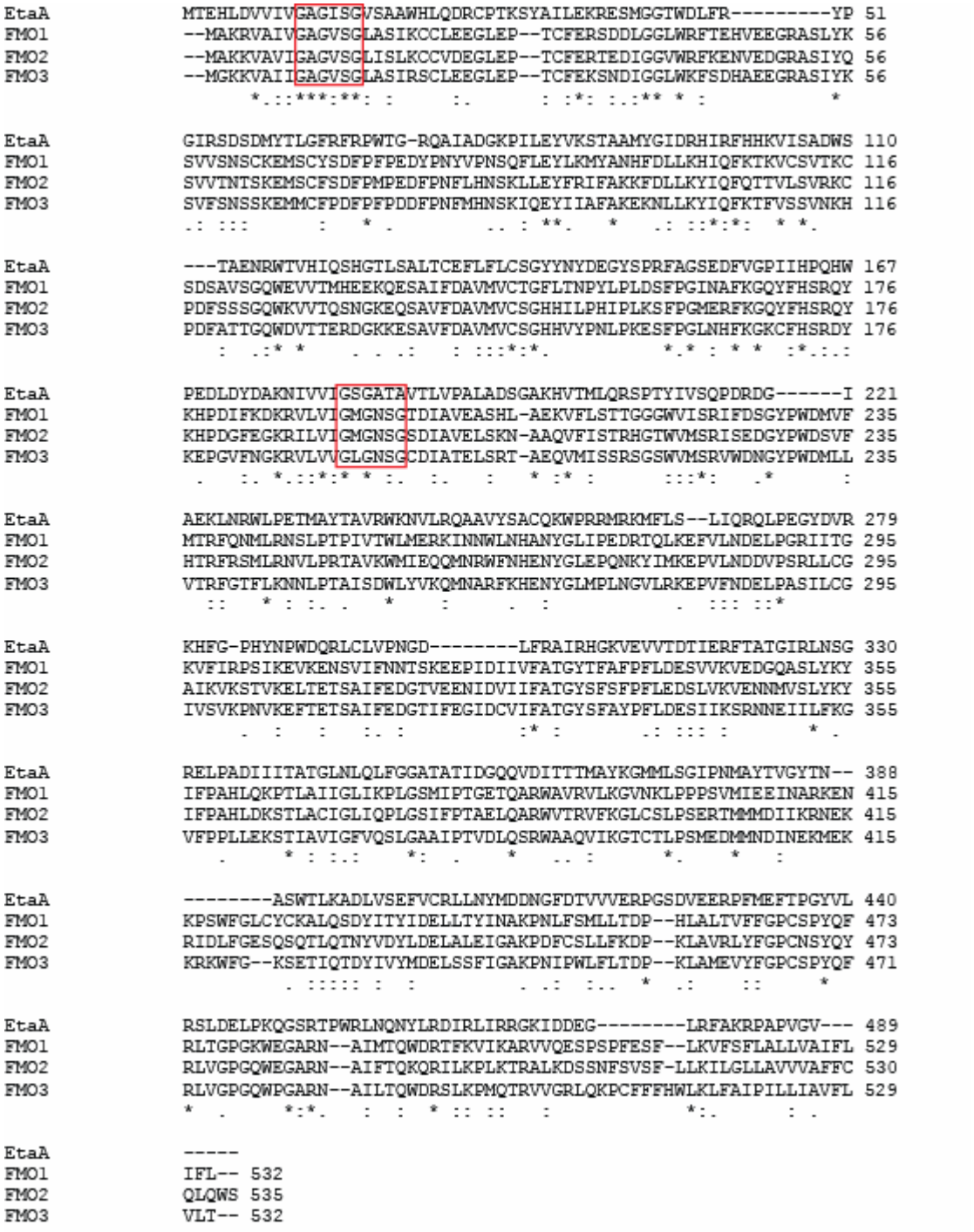


Figure 1.11: A primary sequence alignment of EtaA and human FMOs involved in drug metabolism. The primary sequence of EtaA and FMO1, 2.1 and 3 are aligned. An asterix denotes conserved identical residues, a colon indicates conserved similar residues, and a dot indicates highly similar residues. The 'GXGXXG/A' FAD and NADPH binding moieties are boxed in red. Primary sequences were aligned using the ClustalW2 tool available at http://www.ebi.ac.uk/Tools/clustalw2/index.html.

(Qian *et al.* 2006). Human FMO1 and FMO3 are also capable of catalysing the two step oxidation of TAZ to TAZ-sulphinic acid and carbodiimide the same products identified for EtaA (Qian *et al.* 2006). This finding suggests that upon administration of TAZ to humans, oxidation of TAZ by eukaryotic monooxygenases may result in the production of potentially toxic metabolites and/or the inactivation of the drug leading to reduced drug efficacy. It is therefore crucial to determine the role of mammalian monooxygenases in the activation of TAZ and other thiocarbamide drugs and this provides the focus of this investigation.

EtaA is under the transcriptional control of a regulatory protein that is encoded by the gene, Rv3855. *In vitro* studies demonstrate that over-expression of Rv3855 generates ETA resistance. The protein encoded by this gene displays homology to members of the TetR family of transcriptional repressors and thus is thought to be a negative repressor of EtaA expression and has been designated as EtaR. Supporting this finding is the observation that chromosomal inactivation of Rv3855 in *M. bovis* results in the hyper-sensitivity of *Mycobacteria* to ETA and TAZ (Baulard *et al.* 2000; Dover *et al.* 2007). Not only do these findings confirm the regulation of *EtaA* by EtaR but also confirm EtaA as the enzyme responsible for activating ETA and TAZ. Clinical isolates resistant to TAZ and ETA are also resistant to thiocarlide (a second-line antibiotic) treatment. It thus appears that EtaA may be a common activator for thiocarbamide containing drugs (Dover *et al.* 2007).

1.5 Pharmacogenetics

Pharmacogenetics is the discipline that studies the influence of genetic variation on drug response. The field has generated recent interest owing to the fact that around 100,000 deaths occur in the United States every year because of adverse-drug reactions exhibited by patients on medication (Lazarou 1998; Abbott 2003).

The first observation of genetic variation on drug response was in the 1950s for suxamethonium chloride, a muscle relaxant administered during general anaesthesia (Kalow 1952; Evans *et al.* 1953; Kalow 1961 reviewed by Gardiner *et al.* 2006). Doctors observed that upon drug administration, some patients remained paralysed for longer than others and some suffered from life-threatening respiratory arrest (Abbott 2003; Kalow 2004). It was later established that homozygousity for mutations on the *butyrlcholinesterase* gene led to this phenomena (Kalow 1957; Jensen *et al.* 1995). Cholinesterase is the enzyme that hydrolyses the drug to an inactive metabolite and in the event when this process is impaired, the resultant accumulation of succinylcholine results in the prolonged paralysis of muscles including those that are involved in breathing (Jensen *et al.* 1995; Yen *et al.* 2003 and reviewed by Lee 2009).

Of particular interest to pharmacogenetic studies are families of drug metabolising enzymes, in particular the cytochrome P450s (CYPs). It is estimated that more than 80% of serious adverse drug reactions are the consequence of polymorphic variants of CYPs (Abbott 2003). Though it is beyond the scope of this section to describe in detail the polymorphic nature of CYPs, a few examples of altered drug metabolism by CYP variation will briefly be described here. For an extensive review see Gardiner *et al.* 2006.

Codeine, a pain killer commonly given to post-operative patients to manage pain is ineffective in about 10% of individuals. The drug is oxidised to morphine by CYP2D6, but in people possessing polymorphic variants of this enzyme, the drug is ineffective and patients

do not get any pain relief (reviewed by Lotsch *et al.* 2009 and Thorn *et al.* 2009). *CYP2D6* is in fact the most polymorphic of *CYP* genes and genetic variations result in individuals being rapid, normal or slow metabolisers of drugs that are substrates for this enzyme (reviewed in (Gardiner *et al.* 2006)). Therapeutics metabolised by CYP2D6 for which a pharmacogenetic link has been established include the anti-hypertensive agent debrisoquine (Tucker *et al.* 1977; Wolf *et al.* 1992), the anti-depressant nortriptyline (Dalen *et al.* 1998; Dalen *et al.* 2003) and the anti-angina drug perhexilline (reviewed by Ashrafian *et al.* 2007).

The anti-coagulant Warfarin is metabolised by CYP2C9. Variant alleles that reduce the enzymes capacity to clear this drug, such as *CYP2C9*2* and *CYP2C9*2* cause Warfarin hypersensitivity that can lead to life-threatening bleeding and a higher risk of stroke (Rettie *et al.* 1994; Haining *et al.* 1996). Allelic variants of *CYP2C8* have been implicated in altered oxidation of the mitotic inhibitor paclitaxel (Dai *et al.* 2001; Bahadur *et al.* 2002).

The ultimate goal of pharmacogenetic research is a healthcare service that would consider the genetic profile of a patient so that therapeutics can be tailored to maximise drug efficacy and minimise adverse drug reactions. No matter how beneficial the concept of 'personalised medicine' may sound it is unlikely to happen in the foreseeable future given the complex nature of drug metabolism that usually involves the inter-play of several enzymes and not to mention the cost and time involved in mass biochemical and genetic testing. Even if it is viable in affluent parts of the industrialised world, it may be unethical to broaden the gap between the rich and poor in terms of healthcare. At this moment, it is feasible to consider using genomic data to avoid prescribing certain drugs to people that may be predisposed to an adverse drug reaction. Whether pharmacogenetic information will be used in such a way is still to be seen. Experiments described in this thesis attempt to elucidate the basic biochemistry behind the metabolism of anti-tubercular drugs by polymorphic variants

of human FMOs, by doing so we try to explain the underlying cause of toxicity that is exhibited by these drugs in so many TB patients.

1.6 AIMS

The aims of this investigation were as follows.

- To establish whether TAZ and ETA were substrates for human FMO2.1 and to identify the products of this reaction. To compare the products of TAZ oxidation by human FMO2.1 to those generated by EtaA-, FMO1- and FMO3- catalysed reactions. To determine the kinetic parameters of the reaction of human FMO2.1 and TAZ through the development of a spectroscopic assay. To compare the kinetic constants obtained for FMO2.1 to those of human FMO1, FMO3 and EtaA.
- To determine the catalytic consequences of human FMO3 variation, particularly focussing on polymorphisms that increase or abolish catalytic activity and those that are prevalent in Africa. Kinetic performance of FMO3 variants was assessed using an established enzyme assay for a typical FMO substrate and the novel spectroscopic assay for TAZ.
- To attempt to understand the relative contribution of FMOs and CYPs to TAZ metabolism *in vitro* by using microsomal samples isolated from mouse lung and liver. Microsomes isolated from wild type and FMO knockout mouse lines were employed to determine the role of individual FMO isoforms to TAZ metabolism.
- To attempt to gain an understanding of the *in vivo* metabolism and disposition of TAZ by FMOs using a mouse model.

Chapter 2: Materials and Methods

2.1 Chemicals

All chemicals, except tissue culture material, where purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Tissue culture materials were purchased from Invitrogen (Carlsband, CA, USA) and were of cell culture grade. Plastic ware for cell culture was purchased from VWR International (West Chester, PA, USA). Materials used for HPLC and LC-MS were purchased from Fisher Scientific (Hampton, NH, USA) and were of HPLC grade.

2.2 Cell Culture Conditions

2.2.1 *Spodoptera frugiperda*, (*Sf*9) Cells

Solutions:

- SF-900 Serum-Free Media (Media/SFM) (Invitrogen): Stored at 4˚C in the dark.
- Gentamicin, tissue culture grade: Stored at 4˚C.
- Fetal Bovine Serum (FBS), (Invitrogen): Stored at -20˚C and thawed on ice before use.

Frozen stocks of *Sf*9 cells were previously prepared in the laboratory and stored in liquid nitrogen. These stocks contained 10% Fetal Bovine Serum (FBS) (Invitrogen). Maintaining cells in serum has been reported to make cell cultures prone to contamination and also results in higher costs. The cells were therefore adapted to grow in serum-free conditions. Cells were also adapted to grow in suspension cultures as higher levels of protein expression can be achieved in this way. A frozen *Sf*9 cell aliquot was removed from liquid nitrogen and rapidly thawed by rubbing the tube repeatedly between two hands. As soon as the cells started to thaw the contents of the tube were poured into a T25 attachment flask (Nunc™ VWR, UK) containing 5mL of pre-warmed SF-900 serum-free media and 10 µg/mL gentamicin. The

cells were allowed to attach to the surface of the flask in a nonhumified incubator (Innova™ 4230, New Brunswick Scientific, USA) at 27˚C and were inspected after 1 hour. After 1 hour the cells had attached to the surface, this was observed by viewing the flask under a light microscope and gently moving the flask from side to side. Cells which have attached remain stationary whereas unattached cells 'float' in the medium. The cells were incubated at 27˚C until they reached confluence. This took approximately 48 hours. At this point the cells were seeded. To seed the cells, medium from the flask was removed and replaced with 5mL of fresh media containing gentamicin. The flask was then firmly hit against the palm of the hand three times to dislodge cells from the surface. 2.5 mL of culture was transferred into a new T25 flask containing 2.5 mL of media, gentamicin and 10% FBS. This culture was maintained in serum and was used as a 'stock' while cells where adapted to suspension cultures. The remainder 2.5 mL of the original culture was transferred into a 125 mL shaking flask (sterile, disposable Erlenmeyer flask (VWR Intl.), containing 23 mL of SF-900 serum-free media and 10 µg/mL gentamicin. To start off, cells were shaken at 110 rpm. Cells were counted every 24 hours to assess cell viability at the rotational speed. If cell death was apparent then the speed of shaking was reduced by 5 rpm until cells reached a mid-log phase ($2x10^6$ cells/mL). It was found that at this initial stage, slower rates of shaking (<110 rpm) led to cell death, as did a rate over 115 rpm. If these cell batches reached cell densities of $2x10^6$ cell/mL, they were re-seeded to a density of $8x10^5$cell/mL and shaken at 115 rpm. Cell batches were monitored in this way until they had adapted to grow at 125 rpm. This speed is the optimum speed given in the supplier's instructions (Invitrogen). The cells took a total of 5 weeks to adapt to the new conditions.

Sf9 cells adapted to serum-free and suspension cultures were maintained in SF-900 serum-free media containing 10 µg/mL gentamicin in suspension cultures. Cells were seeded at a

density of $2x10^6$ to $5x10^5$ cell/mL every 2-3 days in 25 mL or 50 mL suspension cultures in sterile 125 mL or 250 mL Erlenmeyer flasks respectively. Cultures were grown in a nonhumified incubator at 27ºC and at 125 rpm shaking.

2.2.2 Cell Counting and Seeding

Solutions:

- Dulbecco's Phosphate - Buffered Saline (D-PBS)
- 0.4% (w/v) Trypan Blue Stain

To count the cells, a 1 in 10 dilution was prepared. 900 µL of D-PBS, 100 µL of Trypan blue stain and 100 µL of cells were mixed in a 1.5mL microfuge tube. 100µL of this sample was then mounted onto a hemocytometer (VWR, Intl.) and viewed under a light microscope. Cells within the grid of the cytometer were counted. In the presence of the stain, viable cells appear round and have a bright silver–white outline. Dead cells appear blue and usually have lost their characteristic round shape. Only viable cells (i.e. white and round) are counted. In this way, the stain allows one to assess the viability of cell cultures. Using equation 1, the volume of cells required for seeding is calculated and cultures made up to the final volume (25 mL or 50 mL) with SFM containing gentamicin. Media was incubated at 27˚C for 30 minutes before use.

Equation 1:

Volume of cells (mL) = (Final cell density / No. of viable cells $x10^6$) X Final Volume

If a batch has <98% viability it is not used for transfection or infection with baculovirus. Cell batches were counted every 2-3 days.

2.2.3 Culture of Bacteria

Solutions

- Luria-Bertani Agar medium (LB – Agar) (Q-Biogene Inc., USA). Formulation/L: 10g Tryptone – B, 5g Yeast Extract B, 10g NaCl, 15g Agar-B. Tablets were dissolved in deionised water and autoclaved.
- Luria-Bertani Medium (formulation/L: 10g bactotryptone, 5g yeast extract, 5 g NaCl). Forty tablets were dissolved in 1 L of deionised water and autoclaved.
- Ampicillin: A 50 mg/mL stock solution was prepared by weighing out the appropriate amount of solid ampicillin and dissolving it in deionised water.
- Tetracycline: A 50 mg/mL stock solution was prepared by weighing out the appropriate amount of solid tetracycline and dissolving it in ethanol. Aliquots were stored at -20˚C.
- Gentamicin: A 10 mg/mL stock solution was prepared by weighing out the appropriate amount of solid gentamicin and dissolving it in water. Aliquots were stored at -20˚C.
- Kanamycin: A 50 mg/mL stock solution was prepared by weighing out the appropriate amount of solid kanamycin and dissolving it in water. Solution aliquots were stored at -20˚C.

Forty tablets of LB-Agar were dissolved in 1 litre of deionised water and autoclaved on the day of use. The solution was allowed to cool until it could be held by the hand comfortably but not long enough for the agar to set. At this point the appropriate antibiotics were added and the solution gently swirled by the wrist. Approximately 30 mL of medium was poured into 82 mm Petri dishes or till they were half full.

The agar was allowed to set at room temperature. The plates were briefly kept at 37˚C and then stored at 4˚C.

Bacteria was streaked on to plates and incubated at 37˚C overnight. The following day, a singly colony was picked and inoculated into 5mL of LB-medium containing the appropriate antibiotics. Cultures were grown for 6-8 hours or overnight with shaking at a rotational speed of 250 rpm.

2.3 Bac-to-Bac® Baculovirus Expression system (Invitrogen)

The Bac-to-Bac® Baculovirus Expression system (Invitrogen) allows high expression of a gene in insect cells by producing recombinant baculovirus. The system provides a faster route to recombinant protein expression compared to traditional baculovirus methods as a homologous recombination event takes place in *E.coli* rather than in insect cell. The system uses a pFastBac™ plasmid which encodes an insect cell- specific promoter (i.e. a polyhedrin promoter) for high expression in insect cells. The main features of the plasmid include multiple cloning sites to facilitate cloning and an SV40 poly-A sequence to promote transcription termination and polyadenalation of the mRNA transcript in insect cells. The plasmid also contains sites to which the bacterial Tn7 transposon (provided by a helper plasmid) can attach and allow transposition in bacmid DNA (a baculovirus vector). The presence of a gentamicin resistance gene in pFastBac™ allows for the selection of recombinant *E.coli* DH10Bac™ cells (these cells provide the bacmid DNA). Upon transformation of competent *E.coli* DH10Bac™ cells with pFastBac™, a homologous recombination event takes place between the mini-T7 element from the pFastBac™ plasmid and the mini–*att*Tn7 site in the bacmid. Successful transposition results in the disruption of the *lacZα* gene in the bacmid, so *E.coli* colonies containing recombinant bacmid appear white and non-recombinants appear blue when grown in the presence of a gratuitous inducer (Isopropyl-β-D-thiogalactopyranoside) and a chromogenic substrate (i.e. X-Gal).

Recombinant bacmid is used to transfect insect cells which will produce budding virus (baculovirus) harbouring the gene of interest. This virus is used to infect insect cells which will express the recombinant protein. A schematic diagram outlining the main steps is shown in Figure 2.1.

2.3.1 Generation of Recombinant Bacmid DNA

Human FMO1 cDNA was previously cloned into pFastBac™ 1 and maintained in *E.coli* DH5α™ glycerol stocks. Recombinant bacmid containing human FMO1 cDNA was prepared from this stage as follows.

2.3.1.1 Small Scale Isolation of plasmid DNA

Solutions:

The following solutions were obtained from the QIAprep miniprep Kit (Qiagen, Hilden, Germany):

- P1 – Re-suspension Buffer : 50mM Tris-Cl (pH 8), 10mM EDTA, 100 µg/mL RNaseA
- P2 – Lysis Buffer: 200 mM NaOH, 1% (w/v) SDS
- N3 – Neutralisation Buffer
- PE - Wash Buffer

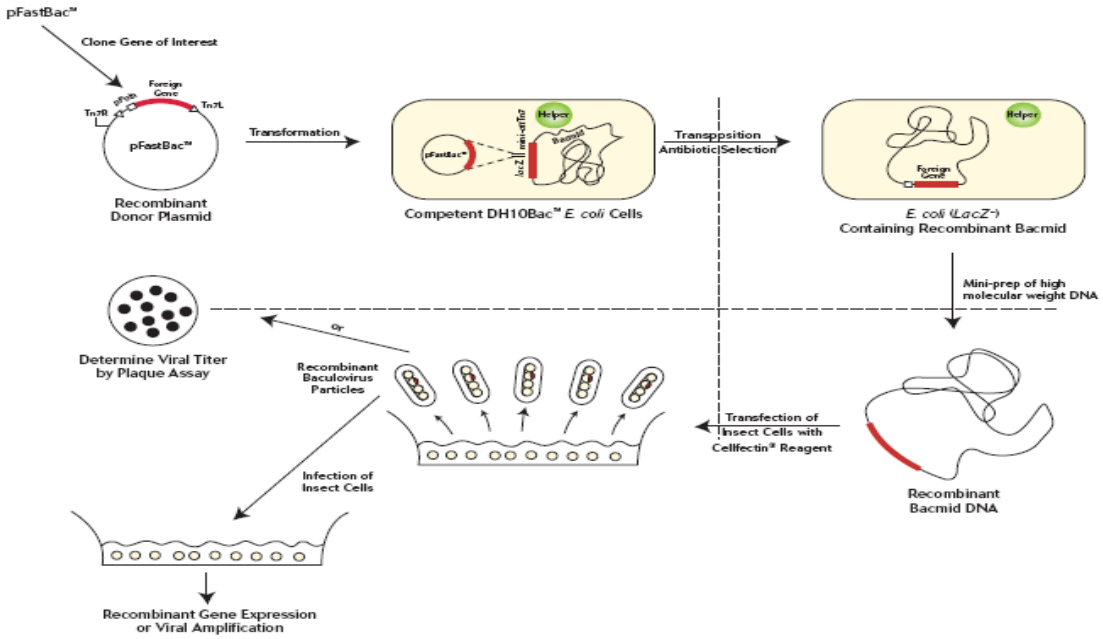


Figure 2.1: Generation of recombinant baculovirus using the Bac-to-Bac expression system. Taken from Bac-to-Bac® Baculovirus Expression System User's Manual

LB-agar plates were prepared containing ampicillin at a final concentration of 50 μg/mL as described in section 2.2.3. Glycerol stocks previously prepared in the laboratory had been stored at -80˚C and it is important that during their use the glycerol sample does not thaw as the bacteria will die as a result of the rapid temperature change. An agar plate was streaked with bacteria from the glycerol stock and incubated at 37˚C overnight. The glycerol stock sample was returned to -80˚C before it had thawed. The following day, a single colony was picked and used to inoculate 5 mL of LB-medium containing ampicillin at a final concentration of 50 μg/mL. The culture was grown for 6-8 hours as described in section 2.2.3.

Plasmid DNA was isolated from the bacterial culture using the QIAprep Miniprep Kit (QIAGEN) and was performed as described in the QIAprep Miniprep Kit handbook. Briefly, 1.5 mL of bacterial culture in mid-log phase was transferred into a 1.5 mL microfuge tube and centrifuged for 5 minutes at 13, 000*g* at 4˚C using a bench top microcentrifuge (Eppendorf). The following steps were carried out at room temperature. The bacterial pellet was re-suspended in 250 μL Buffer P1 by pipetting up and down until no clumps were visible. 250 μL of Buffer P2 was added to the tube. The solution was mixed by inverting the tube several times and until the solution had turned a homogenous blue. 350 μL Buffer N3 was added drop wise and mixed thoroughly by inverting the tube several times to avoid localised precipitation. This was done until the solution had turned white. The sample was centrifuged at 13,000*g* for 10 min. The supernatant from this stage was applied to the QIAprep spin column using a pipette (and avoiding the pellet) and centrifuged (30 s). The flow through was discarded. The column was washed again using 0.75 mL of Buffer PE by centrifuging for 30 s and discarding the flow through. The column was centrifuged again for 1 min at high speed to remove any residue of Buffer PE. The

QIAprep column was placed in a fresh 1.5 mL microfuge tube and 50µL of sterile deionised water added to the centre of the column. The column was allowed to stand for 1 min and plasmid eluted by centrifugation for 1 min at high speed. Isolated plasmid DNA was stored at 4˚C until further use.

2.3.1.2 Quantification of Isolated plasmid DNA

Plasmid DNA was quantified using a Nanodrop Spectrophotometer 1000 (Nanodrop). 1µL of deionised water was applied to the platform to calibrate the machine and to make a 'blank' measurement. The platform was wiped clean and 1µL of isolated plasmid DNA sample was applied to the platform. The absorbance of the samples was measured at 260 nm.

Presence of the plasmid was confirmed by running the DNA sample on a 1% (w/v) agarose gel.

2.3.1.3 Agarose Gel Electrophoresis of DNA

Solutions

- 10X Tris-Borate-EDTA Buffer (TBE): 0.89 M Tris-base, 0.09 M Boric acid, 20 mM EDTA (pH 8)
- 6X loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% (v/v) glycerol.
- Ethidium Bromide (EtBr): 10 mg/mL stock solution (Fisher Scientific, UK)

For a 1% (w/v) agarose gel, 1g of agarose was dissolved in 100 mL of 1X TBE by heating the solution in a microwave oven for 2 min or until the solution was clear. 2µL of Ethidium bromide was added to the solution once it had cooled but before the

agarose had started to set. The agarose was poured into a gel mould and an appropriate comb was positioned into place. The gel was allowed to set for at least 1 hour. Once the gel had set, the gel mould was positioned into an electrophoresis tank and submerged in 1xTBE. The comb was removed and 10µL of HyperLadder 1 (Bioline Ltd, UK) was loaded into a well as a molecular weight marker. Sample DNA was mixed with 1X loading dye and loaded into the wells. The gel was run at 100 volts to separate the DNA. After electrophoresis the gel was viewed and photographed using a UV transilluminator (Syngene, GeneSnap).

2.3.1.4 Transformation of Competent E.coli DH10Bac™ and the Blue-White Assay

Solutions

- 1M Isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen): prepared fresh on the day in water and filter-sterilised.
- 2% (w/v) 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) prepared fresh on the day in dimethyl sulfoxide (DMSO) and stored in the dark.
- SOC Medium provided with MAX Efficiency® DH10Bac™ competent cells (Invitrogen)

A 50 µL aliquot of competent MAX Efficiency® DH10Bac™ cells (Invitrogen) was removed from -80ºC and thawed on ice. The cells were transformed with 100 ng of plasmid by heat shock treatment for 45 s using a water bath set at 42˚C. 900µL of SOC medium was added to the transformed cells and incubated at 37˚C for four hours in a shaking water bath. During this time, agar plates containing gentamicin, tetracycline and kanamicin at a final concentration of 7 µg/mL, 10 µg/mL and 50

µg/mL respectively were prepared as described in section 2.2.3. 50 µL X-Gal (0.02µg) and 10µL IPTG (10µmol) were spread on to these plates and allowed to dry. Serial dilutions of the transformed cell culture were prepared (10^{-1}, 10^{-2}, 10^{-3} 10^{-4}) and spread onto the agar plates and incubated for 24 hours at 37˚C. The following day the plates where transferred to 4˚C for a further 24 hours to enhance the colour of the blue colonies. Three white colonies and one blue colony were picked and inoculated into 5 mL of LB medium containing 7 µg/mL gentamicin, 10 µg/mL tetracycline and 50 µg/mL kanamycin as described in section 2.2.3. Bacmid DNA was isolated from these cultures.

2.3.1.5 Isolation of recombinant Bacmid DNA

Solutions:

- Propan-2-ol
- Ethanol (70%, (v/v))

The following solutions were provided with the QIAprep miniprep Kit (Qiagen):

- P1 – Re-suspension Buffer : 50 mM Tris–Cl (pH 8) , 10 mM EDTA, 100 µg/mL RNase A.
- P2 – Lysis Buffer: 200 mM NaOH, (1%) SDS.
- P3 – Neutralisation Buffer: 3M potassium acetate (pH 8).

<u>Human FMO2.1 and FMO3</u>

E.coli DH10Bac™ cells containing recombinant bacmid were previously prepared in the laboratory and maintained in glycerol stocks. *E.coli* DH10Bac™ cells containing either human FMO2.1 or FMO3 cDNA were streaked on to agar plates containing 7 µg/mL gentamicin, 10 µg/mL tetracycline and 50 µg/mL kanamicin and incubated at

37ºC overnight as described in section 2.2.3. A single colony was used to inoculate 5 mL of LB medium containing 7 µg/mL gentamicin, 10 µg/mL tetracycline and 50 µg/mL kanamycin overnight at 37ºC with shaking. The following day, recombinant bacmid was isolated.

Human FMO1, FMO2.1 and FMO3

Bacmid DNA was isolated using the method of alkaline lysis as described by (Janmohamed *et al.* 2006). Briefly, 1.5 mL of bacterial culture growing in mid-log phase in the presence of antibiotics was transferred into a 1.5 mL microfuge tube and centrifuged at 14,000*g* for 10 min at 4ºC in a bench top microcentrifuge (Eppendorf, Germany). The pellet was re-suspended in 300µL Buffer P1 by pipetting up and down. 300µL of Buffer P2 was added to the tube and mixed by inverting the tube. The mixture was allowed to stand at room temperature for 5 minutes. Buffer P3 was slowly added to the tube, mixed gently and centrifuged at 14, 000*g* for 10 min at room temperature. The supernatant was transferred into a 2mL microfuge tube and 800µL of propan-2-ol was added. The tube was inverted several times and placed on ice for 10 min, then centrifuged at 14, 000*g* at room temperature. The supernatant was removed and 500µL of 70% (v/v) ethanol was added to the pellet. The tube was inverted several times to wash the pellet and centrifuged at 14, 000*g* for 15 min at room temperature. The supernatant was removed, initially by decanting the liquid and then using a glass Pasteur pipette to remove all the ethanol, which can reduce transfection efficiency. The pellet was air dried for 10 min. The DNA was dissolved in 50µL of sterile deionised water. The tube was gently tapped to dissolve the DNA - vortexing at this stage would cause the DNA to shear. Bacmid DNA was stored at -20ºC.

2.3.1.6 Polymerase Chain Reaction (PCR) (Standard)

All standard PCR reactions were carried out using a Genius PCR machine (Techne Ltd., UK).

Solutions

- BIO-X-ACT™ Short DNA Polymerase (4U/ μL) (Bioline Ltd, UK)
- OptiBuffer™ (10x Reaction buffer): 10 mM Tris-Cl (pH8.3), 15 mM $MgCl_2$, 500 mM KCl (Bioline Ltd, UK)
- dNTP mix (10 mM) (Bioline Ltd, UK)
- 50 mM Magnesium Chloride (Bioline Ltd, UK)
- Forward and Reverse Primers

PCR consisted of; 10 ng template DNA, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$ and 0.5 μM reverse and forward primers. The reaction volume was made up to 50 μL with sterile water. Four units of BIO-X-ACT™ Short DNA Polymerase were added to the reaction. Amplification parameters are shown in Figure 2A for BIO-X-ACT™ polymerase catalysed PCR. The annealing temperature depends on the melting temperature (Tm) of the primers which are to be used. The Tm and annealing temperature were calculated using formulae given in Figure 2B. Primer sequences have been given in Table 1 of Appendix 1. PCR products were electrophoresed on a 1% (w/v) agarose gel and viewed as described in section 2.3.1.3.

(A)

PCR Conditions

Initial Denaturation	95 ˚C	2 min	1 Cycle
Denaturation	95 ˚C	30 sec	40 Cycles
Annealing	55 ˚C	30 sec	
Extension	72 ˚C	30 sec	
Final Extension	72 ˚C	5 min	1 Cycle

(B)

$$Tm = 69.3 + (0.41 \times (\%G+C)) - 650/\text{length (nt)}$$

$$\text{Annealing temperature} = \frac{Tm1 + Tm2}{2} - 6$$

Figure 2.2: (A) General PCR programme for BIO-X-ACT™. (B) Formulae for determining the Tm and annealing temperatures.

Human FMO1

A PCR was carried out to confirm the presence of human FMO1 cDNA insert in the recombinant bacmid DNA using M13 forward and reverse primers as described above. M13 sequences are present in the regions flanking the site of pFastBac™ 1 transposition (i.e. the cDNA insert site) in the bacmid.

2.3.1.7 Preparation of Glycerol Stocks

Solution:

- Glycerol.

For long term storage of *E.coli* transformed with recombinant bacmid DNA, glycerol stocks were prepared. 1mL of bacterial culture (grown overnight at 37˚C with shaking) was mixed with 100 µL of glycerol in a 1.5 mL microfuge tube. The contents were inverted several times to mix the solutions thoroughly and immediately stored at -80˚C.

2.4 Generation of Recombinant Baculovirus

Solutions:

- SF-900 Serum-Free Media (SFM / media)
- Tissue culture grade gentamicin
- CELLFECTIN® Reagent

Transfection was carried out essentially as described by (Janmohamed *et al.* 2006). Briefly, *Sf*9 cells in mid-log phase ($2x10^6$ cell/mL) were seeded at a density of $9x10^5$ cell/mL in 2 mL of media containing gentamicin into a 35-mm dish. It is important

that cell cultures with a viability of >98% are used for this stage; otherwise transfection efficiency is reduced. The cells were allowed to attach to the dish by incubating the dish at 27ºC for 1 hour. During this time the transfection mixture was prepared which consists of solution A and B. Solution A: 5µL of miniprep bacmid DNA and 100 µL SFM without gentamicin. Solution B: 6µL CELLFECTIN® Reagent (Invitrogen) and 100 µL SFM without gentamicin. The CELLFECTIN® reagent tube was inverted several times before use as lipids can settle to the bottom of the tube. Solutions A and B were combined and incubated for 30 min at room temperature. Media was removed from the attached cells by gently tilting the dish to one side and pipetting the media away from the dish without touching the surface. The cells were washed once in the same way with media without gentamicin. Media (788 µL) without gentamicin was added to the mix of solution A and B to give a final volume of 1mL. This transfection mixture was gently pipetted over the attached cells. The dish was placed on a bed of tissues dampened with sterile water in an airtight container. This humidified container was incubated at 27ºC for 5 h. The transfection mixture was then removed and replaced with 2 mL of media containing gentamicin. The cells were incubated for a further 72 h at 27ºC in a fresh humidified box. The virus (in the supernatant) was harvested by transferring the medium in the dish into a tube (Falcon) and centrifuged at 100*g* for 10 min at 4ºC. The virus is present in the supernatant and was filter-sterilised using a 0.22 micron filter into a fresh tube. The virus was stored at 4ºC in the dark.

2.4.1 Amplification of Baculovirus

Virus harvested from the transfection stage is of low titre and needs to be amplified. Virus was amplified as described by (Janmohamed *et al.* 2006). 50µL of the

transfection stage virus was used to infect a 25mL batch of cells which were at a cell density of $2x10^6$ cell/mL. Cells were infected for 72 hours at 27ºC in shaking. The cell culture was then transferred into a sterile tube (Falcon) and centrifuged for 10 min at 4000 rpm at 4ºC. The supernatant represents the amplified high titre virus. The supernatant was filter-sterilised into a fresh tube and stored at 4˚C in the dark. Insect cell microsomes were prepared from the cell pellet and analysed for FMO expression.

2.5 Expression & Analysis of Recombinant Protein in *Sf*9 Cells

2.5.1 Baculovirus Infection of *Sf*9 cells

The amount of virus required for optimum FMO expression was investigated using a range of volumes of amplified virus. To begin with, 100, 250 and 500µL of virus were used to infect 50 mL of insect cell cultures which were at a cell density of $2x10^6$ cell/mL for 72 or 96 hours. Expression and catalytic activity was then analysed from microsomes prepared from these cells using western blotting and the methimazole assay.

2.5.2 Insect cell Microsome Preparation

Solution:

- HEPES Buffer: 10 mM HEPES (Sodium salt) (pH 7.4), 0.154 M KCl, 0.1 mM EDTA (pH 8), 20% (v/v) glycerol.

Microsomes were prepared as described by (Janmohamed *et al.* 2006) with slight modification. *Sf*9 cells were transferred into a tube (50 mL Falcon) and centrifuged at 4000 rpm for 10 min at 4ºC (Eppendorf, Rotor 5810 R). The cell pellet was re-

suspended in chilled HEPES buffer (which was three times the volume of the pellet) by pipetting up and down. The cells were lysed by sonification; three 12 s bursts with 5 s pauses in between each burst were found to cause sufficient cell lysis and minimum protein degradation. Cells were placed on ice during the sonification process. The homogenate was centrifuged at 1000*g* for 10 min at 4ºC. The supernatant obtained from the previous step was transferred into pre-chilled ultracentrifuge tubes and centrifuged at 50,000 rpm for 1 h at 4ºC in a Beckman Coulter Optima Max Ultracentrifuge (Fullerton, CA, USA) (rotor TLA-110K). The pellet was re-suspended in chilled HEPES buffer using a glass hand held homogeniser on ice to give a total protein concentration of around 5 µg/µL and the total protein concentration was determined by the assay of Lowry (see next section). The microsomes were aliquoted into 500µL samples in 1.5mL microfuge tubes to avoid freeze-thawing large volumes as this leads to loss of activity. Samples were immediately stored at -80ºC.

2.5.3 Determination of Total Protein Concentration

Protein concentration was determined by the assay of Lowry (Lowry *et al.* 1951) using the Bio-Rad D_C Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Solutions:

- Bovine Serum Albumin (BSA) (Bio-Rad) : Pre-weighed BSA was dissolved in 20 mL of sterile deionised water and gave a total concentration of 1.47 mg/mL

The following solutions were obtained from Bio-Rad D_C Protein Assay Kit

- Reagent A

- Reagent B

The assay was carried out as described in the Bio-Rad Protein Assay Kit hand book with slight modification. Briefly, 6 BSA standard concentrations ranging from 0 – 147 mg/mL were prepared in HEPES buffer. 2µL and 5µL of experimental protein sample were dissolved in HEPES buffer to give a final volume of 100µL. Each assay was carried out in duplicate. 0.5 mL of Solution A was added to each standard and sample tube. These were mixed by vortexing and 4 mL of solution B was then added to each tube and the samples mixed immediately (by vortexing). The tubes were allowed to stand for 20 min. 1 mL of each standard and sample were transferred into plastic cuvettes and their absorbance was measured at 595 nm using a GeneQuant spectrophotometer. The spectrophotometer deduced the protein concentration of standards and sample and this was recorded.

2.5.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Solutions:

- Protogel Solution: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (National Diagnostics).
- 10% (w/v) Ammonium persulfate (APS): The appropriate amount of solid APS was weighed out and dissolved in deionised water on the day of use.
- Resolving buffer: 1.5 M Tris-HCl (pH 8.4), 0.4% (w/v) SDS. pH adjusted with HCl.
- Stacking buffer: 0.5 M Tris-HCl (pH 6.8), 0.4% (w/v) SDS. pH adjusted with HCl.
- TEMED (Bio-Rad).

- NuPAGE LDS Loading Dye (Invitrogen).
- SDS Running Buffer: 0.025 M Tris-base, 0.192 M Glycine, 01% (w/v) SDS prepared on the day of use.
- β-Mercaptoethanol.
- Coomassie Blue Stain: 0.1% (w/v) Coomassie Brilliant Blue Dye, 40% (v/v) methanol, 10% Acetic acid. The solution was filtered through 3MM Whatmann paper before use and stored at room temperature.
- Destain: 30% (v/v) methanol, 10% (v/v) acetic acid, 60% (v/v) deionised water.

25 mL of a 10% resolving gel was prepared with the following materials; 8.33 mL Protogel, 6.25 mL resolving buffer, 0.25 mL APS and 10.14 mL deionised water. This solution was mixed thoroughly and the gel running mould was set up and tested for leaks using water. Only then was 25 µL of TEMED added to the solution, which was mixed and immediately pipetted into the gel mould. The solution was overlaid with propan-2-ol to ensure a flat surface. The resolving gel was allowed to set for at least 2 hours. 10 mL of a 3% stacking gel was prepared with the following materials; 1 mL Protogel, 2.5 mL stacking buffer, 100 µL APS and 6.40 mL of deionised water. Once the resolving gel had set, the propan-2-ol was washed away by tilting the gel mould slightly to one side. Water was used to wash out any residual propan-2-ol. 10 µL of TEMED was then added to the stacking gel mixture and immediately pipetted into the gel mould over the resolving gel. A comb of an appropriate size was positioned to place immediately and the gel was allowed to set for at least one hour. Protein samples were mixed with up to 4µL of NuPAGE loading dye and were boiled for 5 min at 95˚C.

β-Mercaptoethanol was added to the sample to give a final concentration of 20% (v/v) and samples were centrifuged for 1 min . The gel mould was placed into the electrophoresis tank and SDS-running buffer was poured over this so that the electrodes were submersed in buffer. The comb was then removed. 20µL of molecular weight marker (See-Blue 2, Invitrogen) was loaded into the well and so were the samples. The gel was electrophoresed with SDS-Running buffer at 25 mA until proteins had stacked upon the resolving gel and then run at 35 mA till the samples had resolved – i.e. till the bands had travelled to the bottom of the resolving gel. The gel was immersed in Coomassie staining solution for at least 24 hours. The stain was removed by replacing the staining solution with destain. Fresh destain solution was applied until protein bands were clearly visible. Using the geneSNAP tool, the gel was photographed (Syngene).

2.5.5 Western Blot Analysis

Solutions:

- Transfer/Blotting Buffer: 192 mM Glycine, 25 mM Tris, 20% (v/v) methanol. The transfer/blotting buffer was prepared fresh.
- 5X TBS: 100 mM Tris-Cl (pH 7.5), 2.5 M NaCl. The pH was adjusted with HCl.
- 1X TTBS: 1X TBS, 0.1% (v/v) Tween 20. A 100 µL of Tween 20 was used for every 100mL of 1x TBS.
- I-Block Solution: 0.2% (w/v) I-Block (TROPIX Inc., USA) in 100mL 1X TBS. The solution was heated in a microwave oven to melt the solid. The solution was cooled to room temperature before 100µL of Tween 20 was added.

- Primary Antibody Solution: Primary antibodies were used as a 1 in 3000 dilution made up in I-Block solution and were stored at -20˚C. Primary antibody solution used to detect human FMO1, FMO2.1 and FMO3 were goat anti-(rabbit FMO1), goat anti-(rabbit FMO2) or goat anti-(rabbit FMO3) serum respectively. Primary antibodies were a kind gift from Dr. R. Philpot.
- Secondary Antibody Solution: anti-goat IgG (whole molecule) antibody-Alkaline Phosphatase conjugate (Sigma-Aldrich) was used as a 1 in 30, 000 dilution and dissolved in I-Block solution. This was prepared on the day of use.

The following solutions were obtained with the AP Conjugate Kit (Bio-Rad)

- 25X Alkaline Phosphatase (AP) Colour Development Solution. This solution is stored at 4˚C.
- Reagent A: stored at -20˚C.
- Reagent B: stored at -20˚C.

2.5.6.1 Protein Transfer

Proteins from SDS-PAGE gels were transferred onto a supported nitrocellulose 0.45μM membrane (BDH) using a TRANS-BLOT Cell (Bio-Rad). Once protein samples had been electrophoresed, the gel mould was dissembled and the approximate size (length and width) of the gel was estimated. 4 sheets of 3MM Whatman filter paper and 1 sheet of blotting membrane were cut to size. Filter papers and blotting pads were soaked in transfer buffer before use. The gel was 'sandwiched' between filter paper and blotting membrane as shown in Figure 3. It is important not to move the gel once it has come in contact with the membrane, as transfer of protein may

have occurred. Any bubbles between the gel and the membrane were removed by running a pipette gently over the gel. The 'sandwich' was placed within a blotting cassette and loaded into the transfer cell. The cell was filled with transfer buffer and run overnight at 100 mA. The next day the voltage was turned up to 200 mA for 2 hours.

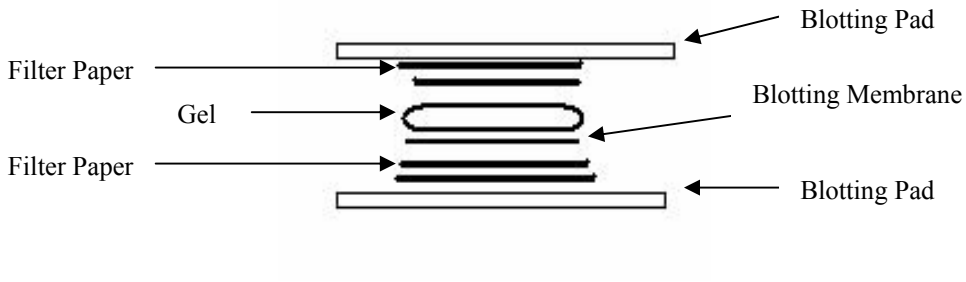


Figure 2.3: 'Sandwich' arrangement of the gel during transfer

2.5.6.2 Antigen Detection

Antigen detection was carried out by a series of incubations and washes as described below. All incubations were carried out at room temperature and on shaking. Blotting membrane was washed in I-Block Solution for 1 h and the solution was discarded after use. Primary antibody solution was poured over the membrane and incubated for 1 h, this solution can be re-used. The membrane was washed for 5 min with 1X TTBS, three times. The solution was discarded. A 1 h incubation with secondary antibody solution was carried out and the solution was discarded. The membrane was washed three times in 1X TTBS for 5 min and once in 1x TBS. The solution was discarded.

2.5.6.3 Development of Western Blot

An Alkaline Phosphatase Conjugate Kit (Bio-Rad) detection method was used to detect the presence of antigen. 1X AP Colour Development solution was prepared

just before use, to which Reagents A and B were added. 1mL of Reagents A and B were used for every 100 mL of 1X AP solution. This mixture was poured over the membrane and observed for the appearance bands. As soon as bands started to develop the reaction was quenched by replacing the colour detection mixture with deionised water – this will prohibit the blotting membrane to overdevelop. The blot was scanned (Epson Scanner, Epson, Naguna, Japan).

2.6 Quantification of Recombinant FMO in Insect Cell Microsomes

2.6.1 Quantitative Western Blotting

Various amounts of authentic rabbit FMO1, FMO2 or human FMO3 of known amounts were loaded along side *Sf* 9 insect cell membranes containing heterologously expressed FMO of unknown concentrations as described in section 2.5.6. SDS-PAGE was performed as described in section 2.5.4 and a Western blot was performed on these gels as described in section 2.5.5. The developed Western blot was scanned at a resolution of 1200 dpi and the image was saved in TIFF format. The amount of FMO in the recombinant insect membranes was quantified by scanning densitometry using Image Gauge software, version 4.2.1 (Science Lab, FujiFilm, Tokyo, Japan) and by comparison with a standard curve of standard FMO vs band intensity. Values determined for FMO quantity were obtained from samples tested in quadruplicate from two independent Western blots. As authentic standards of FMO1 and FMO2 were from rabbit and authentic FMO3 was from human, relative abundance of FMOs was analysed by SDS-PAGE analysis to ensure that there was no difference in antigenicity. The amount of FMO in insect cell microsomes was measured by comparison of intensity of Coomassie blue stained protein bands of authentic FMO.

2.7 HPLC and LC-MS Analysis of FMO Catalysed Reactions

2.7.1.1 Incubations with Thiacetazone (TAZ)

Solutions:

- 100 mM TAZ: TAZ was dissolved in dimethyl sulphoxide (DMSO)
- Assay buffer: 100 mM potassium phosphate buffer, pH 7.5 contained:
- 100 U/ mL Catalase: prepared by weighing the appropriate amount of solid Catalase powder in the appropriate amount of sterile filtered water to give a working stock concentration of 40 U/mL. The solution was stored at -20˚C and re-used.
- 100 U/mL Superoxide dismutase (SOD): prepared by weighing the appropriate amount of solid SOD in the appropriate amount of sterile filtered water to give a working stock concentration of 40 U/mL. The solution was stored at -20˚C and re-used.
- 0.1 mg/mL Bovine serum albumin (BSA): prepared by weighing the appropriate amount of solid SOD in the appropriate amount of sterile filtered water. The solution was stored at -20˚C and re-used.
- NADPH regenerating system consisting of: 2 U/mL of glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$ and 2.5 mM glucose-6-phosphate. Working solutions were prepared for each component in sterile filtered water and stored at -20˚C.

Incubations of TAZ and FMO were performed as described by (Qian *et al.* 2006) but with modifications. The assay buffer was prepared to give a reaction volume of 1 mL. Assay buffer was incubated at 37 ˚C for 10 min to allow the formation of NADPH

from the regenerating system to occur. TAZ was added at a final concentration of 100 μM to the assay buffer and mixed. Reactions were initiated by adding *Sf*9 insect cell microsomes containing heterologously expressed human FMO1, FMO2.1 or FMO3 (at a final concentration of 500 nM) or purified EtaA (1 μM final concentration). Reactions were incubated at 37 ˚C for 90 min and quenched by addition of an equal volume of ice-cold acetonitrile (CH_3CN). Mixtures were centrifuged at 10,000*g* for 5 min at 4 ˚C and analysed by reverse phase HPLC as described below.

2.7.1.2 Incubations with Ethionamide (ETA)

*Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 were incubated with ETA (final concentration 100 μM) for 60 min in assay buffer as described for TAZ. Reactions were analysed by Liquid Chromatography- Mass Spectroscopy (LC MS) as described below.

2.7.2 Reverse Phase HPLC analysis

HPLC analysis was done as described previously (Qian *et al.* 2006). The supernatants were diluted to a final concentration of 5% CH_3CN and then analysed by HPLC on a Hewlett Packard II instrument (Hewlett-Packard Company, CA, USA) equipped with a photodiode array detector and a reverse-phase C18 column (Waters, 3.5 μm particle size, 4.6 mm i.d x 150mm, Symmetry) employing two buffers: A, H_2O and 0.1% formic acid (FA); and B, CH_3CN and 0.1% FA. The solvent flow rate was 0.2 mL/min and the eluent was spectrophotometrically monitored using two bandwidths [330 +/- 60 nm and 260 +/- 4 nm]. The column was eluted from 0 to 25 min with a linear gradient from 5 to 20% buffer B. For spectral analysis of metabolites eluent peaks were 'monitored' between 200 and 500 nm. 20 μL of sample was injected.

2.7.3 Liquid Chromatography-Mass Spectroscopy (LC-MS) Analyses

Samples for LC-MS analysis were prepared as described for HPLC. LC-MS was carried out as described previously (Qian *et al.* 2006). Briefly, LC-MS analysis of TAZ metabolites was performed on a Waters Micromass ZQ coupled to a Waters Alliance HPLC system (Waters Corporation, MA, USA). The system was equipped with a 2695 separations module, a Waters 2487 Dual λ Absorbance detector and a reverse phase C18 column (Waters, 3.5 μm particle size, 4.6 mm i.d x 150mm, Symmetry). The reverse-phase column was eluted with a flow rate of 0.2 mL/min (buffer A, H_2O and 0.1% FA; and buffer B, CH_3CN and 0.1% FA) with the following protocol: 0 - 16 min, 5-30% buffer B (linear gradient). The eluent was monitored at 310 nm. The eluent was monitored at 280 and 320 nm. The mass spectrometer settings were as described previously (Qian *et al.* 2006). The mass spectrometer settings were as follows: mode, ES^+, capillary voltage, 3.5 kv, cone voltage, 25 v, desolvation temperature, 250 ˚C.

LC-MS analysis of ETA metabolites produced by FMO2.1 was performed as described above, with the following modifications. The column was eluted at a flow rate of 0.2 mL/min (buffer A, H_2O and 0.1% FA ; and B, CH_3CN and 0.1% FA with the following protocol: 0-15 min with 1% buffer B (isocratic). The eluent was monitored at 350 nm.

2.8 FMO Assays

2.8.1 Methimazole Assay of FMO activity (Dixit *et al.* 1984)

Methimazole oxidation by human FMOs was monitored spectrophotometrically using a dual beam spectrophotometer (Varian Cary 100, Varian Inc., CA, USA). The assay

consists of a coupled reaction in which Dithiothreitol (DTT) and 5-Dithio-bis (2-nitrobenzoic acid) (DTNB) initially react to form nitro-5-thiobenzoate (TNB), a yellow compound which absorbs light in the visible range. In the presence of NADPH and molecular oxygen, FMOs catalyse the oxidation of methimazole to methimazole *S*-oxide, this will oxidise TNB to a colourless compound (Dixit *et al.* 1984). The rate of colour change from yellow to colourless is monitored spectrophotometrically at 412 nm and is proportional to the rate of methimazole oxidation by FMO.

Solutions:

Assay Buffers

- Human FMO1 and 3: 0.1 M Tris-Cl (pH 8.4), 1 mM EDTA

  Buffer was prepared on the day of use. The pH was adjusted to 8.4 using concentrated HCl. Prior to use, the buffer was aerated for 30 min by placing the bottle in a shaking water bath set to 37 ˚C .

- Human FMO2.1: 0.1 M Tricine (pH 9.5), 1 mM EDTA.

  Buffer was prepared on the day of use. The pH was adjusted to 9.5 using 5 M NaOH. Prior to use, the buffer was aerated for 30 min by placing the bottle in a shaking water bath set to 37 ˚C .

Methimazole

- 200 mM Methimazole: Stock solution was prepared fresh by adding the appropriate amount of solid methimazole to the assay buffer.

- 10 mM Methimazole: Stock solution was prepared fresh by adding the appropriate amount of solid methimazole to the assay buffer

NADPH

- 20 mM NADPH reduced (sodium salt): Stock solution was prepared fresh by adding the appropriate amount of solid NADPH to chilled assay buffer and stored on ice.
- NADPH regenerating system:
    - 2 U/mL Glucose-6-Phosphate Dehydrogenase
    - 3.0 mM Glucose-6-Phosphate
    - 0.375 mM $NADP^+$

All components where prepared on the day in the appropriate assay buffer which was chilled before use.

- 4 mM Dithiothreitol (DTT): DTT solution was prepared fresh by adding the appropriate amount of solid DTT to assay buffer.

- 12 mM 5, 5-Dithio-bis (2-nitrobenzoic acid) (DTNB): Prepared fresh by adding the appropriate amount of solid DTNB to 100% ethanol.

Assay

Reactions were carried out in a final volume of 1 mL and at 37ºC. The final concentrations of DTNB and DTT were 0.06 µM and 0.02 mM respectively. Two identical 'blank' cuvettes were prepared with the following components, 5 µL NADPH or 25µL of glucose-6-phosphate, 25µL of NADP+, 7.5µL of glucose-6-

phosphate dehydrogenase when a regenerating system was used, 5µL of DTT, 5µL of DTNB and 15-70 µL of human FMO expressing microsomes (no more than 1 mg/mL total protein) in assay buffer to give a total volume of 1 mL. The contents were mixed by inverting the cuvettes. Cuvettes were placed in cell 1 or cell 7 of a Varian Cary 100 dual beam spectrophotometer and a blank measurement was taken. Using the Kinetics application, the spectrophotometer was set to blank correction mode. Methimazole was added at the appropriate concentration to the sample cuvette and the equivalent volume of assay buffer was added to the reference cuvette. The rate of decrease in optical density at 412 nm was monitored after one minute for 4 minutes.

Kinetic studies were performed on triplicate preparations of enzymes at concentrations of methimazole ranging from 10-2000 µM for human FMO1 and FMO3 and 200-6000 µM for human FMO2.1. Steady-state parameters, *V*max, K_M and *k*cat were determined as described in section 2.8.3.1.

2.8.2 TAZ Oxygenation by Human FMOs and EtaA

2.8.2.1 Development of a UV-Spectroscopic Assay to monitor the rate of TAZ oxygenation

Solutions:

- Assay Buffer: 0.1 M Tris-Cl (pH 8.4). Buffer was prepared on the day of use. The pH was adjusted to 8.4 using concentrated HCl. Prior to use, the buffer was aerated for 30 min by placing the bottle in a water bath set to 37 ˚C with shaking.

- 20 mM TAZ: A stock solution was prepared fresh in dimethyl sulphoxide (DMSO). Serial dilutions of TAZ were prepared from the 20 mM stock and in DMSO.

Two quartz cuvettes containing 999 µL of assay buffer were placed in cell 1 and cell 7 of a Varian Cary 100 dual-beam spectrophotometer (Varian Inc., USA). Using the Varian Scan application, the spectrophotometer was set to blank correction mode. 1 µL of TAZ (in DMSO) was added to the cuvette in cell 7 (sample cuvette) and 1 µL of DMSO to the cuvette in cell 1 (reference). The final organic solvent concentration in each cuvette was held at 0.1% (v/v). Using the Varian Scan application, samples were scanned from 200 to 500 nm over a range of TAZ concentrations between 1 and 20 µM. These measurements were done in triplicate using three independently prepared stock solutions of TAZ (20 mM) that were diluted accordingly in DMSO. The absorbance of TAZ, measured at its λmax (328 nm) was plotted against concentration. The molar extinction coefficient of TAZ in 0.1% DMSO was determined from the gradient of this graph and the Beer-Lambert equation (equation 2).

$$\text{Molar Extinction Co-efficient } (\varepsilon) = \frac{A}{\ell . C}$$

Equation 2

Where,

A = Absorbance of a given solution at a particular wavelength

C = concentration of the solution

ℓ = the distance light has to travel through a solution

2.8.2.2 UV-Spectrophotometric Assay to Monitor FMO-dependent TAZ oxidation

Solutions:

Assay Buffers:

- Human FMO1 and 3: 0.1 M Tris-Cl (pH 8.4), 1 mM EDTA. Buffer was prepared on the day of use. The pH was adjusted to 8.4 using concentrated HCl. The buffer was placed in a water bath set to 37 ˚C. Prior to use, the buffer was aerated for 30 min by placing the bottle in a shaking water bath set to 37 ˚C.
- Human FMO2 and EtaA: 0.1 M tricine (pH 9.5), 1 mM EDTA. Buffer was prepared on the day of use. The pH was adjusted to 9.5 using 5M NaOH. The buffer was placed in a water bath set to 37 ˚C. Prior to use, the buffer was aerated for 30 min by placing the bottle in a shaking water bath set to 37 ˚C.
- 20 mM NADPH: prepared fresh by adding the appropriate amount of solid NADPH to assay buffer. The solution was stored on ice.
- 20 mM TAZ: solution was prepared fresh by adding the appropriate amount of solid TAZ to in dimethyl sulphoxide (DMSO). Serial dilutions ogf TAZ were prepared from the 20 mM stock solution in DMSO. TAZ solutions were kept at room temperature.

