The interaction of antiparasitic drugs with the drug-metabolising cytochrome P450s (CYPs) and characterisation of a CYP variant unique to African populations

by

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ABSTRACT

The cytochrome P450 (CYP) superfamily of enzymes is now known to be an important determinant of the outcome of drug therapy. Consequently, drug-CYP interaction studies that have led to an improvement in production of safer and more effective drug therapies constitute a critical component of modern drug discovery and development programs. There is, however, a general paucity of data on the interactions of CYPs with antiparasitic drugs, most of which were discovered a long time ago and whose use is associated with numerous side effects. In addition, the enzyme kinetic profiles of CYP variants unique to African populations and important in drug metabolism are poorly characterised. In this study, therefore, the inhibitory effects of twenty-nine clinically important antiparasitic drugs on the major drug-metabolising CYPs were investigated *in vitro* using the well-established HPLC-based assays with human liver microsomes. Studies also involved the relatively new fluorescence-based high throughput screening (HTS) inhibition assays and human CYPs heterologously expressed in *Saccharomyces cerevisiae*. In addition, the inductive effects of the antiparasitic drugs on CYPs 1A1 and 1A2 were studied *in vitro* using the human hepatoma (HepG2) cell line as a model. The potential implications of the African specific *CYP2D6*17* allele for phenotyping studies and clinical use of CYP2D6 substrate drugs in African populations were investigated with CYP2D6.17, heterologously expressed in *Saccharomyces cerevisiae*, by studying its enzyme kinetic profile towards CYP2D6 probe drugs and its capacity to clear CYP2D6 substrate drugs *in vitro*. To investigate the effects of amino acid exchanges in CYP2D6.17 on the structure of the enzyme, homology models were built using the CYP2C5 crystal structure as a template.

Results from the HTS assays utilising recombinant CYPs were comparable with those from HPLC-based assays using human liver microsomes and validate inhibition data from fluorescence-based assays with recombinant CYPs. The majority of antiparasitic drugs are not expected to pose any risk for clinically significant effects based on their interactions with CYPs although some of them, however, could result in undesirable interactions with CYPs 1A1, 1A2 and 2D6. Potent inhibitors of CYP1A2 included artemisinin, niclosamide, thiabendazole, primaquine and dihydroartemisinin. Of these CYP1A2 inhibitors, thiabendazole, artemisinin and primaquine could give rise to clinically significant interactions as they were predicted to cause 98, 76 and 67 % inhibition of CYP1A2 *in vivo* respectively. Quinine, albendazole and primaquine induced the activities and mRNA levels of CYP1A1 and CYP1A2 with the induction by quinine and albendazole likely to be of significance *in vivo*. While cycloguanil, quinine, amodiaquine, desethylamodiaquine and proguanil were potent inhibitors of CYP2D6 *in vitro*, the inhibition is not expected to be of significance *in vivo*. For clinicians, knowledge of these possible antiparasitic drug-CYP interactions will be useful in designing appropriate interventions to address the effects of the drug-CYP interactions when they are encountered in the clinic.

CYP2D6.17 exhibited a generally reduced capacity for clearing CYP2D6 substrates compared to CYP2D6.1 (wildtype) with the extent of reduction being dependent on the drug. This suggests the need for a general reduction in dosage of some CYP2D6 substrate drugs in African populations, particularly for drugs with narrow therapeutic indices. Results of homology modelling showed the arrangement of active-site residues in CYP2D6.17 to be different from those in CYP2D6.1 and could explain the substrate-dependent reduced capacity of CYP2D6.17 to clear CYP2D6 substrates.

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LIST OF ABBREVIATIONS AND SYMBOLS

CYP	Cytochrome P450
rCYP	Recombinant cytochrome P450
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
cDNA	Complementary deoxyribonucleic acid
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
mRNA	Messenger ribonucleic acid
dNTP	Deoxynucleotide triphosphate
AhR	Aryl hydrocarbon receptor
Arnt	Aryl hydrocarbon receptor nuclear translocator
Hsp90	Heat shock protein 90
XRE	Xenobiotic response element
CAR	Constitutive androstane receptor
PXR	Pregnane X receptor
DR4	DNA response element 4
PPAR	Peroxisome proliferator-activated receptor
V_{max}	Maximum velocity
K_m	Substrate concentration at half maximal velocity (Michaelis-Menten constant)
RFLP	Restriction fragment length polymorphism
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
MR	Metabolic ratio
PM	Poor metaboliser
EM	Extensive metaboliser
DDD	Drug discovery and development
HTS	High throughput screening
HLM	Human liver microsomes
RAF	Relative activity factor
IC_{50}	Inhibitor concentration causing a reduction of enzyme activity by half
EC_{50}	Concentration of drug that provokes a response halfway between the baseline and maximum response
K_i	Inhibitor constant
HPLC	High performance liquid chromatography
[I]	Inhibitor concentration
[S]	Substrate concentration
cam	Camphor
GOLD	Genetic optimisation for ligand