Assay

TAZ oxidation by human FMO or EtaA was measured by monitoring the rate of decrease in TAZ absorbance at 328 nm using a dual beam spectrophotometer (Varian Cary 100). The spectrophotometer was set to blank correction mode and the Peltier

module to 37˚C. Two identical 'blank' quartz cuvettes were prepared with the following components; 100 μM NADPH, insect cell microsomes containing heterologously expressed human FMO1 (320 nM), FMO2.1 (5 nM), FMO3 (230 nM), purified ETA (1 μM) or the equivalent amount of uninfected *Sf*9 cell microsomes in the appropriate assay buffer (see next section) to give a final reaction volume of 1 mL. The contents of the cuvettes were mixed thoroughly and placed in cell 1 (reference) or cell 7 (sample) of the spectrophotometer and a 'blank' measurement was made. The samples were allowed to equilibriate at 37˚C for 1 min. 1 μL of TAZ from an appropriate stock solution was added to the sample cuvette and 1 μL of DMSO was added to the blank cuvette. The contents were mixed by inverting the cuvette and the cuvettes were immediately replaced to their respective cells. The rate of decrease in optical density at 328 nm was monitored after one minute for 4 minutes.

2.8.2.2.1 Determining pH dependence of FMO and EtaA activity

The pH optimum for human FMOs and ETA was determined using the following reaction buffers:

- 0.1 mM potassium phosphate (pH 7.5), 1 mM EDTA
- 0.1 M Tris-HCl (pH 8.5), 1mM EDTA
- 0.1 M Tricine-OH (pH 9.5), 1 mM EDTA.

Buffers were prepared fresh on the day and were aerated for 30 min at 37 ˚C in a shaking water bath before use. Optimum pH for enzymes was determined using the UV-spectroscopic assay detailed in the preceding section using TAZ at a final concentration of 10 μM.

2.8.3 Kinetic Studies of TAZ oxygenation using a UV-spectroscopic Assay

Kinetic studies were performed on triplicate preparations of enzymes at concentrations of TAZ ranging from 1 to 20 μM. The final organic solvent concentration was held at 0.1% (v/v). Assays were carried out in triplicate on each enzyme preparation as described in section 2.7.2 in the optimum pH buffer for each enzyme: pH 8.5 for FMO1 and FMO3 and 9.5 for FMO2.1 and EtaA.

2.8.3.1 Determination of Steady-State Kinetic Parameters

Enzyme velocity was converted from absorbance min^{-1} to μmol L^{-1} min^{-1} using the Molar extinction co-efficient of TAZ (determined to be 38,300 ± 2,320 M^{-1} cm^{-1}) or TNB (13,600 M^{-1} cm^{-1}). Steady-state kinetic parameters, *V*max and K_M were estimated by non-linear regression using the Enzyme Kinetics module (v.1.3) of the SigmaPlot (v 10.0) program (Systat Software Inc., CA, USA). Non-linear regression has been chosen to determine kinetic parameters as it is superior to methods which transform Michaelis-Menten equation in a linear fashion. Linearity of data was confirmed by Hanes-Woolf (Hanes 1932) linear transformations of the Michaelis-Menten equation using the Enzyme Kinetics module. Turnover number (*k*cat) was determined using the following equation.

$$kcat = Vmax / [E]$$

Equation 3

Where *V*max is the maximal enzyme velocity of enzyme catalysis and [E] is the final enzyme concentration in the reaction.

2.8.4 Kinetic Studies of TAZ oxygenation using HPLC analysis

*Sf*9 cell microsomes containing heterologously expressed human FMO2.1 were incubated in 0.1 M Tricine-OH (pH 9.5), 1 mM EDTA, 0.1 mM NADPH and TAZ (concentrations ranged from 1 - 50μM in DMSO). A duplicate set of samples was prepared but without the addition of enzyme. The final organic solvent concentration was held at 0.1% (v/v). Mixtures were incubated at 37 ˚C for 5 min and reactions were quenched with an equal volume of ice-cold CH_3CN. ETA was added as an internal standard at a final concentration of 100 μM (in DMSO) and mixtures were prepared for HPLC analysis as described above. A standard curve was generated by plotting the ratio of the integrated HPLC peak areas of TAZ and ETA (from the sample set without added enzyme), against the range of TAZ concentrations used. The ratio of the integrated HPLC peak areas of TAZ and ETA, in the sample set with added enzyme, was calculated and the amount of unmetabolised TAZ determined from the standard curve. This value was subtracted from the input concentration of TAZ to calculate the amount of TAZ metabolized by the enzyme. *V*max, K_M and *k*cat were determined as described in section 2.8.3.1.

2.9 Generation of human FMO3 Amino Acid Variant cDNAs

Human FMO3 cDNAs containing the desired mutations were previously prepared in the laboratory as described elsewhere (Allerston *et al.* 2009). Each human FMO3 cDNA variant was cloned in pET21b and transformed into *E.coli* DH5α™ and maintained as glycerol stocks. This section describes the sub-cloning of FMO3 cDNA from pET21b into pFastBac1™ for subsequent expression in *Sf*9 cells. For

simplicity human FMO3 cDNA for wild type and FMO3 polymorphic variants will be referred to as 'human FMO3'.

E.coli DH5α™ cells containing FMO3-pET21b plasmid DNA were streaked on to agar plates containing 50 µg/mL ampicillin and incubated at 37ºC overnight as described in section 2.2.3. A single colony was used to inoculate 5 mL of LB-medium containing 50 µg/mL ampicillin. The culture was incubated overnight at 37ºC with shaking. The following day, plasmid was isolated from 3 mL of bacterial culture using the QIAprep Miniprep Kit (QIAGEN) as described in section 2.3.1.1. Plasmid DNA was quantified as described in section 2.3.1.2.

pFastBac1™ was purchased from Invitrogen and was at a concentration of 1.3 µg/µL.

2.9.1 *Bam*HI and *Hind*III Restriction Digest of human FMO3-pET21b and pFastBac1™

Solutions:

- 10X NEBUFFER 2 (New England Biolabs, MA, USA)
- 100X Bovine Serum Albumin (BSA) (New England Biolabs, MA,USA)
- Sterile water

*Bam*HI and *Hind*III were used to excise FMO3 cDNA from pET21b. 1 µg of plasmid DNA was digested at 37ºC overnight with 5 U of *Bam*HI and 5 U of *Hind*III, in a solution containing 1X NEBuffer 2, 1X BSA and sterile water according to NEB instructions. pFastBac1™ was also digested with *Bam*HI and *Hind*III to produce complementary DNA sequences or 'sticky ends' to the DNA sequence of digested FMO3 cDNA.

2.9.2 Gel Extraction of DNA

Solutions:

- SYBR® Green 100,000X Concentrate (Invitrogen)
- Propan-2- ol
- Sterile Water

The following solutions were obtained from the QIAquick Gel Extraction Kit (Qiagen)

- Buffer QG
- Buffer PE

After digestion of pET21b, human FMO3 cDNA was isolated using the QIAquick gel Extraction kit (QIAGEN). Samples prepared as described in section 2.9.1 were separated on a 1% agarose gel as described in section 2.3.1.3 with the exception that 1 µL of SYBR® Green was added to molten agarose instead of ethidium bromide. This allowed DNA to be visualised using a light box with an orange filter rather than exposing it to UV. Using the molecular weight marker as a guide, FMO3 cDNA was excised from the gel using a scalpel. The piece of agarose was weighed and placed in a 1.5 mL microfuge tube. Three volumes of Buffer QG was added to one volume of agarose (1 volume =100 µL of agarose or 100 mg) and incubated at 50˚C for 10 min or until the agarose had completely dissolved. The following steps were carried out at room temperature. One 'gel' volume of propan -2-ol was added and the tube was inverted. A QIAquick spin column was placed in a 2 mL collection tube and 800 µL of sample was applied to the column. Samples were centrifuged at 13,000*g* for 1 min

in a microcentrifuge, the flow through was discarded and the column was placed in the same collection tube. 0.5 mL of Buffer QG was added to the column to remove traces of agarose. The column was centrifuged for 1 min at 13,000*g* and the flow through was discarded. The column was washed by adding 0.75 mL of Buffer PE, the sample was centrifuged for 1 min and the flow through was discarded. The column was centrifuged for an additional 1 min to ensure all residual ethanol from Buffer PE was removed. For the elution step, the column was placed into a fresh 1.5 mL microfuge tube and 30 µL of sterile water was applied to the column. The column was allowed to stand for 1 min and then centrifuged for 1 min at 13,000*g*. For short term storage, human FMO3 cDNA was kept at 4˚C.

2.9.3 DNA Ligation

Solutions:

The following solutions were provided with the Quick Stick Ligase Kit (Bioline Ltd, UK).

- 4X QS Buffer
- Sterile water

DNA Ligation was carried out using the Quick Stick Ligase Kit (Bioline Ltd, UK) Human FMO3 cDNA isolated in the previous section was ligated to pFastBac1™. Prior to this step, pFastBac1™was digested with *Bam*HI and *Hind*III as described in section 2.9.1 and a 1 in 100 dilution was prepared. For each ligation reaction, two tubes were set up: Vector Only (digested pFastBac1™) and Vector plus Insert (digested pFastBac1™and digested FMO3 cDNA). Vector only tubes contained: 1 µL of digested pFastBac1™, 1 µL of Quick Stick Ligase enzyme, 5 µL of 4X QS

buffer (which was vortexed before use) and 13 µL of sterile water. Vector plus Insert tubes contained: 1 µL of digested pFastbac1, 1 µL of Quick Stick Ligase enzyme, 5 µL of 4X QS buffer (which was vortexed before use) and 13 µL of digested human FMO3 cDNA. The tubes were incubated overnight at 16˚C.

2.9.4 Tranformation of *E.coli* OneShot™ TOP10 competent cells with Ligation Products

In order to confirm that the ligation reaction between human FMO3 cDNA and pFastBac1™ was successful, *E.coli* OneShot™ TOP10 chemically competent cells Invitrogen) were transformed with the ligation reaction mixtures. A 50 µL vial of OneShot™ cells was used per ligation. OneShot™ cells were thawed by gently rubbing the vial in the hands and as soon as thawing was observed, the samples were placed on ice and 1 µL of ligation reaction mixture was added. The contents were mixed by gently tapping and inverting the vial as pipetting can reduce transformation efficiency. The vials were incubated on ice for 30 min. Vials were incubated for exactly 30 sec at 42˚C using a heat block. The vials were then placed on ice. 250 µL of pre-warmed SOC solution was added to each vial. Samples were incubated with shaking in a heat block for exactly 1 hr at 37˚C. During this time, agar plates containing ampicilin (prepared as described in section 2.2.3) were placed in a 37˚C incubator to warm up. 50 µL of transformed cells from each vial were spread on an agar plate, the plates were inverted and incubated at 37˚C overnight. Colonies were counted the following day and the ratio of colonies formed from 'Vector only' and 'Vector plus Insert' vials was noted. 5 colonies were picked from each Vector plus Insert plate and 1 colony from Vector only plates. Each colony was inoculated into 5mL of LB-medium containing 50 µg/ mL of ampicillin. Cultures were grown for 6-8

hours or overnight with shaking at a rotational speed of 250 rpm at 37˚C. Plasmid DNA was isolated using the QIAprep Miniprep kit (QIAGEN) as described in section 2.3.1.1.

2.9.5 Analysis to Confirm Successful ligation of human FMO3 cDNA and pFastBac1™

10 µL of plasmid DNA was digested with *Bam*HI and *Hind*III as described in section 2.9.1. Digested products were run on a 1% agarose gel as described in section 2.3.1.3. If ligation was successful, two DNA bands were expected to be observed, representing pFastBac1™ (4.8kb) and human FMO3 cDNA (~2kb). In order to confirm that the vector was indeed pFastBac1™ (and not the original vector, pET21b), a restriction enzyme was chosen which could differentiate between the two plasmids. *Bgl* II was used as it has two restriction sites in pFastBac1™ but only one in pET21b. Reactions contained 5 µL of plasmid DNA, 1 µL of *Bgl* II (NEB, England), NEBuffer 3 (NEB, England) and 3 µL of water. Samples were incubated for 2 hours at 37˚C. Digested products were visualised by agarose gel electrophoresis as described in section 2.3.1.3.

2.9.6 DNA Sequencing

DNA sequencing was performed by Eurofins-MWG Biotech (Germany) using the 'value read' service. The service required 1 µg of plasmid DNA per sequencing reaction. Sequences were analysed by comparison to the wild type human FMO3. DNA was sequenced in both forward and reverse orientations using primers annealing to sequences in the pFastBac1™ vector, these primers were available at MWG-

Biotech. Primer sequences used for sequencing wild type and variant human FMO3 DNA are given in appendix 1.

The remainder of the method is the same as that described for human FMO1, FMO2.1 and FMO3. *E.coli* DH10Bac™ cells were transformed with human FMO3 cDNA as described in section 2.3.1.4 and the resulting bacmid DNA was isolated as described in section 2.3.1.5. Generation of baculovirus containing human FMO3 DNA was done as described in section 2.4. Expression of recombinant human wild-type FMO3 protein and FMO3 amino acid variants was carried out as described in section 2.5. Quantitation of FMO3 in insect cell microsomal membranes was done as described in section 2.6 using human FMO3 containing insect cell microsomes of known FMO3 amounts as a standard.

2.9.7 Assays of Variant human FMO3 Activity

Activity of FMO3 amino acid variants was determined towards methimazole and TAZ.

2.9.7.1 Methimazole Assay of FMO3 Amino Acid Variant activity

Kinetic studies on human FMO3 amino acid variants and methimazole were performed at pH 8.4 and essentially as described in section 2.8.1. NADPH was supplied to the reaction using a 20 mM NADPH solution prepared in assay buffer. 200-500 pmol of FMO3 protein was used in the assay depending on the variant. Methimazole was tested at concentrations ranging from 10-1000 μM on batches of microsomes isolated from three independent infections of *Sf*9 cells. Steady-state kinetic parameters were determined from assays performed in triplicate for each

FMO3 protein (as described in section in 2.8.3.1) and were compared to those obtained for wild type FMO3. Statistical significance was assessed using an unpaired, two-tailed t-test performed using the Microsoft Excel application of Microsoft Office 2003.

2.9.7.2 TAZ Assay of FMO3 amino Acid Variant activity

Kinetic studies on human FMO3 amino acid variants and TAZ were performed at pH 8.4 and essentially as described in section 2.8.2.2. 200-500 pmol of FMO3 protein was used in the assay depending on the variant. NADPH was supplied to the reaction using a 20 mM NADPH solution prepared in assay buffer. TAZ was tested at concentrations ranging from 1-20 μM on batches of microsomes isolated from three independent infections of *Sf*9 cells. Steady-state kinetic parameters were determined from assays performed in triplicate for each FMO3 protein (as described in section in 2.8.3.1) and were compared to those obtained for wild type FMO3. Statistical significance was assessed using an unpaired, two-tailed t-test performed using the Microsoft Excel application of Microsoft Office 2003.

2.10 Isolation and Preparation of Mouse Tissue Microsomes

Age-matched male and female wild-type C57BL/6 and knockout mice back-crossed for eight generations onto the C57BL/6 line were used in this investigation. The construction of the *Fmo1* (-/-), *Fmo2* (-/-) *Fmo4* (-/-) knockout mouse has been described (Hernandez et al 2009). The production of the *Fmo*5 (-/-) knockout line has not yet been published but preliminary data on its construction can be found in the Ph D thesis of A. Melloni and in (Hernandez *et al.* 2006).

2.10.1 Dissection

Liver and lungs were obtained from 8-10-week old C57BL/6 mice, *Fmo1* (-/-), *Fmo2* (-/-) *Fmo4* (-/-) mice and *Fmo5* (-/-) mice as described in this section. Mice had access to food and water *ad libitum* and were housed with appropriate animal husbandry facilities.

Solutions:

- 70% Ethanol
- Buffer A: 0.1 M Tris-Acetate (pH 7.4), 0.1 M KCl, 1 mM EDTA. Buffer was prepared the day before dissection was to take place and stored at 4˚C.

On the morning of the procedure, dissection equipment was ensured to be clean and was wiped with 70% ethanol. Mice were sacrificed in the morning between 9 and 11 am by carbon dioxide asphyxiation. For this, the asphyxiation chamber was first placed on its side to allow any residual CO_2 to be removed and then returned to its upright position. Mice (no more than 4 at a time) were placed in the box and the CO_2 concentration was slowly increased to avoid stress to the animals. After 10 min, mice were taken out of the box and their feet were pinched to observe pedal reflex. If no response was observed, the abdomen was swabbed with 70% ethanol and opened through a U-shaped incision. The intestines were moved to one side of the torso and the liver was excised. The liver was weighed and submerged in three times the volume of chilled Buffer A that was placed on ice. The abdomen was inspected to ensure that all three lobes of the liver were excised. To excise the lungs, the

diaphragm was cut and so was the rib cage. Again inspections were made to ensure that the entire lung was obtained. Lungs were weighed and submerged in three times the volume of chilled Buffer A. The tissues were minced in this buffer using scissors. The steps described in the next section were performed immediately.

2.10.2 Isolation of Mouse Tissue Microsomes

Solution:

- Storage Buffer: 10 mM potassium phosphate (pH7.5), 1 mM EDTA, 20% Glycerol. Buffer was prepared a day before dissection and stored at 4˚C.

Minced tissues from the preceding section were homogenised using a motor powered hand held homogeniser (Citenco, Park Products Ltd, England). The mortar was chilled before transferring minced tissue samples to it and it was kept on ice during the procedure at all times. Tissues were homogenised at speed 7 until the tissue was completely resuspended. The homogenate was then transferred to a chilled centrifuge tube and centrifuged at 10,000g for 10 min at 4˚C. The supernatant from this step was centrifuged at 105,000g for 90 min at 4˚C to give a pale yellow pellet. The pellet obtained from this step is a crude preparation of smooth and rough endoplasmic reticulum membranes. The pellet was immediately placed on ice and resuspended in Storage buffer using a glass-glass hand held homogeniser. The desired final concentration of the suspended microsomes was about 10-25 µg/µL. Microsomes were frozen at -80˚C in 500 µL aliquots to avoid freeze-thawing of large volumes as this leads to reduced activity. Microsomal protein concentration was determined by the method of Lowry as described in section 2.5.3.

2.10.3 Kinetic Studies of TAZ Metabolism by Mouse Tissue Microsomes

2.10.3.1 TAZ metabolism by Mouse Lung and Liver Microsomes

Solution

- Assay Buffer: 100 mM potassium phosphate (pH 7.5), 1 mM EDTA. Buffer was made on the day from a pre-made 1M potassium phosphate solution (pH7.5) that was stored at 4˚C. The buffer was aerated for 30 min in a 37˚C water bath with shaking.
- NADPH regenerating system:
    - 2 U/mL Glucose-6-Phosphate Dehydrogenase (7.5 µL was used in a 1 mL reaction)
    - 3.0 mM Glucose-6-Phosphate (25 µL was used in a 1 mL reaction)
    - 0.375 mM $NADP^+$ (25 µL was used in a 1 mL reaction)

  All components where prepared on the day in the appropriate assay buffer which was chilled before use.

The rate of TAZ metabolism by mouse liver and lung microsomes was determined essentially as described in section 2.8.2.2 but using TAZ at a final concentration of 10 µM and using an NADPH regenerating system. The relative contribution of FMOs and CYPs was to be determined by this method and so reactions were performed at pH 7.5 because a) this resembles physiological pH and b) because CYP function is inhibited in alkaline conditions. NADPH was supplied through an NADPH regenerating system for which the components are described above. 500 µg of microsomal protein was used in the reaction and enzyme velocity was converted from

absorbance min^{-1} to μmol L^{-1} min^{-1} using the Molar extinction co-efficient of TAZ (determined to be 38,300 ± 2,320 M^{-1} cm^{-1}). Michaelis-Menten plots (initial enzyme velocity vs concentration of substrate) were plotted for microsomal samples isolated from liver and lung from female mice.

The rate of TAZ metabolism obtained for liver and lung microsomal samples was compared between mouse lines and to results obtained for enzyme inhibition studies (see following section). Statistical significance was assessed using an unpaired two-tailed t-test when results were compared between samples (same test condition, different samples), when results were compared within a sample (i.e. different test conditions, one sample) a paired two-tailed t-test was employed.

2.10.4 Control Experiments used to Assess Activity of Mouse Tissue Microsomes

It was necessary to check that the microsomes displayed activity towards control substrates of the FMOs and CYPs to ensure that they had not become inactivated during the isolation procedure. FMO activity was assessed using the assay of methimazole and CYP activity was assessed by monitoring the rate of cytochrome *c* reduction by NADPH-dependent cytochrome P450 reductase.

2.10.4.1 Methimazole Oxidation by Mouse Tissue microsomes

To ensure that FMOs were not inactivated during the isolation procedure their capacity to oxidise methimazole was assessed.

The rate of methimazole oxidation by mouse liver and lung microsomes was determined essentially as described in section 2.8.1. Reactions were performed in pH 8.4 and NADPH was supplied through a NADPH regenerating system. 50-200μg of microsomal protein was used in the reaction depending on the tissue from which the

microsomes were isolated from. Enzyme velocity was converted from absorbance min^{-1} to μmol L^{-1} min^{-1} using the Molar extinction co-efficient of TNB (13,600 M^{-1} cm^{-1}). Though kinetic parameters were not calculated for microsomes because of the shortage of sample, the relative rate of TNB oxidation was used as a measure of FMO activity.

2.10.4.2 Activity of NADPH Cytochrome P450 Reductase in Mouse Liver Microsomes

Although CYPs are relatively robust enzymes that are stable at room temperature and can withstand incubations of up to several minutes at 45-50˚C, it was necessary to ensure that they were active during the experiment. NADPH cytochrome P450 reductase is an enzyme that is necessary for the activity of CYPs and the activity of this enzyme was used as an assessment of CYP activity. Details regarding its function will be given in section 3.3. It is appreciated that factors other than CRP activity may affect CYP activity; however these are uncommon under the conditions used in this experiment.

Solutions:

- Assay Buffer: 100 mM potassium phosphate buffer (pH 7.5) prepared on the day from a pre-made 1M potassium solution that was stored on ice.
- 0.2 mM Cytochrome *c*: Stock solution was prepared by weighing the appropriate amount of solid Cytochrome *c* from *Saccharomyces cerevisiae* and adding it to assay buffer.

- 20 mM NADPH reduced (sodium salt): Stock solution was prepared fresh by adding the appropriate amount of solid NADPH to chilled assay buffer and was stored on ice.

The rate of cytcochrome *c* reduction by CRP was measured spectrophotometrically at 25˚C as described by Omura and Takesue (Omura *et al.* 1970). Two identical 'blank' cuvettes were prepared with the following components; 200 µg of microsomes isolated from mouse lung or liver and cytochrome *c* at a final concentration of 0.2 µM. The cuvettes were capped and inverted to mix the contents before placing them in cell 1 (reference) or cell 7 (sample) of a Varian Cary 100 dual beam spectrophotometer. The Peltier module was set to 25˚C. Using the Kinetics application, the spectrophotometer was set to blank correction mode and a blank measurement was taken. Reactions were initiated by the addition of 100 µM NADPH. The increase in optical density at 550 nm was measured for 10 min.

2.10.5 Inhibition Studies on Mouse Tissue Microsomes.

In this section the rate of TAZ metabolism was determined for microsomes that have been treated to inhibit FMO activity or CYP activity. Sections 2.10.5.1 through 2.10.5.3 describe how FMOs and CYPs were inactivated. Samples were then assessed for their ability to metabolise 10 µM TAZ as described in section 2.10.3.1

2.10.5.1 Competitive Inhibition of FMO activity

Solutions:

- Assay Buffer: 100 mM potassium phosphate (pH 7.5), 1 mM EDTA. Buffer was made on the day from a pre-made 1M potassium phosphate solution (pH

7.5) that was stored at 4C. The buffer was aerated for 30 min in a 37˚C water bath with shaking

- 200 mM Methimazole: Stock solution was prepared fresh by adding the appropriate amount of solid methimazole to assay buffer.

FMO activity was inhibited by pre-incubating mouse tissue microsomes with methimazole, a probe substrate of the FMOs that acts as a competitive inhibitor. The concentration of methimazole chosen in this experiment was determined by reviewing literature of the saturating concentration of methimazole.

Tubes (prepared in duplicate) containing 500 µg microsomal protein, 1 mM methimazole and NADPH regenerating components were incubated at 37˚C for 2 min. The contents of the tubes were transferred to two quartz cuvettes where one would be treated with TAZ and the other would serve as a blank in the assay. The rate of TAZ metabolism after FMO inhibition was measured as described in section 2.8.2.2. Assays were carried out in triplicate on two batches of mouse tissue microsomal samples and statistical significance was assessed as described in section 2.10.4.

2.10.5.2 Heat Inactivation of FMO Activity

FMOs are heat labile and can be inactivated by short durations of heat treatment at 45-50˚C in the absence of NADPH (Rawden *et al.* 2000; Stormer 2000; Virkel *et al.* 2006; Siddens *et al.* 2008).

Tubes (prepared in duplicate) containing 500 µg microsomal protein in assay buffer (to give a final volume of 942 µL) were incubated at 45˚C for 5 min. After heat treatment, tubes were returned to ice for one min and components of the NADPH regenerating system were added. Tubes were inverted to ensure adequate mixing. The

contents of the tubes were transferred to two quartz cuvettes where one would be treated with TAZ and the other would serve as a blank in the assay. The rate of TAZ metabolism after FMO inhibition was measured as described in section 2.8.2.2. Assays were carried out in triplicate on two batches of mouse tissue microsomal samples and statistical significance was assessed as described in section 2.10.4.

2.10.5.3 Inhibition of CYP Inactivity

NADPH-dependent Cytochrome P450 Reductase (CRP) anti-serum was employed to abolish the activity of CYPs in the microsomal samples.

The amount of CRP-anti-serum was optimised as follows: tubes (prepared in duplicate) containing 500 μg microsomal protein in assay buffer and increasing volumes of CRP-anti-serum or control non-immune serum (0-50 μL) were incubated on ice for 15 min. The components of the NADPH regenerating system were applied to these tubes and these were inverted to mix the contents. The contents of the tubes were transferred to two quartz cuvettes where one would be treated with TAZ and the other would serve as a blank in the assay. The rate of TAZ metabolism after CYP inhibition was measured as described in section 2.8.2.2. Assays were carried out in triplicate on two batches of mouse tissue microsomal samples and statistical significance was assessed as described in section 2.10.4.

chapter 3: Results and Discussion

3.1 Analysis of the Reaction between Human FMO2.1 and the Anti-Tubercular Drugs Thiacetazone and Ethionamide.

The work described in this section has been published.

Francois, A.A., Nishida, C.R., Ortiz de Montellano, P.R., Phillips, I.R., Shephard, E.A. (2009) Human Flavin-Containing Monooxygenase 2.1 Catalyzes Oxygenation of the Antitubercular Drugs Thiacetazone and Ethionamide. *Drug Metabolism and Disposition.* 37(1):178-186.

Introduction

This section describes the reaction between human FMO2.1 and the anti-tubercular drugs Thiacetazone (TAZ) and Ethionamide (ETA). Metabolites generated for TAZ by FMO2.1 were compared to those formed by the action of human FMO1, FMO3 and the *Mycobacterial* FMO, EtaA. Kinetic parameters describing the reaction between FMO2.1 and TAZ were determined using a novel UV-spectroscopy assay. Kinetic parameters for human FMO1, FMO3 and EtaA were also determined for comparison.

Results

Protein Expression

Human FMO1, FMO2.1 and FMO3 were heterologously expressed in *Sf9* insect cell membranes as described in sections 2.3-2.5. Insect cell microsomes, which represent membranes of the endoplasmic reticulum in suspension, were prepared as described in section 2.5.2.

Figure 3.1.1 shows photographs of polyacrylamide gels obtained from SDS-PAGE analysis of *Sf9* insect cell microsomes containing heterologously expressed

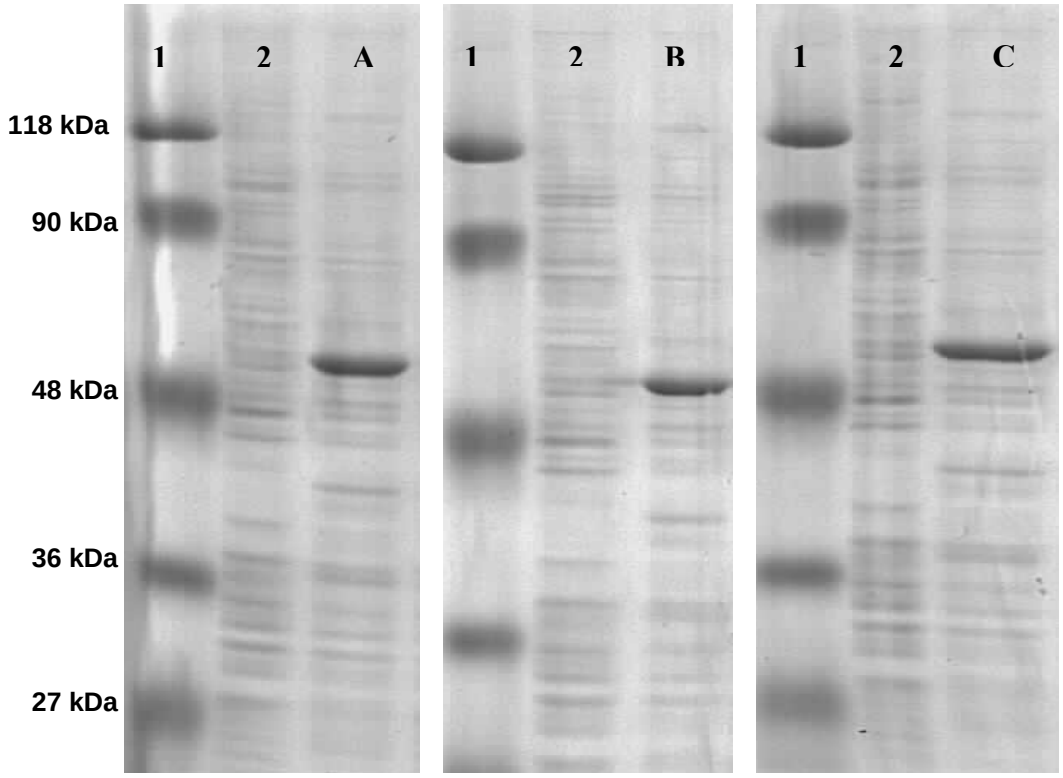


Figure 3.1.1: SDS-PAGE gel illustrating the expression of human FMO1, FMO2.1 and FMO3 expressed in *Sf*9 insect cell microsomes. Lane 1: Molecular weight marker, lane 2 microsomes prepared from non-infected *Sf*9 insect cells (negative control) and *Sf*9 insect cell microsomes containing heterologously expressed human FMO1 (A), FMO2.1 (B) and FMO3 (C).

human FMO1, FMO2.1 or FMO3. The amount of FMO expressed in microsomes was determined by generating a standard curve from authentic FMO standards as described in section 2.6.1. Figure 3.1.2 illustrates an example of a quantitative Western blot and a calibration curve generated to quantify human FMO1 in insect cell microsomes.

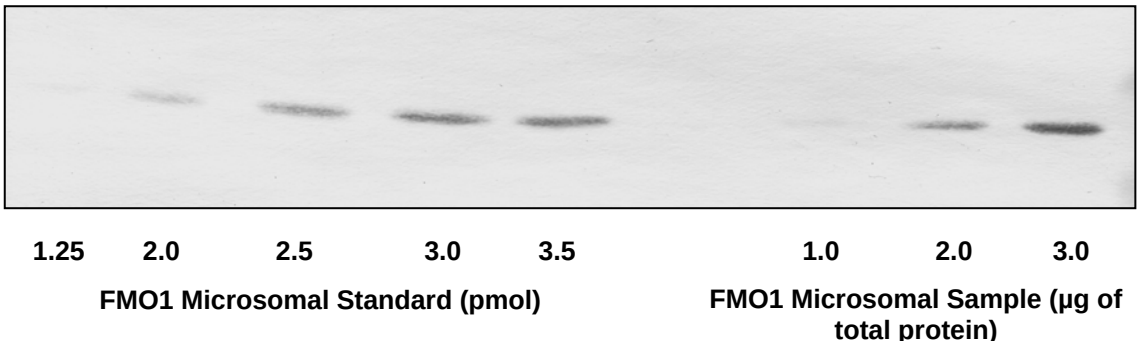
Catalytic Oxidation of TAZ by human FMO2.1

Reactions analysed by reverse phase HPLC and liquid chromatography-mass spectroscopy (LC-MS) were prepared as described in section 2.7.

Incubation of TAZ with *Sf9* insect cell microsomes containing heterologously expressed human FMO2.1, in the presence of NADPH, resulted in the formation of three major metabolites with reverse-phase retention times of 7.5 min (**M1)**, 11.4 min (**M2**) and 14.6 min (**M3**) (Fig. 3.1.3). No products were observed when TAZ was incubated with microsomes isolated from non-infected *Sf9* cells or when NADPH was omitted (results not shown). UV spectral analysis of the metabolites (Fig. 3.1.4) showed that **M1** had a maximal absorption peak at 325 nm and a smaller peak at approximately 230 nm. **M2** had a similar spectrum, with peaks at 320 and 220 nm. The absorption spectrum of **M3** exhibited a main peak at 295 nm and a secondary peak at 220 nm.

To identify the three metabolites, **M1**, **M2**, and **M3,** formed from TAZ by the action of human FMO2.1, were analysed by LC-MS. The mass spectrum of **M1** had a molecular ion $[M + H]^+$ at *m/z* 269.07, with fragment ions at *m/z* 205.14 and 163.12 (Fig. 3.1.5A). The mass of the molecular ion of **M1** is 32 atomic mass units more than that of the molecular ion of TAZ (237), suggesting a structure in which TAZ has incorporated two oxygen atoms (Fig. 3.1.5A) and hence supports identification of the

A



B

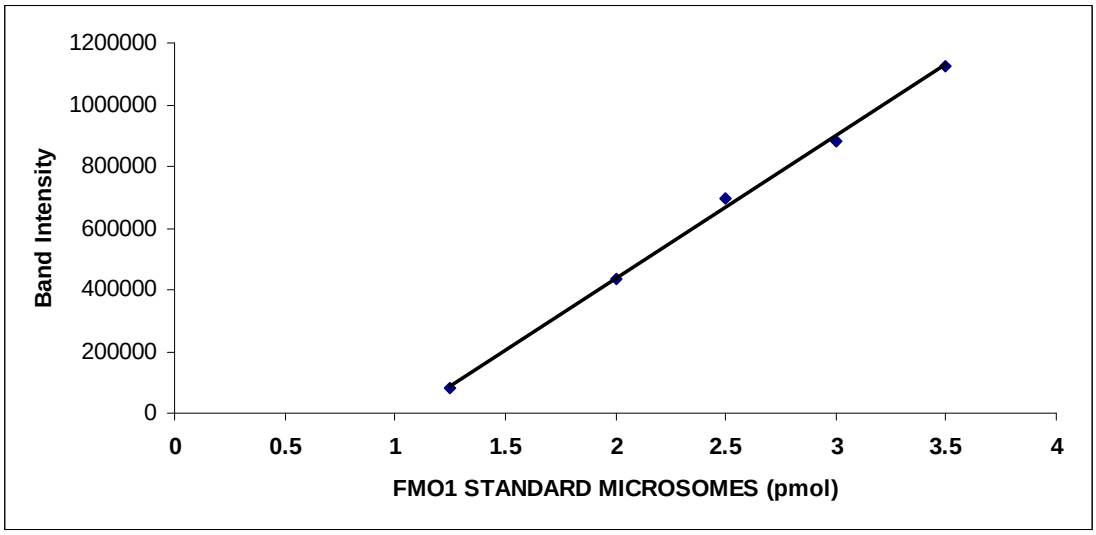


Figure 3.1.2: Quantification of FMO1 amounts in insect cell microsomes. The amount of total FMO1 expressed in microsomal samples was determined as described in section 2.6. (A) Quantitative Western blot illustrating bands of known amounts of FMO1 standard microsomes and FMO1 expressing insect cell microsome sample and (B) a calibration curve generated from the intensities of the standard bands (r^2 = 0.9981).

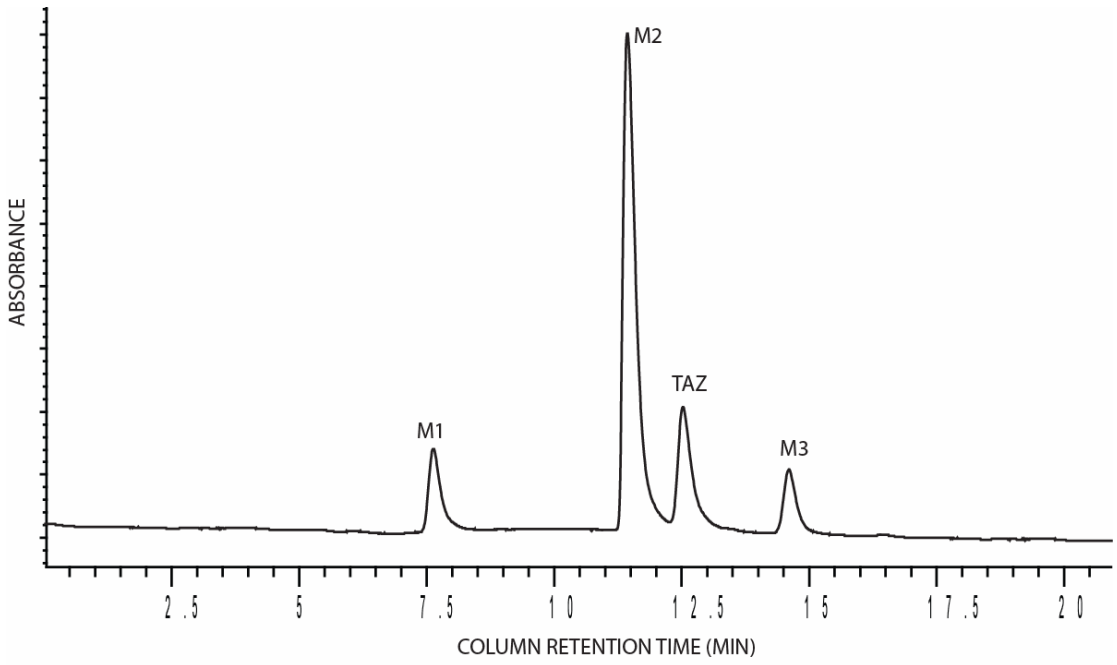


Figure 3.1.3. Metabolites of TAZ generated by human FMO2.1 catalysed oxidation. UV-HPLC chromatogram of M1 (7.6 min), M2 (11.4 min) and M3 (14.5 min) generated from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH. Reactions were carried out at pH9.5 for 90 min at 37 °C

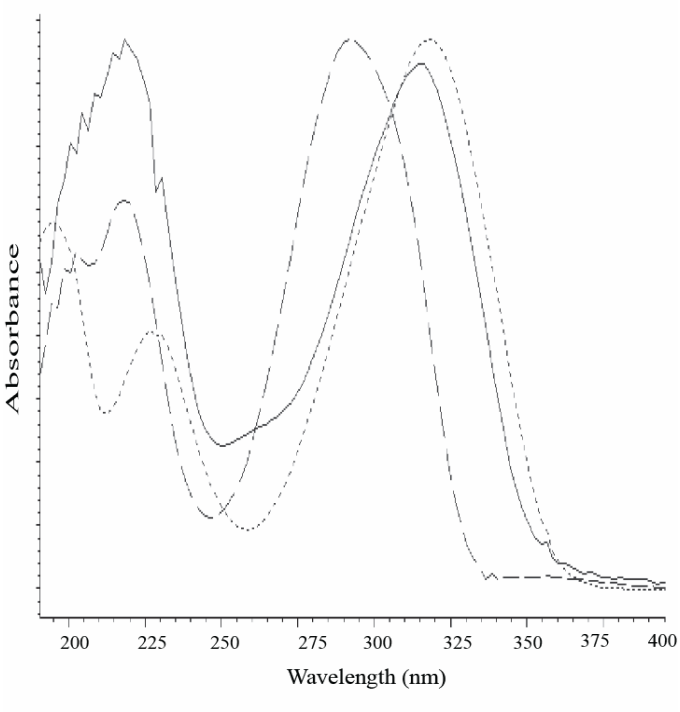


Figure 3.1.4: UV-Absorption spectra of TAZ metabolites generated by human FMO2.1 catalysed oxidation. UV-absorption spectra of the products produced by FMO2.1 metabolism of TAZ. Dotted line, **M1**; solid line **M2**; and dashed line, **M3**.

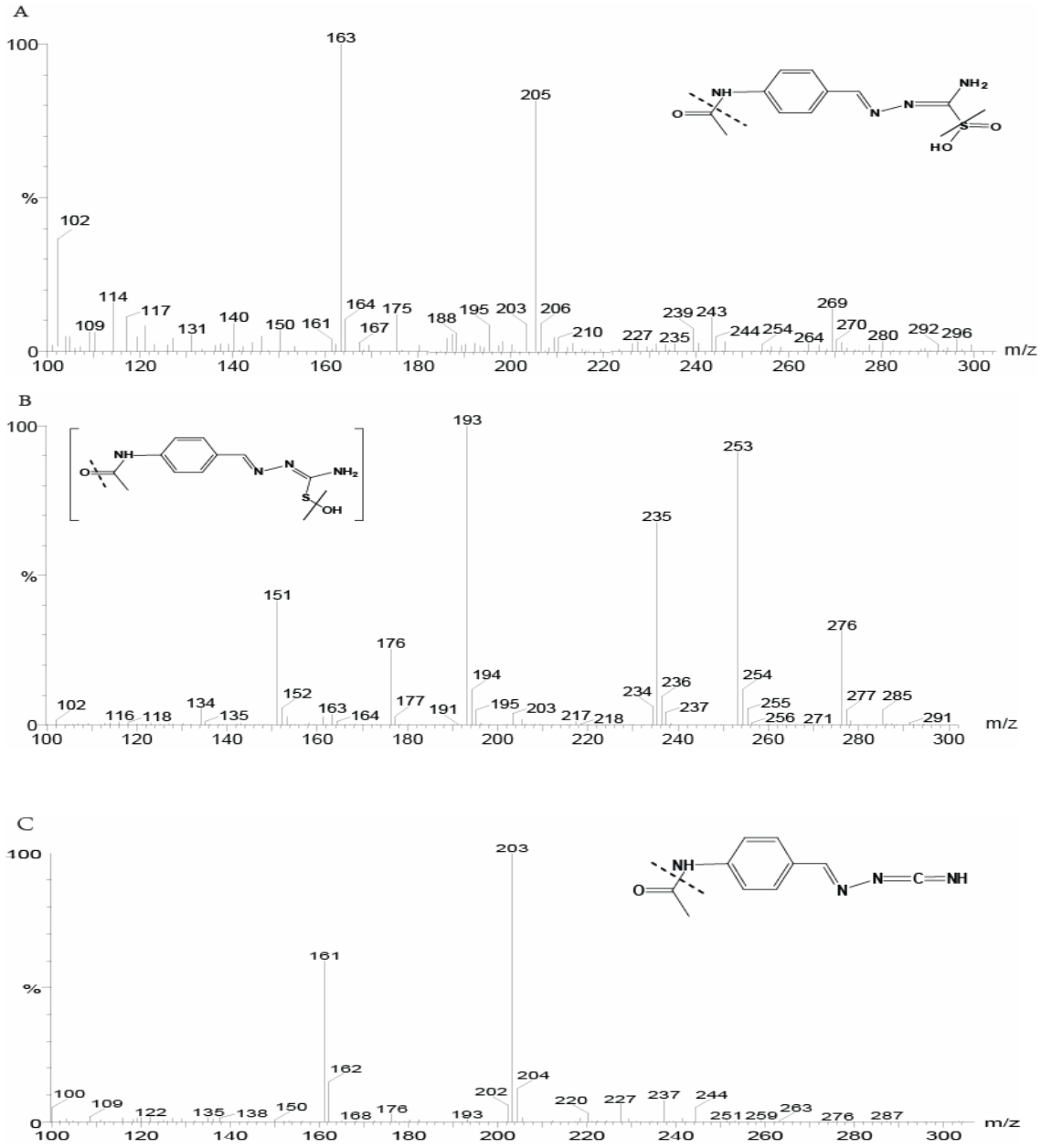


Figure 3.1.5. Mass spectra of TAZ metabolites generated from human FMO2.1 catalysed oxidation. Mass spectra and structures of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH at pH 9.5 for 90 min at 37 °C. (A) **M1,** identified as the sulphinic acid, has a molecular ion [M + H]+ *m*/*z* 269.07, with fragment ions at *m*/*z* 205.14 and 163.12. (B) **M2**, identified as the sulphenic acid, has a molecular ion [M + H] $^{+}$ at *m/z* 253, with fragment ion peaks at *m/z* 235 and 193 and (C) **M3**, identified as the carbodiimide, has a molecular ion [M + H]+) *m*/*z* 203.13 with a fragment ion at *m*/*z* 161.11.

metabolite as the sulphinic acid derivative. The mass spectrum of **M2** had a molecular ion $[M + H]^+$ at *m/z* 253, with fragment ions at *m/z* 235 and 193 (Fig. 3.1.5A). The mass of the molecular ion of **M2** is in accord with a structure in which TAZ has incorporated a single oxygen atom, and thus supports identification of **M2** as the monooxygenated, sulphenic acid derivative. The metabolite **M3** has a molecular ion $[M + H]^+$ at *m/z* 203.13, which suggests that it is the carbodiimide generated by elimination from **M2** of the oxidized sulfur atom (Fig. 3.1.5C). Previously synthesized authentic standards of TAZ-sulphinic acid and TAZ-carbodiimide (Qian *et al.* 2006) were used to confirm the identity of **M1** and **M3**. UV-HPLC chromatograms and UV spectra generated from authentic standards of TAZ-sulphinic acid and TAZ-carbodiimide are given in Figure 3.1.6. Comparison of their HPLC elution times (Fig. 3.1.6), UV spectra (Fig. 3.1.7) and MS spectra (Qian *et al.* 2006) with those of the synthetic standards unambiguously identified the metabolites **M1** and **M3** as the sulphinic acid and carbodiimide derivatives of TAZ, respectively.

The same three metabolites (the sulphinic acid, sulphenic acid and carbodiimide derivatives) were also produced when TAZ was incubated with purified EtaA or with *Sf9* cell microsomes containing heterologously expressed human FMO1 or FMO3 (Fig 3.1.8). Previous work identified **M1** and **M3** as products formed by the action of these enzymes on TAZ(Qian *et al.* 2006). Although the sulphenic acid derivative (**M2)** was not detected, it was postulated as an intermediate in the enzyme-catalyzed metabolism of TAZ (Qian *et al.* 2006). To confirm this, incubations of TAZ with heterologously expressed human FMO2.1 were quenched at different time points (Fig. 3.1.9). It is clear from the results that formation and accumulation of **M2** precedes that of **M1** and **M3**, indicating that **M2** is an intermediate in the formation of the latter two metabolites.

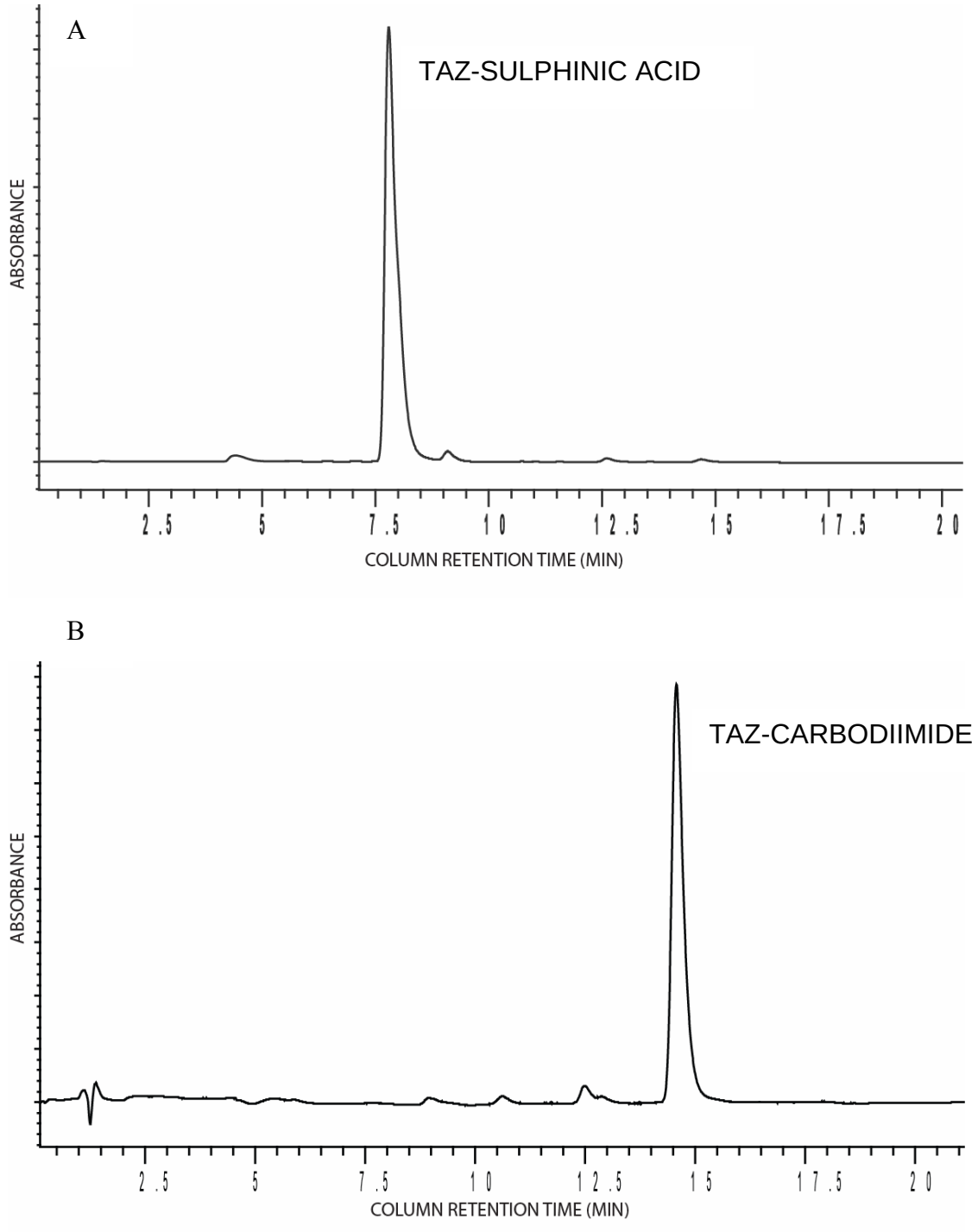


Figure 3.1.6. UV-HPLC Chromatograms of TAZ metabolite standards. Reverse phase chromatograms obtained for (A) TAZ-sulphinic acid (7.6 min) and (B) TAZ-carbodiimide standard (14.5 min).

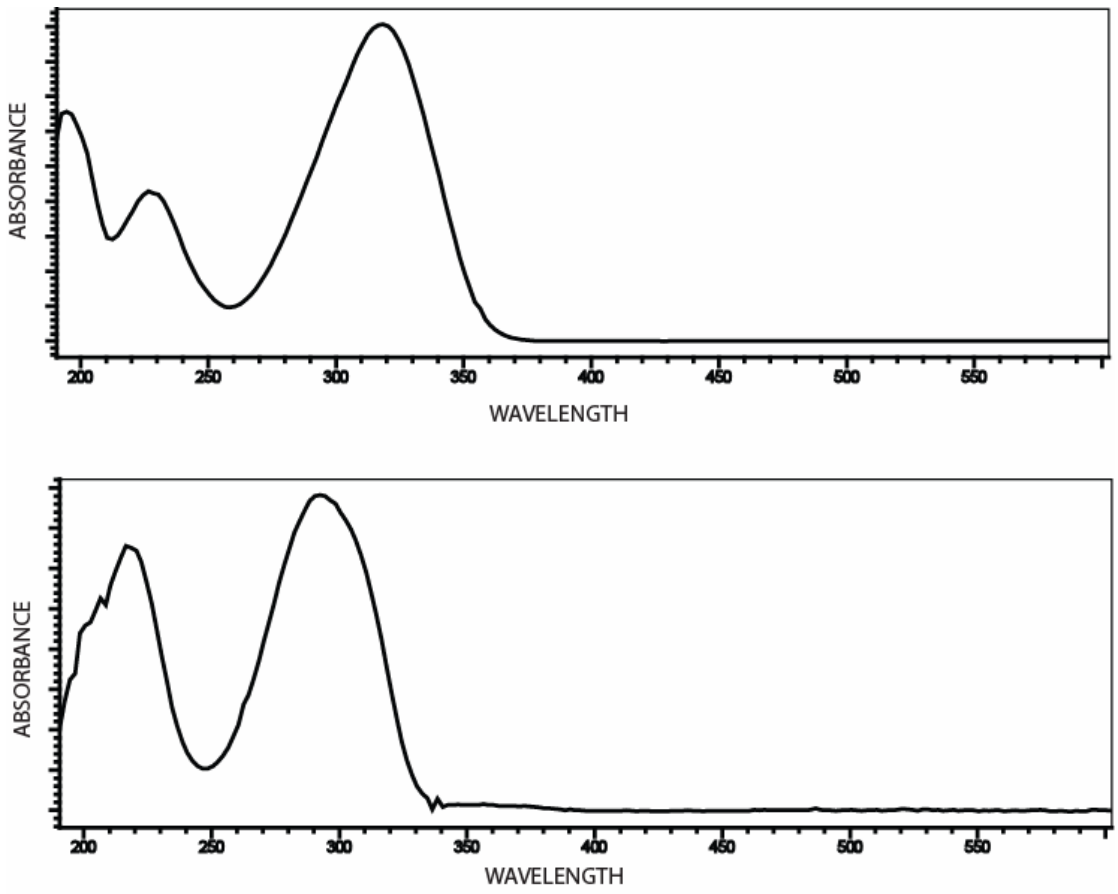


Figure 3.1.7: UV-spectra of TAZ metabolite standards. TAZ-sulphinic acid standard (top) gives a major peak at 325 nm and a smaller peak at approximately 220 nm. (Bottom) TAZ-carbodiimide standard gives a major peak at 295 and 220 nm.

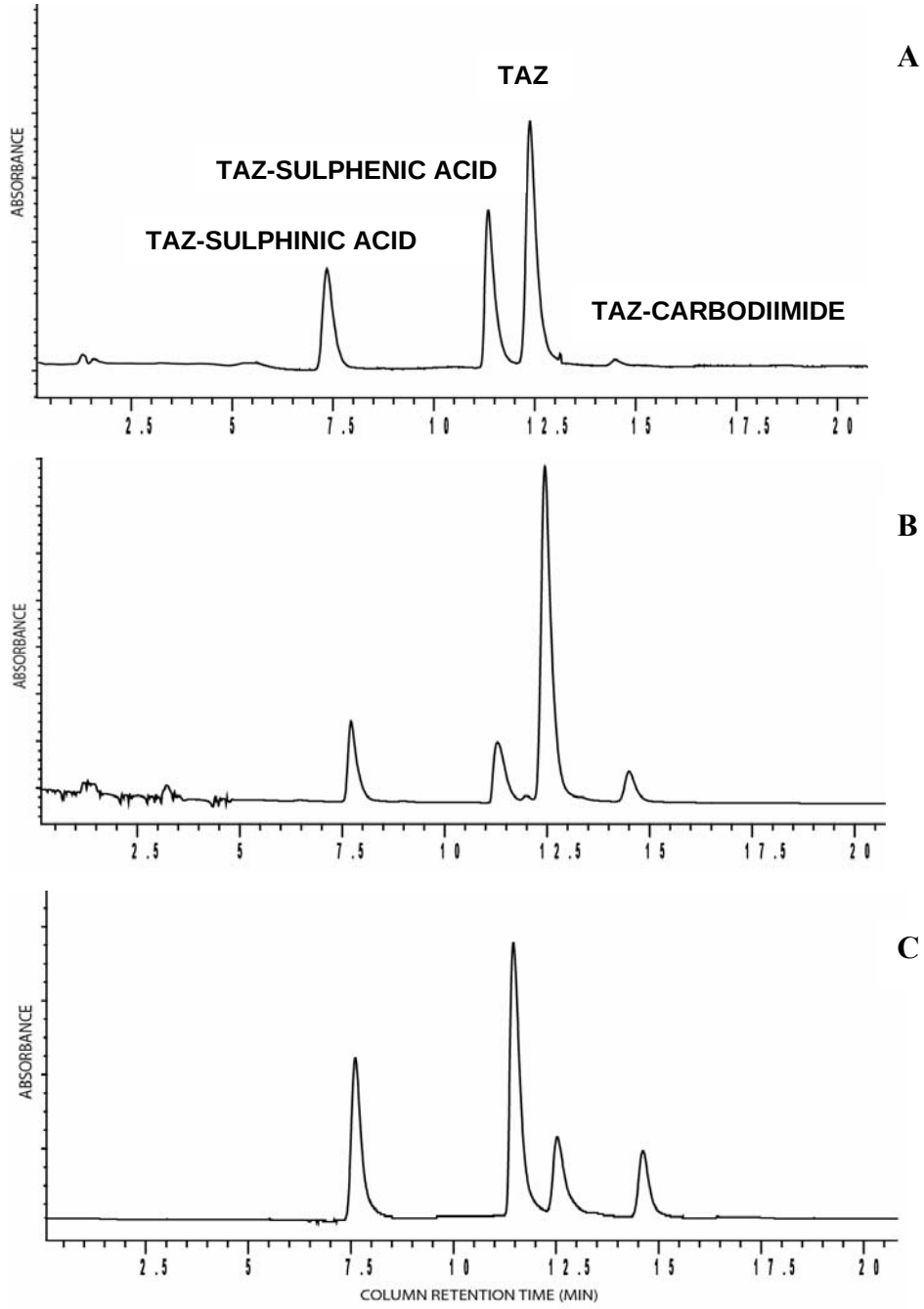


Figure 3.1.8: Metabolites of TAZ generated by human FMO1, FMO3 and EtaA catalysed oxidation. UV-HPLC chromatogram of TAZ-sulphinic acid (7.6 min), TAZ-sulphenic acid (11.4 min) and TAZ-carbodiimide (14.5 min) generated from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed (A) human FMO1 (B) human FMO3 and (C) EtaA and NADPH in pH 8.4 (for FMO1 and FMO3) or 7.5 (EtaA) for 90 min at 37 °C.

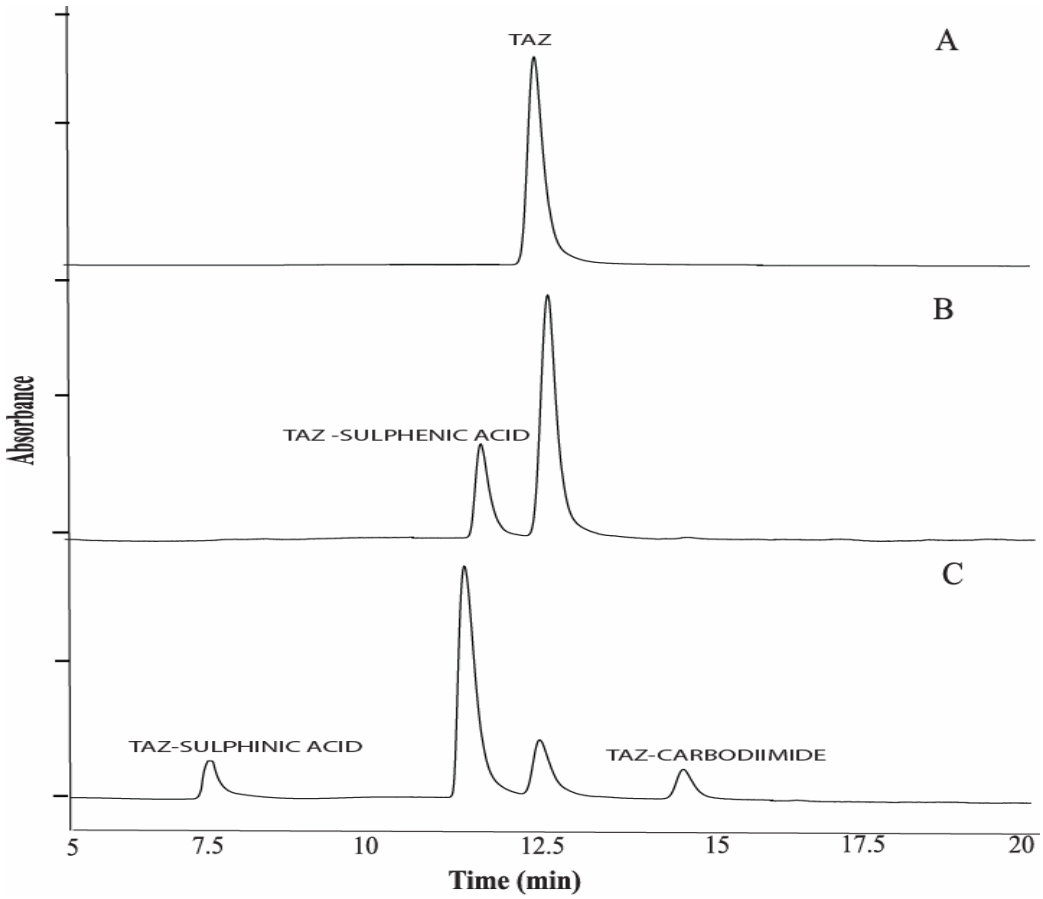


Figure 3.1.9: Time Course Experiment of TAZ oxidation by human FMO2.1. UV-HPLC chromatograms of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH at pH 9.5 and 37 °C for 0 min (A) 10 min (B) or 20 min (C).

Catalytic oxidation of ETA by human FMO2.1.

ETA and FMO2.1 reactions were analysed by liquid chromatography-mass spectroscopy (LC-MS) as described in section 2.7.

Incubation of ETA with *Sf9* microsomes containing heterologously expressed human FMO2.1, in the presence of NADPH, resulted in the formation of a major product, **P1**, with LC retention time of 6.2 min (Fig. 3.1.10A). The mass spectrum of **P1** had a molecular ion $[M + H]^+$ at *m/z* 183, with fragment ions at *m/z* 151 and 133 (Fig. 3.1.10B). The mass of the molecular ion of **P1** is 16 atomic mass units more than that of the molecular ion of ETA (166), suggesting a structure in which ETA has incorporated one oxygen atom (Fig. 3.1.10B). This supports identification of **P1** as the *S*-oxide of ETA. The mass spectrum of **P1** is identical to that of authentic ETA *S*-oxide (Vannelli *et al.* 2002), therefore unambiguously identifying this metabolite as the *S*-oxide of ETA. A mass spectrum of the broad peak with a LC retention time of approx. 3 min (Fig. 3.1.10A) could not be obtained.

Development of a spectrophotometric assay of TAZ oxidation.

Although, reverse phase HPLC was used to identify metabolites generated by FMOs, access to this method was not always available. For this reason a UV-spectroscopy based enzyme assay was developed to determine the kinetic parameters of TAZ and FMOs.

An assay was developed to monitor the rate of decrease in TAZ spectrophotometrically as a measure of its oxygenation by FMOs (described in section 2.8.2.1). First, the UV properties of TAZ were investigated. A UV absorption

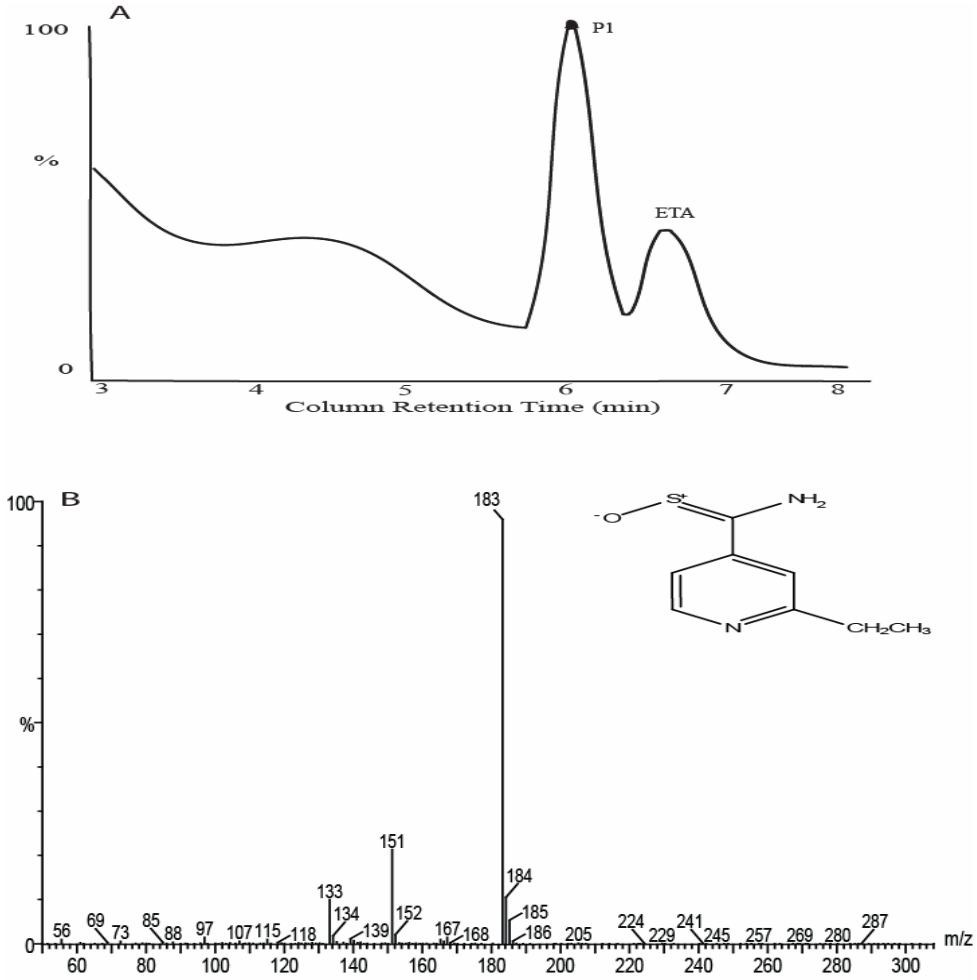


Figure 3.1.10. LC-MS analysis of ETA metabolites generated by human FMO2.1 catalysed oxidation. (A) LC chromatogram (350 nm) of the products from incubation of ETA, *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH in pH 7.5 for 60 min at 37 °C. (B) The mass spectrum and structure of the product **P1**. **P1** has a molecular ion $[M + H]^+$ at *m/z* 183, with fragment ion peaks at *m/z* 151 and 133.

spectrum of TAZ revealed an absorption maximum at 328 nm and a smaller peak at approximately 220 nm (Fig. 3.1.11). The absorbance of TAZ at 328 nm was linear between 1 and 20 μM (Fig. 3.1.12) and at this wavelength the molar extinction coefficient of TAZ in 0.1% DMSO was determined as 38,300 ± 2,320 $M^{-1}cm^{-1}$.

Incubation of TAZ, in the presence of NADPH, with *Sf*9 cell microsomes containing heterologously expressed human FMO1, FMO2.1 or FMO3, or with purified EtaA, resulted in a decrease in TAZ absorbance at 328 nm that was linear over time (Fig. 3.1.13A and B) and with respect to enzyme concentration (data not shown). No decrease in TAZ absorbance was observed in the absence of EtaA or human FMOs, or when microsomes prepared from non-infected *Sf*9 cells were used (Fig. 3.1.14). Omission of NADPH from reaction mixes containing heterologously expressed human FMOs resulted in a very small (<1% of that observed in the presence of NADPH) and short-lived (<2 min.) decrease in TAZ absorbance (Fig. 3.1.15). This is due to the presence of endogenous NADPH in the insect cell microsomes. This spectrophotometric assay was used to determine the pH optima and kinetic parameters of enzyme-catalyzed oxidation of TAZ.

Effect of pH on TAZ oxidation catalyzed by human FMOs and EtaA.

FMO1, FMO2.1 and FMO3 display different pH optima for enzymatic activity (Krueger *et al.* 2002; Qian *et al.* 2006; Siddens *et al.* 2008). The dependence of FMO activity on pH with respect to TAZ oxidation was measured spectrophotometrically as described in section 2.7.2.1.1.

The activity of EtaA and heterologously expressed human FMO1, FMO2.1 and FMO3 towards TAZ was assessed in buffers of pH 7.5, 8.5 or 9.5. All enzymes displayed activity towards TAZ throughout the tested range. Each FMO however,

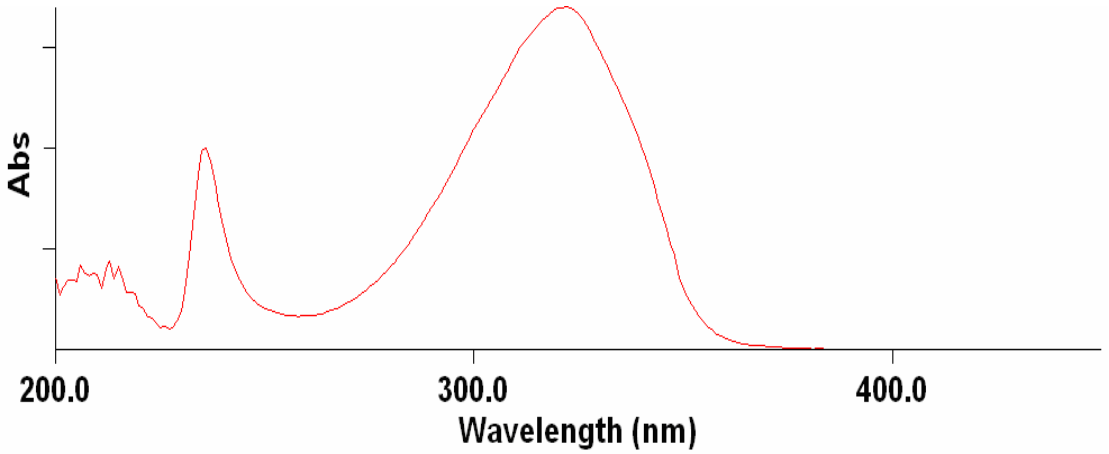


Figure 3.1.11: UV-spectra of TAZ. TAZ in DMSO gives a major peak at 328 nm and a smaller peak at approximately 220 nm as measured using a UV-spectrophotometer.

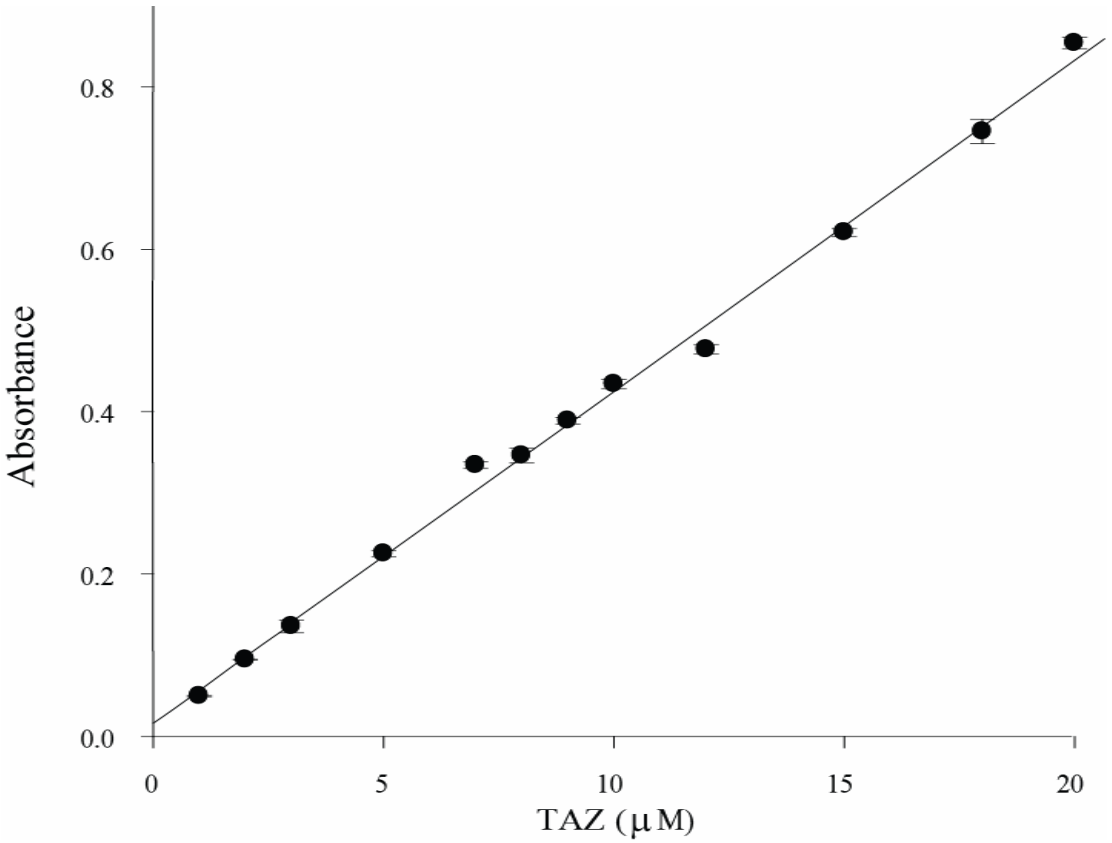


Figure 3.1.12: Linear absorbance of TAZ . The absorbance of TAZ at 328 nm was determined over a range of concentrations. The molar extinction coefficient for TAZ was calculated using the gradient of the straight line (r2 = 0.996).

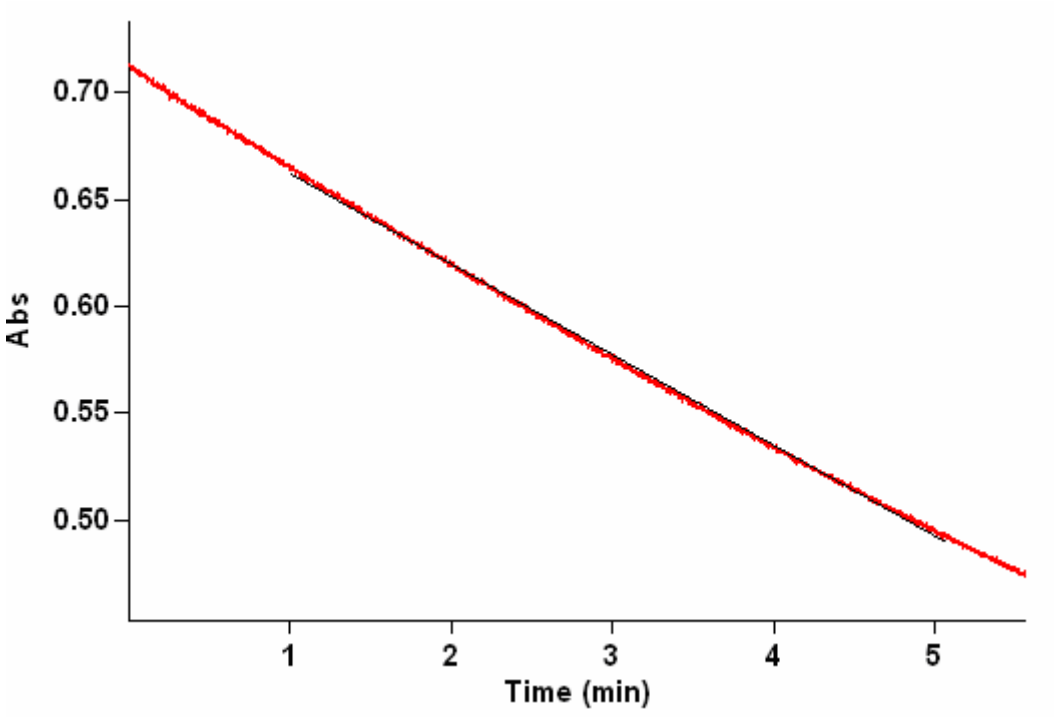


Figure 3.1.13A: Human FMO2.1- and NADPH-dependent decrease in TAZ absorbance at 328 nm is linear. TAZ (20 μM) was incubated with insect cell microsomes containing heterologously expressed human FMO2.1, NADPH at pH 9.5 and 37 °C. Rate of TAZ oxidation was determined from 1-5 minutes.

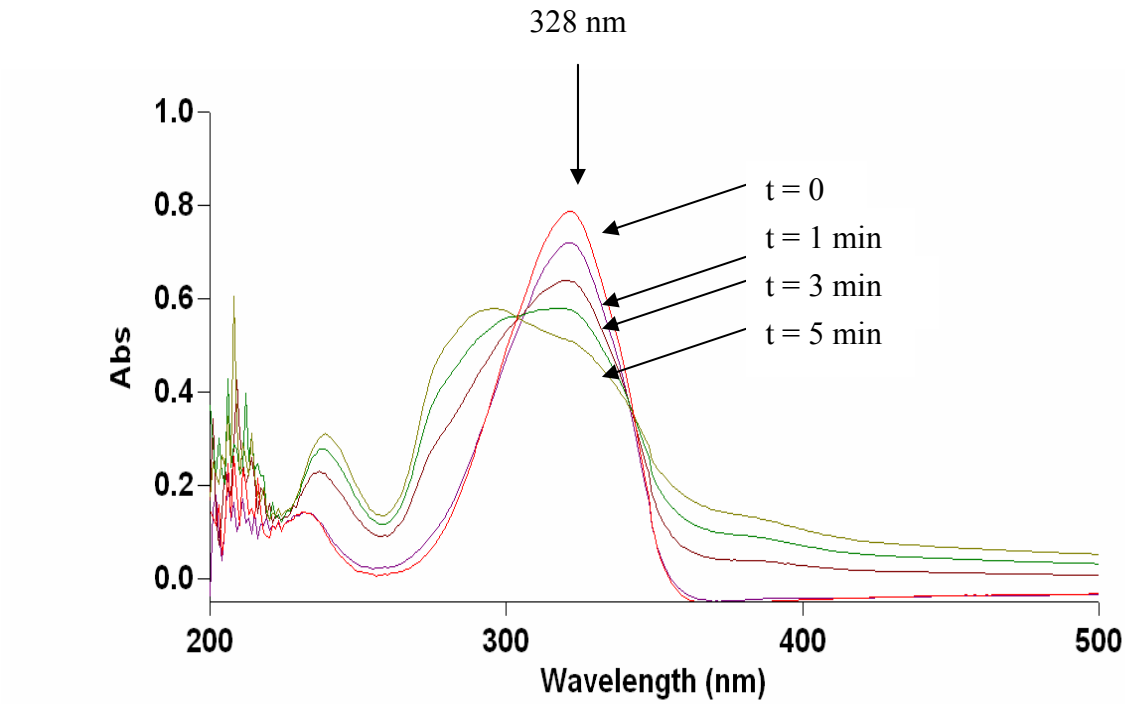


Figure 3.1.13B: Human FMO2.1- and NADPH-dependent decrease in TAZ absorbance at 328 nm over time.

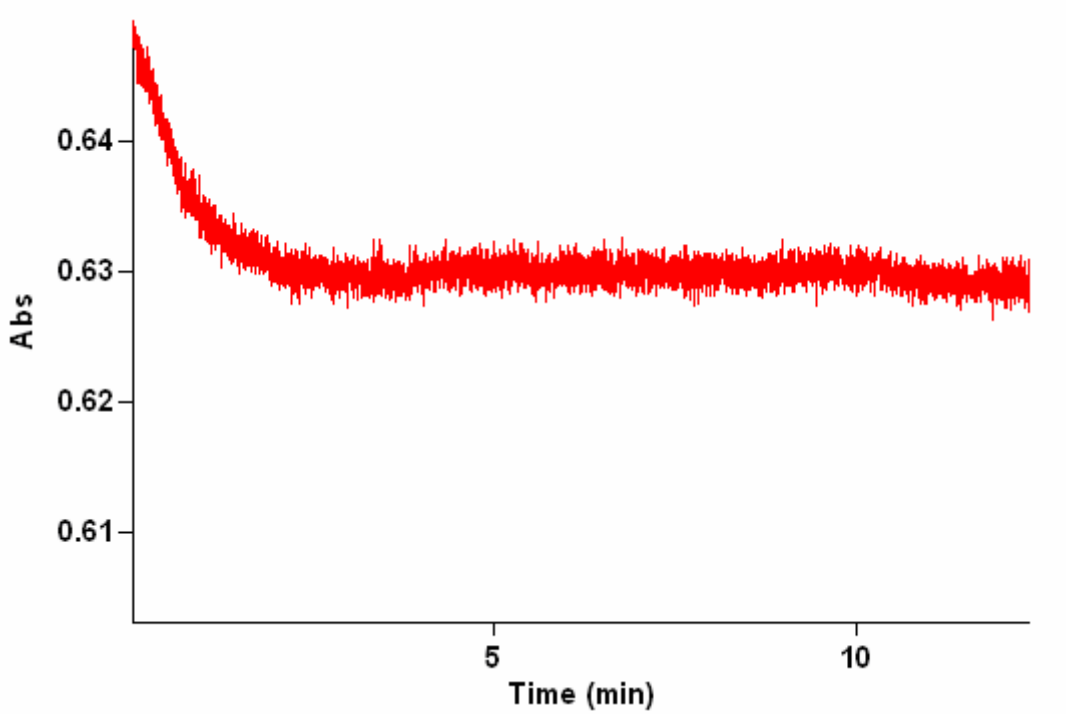


Figure 3.1.14: UV-spectra at 328 nm in the presence of non-infected insect cell microsomes and NADPH. No decrease in TAZ absorbance was observed in the presence of non-infected insect cell microsomes and NADPH at pH 9.5 at 37 °C. The change in absorbance during the first minute represents scattering.

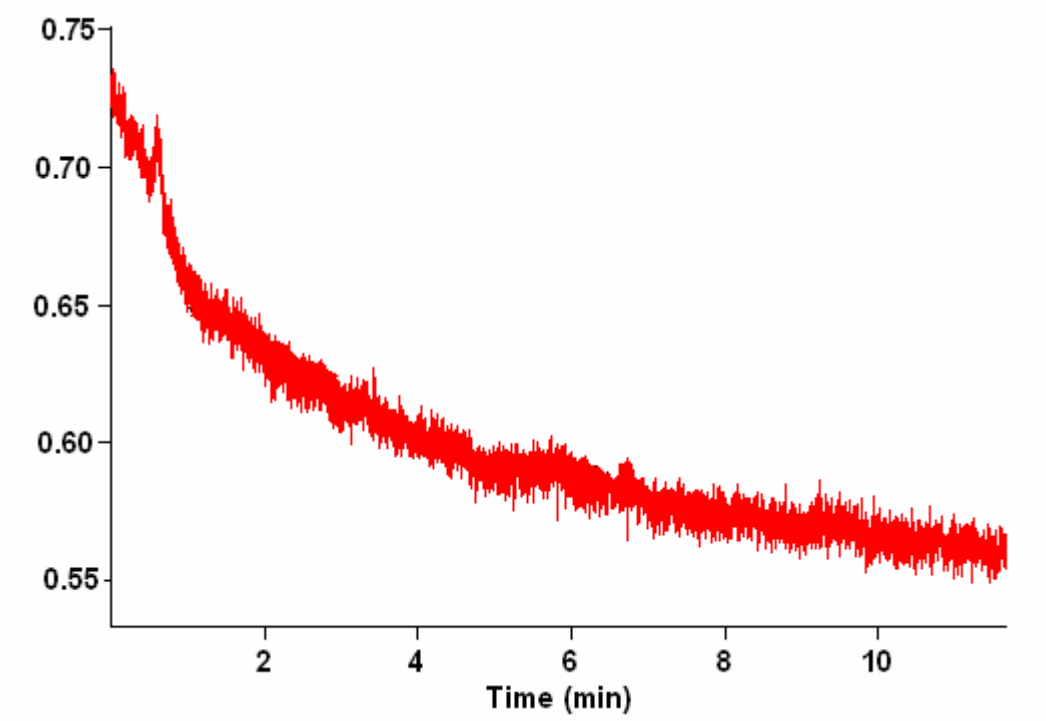


Figure 3.1.15: Human FMO2.1-dependent decrease in TAZ absorbance in the absence of NADPH at 328 nm. TAZ (20 μM) was incubated with insect cell microsomes containing heterologously expressed human FMO2.1 in the absence of NADPH at pH 9.5 and 37 °C.

had a particular pattern of pH-dependent activity. The pH optimum for the oxidation of TAZ was 8.5 for human FMO1 and FMO3, and 9.5 for human FMO2.1 and EtaA (Fig. 3.1.16).

Assessing FMO catalytic capacity using methimazole as a control substrate.

Human FMOs were first tested for catalytic activity towards methimazole to ensure that the *Sf9* insect cell microsomes were of good quality. Kinetic parameters were determined for FMO1-, FMO2.1- and FMO3-catalyzed oxygenation of methimazole, a probe substrate of the FMOs. The rate of methimazole *S*-oxygenation was monitored spectrophotometrically by monitoring the rate of decrease of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm. Steady state kinetic parameters were determined for human FMOs using the non-linear regression application of the Kinetics module (v.3.1) of SigmaPlot (v.10)

K_M values obtained for FMO1, FMO2.1 and FMO3 were in good agreement with previously published data (Dolphin *et al.* 1998; Krueger *et al.* 2002; Furnes *et al.* 2004) (Table 3.1.1). This confirms microsomes containing heterologously expressed FMO1, FMO2.1 or FMO3 were suitable for determining the kinetic parameters for TAZ.

Kinetics of TAZ oxidation by human FMOs and EtaA.

The kinetics of TAZ oxidation catalyzed by EtaA or by heterologously expressed human FMO1, FMO2.1 or FMO3 were evaluated by determining the initial rates of TAZ oxidation, measured spectrophotometrically, over a range of TAZ concentrations (Fig. 3.1.17 and data not shown) as described in section 2.8.2.2. Assays were performed at the optimum pH for each enzyme (see above). Steady state kinetic

parameters were determined for human FMOs and EtaA using the non linear regression application of the Kinetics module (v.3.1) of Sigmaplot (v.10).

Human FMOs and EtaA displayed hyperbolic Michaelis-Menten relationship between enzyme activity and TAZ concentration. Michaelis-Menten and Hanes-Woolf plots obtained for human FMO catalysed TAZ oxygenation is given in Figure 3.1.17.

TAZ was an excellent substrate for the human FMOs and EtaA (Table 3.1.2). The K_M values for TAZ oxidation catalyzed by human FMO1, FMO2.1, FMO3 and EtaA are in the low micromolar range and very similar to each other (Table 3.1.2). The *k*cat of the FMO2.1-catalyzed reaction is much higher than that of reactions catalyzed by human FMO1, FMO3 or EtaA (Table 3.1.2). Consequently, $k\text{cat}/K_M$ (the specificity constant) for TAZ is much higher for human FMO2.1 than for the other three enzymes (Table 3.1.2).

Kinetic analysis of the reaction between human FMO2.1 and TAZ was evaluated by reverse phase HPLC in order to validate the UV-spectroscopy assay. ETA was used as an internal standard. The kinetic analysis methodology using HPLC is detailed in section 2.8.4. A standard curve corresponding to the ratio of the integrated HPLC peak of TAZ and ETA plotted against TAZ concentration was used to determine the amount of unmetabolised TAZ in the reaction (Fig. 3.1.18). Table 3.1.3 summarises the kinetic parameters for human FMO2.1 and TAZ determined by the HPLC and UV methods. There was no statistical difference observed in K_M or *k*cat values determined from the use of an HPLC- or the UV-based assay, thus validating the UV spectrophotometric method ($p>0.05$).

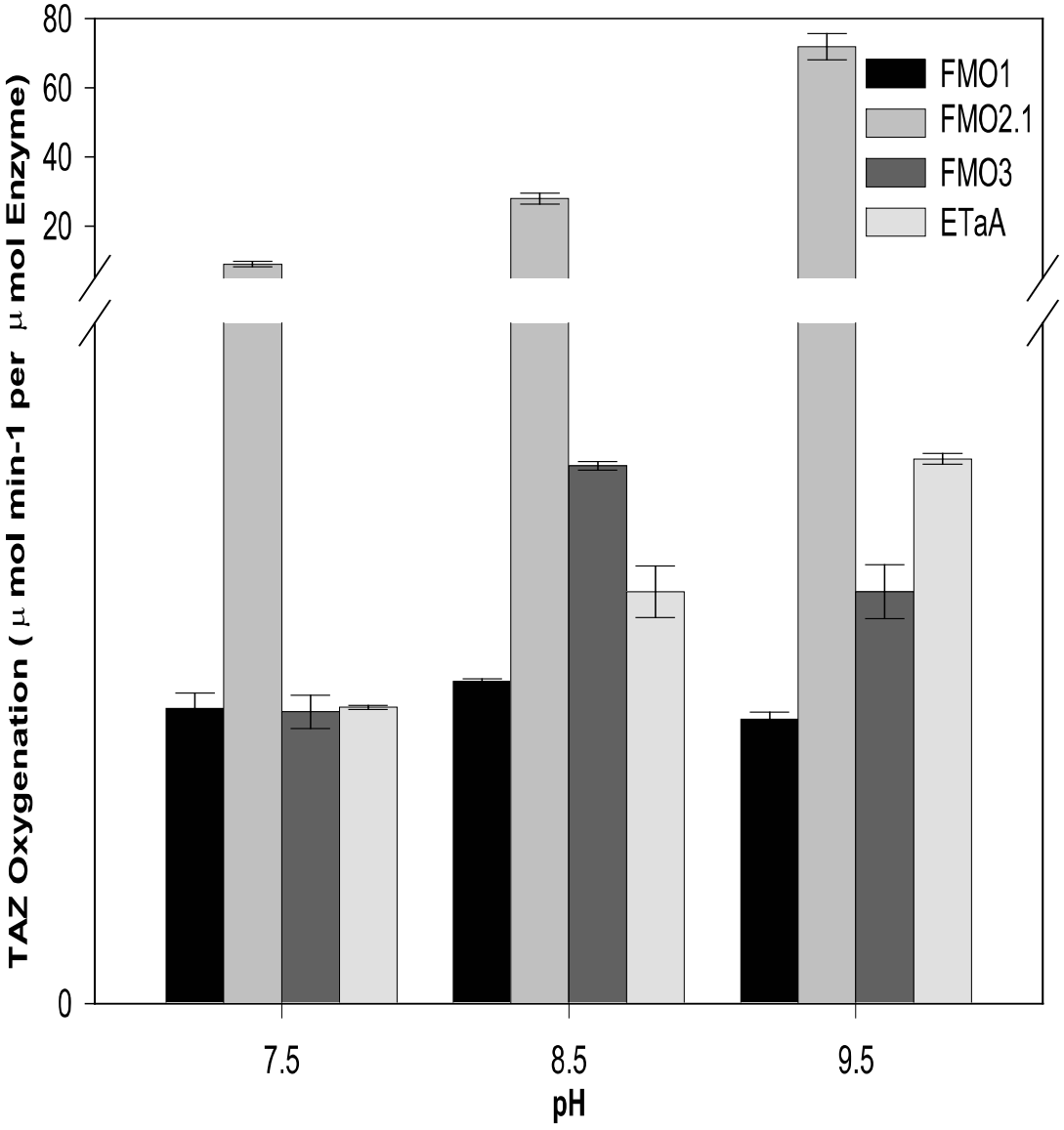


Figure 3.1.16. Effect of pH on the rate of TAZ oxygenation by purified EtaA or *Sf9* insect cell microsomes containing heterologously expressed human FMO1, FMO2.1 or FMO3. The decrease in TAZ over time was measured at 328 nm in the presence of NADPH in buffers at pH 7.5, 8.5 or 9.5.

Table 3.1.1: Kinetic Parameters of enzyme catalysed oxidation of Methimazole.

Enzyme	$\mathbf{K_M}$ **(μM)**	***k*cat** **(min^{-1})**	***k*cat/ $\mathbf{K_M}$** **($min^{-1}M^{-1}$) ($x10^4$)**
FMO1	8.08 ± 2.35	2.27 ± 0.30	28.1 ± 7.95
FMO2.1	575 ± 60.02	31.5 ± 2.11	5.48 ± 0.68
FMO3	29 ± 4.06	2.60 ± 0.38	8.97 ± 1.81

The *S*-oxygenation of methimazole by human FMOs was determined as described in section 2.8.1. K_M (μM), *k*cat (min^{-1}), *k*cat / K_M (min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from a single infection of *Sf9* cells.

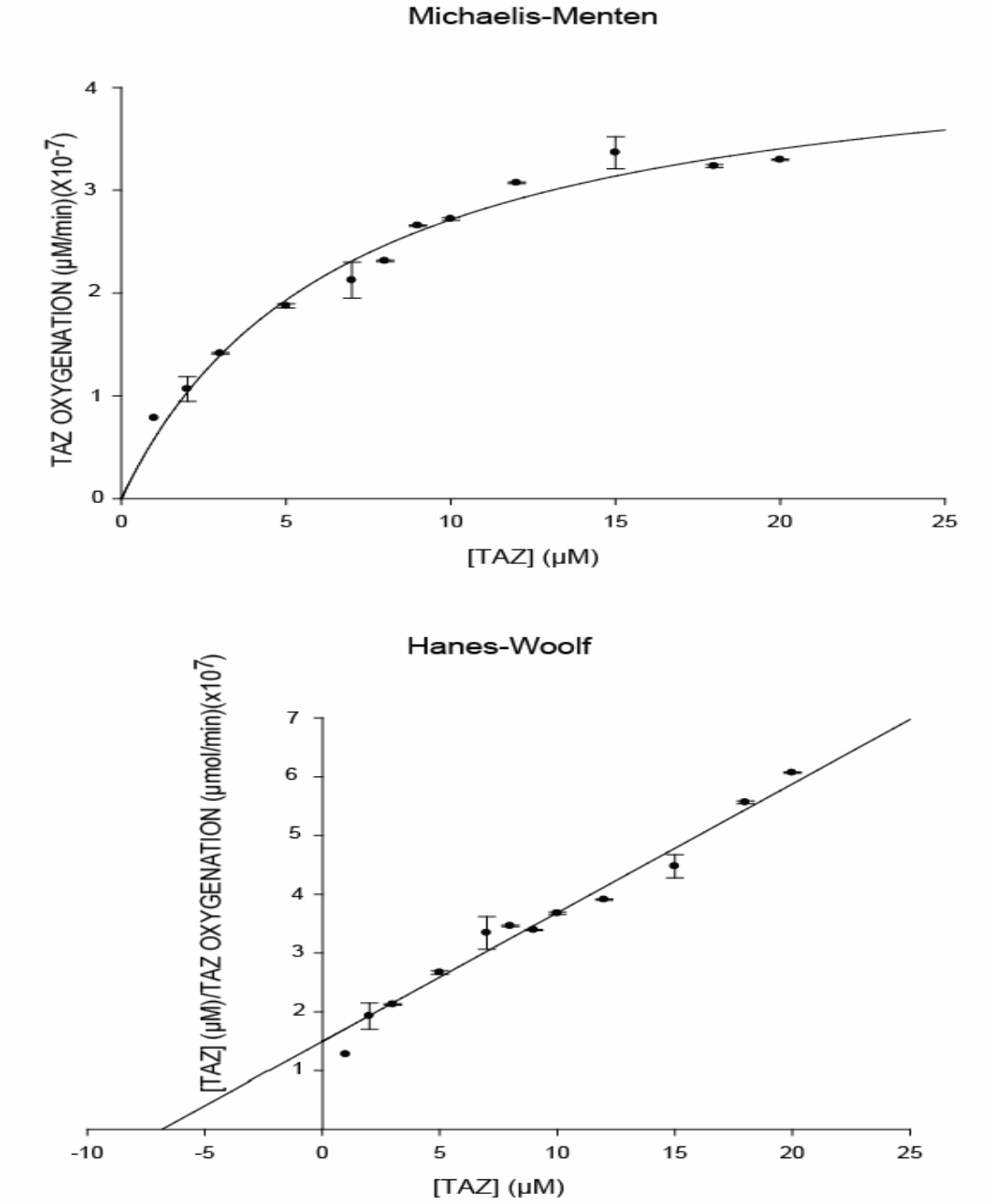


Figure 3.1.17A: Non-linear regression and linear transformation of the Michaelis-Menten equation for human FMO1 catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed human FMO1 in the presence of NADPH and a buffer at pH 8.5. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

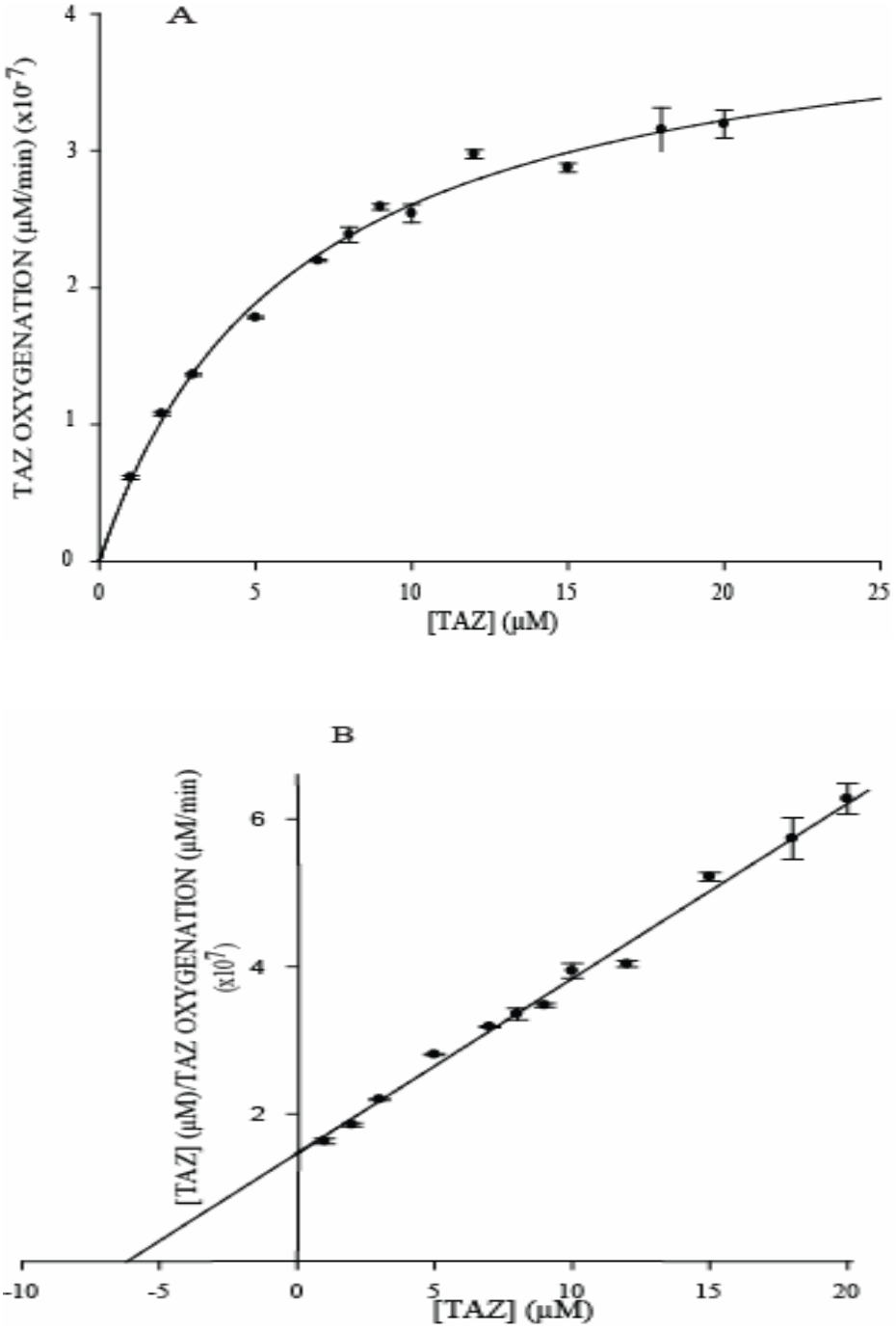


Figure 3.1.17B: Non-linear regression and linear transformation of the Michaelis-Menten equation for human FMO2.1 catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 in the presence of NADPH and a buffer at pH 9.5. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

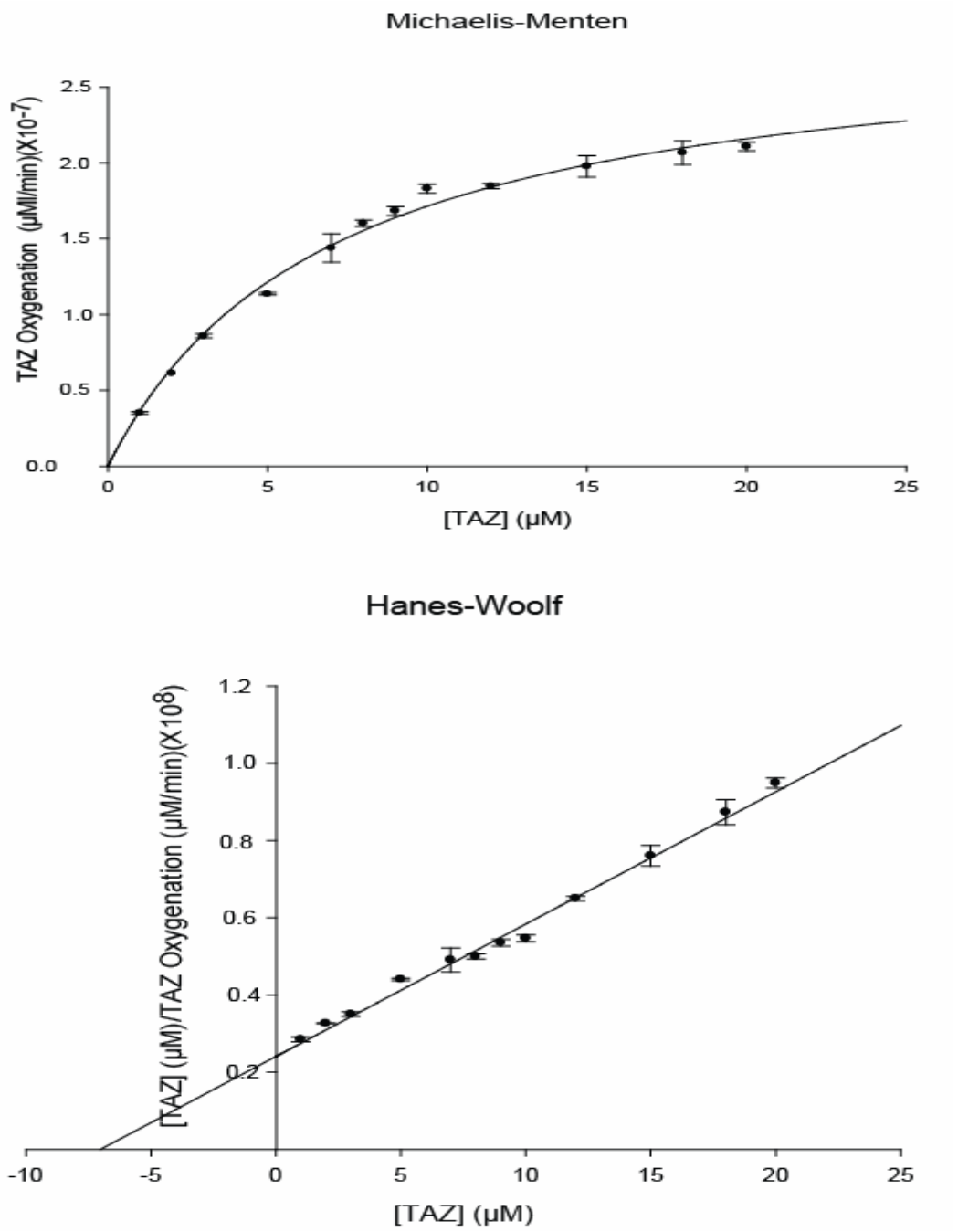


Figure 3.1.17C: Non-linear regression and linear transformation of the Michaelis-Menten equation for human FMO3 catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed human FMO3 in the presence of NADPH and a buffer at pH 8.5. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

Table 3.1.2: Kinetic Parameters of enzyme catalysed oxidation of Thiacetazone.

Enzyme	**K_M** **(µM)**	***k*cat** **(min^{-1})**	***k*cat/ K_M** **($min^{-1}M^{-1}$) ($x10^5$)**
FMO1	6.30 ± 0.80	5.08 ± 0.46	7.9 ± 1.99
FMO2	5.80 ± 0.55	80.10 ± 4.42	142 ± 18.30
FMO3	7.01 ± 0.53	1.37 ± 0.20	1.96 ± 0.39
EtaA	9.05 ± 0.57	3.02 ± 0.30	3.2 ± 0.81

The *S*-oxygenation of TAZ by human FMOs and EtaA is described in section 2.7.2.2. K_M (µM), *k*cat *(*min^{-1}), *k*cat / K_M *(*min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from three independent infections of *Sf9* cells or from a single batch of purified EtaA.

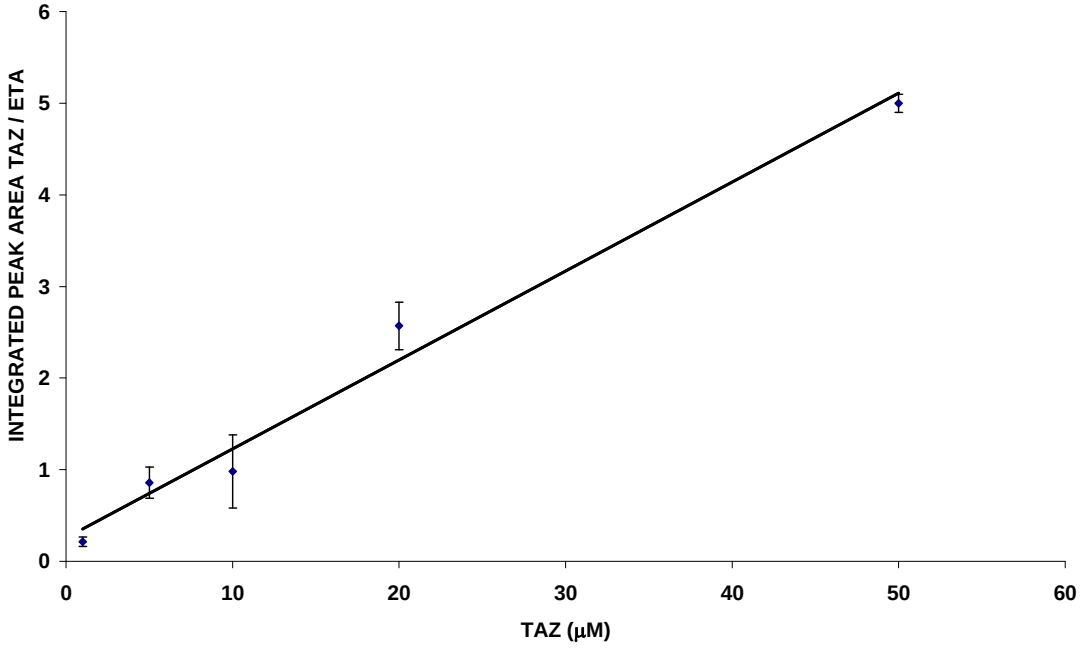


Figure 3.1.18: Standard curve representing the ratio of integrated peak area of TAZ and ETA plotted against TAZ concentration. The standard curve was used to determine the amount of unmetabolised TAZ after incubation with human FMO2.1 and NADPH.

Table 3.1.3: Comparison of kinetic parameters of human FMO2.1 catalysed TAZ oxidation determined by HPLC and UV-spectroscopy.

Method	K_M (μM)	*k*cat (min^{-1})
UV-Spectroscopic Assay	5.80 ± 0.55	80.10 ± 4.42
HPLC Assay	5.90 ± 0.52	71.47 ± 6.80

The *S*-oxygenation of TAZ by human FMO2.1 is described in section 2.8.3 and 2.8.4. Kinetic parameters determined by a UV-spectroscopy based assay and by reverse phase HPLC (K_M (μM) and *k*cat (min^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from a single infection of *Sf9* cells.

In summary, this section has established TAZ to be an excellent substrate for human FMO2.1 and identifies the products of this reaction to be TAZ-sulphinic acid, TAZ-carbodiimide formed from a common intermediate precursor, a sulphenic acid of TAZ. Kinetic studies confirm this enzyme to be catalytically more efficient at this reaction than is the bacterial FMO, EtaA or human FMO1 or 3.

Discussion-Analysis of the reaction between human FMO2.1 and the Anti-tubercular drugs Thiacetazone and Ethionamide.

Thiacetazone (TAZ) and Ethionamide (ETA) are second-line antibiotics prescribed in the treatment of pulmonary tuberculosis. TAZ and ETA are pro-drugs which require metabolic activation by the *mycobacterial* enzyme EtaA, to exert a cytotoxic effect.

The aim of this section was to establish whether anti-tubercular drugs, thiacetazone (TAZ) and ethionamide (ETA) are substrates for human FMO2.1 and whether this enzyme catalysed the same reaction as the *Mycobacterial* FMO, EtaA.

Experiments carried out in this investigation have shown human FMO2.1 to be capable of catalysing the *S*-oxygenation of TAZ. The metabolites generated, the sulphinic acid, sulphenic acid and carbodiimide of TAZ are the same as those identified for EtaA, FMO1 and FMO3 in this investigation. Although, a previous study had identified the sulphinic acid and carbodiimide as the products of EtaA, FMO1 and FMO3 catalysed oxidation, a sulphenic acid intermediate was postulated to be the precursor of these metabolites (Qian *et al.* 2006). Experiments carried out as part of this investigation have for the first time, identified the intermediate as the sulphenic acid of TAZ.

Human FMO2.1 catalysed the oxidation of ETA to generate the corresponding *S*-oxide. Though the *S*-oxide has been identified as the product of EtaA oxidation (DeBarber *et al.* 2000; Vannelli *et al.* 2002; Hanoulle *et al.* 2006), these studies also identify the corresponding amide and nitrile of ETA. According to our results, human FMO2.1 does not generate these products and this finding is supported by others (Henderson *et al.* 2008). Given that *S*-oxides are generally pharmacologically less toxic than the parent compound, it can be presumed that individuals prescribed ETA and who express FMO2.1 in the lung will not be at a higher risk of drug toxicity.

Conversely, given that FMO2.1 can oxidise ETA, the inactivation of the drug before it reaches *Mycobacteria* may result in reduced drug efficacy. This suggests that individuals expressing this enzyme are at a genetic disadvantage with regard to ETA metabolism.

Analyses of kinetic parameters indicate FMO2.1 to be more efficient at TAZ oxygenation than EtaA, human FMO1 and FMO3 as a measure of the specificity constant ($k$cat/K_M). K_M determined for the enzymes was similar indicative of them having similar affinities for this substrate. The high value of the specificity constant of FMO2.1 was therefore a result of a higher $k$cat rather than a lower K_M. Although kinetic parameters were determined at optimum pH for each enzyme, human FMO2.1 was still the most effective at TAZ oxygenation at a more physiological pH, pH 7.5 (Fig 3.1.16) .

The catalytic cycle of FMOs described in section 1.2.2 illustrates how the rate-limiting step in an FMO-catalysed reaction is not the binding of a substrate. In a mechanism unique to FMOs, the enzyme is 'ready' to oxygenate a substrate before it (the substrate) has bound. Tight binding between enzyme and substrate is therefore not required but a single point of contact with the active site (the 4a-peroxyflavin intermediate) is sufficient for the substrate to be oxidised. The rate-limiting step is therefore the release of one molecule of water and the subsequent recycling of FAD to peroxyflavin *after* the oxygenation of the substrate. This implies that a given FMO would have similar $k$cat values for all of its oxidisable substrates. Comparison of $k$cat values obtained for TAZ and methimazole for FMO1, FMO2.1 or FMO3 respectively are in the same order of magnitude and conform to this expectation (Table 3.1.1 and 3.1.2). Comparison of $k$cat values determined for human FMO2.1 towards thiourea and ethylene-thiourea (Henderson *et al.* 2004) are similar to that obtained for TAZ in

this investigation. Similarly the relatively low *k*cat values determined for human FMO1 and FMO3 are comparable to those obtained for the oxygenation of a panel of thioureas by these enzymes (Onderwater *et al.* 2006). The high *k*cat value obtained for human FMO2.1 in this section implies that the enzyme is more efficient at catalysing the rate-limiting step than is FMO1 or FMO3. The high K_M and high *k*cat determined for the reaction between methimazole and human FMO2.1 (Table 3.1.1) is comparable to that observed for purified and heterologously expressed rabbit FMO2 and this substrate (Lawton *et al.* 1991).

FMOs generally catalyse the detoxification of xenobiotics. The prototypic product of FMO reactions is the non-toxic and non-reactive *N*- and *S*-oxide. But thiourea compounds undergo FMO-dependent bioactivation in which the metabolites generated are more toxic than the parent compound (Smith *et al.* 2002; Henderson *et al.* 2004; Onderwater *et al.* 2004; Onderwater *et al.* 2006) . Sulphenic acids of thioureas (generated by FMO oxygenation) are electrophiles that can react reversibly with glutathione (GSH) an important anti-oxidant in a mammalian cell (Ziegler-Skylakakis *et al.* 1998; Henderson *et al.* 2004). The sulphenic acid of TAZ can react with GSH to give rise to TAZ and to oxidised GSH (GSSG) (Qian *et al.* 2006). In the presence of GSH reductase, a concurrent cycle may establish with the net effect being the depletion of GSH in a cell, and regeneration of the parent compound subsequently leading to oxidative stress and cell toxicity (Fig. 3.1.19) (Krieter *et al.* 1984; Henderson *et al.* 2004; Onderwater *et al.* 2004). Sulphenic acid metabolites can react covalently with other thiol-containing molecules like cysteine residues in proteins (Decker *et al.* 1992). This can perturb the function of proteins and enzymes directly and is likely to be detrimental for cellular functions. TAZ-carbodiimide can

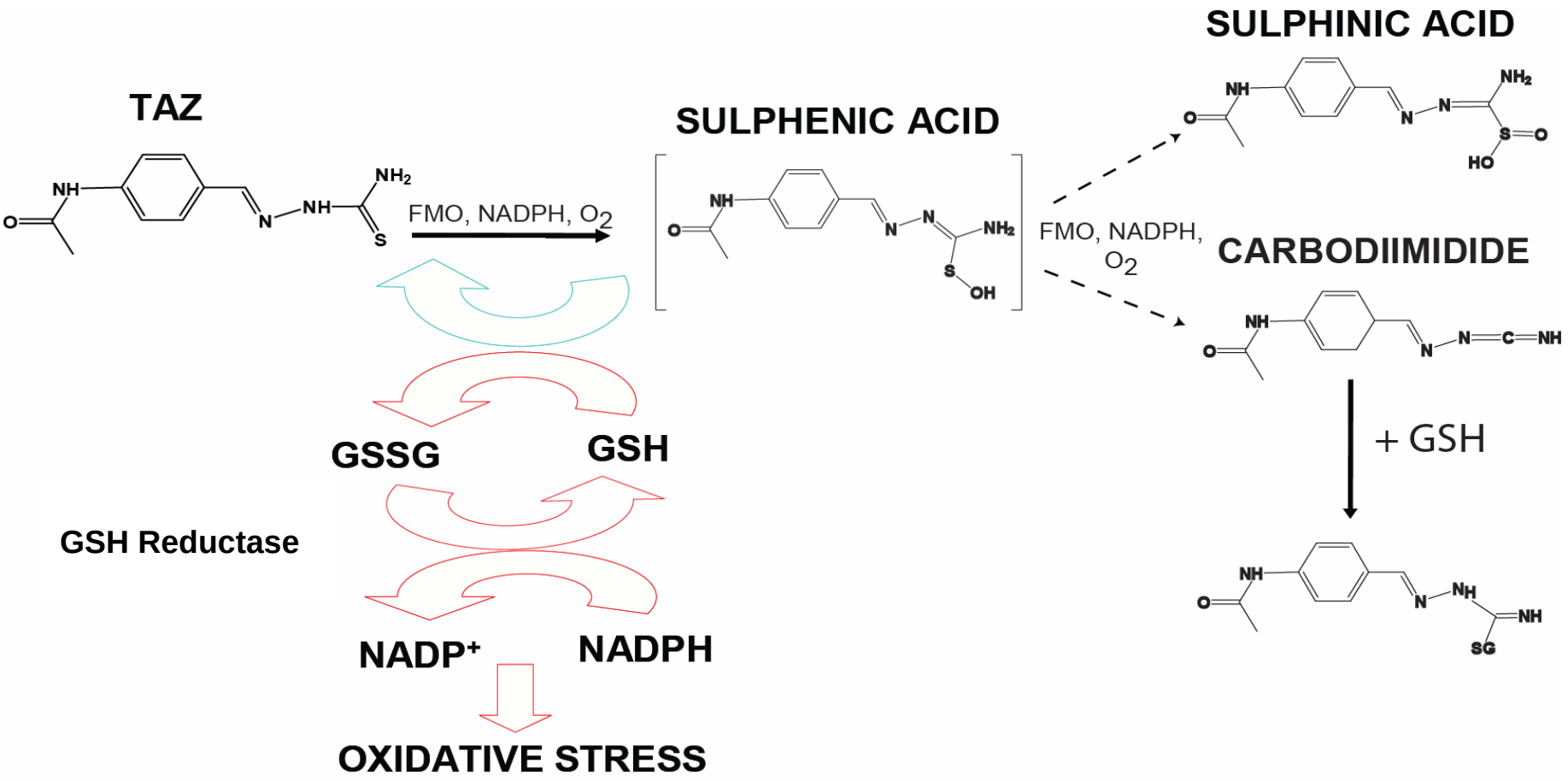


Figure 3.1.19: Diagram depicting TAZ metabolites generated by the oxidation of FMO and the interaction with glutathione (GSH). Adapted and revised from Qian *et al.* 2006.

form covalent bonds with GSH thus reducing the amount of an anti-oxidant in a cell (Qian *et al.* 2006). Human FMO1, FMO2.1 and FMO3 are therefore able to catalyse the oxygenation of TAZ to give rise to metabolites which are known to be toxic to a mammalian cell.

Given that human FMO1, FMO2.1 and FMO3 catalyse the bioactivation of TAZ may explain the basis of the adverse drug reactions observed in patients prescribed with this drug (Harland 1962; Aquinas 1968; Axton 1971; Hussain *et al.* 1973; Bedi *et al.* 1974; Anonymous 1981; Gupta *et al.* 1983; Fegan *et al.* 1991; Brown 1992; Ipuge *et al.* 1995; Lawn *et al.* 1999; Dieng *et al.* 2001). The role of FMOs in the *in vivo* bioactivation of TAZ is supported by the fact that TAZ-related adverse reactions are observed in tissues in which FMOs are expressed: hepatotoxicity, FMO3 in the liver (Dolphin *et al.* 1996; Hernandez *et al.* 2004); gastrointestinal problems, FMO1 in the small intestine (Yeung *et al.* 2000); and skin rashes, FMO1 and FMO3 in skin (Janmohamed *et al.* 2001). TAZ is not prescribed to patients who suffer from human immunodeficiency virus (HIV) as adverse clinical effects are exasperated. The underlying mechanism of this cytotoxicity is not known but the severity of TAZ-induced toxicity correlates to the level of immunosupression (Nunn *et al.* 1992). It is noteworthy that immunocompromised individuals display impairment in glutathione homeostasis and deficiency of glutathione is established in patients with HIV (Eck *et al.* 1989; Staal *et al.* 1992a; Staal *et al.* 1992b; Staal *et al.* 1992c). Given that glutathione mediates a detoxification process by reducing sulphenic acids of thiourea-containing compounds, it is speculated that the pronounced adverse affects in HIV patients may be due to the accelerated cellular toxicity induced by the accumulation of TAZ-sulphenic acid in the absence of glutathione.

In humans, FMO1 and FMO3 are expressed in the kidney and liver respectively and may contribute to TAZ activation in extra-pulmonary tissues. Inter-individual and inter-racial differences are reported for expression in FMOs. The expression of FMO1 is significantly higher in the kidney of African-Americans than in Caucasians (Krause *et al.* 2003). Significant variations in the amount of FMO1 expressed in the kidney is reported within individuals and is suggested to be a result of upstream promoter variants identified in the *FMO1* gene (Hines *et al.* 2003). Genetic polymorphisms in the human *FMO3* gene result in protein variants with altered catalytic activity (reviewed by Phillips *et al.* 2007 and Phillips *et al.* 2008) one of which, L360P has increased activity (Lattard *et al.* 2003). Amino acid variants of human FMO3 are implicated in the altered metabolism and therapeutic outcome of certain drugs (Stormer 2000; Hisamuddin *et al.* 2004; Mayatepek *et al.* 2004; Hisamuddin *et al.* 2005). Genetic polymorphisms in upstream promoter regions that affect transcription of FMO3 have been identified (Koukouritaki *et al.* 2005; Koukouritaki *et al.* 2007). This suggests that some people may metabolise TAZ more in the liver and kidney than in others. The result of this inter-individual difference may affect the amount of unmetabolised TAZ reaching the lung and thus *Mycobacteria*.

In humans, the expression of FMO2 is ethnic-dependent (Dolphin *et al.* 1998; Whetstine *et al.* 2000). All Europeans and Asians genotyped to date possess the *FMO2**2 allele in which a C>T substitution at position 1414bp replaces a glutamine amino acid residue with a premature stop codon (Dolphin *et al.* 1998). The resulting protein, FMO2.2, is catalytically inactive and is not detected in human lung (Dolphin *et al.* 1998). In sub-Saharan Africa, up to 50% of individuals possess at least one copy of the ancestral allele (*FMO2*1*) and are expected to express catalytically

functional protein (FMO2.1) in the lung (Dolphin *et al.* 1998; Krueger *et al.* 2002; Veeramah *et al.* 2008). The *in vitro* bioactivation of TAZ catalysed by FMO2.1 observed in this investigation suggests that individuals expressing this enzyme in the lung are likely to be predisposed to TAZ-related toxicity in this tissue. The reaction between TAZ and FMO2.1 in the lung represents a non-specific activation of this drug and will consequently lead to less TAZ being activated by EtaA in *Mycobacteria*. The net effect of the reaction is the production of reactive metabolites in lung and the decrease in TAZ efficacy.

Given the high frequency of the ancestral form of FMO2 (FMO2.1) in a region where TB is a major problem, may have implications on the toxicity and efficacy of TAZ and ETA.

3.2 Kinetic Parameters of FMO3 Amino Acid Variants

Introduction

This section describes kinetic studies undertaken on amino acid variants of human FMO3. Variant FMO3 proteins were assayed for TAZ oxygenation activity to assess whether individuals possessing these variants may have altered metabolism of TAZ in the liver and the skin.

Work in this chapter has focused on SNPs which are highly prevalent or unique to the African population. This is because TAZ is only administered in certain parts of Africa due to lack of resources. SNPs which are likely to have a significant impact on the proteins ability to oxygenate TAZ are also investigated, even though they may not be highly prevalent in the African population. The African population has also been chosen as an average of 28% of Sub-Saharan Africans are found to express a functionally active form of FMO2 (FMO2.1) (Dolphin *et al.* 1998; Krueger *et al.* 2002; Veeramah *et al.* 2008). The remainder of the world's population have acquired a polymorphism, which introduces a premature stop codon and thus do not produce functional FMO2 (Dolphin *et al.* 1998; Whetstine *et al.* 2000; Veeramah *et al.* 2008), this aspect will be discussed later.

The four FMO3 SNPs to be investigated in this chapter are shown in Table 3.2.1.

Results – Kinetic Parameters of FMO3 Amino Acid Variants

FMO3 cDNA constructs containing the desired genetic substitutions were previously prepared in the laborotary for bacterial expression (Allerston *et al.* 2009). Sub cloning of these constructs to produce baculovirus for expression in insect cells is described in chapter 2.9. Both strands of each cDNA were sequenced in order to

Table 3.2.1: Genetic polymorphisms of Human FMO3 investigated in this report.

GENETIC SUBSTITUTION	AMINO ACID SUBSTITUTION AND POSITION	SOURCE	SNP ID
g.15167G>A	E158K	(Brunelle *et al.* 1997; Dolphin *et al.* 1997; Treacy *et al.* 1998; Zschocke *et al.* 1999; Furnes *et al.* 2003)	rs2266782
g.21443A>G	E308G	(Treacy *et al.* 1998; Zschocke *et al.* 1999)	rs2266780
g.15167G>A, g.21443A>G	E158K/E308G	(Furnes *et al.* 2003; Allerston *et al.* 2007)	rs2266782 rs2266780
g.21599T>C	L360P	(Furnes *et al.* 2003; Lattard *et al.* 2003)	rs28363581

Genetic and amino acid substitutions are given for *FMO3* polymorphic variants studied in this section. Publications which identified the polymorphism or characterised the functional effect have been given. SNP id references are provided.

confirm the presence of the SNP and to ensure that other mutations were not introduced during the cloning procedure. The original cDNA for FMO3 which has been used in this section and in section 3.1 encodes methionine at position 486 (Dolphin *et al.* 1997). Subsequent studies have found isoleucine to be more common at this position and thus the methionine residue has been reported as a polymorphism (Cashman 2002). Prior to this knowledge, constructs had been prepared on the methionine and isoleucine background. No population frequency data is available on these SNPs and there are no reports of catalytic differences between the I486 and M486 proteins. In this chapter the catalytic activities of heterologously expressed FMO3 I486 and M486 have also been compared. Henceforth, FMO3 M486 will be referred to as wild type (M486) and FMO3 I486 as wild type (I486). Figure 3.2.1 illustrates whether a SNP has been constructed on the I486 background or on the M486 background. Amino acid variants G308 and P360 will be compared to wild type (M486) whereas K158 and K158 / G308 will be compared to wild type (I486).

The FMO3 variants, K158, G308, K158/G308, P360 and wild type (I486) and (M486) have been investigated by expressing each variant FMO3 protein using the baculovirus/insect cell system. Insect cell microsomal membranes were isolated and characterised for their ability to oxygenate TAZ. This was done using the UV spectroscopic assay described in section 3.1. Previous reports have characterised the effects of these SNPs towards methimazole, a probe substrate of the FMOs and therefore, the activity of the variant FMO3 proteins towards methimazole was also tested.

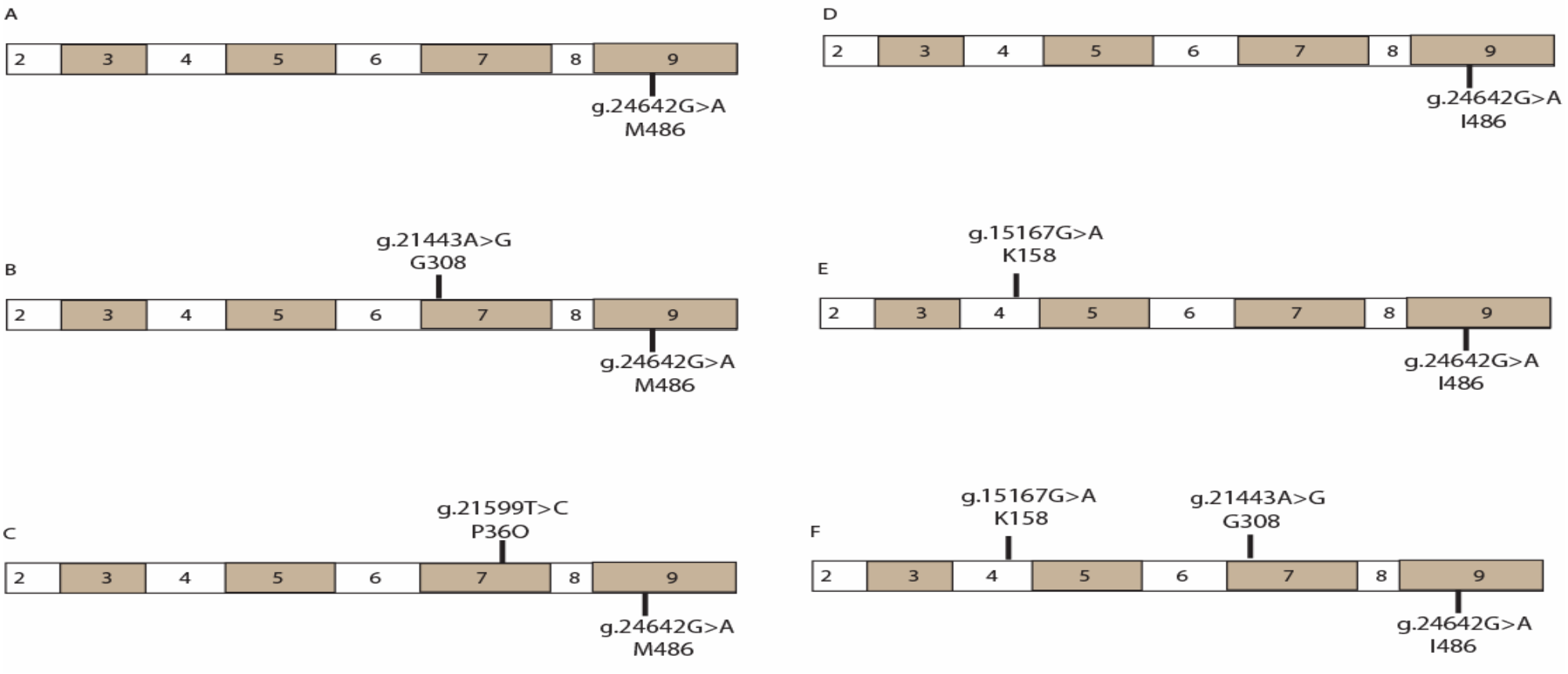


Figure 3.2.1: Polymorphic Variants of Human FMO3 investigated in this Chapter

Positions of genetic and amino acid substitutions are shown above. Exons are numbered. G308 (B) and P360 (C) polymorphic variants were constructed on the M486 background (A), whereas K158 (E) and K158/G308 (F) were on the I486 background (D).

Methimazole *S*-Oxygenation by human FMO3 Amino Acid Variants

All protein variants were first tested for catalytic activity towards methimazole to ensure that the insect cell microsomes were of good quality. The rate of methimazole *S*-oxygenation was monitored spectrophotometrically by monitoring the rate of decrease of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm. Methimazole assays were performed in triplicate on batches of microsomes isolated from three independent infections of *Sf*9 cells. Steady state kinetic parameters, K_M and *V*max were determined for wild type and variant FMO3 proteins using the non-linear regression application of the Kinetics module (v.3.1) of SigmaPlot (v.10). Turnover number was calculated using *V*max values and enzyme concentration as described in chapter 2.9.8.2. The findings are summarised in Table 3.2.2 and 3.2.3.

All variant proteins catalysed methimazole *S*-oxygenation and a hyperbolic Michaelis-Menten relationship was observed between methimazole concentration and the initial rate of TNB disappearance.

Wild type (M486) (Fig. 3.2.1A) *S*-oxygenated methimazole with a turnover number (*k*cat) and Michaelis constant (K_M) of 2.95 ± 0.25 min^{-1} and 31.45 ± 2.05 μM respectively. These finding are in agreement with previously published data (Dolphin *et al.* 1997) (Table 3.2.2). Table 3.2.2 summarises kinetic data for FMO3 G308 (Fig. 3.2.1B) and FMO3 P360 (Fig. 3.2.1C) in comparison to wild type (M486) (Fig 3.2.1A). FMO3 G308 catalysed methimazole oxygenation with a *k*cat of 2.03 ± 0.83 min^{-1} and a K_M of 49.50 ± 5.45 μM. This represents a 30% decrease in the turnover number of FMO3 G308 when compared to that of wild type (M486) ($p<0.05$). The K_M of FMO3 G308 is observed to be higher than that of wild type (M486) but this difference was not statistically significant ($p>0.05$). Although direct comparison to published data is not possible (as published work refers to this SNP expressed on the

I486 background), the general effect of this SNP is in agreement with published data. Lattard and co-workers report a ~25% decrease in catalytic activity and a 2.4-fold increase in K_M, similar to findings in this study (Lattard *et al.* 2003). Kinetic analysis of FMO3 P360 showed this protein to be significantly more efficient at methimazole oxygenation than wild type (M486) ($p<0.05$) with a *k*cat of 35.45 ± 3.50 min^{-1}. The turnover number for this variant is 4 -fold greater than that found for wild type (M486). There was no significant difference however, in the K_M values determined for the two proteins ($p>0.05$). This trend is in good agreement with work in previous publications in which a 4-fold increase in *V*max was observed (Lattard *et al.* 2003).

Table 3 summarises kinetic data for FMO3 K158 (Fig 3.2.1E) and FMO3 K158/G308 (Fig 3.2.1F) in comparison to wild-type (I486) (Fig 3.2.1C). Wild type (I486) catalysed methimazole oxygenation and displayed a *k*cat of 6.26 ± 1.55 min^{-1} and a K_M of 24.50 ± 1.70 μM. These kinetic parameters are within a similar range to those already published (Lattard *et al.* 2003; Koukouritaki *et al.* 2007). *k*cat and K_M values for FMO3 K158 catalysed methimazole oxygenation was found to be 7.25 ± 2.91 min^{-1} and 39.40 ± 2.41 μM respectively. No significant difference was observed in the turnover number between FMO3 K158 and wild type (I486) ($p>0.05$). This data supports previous findings (Lattard *et al.* 2003). The double mutant, K158 / G308 had significantly reduced activity compared to that of wild type (I486). The double mutant catalysed methimazole *S*-oxygenation with a *k*cat of 0.60 ± 0.09 min^{-1} and a K_M of 570 ± 26 μM. These kinetic parameters are significantly different from those determined for wild type I486. The trend is in agreement with previously published data, whereby the double mutant has diminished activity compared to that of wild type FMO3, and the single variants K158 and G308. The K_M of methimazole

Table 3.2.2: Methimazole *S*-oxygenation by human FMO3 (M486) and FMO3 amino acid variants

FMO3 Variant	K_M (μM)	*k*cat (min^{-1})	*kcat* / K_M (min^{-1} M^{-1}) (x10^4)	Relative Activity (%)
Wild type (M486)	31.45 ± 2.05	2.95 ± 0.25	9.2 ± 2.10	100%
FMO3 G308	49.50 ± 5.45	2.03 ± 0.83	4.10 ± 0.67	45%*
FMO3 P360	35.45 ± 3.50	12.20 ± 3.45	34.5 ± 0.21	375%*

The *S*-oxygenation of methimazole by human FMO3 variants was determined as described in section 2.9.8.1. K_M (μM), *k*cat (*(*min^{-1}), *k*cat / K_M (*(*min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from three independent infections of *Sf9* cells. Relative catalytic activity compared to that of wild type FMO3 is displayed (%).

*$p<0.05$ compared to the result of obtained for wild type (M486)

Table 3.2.3: Methimazole *S*-oxygenation by human FMO3 (I486) and FMO3 amino acid variants

FMO3 Variant	**K_M (μM)**	***k*cat (min^{-1})**	***k*cat / K_M ($min^{-1} M^{-1}$) ($x10^5$)**	**Relative Activity (100%)**
Wild type (I486)	24.50 ± 1.70	6.26 ± 1.55	2.56 ± 0.66	100%
FMO3 K158	39.4 ± 2.6	7.25 ± 2.91	1.85 ± 0.78	70%
FMO3 K158 / G308	570 ± 26	0.66 ± 0.04	0.12 ± 0.08	5%**

The *S*-oxygenation of methimazole by human FMO3 variants is described in section 2.9.8.1. K_M (μM), *k*cat *(*min^{-1}), *k*cat / K_M *(*min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from three independent infections of *Sf9* cells. Relative catalytic activity compared to that of wild type FMO3 is displayed (%).

***p<0.005* compared to the result of obtained for wild type (I486)

was found to be several orders of magnitude higher than that reported by Lattard and co-workers (Lattard *et al.* 2003). This group also report a higher *V*max for the reaction than was observed in this investigation. However, overall catalytic efficiency of K158/G308 determined in this chapter (as *k*cat /K_M) and by Lattard *et al.* (as *V*max / K_M) are very similar (5% and 9% of wild type FMO3 activity respectively).

TAZ *S*-Oxygenation by human FMO3 Amino Acid Variants

Rate of TAZ oxygenation catalysed by FMO3 protein variants was assessed by monitoring the rate of decrease in TAZ at 328 nm (described in chapter 2.9.8.2). TAZ assays were performed in triplicate on batches of microsomes isolated from three independent infections of *Sf* 9 cells. Steady state kinetic parameters, K_M and *V*max were determined for wild type and variant FMO3 proteins using the non linear regression application of the Kinetics module (v.3.1) of SigmaPlot (v.10). Turnover number was calculated using *V*max values and enzyme concentration as described in chapter 2.9.8.2. These findings are summarised in Table 3.2.4 and 3.2.5.

FMO3 wild type (M486) (Fig.3.2.1A), FMO3 G308 (Fig 3.2.1B) and FMO3 P360 (Fig 3.2.1C) were capable of TAZ oxygenation. Analysis of Michaelis-Menten plots confirmed a hyperbolic relationship between enzyme activity and initial rates of TAZ disappearance (Fig. 3.2.2 A-C).

The reaction between TAZ and FMO3 wild type (M486) was found to have a *k*cat and K_M of 1.47 ± 0.37 min^{-1} and 8.2 ± 0.70 μM respectively. This data is in good agreement with kinetic parameters determined in section 3.1. FMO3 G308 catalysed TAZ oxygenation with an observed *k*cat of 0.35 ± 0.09 min^{-1} and a K_M of 14.40 ± 3.52.

Table 3.2.4: *S*-oxygenation of TAZ by Human FMO3 (M486) and FMO3 amino acid variants

FMO3 Variant	K_M (μM)	*k*cat (min^{-1})	*k*cat / K_M (min^{-1} M^{-1}) (x10^5)	Relative Activity (%)
Wild type (M486)	8.2 ± 0.70	1.47 ± 0.37	1.80 ± 0.34	100%
FMO3 G308	14.40 ± 3.52	0.35 ± 0.09	0.24 ± 0.13	14%**
FMO3 P360	8.53 ± 0.83	4.05 ± 0.57	4.75 ± 0.81	263%*

The *S*-oxygenation of TAZ by human FMO3 variants is described in section 2.9.8.2. K_M (μM), *k*cat (*(*min^{-1}), *k*cat / K_M (*(*min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from three independent infections of *Sf9* cells. Relative catalytic activity compared to that of wild type FMO3 is displayed (%).

**p<0.05* compared to the result of obtained for wild type (M486)

***p<0.005* compared to the result of obtained for wild type (M486)

Table 3.2.5: *S*- oxygenation of TAZ by Human FMO3 (I486) and FMO3 amino acid variants

FMO3 Variant	K_M (μM)	*k*cat (min^{-1})	*k*cat / K_M (min^{-1} M^{-1})(x10^5)	Relative Activity (%)
Wild type (I486)	10.80 ± 1.33	1.70 ± 0.25	1.57 ± 0.30	100%
FMO3 K158	8.80 ± 0.57	1.87 ± 0.19	2.12 ± 0.26	135%
FMO3 KI58 / G308	N.D	N.D	N.D	N.D

The *S*-oxygenation of TAZ by human FMO3 variants is described in section 2.9.8.2. K_M (μM), *k*cat (*(*min^{-1}), *k*cat / K_M (*(*min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from three independent infections of *Sf9* cells. Relative catalytic activity compared to that of wild type FMO3 is displayed (%).

N.D: Enzyme activity was not detected.

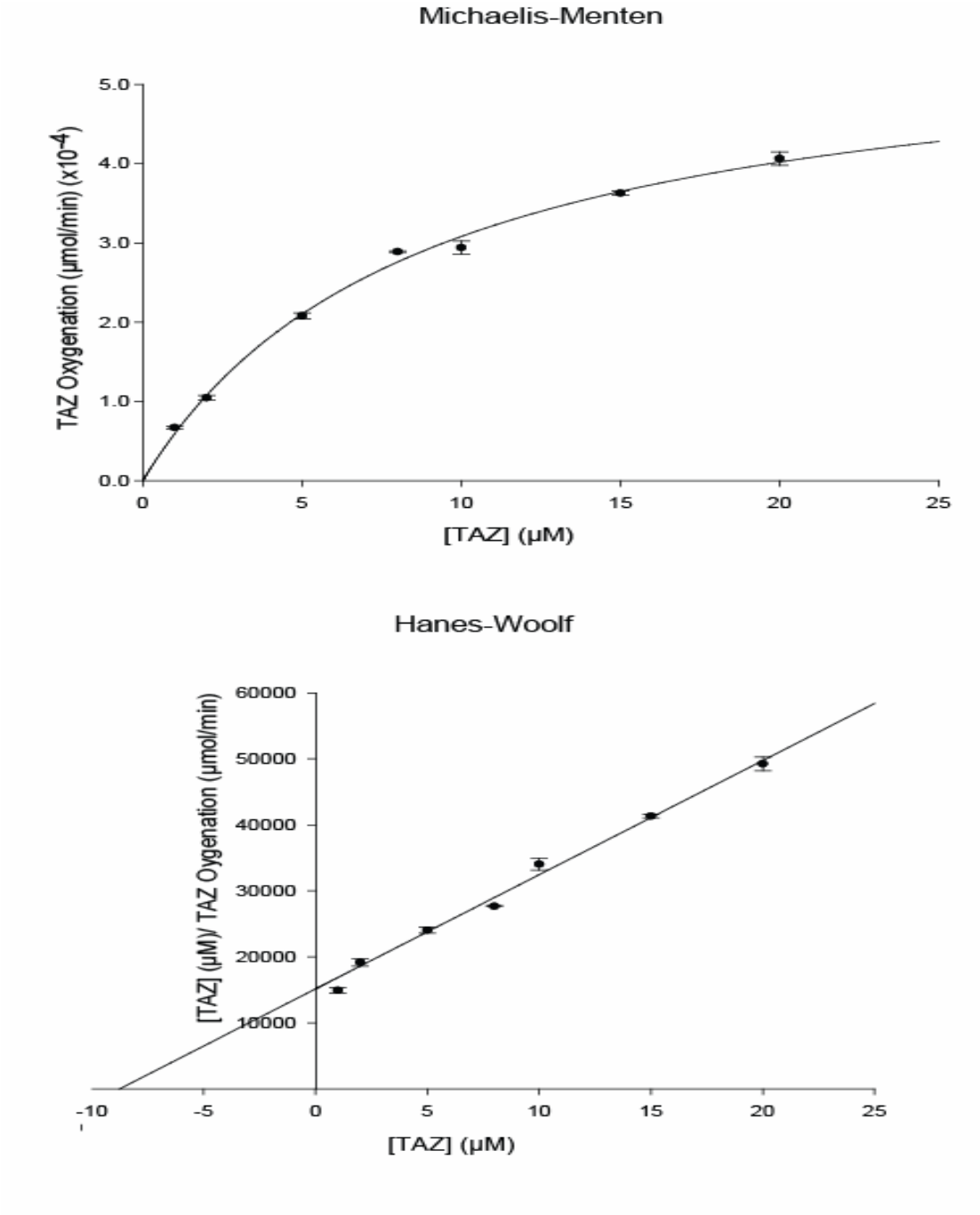


Figure 3.2.2A: Non-linear regression and linear transformation of the Michaelis-Menten equation for FMO3 wild type (M486) catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed FMO3 wild type (M486) in the presence of NADPH and a buffer at pH 8.4. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

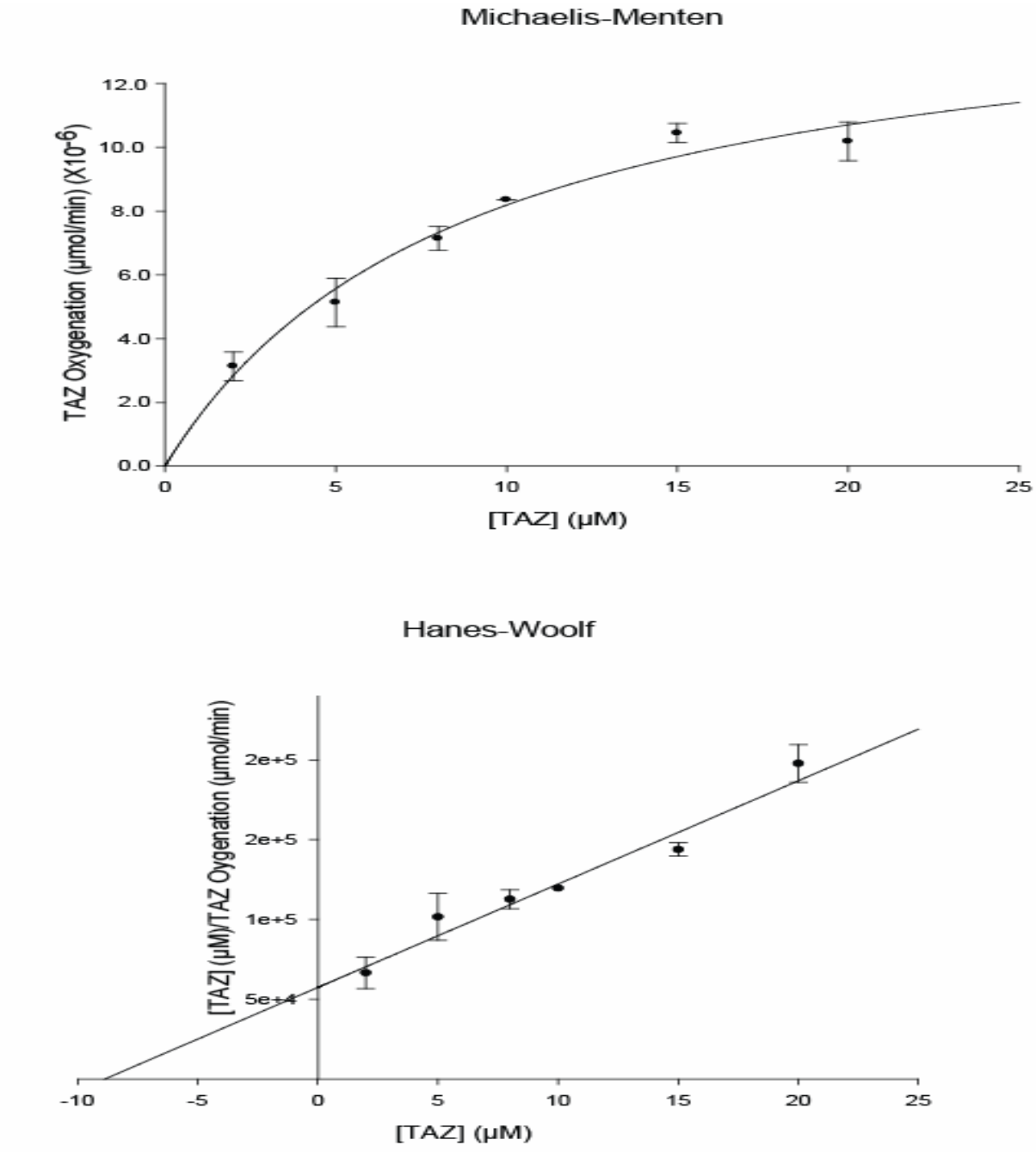


Figure 3.2.2B: Non-linear regression and linear transformation of the Michaelis-Menten equation for FMO3 G308 catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed FMO3 G308 in the presence of NADPH and a buffer at pH 8.4. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

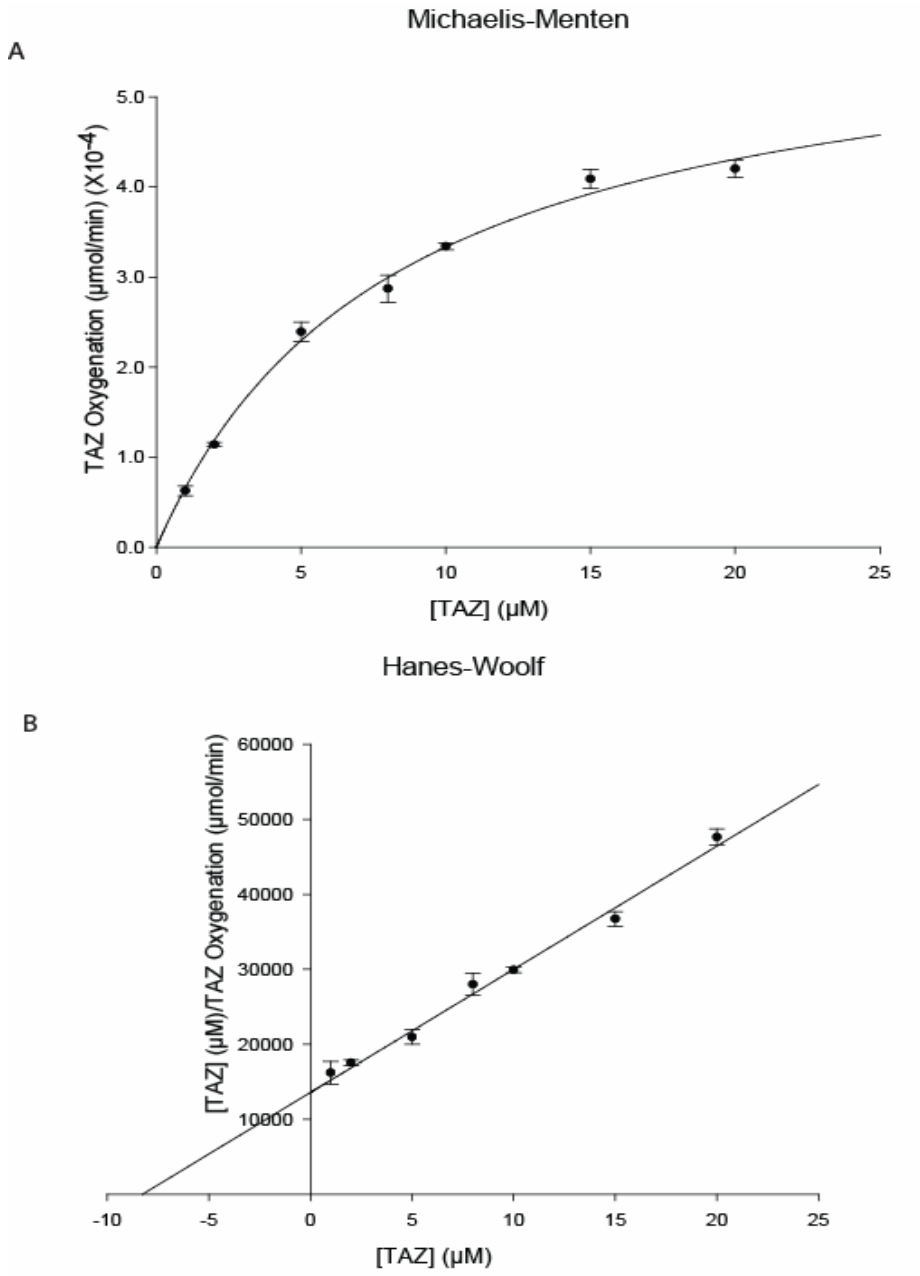


Figure 3.2.2C: Non-linear regression and linear transformation of the Michaelis-Menten equation for FMO3 P360 catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed FMO3 P360 in the presence of NADPH and a buffer at pH 8.4. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

Compared to wild type (M486), this variant shows a significant decrease in TAZ oxygenation activity. The *k*cat / K_M of this reaction is approximately 85% lower than that of wild type (M486) (*p<0.005)*. A small increase in the K_M of the reaction between FMO3 G308 and TAZ was observed when compared to that of wild type (M486) and TAZ, however this is not of statistical significance (*p>0.05*). These findings suggest that individuals expressing FMO3 G308 in the liver may metabolise TAZ at a much lower rate than those who possess the wild type form. Analysis of FMO3 P360 found this enzyme to be significantly more efficient at TAZ oxygenation than wild type (M486) (*p< 0.05)*. The *k*cat of the FMO3 P360 catalysed reaction was found to be 4.05 ± 0.57 min^{-1}, 2.7-fold greater than that observed for wild type (M486). No significant difference was observed in the K_M of TAZ and FMO3 P360 (8.53 ± 0.83 μM) compared to that of wild type (M486) and TAZ (*p>0.05*). These findings suggest that individuals possessing this variant in the liver are likely to metabolise TAZ much faster than those who possess the wild type form.

FMO3 wild type (I486) (Fig.3.2.1D) and FMO3 K158 (Fig 3.2.1E) were capable of TAZ oxygenation. Analysis of Michaelis-Menten plots confirmed a hyperbolic relationship between enzyme activity and initial rates of TAZ disappearance (Fig. 3.2.3A and B). No TAZ oxygenation activity was observed for the K158/G308 variant of FMO3.

*k*cat values for wild type (I486) and FMO3 K158 catalysed TAZ oxygenation were determined as 1.70 ± 0.25 and 1.87 ± 0.19 min^{-1} respectively (see Fig. 3.2.1 D and E). K_M values for wild type (I486) and FMO3 K158 towards TAZ were found to be 10.80 ± 1.33 and 8.80 ± 0.57 μM respectively (Table 3.2.5). *k*cat and K_M values for FMO3 K158 were not statistically different to those determined for wild type (I486) (Table 3.2.5). This suggests that individuals expressing this variant in the liver

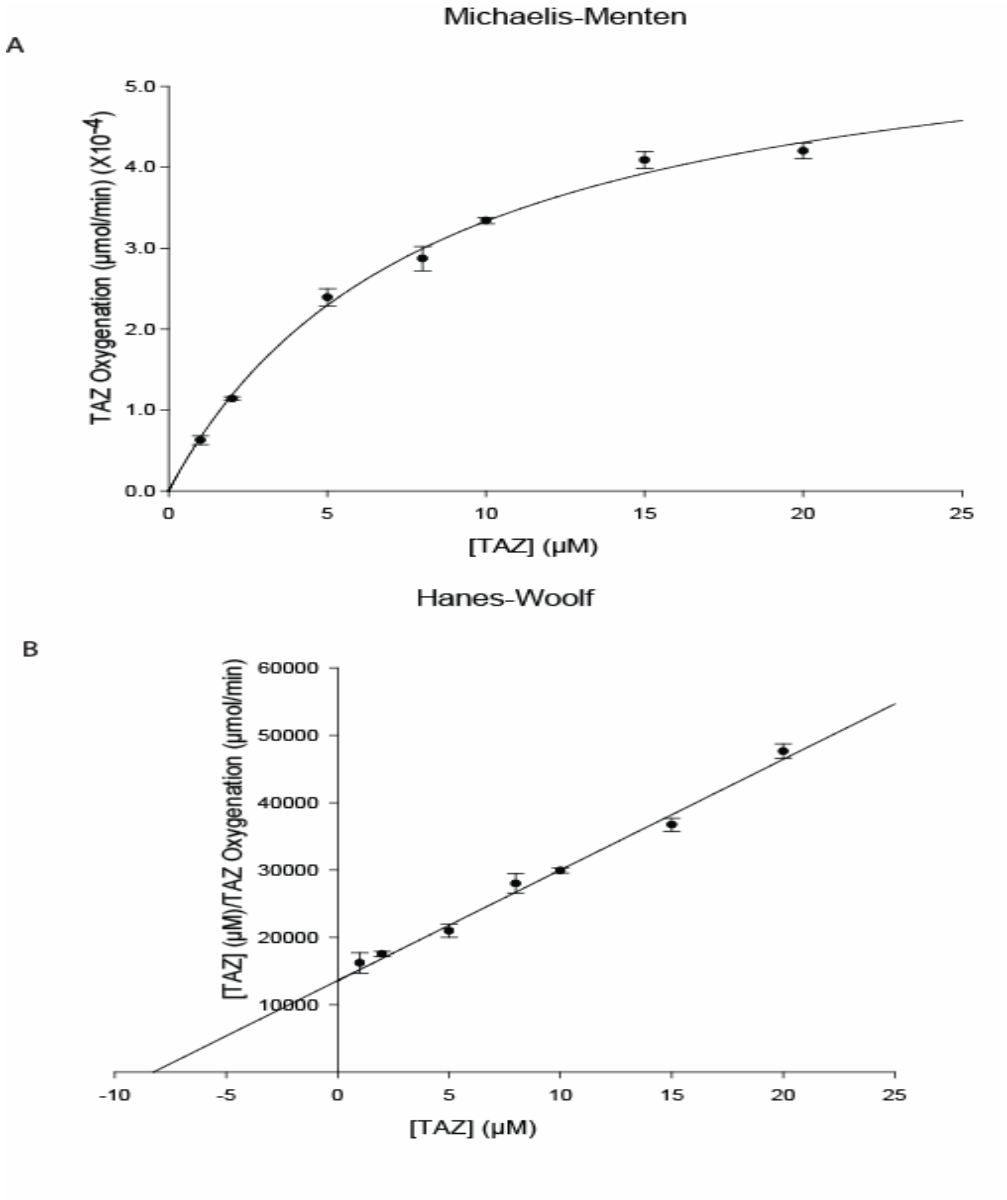


Figure 3.2.3A: Non-linear regression and linear transformation of the Michaelis-Menten equation for FMO3 wild type (I486) catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed FMO3 wild type (I486) in the presence of pH NADPH and a buffer at pH 8.4. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

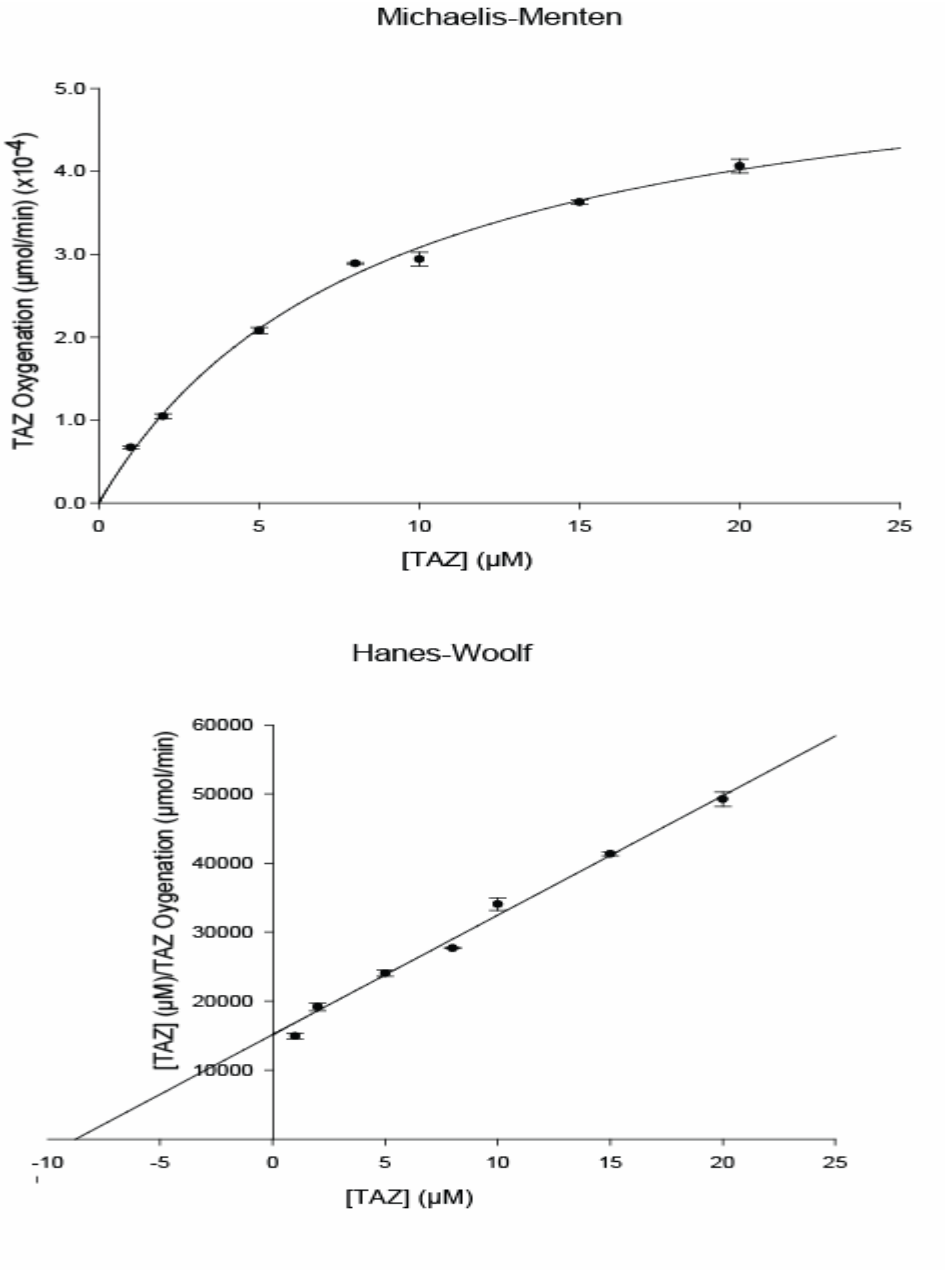


Figure 3.2.3B: Non-linear regression and linear transformation of the Michaelis-Menten equation for FMO3 K158 catalysed TAZ oxidation (A) Michaelis-Menten curve of TAZ oxygenation catalysed by *Sf*9 insect cell microsomes containing heterologously expressed FMO3 K158 in the presence of NADPH and a buffer at 8.4. (B) Linear transformation of Michaelis-Menten data using Hanes-Woolf regression.

are likely to metabolise TAZ at similar rates to those possessing the wild type form. No activity was detected for FMO3 K158 /G308 towards TAZ at the tested TAZ concentrations (Table 3.2.5). This suggests that the double mutant is either, not capable of TAZ oxygenation, or that the reaction has a significantly higher Michaelis constant for TAZ.

Finally, kinetic parameters obtained for TAZ and methimazole *S*-oxygenation catalysed by wild type M486 and I486 were compared. Table 3.2.6 summarises kinetic data determined for FMO3 wild type (M486) and wild type (I486) with methimazole.

Data shows a significant difference in *k*cat values between wild type (M486) and wild type (I486) (*p<0.005)*. Wild type (I486) is approximately twice as efficient at methimazole oxidation as wild type (M486). No significant difference in K_M values was observed between the two proteins (*p>0.05)*. In terms of TAZ oxygenation, wild type (486M) catalysed this reaction with a *k*cat of 1.47 ± 0.37 min^{-1}, whereas a *k*cat of 1.70 ± 0.25 min^{-1} was observed for wild type (I486) (Table 3.2.7). Therefore no significant difference was found in the rate of TAZ oxygenation by wild type (M486) and wild type (I486) (*p>0.05)*. These findings suggest that the M486 mutation is reducing its catalytic efficiency in a substrate-specific manner. It is therefore important for this mutation to be addressed in future work in this field.

HapMap Frequency Analysis of human FMO2 and FMO3 Variations

Tables 3.2.4 - 6 illustrate how single nucleotide polymorphisms in the *FMO3* gene can result in the alteration of enzyme activity towards a given substrate. This may a

affect the metabolism of a therapeutic drug and furthermore have an impact on the outcome of such a compound *in vivo*. In the second part of this section, analysis of HapMap was undertaken to establish whether individuals possessing the 'rapid' variant of FMO3 might also possess the ancestral allele encoding the functional FMO2.1 protein. The reason why this is deemed important is because functional FMO2.1 is expressed only in a proportion of sub-Saharan African and Hispanic individuals and the enzyme, as demonstrated in section 3.1, is capable of oxidising TAZ to metabolites that are expected to be detrimental to a cell. Therefore individuals expressing this protein in the lung and FMO3 P360 in the liver may be at higher risk of adverse drug reactions.

HapMap is an international collaboration that catalogues genetic differences and similarities in humans of different ethnic populations. This allows one to investigate variations in several genes in a given individual. Table 3.2.8 depicts information derived from HapMap on individuals from three African populations who possess at least one copy of g.21599T>C (L360P) SNP in *FMO3*. Table 3.2.8 also identifies which variant of *FMO2* these individuals possess.

Table 3.2.6: *S*-oxygenation of methimazole by Human FMO3 (M486) and FMO3 (I486)

FMO3 Variant	K_M (μM)	*k*cat (min^{-1})	*k*cat / K_M (min^{-1} M^{-1}) (x10^5)
Wild type (M486)	31.45 ± 2.05	2.95 ± 0.25	9.2 ± 2.10
Wild type (I486)	24.50 ± 1.70	6.26 ± 1.55**	2.56 ± 0.66

The *S*-oxygenation of methimazole by human FMO3 (M486) and (I486) is described in section 2.9.8.1. K_M (μM), *k*cat (*(*min^{-1}), *k*cat / K_M (*(*min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from three independent infections of *Sf*9 cells.

***p<0.005* compared to the result obtained for wild type (M486)

Table 3.2.7: *S*- oxygenation of TAZ by Human FMO3 (M486) and FMO3 (I486)

FMO3 Variant	K_M (μM)	*k*cat (min^{-1})	*k*cat / K_M (min^{-1} M^{-1}) (x10^5)
Wild type (M486)	8.2 ± 0.70	1.47 ± 0.37	1.80 ± 0.34
Wild type (I486)	10.80 ± 1.33	1.70 ± 0.25	1.57 ± 0.30

The *S*-oxygenation of TAZ by human FMO3 variants is described in section 2.9.8.2. K_M (μM), *k*cat (*(*min^{-1}), *k*cat / K_M (*(*min^{-1} / M^{-1}) are reported as mean ± standard error obtained from enzyme assays performed in triplicate on microsomes isolated from three independent infections of *Sf9* cells.

Table 3.2.8: Data extracted from HapMap for *FMO3* g.21599T>C (FMO3 360Pro/Leu) and *FMO2* g.23238C/T (FMO2-472Gln/STOP).

HapMap Chromosome Identifier No.	*FMO3* g.21599T>C (FMO3 360Pro/Leu)	*FMO2* g.23238C>T FMO2 – 472Gln/STOP	Population
NA20340_c1 NA20340_c2	C (360Leu) T (360Pro)	T 472 STOP C 472 Gln	ASW
NA19438_c1 NA19438_c2	T (360Pro) C (360Leu)	T 472 STOP T 472 STOP	LKW
NA19182_c1 NA19182_c2	T (360Pro) C (360Leu)	T 472 STOP T 472 STOP	YRI
NA18855_c1 NA18855_c2	T (360Pro) C (360Leu)	T 472 STOP T 472 STOP	YRI
NA19093_NA19092_c1 NA19093_NA19092_c2	C (360Leu) T (360Pro)	T 472 STOP NO DATA AVAILABLE	YRI

(HapMap Genome Browser (Phase 3 - genotypes, frequencies & LD, www.hapmap.org). ASW = African ancestry in Southwest USA (population sampled: 82 individuals). YRI = Yoruba in Ibadan, Nigeria (population sampled: 256 individuals). LKW = Luhya in Webuye, Kenya (population sampled: 268 individuals).

Given that the g.21599T>C (L360P) polymorphisms encodes a protein with higher catalytic activity, it is of particular interest to assess the frequency at which it occurs in *cis* with *FMO2* g.23238C (Q472, FMO2.1). Such individuals will express functional FMO2 in their lungs and a rapid metaboliser of TAZ in the liver. Analysis of the African data set on HapMap identifies one individual (out of a sample of 82), of African-American descent to be heterozygous for g.21599T>C (L360P) and *FMO2* g.23238C (Q472). Given the small sample size (82 individuals) and the fact that L360P is a rare polymorphism it is of no surprise that only one individual was identified. Nevertheless, this finding is of importance as it confirms that the two polymorphisms can and do occur together. Allelic frequencies cannot be calculated from data obtained from one individual and so we have considered frequency data of g.21599T>C (L360P) and *FMO2* g.23238C (Q472) that have been published by studies employing larger samples. The number of individuals estimated to possess at least one copy of the ancestral allele of FMO2.1 is 28% in African-Americans (Dolphin 1998, Whetstine 2000). The frequency of individuals heterozygous for the g.21599T>C (L360P) polymorphism has been determined as 2% in an African-American population (Furnes and Schlenk 2003). Thus assuming that the variations assort independently, the frequency at which they occur in *cis* can be estimated by multiplying the individual frequencies. By doing so, it is estimated that 0.56% of African-American individuals will encode FMO3 P360 and FMO2.1 in their liver and lungs respectively. Given that the population of African-Americans is approximately 36 million, the number of individuals possessing at least one copy of these polymosphisms in *cis* is estimated to be around 20,000. Though not a considerably high number, it must be noted that this figure may be in the order of several million in

sub-Saharan Africa (where these individuals have originally come from) since the population of this area is in excess of 800 million.

Discussion – Kinetic Parameters of FMO3 Amino Acid Variants

The impact of pharmacogenetics on anti-tubercular drug metabolism has been established. Isoniazid (INH), an anti-tubercular prescribed since the 1950's is a pro-drug that requires metabolic activation by the *Mycobacterial* catalase-peroxidase KatG to generate an irreversible inhibitor of the FAS II enzyme, InhA(Zhang *et al.* 1992; Banerjee *et al.* 1994). Variant alleles of human *arylamine N-acetyltransferase 2* are implicated in the altered metabolism of Isoniazid. The drug undergoes acetylation in the liver by arylamine N-acetyltransferase 2 (NAT2) and this process represents the inactivation of the drug (reviewed in (Preziosi 2007)). The human population can be divided into 'rapid', 'moderate' and 'slow' acetylators depending on which variant allele of NAT2 they possess(Parkin *et al.* 1997; Singh *et al.* 2009). 'Slow' acetylators have slower rates of INH acetylation in the liver and are more likely to develop neuropathy and hepatotoxicity as a result of the accumulation of toxic intermediates of INH than those who are 'rapid' acetylators (Ellard *et al.* 1976; Timbrell *et al.* 1977; Ellard *et al.* 1981; Possuelo *et al.* 2008).

Thiacetazone (TAZ) was once a widely prescribed drug against tuberculosis in Africa, Asia, South America and Germany (Anonymous 1963; Anonymous 1968; Heffernan 1968; Anonymous 1971; Mame Thierno, On et al. 2001). But numerous reports of TAZ-induced dermatological reactions and hepatotoxicity led to this drug being discontinued in the industrialised world (Narang ; Harland 1962; Aquinas 1968; Axton 1971; Hussain *et al.* 1973; Bedi *et al.* 1974; Gupta *et al.* 1977; Fegan *et al.* 1991; Fegan *et al.* 1991; Chintu *et al.* 1993; Ipuge *et al.* 1995; GOTHI 1998 ; Dieng *et al.* 2001; Mame Thierno *et al.* 2001). Severe cutaneous rashes including Stevens-Johnson syndrome have frequently been reported for patients taking TAZ and early studies on this drug have shown ethnic-dependent side effects (Miller *et al.* 1966).

Despite the availability of more effective and 'safer' antibiotics, certain parts of Africa still receive TAZ as part of treatment for TB because of the lack of resources (Lawn *et al.* 1999; (Keus *et al.* 2003). To date the underlying cause of the observed adverse reactions has not been explained. It has been suggested that the toxicity may be driven by the parent compound, by a reactive metabolite, impairment in detoxification processes or by any combination of these factors (Park *et al.* 1990).

In humans the major FMO involved in hepatic drug metabolism is FMO3. Expression of FMO3 while largely restricted to the liver in humans is expressed in the skin in amounts comparable to other Phase I metabolising enzymes such as CYPs 2A6, 2B6 and 3A4 (Janmohamed *et al.* 2001). FMO3 is encoded from a highly polymorphic gene and several polymorphisms give rise to amino acid variants with altered catalytic capacity (reviewed in (Phillips *et al.* 2008)). This section of the thesis describes five amino acid variants of human FMO3 and their capacity to catalyse TAZ oxygenation. It is important to assess the catalytic activity of commonly occurring amino acid variants of FMO3 towards TAZ oxygenation given that the *in vitro* reaction between FMO3 and TAZ represents one of bioactivation as it generates metabolites that represent chemically reactive species (discussed in section 3.1). It is expected that the catalytic efficiency of an FMO3 variant will dictate the amount of reactive products produced (discussed in section 3.1).

The most common polymorphism g.15167G>A (E158K) has an allelic frequency ranging from 19-50% depending on the population sampled (Dolphin, Riley et al. 1997; Park, Chung et al. 1999; Zschocke, Kohlmueller et al. 1999; Cashman and Zhang 2002; Park, Kang et al. 2002; Lattard, Zhang et al. 2003; Koukouritaki, Poch et al. 2005; Hao, Sun et al. 2007). In African-Americans, the frequency ranges from 45-50% and is the most common FMO3 polymorphism found

in this ethnic group (Dolphin *et al.* 1997; Lattard *et al.* 2003; Hao *et al.* 2007). Heterozygotes for this polymorphism showed no significant decrease in TMA *N*-oxidation as judged by the excretion of free TMA in urine (Zschocke *et al.* 1999). *In vitro* studies by other groups have also shown the lack of significant effect of E158K amino acid substitution on FMO3 catalysis towards several substrates (Lattard *et al.* 2003; Zhang *et al.* 2003; Koukouritaki *et al.* 2005; Shimizu *et al.* 2007). This finding is confirmed in this investigation where no significant difference was observed in the E158K protein ability to catalyse *S*-oxygenation of TAZ and methimazole. This suggests that individuals possessing only this polymorphism of FMO3 are not likely to metabolise TAZ less efficiently than those possessing the wild type enzyme.

A relatively common polymorphism g.21443A>G (E308G) has an allelic frequency of 5-26% depending on the population sampled (Akerman *et al.* 1999; Park *et al.* 1999; Cashman *et al.* 2001; Dolan *et al.* 2005; Koukouritaki *et al.* 2005; Hao *et al.* 2007). The E308G mutation, unlike E158K, is less prevalent in African-Americans, occurring in approximately 5% of individuals (Koukouritaki *et al.* 2007). *In vivo* studies have shown the variant to have little consequence catalytically to FMO3 efficiency (Sachse *et al.* 1999; Lambert *et al.* 2001). Kinetic data determined for heterologously expressed G308 have shown moderate but substrate specific effects of this mutation on enzyme catalysis (Stormer 2000; Lattard *et al.* 2003). In this investigation we observe a significant decrease in enzyme efficiency. In the case of methimazole *S*-oxygenation, a decrease of 55% was observed (Table 3.2.2) ($P<0.05$). In the case of TAZ oxygenation, the E308G variant retained just 20% of the wild type FMO3 capacity ($p<0.005$) as a measure of $k\text{cat}/K_M$. This finding supports previous reports that G308 displays substrate-specific catalytic efficiency. In terms of TAZ metabolism, individuals expressing the G308 variant are likely to metabolise TAZ at a

slower rate in the tissues that normally express FMO3, the liver and skin, compared to those who possess wild type FMO3. This may be beneficial for individuals receiving TAZ as chemotherapy. The advantages of possessing a 'slower' enzyme are discussed below.

The two polymorphisms g.15167G>A (E158K) and g.21443A>G (E308G) occur in *cis* at an allelic frequency ranging from 1-16% depending on the population sampled (Cashman *et al.* 2001; Hao *et al.* 2007; Koukouritaki *et al.* 2007). In African- Americans, a small proportion (~1 %) of individuals possess a haplotype in which g.15167G>A (E158K) and g.21443A>G (E308G) exist in *cis* (Allerston *et al.* 2007; Koukouritaki *et al.* 2007). The K158/G308 double variant when found in *cis* has previously been shown to reduce FMO3 activity by around 50% *in vivo* (as measured by the amount of free TMA excreted in urine samples compared to control subjects) (Akerman *et al.* 1999; Akerman *et al.* 1999; Zschocke *et al.* 1999; Zschocke *et al.* 2000). Another *in vivo* study investigating the metabolism of the H_2-receptor antagonist ranitidine, showed less ranitidine *N*-oxide, compared to control subjects, in the urine of individuals possessing the K158/G308 variant (Park *et al.* 2002). The activity of *in vitro* expressed K158/G308 shows a dramatic reduction in catalytic activity when compared to either the K158 or G308 single variants (Park *et al.* 2002; Lattard *et al.* 2003). The kinetic data presented in this investigation supports these findings. The double variant displayed a significant reduction in enzyme efficiency, retaining just 5% of FMO3 activity towards the *S*-oxygenation of methimazole (as a measure of $k\text{cat}/K_M$) ($p<0.005$). TAZ oxygenation catalysed by this variant was not observed at the tested TAZ concentrations. This may indicate that the enzyme has lost the ability to oxygenate TAZ as an effect of the amino acid substitutions, or that the variant has a considerably higher Michaelis constant for this substrate. The

finding that the double variant has reduced activity towards TAZ and methimazole is of concern to FMO3-substrate metabolism with particular reference to substrates of therapeutic importance. FMO3-substrate metabolism shown to be affected by the reduction of in catalytic efficiency of the K158/G308 variant occurring in *cis* includes the H_2-receptor antagonist ranitidine (Park *et al.* 2002) and the non-narcotic, non-steroidal anti-inflammatory drug, sulindac (Hamman *et al.* 2000; Hisamuddin *et al.* 2004; Hisamuddin *et al.* 2005). In the latter case, a less active FMO3 enzyme has been shown to be beneficial. The K158/G308 double variant is implicated in having a protective effect on the development of polyps in familial adenomatous polyposis patients who received sulindac as part of primary chemoprevention. The prodrug sulindac, is converted by gut bacteria to the active metabolite sulindac sulphide. FMO3 inactivates the drug by converting it to sulindac sulphoxide. The beneficial outcome for a patient with an FMO3 protein with reduced enzyme activity is presumed to be the result of the reduced ability of this enzyme to inactivate the drug and thus leading to prolonged exposure to the active form of sulindac, sulindac sulphide (Hisamuddin *et al.* 2004; Hisamuddin *et al.* 2005). The K158/G308 variant protein therefore has the potential to affect the efficacy of a given therapeutic agent. In the case of TAZ, the double variant K158/G308 and the single variant FMO3 G308 (described above) may both be advantageous to those receiving TAZ as part of TB treatment. *Mycobacterial* infections are treated when TAZ is activated by the bacterial FMO, EtaA (Qian *et al.* 2006; Alahari *et al.* 2007). This results in cell death. The reaction of FMO3 and TAZ *in vivo* would therefore represent a 'non-specific' TAZ-activation event that would not just give rise to chemically reactive species in a mammalian cell (which we wish not to harm) but would also reduce the amount of pro-drug reaching the *Mycobacteria* at the site of infection. This scenario is likely to

not only reduce the efficacy of the drug but also to produce harmful side effects in the patient. So an individual who possesses an FMO3 variant which is catalytically inefficient may be at a lower risk of TAZ bioactivation in the liver and skin and is likely also to have increased drug delivery to the site of infection and thus increase and prolong the exposure of the *Mycobacteria* to TAZ.

A rare polymorphism, g.21599T>C (L360P), is observed at an allelic frequency of about 2% in African-American populations (Furnes *et al.* 2003; Lattard *et al.* 2003). The polymorphism is thought to be unique to this ethnic group as it has not been identified in Caucasians and Asians (Furnes *et al.* 2003; Lattard *et al.* 2003). *In vitro* studies of heterologously expressed protein have shown that FMO3 P360 is the only known variant of FMO3 to display an increased catalytic efficiency to substrates. The substrates investigated were methimazole, TMA and 10-(*N*, *N*-dimethylaminopentyl)-2-(trifluoromethyl) phenothiazine (5-DPT) (Lattard *et al.* 2003). This variant has therefore both increased *N*- and *S*-oxygenation activity. In the studies we undertook, the P360 variant showed significantly higher catalytic efficiencies towards both TAZ and methimazole when compared to wild type FMO3 ($p<0.05$). No data has been published on the *in vivo* consequence of this polymorphism for drug metabolism. With respect to TAZ metabolism, we speculate that individuals expressing the P360 variant may be at higher risk of mediating TAZ activation in the liver and skin for reasons described above. The formation of chemically reactive and oxidising metabolites (described in section 3.1) and depletion of TAZ may be detrimental to individuals possessing this variant.

The functional effects of the g.24642G>A (M486) polymorphism have been investigated. The allelic frequency of this polymorphism is yet to be determined. Kinetic data obtained as part of this investigation has shown this variant to have

significantly reduced catalytic efficiency towards methimazole (but not to TAZ) when compared to wild type FMO3 (I486), as a measure of the turnover number ($p<0.05$). This finding supports previously described data regarding the substrate-specific effects of certain amino acid substitutions on FMO3 activity.

In this section, the effect of five polymorphic variants on FMO3 enzyme catalysis has been discussed, with particular reference to methimazole and TAZ. Work in this section has focussed on functional effects of genetic polymorphisms identified in the coding region of the human *FMO*3 gene and discussed how they might influence the metabolism of an anti-tubercular *in vivo*. However, it must be noted that genetic variations in upstream sequences (promoter and enhancer) of the gene have not been analysed. It is possible than an individual expressing a 'slow' variant of FMO3 like G308 or K158/G308 may have additional polymorphisms in the promoter region of this gene, whereby the expression of the protein is upregulated. The production of more 'slow enzyme' would therefore be expected to have a compensatory effect. Promoter variations have been identified by Koukouritaki and co-workers and are shown to vary within ethnic groups(Koukouritaki *et al.* 2005; Koukouritaki *et al.* 2007). Interestingly the group reported an inferred frequency of 5.6% of African-American individuals to possess a haplotype which encodes a high activity promoter and at least one of the g.15167G>A (E158K) and/or g.21443A>G (E308G) polymorphisms. Haplotype analysis has not been reported for the g.21599T>C (L360P) polymorphism, it is yet to be seen whether an upstream polymorphism will mask or exacerbate the functional effects of this variant.

In the second part of this section, an attempt was made to estimate the frequency of individuals that possess the g.21599T>C (L360P) and the g.23238C (Q472) alleles in *cis*. Though just a rough estimate, it was determined that more than

20,000 Africans in America and in excess of 4 million people in sub-Saharan Africa may express FMO2.1 *and* FMO3 P360 in their lungs (FMO2.1), liver and skin (FMO3). This may have implications regarding the metabolism of TAZ and other xenobiotic compounds that are substrates for these enzymes.

Although, to elucidate the cause(s) or mechanism of TAZ-induced toxicity are beyond the scope of this investigation, this section has described significant catalytic differences in commonly found variants of human FMO3 which may be involved in the inter-individual difference observed for the response towards TAZ treatment.

3.3 Metabolism of Thiacetazone by Mouse Tissue Microsomes

Introduction

Sections 3.1 and 3.2 described the *in vitro* metabolism of TAZ by heterologously expressed human FMOs in microsomes prepared from insect cells. In this section, microsomes prepared from mouse tissue have been used to investigate the relative contribution of the flavin containing monooxygenases (FMOs) and cytochrome P450s (CYPs) to TAZ metabolism.

Microsomes prepared from tissue are fragments of membranes of the endoplasmic reticulum in suspension, they reflect (among other things) the tissue-specific expression of Phase I drug metabolising enzymes. This makes them an invaluable tool to investigate the contribution of such enzymes in the metabolism of a given substrate *in vitro*. A UV spectroscopy assay (described in section 3.1 and 2.8.2.2) was used to analyse the rate of TAZ metabolism catalysed by microsomes isolated from mouse liver and lung. Mouse liver was investigated for its capacity to metabolise TAZ as the first site of metabolism of an orally administered drug is thought to be the liver. The lung was analysed as this is thought to be the site of activation of TAZ (by *Mycobacterium tuberculosis*) in the case of pulmonary tuberculosis.

The expression of FMOs is known to be sex- and tissue-specific, the key sex-difference in mice is the absence of FMO3 expression in the liver of adult males (Falls *et al.* 1995; Falls *et al.* 1997). FMO3 continues to be expressed in adult, male lung (Janmohamed *et al.* 2004). Lung and liver microsomes from male and female mice were therefore analysed. Microsomes from liver and lung were prepared from three mouse lines; wild type (C57BL/6 mice), *Fmo1* (-/-), *2* (-/-), *4*(-/-) line and an *Fmo 5*(-

/-) line. Each of the *Fmo* knockout lines was produced by back-cross breeding onto the C57BL/6 line for 8 generations. Preparation of microsomes from the three mouse lines allowed the investigation of the relative contribution of different members of the FMO family of enzymes to TAZ metabolism to be analysed.

Results

Metabolism of TAZ by Liver Microsomes

Liver microsomes were prepared from wild type C57BL/6 mice, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo 5* (-/-) mouse line as described in section 2.10.1. The rate of TAZ metabolism catalysed by mouse liver microsomes was monitored spectrophotometrically and is described in section 2.10.2. Data represent mean ± standard deviation obtained from two independent isolations of microsomes. All assays were carried out in triplicate on each batch of microsomes.

Figures 3.3.1-3 represent Michaelis-Menten plots (Vo versus [TAZ]) obtained for female liver microsomes prepared from the three mouse lines. Liver microsomes from all three mouse lines catalysed TAZ metabolism and showed a hyperbolic Michaelis-Menten relationship between initial rate of enzyme activity and substrate concentration.

There are five FMO proteins to consider in the mouse. Studies on FMO expression in mouse tissues have reported FMO1, FMO3 and FMO5 to be highly expressed in female liver whereas FMO2 and FMO4 are not expressed (Falls *et al.* 1995; Janmohamed *et al.* 2004). Therefore in the case of wild type liver microsomes, TAZ metabolism could be due to FMO1, FMO3 or FMO5 (Fig. 3.3.1). Experiments shown in Fig. 3.3.2 implicate FMO1 in TAZ metabolism as there is a marked decrease

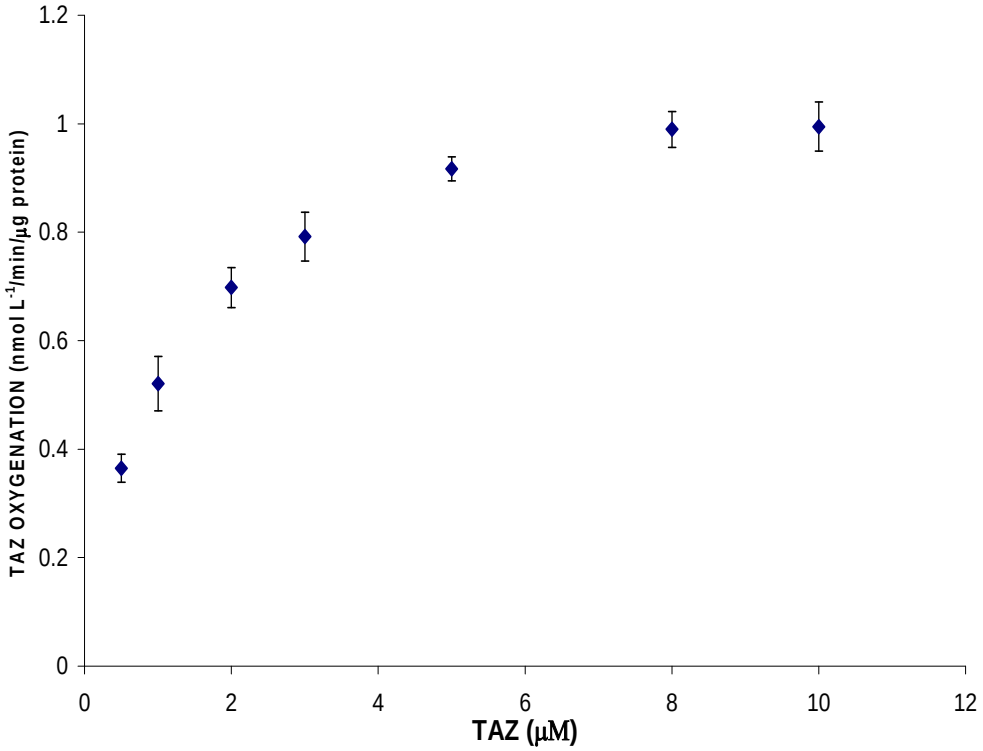


Figure 3.3.1: vo versus [TAZ] plot for TAZ metabolism catalysed by wild type female mouse liver microsomes. The rate of TAZ metabolism by wild type female mouse liver microsomes was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from assays carried out in triplicate.

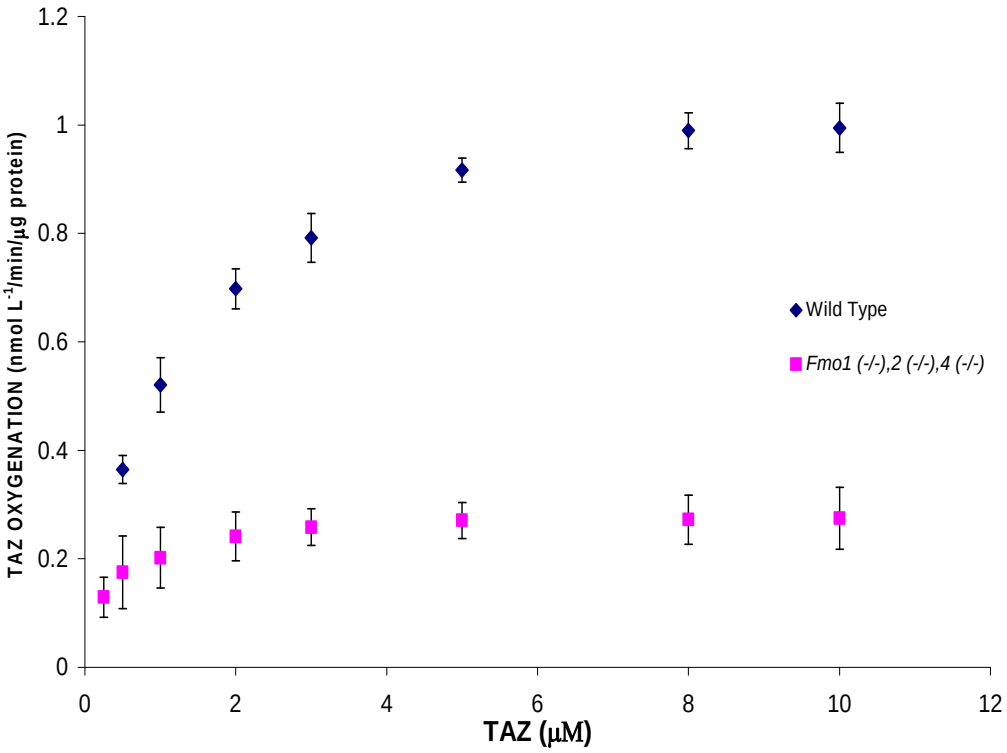


Figure 3.3.2: vo versus [TAZ] plot catalysed by wild type and *Fmo1* (-/-), *2* (-/-), *4* (-/-) female mouse liver microsomes. The rate of TAZ metabolism by wild type and *Fmo1* (-/-), *2* (-/-), *4*(-/-) female mouse liver microsomes was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from assays carried out in triplicate.

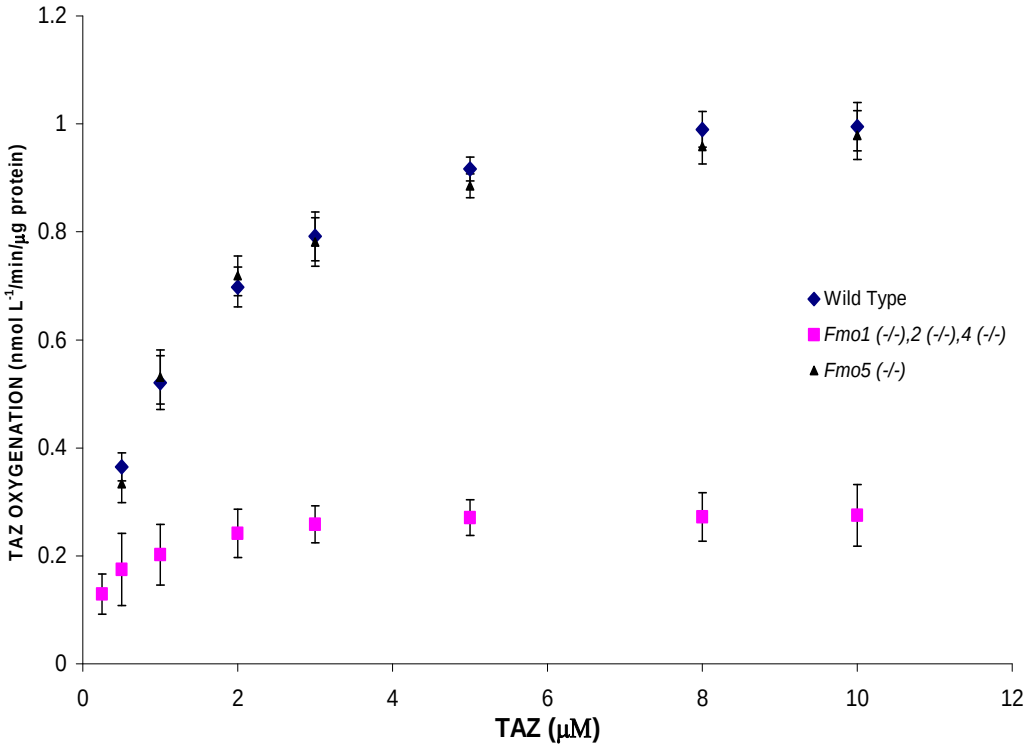


Figure 3.3.3: vo versus [TAZ] plot catalysed by wild type, *Fmo1* (-/-), *2* (-/-), *4*(-/-) and *Fmo5* (-/-) female mouse liver microsomes. The rate of TAZ metabolism by wild type, *Fmo1* (-/-), *2* (-/-), *4*(-/-) and *Fmo5 (-/-)* female mouse liver microsomes was determined as described in section 2.10.2. Data are mean ± standard deviation from assays carried out in triplicate.

in TAZ metabolism by microsomes isolated from the mouse line that has the *Fmo1* gene deleted. In contrast, microsomes isolated from the *Fmo 5* (-/-) line showed similar metabolism to that of wild type microsomes (Fig 3.3.3). FMO5 therefore does not contribute to hepatic TAZ metabolism. To establish whether these differences were significant, rates of TAZ metabolism were analysed at saturating TAZ concentrations. Figure 3.3.4 illustrates a significant decrease in TAZ metabolism by microsomes isolated from liver of the *Fmo1* (-/-), *Fmo2* (-/-), *Fmo 4* (-/-) line compared to wild type liver microsomes(*P<0.005)*. There is no significant difference, however between wild type and *Fmo 5* (-/-) liver microsomes (*P>0.05*). FMO3 is expressed in liver microsomes from all three mouse line and its contribution needs to be addressed. In the liver of a mouse, FMO3 is expressed in a sex-specific manner whereby its expression is 'turned off' in males as a result of high testosterone levels (Falls *et al.* 1995; Falls *et al.* 1997). In order to distinguish between the contribution of FMO1 and FMO3 to the rate of TAZ metabolism, microsomes from male liver were prepared. As shown in Figure 3.3.5 there is no significant difference between male and female liver microsomes isolated from wild-type mice, indicative that FMO3 is unlikely to contribute to the activity shown by female liver microsomes (Fig. 3.3.1-4). Comparison between male, wild-type and *Fmo5* (-/-) liver microsomes, shows no significant difference in TAZ metabolism (Fig. 3.3.5) indicating that FMO5 is unlikely to contribute to this reaction. A small decrease in TAZ metabolism is observed in male *Fmo 1* (-/-), *2* (-/-), *4* (-/-) liver microsomes when compared to male wild-type liver, however this difference is not statistically significant (*p>0.05)* (Fig. 3.3.5). Compared to female *Fmo 1* (-/-), *2* (-/-), *4* (-/-) liver microsomes, male microsomes from the same mouse line showed a significant increase in TAZ

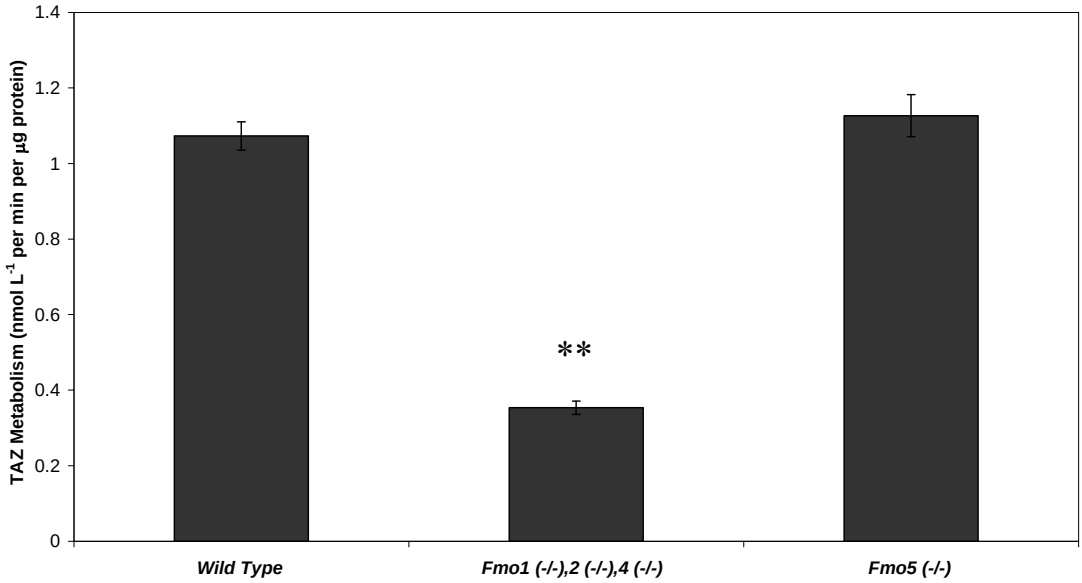


Figure 3.3.4: Rate of TAZ metabolism observed for female mouse liver microsomes. The rate of TAZ metabolism catalysed by liver microsomes prepared from female wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

***p<0.005* compared to the result obtained from wild type female liver microsomes.

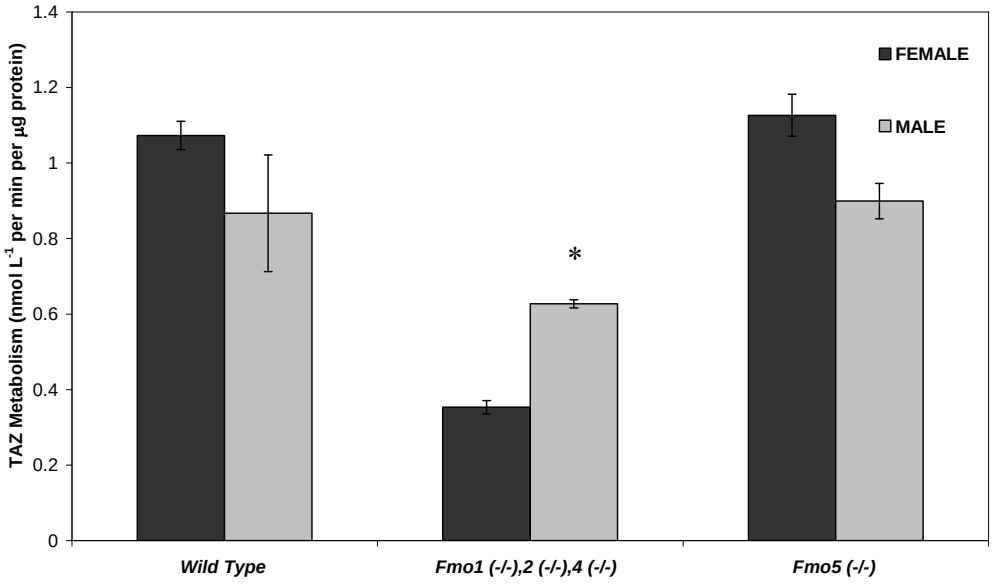


Figure 3.3.5: Rate of TAZ metabolism observed for female and male mouse liver microsomes. The rate of TAZ metabolism catalysed by liver microsomes prepared from female and male wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

*$p<0.05$ compared to the result obtained from female *Fmo1* (-/-), *2* (-/-), *4* (-/-) liver microsomes.

metabolism ($p<0.005$). These findings suggest the contribution of FMOs to TAZ metabolism in male mice may be less than in female mice.

Although FMO contribution to TAZ metabolism has been confirmed, it was important to assess whether FMOs are responsible for all of the TAZ metabolism observed in the liver. The relative amount of FMO contribution to TAZ metabolism was tested by using methods to distinguish between FMO and CYP activity.

Assessing FMO Contribution to TAZ Metabolism in Mouse Liver

Methimazole is used as a probe substrate and as a competitive inhibitor for FMO activity (Dixit *et al.* 1984; Chung *et al.* 1997; Rawden *et al.* 2000; Stormer 2000; Virkel *et al.* 2006). By acting as a competitive inhibitor, methimazole can be used to inhibit FMO-dependent TAZ metabolism. Figure 3.3.6 shows the effect of pre-incubation of female mouse liver microsomes with methimazole before the addition of TAZ. In the presence of methimazole, a significant decrease in TAZ metabolism is observed in the wild type ($p<0.005$) and *Fmo5* (-/-) ($p<0.05$) female liver microsomes, with a reduction of 48% and 35% in enzyme activity respectively. This confirms that FMOs contribute to TAZ metabolism. In the *Fmo 1* (-/-), *2* (-/-), *4* (-/-) female liver microsomes, however, there is no significant difference between methimazole treated and untreated samples ($p>0.05$). Given that there was no significant decrease in TAZ metabolism in microsomes in which the *Fmo1* gene had been deleted further supports the role of FMO1 in the metabolism of this drug. In male, microsomal liver samples, a decrease in TAZ metabolism activity was observed for wild type, *Fmo1* (-/-), *Fmo2* (-/-), *Fmo4* (-/-), and *Fmo5* (-/-) knockout lines in the presence of methimazole (Fig. 3.3.7), however this was only significant for wild-type

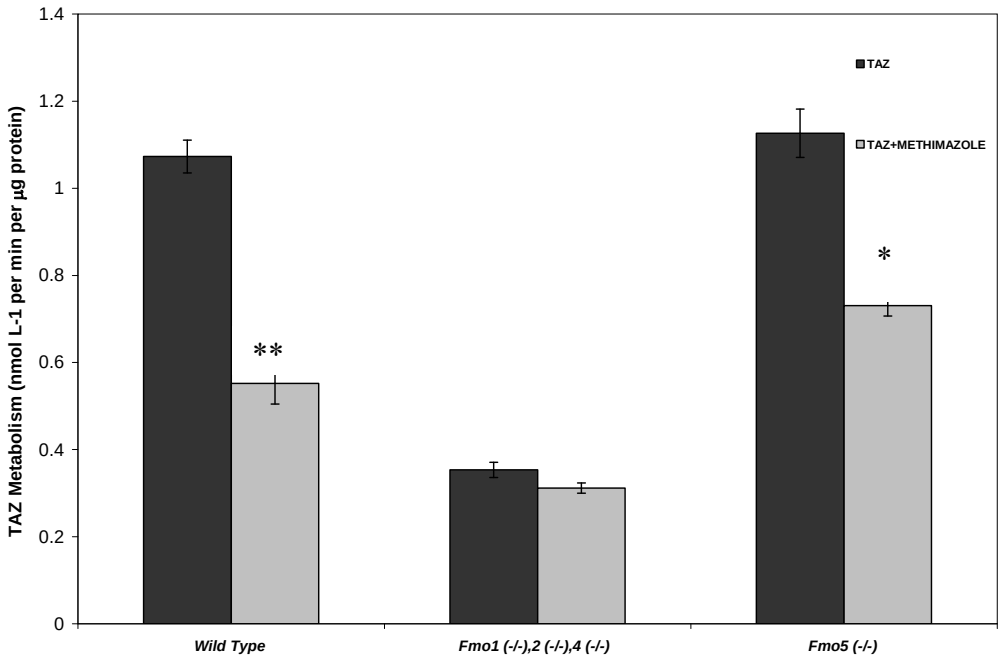


Figure 3.3.6: The effect of pre-treatment of female mouse liver microsomes with methimazole on the rate of TAZ metabolism. Pre-treatment of liver microsomes with methimazole is described in section 2.10.3.1. The rate of TAZ metabolism catalysed by liver microsomes prepared from female and male wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

*$p<0.05$ compared to the result obtained from untreated *Fmo5 (-/-)* female liver microsomes.

*$p<0.005$ compared to the result obtained from untreated female wild type liver microsomes.

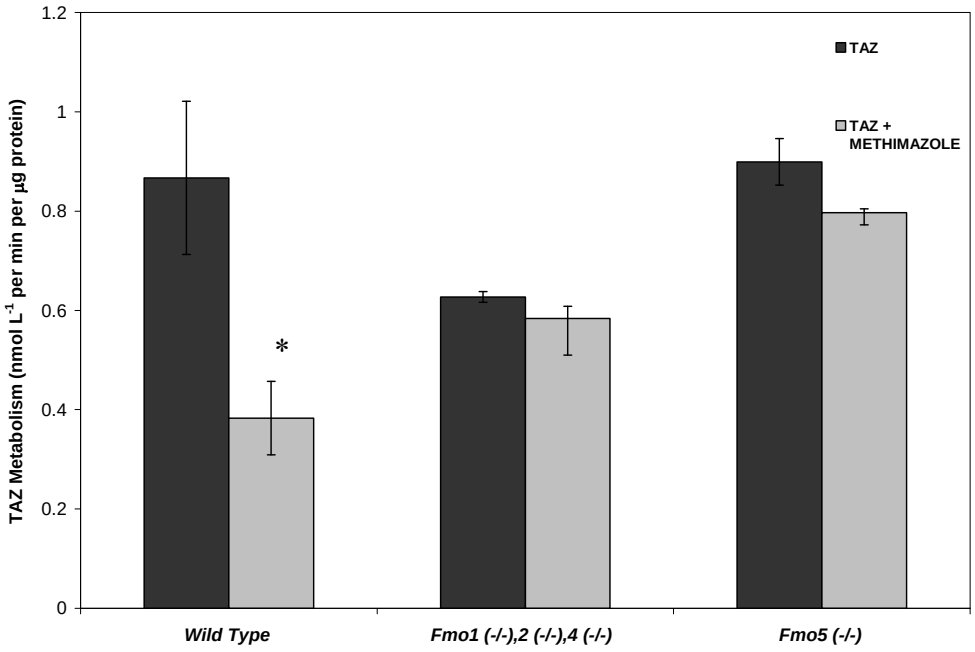


Figure 3.3.7: The effect of pre-treatment of male mouse liver microsomes with methimazole on the rate of TAZ metabolism. Pre-treatment of liver microsomes with methimazole is described in section 2.10.3.1. The rate of TAZ metabolism catalysed by liver microsomes prepared from male wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

**p<0.05* compared to the result obtained from untreated wild type male liver microsomes.

microsomes ($p<0.05$) and not for the *Fmo* knockout lines. This may indicate a lesser contribution of FMOs to TAZ metabolism in male mice.

Although the use of methimazole as a measure of FMO contribution is accepted, methimazole is not a substrate for FMO5 and therefore will not affect its activity towards TAZ (Cherrington *et al.* 1998). Some reports also claim that methimazole may inhibit the activity of certain CYP isoenzymes (Guo *et al.* 1991). To gain more of an understanding of FMO contribution to TAZ metabolism, liver microsomes were pre-heated to inactivate FMOs. Unlike CYPs, FMOs are relatively heat labile and this property has been exploited to distinguish between FMO and CYP activity *in vitro* (Rawden *et al.* 2000; Stormer 2000; Virkel *et al.* 2006). Figure 3.3.8 illustrates the effects of heat inactivation on TAZ metabolism activity for female liver microsomes. There is a significant reduction in activity for wild type ($p<0.005$) and *Fmo5* (-/-) ($p<0.05$) female liver microsomes, but not for microsomes isolated from livers of *Fmo1* (-/-), *2* (-/-), *4*(-/-) mice ($p>0.05$). A 42% and 36% decrease in activity is seen for wild type and *Fmo5* (-/-) female liver microsomal samples respectively. For male liver microsomes, there was no significant difference in TAZ metabolism by liver microsomes isolated from wild type, *Fmo1* (-/-), *2* (-/-), *4*(-/-) or *Fmo5* (-/-) mouse lines after heat treatment ($p>0.05$) (Fig. 3.3.9). The reduction in activity in wild type male liver microsomes observed after methimazole treatment (Fig. 3.3.7) may therefore indicate the inhibition of a CYP rather than an FMO. These findings suggest a sex-specific difference in TAZ metabolism in liver of a mouse. To ensure that FMO activity had fully diminished after heat inactivation, samples were tested for activity towards methimazole (see section 2.10.3.1) before and after heating. No TNB oxidation was observed after heat treatment (data not shown) indicating that FMOs were no longer active.

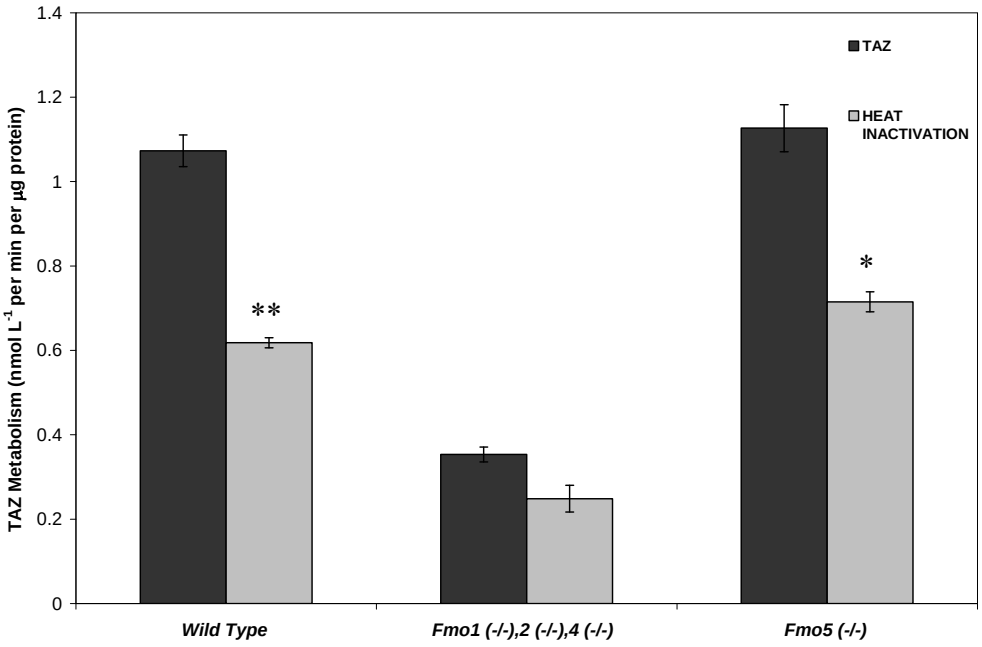


Figure 3.3.8: The effect of pre-heat treatment of female liver microsomes on TAZ metabolism. Heat inactivation of FMOs in liver microsomes is described in section 2.10.3.2. The rate of TAZ metabolism catalysed by liver microsomes prepared from female wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

**p<0.05* compared to the result obtained from untreated *Fmo5 (-/-)* female liver microsomes.

***p<0.005* compared to the result obtained from untreated wild type female liver microsomes.

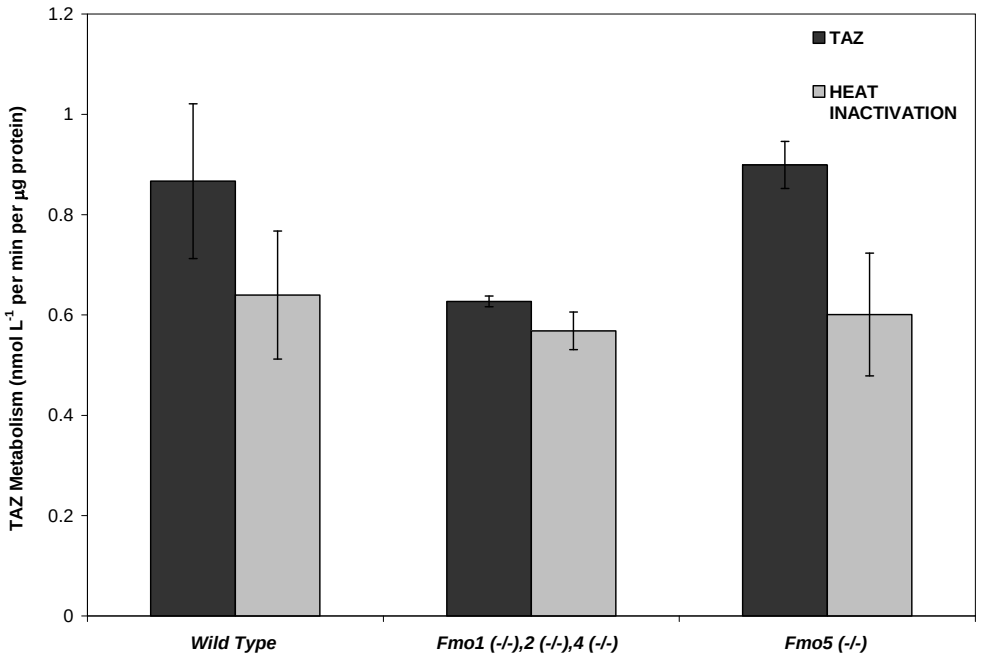


Figure 3.3.9: The effect of pre-heat treatment of male liver microsomes on TAZ metabolism. Heat inactivation of FMOs in liver microsomes is described in section 2.10.3.2. The rate of TAZ metabolism catalysed by liver microsomes prepared from male wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

As TAZ metabolism was not completely abolished by methimazole treatment or heat inactivation, it indicates that another enzyme or group of enzymes are involved in the metabolism of TAZ.

Assessing CYP Contribution in Liver Microsomes to TAZ Metabolism

The relative contribution of CYPs was investigated using an antibody specific to NADPH-dependent Cytochrome P450 reductase. This antibody will inactivate all liver microsomal CYP activity regardless of the CYP form. NADPH-dependent Cytochrome P450 reductase (CRP) are FAD and FMN containing enzymes which transfer electrons to CYPs. By inhibiting CRP, CYPs can no longer function as there is no electron donor from which they can accept an electron (as a result of the absence of reduced cytochrome P450 reductase). Therefore the use of an antibody against CRP is a useful tool to investigate the relative contribution of CYP to a given substrate *in vitro*. The amount of NADPH-dependent Cytochrome P450 Reductase antiserum was optimised by testing increasing volumes of antiserum on rates of TAZ metabolism catalysed by microsomal samples until no further change in the rate of TAZ metabolism was observed (see section 2.10.3.4). Non-immune sera was used to approximate the amount of non-specific inhibition. Non-specific inhibition of TAZ metabolism was found to be ~20%. The actual amount of inhibition by NADPH-dependent Cytochrome P450 Reductase antiserum was corrected for the non-specific inhibition (see section 2.10.3.4). Figure 3.3.10 shows the effect of CYP inactivation on the rate of TAZ metabolism in isolated female liver microsomes. CYP inactivation caused a significant decrease in TAZ metabolism by the wild type and two *Fmo* knockout mouse lines. A 44%, 63% and 52% decrease in

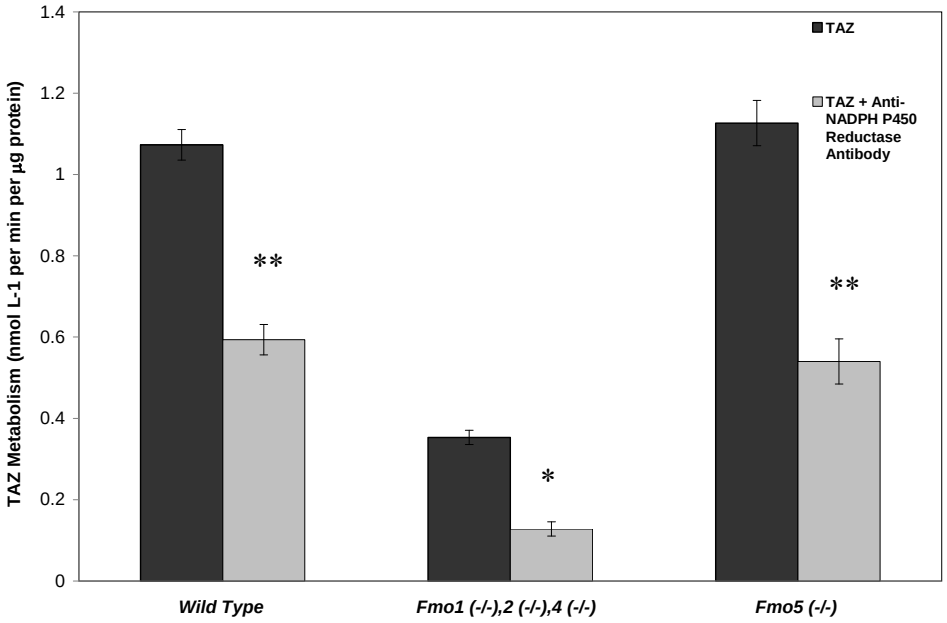


Figure 3.3.10: The effect of Anti-NADPH-dependent Cytochrome P450 reductase serum on TAZ metabolism by female liver microsomes. Inactivation of P450s in liver microsomes is described in section 2.10.3.4. The rate of TAZ metabolism catalysed by liver microsomes prepared from female wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

* $p<0.05$ compared to the result obtained from untreated female *Fmo1 (-/-),2 (-/-),4 (-/-)* liver microsomes.

** $p<0.005$ compared to the result obtained from untreated female wild type or *Fmo5 (-/-)* female liver microsomes respectively.

TAZ metabolism was observed for microsomes from wild type ($p<0.005$), *Fmo1* (-/-), *2*(-/-), *4*(-/-), ($p<0.05$) and *Fmo5* (-/-) mice ($p<0.005$) respectively. In males, inactivation of CYPs caused a significant decrease in TAZ metabolism by liver microsomes isolated from all three mouse lines. Wild type, *Fmo1* (-/-), *2* (-/-), *4*(-/-) and *Fmo5* (-/-) microsomes had a reduction of 61%, 64% and 75% respectively (Fig. 3.3.11). This confirms that CYPs, as well as FMOs contribute towards the metabolism of TAZ by mouse liver microsomes.

Figure 3.3.12 summarises the results obtained after heat inactivation (FMO inactivation) or after anti-CRP incubation (CYP inactivation) in male and female wild type liver microsomes. The reduction in TAZ metabolism after FMO or CYP inactivation in female samples was 42% and 44% respectively whereas in male liver microsomes CYP inactivation led to a 61% decrease in enzyme activity but no significant difference was observed after FMO inactivation. When FMO and CYPs were both inactivated (heat inactivation and anti-CRP incubation) the effect was additive. In the case of female liver microsomes inactivation of FMO and CYPs resulted in an insignificant amount of enzyme activity being observed but in the male microsomal samples, approximately 23% of TAZ metabolism was still present (data not shown).

Data reported here suggests that FMO and CYPs are involved to similar degrees in the metabolism of TAZ in liver microsomes of female wild type mice, but not in liver microsomes of male mice. In male liver microsomal samples (of wild type mice), data suggests a CYP-mediated enzymatic reaction as the major pathway and the possibility of a third, unidentified enzyme or group of enzymes to contribute as well. Analysis of data from the two knockout lines implicates FMO1 as the major FMO

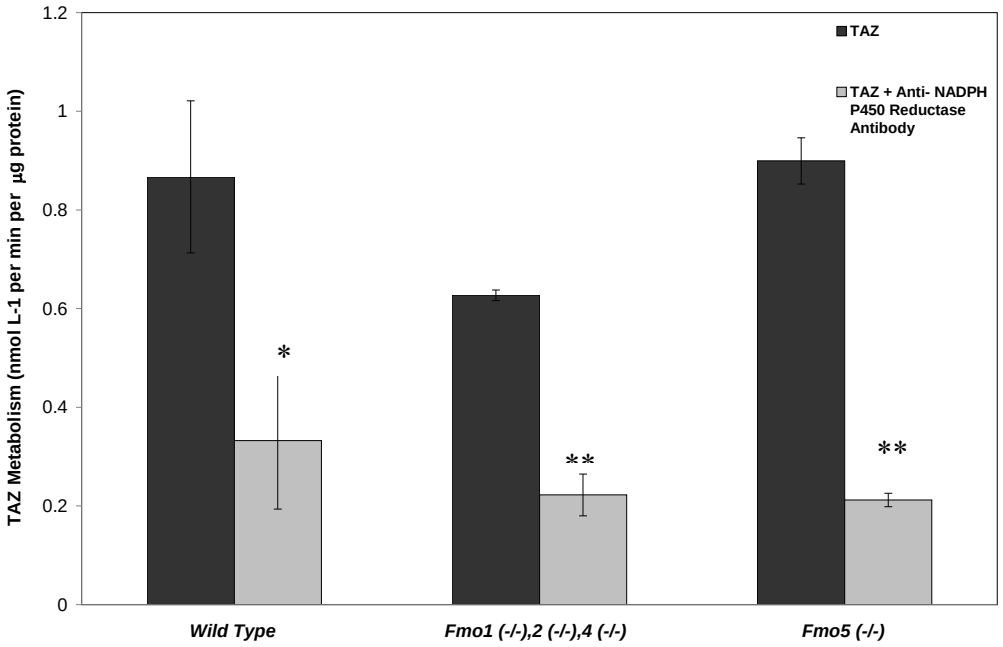


Figure 3.3.11: The effect of Anti-NADPH-dependent Cytochrome P450 reductase serum on TAZ metabolism by male liver microsomes. Inactivation of P450s in liver microsomes is described in section 2.10.3.4. The rate of TAZ metabolism catalysed by liver microsomes prepared from male wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

* $p<0.05$ compared to the result obtained from untreated male wild type liver microsomes.

** $p<0.005$ compared to the result obtained from untreated male *Fmo1* (-/-), *2* (-/-), *4* (-/-) or *Fmo5 (-/-)* female liver microsomes respectively.

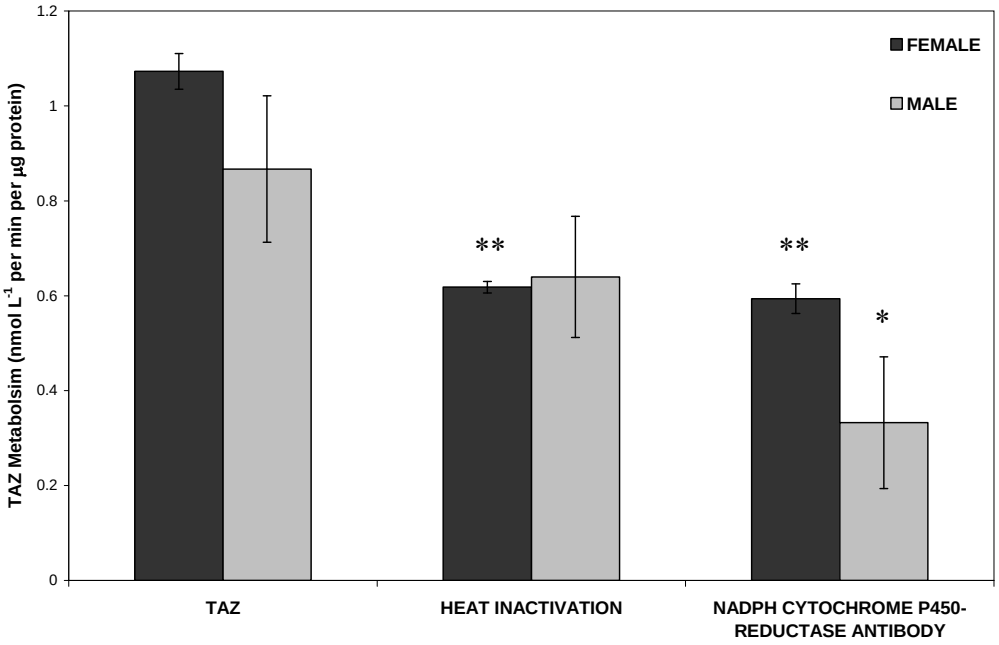


Figure 3.3.12: Comparison of the effects of pre-heat treatment and Anti-NADPH-dependent Cytochrome P450 reductase serum incubation on the rate of TAZ metabolism by male and female liver microsomes. Pre-heat treatment and incubation with NADPH-dependent cytochrome P450-Reducatse antibody of liver microsomes is described in section 2.10.3. The rate of TAZ metabolism catalysed by liver microsomes prepared from male and female wild type, *Fmo1* (-/-), *2* (-/-), *4* (-/-) and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

*$p<0.05$ compared to the result obtained from untreated male liver microsomes.

**$p<0.005$ compared to the result obtained from untreated female liver microsomes.

isoform to be involved in TAZ metabolism by female liver microsomes but not by male liver microsomes.

TAZ Metabolism by Lung Microsomes

Lung microsomes were prepared from wild type (C57BL/6 mice), *Fmo1* (-/-), *2* (-/-), *4* (-/-) and an *Fmo 5* (-/-) mouse line as described in section 2.10.1. The rate of TAZ metabolism catalysed by mouse liver microsomes was monitored spectrophotometrically and is described in section 2.10.2.2. Data represent mean ± standard deviation obtained from two independent microsomal isolations. All assays were carried out in triplicate on each batch of microsomes.

Figure 3.3.13 represents Michaelis-Menten plots (vo versus [TAZ]) obtained for female lung microsomes prepared from the three mouse lines. Lung microsomes prepared from wild type and *Fmo5* (-/-) mice catalysed TAZ metabolism and showed a hyperbolic Michaelis-Menten relationship between initial rate of enzyme activity and substrate concentration. Microsomes prepared from *Fmo1* (-/-), *2* (-/-), *4* (-/-) mouse lung however were not found to metabolise TAZ, suggesting that one or all of the three genes deleted (*Fmo1, 2 or 4)* are important in TAZ metabolism in the lung. To test whether the lack of activity towards TAZ is because of the absence of FMO protein or because the lung microsomal samples were inactivated during isolation, a control experiment was carried out. In the control experiment the activity of NADPH-dependent Cytochrome P450 reductase was measured using the substrate cytochrome *c*. The methimazole assay was not used because mouse lung have low amounts of FMO3, therefore in the absence of FMO1, FMO2 and FMO4, little or no activity towards this substrate would be expected. The activity of NADPH-dependent

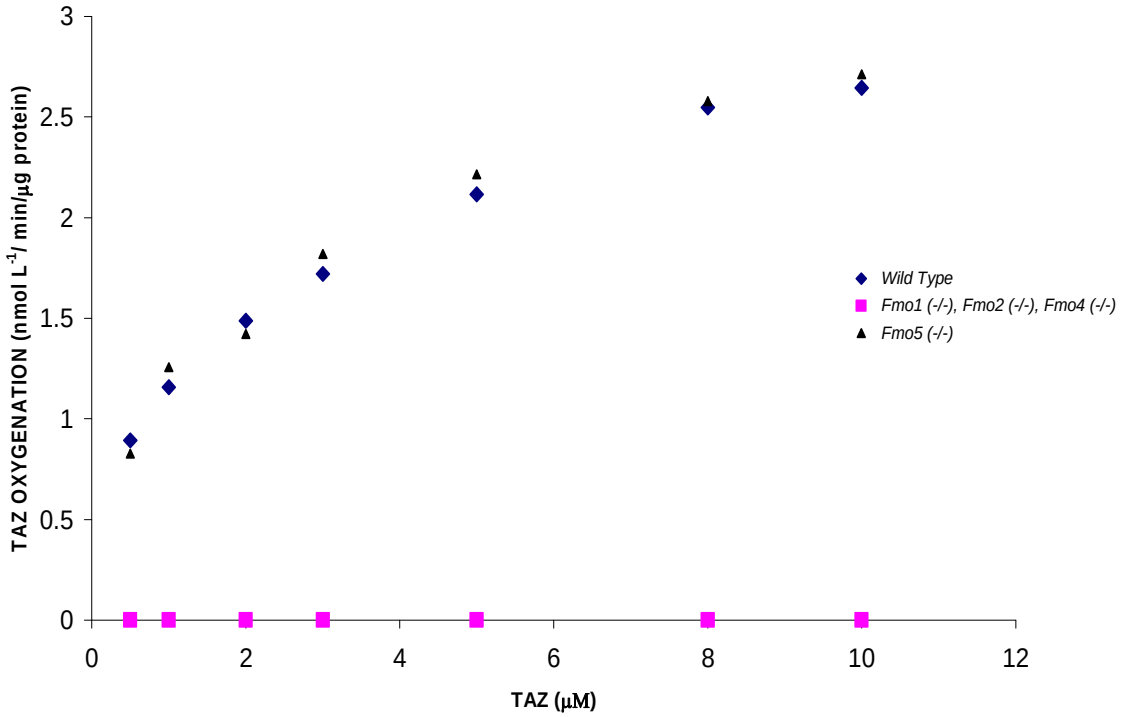


Figure 3.3.13: vo versus [TAZ] plot for TAZ metabolism catalysed by female mouse lung microsomes. The rate of TAZ metabolism catalysed by wild type female mouse lung microsomes was determined as described in section 2.10.2. Data are mean ± standard deviation from assays carried out in triplicate.

NADPH-dependent Cytochrome P450 reductase was assayed by measuring the initial rate of cytochrome *c* reduction [Omura *et al.* 1970]. Figure 3.3.14 shows the activity of lung microsomes isolated from female *Fmo1* (-/-), *2*(-/-), *4*(-/-) and female wild type mice. The rate of cytochrome *c* reduction by *Fmo1* (-/-), *2*(-/-), *4*(-/-) lung microsomes is similar to that catalysed by wild type microsomes and therefore shows that the microsomes from the knockout line are active.

Wild type and *Fmo5* (-/-) lung microsomes showed Michaelis-Menten behaviour over the tested TAZ concentrations (Fig. 3.3.13). At saturating TAZ concentrations, lung microsomes from female and male wild type and *Fmo5* (-/-) mice showed a significant difference in TAZ metabolism *($p<0.05$)* (Fig. 3.3.15). Female lung microsomal samples from both mouse lines were more efficient at TAZ metabolism than were microsomes from male lung samples (Fig. 3.3.15). There was no significant difference in TAZ metabolism between female wild type and female *Fmo5* (-/-) lung microsomes (Fig. 3.3.15) indicating that FMO5 is unlikely to be contributing to TAZ metabolism in female lung microsomes. In males however, lung microsomal samples from *Fmo5* (-/-) mice showed a significant decrease in TAZ metabolism compared to the wild type microsomes *($p<0.005$)* (Fig. 3.3.15). The results indicate a sex-dependent difference in mouse lung towards TAZ and also indicate a role for FMO5 in male lung microsomes. A possible explanation for the observed activity in the *Fmo*5 (-/-) microsomal samples may be that the lack of FMO5 in lung leads to an up-regulation of another FMO or CYP to compensate for the role FMO5 would normally play in the lung.

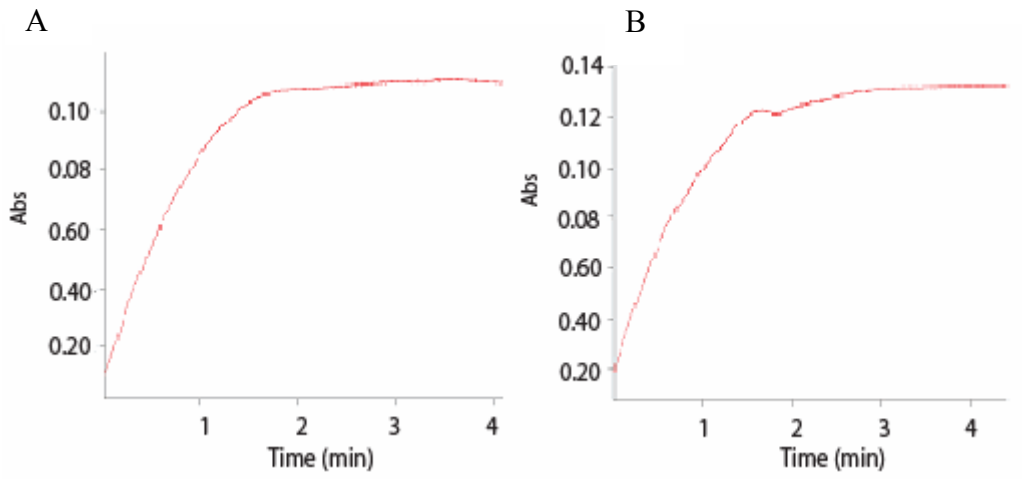


Figure 3.3.14: The reduction of cytochrome *c* catalysed by wild type and *Fmo1* (-/-), *2* (-/-), *4* (-/-) lung microsomes. The rate of cytochrome *c* reduction was monitored as described in section 2.10.4. The change in absorbance signal at 550 nm representing the reduction of cytochrome *c* by lung microsomes prepared from wild type female (A) and *Fmo 1* (-/-), *Fmo2* (-/-), *Fmo4* (-/-) female mice (B) are shown.

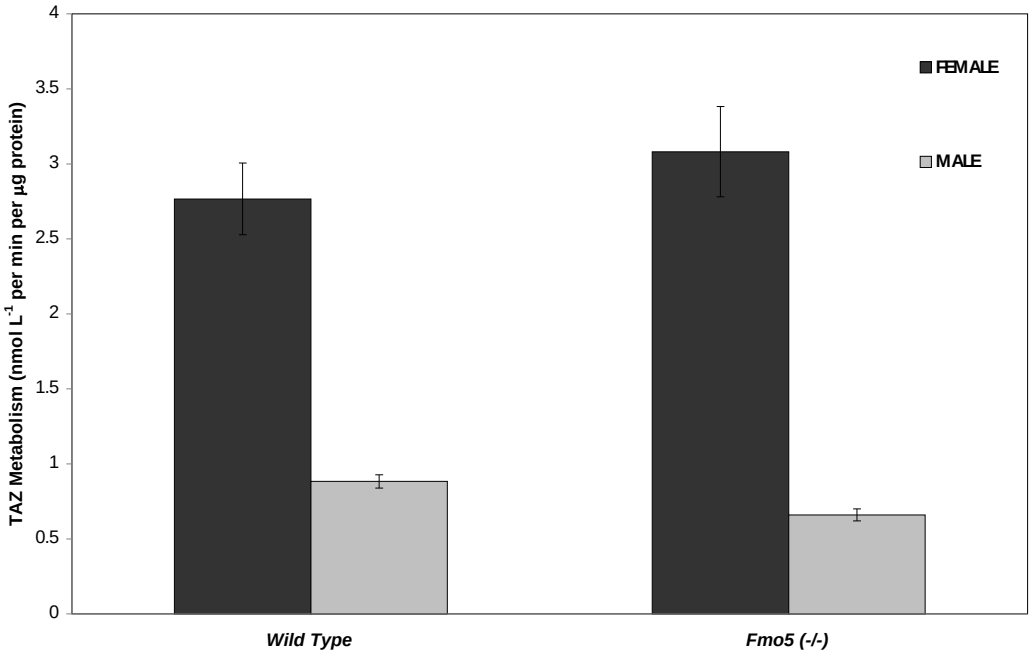


Figure 3.3.15: Rate of TAZ metabolism observed for female and male mouse lung microsomes. The rate of TAZ metabolism catalysed by lung microsomes prepared from female and male wild type and *Fmo5* (-/-) mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

***p<0.05* compared to the result obtained from female wild type or *Fmo5* (-/-) lung microsomes respectively.

Assessing FMO Contribution to TAZ Metabolism in Mouse Lung Microsomes

Methimazole was used as a competitive inhibitor to determine the contribution of FMOs to TAZ metabolism as described above. Female lung microsomes showed a significant difference in TAZ metabolism after treatment with methimazole; a 63 and 65% decrease in activity was observed for wild type and *Fmo5* (-/-) microsomes respectively (Fig. 3.3.16). For male lung microsomes treated with methimazole; a 32% and 47% decrease in activity in TAZ metabolism was observed for wild type ($p<0.005$) and *Fmo5* (-/-) ($p<0.05$) lung microsomes respectively (Fig. 3.3.17). These findings suggest that although FMOs are contributing to TAZ metabolism in the lung, FMO contribution is higher in the female microsomes than the male microsomes of this organ. This is supported by experiments shown in Figure 3.3.15 where female lung microsomal samples are observed to be more efficient at TAZ metabolism than male lung microsomes. The role of FMO5 is not confirmed by these findings (shown in Fig. 3.3.17). FMO5, unlike other members of the FMO family, does not metabolise methimazole, therefore, if one assumes the difference in TAZ metabolism between male wild type and male *FMO5* (-/-) microsomes (shown in Fig. 3.3.16) is because of the absence of FMO5, then methimazole treatment should have no effect on the rate of TAZ metabolism catalysed by *Fmo5* (-/-) lung microsomes. Figure 3.3.17 shows, however a significant decrease in TAZ metabolism in male *FMO5* (-/-) lung microsomes compared to TAZ 'only' treated *Fmo5* (-/-) microsomes ($p<0.05$). This suggests that FMO5 is unlikely to influence TAZ metabolism in the male lung microsomes. Following heat inactivation of the FMOs, a significant difference in TAZ metabolism was observed for female lung microsomes (Fig. 3.3.18). Compared to unheated lung microsomes; heating caused a reduction in TAZ metabolism of 61% and 68% by wild type and *Fmo5* (-/-) female lung microsomes

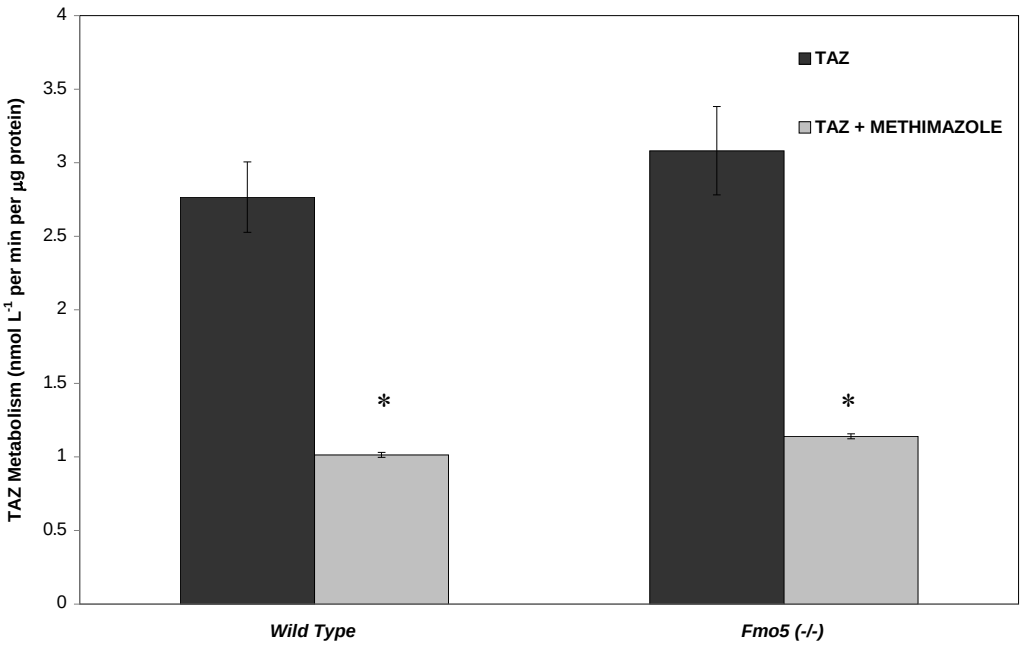


Figure 3.3.16: The effect of pre-treatment with methimazole on the rate of TAZ metabolism by female mouse lung microsomes. Pre-treatment of lung microsomes with methimazole is described in section 2.10.3.1. The rate of TAZ metabolism catalysed by lung microsomes prepared from female wild type and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

*$p<0.05$ compared to the result obtained from untreated female wild type or *Fmo5* (-/-) lung microsomes respectively.

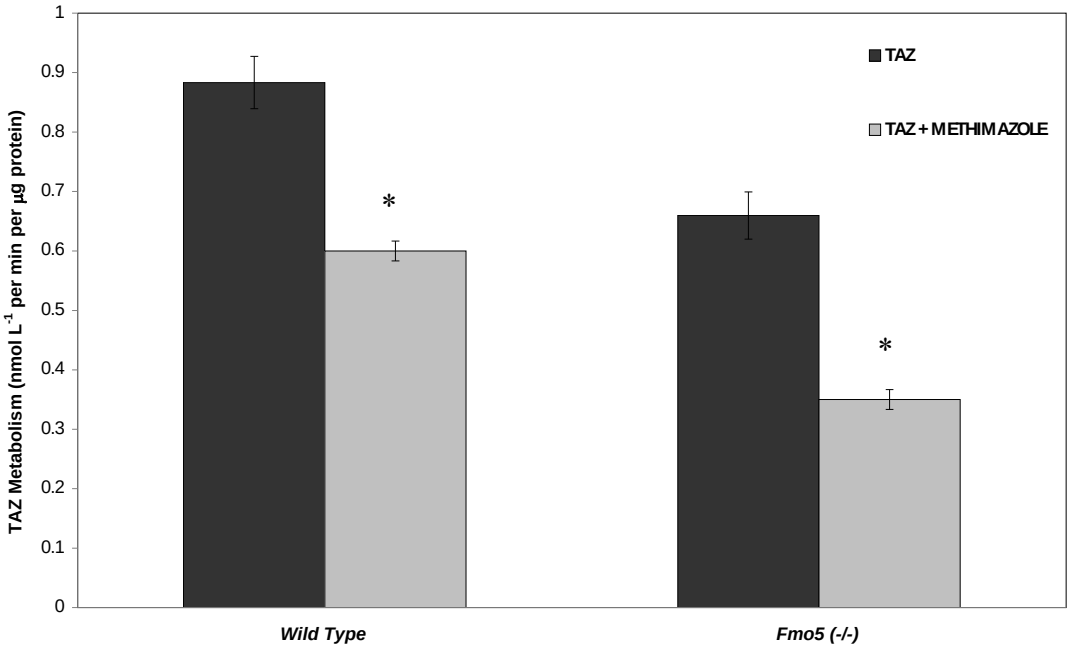


Figure 3.3.17: The effect of pre-treatment with methimazole on the rate of TAZ metabolism by male mouse lung microsomes. Pre-treatment of lung microsomes with methimazole is described in section 2.10.3.1. The rate of TAZ metabolism catalysed by lung microsomes prepared from male wild type and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

*$p<0.05$ compared to the result obtained from untreated male wild type or *Fmo5 (-/-)* lung microsomes respectively.

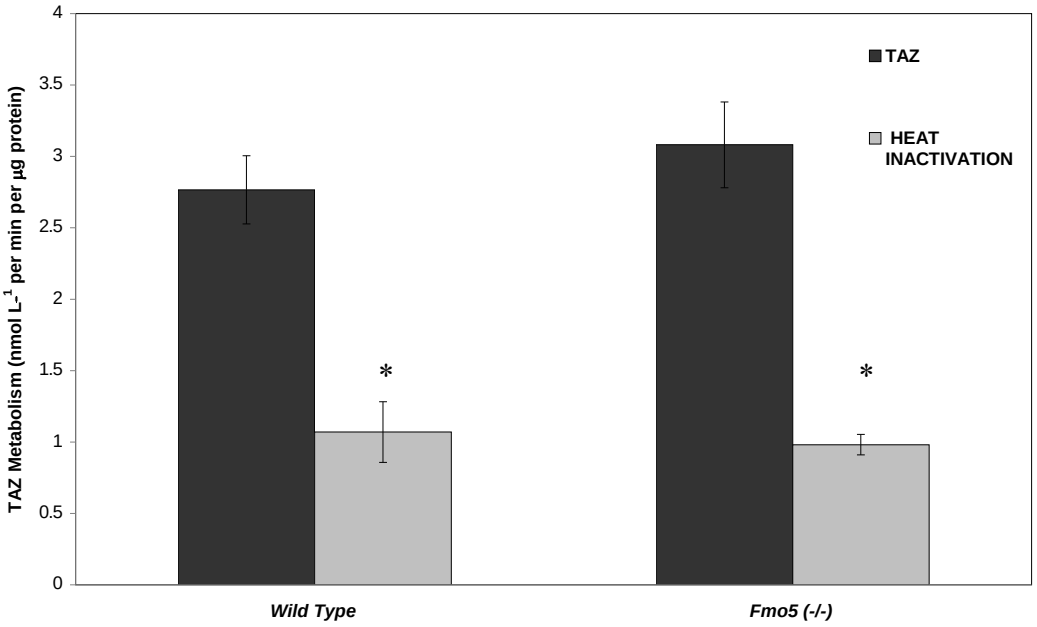


Figure 3.3.18: The effect of pre-heat treatment on TAZ metabolism observed by lung microsomes. Heat inactivation of FMOs in lung microsomes is described in section 2.10.3.2. The rate of TAZ metabolism catalysed by lung microsomes prepared from female wild type and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

*$p<0.05$ compared to the result obtained from untreated female wild type or *Fmo5* (-/-) lung microsomes respectively.

This data is in agreement with findings from methimazole treated microsomes. Heat inactivation of FMOs in male lung microsomes caused a significant decrease in TAZ metabolism by wild type lung microsomes ($p<0.05$) but not however, for *Fmo5* (-/-) lung microsomes (Fig. 3.3.19). This indicates that FMO contribution is less in the lung microsomes of the male *Fmo5* (-/-) mouse line.

Assessing CYP Contribution to TAZ Metabolism in Mouse Lung Microsomes

As described earlier, anti-sera to NADPH-dependent Cytochrome P450 Reductase is a tool which can be used to inactivate CYP activity. Here it has been used to deduce the relative contribution of CYPs to TAZ metabolism in mouse lung microsomes. Non- specific inhibition of TAZ metabolism by serum addition was found to be ~20%.

The effects of pre-incubating microsomes with NADPH-dependent Cytochrome P450 Reductase antibody shows that in both male and female lung samples a significant decrease in TAZ metabolism had occurred ($p<0.05$) (Fig. 3.3.20 and 3.3.21). For female lung microsomes there was a 31 and 57% reduction in activity for wild type and *Fmo5* (-/-) mouse lines respectively and in males a 34 and 31% decrease in activity of wild type and *Fmo5* (-/-) lungs respectively. These findings confirm that CYPs contribute to the metabolism of TAZ in mouse lung microsomes.

Figure 3.3.22 summarises the results obtained after heat inactivation (FMO inactivation) or after anti-CRP incubation (CYP inactivation) in male and female wild type lung microsomes. The reduction in TAZ metabolism after FMO or CYP inactivation in female samples was 63% and 31% respectively whereas in male liver

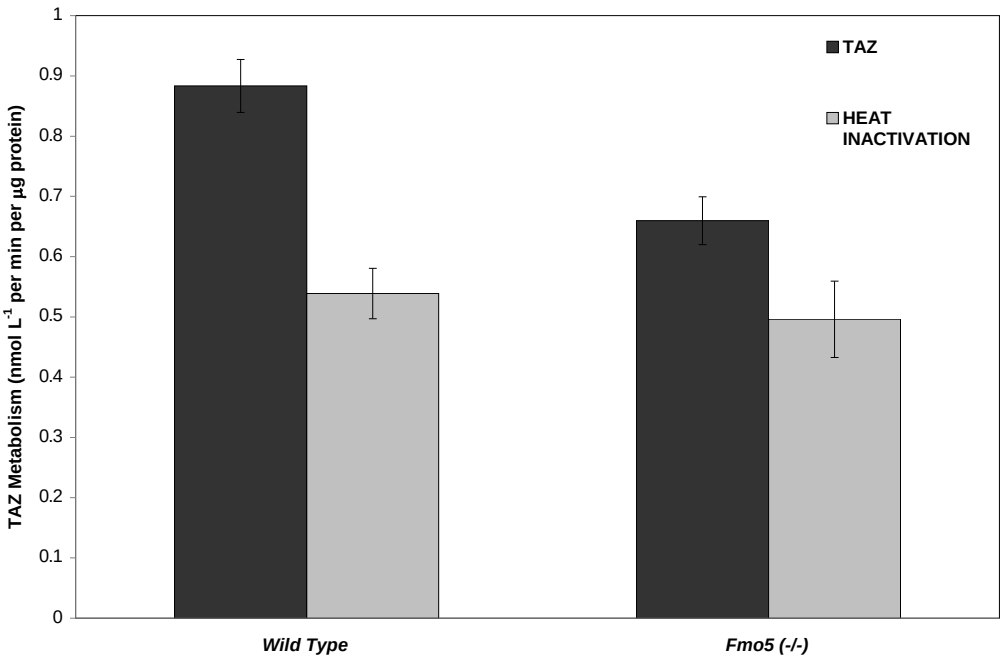


Figure 3.3.19: The effect of pre-heat treatment on TAZ metabolism by male lung microsomes. Heat inactivation of FMOs in lung microsomes is described in section 2.10.3.2. The rate of TAZ metabolism catalysed by lung microsomes prepared from male wild type and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

*$p<0.05$ compared to the result obtained from untreated male wild type lung microsomes.

microsomes FMO and CYP inactivation led to a 32% and 34% decrease in enzyme activity respectively. When FMO and CYPs were both inactivated (heat inactivation and anti-CRP incubation) the effect was additive. In the case of female liver microsomes inactivation of FMO and CYPs resulted in an insignificant amount of enzyme activity being observed but in the male microsomal samples, approximately 25% of TAZ metabolism was still present (data not shown). Data reported here suggests that FMO is the major enzymatic pathway involved in the metabolism of TAZ in the lung microsomes of female wild type mice, but not in lung microsomes of male mice. In male lung microsomal samples (of wild type mice) the contribution of FMOs and CYPs is similar, the sum of FMO and CYP contribution however does not equal the total amount of TAZ metabolism observed in this tissue. This may indicate a role of another enzyme in the metabolism of TAZ in male lung microsomes of mice. The relative contribution of FMO1 and FMO2 to TAZ metabolism could not be deduced, mainly because methimazole is a substrate for both isoforms. A probe substrate for FMO1 could have been imipramine (Kim *et al.* 2000; Hernandez *et al.* 2004), however this compound is also a substrate for some CYPs. Further studies are required to elucidate the relative contribution of FMO1 and FMO2 to TAZ metabolism *in vitro*

Comparison between Liver and Lung Microsomes With Respect to TAZ Metabolism

Figure 3.3.23 shows the rate of TAZ metabolism for female liver and lung microsomes. There is a significant difference between activities of lung and liver microsomes isolated from female wild type mice ($p<0.005$). Lung microsomes are more efficient at TAZ metabolism than liver microsomes from female wild type mice.

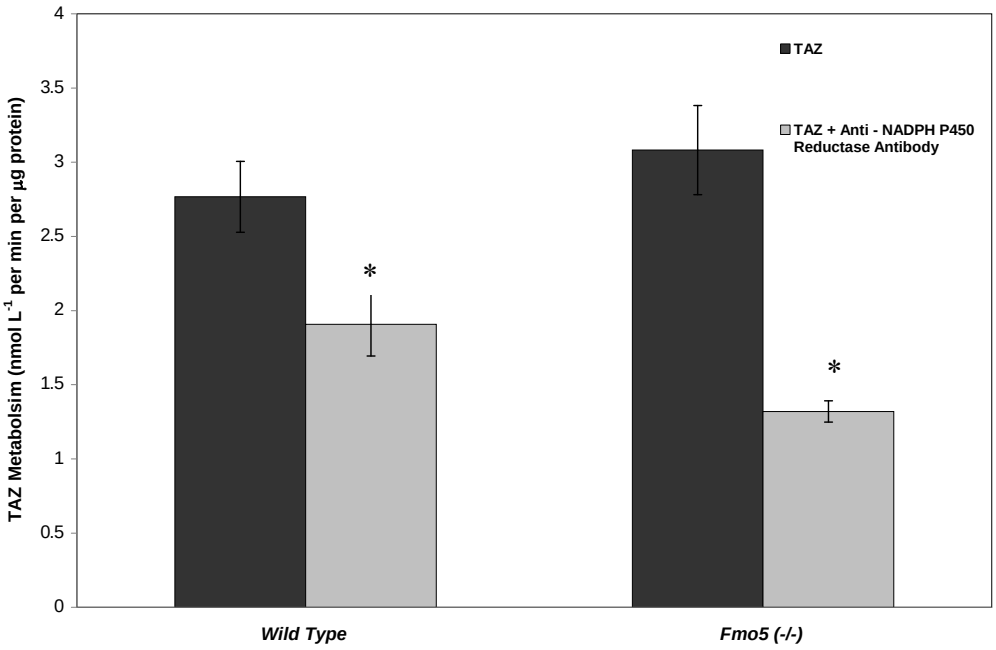


Figure 3.3.20: The effect of Anti-NADPH-dependent Cytcrome P450 reductase serum on TAZ metabolism observed for female lung microsomes. Inactivation of NADPH-dependent Cytochrome P450 reductase in lung microsomes is described in section 2.10.3.3. The rate of TAZ metabolism catalysed by lung microsomes prepared from female wild type and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

* $p<0.05$ compared to the result obtained from untreated female wild type or *Fmo5 (-/-)* lung microsomes respectively.

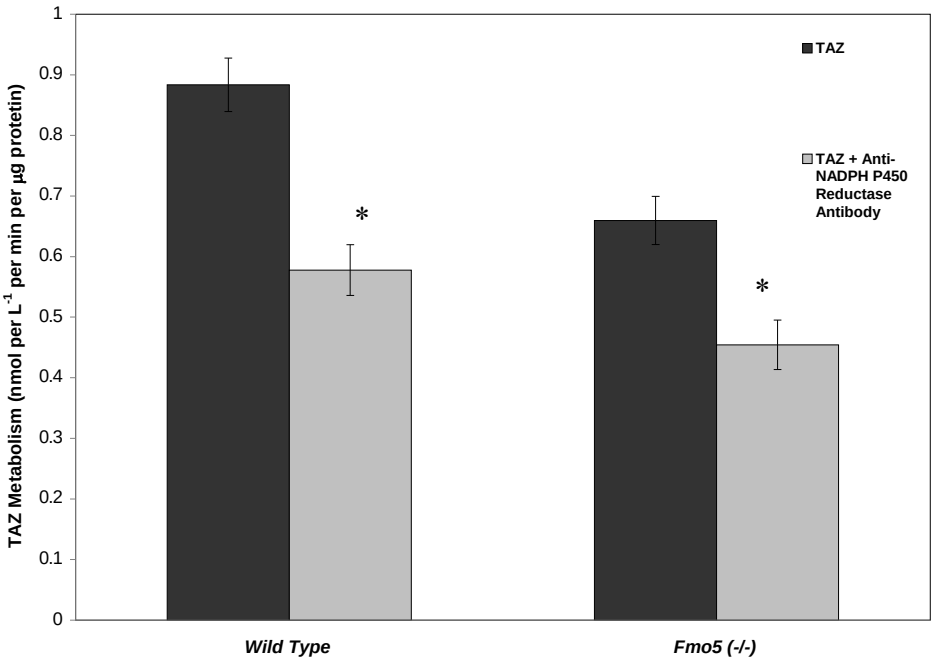


Figure 3.3.21: The effect of Anti-NADPH-dependent Cytochrome P450 reductase serum on TAZ metabolism by male lung microsomes. Inactivation of P450s in lung microsomes is described in section 2.10.3.3. The rate of TAZ metabolism catalysed by lung microsomes prepared from male wild type and *Fmo5 (-/-)* mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

* $p<0.05$ compared to the result obtained from untreated male wild type or *Fmo5 (-/-)* lung microsomes respectively.

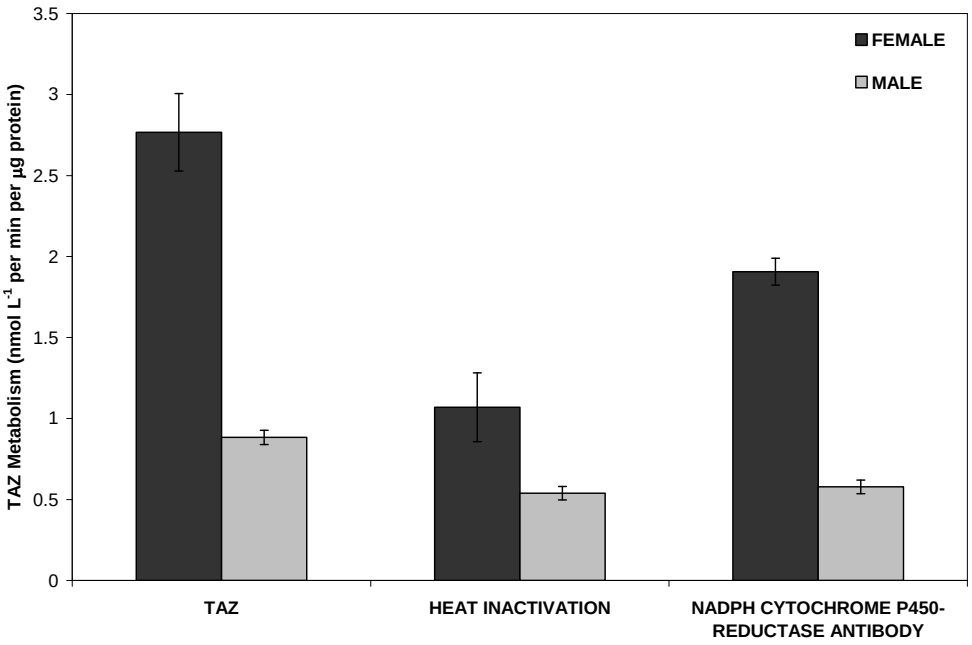


Figure 3.3.22: Comparison of the effects of pre-heat treatment and Anti-NADPH Cytochrome P450 reductase antibody incubation on the rate of TAZ metabolism observed for male and female lung microsomes. Pre-heat treatment and incubation with anti-NADPH-dependent Cytochrome P450-Reductse antibody of liver microsomes is described in section 2.10.3.2 and 2.10.3.3. The rate of TAZ metabolism catalysed by lung microsomes prepared from male and female wild type and *Fmo5* (-/-) mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

However, there is no significant difference in TAZ metabolism by male lung and liver microsomes isolated from wild type mice. This indicates a sex-dependant difference within the capacity to metabolise TAZ in mouse lung and liver.

In summary, experiments carried out in this section describe the relative contribution of FMOs and CYPs to the metabolism of TAZ in microsomes prepared from lung and liver of mouse. The data confirms a role for both FMOs and CYPs in the *in vitro* metabolism of TAZ. Sex- and tissue-specific differences in TAZ metabolism have been identified.

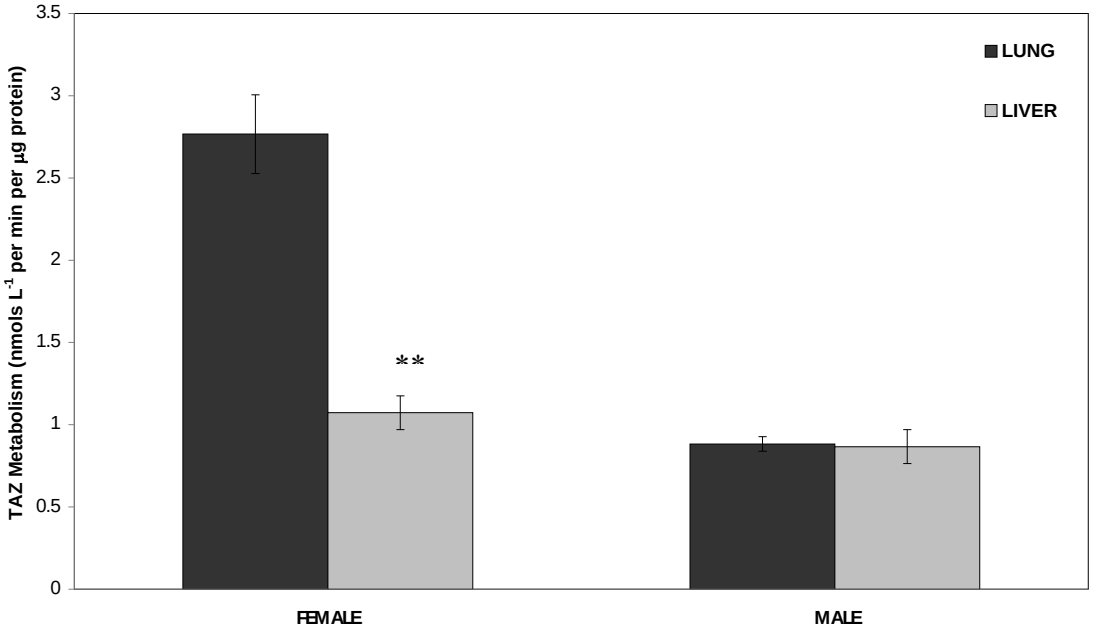


Figure 3.3.23: Comparison of the rate of TAZ metabolism by lung and liver microsomes prepared from male and female wild type mice. The rate of TAZ metabolism catalysed by lung and liver microsomes prepared from male and female wild type mice was determined as described in section 2.10.2. Data are mean ± standard deviation obtained from two independent experiments. Enzyme assays were carried out in triplicate.

***p<0.005*

Discussion - Metabolism of Thiacetazone by Mouse Tissue Microsomes

Studies of drug metabolism traditionally examine the role of Cytochrome P450s (CYPs) in this process. CYPs are a large family of enzymes and considered to be the most important group in Phase I metabolism. Members of this family exhibit broad and overlapping substrate specificities towards therapeutic compounds and other xenobiotics. In recent times however, the role of flavin containing monooxygenases (FMOs) has become of increasing interest.

Numerous substrates of therapeutic importance are metabolised by both CYPs and FMOs, these include the estrogen receptor modulator, Tamoxifen prescribed in the treatment of breast cancer (Parte *et al.* 2005), the anti-depressant imipramine (Kim *et al.* 2000; Hernandez *et al.* 2004) and Triclabendazole a compound used to treat fascioliasis (Virkel *et al.* 2006).

In this section, microsomes prepared from mouse liver and lung were employed to represent the pool of CYPs and FMOs expressed in a tissue *in vivo*. The relative contribution of CYPs and FMOs was investigated with reference to TAZ using a combination of approaches; inhibition of CYPs with an antiserum directed against NADPH-dependent Cytochrome P450 reductase, inactivation and chemical inhibition of FMOs, and distinguishing between different FMOs using our knowledge of their sex- and tissue-specific expression. Microsomes prepared from two *Fmo* knockout lines were used to gain further understanding of the contribution of specific FMO isoforms to TAZ metabolism.

Inhibition experiments performed on microsomes prepared from wild type female liver suggests that FMOs and CYPs are responsible for TAZ metabolism *in vitro* and that the contribution of FMOs and CYPs is similar. The metabolism of TAZ

by male liver microsomes prepared from wild type mice catalysed the reaction to a similar extent to female wild type microsomal samples. Inhibition studies however unmasked significant differences between the contribution of FMOs and CYPs to this reaction by the two sexes. In males, CYP contribution was determined to be greater than that of FMOs with the latter enzymatic contribution being insignificant. Analysis of data obtained from both sexes and the two knockout mouse lines and between both sexes implicated FMO1 to be important in the metabolism of TAZ in females but not in males. A survey of literature describing the sexual dimorphic expression of FMOs and CYPs was undertaken to try to explain this finding. Examples of sex-specific expression of CYPs and FMOs in the liver are documented. In the mouse, CYP2D9 is highly expressed in the liver of males but is repressed in the female (Sakuma *et al.* 2004), whereas hepatic expression of CYP2A4 and CYP2B9 is predominant in the liver of females (reviewed by Waxman *et al.* 2009). Studies of FMO mRNA expression have reported FMO1 transcript levels to be significantly higher in the liver of female mouse (Janmohamed *et al.* 2004) whereas FMO3 is not expressed in the liver of adult males (Cherrington *et al.* 1998). Given that liver microsomes obtained from male *Fmo1*(-/-), *2* (-/-), *4* (-/-) mice showed significantly higher levels of TAZ metabolism than female liver microsomes and the insignificant effect of heat inactivation on these samples, suggests that FMOs (and FMO1 in particular) are not responsible for the metabolism of TAZ observed in male microsomal samples. Therefore it is likely that the sex-specific expression of a CYP isoform was responsible for the difference in TAZ metabolism observed in between male and female liver microsomes. It was beyond the scope of this investigation to determine the exact CYP isoform responsible for the sex-specific difference.

After CYP and FMO inactivation in male liver microsomal samples, enzyme activity representing approximately a third of the control was still observed, indicating the contribution of a third enzyme present in male liver but not female liver microsomal samples.

Lung microsomal samples prepared from female wild type and *Fmo5* (-/-) mice were significantly more efficient at TAZ metabolism than male microsomal samples of this tissue. The contribution of FMOs to TAZ metabolism by male lung microsomes was identified but this was about half of that determined for female lung microsomes. This finding, like that observed in the liver microsomes suggests a sex-specific difference in TAZ metabolism in the lung microsomes of male mice. A literature survey of sex-specific expression of FMO and CYP abundance in lung reports significantly higher levels of FMO1 in the female mouse than in males (Janmohamed *et al.* 2004). Gene expression studies have identified 24 CYP forms expressed in mouse lung, among these *Cyp 2g1* is specific to this tissue and *Cyp 2f2* is the most highly expressed (Choudhary *et al.* 2005), however literature relating to sex-specific expression of CYPs in the lung was not found. After both FMO and CYP inactivation in male lung microsomes significant activity towards TAZ is observed. This was not the case for female lung microsomes whose activity towards TAZ was abolished when both FMO and CYP activity were inhibited. The findings illustrate a sex-specific difference in both lung and liver microsomes of male mice and implicate a third enzyme in TAZ metabolism.

Lung microsomes from male and female *Fmo*1 (-/-), *Fmo*2 (-/-), *Fmo*4 (-/-) mice showed no activity towards TAZ. The microsomes demonstrated the ability to reduce cytochrome *c* and therefore were 'active' with respect to CYP activity. From this finding it was expected that one or all of the deleted *Fmo*s (expressing FMO1,

FMO2 and FMO4) would be responsible for the total amount of TAZ metabolism observed in wild type lung microsomes. But as described above, metabolism of TAZ was still observed after FMO inactivation by heat or methimazole treatment. This result is perplexing, it cannot be explained why microsomes of the knockout line did not show CYP-mediated TAZ metabolism when this enzyme group is contributing to the reaction in wild type microsomes.

In the lung of mouse, FMO1 and FMO2 are considered the major pulmonary isoforms (Janmohamed *et al.* 2004; Siddens *et al.* 2008). In this investigation it was not possible to distinguish the relative contribution of these isoforms in microsomes of this tissue, the reason being the lack of an appropriate probe substrate to distinguish between FMO1 and FMO2 activity. Some reports have claimed that mouse FMO2 is considerably more resistant to heat inactivation than other members of this family and that this property can be used to distinguish FMO2 activity (Siddens *et al.* 2008). This was not observed in experiments carried out as part of this investigation as microsomes were tested for their ability to oxygenate methimazole before and after heat treatment. TNB oxidation was completely abolished after heat treatment and suggests that all FMOs were inactivated.

Lung and liver microsomes prepared from wild type female mice displayed a hyperbolic relationship between enzyme activity and TAZ concentration, indicating Michaelis-Menten kinetics. Given that FMOs and CYPs were contributing to TAZ metabolism in these samples, may suggest that the affinity of this substrate for the two enzymes is similar at the tested concentrations.

In summary, this section has described the *in vitro* metabolism of TAZ by liver and lung microsomes prepared from mouse. The relative contribution of CYPs and FMOs has been determined and an insight into the possible FMO isoform

involved in this reaction has been gained. Further studies are required to identify CYP isoforms involved in this reaction and also to identify other enzymes which are likely to be involved in TAZ metabolism. Additional studies are required to identify the products generated by CYP-mediated metabolism of TAZ.

Chapter 4: Summary

4.1 Summary

TAZ and ETA are anti-tubercular drugs prescribed to treat TB in the poorest parts of the world and in industrialised countries respectively. With TB announced as a pandemic by the WHO and the dramatic increase in MDR- and X-MDR- TB cases, development of new and effective drugs is a race against time. Some researchers and medical professionals alike have resorted to the re-introduction of TAZ to chemotherapy regimens in an attempt to use whatever is at hand. There are two main obstacles in the way of 'reinstating' this cheap and effective drug, first the toxicity that TAZ exerts in a significant proportion of individuals and secondly the scarce amount of information regarding its metabolism in man and bacteria. The metabolism of TAZ in the latter has provided the focus of recent literature that has subsequently determined the nature of TAZ as a prodrug (Alahari *et al.* 2007), the enzyme responsible for its activation (Baulard *et al.* 2000; DeBarber *et al.* 2000; Qian *et al.* 2006; Dover *et al.* 2007; Alahari *et al.* 2009), the bacterial target of TAZ action (Alahari *et al.* 2007) and the mechanism by which it debilitates *Mycobacteria* (Alahari *et al.* 2007; Dover *et al.* 2007). Though essential pieces of information, they do not explain why or how TAZ induces derma- and hepatic toxicity. This will become clear once the *in vivo* metabolism of TAZ in man is determined and understood.

A pharmacogenetic investigation was undertaken to assess the effect of a recently identified genetic variation in FMOs in individuals of African-decent. A SNP in the human *FMO2* gene that occurs at a frequency of 100% of Asians and Europeans results in the production of a non-functional enzyme (Dolphin *et al.* 1998). Interestingly, a significant proportion of Africans and Hispanics of African-decent encode the ancestral form of the gene and consequently express functionally active FMO2 in the lungs (Dolphin *et al.* 1998; Krueger *et al.* 2002; Krueger *et al.* 2005;

Veeramah *et al.* 2008). The pharmacological implications of human FMO2.1 are yet to be identified, but given that FMOs are important in drug metabolism it is plausible to assume that individuals expressing this enzyme in the lung will display an altered metabolic profile with regard to xenobiotics in this tissue. Experiments in this investigation were used to assess the pharmacological consequences that this enzyme might have on the metabolism of TAZ and ETA.

Human FMO2.1, at least *in vitro* can catalyse the oxidation of both TAZ and ETA. In the case of ETA, FMO2.1 forms one of the products that has been identified for EtaA, that is the *S*-oxide. Given the non-toxic nature of such a metabolite, it is likely that FMO2.1 may catalyse the detoxification of this drug and in effect mediate the 'inactivation' of ETA thus decreasing the effective concentration of ETA available to the bacteria. This would result in reduced drug efficacy in such individuals and therefore may represent a genetic disadvantage. Conversely, FMO2.1 can catalyse two independent oxidation steps of TAZ to generate the same products as EtaA. Data presented in this thesis has for the first time identified the intermediate of this reaction as the sulphenic acid of TAZ. The potential implications of our findings are that, those patients given TAZ as part of TB treatment and that also express the FMO2.1 enzyme in their lung will have two activation events taking place; one by EtaA in the *Mycobacteria* and the other by FMO2.1 in healthy lung cells. The net effect will be the production of toxic, highly reactive electrophilic species and the concurrent decrease in TAZ available to *Mycobacteria*. Thus it is feasible to assume that individuals expressing FMO2.1 in the lung are likely to be at a therapeutic disadvantage if prescribed TAZ. To provide a direct link between FMO2.1 expression and drug toxicity, analysis of lung injury would be required. Although this would be important to establish, the finding will be complicated because it would be

difficult to distinguish between lung injury caused by *Mycobacterial* infection and that caused by drug toxicity as a result of TAZ-activation by FMO2.1. *Mycobacterial* infections cause lesions in the lung and further injury is caused by the oxidative activation of macrophages.

This study also assayed variant forms of the major human hepatic FMO, FMO3 for their capacity to catalyse TAZ oxygenation. The results demonstrate that some polymorphic variants of FMO3 displayed reduced catalytic activity whereas others displayed an increase in catalytic efficiency. The importance of this finding to human health is two-fold. First, in the case of the double variant K158/G308, individuals may be protected from TAZ toxicity as the enzyme has significantly reduced capacity at oxidising this compound and so more TAZ reaches the site of infection. In the second scenario those expressing the 'hyper-active' variant P360 might be predisposed to hepato- and dermatological toxicity and may require higher doses of TAZ to treat the infection. The effect of polymorphic FMO3 variants on sulindac and benzydamine metabolism has been discussed in section 3.2 and demonstrates how the outcome of a therapeutic regime may be affected by the catalytic efficiency of an FMO3 variant. Data provided in this thesis supports and strengthens this knowledge.

Although products of the reaction were not analysed, both CYPs and FMOs were found to contribute to TAZ metabolism by microsomes isolated from mouse lung and liver. There is no evidence to date that demonstrates a role for CYPs in the meabolism of TAZ in humans. Inferences from microsomes prepared from male and female mice of FMO knockout lines suggested that in the mouse, FMO1 is the major FMO involved in TAZ metabolism. However, it should be noted that species differences exist with respect to the tissue distribution of FMOs in humans and

mouse. The most notable difference being that the *FMO1* gene is switched off in human liver at birth. Thus in humans, FMO3 is the enzyme expected to produce hepatic toxicity as a result of TAZ metabolism.

Though not detailed in this thesis, preliminary *in vivo* studies of TAZ metabolism in mouse identified, for the first time, TAZ-sulphinic acid, TAZ-carbodiimide and TAZ-sulphenic acid in the kidney. This indicated a role for FMOs in the extra-hepatic metabolism of this drug. Though unexpected, this finding supports the role of FMO1 as the predominant TAZ metaboliser in mouse. Extrapolation of mouse data to humans should always be done with caution especially in the case of FMOs because of the difference in the tissue-specific expression of these enzymes stated above. In mouse and all other mammals, FMO1 is the major hepatic FMO isoform whereas humans express FMO3 in the liver and FMO1 expression is largely restricted to the kidney and skin.

It is increasingly being acknowledged that FMOs, though a small family of enzymes are still important players in the metabolism of xenobiotics and endogenous compounds when compared to the vast family of CYPs. The high expression in tissues that are vital for drug metabolism and the unique mechanism by which FMOs catalyse the oxidation of a plethora of foreign compounds provides a remarkably wide substrate palette for these enzymes. Experiments undertaken as part of this investigation have attempted to broaden the understanding of FMO catalysed reactions and to delineate the contribution of individual FMO enzymes.

Clearly the data presented in this thesis can be considered as the tip of the iceberg; nevertheless it is envisaged that the basic biochemical details regarding the metabolism of TAZ and ETA given here will provide a foundation for future work in this field. The knowledge gained of the contribution and inter-play of different FMO

genetic variants to the metabolism of a therapeutic drug may help to rationalise drug treatment regimes and we hope contribute to better health care especially in the poorer areas of the world.

Chapter 5: References

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Appendix 1

Table 1: Primer sequences used in standard PCR.

Name of primer	Orientation	Sequence (5' – 3')	Source of sequence
M13F	Forward	TCCCAGTCACGACGTCGT	pUC
M13R	Reverse	GGAAACAGCTATGACCATG	pUC

Table 2: Gene Specific Primers used in qRT-PCR

Gene	Orientation	Primer Sequence (5'-3')	Amplicon size (bp)	Source
Fmo1	Forward Reverse	TGTCTCTGGACAGTGGGAAGT CATTCCAACTACAAGGACTCG	166	(Janmohamed *et al.* 2001)
Fmo2	Forward Reverse	AGGCTCCATCTTCCCAACCGTA CCGGGTCTTTAAGGGTTTCAGG	382	(Siddens *et al.* 2008)
Fmo3	Forward Reverse	ACAACTTACCCACCGCCATCTC GGCATAACCATAGCCTGTGGCAAA	269	(Hernandez *et al.* 2009)
GAPDH	Forward Reverse	TTCACCACCATGGAGAAGGC GGCATGGACTGTGGTCATGA	237	Bioline Reference Gene Panel
18S rRNA	Forward Reverse	TTGACGGAAGGGCACCACCAG GCACCACCACCCACGGAATCG	130	Bioline Reference Gene Panel
G-Actin	Forward Reverse	AGGAGATCACAGCCCTAGCA ACATCTGCTGGAAGGTGGAC	189	Bioline Reference Gene Panel

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Human Flavin-Containing Monooxygenase 2.1 Catalyzes Oxygenation of the Antitubercular Drugs Thiacetazone and Ethionamide

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ABSTRACT:

The second-line antitubercular drugs thiacetazone (TAZ) and ethionamide (ETA) are bioactivated by the mycobacterial enzyme EtaA. We report here that human flavin-containing monooxygenase 2.1 (FMO2.1), which is expressed predominantly in the lung, catalyzes oxygenation of TAZ. The metabolites generated, the sulfenic acid, sulfinic acid, and carbodiimide derivatives, are the same as those produced by EtaA and human FMO1 and FMO3. Two of the metabolites, the sulfenic acid and carbodiimide, are known to be harmful to mammalian cells. FMO2.1 also catalyzes oxygenation of ETA, producing the *S*-oxide. We have developed a novel spectrophotometric assay for TAZ oxygenation. The assay was used to determine kinetic parameters for TAZ oxygenation catalyzed by human FMO1, FMO2.1, and FMO3 and by EtaA. Although the K_M values for the four enzyme-catalyzed reactions are similar, k_{cat} and, consequently, k_{cat}/K_M (the specificity constant) for FMO2.1-catalyzed TAZ oxygenation are much higher than those of FMO1, FMO3, or EtaA. This indicates that FMO2.1 is more effective in catalyzing TAZ oxygenation than are the other three enzymes and thus is likely to contribute substantially to the metabolism of TAZ, decreasing the availability of the prodrug to mycobacteria and producing toxic metabolites. Because of a genetic polymorphism, Europeans and Asians lack FMO2.1. However, in sub-Saharan Africa, a region in which tuberculosis is a major health problem, a substantial proportion of individuals express FMO2.1. Thus, our results may explain some of the observed interindividual differences in response to TAZ and ETA and have implications for the treatment of tuberculosis in sub-Saharan Africa.

Pulmonary tuberculosis (TB) is a serious respiratory disease caused by the opportunistic bacterium *Mycobacterium tuberculosis*. The World Health Organization estimated 9.2 million new cases of TB infection worldwide in 2006, of which 31% were in Africa. The appearance of strains of *M. tuberculosis* that are resistant to more than one first-line antitubercular drug has required the use of second-line drugs (Peloquin, 1993), such as the thiourea thiacetazone (TAZ; 4′-formylacetanilide thiosemicarbazone) and the thioamide ethionamide (ETA; 2-ethylpyridine-4-carbothioamide). TAZ has been widely used in the developing world (Brown, 1992). Although an effective treatment for multidrug-resistant TB, it can produce adverse effects such as liver toxicity, gastrointestinal disturbances, and life-threatening skin reactions, particularly in human immunodeficiency virus patients (Teklu, 1976; Brown, 1992; Peloquin, 1993; Ipuge et al., 1995), and, consequently, its use has been discontinued in several countries (Brown, 1992). ETA continues to be prescribed in both developed and developing countries.

Both TAZ and ETA are prodrugs that are converted to their active forms by the mycobacterial enzyme EtaA (Baulard et al., 2000; DeBarber et al., 2000; Qian and Ortiz de Montellano, 2006), a flavin-containing monooxygenase (FMO) (Vannelli et al., 2002). EtaA activates TAZ by two sequential oxidation steps to form a sulfinic acid and a carbodiimide via a postulated sulfenic acid intermediate (Qian and Ortiz de Montellano, 2006). TAZ treatment affects mycolic acid biogenesis in mycobacteria (Alahari et al., 2007; Dover et al., 2007), and this may be the mechanism by which the drug exerts its antimicrobial effect.

The FMOs (EC 1.14.13.8) of mammals catalyze the oxidative metabolism of numerous xenobiotics, including pesticides, fertilizers, and therapeutic drugs (Krueger and Williams, 2005; Cashman and Zhang, 2006; Phillips et al., 2007; Phillips and Shephard, 2008). Humans express five functional FMOs, FMOs 1 through 5 (Phillips et al., 1995; Hernandez et al., 2004). FMO1, FMO2, and FMO3 can

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ABBREVIATIONS: TB, tuberculosis; TAZ, thiacetazone; ETA, ethionamide; FMO, flavin-containing monooxygenase; HPLC, high-performance liquid chromatography; *Sf*, *Spodoptera frugiperda*; LC/MS, liquid chromatography/mass spectrometry; FA, formic acid; DMSO, dimethyl sulfoxide; GSH, glutathione.

bioactivate thiourea-based drugs (Smith and Crespi, 2002; Henderson et al., 2004; Onderwater et al., 2006), and FMO1 and FMO3 have been shown to catalyze oxygenation of TAZ in vitro, forming the same products as EtaA (Qian and Ortiz de Montellano, 2006).

A genetic polymorphism of the *FMO2* gene, g.23238C>T (Q472X), gives rise to an allele, *FMO2*2*, which encodes a truncated, nonfunctional protein (FMO2.2) (Dolphin et al., 1998). Essentially all Europeans and Asians are homozygous for *FMO2*2* and thus do not express functional FMO2 (Dolphin et al., 1998; Whetstine et al., 2000). However, in sub-Saharan Africa, and in populations recently descended from this region, a substantial proportion of individuals possess at least one copy of the ancestral *FMO2*1* allele, which encodes a full-length functional protein (FMO2.1) (Dolphin et al., 1998; Whetstine et al., 2000; Veeramah et al., 2008). In contrast to *FMO1* and *FMO3*, which in the adult human are expressed primarily in kidney and liver, respectively (Dolphin et al., 1996; Yeung et al., 2000; Hernandez et al., 2004; Cashman and Zhang, 2006), the main site of expression of *FMO2* is the lung (Dolphin et al., 1998; Krueger et al., 2002; Hernandez et al., 2004; Cashman and Zhang, 2006). Expression of functional FMO2.1 has been confirmed in lung microsomes from an individual heterozygous for the *FMO2*1* allele (Krueger et al., 2002).

Because TAZ and ETA act against mycobacteria in the lung, we investigated the ability of human FMO2.1 to catalyze the oxygenation of these antitubercular drugs. In this article, we show that the protein encoded by the *FMO2*1* allele, FMO2.1, catalyzes oxygenation of both TAZ and ETA, forming the same metabolites as those produced by human FMO1 and FMO3 and by the mycobacterial enzyme EtaA. Furthermore, we show that the specificity constant of FMO2.1 for TAZ is higher than that of any of the other three enzymes. Our results provide a potential explanation for some of the observed interindividual differences in the efficacy of and response to TAZ and ETA and have implications for the treatment of TB in sub-Saharan Africa and in individuals of recent African descent.

Materials and Methods

Materials. Chemical reagents, enzymes, and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Plasticware for insect cell culture was obtained from VWR (West Chester, PA). Reagents for high-performance liquid chromatography (HPLC) were obtained from Fisher Scientific (Waltham, MA) and were of HPLC grade.

Protein Expression. Recombinant bacmids encoding full-length human FMO2 (FMO2Q472; FMO2.1) and FMO3 were as described previously (Dolphin et al., 1997, 1998). A recombinant bacmid encoding human FMO1 was prepared from an FMO1 cDNA (Dolphin et al., 1991) (accession number Q01740) via site-specific transposition in *Escherichia coli*, through the use of the Bac-to-Bac system (Invitrogen, Carlsbad, CA) as described previously (Janmohamed et al., 2006). Bacmid DNAs were isolated using a modified alkaline lysis method. Production of baculovirus and expression of FMOs in *Spodoptera frugiperda* (*Sf*) 9 cells were as described previously (Janmohamed et al., 2006), except that after infection with recombinant baculovirus, cells were cultured for 96 h before harvesting. EtaA was expressed and purified as described previously (Vannelli et al., 2002).

Isolation of Microsomal Membranes. *Sf*9 cells were harvested and resuspended in HEPES buffer [0.154 M KCl, 10 mM HEPES, pH 7.4, 1 mM EDTA, 20% (v/v) glycerol]. Cells were lysed by three 12-s bursts of sonication on ice. Cell lysates were centrifuged at 1000*g* for 10 min at 4°C. The resulting supernatant was centrifuged at 100,000*g* for 1 h at 4°C. The pellet was resuspended in HEPES buffer by hand, using a glass-glass homogenizer placed on ice, and stored in aliquots at −80°C. Protein concentration was determined using the method of Lowry (D_C Protein Assay kit; Bio-Rad, Hercules, CA) and bovine serum albumin (Bio-Rad) as a standard.

Quantification of FMOs. Antibodies to FMO1, FMO2, and FMO3 were a gift from Dr. R. Philpot. Heterologously expressed FMOs were quantified by Western blotting essentially as described previously (Dolphin et al., 1997, 1998). Blots were incubated with goat anti-(rabbit FMO1), goat anti-(rabbit FMO2), or goat anti-(rabbit FMO3) serum (1 in 3000 dilution), then with a rabbit anti-(goat IgG)-alkaline phosphatase conjugate (1 in 30,000 dilution). Antigen was visualized through the use of a color development kit (AP Conjugate Substrate Kit; Bio-Rad). The concentration of each expressed FMO was determined by scanning densitometry and Image Gauge software, version 4.2.1 (Science Lab, FujiFilm, Tokyo, Japan) using a standard curve of authentic rabbit FMO1 and FMO2 or human FMO3 (which were gifts from Dr. R. Philpot). As a comparison, the relative abundance of heterologously expressed FMOs was estimated from scans of Coomassie Blue-stained SDS-polyacrylamide gels of a range of amounts of microsomal protein isolated from *Sf*9 cells infected with recombinant baculoviruses (data not shown). The results corresponded well with those obtained by Western blotting, indicating that there was no appreciable difference in cross-reactivity among the antibodies.

Enzyme Incubations with TAZ or ETA. *Sf*9 insect cell microsomes containing heterologously expressed human FMO1, FMO2.1, or FMO3 (at a final concentration of 500 nM) or purified EtaA (1 μM final concentration) were incubated with TAZ (100 μM final concentration) at 37°C for 90 min in the buffer described previously (Qian and Ortiz de Montellano, 2006). Reactions were initiated by the addition of enzyme. Reactions were stopped by addition of an equal volume of ice-cold CH_3CN. Mixtures were centrifuged at 10,000*g* for 5 min at 4°C and analyzed by HPLC as described below.

*Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 were incubated with ETA (final concentration 100 μM) for 60 min as described previously (Qian and Ortiz de Montellano, 2006). Reactions were analyzed by liquid chromatography/mass spectroscopy (LC/MS) as described below.

HPLC. The supernatants were diluted to a final concentration of 5% CH_3CN and then analyzed by HPLC on a reverse-phase C18 column (3.5-μm particle size, 4.6 mm i.d. × 150 mm, Symmetry; Waters, Milford, MA) using two buffers: A, H_2O and 0.1% formic acid (FA) and B, CH_3CN and 0.1% FA. The solvent flow rate was 0.2 ml/min, and the eluent was spectrophotometrically monitored using two bandwidths (330 ± 60 and 260 ± 4 nm). The column was eluted from 0 to 25 min with a linear gradient from 5 to 20% buffer B. For spectral analysis of metabolites, eluent peaks were monitored between 200 and 500 nm.

LC/MS Analyses. For analysis of TAZ metabolites, LC/MS was performed as described previously (Qian and Ortiz de Montellano, 2006), with the exception of the following modifications. The reverse-phase column was eluted with a flow rate of 0.2 ml/min (buffer A, H_2O and 0.1% FA; buffer B, CH_3CN and 0.1% FA) with the following protocol: 0 to 16 min, 5 to 30% buffer B (linear gradient). The eluent was monitored at 310 nm. The mass spectrometer settings were as described previously (Qian and Ortiz de Montellano, 2006).

LC/MS analysis of ETA metabolites produced by FMO2.1 was performed as described above, with the following modifications. The column was eluted at a flow rate of 0.2 ml/min (buffer A, H_2O and 0.1% FA; buffer B, CH_3CN and 0.1% FA) with the following protocol: 0 to 15 min with 1% buffer B (isocratic). The eluent was monitored at 350 nm.

Determination of Kinetic Parameters by Spectrophotometric Analysis. The molar extinction coefficient of TAZ was determined as follows. Two cuvettes containing 1 ml of Tris-HCl, pH 8.5, 1 mM EDTA were placed in a Varian Cary 100 dual-beam spectrophotometer (Varian, Inc., Palo Alto, CA). The spectrophotometer was set to blank correction mode. TAZ [in dimethyl sulfoxide (DMSO)] was added to the sample cuvette, and DMSO was added to the reference cuvette. The final organic solvent concentration in each cuvette was held at 0.1% (v/v). Using the Varian Scan application, samples were scanned from 200 to 500 nm over a range of TAZ concentrations between 1 and 20 μM. These measurements were carried out in triplicate using three independently prepared stock solutions of TAZ (20 μM) that were diluted accordingly in DMSO. The absorbance of TAZ, measured at its λ_{max} (328 nm), was plotted against concentration. The molar extinction coefficient of TAZ in DMSO was determined from the gradient of this graph and the Beer-Lambert equation.

Enzyme-catalyzed oxidation of TAZ was monitored by measuring the rate of decrease in the absorbance of TAZ at 328 nm using a dual-beam spectro-

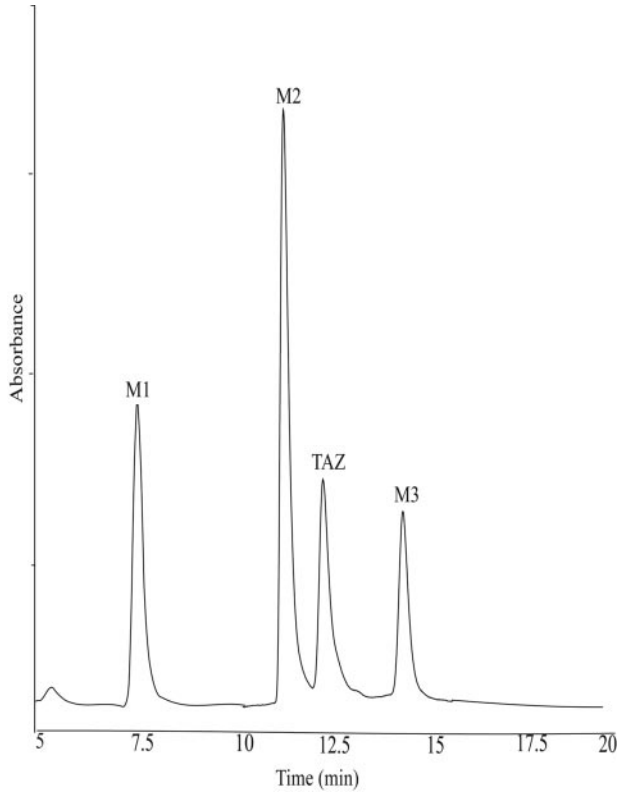

FIG. 1. UV-HPLC chromatogram of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 in the presence of NADPH. Reactions were carried out at pH 9.5 for 90 min at 37°C.

photometer (Varian Cary 100, kinetics module). The pH optima for human FMOs and EtaA were determined using the following reaction buffers: 0.1 mM potassium phosphate, pH 7.5, 1 mM EDTA; 0.1 M Tris-HCl, pH 8.5, 1 mM EDTA; and 0.1 M Tricine-OH, pH 9.5, 1 mM EDTA. Immediately before use buffers were aerated at 37°C for 30 min in a shaking water bath. Assays were performed in a volume of 1 ml in reaction buffer containing 0.1 mM NADPH and either *Sf*9 cell microsomes containing human FMO1 (320 nM), FMO2.1 (5 nM), or FMO3 (230 nM) or purified EtaA (1 μM). Reaction mixtures were allowed to equilibrate for 1 min at 37°C. Reactions were initiated by the addition of TAZ in DMSO (to a final TAZ concentration of 10 μM) to the sample cuvette and DMSO to the reference cuvette. Initial rates were recorded between 1 and 5 min.

For determination of kinetic parameters, assays were performed on triplicate preparations of enzymes at concentrations of TAZ ranging from 1 to 20 μM. The final organic solvent concentration was held at 0.1% (v/v). Assays were carried out in triplicate as described above in the optimum pH buffer for each enzyme: pH 8.5 for FMO1 and FMO3 and pH 9.5 for FMO2.1 and EtaA.

K_M and V_{max} values were determined from vi versus [S]o data using nonlinear regression and the kinetics module 3.1 of Sigma Plot version 10 (SPSS Inc., Chicago, IL). For calculation of k_{cat} values, enzyme concentration was determined as described above.

Determination of Kinetic Parameters by HPLC Analysis. *Sf*9 cell microsomes containing heterologously expressed human FMO2.1 were incubated in 0.1 M Tricine-OH, pH 9.5, 1 mM EDTA, 0.1 mM NADPH, and TAZ (concentrations ranged from 1–50 μM in DMSO). A duplicate set of samples was prepared but without the addition of enzyme. The final organic solvent concentration was held at 0.1% (v/v). Mixtures were incubated at 37°C for 5 min, and reactions were quenched with an equal volume of ice-cold CH_3CN. ETA was added as an internal standard at a final concentration of 100 μM (in DMSO), and mixtures were prepared for HPLC analysis as described above. A standard curve was generated by plotting the ratio of the integrated HPLC peak areas of TAZ and ETA (from the sample set without added enzyme) against the range of TAZ concentrations used. The ratio of the integrated HPLC peak areas of TAZ and ETA, in the sample set with added enzyme, was calculated, and the amount of unmetabolized TAZ was determined from the standard curve. This value was subtracted from the input concentration of TAZ to calculate the amount of TAZ metabolized by the enzyme. V_{max}, K_M, and k_{cat} were determined as above.

Enzyme Incubations with Methimazole. *Sf*9 insect cell microsomes containing heterologously expressed human FMO1, FMO2.1, or FMO3 were assayed for activity toward methimazole by the method of Dixit and Roche (1984) as described previously (Dolphin et al., 1998). Assays for FMO1 and FMO3 activity were carried out at pH 8.5, and those for FMO2.1 were done at pH 9.5.

Results

Catalytic Oxidation of TAZ by Human FMO2.1. Incubation of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1, in the presence of NADPH, resulted in the formation of three major metabolites with reverse-phase retention times of 7.5 min (**M1**), 11.4 min (**M2**), and 14.6 min (**M3**) (Fig. 1). No products were observed when TAZ was incubated with microsomes isolated from noninfected *Sf*9 cells or when NADPH was omitted (results not shown). UV spectral analysis of the metabolites (Fig. 2) showed that **M1** had a maximal absorption peak at 325 nm and a smaller peak at approximately 230 nm. **M2** had a similar spectrum, with peaks at 320 and 220 nm. The absorption spectrum of **M3** exhibited a main peak at 295 nm and a secondary peak at 220 nm.

To identify the three metabolites, **M1**, **M2**, and **M3**, formed from TAZ by the action of human FMO2.1, they were analyzed by LC/MS. The mass spectrum of **M1** had a molecular ion $[M + H]^+$ at *m/z* 269.07, with fragment ions at *m/z* 205.14 and 163.12 (Fig. 3A). The mass of the molecular ion of **M1** is 32 atomic mass units more than that of the molecular ion of TAZ (237), suggesting a structure in which TAZ has incorporated two oxygen atoms (Fig. 3A) and hence supports identification of the metabolite as the sulfinic acid derivative. The mass spectrum

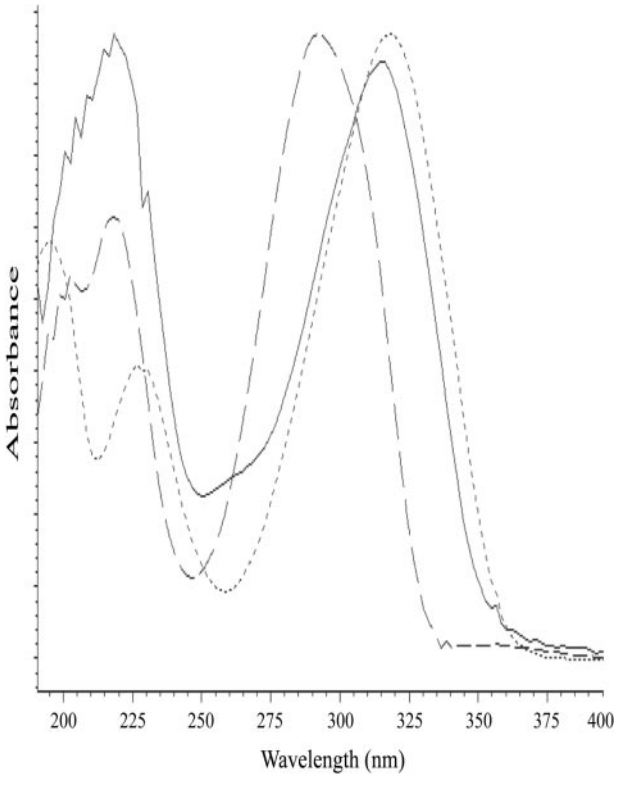

FIG. 2. UV-absorption spectra of the three metabolites produced from the incubation of TAZ with heterologously expressed human FMO2.1 and NADPH. Dotted line, **M1**; solid line, **M2**; and dashed line, **M3**.

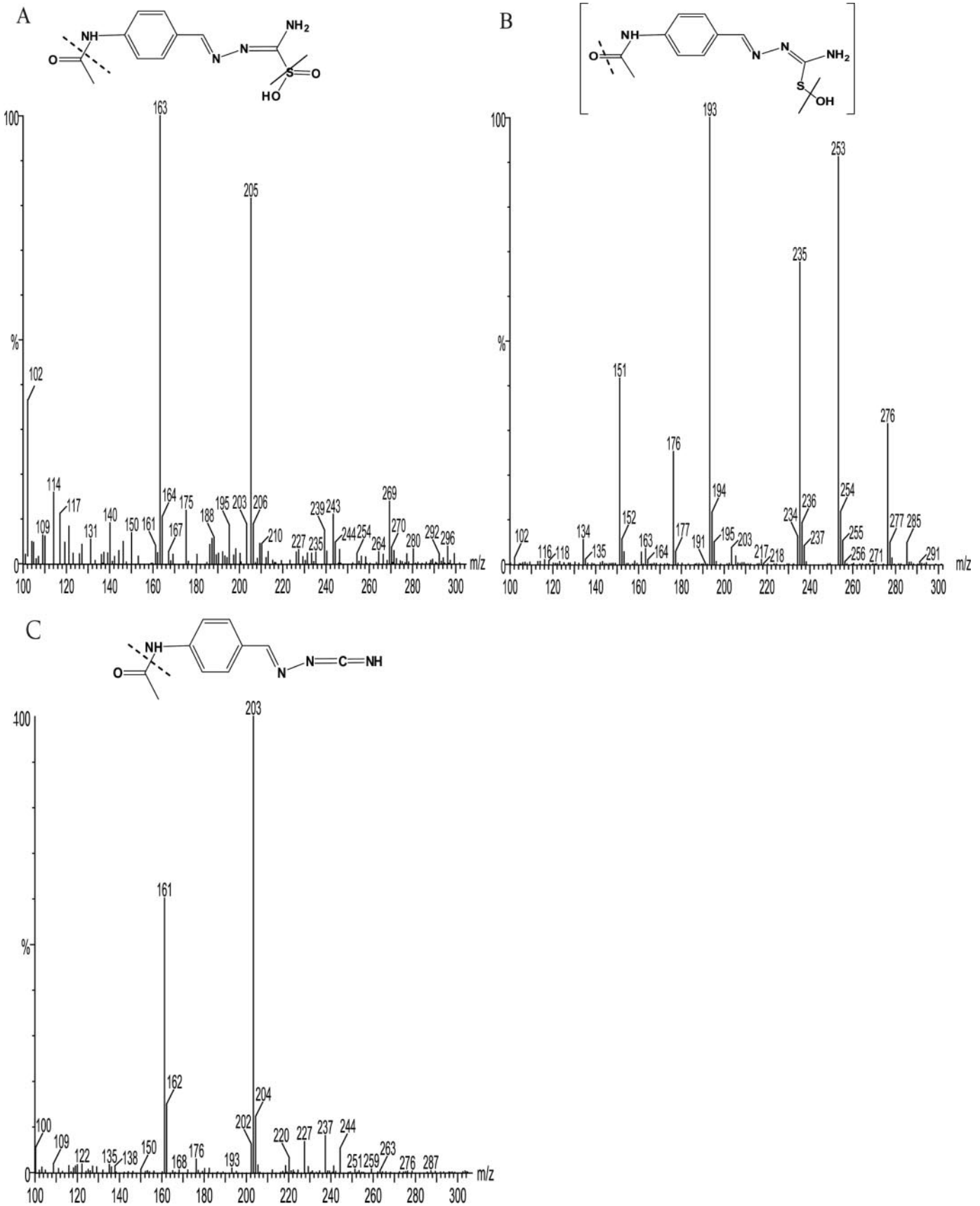

FIG. 3. Mass spectra and structures of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 in the presence of NADPH in 100 mM tricine buffer, pH 9.5, for 90 min at 37°C. A, **M1**, identified as the sulfinic acid, has a molecular ion $[M + H]^+ = m/z$ 269.07, with fragment ions at m/z 205.14 and 163.12. B, **M2**, identified as the sulfenic acid, has a molecular ion $[M + H]^+ = m/z$ 253, with fragment ions at m/z 235 and 193. C, **M3**, identified as the carbodiimide, has a molecular ion $[M + H]^+ = m/z$ 203.13, with a fragment ion at m/z 161.11.

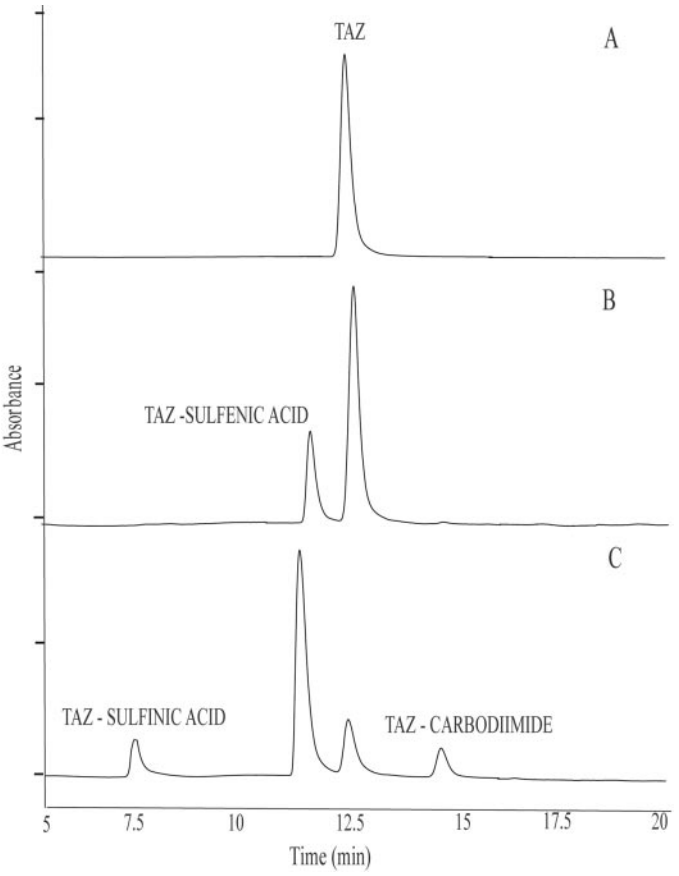

FIG. 4. UV-HPLC chromatograms of the products from incubations of TAZ with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 in the presence of NADPH. Reactions were carried out at pH 9.5 and 37°C for 0 min (A), 10 min (B), or 20 min (C).

of **M2** had a molecular ion $[M + H]^+$ at *m/z* 253, with fragment ions at *m/z* 235 and 193 (Fig. 3B). The mass of the molecular ion of **M2** is in accord with a structure in which TAZ has incorporated a single oxygen atom, and thus supports identification of **M2** as the monooxygenated, sulfenic acid derivative. The metabolite **M3** has a molecular ion $[M + H]^+$ at *m/z* 203.13, which suggests that it is the carbodiimide generated by elimination from **M2** of the oxidized sulfur atom (Fig. 3C). Previously synthesized authentic standards of TAZ-sulfinic acid and TAZ-carbodiimide (Qian and Ortiz de Montellano, 2006) were used to confirm the identity of **M1** and **M3**. Comparison of their HPLC elution times, UV spectra, and MS spectra with those of the synthetic standards unambiguously identified the metabolites **M1** and **M3** as the sulfinic acid and carbodiimide derivatives of TAZ, respectively.

The same three metabolites (the sulfinic acid, sulfenic acid, and carbodiimide derivatives) were also produced when TAZ was incubated with purified EtaA or *Sf9* cell microsomes containing heterologously expressed human FMO1 or FMO3 (data not shown). Previous work identified **M1** and **M3** as products formed by the action of these enzymes on TAZ (Qian and Ortiz de Montellano, 2006). Although the sulfenic acid derivative (**M2**) was not detected, it was postulated as an intermediate in the enzyme-catalyzed metabolism of TAZ (Qian and Ortiz de Montellano, 2006). To confirm this, incubations of TAZ with heterologously expressed human FMO2.1 were quenched at different time points (Fig. 4). It is clear from the results that formation and accumulation of **M2** precede that of **M1** and **M3**, indicating that **M2** is an intermediate in the formation of the latter two metabolites.

Catalytic Oxidation of ETA by Human FMO2.1. Incubation of ETA with *Sf9* microsomes containing heterologously expressed human FMO2.1, in the presence of NADPH, resulted in the formation of a major product, **P1**, with LC retention time of 6.2 min (Fig. 5A). The mass spectrum of **P1** had a molecular ion $[M + H]^+$ at *m/z* 183, with fragment ions at *m/z* 151 and 133 (Fig. 5B). The mass of the molecular ion of **P1** is 16 atomic mass units more than that of the molecular ion of ETA (166), suggesting a structure in which ETA has incorporated one oxygen atom (Fig. 5B). This supports identification of **P1** as the *S*-oxide of ETA. The retention time and mass spectrum of **P1** are identical to those of authentic ETA *S*-oxide (Vannelli et al., 2002), therefore unambiguously identifying this metabolite as the *S*-oxide of ETA. A mass spectrum of the broad peak with an LC retention time of approximately 4 min (Fig. 5A) could not be obtained.

Development of a Spectrophotometric Assay of TAZ Oxidation. A UV absorption spectrum of TAZ revealed an absorption maximum at 328 nm and a smaller peak at approximately 220 nm (data not shown). The absorbance of TAZ at 328 nm was linear between 1 and 20 μM (data not shown), and at this wavelength the molar extinction coefficient of TAZ in DMSO was determined as 38,300 $\pm$ 2320 $M^{-1}cm^{-1}$. Incubation of TAZ, in the presence of NADPH, with *Sf*9 cell microsomes containing heterologously expressed human FMO1, FMO2.1, or FMO3, or with purified EtaA resulted in a decrease in TAZ absorbance at 328 nm that was linear over time (Fig. 6 and data not shown) and with respect to enzyme concentration. No decrease in TAZ absorbance was observed in the absence of EtaA or human FMOs or when microsomes prepared from noninfected *Sf*9 cells were used. Omission of NADPH from reaction mixtures containing heterologously expressed human FMOs resulted in a very small (<1% of that observed in the presence of NADPH) and short-lived (<2 min) decrease in TAZ absorbance. This is because of the presence of endogenous NADPH in the insect cell microsomes. This spectrophotometric assay was used to determine the pH optima and kinetic parameters of enzyme-catalyzed oxidation of TAZ.

Effect of pH on TAZ Oxidation Catalyzed by Human FMOs and EtaA. TAZ was incubated with purified EtaA or with heterologously expressed human FMO1, FMO2.1, or FMO3 in buffers of pH 7.5, 8.5, or 9.5. Oxidation of TAZ was measured spectrophotometrically. The pH optimum for the oxidation of TAZ at a concentration of 10 μM was 8.5 for human FMO1 and FMO3 and 9.5 for human FMO2.1 and EtaA (Fig. 7).

Kinetics of TAZ Oxidation by Human FMOs and EtaA. The kinetics of TAZ oxidation catalyzed by EtaA or heterologously expressed human FMO1, FMO2.1, or FMO3 was evaluated by determining the initial rates of TAZ oxidation, measured spectrophotometrically, over a range of TAZ concentrations (Fig. 8 and data not shown). Assays were performed at the optimum pH determined for each enzyme as described above. Steady-state kinetic parameters were determined from vi versus [S] data by nonlinear regression.

The K_M values for TAZ oxidation catalyzed by human FMO1, FMO2.1, and FMO3 are very similar to each other and slightly lower than that of the EtaA-catalyzed reaction (Table 1). The k_{cat} of the FMO2.1-catalyzed reaction is much higher than that of reactions catalyzed by human FMO1, FMO3, or EtaA (Table 1). Consequently, k_{cat}/K_M (the specificity constant) for TAZ is much higher for human FMO2.1 than for the other three enzymes (Table 1). Very similar kinetic parameters for FMO2.1-catalyzed TAZ oxygenation were obtained through the use of an HPLC-based assay, thus validating the spectrophotometric assay (data not shown).

As a comparison, we determined kinetic parameters for FMO1-, FMO2.1-, and FMO3-catalyzed oxygenation of methimazole, a prototypic substrate for FMOs (Table 1). The k_{cat} of each enzyme was similar for TAZ and methimazole. However, for FMO2.1 the K_M for

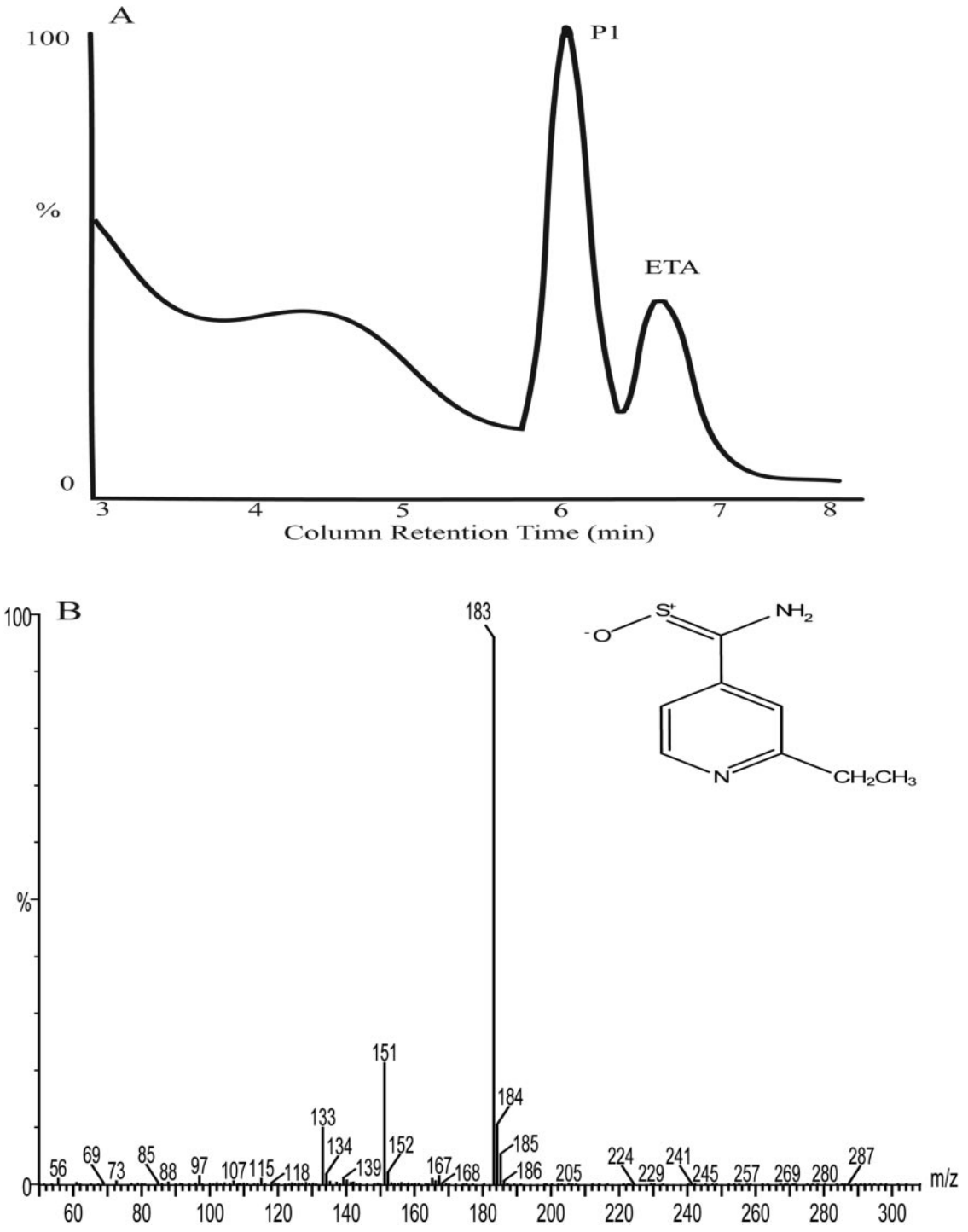

FIG. 5. Analysis of the products from incubation of ETA with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH in 100 mM potassium phosphate buffer, pH 7.5, for 60 min at 37°C. A, LC chromatogram (350 nm). B, the mass spectrum and structure of the product **P1**. **P1** has a molecular ion $[M + H]^+$ at *m/z* 183, with fragment ions at *m/z* 151 and 133.

methimazole was 100-fold greater than that for TAZ. Consequently, for methimazole oxygenation, the k_{cat}/K_M of FMO2.1 is considerably less than that of FMO1 and slightly less than that of FMO3.

Discussion

Our results show that full-length, functional FMO2 of human (FMO2.1) catalyzes oxygenation of the second-line thiourea antitubercular drug TAZ in vitro. The metabolites generated, the sulfenic acid, sulfinic acid, and carbodiimide derivatives, are the same as those produced by the action of human FMO1, human FMO3, or the mycobacterial enzyme EtaA. The sulfinic acid and carbodiimide derivatives have been identified previously as products of TAZ oxygenation catalyzed by FMO1, FMO3, or EtaA (Qian and Ortiz de Montellano, 2006). Although these authors did not detect the sulfenic acid derivative, it was postulated to be an intermediate. Our results confirm that in reactions catalyzed by all three human FMOs and by EtaA, TAZ sulfenic acid is indeed an intermediate in the formation of the sulfinic acid and carbodiimide metabolites.

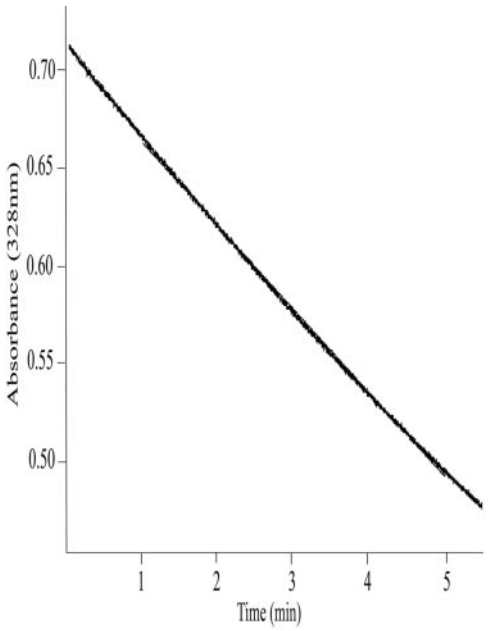

FIG. 6. Linearity of FMO2.1- and NADPH-dependent decrease in TAZ absorbance over time. TAZ (20 μM) was incubated with *Sf*9 insect cell microsomes containing heterologously expressed human FMO2.1 and NADPH in tricine buffer, pH 9.5, at 37°C, and absorbance at 328 nm was monitored over time.

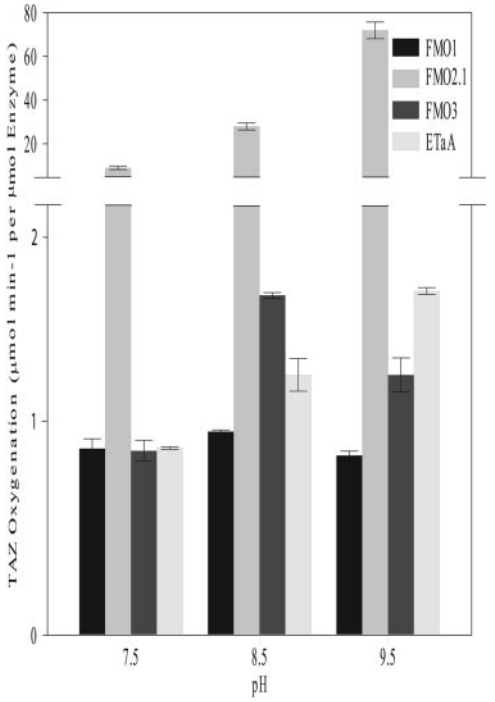

FIG. 7. Effect of pH on the rate of TAZ oxygenation catalyzed by purified EtaA or by heterologously expressed human FMO1, FMO2.1, or FMO3. The initial concentration of TAZ was 10 μM. The decrease in TAZ concentration over time was measured at 328 nm in the presence of NADPH in buffers at pH 7.5, 8.5, or 9.5.

Human FMO2.1 also catalyzes the oxygenation of ETA, a thioamide second-line antitubercular. The single metabolite identified, the *S*-oxide, has been shown to be the product of EtaA-catalyzed metabolism of the drug (Vannelli et al., 2002). However, the second product identified by these authors, the amide of ETA, was not detected in our study.

Our kinetic analyses reveal that k_{cat}/K_M (the specificity constant) for FMO2.1-catalyzed TAZ oxygenation is much higher than that of FMO1, FMO3, or the mycobacterial enzyme EtaA, indicating that FMO2.1 is more effective in catalyzing TAZ oxygenation than are the other three enzymes. In contrast, FMO2.1 is less effective than FMO1 or FMO3 in catalyzing the oxygenation of methimazole. Although kinetic analyses were done at the pH optimum for each enzyme, even at a more physiological pH, 7.5, as shown in Fig. 7, FMO2.1 is the most effective of the enzymes studied in catalyzing TAZ oxygenation. The higher value of the specificity constant of FMO2.1 for TAZ is a consequence of the higher k_{cat} value rather than a lower K_M value, which is similar to those of the other enzymes with lower k_{cat} values (Table 1). Similar high k_{cat} values have been reported for human FMO2.1-catalyzed oxygenation of other thioureas, including thiourea and ethylene-thiourea (Henderson et al., 2004). The relatively low k_{cat} values determined for TAZ oxygenation catalyzed by FMO1 and FMO3 are comparable to those reported for oxygenation of a panel of thioureas by these enzymes (Onderwater et al., 2006). The high values of K_M and k_{cat} determined for FMO2.1-catalyzed oxygenation of methimazole are similar to the values reported for purified or heterologously expressed FMO2 of rabbit (Lawton et al., 1991).

Spectrophotometric and kinetic studies indicate that the rate-limiting step for FMO-catalyzed reactions occurs after substrate oxygenation (reviewed in Ziegler, 2002) and thus is independent of oxidizable substrate. Consequently, for a particular FMO, the value of k_{cat} would be expected to be similar for all its substrates. Although our results conform to this expectation, they indicate that the k_{cat} of human FMO2.1 is higher than that of FMO1 or FMO3, suggesting that FMO2.1 is more effective in catalyzing the rate-limiting step of the reaction, the elimination of H_2O, than is either of the other two FMOs investigated.

Substrate oxygenation by FMOs is usually a detoxification process. However, in the case of thioureas, the products of FMO-catalyzed oxygenation are typically more toxic than the parent compound (Smith and Crespi, 2002; Henderson et al., 2004; Onderwater et al., 2004, 2006). TAZ sulfenic acid is an electrophile that can react with glutathione (GSH) (Qian and Ortiz de Montellano, 2006) to regenerate the parent compound and convert GSH to its oxidized form (GSSG). In the presence of GSH reductase, a redox cycle may be established that depletes GSH, thus causing oxidative stress and cellular injury (Krieter et al., 1984; Henderson et al., 2004; Onderwater et al., 2004). Sulfenic acid metabolites of thioureas can react covalently with other thiol-containing molecules, such as cysteine residues in proteins, and thus directly perturb protein function (Decker and Doerge, 1992). The TAZ carbodiimide also has the potential to form covalent products with cysteine residues in proteins. Thus, each of the human FMOs that we have investigated—FMO1, FMO2.1, and FMO3—is able to catalyze oxygenation of TAZ, producing metabolites that are known to be harmful to mammalian cells.

The FMO-catalyzed production of TAZ metabolites that are known to be harmful to mammalian cells may be the basis for the adverse clinical reactions associated with this drug (Teklu, 1976; Brown, 1992; Peloquin, 1993; Ipuge et al., 1995). This is supported by the fact that all the adverse effects associated with TAZ occur in tissues in which FMOs are expressed: hepatotoxicity, FMO3 in the liver (Dolphin et al., 1996; Hernandez et al., 2004); gastrointestinal problems, FMO1 in the small intestine (Yeung et al., 2000); and skin rashes, FMO1 and FMO3 in skin (Janmohamed et al., 2001).

Human FMO1 and FMO3 are expressed primarily in the kidney and liver, respectively, and thus may contribute to the extrapulmonary metabolism of TAZ. FMO1 displays interindividual variation in its expression (Yeung et al., 2000; Koukouritaki et al., 2002), and several nonsynonymous polymorphic variants of FMO3 have been identified

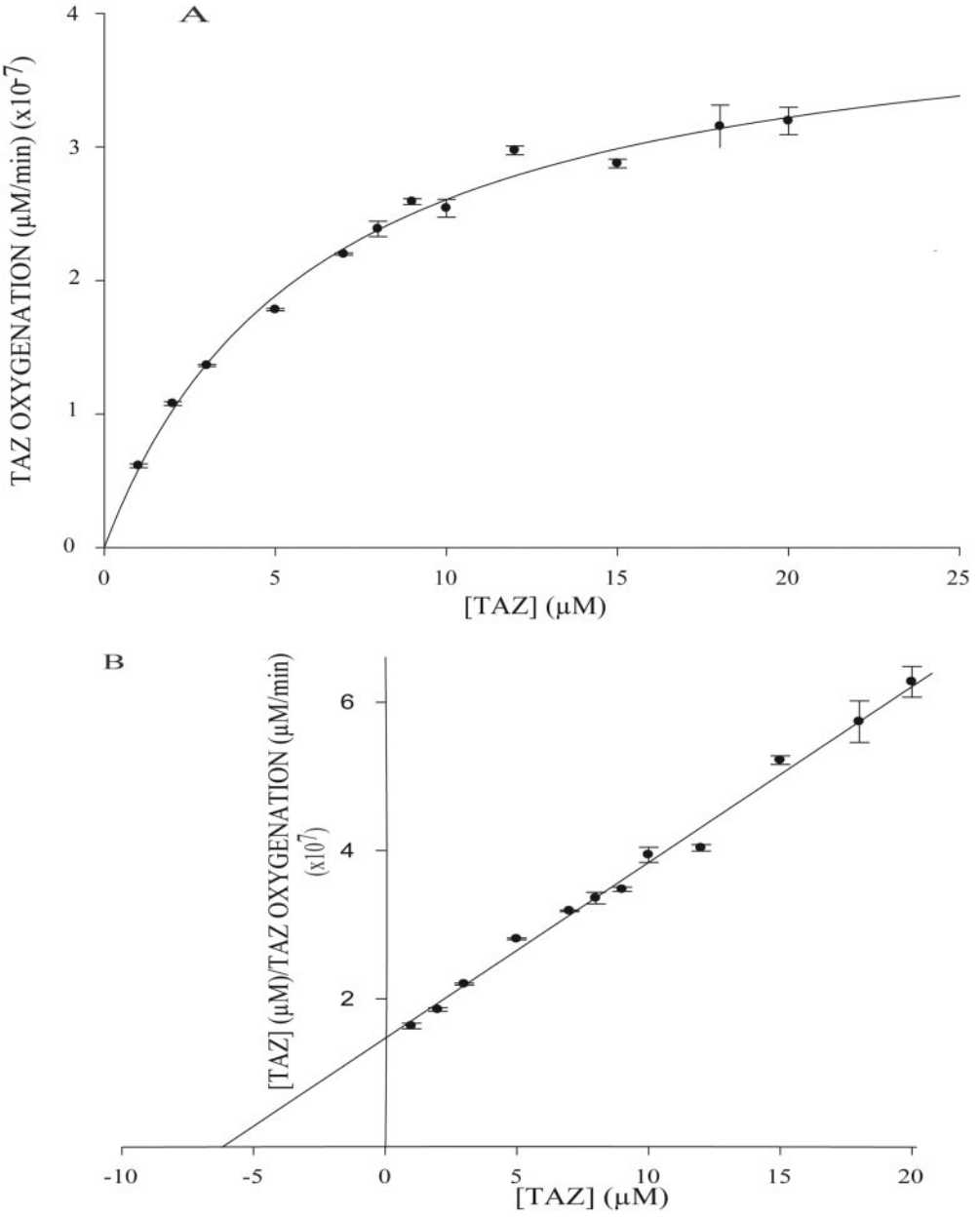

FIG. 8. A, Michaelis-Menten plot of TAZ oxygenation catalyzed by heterologously expressed human FMO2.1 in the presence of NADPH at pH 9.5. B, linear transform of Michaelis-Menten data using Hanes-Woolf regression (r^2 = 0.961).

TABLE 1

Kinetic parameters of enzyme-catalyzed oxygenation of TAZ and methimazole

TAZ assays were performed in triplicate on batches of microsomes isolated from three independent infections of *Sf*9 cells (i.e., nine measurements per FMO). Methimazole assays were performed in triplicate on a single batch of microsomes for each heterologously expressed human FMO. EtaA assays were performed in triplicate on purified protein. Kinetic parameters are reported as mean ± S.E.

Enzyme	Thiacetazone			Methimazole		
	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M
	μM	min^{-1}	$min^{-1}M^{-1}$ ($\times 10^5$)	*μM*	min^{-1}	$min^{-1}M^{-1}$ ($\times 10^4$)
FMO1	6.30 ± 0.80	5.08 ± 0.46	7.94 ± 1.99	8.08 ± 2.35	2.27 ± 0.30	28.11 ± 7.95
FMO2.1	5.80 ± 0.55	80.10 ± 4.42	142.56 ± 18.30	575.75 ± 60.02	31.50 ± 2.11	5.48 ± 0.68
FMO3	7.01 ± 0.53	1.37 ± 0.20	1.96 ± 0.39	29.30 ± 4.06	2.60 ± 0.38	8.97 ± 1.81
EtaA	9.05 ± 0.57	3.02 ± 0.30	3.29 ± 0.81	N.D.	N.D.	N.D.

N.D., not determined.

(reviewed in Phillips et al., 2007; Phillips and Shephard, 2008), one of which, L360P, increases enzyme activity (Lattard et al., 2003), whereas others, such as E158K and E308G, when present together in *cis*, decrease enzyme activity (reviewed in Phillips et al., 2007; Phillips and Shephard, 2008). Promoter variants that affect transcription have also been identified (Koukouritaki et al., 2005). Therefore, individuals who have lower expression or activity of FMO1 or FMO3 would be less able to metabolize TAZ in extrapulmonary tissues,

whereas those with higher expression or activity would metabolize TAZ more effectively, thus reducing the amount of prodrug reaching the lung and increasing the amounts of metabolites toxic to the host. Polymorphic variants in *FMO3* have been shown to influence the metabolism and therapeutic outcomes of drugs such as benzydamine and sulindac (Störmer et al., 2000; Hisamuddin et al., 2005).

TAZ and ETA are prodrugs that are converted to their active forms in mycobacteria. Therefore, for treatment to be effective, a sufficient amount of unmetabolized drug must reach mycobacteria in the lung. Although Europeans and Asians lack functional FMO2, a substantial proportion of sub-Saharan Africans and individuals of recent African descent possess an ancestral *FMO2*1* allele (Whetstine et al., 2000; Veeramah et al., 2008) and thus would be expected to express functional FMO2 in the lung (Krueger et al., 2002). The relatively high specificity constant for FMO2.1-catalyzed TAZ oxygenation suggests that, when present in lung, FMO2.1 is likely to contribute substantially to the metabolism of TAZ in this tissue, thus decreasing the availability of the prodrug to mycobacteria and producing metabolites toxic to the host. Therefore, patients with multidrug-resistant TB who express FMO2.1 may respond less well to treatment with second-line antitubercular drugs such as TAZ and ETA and may be more prone to adverse clinical reactions to these drugs. The relatively high frequency of the *FMO2*1* allele in sub-Saharan Africa, a region in which TB is a major health problem, has implications for the efficacy of and response to antitubercular drugs, such as TAZ and ETA, that are substrates for FMO2.1.

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The Flavin-Containing Monooxygenases (FMOs): Genetic Variation and its Consequences for the Metabolism of Therapeutic Drugs

FINAL

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Abstract: Flavin-containing monooxygenases (FMOs) are a family of enzymes involved in the metabolism of foreign chemicals, including many therapeutic drugs. In this review we focus on the functional FMOs of humans (FMOs 1, 2, 3, 4 and 5). For each FMO we describe its gene organization, developmental- and tissue-specific pattern of expression, substrate specificity and the identity, frequency and functional effect of polymorphic variants. We also review the consequences of genetic variation in the FMOs for the metabolism of therapeutic drugs and the implications of this for drug efficacy and response. Some key points are: the majority of humans are homozygous for an allele (FMO2*2) that encodes a truncated, non-functional polypeptide, but a substantial proportion of individuals of African descent possess a copy of the functional ancestral (FMO2*1) allele and thus are predicted to respond differently to drugs and other foreign chemicals that are substrates for FMO2; *FMO3* polymorphisms that decrease catalytic activity have been linked to increased drug efficacy; rare mutations in *FMO3* are causative of the disorder trimethylaminuria; and the role of FMO1 and FMO3 in the oxidation of the antiestrogen tamoxifen and the antitubercular drug thiacetazone are discussed.

INTRODUCTION

Flavin-containing monooxygenases (FMOs; EC 1.14.13.8) of eukaryotes are located in the membranes of the endoplasmic reticulum. The enzymes contain flavin adenine dinucleotide (FAD), as a prosthetic group, and require NADPH and molecular oxygen to catalyze the oxidative metabolism of numerous foreign chemicals, including therapeutic drugs, dietary-derived compounds and pesticides [Ziegler 1993; Krueger and Williams, 2005; Cashman and Zhang, 2006]. Preferred substrates contain, as the site of oxidative attack by the enzyme, a soft nucleophile, typically a nitrogen, sulfur, phosphorus or selenium atom, and include chemicals as diverse as hydrazines [Prough *et al.* 1981], phosphines [Smyser and Hodgson, 1985], iodide boron-containing compounds [Jones and Ballou,1986], sulfides [Hamman *et al.* 2000], selenides [Ziegler *et al.* 1992] and many secondary and tertiary amines [Ziegler 1980]. The products of FMO-catalyzed reactions are relatively polar, readily excretable and generally less toxic or pharmacologically active than the parent compounds. However, compounds such as *N*-alkylarylamines are converted into more reactive, carcinogenic products [Ziegler 1991]. FMOs thus have an important pharmacological and toxicological relevance. A limited number of endogenous compounds, including methionine [Duescher *et al.* 1994], cysteamine and cysteine- and homocysteine-*S*-conjugates, may function as substrates for FMOs [Ziegler 1993].

FMOs differ from other monooxygenases because activation of oxygen, in the form of the C(4a) hydroperoxide derivative of FAD, does not require binding of the substrate capable of being oxygenated [Ziegler 1993]. Consequently, the enzyme is present within the cell in its activated form and has been likened to a "loaded, cocked gun," capable of oxidizing any soft nucleophile able to gain access to the active site. The unique mechanism of FMOs thus accounts for their extremely broad range of substrates.

In humans, five functional *FMO* genes, designated *FMO1-FMO5*, have been identified [Lawton *et al.* 1994; Phillips *et al.* 1995; Hernandez *et al.* 2004]. *FMO1* to *FMO4* are located within a cluster spanning ~245 kb on chromosome 1, in the region q24.3 [Hernandez *et al.* 2004]. The cluster contains an additional *FMO* gene, *FMO6P*, which is classified as a pseudogene because it is unable to produce a correctly spliced mRNA [Hines *et al.* 2002]. The order of the genes within the cluster is *cen-FMO3-FMO6P-FMO2-FMO1-FMO4-tel*, with no other genes being present. The gene encoding FMO5 is located ~26 Mb closer to the centromere, in the region 1q21.1 [Hernandez *et al.* 2004]. A second cluster of five *FMO* genes has been identified in the region 1q24.2, approximately 4 Mb to the centromeric side of the q24.3 cluster. This cluster, however, consists entirely of pseudogenes, designated *FMO7P-FMO11P* [Hernandez *et al.* 2004]. The human genome therefore contains 11 *FMO* genes, five of which are functional. This review will focus on the five functional *FMO* genes, *FMO1* to *FMO5*, and the proteins which they encode. Functional *FMO* genes contain eight coding exons and either one or two 5' non-coding exons. In each case, the mature mRNA is produced by splicing the coding exons to a single non-coding exon.

FMOs 1, 2, 3, 4 and 5 have 51 to 57% amino acid sequence identity, whereas FMO6 is 72% identical to FMO3. FMOs of other mammalian species have >80% sequence identity to their human orthologues. Two GXGXXG motifs, characteristic of FAD- and NADPH-pyrophosphate-binding sites are present at identical positions (residues 9-14 and 191-196, respectively) in each human FMO. The FAD-binding site is contained within a "fingerprint" sequence that predicts a βαβ secondary structure, the "Rossman fold,"

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known to be involved in binding dinucleotides [Wierenga *et al.* 1985]. A hydrophobic motif, F(A/T)TGY, characteristic of FMOs, is present at about residue 330. For more information on the structural features of FMOs, see the review by Krueger and Williams [2005]. Recently, a three-dimensional structure of a FMO from *Schizosaccharomyces pombe* has been determined [Eswaramoorthy *et al.* 2006]. In contrast to mammalian FMOs, the yeast enzyme is cytosolic and about 85 residues shorter.

Phylogenetic analysis suggests that the five functional *FMO* genes arose from a common ancestral gene *via* a series of gene duplications, estimated to have occurred between 210 and 275 million years ago [Hernandez *et al.* 2004]. This is much earlier than the divergence of mammals, some 85 million years ago, and therefore it is predicted that all mammals possess the functional *FMO* genes *FMO1* to *FMO5*.

Each member of the *FMO* gene family exhibits a different developmental stage- and tissue-specific pattern of expression, which accounts for the marked qualitative and quantitative differences in FMO activities in tissues such as liver, lung and kidney. In addition, there are species differences in *FMO* gene expression (see below), which have implications for the extrapolation of drug metabolism data from experimental animals to humans.

In this review we focus on the functional FMOs of humans. For each FMO we describe gene organization, developmental- and tissue-specific pattern of expression (and how this may differ from that in other mammals) and substrate specificity. We also describe genetic variants that have been identified, focusing on validated coding-region variants, but including promoter variants that have been shown to have an effect on gene expression, the frequency with which the variants occur in the world's major population groups and their effect on enzyme function. We then discuss consequences of genetic variation in the FMOs for the metabolism of therapeutic drugs and the implications of this for drug efficacy and response.

FMO1

In 1971 Ziegler identified a mixed-function amine oxidase in porcine liver microsomes whose substrates included nitrogen- and sulphur-containing compounds [Ziegler *et al.* 1971]. A cDNA for the corresponding protein of humans was isolated in 1991 [Dolphin *et al.* 1991], and the enzyme is now known as FMO1 [Lawton *et al.* 1994]. The *FMO1* gene, which was mapped to the long arm of chromosome 1 [Shephard *et al.* 1993], encodes a polypeptide of 532 amino-acid residues of molecular mass 60,306.

Tissue Distribution

There is a distinct species difference in the hepatic expression of *FMO1*. In humans, neither FMO1 mRNA [Dolphin *et al.* 1991; Phillips *et al.* 1995; Dolphin *et al.* 1996] nor protein [Yeung *et al.* 2000; Koukouritaki *et al.* 2002] is present in adult liver. This is in marked contrast to all other mammals investigated, in which FMO1 constitutes a major form of the enzyme in adult liver [Lawton *et al.* 1990; Gasser *et al.* 1990; Cherrington *et al.* 1998a; Lattard *et al.* 2002a; Stevens *et al.* 2003]. However, the *FMO1* gene is expressed in fetal human liver [Dolphin *et al.* 1991, 1996; Phillips *et al.* 1995; Yeung *et al.* 2000; Koukouritaki *et al.* 2002]. Expression is highest in the first trimester (7.8 ± 5.3 pmol/mg microsomal protein), then declines during fetal development and by 3 days after birth is completely extinguished [Koukouritaki *et al.* 2002]. *FMO1* is also expressed in fetal human kidney, but in this tissue expression is increased after birth, not switched off as in the liver [Dolphin *et al.* 1991, 1996; Phillips *et al.* 1995; Yeung *et al.* 2000; Krause *et al.* 2003].

In adult human the main site of expression of *FMO1* is the kidney [Dolphin *et al.* 1991, 1996; Phillips *et al.* 1995; Yeung *et al.* 2000]. The gene is also expressed in the small intestine [Yeung *et al.* 2000] and stomach, and in a number of endocrine tissues, including pancreas, adrenal cortex and medulla, thyroid, thymus and testis [Hernandez *et al.* 2004]. The amount of FMO1 in adult human kidney (47 ± 9 pmol/mg microsomal protein) [Yeung *et al.* 2000] is not much lower than that observed in liver for the major hepatic cytochrome P450 (CYP), CYP3A4 (96 ± 51 pmol/mg microsomal protein) [Shimada *et al.* 1994], and is greater than that of the total content of CYPs in adult human kidney [Jakobsson and Cintig, 1973]. Thus, in adult human, FMO1 is likely to be a major contributor to the renal metabolism and clearance of therapeutic drugs. Another study reported lower amounts of FMO1 in adult kidney (3.2 – 11.5 pmol/mg microsomal protein) [Krause *et al.* 2003]. It has been suggested that differences in the amounts of FMO1 detected in these two studies may have been due to the use of different antibodies [Krause *et al.* 2003]. Krause *et al*. [2003] report also that FMO1 amounts are greater in individuals of African descent (African Americans) than in Caucasians. However, it should be noted that the Caucasian kidney samples were obtained from cadavers, whereas the African-American samples were from biopsies.

Interindividual differences in the expression of *FMO1* have been reported. Variations in expression in fetal liver of 10- to 20-fold (n=92), depending on gestational age, were observed [Koukouritaki *et al.* 2002]. Studies of adult human kidney samples found little variation (n=4) [Yeung *et al.* 2000], less than 4-fold variation (n=26) [Krause *et al.* 2003], or less than 5-fold variation (n=13) [Hamman *et al.* 2000], whereas amounts in small intestine varied by up to 5-fold (n=7) [Yeung *et al.* 2000].

Organization of The Human *FMO1* Gene

The human *FMO1* gene has 10 exons. The translation initiation codon is located in exon 2 and the protein-coding sequence is contained within exons 2 to 9. Exons 0 and 1 are upstream non-coding exons. In fetal liver, transcription is initiated from the most 5' exon (exon 0), *via* the P0 promoter. To produce the mature FMO1 mRNA sequences derived from exon 0 are spliced to those derived from exon 2 [Hernandez *et al.* 2004]. Analysis of a cDNA isolated from the small intestine (accession no. AK097039) indicates that in this tissue transcription starts from exon 1, *via* the P1 promoter, and sequences derived from exon 1 are spliced to those derived from exon 2. In the kidney transcription can begin from a site within intron 1, *via* the P2 promoter [Shephard *et al.* 2007]. Therefore the human *FMO1* gene has

three alternative promoters, P0, P1 and P2. In fetal liver only the most upstream, P0, is used [Shephard *et al.* 2007]. Irrespective of the promoter used the FMO1 protein, encoded by alternatively spliced mRNAs, is identical as both exon 0 and 1 are untranslated.

The reasons for the silencing of the *FMO1* gene in adult human liver are not understood. However, a recent study [Shephard *et al.* 2007] using reporter gene assays has shown that sequences upstream of the core liver promoter (upstream of exon 0) act as a powerful transcriptional repressor in transfected HepG2 cells. The repressor sequences contain repetitive elements that are absent from species in which the gene is expressed in adult liver [Shephard *et al.* 2007]. Continued expression of the gene in adult human extra-hepatic tissues can be explained in part by the use of alternative promoters to direct expression in the intestine and kidney [Hernandez *et al.* 2004].

Genetic Variants

Protein-Coding Variants

Fig. (**1**) shows the polymorphic variants identified in the coding region of *FMO1*. Analysis of the *FMO1* gene from 50 unrelated African Americans identified four single-nucleotide polymorphisms (SNPs) that change the amino-acid sequence of the protein [Furnes *et al.* 2003]: g.9614C>G(H97Q), g.23970A>G (I303V), g.23971T>C (I303T) and g.27362C>T(R502X), (Fig. (**1**)) and (Table **1**). Each of the variants was found in only one or two of the 100 chromosomes surveyed. I303V, which is also reported in dbSNP126, has been found in African Americans and Yorubans at frequencies of 10-15%, but is absent from Europeans and Asians. The catalytic activity of each of these variants for *N*- and *S*-oxygenation was assessed in a heterologous expression system using four known substrates for FMO1: methimazole, imipramine, fenthion and methyl *p*-tolyl sulphide [Furnes and Schlenk, 2004]. Three of the variants, H97Q, I303V and I303T, had little if any effect on enzyme activity with any of the substrates. Although R502X was missing 31 residues from the C-terminus, this had only a modest if any effect on activity towards imipramine, fenthion and methyl *p*-tolyl sulphide. However, the variant was completely inactive towards methimazole. The results of this study show that, depending on the substrate, the same SNP can have a markedly different effect on FMO1 activity. R502X was identified in only one of the 100 chromosomes surveyed and thus may represent a rare variant that would have little consequence for the general population.

A further nonsynonymous SNP, g.22739G>A(R223Q), is reported in dbSNP126 (rs16864310), (Fig. (**1**) and Table **1**). The effect of this polymorphism on enzyme activity is not known. However, it is apparently specific to Chinese, in which it occurs at very low frequency (2%). Thus, only five nonsynonymous SNPs have been identified in *FMO1*, each of which is apparently confined to a single population group, in which it occurs at low, or very low frequency.

Three coding variants that do not alter the amino-acid sequence, g.22818C>T(T249T), g.25061A>G(V396V) [Furnes *et al.* 2003] and g.27258A>G(P467P) (dbSNP126, rs28360432) have also been identified in *FMO1*, (Fig. (**1**) and Table **1**). These synonymous SNPs will not affect protein activity; however, it is not known whether such exonic changes will affect the amount of protein produced by influencing the rate of splicing or efficiency of translation of the FMO1 mRNA.

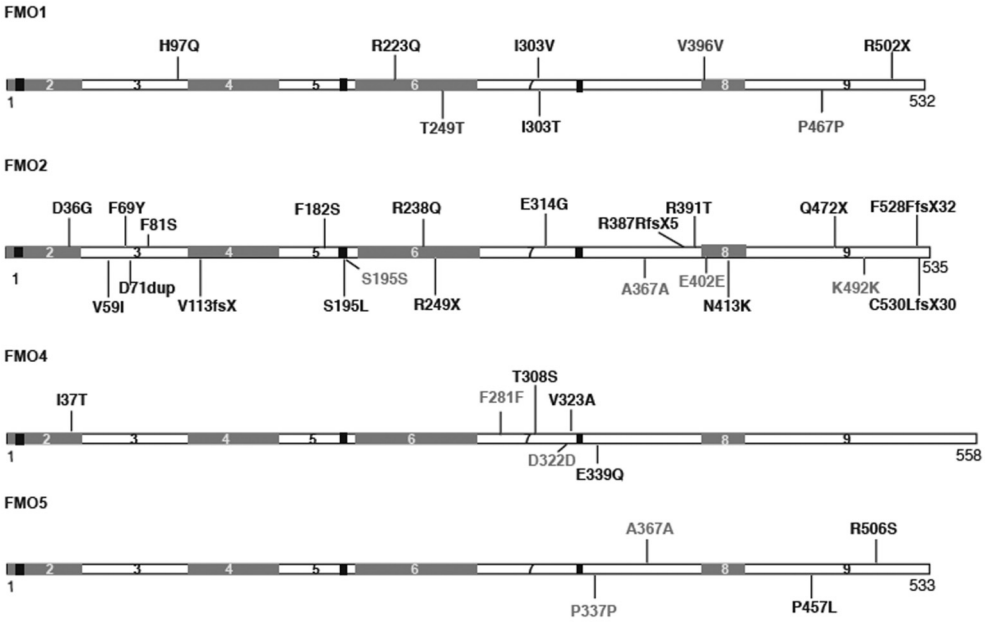

Fig. (1). Genetic variants of FMO1, FMO2, FMO4 and FMO5.

Horizontal bars represent the polypeptide chains. Alternating grey and white boxes represent regions encoded by exons 2 to 9. Black boxes indicate, from left to right, the FAD and NADPH binding sites and the conserved F(A/T)TGY sequence. Nonsynonymous variants are shown in black and synonymous variants in grey.

Table 1. Protein-coding SNPs for *FMOs 1*, *2*, *4* and *5*

Gene	Variant	Exon	Amino Acid Change	Functional Consequence	Reference
FMO1	g.9614C>G	3	H97Q	no effect	Furnes *et al.* 2003; Furnes and Schlenk, 2004
FMO1	g.22739G>A	6	R223Q	n.d.	dbSNP126
FMO1	g.22818C>T	6	T249T	-	Furnes *et al.* 2003; dbSNP126
FMO1	g.23970A>G	7	I303V	no effect	Furnes *et al.* 2003; Furnes and Schlenk, 2004; dbSNP126
FMO1	g.23971T>C	7	I303T	no effect	Furnes *et al.* 2003; Furnes and Schlenk, 2004; dbSNP126
FMO1	g.25061A>G	8	V396V	-	Furnes *et al.* 2003; dbSNP126
FMO1	g.27258A>G	9	P467P	-	dbSNP126
FMO1	g.27362C>T	9	R502X	substrate-dependent decrease	Furnes *et al.* 2003; Furnes and Schlenk, 2004
FMO2	g.107A>G	2	D36G	n.d.	Furnes *et al.* 2003
FMO2	g.7661G>A	3	V59I	n.d.	Furnes *et al.* 2003
FMO2	g.7695T>A	3	F69Y	n.d.	dbSNP126
FMO2	g.7700_7702dupGAC	3	D71dup	loss of function	Furnes *et al.* 2003; Krueger *et al.* 2005
FMO2	g.7731T>C	3	F81S	n.d.	dbSNP126
FMO2	g.10951delG	4	V113fsX	loss of function	Furnes *et al.* 2003; Krueger *et al.* 2005
FMO2	g.13693T>C	5	F182S	n.d.	Furnes *et al.* 2003
FMO2	g.13732C>T	5	S195L	loss of function	Furnes *et al.* 2003; Krueger *et al.* 2005
FMO2	g.13733A>G	5	S195S	-	Furnes *et al.* 2003
FMO2	g.18237G>A	6	R238Q	n.d.	Furnes *et al.* 2003
FMO2	g.18269C>T	6	R249X	n.d. (but likely loss of function)	dbSNP126
FMO2	g.19679A>G	7	E314G	n.d.	dbSNP126
FMO2	g.19839G>A	7	A367A	-	Furnes *et al.* 2003
FMO2	g.19898_19899ins TCAAGCTC	7	R387RfsX5	n.d. (but likely loss of function)	Furnes *et al.* 2003
FMO2	g.19910G>C	7	R391T	n.d.	Furnes *et al.* 2003
FMO2	g.22027G>A	8	E402E	-	Furnes *et al.* 2003
FMO2	g.22060T>G	8	N413K	no effect	Furnes *et al.* 2003; Krueger *et al.* 2005
FMO2	g.23238C>T	9	Q472X	loss of function	Dolphin *et al.* 1998; Whetstine *et al.* 2000
FMO2	g.23300A>G	9	K492K	-	Furnes *et al.* 2003
FMO2	g.23405_23406insT	9	F528FfsX32	n.d.	dbSNP126
FMO2	g.23412_23413insT	9	C530LfsX30	n.d.	Whetstine *et al.* 2000

(Table 1. Contd….)

Gene	Variant	Exon	Amino Acid Change	Functional Consequence	Reference
FMO4	g.110T>C	2	I37T	n.d.	Furnes *et al.* 2003
FMO4	g.14601C>T	7	F281F	n.d.	dbSNP126
FMO4	g.14680A>T	7	T308S	n.d.	dbSNP126
FMO4	g.14724T>C	7	D322D	n.d.	Furnes *et al.* 2003
FMO4	g.14726T>C	7	V323A	n.d.	Furnes *et al.* 2003
FMO4	g.14770G>C	7	E339Q	n.d.	Furnes *et al.* 2003
FMO5	g.23716G>A	7	P337P	n.d.	dbSNP126
FMO5	g. 23806A>G	7	A367A	n.d.	Furnes *et al.* 2003
FMO5	g.37917C>T	9	P457L	n.d.	Furnes *et al.* 2003
FMO5	g.38059G>T	9	R506S	n.d.	dbSNP126

Mutation nomenclature follows that recommended by the Human Genome Organization (http://www.hgvs.org/mutnomen/). n.d., not determined.

Untranslated Coding Variants

Four verified SNPs have been identified in the 3' untranslated region (UTR) of the FMO1 mRNA: g.27568C>T (rs12954) [Hines *et al.* 2003], g.27578 G>A (dbSNP126, rs28360434), g.27590 G>A(dbSNP126, rs28360435;) and g.27664C>T(rs7877) [Hines *et al.* 2003] (Table **1**). Two of these, g.27568C>T and g.27664C>T, are discussed below in the section on FMO1 and disease.

Promoter Variants

A limited number of reports indicate that in rat expression of *FMO1* can be modulated in response to 3-methylcholanthrene [Chung *et al.* 1997], indoles [Katchamart *et al.* 2000] and corticosterone [Chen *et al.* 2005]. However, two of these studies are of liver [Chung *et al.* 1997; Katchamart *et al.* 2000], a tissue in which *FMO1* is not expressed in adult human. Consequently, the relevance of these findings for humans is not clear. The evidence available to date suggests that in humans *FMO1* expression, in contrast to that of *CYP* genes, is not greatly influenced by foreign chemicals. Thus the observed interindividual variations in amounts of FMO1 (see above) are more likely due to genetic rather than environmental factors.

To identify nucleotide changes that might affect the expression of *FMO1*, Hines and co-workers analysed the *FMO1* gene from 177 unrelated individuals representing the world's ethnic diversity: northern Europeans, Africans, Mexican- and native-Americans, and east- and south-Asians [Hines *et al.* 2003]. A region extending about 1 kb upstream of exon 0 [Hernandez *et al.* 2004], exons 0, 2-9 and the immediate flanking intronic sequences were examined for SNPs. This study assessed the novel SNPs identified, together with previously reported SNPs, for any that were likely to influence the expression of *FMO1*. None of the four SNPs in the 5' sequence of *FMO1*, g.-10361T>A, g.-10330C>T, g.-10046A >G and g.-9782C>A, were located in regulatory elements previously identified and implicated in the regulation of transcription of the gene [Luo *et al.* 2001]. However, the SNP g.-9536C>A (in exon 0) was located in a YY1 element previously shown to be involved in regulation of rabbit *FMO1* [Luo *et al.* 2001]. Electrophoretic mobility shift assays using extracts from HepG2 cells revealed a complex set of DNA-protein interactions when 24-bp sequences containing either of the g.-9536C>A SNP variants were used as probes [Hines *et al.* 2003]. The C→A transversion prevented YY1 binding to DNA, but increased the affinity for the transcription factors Oct1, HNF1α and HNF1β. Transfection of HepG2 cells with reporter gene constructs under the control of either the g.-9536C (*FMO1*1*) or g.-9536A (*FMO1*6*) alleles showed no difference when the plasmid contained the *FMO1* minimal promoter. However, constructs containing longer sections of either of the two alleles showed a 2- to 3-fold decrease in reporter gene activity when the g.-9536A was present.

The *FMO1*6* allele is present in African Americans at a frequency of 13%, in northern-European Americans at 11% and in Hispanic Americans at 30%. The effect of this relatively common variant (g.-9536C>A) on *FMO1* gene expression *in vivo* is not clear. Two fetal liver samples, homozygous for the *FMO1*6* allele, showed FMO1 expression levels in the upper quartile range for their age bracket rather than the lower range [Koukouritaki *et al.* 2002] that would be predicted from the results of the transfection experiments described above. It is possible that other transcription factors compensate for the loss of YY1 binding in *FMO1*6* individuals. Therefore at present little is known about SNPs that might influence the amount of FMO1 protein produced.

Two SNPs, g.-11T>C and g.17248T>C, are located within polypyrimidine splice donor sites. However, such pyrimidine for pyrimidine changes are unlikely to affect splicing of the FMO1 transcript and hence would not be expected to influence the amount or type of protein produced.

Therapeutic Drug Substrates For FMO1: Detoxification Versus Bioactivation

Among all FMO isoforms, FMO1 has the broadest range of substrates. A wide range of xenobiotics and foreign com-

pounds are among its substrates, which include therapeutic drugs, pesticides and endogenous compounds. A list of substrates for human FMO1 that are of therapeutic importance is given in Tables **2** and **3**.

A detailed discussion of the activity of FMO1 towards each of these drugs is beyond the scope of this review. For more information on this topic the reader is referred to the review by Krueger and Williams [2005]. However, we have selected two drugs, tamoxifen and thiacetazone, for special mention because of their widespread use in the treatment, respectively, of breast cancer and tuberculosis, and the potential conflict between bioactivation and detoxification with respect to drug metabolism and therapeutic outcome.

Recent work has highlighted interesting results concerning the relationship between FMOs and CYPs with regard to tamoxifen metabolism [Parte and Kupfer, 2005]. Tamoxifen is converted to its *N*-oxide by FMO1 and, less effectively, by FMO3 (see below). *N*-oxygenation of a drug by a FMO is generally considered as a detoxification pathway, with the *N*-oxide being a non-toxic product. In contrast to FMO1-mediated detoxification of tamoxifen, the drug can be bioactivated by CYP-mediated hydroxylation, a reaction catalyzed mainly by CYP3A4, the major hepatic CYP (reviewed in [Krueger *et al.* 2006]). The products of this reaction can bind covalently to DNA and proteins, causing macromolecular damage and potential health risks. Because FMO1 is not present in adult human liver, the ratio of the CYP-bioactivated tamoxifen products to the *N*-oxide detoxification product is expected to be high. However, in tissues in which *FMO1* is expressed, for example, the kidney, in which the amounts of FMO1 more closely resemble that of CYP3A4, tamoxifen-related DNA adducts would be expected to be low. This has been shown to be the case in a study of tissues of monkey treated with tamoxifen [Shibutani *et al.* 2003]. Tamoxifen and its metabolites are widely distributed throughout the body in patients treated with the drug for short or long periods [Lien *et al.* 1991]. The study of Parte and Kupfer [2005] has raised some interesting possibilities with regard to the interplay between CYP and FMO1 metabolism in different tissues. The *N*-oxide of tamoxifen, formed by FMO-mediated action, can be reduced to tamoxifen by CYPs

Table 2. Nitrogen-containing Drugs Oxygenated by Human FMO1

Substrate	Type of Drug and Health Condition	Product	Reference
Benzydamine	nonsteroidal antiinflammatory	*N*-oxide	Lang and Rettie, 2000; Stormer *et al*. 2000
Chlorpromazine	dopamine D2 antagonist (antipsychotic)	-	Kim *et al*. 2000
Deprenyl	monoamine oxidase type B inhibitor (Parkinson's disease)	hydroxylamine	Cashman *et al*. 1999; Szoko *et al*. 2004
Imipramine	5HT/noradrenalin re-uptake inhibitor (antidepressant)	*N*-oxide	Kim and Ziegler, 2000
Itopride	dopamine D2 antagonist (gastroprokinetic)	*N*-oxide	Mushiroda *et al*. 2000
Methamphetamine	psychostimulant	hydroxylamine	Cashman *et al*. 1999; Szoko *et al*. 2004
N-deacetyl ketoconazole*	antifungal agent	*N*-hydroxyl	Rodriguez and Miranda, 2000
Olopatadine	antihistamine	*N*-oxide	Kajita *et al*. 2002
Orphenadrine	anticholinergic (Parkinson's disease)	-	Kim and Ziegler, 2000
SNI-2011	muscarinic receptor agonist (Sjogren's Syndrome)	*N*-oxide	Washio *et al*. 2003
Tamoxifen	estrogen receptor modulator (Breast Cancer Therapy)	*N*-oxide	Parte and Kupfer, 2005
Xanomeline	muscarinic receptor agonist (Alzheimer's Disease)	*N*-oxide	Ring *et al*. 1999

*Major metabolite of the antifungal agent ketoconazole; - Products not identified/unpublished.

In many cases FMO1 is not the only enzyme involved in the metabolism of the drug *in vivo*.

Table 3. Sulphur-containing Drugs Oxygenated by Human FMO1

Substrate	Type of Drug or Health Condition	Product	Reference
Ethionamide	antibiotic (tuberculosis)	-	Krueger and Williams, 2005
Methimazole	thyroperoxidase inhibitor (hyperthyroidism)	*S*-oxide	Furnes *et al*. 2004
S-methyl esonarimod*	cytokine production inhibitor (rheumatism)	*S*-oxide	Ohmi *et al*. 2003
Tazarotenic acid**	retinoic acid receptor modulator (acne/psoriasis)	*S*-oxide	Attar *et al*. 2003
Thiacetazone	antibiotic (tuberculosis)	sulfinic acid/carbodiimide	Qian and Ortiz de Montellano 2006

Products not identified/unpublished; * active metabolite of parent compound esonarimod; ** active metabolite of parent compound tazarotene

In some cases FMO1 is not the only enzyme involved in the metabolism of the drug *in vivo*.

and other heme proteins. Thus, depending on the ratio of FMO1 to CYPs in a particular tissue, reduction of tamoxifen *N*-oxide back to tamoxifen could provide an additional source of the drug for bioactivation by CYPs, or, by acting as a potential store of tamoxifen, the *N*-oxide might increase the amount of available drug and thus lead to greater drug efficacy.

The anti-tubercular drug thiacetazone is a thiocarbamide prodrug, which is activated in *Mycobacterium tuberculosis* by the FMO enzyme EtaA to form a sulfenic acid intermediate [Qian and Ortiz de Montellano, 2006]. This is further oxidized to two metabolites, a sulfinic acid and a carbodiimide. The mechanism of cytotoxicity within the bacterial cell is not known, but it is suggested that metabolites of thiacetazone may lower the concentration of mycothiol (a bacterial antioxidant that has a role similar to that of glutathione in eukaryotic cells), eventually resulting in cell death. Thiacetazone is a substrate *in vitro* for both human FMO1 and FMO3 (see below), the products being the same as those of the reaction catalyzed by the bacterial FMO [Qian and Ortiz de Montellano, 2006]. There is evidence to suggest bioactivation of thiocarbamide drugs by mammalian monooxygenases in both humans and rats [Ruse and Waring, 1991 a, b]. The *in vivo* detoxification routes for thiacetazone are unclear, but FMOs may well play a role in both detoxification and bioactivation pathways. This poses a question concerning the response of individuals to the drug. Will polymorphic variants in *FMO1* that affect the amount or activity of the protein influence the distribution of the drug in extra-hepatic tissues and thus the amount of drug ultimately delivered to the bacterium?

The examples of bioactivation versus detoxification discussed above pose important questions regarding an individual's response to drug therapy. In the case of tamoxifen, tissue distribution of the drug and its metabolites, and hence the balance between drug efficacy and harmful side effects, is likely to be influenced by the combined effects of *FMO1* and *CYP* genetic variants. In the case of thiacetazone, the interplay between the bacterial EtaA protein and FMO1- (or FMO3-) mediated metabolism is predicted to play a role in drug efficacy. However, adverse drug reactions are expected to be due to host FMO-mediated effects.

FMO1 And Disease

Gene expression profiling of human myocardial tissue from patients diagnosed with atrial fibrillation (AF) showed a significant increase in the expression of FMO1 mRNA and of several other mRNAs encoding proteins thought to be involved in oxidative stress [Kim *et al.* 2003]. The role, if any, of specific *FMO1* SNPs in AF is not known. Other DNA profiling experiments have indicated that FMO1 mRNA is under-represented in the spinal cord of patients with amyotrophic lateral sclerosis (ALS) [Malaspina *et al.* 2001]. A recent study [Cereda *et al.* 2006] examined the allelic frequency of two *FMO1* SNPs, g.27568C>T (rs12954) and g.27664C>T (rs7877), located within the 3' UTR of the mRNA, in a group of sporadic ALS patients and a control group. Both SNPs were found to be over-represented in female, but not male, ALS patients. The short distance (97 bp) between the SNPs explains the strong linkage disequilibrium between these two polymorphisms. It is not known whether the two SNPs affect the stability of the mRNA, but this is thought to be unlikely [Hines *et al.* 2003]. The authors suggest that the expression of *FMO1* may be down-regulated in males compared with females and thus the role of FMO1 in neurometabolism may be less important in males than in females. In contrast to the situation in mouse [Cherrington *et al.* 1998b], no significant gender differences in the expression of *FMO1* have been reported for human.

FMO2

The main site of *FMO2* expression is the lung, where in most mammals it constitutes the major form of the enzyme present [Lawton *et al.* 1990; Nikbakht *et al.* 1992; Yueh *et al.* 1997]. In humans the gene is also expressed in skeletal muscle, kidney, prostate gland and blood vessels [Hernandez *et al.* 2004]. In contrast to other FMOs, FMO2 can mediate the *N*-oxygenation of some primary alkylamines to their oximes, *via* an *N*-hydroxylamine intermediate [Tynes *et al.* 1986; Poulsen *et al.* 1986], but is unable to catalyze the oxygenation of certain tertiary amines, such as imipramine and

chlorpromazine [Ohmiya and Mehendale, 1982; Williams *et al.* 1984], that are good substrates for FMO1. In comparison with other FMOs, FMO2 exhibits a greater restriction in the size of its substrates, displaying a marked preference for substrates with a long side chain. This suggests that the active site of FMO2 is located further from the surface of the enzyme than that of other FMOs and is accessed by a relatively narrow channel. In addition, FMO2 is less sensitive to inactivation by elevated temperature and anionic detergents. The gene for FMO2, which was mapped to the long arm of human chromosome 1 [M^{c}Combie *et al.* 1996] comprises 9 exons, the first of which is non-coding.

Genetic Variants

Analysis of cDNAs for human FMO2 revealed that they encoded a polypeptide that, in comparison with FMO2 of rabbit [Lawton *et al.* 1990], guinea pig [Nikbakht *et al.* 1992] and rhesus macaque [Yueh *et al.* 1997], lacks 64 residues from its carboxy terminus [Dolphin *et al.* 1998]. This was the result of C→T mutation that changed a glutamine codon at position 472 to a stop codon. The presence of the premature stop codon in the human *FMO2* gene was confirmed by sequencing genomic DNA from several individuals [Dolphin *et al.* 1998]. The nonsense mutation, g.23238C >T (Q472X), that gave rise to the truncated polypeptide is not present in non-human primates such as chimpanzee (*Pan troglodytes*) and gorilla (*Gorilla gorilla*) [Dolphin *et al.* 1998] and must therefore have arisen in the human lineage some time after the divergence of the *Homo* and *Pan* clades took place some 6 million years ago.

Analysis of individuals of different racial and ethnic backgrounds, namely European Caucasians, Orientals (Japanese and Chinese), Africans (including African Americans and UK Afro-Caribbeans), New-Guinea Aboriginals, Indians and Maoris, revealed that the allele encoding the truncated FMO2, g.23238T (*FMO2*2A*), occurred at a frequency of 100% in all groups investigated, with the exception of individuals of African descent, in which the ancestral g.23238C allele (*FMO2*1*), which encodes a full-length polypeptide (FMO2.1), was present at a frequency of 4% [Dolphin *et al.* 1998]. These results were confirmed and extended by a larger population study [Whetstine *et al.* 2000]. All Europeans (n=79) and Asians (n=120) investigated to date are homozygous for the *FMO2*2A* allele, whereas in African Americans (n=180) the *FMO2*1* allele is present at a frequency of 13%. *FMO2*1* has also been identified in Hispanics [Krueger *et al.* 2002a], and a subsequent study [Krueger *et al.* 2004] found that it is present in Hispanics of Puerto Rican (n=327) and Mexican (n=280) descent at frequencies of 3.5 and 1%, respectively.

Analysis of products of heterologously expressed cDNAs revealed that the truncated protein (X472) encoded by the major allele (*FMO2*2A*) is inactive, whereas the full-length protein (Q472) encoded by the minor allele (*FMO2*1*) is catalytically active [Dolphin *et al.* 1998]. The presence of full-length, active FMO2 in lung microsomes isolated from an individual heterozygous for *FMO2*1/FMO2*2A* was subsequently confirmed [Krueger *et al.* 2002b].

A frame-shift mutation, g.23412_23413insT, has been observed at frequencies of 7 and 13% in African Americans and Caucasians, respectively [Whetstine *et al.* 2000]. This mutation would change the last six amino-acid residues and add 23 residues to the C-terminus of the protein. However, the frame-shift mutation was found to segregate with the g.23238T allele encoding the premature stop codon and thus would have no functional consequence.

A survey of 50 African Americans [Furnes *et al.* 2003] identified an additional 14 coding-region variants in *FMO2*. Four were synonymous; the other ten (seven nonsynonymous, two frame-shifts and one in-frame 3-bp duplication) would result in a change in amino acid sequence. Four of the latter, g.7700_7702dupGAC(D71dup), g.10951delG (V113fsX), g.13732C>T(S195L) and g.22060T>G(N413K), occurred at high frequency (>25%) in this population. The effect on FMO2 activity of these four mutations has been investigated [Krueger *et al.* 2005]. The frame-shift mutation (g.10951delG) results in the replacement of valine at position 113 with a stop codon and thus produces a severely truncated protein. When expressed in a baculovirus-insect cell system the truncated protein was not detected and thus may have been targeted for degradation. The D71dup protein failed to bind FAD and was thus catalytically inactive. Although S195L bound FAD it retained only 2% of the activity of FMO2.1. In contrast, the activity of N413K was similar to that of FMO2.1.

With the exception of g13732C>T(S195L), which is present at high frequency in Africans (35-60%), Asians (45-55%) and Europeans (15-30%), the other mutations are either absent or present in much lower frequency in non-African populations. Of particular importance is whether variants that affect the function of FMO2 occur on the *FMO2*1* allele, which encodes a full-length protein, or on the *FMO2*2A* allele, which encodes a truncated non-functional protein. Haplotype analysis of g.10951delG(V113fsX), g.13732C>T(S195L) and g.22060T>G(N413K) indicated that all three variants segregated with the g.23238T(Q472X) variant and would thus be associated with a protein that is already inactive [Krueger *et al.* 2005]. This implies that individuals who possess a *FMO2*1* allele are likely to express a protein that is catalytically active.

The other coding-region variants identified by Furnes *et al*. [Furnes *et al.* 2003] plus additional ones reported in dbSNP126 are shown in Fig. (**1**) and Table **1**. The functional effect of the nonsynonymous SNPs has not been established. Of the nonsynonymous SNPs, three, g.7695T>A(F69Y), g.18237G>C(R238Q) and g.19910G>C(R391T) are apparently restricted to African populations, where they occur at low frequency (4 to 7%). G.19679A>G(E314G) is particularly common (25-35%) in Asians, whereas g.7731T>C (F81S) is absent from Asians, but relatively common (12-30%) in Africans. Of the synonymous SNPs, g.13733A>G (S195S) occurs at high frequency (25-60%) in all populations, whereas g.19839G>A(A367A) and g.22027G>A(E402E) are restricted to Africans (15-23%).

A large study[1] (more than 1800 individuals from 25 different populations) demonstrated that the *FMO2*1* allele is widely distributed throughout Africa. The allele occurs at

[1] Veeramah *et al*. (submitted).

relatively low frequency (2-6%) in north Africa, but is more common in sub-Saharan Africa, in some regions reaching frequencies as high as 26%, with almost 50% of individuals having at least one *FMO2*1* allele.

FMO2 Substrates

Substrates of FMO2 include the thioether-containing organophosphate insecticides phorate and disulfoton [Henderson *et al.* 2004a]. Organophosphates are bioactivated by CYP-mediated desulfuration to produce the oxon, which is a far more potent inhibitor of acetylcholinesterase than is the parent compound [Kulkarni and Hodgson, 1984]. In contrast, FMO2 catalyzes the *S*-oxygenation of organophosphates [Henderson *et al.* 2004a], which represents a detoxification pathway. Thus, individuals who express functional FMO2 may have a reduced risk of toxicity if exposed to organophosphates.

Of particular concern is the role of FMO2 in bioactivation. FMO2 has been shown to catalyze the *S*-oxygenation of thioureas, including thiourea, 1-phenylthiourea and ethylene thiourea, which are known or suspected lung toxicants, to produce the more toxic sulfenic and/or sulfinic acid metabolites [Henderson *et al.* 2004b]. The sulfenic acid derivatives can combine with glutathione and undergo redox cycling, leading to oxidative stress and toxicity. Thus, individuals who express functional FMO2 would be predicted to be at increased risk of pulmonary toxicity following exposure to thiourea or its derivatives.

Little is known about the role of FMO2 in the metabolism of drugs. However, prochloperazine and trifluoperazine have been shown to be substrates for FMO2 of rabbit [Lomri *et al.* 1993]. Of particular interest is the recent finding that the anti-tubercular drug thiacetazone is a good substrate for human FMO2[2], which has implications for the efficacy and toxicity of this drug in African populations.

Potential Animal Model

A gene encoding a truncated, non-functional FMO2 is present in strains of laboratory rats (Sprague-Dawley and Wistar) and in the species, *Rattus norvegicus*, from which they were derived, but not in the closely related species *Rattus rattus* [Lattard *et al.* 2002b]. However, the mutation that gives rise to the truncated protein, a 2-bp deletion in codon 421, which results in a premature stop codon at position 433, is different from that present in humans, and must have occurred independently after the divergence of *Rattus norvegicus* and *Rattus rattus*. Interestingly, the mutation is polymorphic in wild *Rattus norvegicus* and thus this species may represent a good model for investigating the metabolic and toxicological consequences of the human *FMO2* polymorphism [Hugonnard *et al.* 2004].

Conclusions

Evidence to date indicates that in most of the world's population groups essentially all individuals are homozygous for the *FMO2*2* allele (g.23238C>T[Q472X]) and thus produce no functional FMO2. However, in sub-Saharan Africa and in populations recently descended from this region, a substantial proportion of individuals (almost 50% in some regions) possess at least one copy of the ancestral *FMO2*1* allele and thus express functional FMO2. This has implications for inter-ethnic and, in African populations, interindividual variations in response to drugs, and in susceptibility to toxic chemicals, that are substrates of FMO2, particularly those for which the lung is the target organ or route of entry.

FMO3

A cDNA for human FMO3 was identified in 1992 by Lomri *et al*. [Lomri *et al.* 1992]. The *FMO3* gene was mapped to the long arm of chromosome 1 [Shephard *et al.* 1993]. It comprises 9 exons, of which exon 1 is non-coding [Dolphin *et al.* 1997a], and encodes a polypeptide of 532 amino-acid residues and molecular mass 60,047 [Phillips *et al.* 1995].

Tissue Distribution

FMO3 is the predominant FMO isoform expressed in the liver of the human adult [Lomri *et al.* 1992; Phillips *et al.* 1995; Dolphin *et al.* 1996]. FMO3 mRNA has been detected also in lung, kidney, adrenal medulla and cortex, pancreas, thyroid, gut and brain [Hernandez *et al.* 2004]. *FMO3* expression is switched on after birth in humans [Koukouritaki *et al.* 2002] and in other species such as mouse [Janmohamed *et al.* 2004]. However, small amounts of FMO3 protein have been detected in the human embryonic, but not fetal, liver [Koukouritaki *et al.* 2002]. The mRNA for FMO3 is also not detectable in fetal liver [Dolphin *et al.* 1996]. The mechanisms and factors required to express the *FMO3* gene in the embryo, to silence its expression during fetal development and then to re-activate expression of the gene after birth are unknown. A comprehensive, immunochemical study [Koukouritaki *et al.* 2002] of human liver samples from 240 individuals, ranging in age from 8 weeks gestation to 18 years old, detected small amounts of FMO3 as early as 8 weeks (the embryonic stage), but not in the fetus, 15-40 weeks gestation. Analysis of postnatal samples showed that, during the first 3 weeks postpartum, in most individuals FMO3 was undetectable or present in only small amounts. Three subsequent developmental phases of *FMO3* expression were apparent. In the first phase, between 3 weeks and 10 months of age, amounts of FMO3 increased 5-fold, to 4.7 ± 5.9 pmol/mg microsomal protein. Between 10 months and 11 years of age another 3-fold increase in expression was observed (to 12.7 ± 8.0 pmol/mg microsomal protein). In the third phase, between 11 and 18 years of age, there was a further 2-fold increase (to 26.9 ± 8.6 pmol/mg microsomal protein). The amount of FMO3 produced was not influenced by gender.

The data indicate that birth is necessary, but not sufficient, for the onset of *FMO3* expression in human liver. The age at which *FMO3* expression is switched on varies from birth to up to 2 years old. However, by ten months of age, the majority of individuals are expressing *FMO3*. This contrasts with the extinction of *FMO1* expression in human liver, which is tightly linked to the process of birth, being switched off within a few days of birth, irrespective of gestational age [Koukouritaki *et al.* 2002] (see above). Consequently, during the first year of life many individuals will

[2] Francois, A., Phillips, I.R. and Shephard, E. A. (unpublished).

have no, or very small amounts of drug-metabolizing FMOs in their liver, which has important implications for their ability to metabolize therapeutic drugs that are substrates for these enzymes.

Interindividual variations of 10- to 20-fold in the expression of *FMO3* have been reported [Overby *et al.* 1997; Koukouritaki *et al.* 2002; Cashman and Zhang, 2002]. The amounts of hepatic FMO3 reported by Overby *et al.*, [Overby *et al.* 1997] (60 ± 43 pmol/mg microsomal protein) were higher than those reported by Koukouritaki *et al.* [2002] (see above). However, the samples analysed in the former study were from adult liver, whereas those in the latter study were from livers of 11 to 18 year olds. Therefore, it is possible that the differences reflect a further increase in *FMO3* expression from the teenage years to adulthood. The amounts of FMO3 present in adult liver are therefore similar to those of the most abundant hepatic CYPs, CYP3A4, CYP2C and CYP1A2, with reported specific contents of 96 ± 51, 60 ± 27 and 42 ± 23 pmol/mg microsomal protein, respectively [Shimada *et al.* 1994]. FMO3 expression has been found to be inducible by dioxin in male mouse liver [Tijet *et al.* 2006] and, in humans, may be influenced by physiological factors, e.g., FMO3 declines during menstruation [Zhang *et al.* 1996; Shimizu *et al.* 2007a]. However, the majority of the observed interindividual variation in *FMO3* expression is likely to be due to genetic factors.

Genetic Variants

Trimethylaminuria

Loss-of-function mutations in the *FMO3* gene (MIM 136132) cause the inherited disorder trimethylaminuria (TMAuria) (MIM 60279), also known as fish-odor syndrome. Affected individuals are unable to metabolize dietary-derived trimethylamine (TMA) to its non-odorous *N*-oxide, a reaction catalyzed by FMO3 [Lang *et al.* 1998]. Consequently, they secrete the smelly free amine in their breath, sweat and urine, which imparts a bodily odor reminiscent of rotting fish [Mitchell and Smith, 2001]. Sufferers often display a variety of psychological and antisocial reactions to their condition, including anxiety, chronic depression, and attempted suicide [Mitchell and Smith, 2001]. The disorder is inherited in a recessive manner [Al-Waiz *et al.* 1988].

The incidence of heterozygotes in British Caucasians is estimated to be about 1%, but may be higher in other population groups [Hadidi *et al.* 1995; Mitchell *et al.* 1997; Mitchell and Smith, 2001]. The first report identifying a causative mutation for this disorder, g.15153C>T(P153L), was in 1997 [Dolphin *et al.* 1997b]. The mutant protein, expressed in a baculovirus-insect cell system, was unable to catalyze the *N*-oxygenation of TMA. Subsequently, more than 30 mutations have been identified that cause TMAuria [Mitchell and Smith, 2001; Hernandez *et al.* 2003; Cashman and Zhang, 2006; Phillips and Shephard, 2007], (Fig. (**2**) and Table **4**). The resultant mutant enzymes are unable to catalyze TMA *N*-oxygenation and, with one exception, N61S, are also inactive towards other substrates tested, such as 10-(*N,N*-dimethylaminopentyl)-2-(trifluoromethyl) phenothiazine (5'-DPT), tyramine and methimazole. Although N61S is incapable of catalyzing *N*-oxygenation of TMA, its ability to catalyze *S*-oxygenation of methimazole is unaffected [Dolphin *et al.* 2000].

Individuals displaying symptoms of TMAuria are referred to in the ancient Indian epic the Mahabharata and in Shakespeare's the Tempest [Mitchell and Smith, 2001]. Although we now understand the genetic basis of TMAuria, there has been little improvement in the lives of those affected by this distressing disorder. Antibiotics are sometimes prescribed in an attempt to kill gut flora that produce TMA from dietary precursors such as choline and lecithin. The dietary supplements copper chlorophyllin and charcoal are reported to decrease the amount of TMA in the urine [Yamazaki *et al.* 2004]. However, patients report variable effectiveness of these treatments. Strict diets that limit the intake of foodstuffs rich in precursors of TMA, such as meat, eggs, soya and marine fish, have been reported to help control the condition.

Polymorphic Variants

In the course of studies on the genetic causes of TMAuria, several polymorphic variants of FMO3 have been discovered, (Fig. (**2**) and Table **5**). Common variants that affect enzyme function are of particular interest because of their potential effect on the metabolism of therapeutic drugs and other foreign chemicals by the general population. The first such variant identified was g.15167G>A(E158K) [Brunelle *et al.* 1997; Dolphin *et al.* 1997b]. Although initially identified in TMAuria kindreds, the variant did not segregate with the disorder and was found to be common in the general population. Subsequently, it was found to be present at high frequency in all populations (dbSNP126): 40-45% in Africans, 35-45% in Europeans and 20-25% in Asians. Many studies have investigated the effect of this common variant on catalytic activity, both *in vitro* and *in vivo* (reviewed in [Cashman 2004; Krueger and Williams 2005; Koukouritaki and Hines, 2005 and Cashman and Zhang, 2006); Shimizu *et al.* 2007b]. The results are conflicting. Some reports indicate that the 158K variant has a lower catalytic activity towards some substrates, whereas others indicate no effect, or substrate-dependent effects.

Another variant, g.21443A>G(E308G) [Treacy *et al.* 1998], is relatively common in Asians and Europeans (at allele frequencies of 15-25%), but is rare in Africans (frequency of 1 to 2%) (dbSNP126). As is the case for E158K (see above), studies on the effect of E308G on enzyme activity report conflicting results [Cashman 2004; Krueger and Williams 2005; Koukouritaki and Hines, 2005; Cashman and Zhang, 2006]. In Asians and Europeans, the E158K and E308G variants are often linked, occurring on the same haplotype [Sachse *et al.* 1999; Kang *et al.* 2000; Cashman *et al.* 2001; Park *et al.* 2002]. Evidence from several studies indicates that the effect on enzyme activity is greater when both mutations are present than when either is present alone [Lattard *et al.* 2003; Cashman 2004; Krueger *et al.* 2005; Koukouritaki and Hines, 2005; Cashman and Zhang, 2006; Shimizu *et al.* 2007b]. Individuals homozygous for the linked E158K/E308G variants may exhibit symptoms of 'mild' or transient TMAuria [Mayatepek and Kohlmüller,

Table 4. Rare *FMO3* Mutations Causative of TMAuria or Implicated in the Disorder

Mutation	Exon	Amino Acid Change	Reference
g.-2092 to 10145del			Forrest *et al.* 2001
g.94G>A	2	E32K	Zhang *et al.* 2003
g.110T>C	2	I37T	Teresa *et al.* 2006
g.11145A>G	3	R51G	Mazon Ramos *et al.* 2003
g.11148G>T	3	A52T	Akerman *et al.* 1999a
g.11166G>A	3	V58I	Kubota *et al.* 2002
g.11177A>G	3	N61S	Dolphin *et al.* 2000
g. 11185delA	3	K64KfsX2	Zhang *et al.* 2003
g.11192G>T	3	M66I	Cashman *et al.* 1997; Akerman *et al.* 1999b; Forrest *et al.* 2001
g.11239T>C	3	M82T	Murphy *et al.* 2000
g.15036A>G	4	N114S	Shimizu *et al.* 2007c
g.15123T>A	4	V143E	Basarab *et al.* 1999
g.15137G>T	4	G148X	Park *et al.* 1999
g.15153C>T	4	P153L	Dolphin *et al.* 1997b; Cashman *et al.* 1997
g.15526_15527delTG	5	C197fsX	Yamazaki *et al.* 2007
g.15531T>A	5	D198E	Fujieda *et al.* 2003
g.15533T>C	5	I199T	Zschocke *et al.* 1999
g.15539C>A	5	T201K	Shimizu *et al.* 2006
g.18177G>A	6	R223Q	Preti *et al.* 2002
g.18225G>C	6	R238P	Teresa *et al.* 2006
g.21429G>T	7	E305X	Treacy *et al.* 1998; Akerman *et al.* 1999a
g.21460G>T	7	E314X	Akerman *et al.* 1999a
g.21680G>T	7	R387L	Akerman *et al.* 1999a
g.21684G>A	7	W388X	Shimizu *et al.* 2007c
g.21702delG	7	K394KfsX11	Teresa *et al.* 2006
g.23580delG	8	M405IfsX	Teresa *et al.* 2006
g.24486G>A	9	M434I	Dolphin *et al.* 2000
g.24592C>T	9	Q470X	Shimizu *et al.* 2007c
g.24608G>A	9	G475D	Zschocke *et al.* 1999
g.24658C>T	9	R492W	Akerman *et al.* 1999b; Dolphin, 2000
g.24682C>T	9	R500X	Shimizu *et al.* 2006

K64KfsX2 has also been referred to as M66X

Mutation nomenclature follows that recommended by the Human Genome Organization (http://www.hgvs.org/mutnomen/)

1998; Zschocke *et al.* 1999]. However, many of the cases reported are of young children, in whom low expression of *FMO3* [Koukouritaki *et al.* 2002] (see above) may contribute to symptoms.

A third variant, g.18281G>A(V257M) [Treacy *et al.* 1998], is relatively common in Asian (allele frequency of 16 to 28%), but less so in African (0 to 4%) or European (4 to 7%) populations (dbSNP126). V257M has little effect on enzyme activity [Dolphin *et al.* 2000; Cashman 2004;

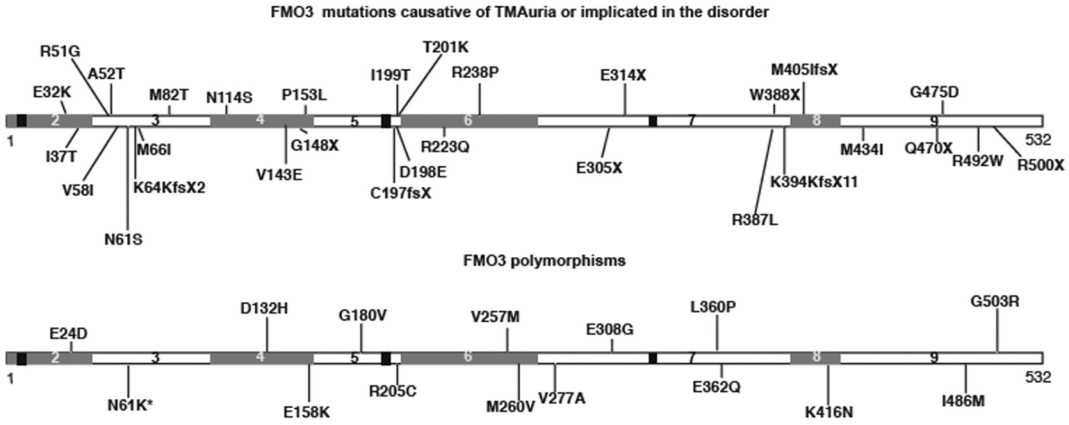

Fig. (2). Genetic variants of FMO3.

Horizontal bars represent the polypeptide chains. Alternating grey and white boxes represent regions encoded by exons 2 to 9. Black boxes indicate, from left to right, the FAD and NADPH binding sites and the conserved FATGY sequence. The upper bar shows the identity and position of rare mutations that are causative of TMAuria, or implicated in the disorder. The lower bar shows the identity and position of non-synonymous polymorphic variants. *SNP likely to be causative for TMAuria.

Krueger and Williams 2005; Koukouritaki and Hines, 2005; Cashman and Zhang, 2006; Shimizu *et al.* 2007b].

A further 20 coding-region SNPs, 8 synonymous and 12 nonsynonymous, have been identified in *FMO3* [Dolphin *et al.* 2000; Cashman 2002; Furnes *et al.* 2003; Fujieda *et al.* 2003; Koukouritaki *et al.* 2005b; and dbSNP126], (Fig. (**2**) and Table **5**). Of the nonsynonymous SNPs only g.21350T>C(V277A) is relatively common, occurring at a frequency of 13% in Africans and ~20% in Asians, but it is absent from Europeans. Another, g.11177C>A(N61K), is present in Africans and Europeans at frequencies of 3.5 and 5%, respec-

Table 5. Polymorphic Variants of *FMO3*

Variant	Exon	Amino Acid Change	Functional Consequence	Reference
g.72G>T	2	E24D	limited	Koukouritaki *et al.* 2007
g.11177C>A	3	N61K*	reduced or abolished	Koukouritaki *et al.* 2007
g.15089G>C	4	D132H	substrate-dependent decrease	Kobayashi *et al.* 2001; Furnes *et al.* 2003; Lattard *et al.* 2003
g.15167G>A	4	E158K	moderate, substrate- dependent decrease	Dolphin *et al.* 1997b; Brunelle *et al.* 1997; Treacy *et al.* 1998; Zschocke *et al.* 1999; Furnes *et al.* 2003
g.15475G>T	5	G180V	no effect	Dolphin *et al.* 2000
g.15550C>T	5	R205C	moderate decrease	Fujieda *et al.* 2003
g.18281G>A	6	V257M	no effect or limited substrate dependent decrease	Treacy *et al.* 1998; Dolphin *et al.* 2000; Furnes *et al.* 2003
g.18290A>G	6	M260V	n.d.	Shimizu *et al.* 2006
g.21350T>C	7	V277A	n.d.	Cashman 2002
g.21443A>G	7	E308G	moderate substrate dependent decrease	Treacy *et al.* 1998; Zschocke *et al.* 1999
g.21599T>C	7	L360P	increased activity	Furnes *et al.* 2003; Lattard *et al.* 2003
g.21604G>C	7	E362Q	n.d.	Cashman 2002; Furnes *et al.* 2003
g.23613G>T	8	K416N	limited	Koukouritaki *et al.* 2007
g.24642G>A	9	I486M	n.d.	Cashman 2002
g.24691G>C	9	G503R	n.d.	Furnes *et al.* 2003

*likely to be causative for TMAuria

tively. The remaining SNPs occur at low frequency (<4%, in some cases <1%) and are confined to a single population group.

G180V [Dolphin *et al.* 2000], K416N and E24D [Koukouritaki *et al.* 2007] have little or no effect on catalytic activity. The effect of D132H appears to be substrate-dependent, decreasing *N*-oxygenation of TMA and *S*-oxygenation of methimazole, but not *N*-oxygenation of 5'DPT [Koukouritaki *et al.* 2007]. R205C has a moderate effect on enzyme activity and, interestingly, exhibits substrate-inhibition of sulindac sulfide *S*-oxygenation [Shimizu *et al.* 2007b]. N61K either abolished or dramatically reduced enzyme activity towards four different substrates [Koukouritaki *et al.* 2007]. In contrast, L360P causes a 2- to 5-fold increase in catalytic activity [Lattard *et al.* 2003], and is the only genetic variant of FMO3 known to substantially increase enzyme activity. As such, it could increase the rate of drug metabolism, with a consequent decrease in efficacy. However, it has been detected only in African populations and then at low frequency (2 to 4%). Consequently, it is of limited significance for the general population. The functional consequence of V277A, which is relatively common in Asian and African populations, but absent from Europeans, has not been investigated. However, valine is not conserved in FMOs at this position and thus the substitution, which represents a conservative change, is not expected to affect activity.

Polymorphisms in Upstream Regulatory Region

Resequencing of 24 DNA samples from the Coriell Polymorphism Discovery Resource [Koukouritaki *et al.* 2005b] identified seven variants in the *FMO3* 5'-flanking sequence: g.-2650C>A, g.-2589C>T, g.-2543T>A, g.-2177G>C, g.-2106G>A, g.-2099A>G and g.-1961T>C. Based on the frequencies of individual alleles in northern-European Whites, African Americans or Hispanic Americans of Mexican descent, 15 haplotypes were inferred. Seven haplotypes were considered common (frequency >1%) and were found in one or more of the ethnic groups at significantly different frequencies. The effect of individual haplotypes on gene expression was measured in HepG2 cells transfected with different *FMO3*-reporter constructs [Koukouritaki *et al.* 2005b]. Haplotype 2, (g.-2650C>A, g.-2543T>A, g.-2177G>C) caused an 8-fold increase in reporter gene activity compared with haplotype 1 (the reference sequence). Haplotype 2 was present at frequencies of 29, 11 and 6%, respectively, in the Hispanic, African-American and White groups. Two haplotypes, 8 (g.-2589C>T, g.-2106G>A) and 15 (g.-2106G>A, g.-1961T>C), resulted in very low reporter gene activity. Haplotype 8 (-2589C>T, -2106G>A) is present only in the White group, at a frequency of 4%, whereas haplotype 15 (-2106G>A, -1961T>C) was found only in the African-American group (frequency 1.5%). The effects on reporter gene activity of haplotypes 3 (-2650C>G), 4 (-2650C>G, -2543T>A) and 11 (-2543T>A) were similar to those of the reference haplotype 1.

Individuals with haplotype 2, particularly homozygotes, would be expected to metabolize FMO3 substrates more rapidly if the increased promoter activity observed *in vitro* [Koukouritaki *et al.* 2005b] translates into increased production of FMO3 protein in the liver. Similarly, those individuals homozygous for haplotypes 8 or 15 are predicted to produce lower amounts of the protein. If the relative promoter activities seen in the HepG2 cell transfection experiments are similar to those *in vivo* then individuals homozygous for haplotypes 8 or 15 would be expected to suffer some of the symptoms of TMAuria, due to the low amount of FMO3 protein produced in their liver. Individuals who are compound heterozygotes for haplotypes 2 and 15 (African American) or haplotypes 2 and 8 (White) would be expected to have amounts of the protein closer to that produced under the influence of haplotype 1.

This study was extended over a longer region of the *FMO3* gene to examine linkage between the promoter SNP clusters (see above) and downstream coding variants, in 404 Hispanic (Mexican), 400 Latino White and 402 African Americans [Koukouritaki *et al.* 2007]. Haplotype 2 [Koukouritaki *et al.* 2005b], which caused an 8-fold increase in reporter gene activity (see above) was found to be associated with E158K, E308G or the linked variant E158K/E308G. The combination of a high activity promoter with a coding variant that causes substrate-dependent reduction in enzyme activity may balance out and result in the production of sufficient protein to compensate for an enzyme with lower activity. The haplotype analysis undertaken by Koukouritaki *et al*. [Koukouritaki *et al.* 2007] underlines the importance, and the difficulties we face, in understanding the complete relationship between genotype and phenotype *in vivo*.

Human FMO3 Variation And Clinical Implications

TMAuria sufferers lack functional FMO3 and, consequently, are expected to have impaired ability to metabolize therapeutic drugs that are substrates for FMO3. Owing to the relatively small number of patients identified to date, little systematic investigation of this has been undertaken. However, one study (Mayatepek *et al.* 2004) found that TMAuria sufferers have a markedly reduced capacity for *N*-oxygenation of benzydamine, a non-steroidal anti-inflammatory, and there are numerous anecdotal accounts of patients experiencing poor responses to other drugs. Tables **6** and **7** summarize the known drug substrates for FMO3.

In this section we highlight some recent studies of the effect of polymorphic *FMO3* variants on drug metabolism. The first study to show that *FMO3* variants influence clinical outcomes was in a group of patients treated with sulindac [Hisamuddin *et al.* 2004], a nonsteroidal anti-inflammatory drug prescribed to patients with familial adenomatous polyposis (FAP) or to individuals who are perceived to be predisposed to this condition, judged by mutations in the *APC* gene. Sulindac is a prodrug and is converted by gut flora to its active metabolite, sulindac sulfide, which is then absorbed [Duggan *et al.* 1977; Etienne *et al.* 2003]. Human FMO3 catalyzes the oxygenation of sulindac sulfide to its sulfoxide [Hamman *et al.* 2000], which is virtually inactive [Duggan *et al.* 1977]. Forty one individuals who were known to have mutations in the *APC* gene, but had not developed polyps at the start of the study, were divided into two groups. One group was given sulindac for 4 years, while the other received a placebo. At the end of the trial 57% of the sulindac-

Table 6. Nitrogen-containing Drugs Oxygenated by Human FMO3

Substrate	Type of Drug or Health Condition	Product(s)	Reference
Amphetamine	dopamine transporter ligand (antipsychotic)	hydroxylamine	Cashman *et al*. 1999; Szoko *et al*. 2004
Benzydamine	nonsteroidal antiinflammatory (rheumatism)	*N*-oxide	Lang and Rettie, 2000; Stormer *et al*. 2000
Clozapine	antipsychotic agent	*N*-oxide	Tugnait *et al*. 1997
Deprenyl	monoamine oxidase type B inhibitor (Parkinson's disease)	hydroxylamine	Szoko *et al*. 2004
Itopride	dopamine D2 antagonist (gastroprokinetic agent)	*N*-oxide	Mushiroda *et al*. 2000
K11777	cysteine protease inhibitor agent against *Trypanosoma cruzi*	*N*-oxide	Jacobsen *et al*. 2000
Methamphetamine	psychostimulant	hydroxylamine	Cashman *et al*. 1999; Szoko *et al*. 2004
N-deacetyl ketoconazole*	antifungal	*N*-hydroxy/nitrone	Rodriguez and Miranda, 2000
Nicotine	stimulant	Trans *N*-oxide	Park *et al*. 1993
Olopatadine	antihistamine	*N*-oxide	Kajita *et al*. 2002
Pyrazolacridine	antitumour	*N*-oxide	Reid *et al*. 2004
Ranitidine	Antihistamine (stomach ulcers/ Zollinger Ellison syndrome)	*N*-oxide	Overby *et al*. 1997; Chung *et al*. 2000
S16020	topoisomerase II inhibitor (antitumour)	*N*-oxide	Pichard-Garcia *et al*. 2004
Tamoxifen	estrogen receptor modulator (breast cancer therapy)	*N*-oxide	Parte and Kupfer, 2005
Xanomeline	muscarinic receptor agonist (Alzheimer's Disease)	*N*-oxide	Ring *et al*. 1999

*Major metabolite of antifungal agent ketoconazole.

In many cases FMO3 is not the only enzyme involved in the metabolism of the drug *in vivo*.

treated group were polyp-free compared with 45% of the placebo-treated group. When the individuals were genotyped for common *FMO3* polymorphic variants, those homozygous for E158K (33%) or E308G (17%), and who had received sulindac, had not developed polyps. Individuals who had developed polyps were not homozygous for either of the *FMO3* variant alleles [Hisamuddin *et al.* 2004]. A follow-on study with 19 individuals already suffering from FAP assessed whether *FMO3* genotype correlated with polyp regression following sulindac treatment [Hisamuddin *et al.* 2005]. All individuals homozygous or heterozygous for the E158K allele or heterozygous for both E158K and E308G alleles showed a reduction in polyp number. No individuals homozygous for E308G were included in this study.

The reasons for the increased sulindac efficacy in patients with E158K and E308G variants might be due to the reduction in the conversion by FMO3 of sulindac sulfide to inactive metabolites. Thus the amount of sulindac sulfide available to the patient's circulation would be expected to increase and the longer duration of the active drug may contribute to the regression of polyps and/or more effectively prevent their formation.

This study raises an important question with respect to sulindac efficacy and other *FMO3* polymorphisms. Recently, the N61K variant was shown to have a 30-fold decrease in catalytic activity towards sulindac [Koukouritaki *et al.* 2007]. It remains to be seen whether sulindac will be a less effective treatment against FAP in individuals with *FMO3* variants that increase promoter activity [Koukouritaki *et al.* 2005b] or enzyme activity (L360P) [Lattard *et al.* 2003] (see above).

Clozapine, an antipsychotic, is metabolized to its *N*-oxide by both FMO3 [Tugnait *et al.* 1997] and CYP3A4 [Fang *et al.* 1998]. A study of 396 individuals of German ethnicity found no correspondence between serum *N*-oxide concentrations and *FMO3* haplotypes [Sachse *et al.* 1999].

Most studies on the effect of *FMO3* polymorphisms have been carried out *in vitro*. An interesting example of the effect of the E158K polymorphism *in vitro* is with respect to

Table 7. Sulfur-containing Drugs Oxygenated by Human FMO3**ubstrate**	**Type of Drug Or Health Condition**	**Product(s)**	**Reference**
Ethionamide	antibiotic (tuberculosis)	-	Krueger and Williams, 2005
MK-0767 methyl sulfide	peroxisome proliferator receptor activator (diabetes)	*S*-oxide	Karanam *et al.* 2004
Methimazole	thyroperoxidase inhibitor (hyperthyroidism)	*S*-oxide	Overby *et al.* 1997
Ranitidine	Antihistamine (stomach ulcers/ Zollinger Ellison syndrome)	*S*-oxide	Chung *et al.* 2000
S-methyl esonarimod *	cytokine production inhibitor (rheumatism)	*S*-oxide	Ohmi *et al.* 2003
Sulindac sulfide**	nonsteroidal anti-inflammatory (colorectal cancer)	*S*-oxide	Hamman *et al.* 2000
Tazarotenic Acid***	retinoic acid receptor modulator (acne/psoriasis)	*S*-oxide	Attar *et al.* 2003
Thiacetazone	antibiotic (tuberculosis)	sulfinic acid/ carbodiimide	Qian and Ortiz de Montellano, 2006

- Products not identified/unpublished; * active metabolite of parent drug esonarimod; **active metabolite of parent drug sulindac; *** active metabolite of parent drug tazarotene. In many cases FMO3 is not the only enzyme involved in the metabolism of the drug *in vivo*.

tamoxifen *N*-oxidation: when catalyzed by baculovirus-expressed *FMO3* variants this was 5-fold greater with FMO3(K158) than with FMO3(E158) [Krueger *et al.* 2006]. Therefore the K158 variant would be expected to increase the amount of tamoxifen *N*-oxide in the liver, which may be important in the therapeutic response (see FMO1 section above).

Benzydamine, an anti-rheumatic drug is metabolized by FMO3 to its *N*-oxide [Lang and Rettie, 2000]. A study involving human liver microsomes from individuals genotyped for *FMO3* variants, found that microsomes from individuals homozygous for the K158 variant showed significantly reduced activity compared with those from individuals with at least one copy of the E158 variant [Stormer *et al.* 2000]. However, a study using recombinantly expressed E158 and K158 variants found no difference in oxygenation of benzydamine [Shimizu *et al.* 2007b]. The different results obtained by different groups when studying the effect of *FMO3* variants on enzyme activity indicates the need for the validation of methods used to express the recombinant proteins and to assay their activity.

FMO3, like FMO1 (see above), metabolizes thiacetazone, an anti-tubercular drug prescribed in certain parts of Africa because it is cheap. Thiacetazone is a thiocarbamide prodrug and is a substrate for human FMO3. The main products of the reaction are the sulfinic and sulfenic acids of the drug [Qian and Ortiz de Montellano, 2006], which have been shown to be genotoxic in mammalian cells [Ziegler-Skylakakis *et al.* 1998] and are thought to affect the redox state [Qian and Ortiz de Montellano, 2006]. The use of this drug in areas of the world other than Africa is limited, due to severe hepatotoxic effects. It would be of interest to know whether polymorphic variants of *FMO3* contribute to this toxicity. Although the large-scale genotyping of individuals in developing countries may not be realistic on economic grounds, it may be worth considering in cases where a common polymorphism has been identified that has a significant effect on response to a commonly prescribed drug.

FMO4

When cDNA clones for human FMO4 were first isolated [Dolphin *et al.* 1992] the isoform was designated FMO2. The nomenclature was subsequently changed to FMO4 [Lawton *et al.* 1994]. FMO4 is 558 amino-acid residues long and has a molecular mass of 63,338. Other members of the FMO family, with the exception of the truncated form of FMO2, encoded by the *FMO2*2A* allele (see above), contain between 532 and 535 residues [Phillips *et al.* 1995]. From sequence comparisons, it is apparent that the additional residues in FMO4 are contained in a single block located at the C-terminus of the polypeptide [Dolphin *et al.* 1992]. Inspection of the nucleotide sequence of the FMO4 cDNA lead these authors to suggest that the extension may have been the result of a single point mutation in the termination codon of the ancestral *FMO4* gene. A similar extension is present in FMO4s of other mammals and thus the predicted mutation would have occurred before the radiation of mammals.

The *FMO4* gene was mapped to the long arm of human chromosome 1 [Shephard *et al.* 1993]. It contains ten exons, of which eight are coding [Hernandez *et al.* 2004]. In contrast to other FMO mRNAs the mRNA for FMO4 contains sequences derived from 10, not 9 exons, the first two of which are entirely non-coding. *FMO4* is expressed in relatively low amounts in several tissues, the main sites of expression being liver and kidney [Dolphin *et al.* 1996; Hernandez *et al.* 2004; Zhang and Cashman, 2006; Cashman and

Zhang, 2006]. Alternative splice variants of FMO4 have been reported [Lattard *et al.* 2004]. However, the relevance of this for the metabolism of foreign chemicals is not known.

Polymorphic Variants

A survey of 50 African Americans identified four coding-region SNPs, Fig. (**1**) and Table **1** [Furnes *et al.* 2004]. One was synonymous. The other three, g.110T>C(I37T), g.14726T>C(V323A) and g.14770G>C(E339Q), were nonsynonymous. Of these, I37T and E339Q each occurred only once in the 100 chromosomes surveyed and thus may represent rare mutations. However, V323A, which is also reported in dbSNP126, is common in Africans (20-33%), but is absent from Europeans and Asians. Although the functional consequence of this SNP has not been investigated, valine at position 323 is not conserved among FMOs and the change of amino acid (val→ala) is conservative. This SNP is therefore unlikely to have a significant effect on protein function. A further two SNPs are reported in dbSNP126. One of these is a synonymous SNP, g.14601C>T(F281F), which is present at low frequency (4 to 7%) in Asians, but is absent from Africans and Europeans. The other, which is nonsynonymous, g.14680A>T(T308S), is present in Japanese, at frequency ~1%, but has not been detected in Africans or Europeans.

Although *FMO4* is expressed in many tissues, in each case expression is low [Dolphin *et al.* 1996]. Furthermore, expression of the protein in a stable form in heterologous systems has proved difficult [Itagaki *et al.* 1996] and, consequently, little is known about the substrate specificity of FMO4. Nevertheless, it has been shown that FMO4 is capable of oxidizing methimazole [Itagaki *et al.* 1996], L-methionine and S-allyl-L-cysteine [Ripp *et al.* 1999].

FMO5

A cDNA for FMO5 of human was isolated in 1995 [Overby *et al.* 1995; Phillips *et al.* 1995]. The *FMO5* gene is not part of the *FMO* gene cluster on human chromosome 1q24.3, but is located ~26Mb closer to the centromere, at 1q21.1 [Hernandez *et al.* 2004]. However, the internal organization of the gene is similar to that of other *FMO* genes: it comprises nine exons, the first of which is non-coding, and encodes a polypeptide of 533 amino-acid residues and has a molecular mass of 60,225. *FMO5* is expressed in many fetal and adult tissues, but in human its main site of expression is adult liver [Janmohamed *et al.* 2001; Hernandez *et al.* 2004; Cashman and Zhang, 2006; Zhang and Cashman, 2006], where, along with FMO3, it is a major form of the enzyme present [Overby *et al.* 1997; Cashman and Zhang, 2006; Zhang and Cashman, 2006]. It is also the most abundantly expressed FMO in the small intestine [Cashman and Zhang, 2006; Zhang and Cashman, 2006] and skin [Janmohamed *et al.* 2001].

FMO5 exhibits little catalytic activity towards compounds, such as methimazole, that are good substrates for other FMOs [Rettie *et al.* 1994; Overby *et al.* 1995; Cherrington *et al.* 1998a; Krueger and Williams, 2005], but does catalyze the *N*-oxygenation of short-chain aliphatic primary amines such as *N*-octylamine [Overby *et al.* 1995]. Aliphatic primary amines are substrates of FMO2, but this enzyme has a marked preference for molecules with longer chains (see above). More recently, FMO5 has been reported to catalyze the oxygenation of other compounds, for instance, thioethers with proximal carboxylic acids that are not utilized as substrates for other FMOs. For example, esonarimod, an anti-rheumatic drug is converted to the active metabolite *S*-methyl esonarimod by FMO5 [Ohmi *et al.* 2003].

Thus, with respect to substrate specificity, FMO5 is regarded as an atypical FMO and, despite its high level of expression in adult human liver, the enzyme is not thought to play a significant role in the metabolism of drugs, with the possible exception of esonarimod.

Polymorphic Variations

In a survey of 50 African Americans [Furnes *et al.* 2003] only two coding-region mutations were identified, of which only one, g.37917C>T(P457L), results in a change in amino-acid sequence, Fig. (**1**) and Table **1**. Although the effect of this change on protein function has not been investigated, a proline at this position is conserved in all mammalian FMOs sequenced to date. However, the variant was identified in only one of the 100 chromosomes surveyed and thus may represent a rare mutation that would have little consequence for the general population. The other mutation detected in this study, g.23806A>G(A367A), was synonymous. Two additional coding-region SNPs are reported in dbSNP126. One is a synonymous SNP, g.23716G>A(P337P). The other, g.38059G>T(R506S), is nonsynonymous, but is found only in Yorubans, at a frequency of 9%.

Physiological And Xenobiotic Factors Influence *FMO5* Expression

Interindividual variations of 10-fold in the amount of FMO5 in adult human liver (3.5 to 34 pmol/mg microsomal protein) have been reported [Overby *et al.* 1997]. Although no polymorphisms have been identified that affect FMO5 catalytic activity, it is possible that promoter variants exist that influence the amount of FMO5 produced. Unlike other human FMOs, FMO5 is inducible and interindividual variations in amounts of the protein may be due in part to physiological effects or to exposure to xenobiotics. The synthetic progestin R5020 increased FMO5 expression in a breast cancer cell line stably expressing the progesterone B-receptor [Miller *et al.* 1997]. The authors suggested that increased expression of FMO5 might lead to increased carcinogenicity of tamoxifen in target tissues that overexpress progesterone B-receptors. However, FMO5, unlike FMO1 and FMO3, does not metabolize tamoxifen [Hodgson *et al.* 2000]. A recent profiling study of primary breast tumours showed that FMO5 expression may be up-regulated by estrogen receptor α (ERα) [Bieche *et al.* 2004]. FMO5 mRNA was increased in ERα-positive tumours, but was not detected in ERα-negative tumours. There was a strong correlation in this study between *FMO5* over-expression and *N*-acetyl transferase 1 (*NAT-1*) over-expression, with regard to relapse-free survival. However, statistical analysis found that only *NAT-1* expression was of prognostic value [Bieche *et al.* 2004]. FMO5 is increased by the xenobiotic hyperforin [Krusekopf and Roots, 2005], a component of St. John's Wort, and the antibiotic rifampicin [Rae *et al.* 2001]. Both compounds are known to increase CYP3A4 through the action of the preg-

nane X receptor (PXR) [Kliewer *et al.* 1999; Moore *et al.* 2000]. No study on the mechanism of action of *FMO5* gene activation, by any of the compounds mentioned, has been published.

Extensive expression profiling of human liver samples found that FMO5 mRNA was markedly down-regulated in individuals with type-2 diabetes compared with unaffected individuals [Takamura *et al.* 2004]. As knowledge of the role of FMO5 in both endogenous and xenobiotic metabolism is limited, the significance, if any, for drug treatment of those with type-2 diabetes is unknown.

CONCLUDING REMARKS

Five functional FMOs, FMO1, 2, 3, 4 and 5, are present in humans. Based on substrate-specificity and site and amount of expression, FMO1 and FMO3 are the most important for the metabolism of therapeutic drugs. For both of these FMOs the pattern of expression in humans is markedly different from that in other mammals, particularly rodents used as animal models for the investigation of drug metabolism. In humans, the expression of the *FMO1* gene is switched off after birth and FMO1 is undetectable in adult liver. This is in contrast to other mammals, in which FMO1 represents the major form of the enzyme present in adult liver. FMO3 is the major drug-metabolizing FMO in adult human liver. These species differences in the tissue-specific pattern of expression of *FMO1* and *FMO3* have important consequences for the extrapolation of drug metabolism data from experimental animals to humans.

Essentially all individuals of European or Asian origin express no functional FMO2, which in most other mammals is the major form of the enzyme present in the lung. However, in sub-Saharan Africa, and in populations recently descended from this region, a substantial proportion of individuals do express functional FMO2 in the lung. This has implications for inter-ethnic and, in African populations, interindividual variations in response to drugs and toxic chemicals that are substrates for FMO2.

There are very few examples of induction or inhibition of FMOs by endogenous or foreign chemicals, suggesting that drug-drug interactions would not be an important feature of therapeutic agents that are metabolized predominantly by FMOs. Variations in the expression and activity of FMOs are thus mainly due to genetic factors. An understanding of the pharmacogenetics of FMOs would help identify individuals who would respond favourably to a particular drug and those who would be at risk of experiencing harmful side effects, and thereby contribute significantly to drug development by decreasing the failure rate of new candidate drugs and reducing the time taken for approval of a drug by regulatory authorities.

The number of validated nonsynonymous SNPs identified in *FMO2* and *FMO3* is considerably more than in *FMO1*, *FMO4* or *FMO5* (see Tables **1** and **5**). In the case of *FMO2* and *FMO3*, several variants are present in high frequency in some, or all, major population groups and are known to affect enzyme activity. In *FMO1*, *FMO4* and *FMO5*, however, all variants are confined to only one population group and, with the exception of V323A in *FMO4*, which is unlikely to affect enzyme function, are present at low or very low frequency. The differences in the number of nonsynonomous variants identified in various members of the *FMO* family may reflect greater interest in *FMO3*, as a consequence of its role in the inherited disorder TMAuria, and in *FMO2*, as a result of the discovery of the common Q472X polymorphism. This seems unlikely, however, because the total number of genetic variants, i.e., coding and noncoding, reported in dbSNP126 for *FMO1* and *FMO5* is greater than for *FMO3* and is comparable with that for *FMO2*.

The relatively high number and frequency of nonsynonomous SNPs in *FMO2* may be the result of an accumulation of potentially deleterious mutations in an allele, *FMO2*2*, which is already defective. In *FMO3*, the ratio of nonsynonomous to synonomous polymorphisms is significantly more than expected under a neutral model of evolution, providing evidence that the gene has been the subject of balancing natural selection (Allerston *et al.* 2007). In the case of *FMO1* and *FMO5*, the relative paucity and low frequency of nonsynonymous SNPs may be indicative of an important physiological role for the proteins encoded by these genes.

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ABBREVIATIONS

ALS	=	Amyotrophic lateral sclerosis
AF	=	Atrial fibrillation
CYP	=	Cytochrome P450
5'-DPT	=	10-(*N,N*-dimethylaminopentyl)-2-(trifluoromethyl) phenothiazine
ERα	=	Estrogen receptor α
FAD	=	Flavin adenine dinucleotide
FAP	=	Familial adenomatous polyposis
FMO	=	Flavin-containing monooxygenase
NAT-1	=	*N*-acetyl transferase 1
SNP	=	Single-nucleotide polymorphism
TMA	=	Trimethylamine
TMAuria	=	Trimethylaminuria
UTR	=	untranslated region

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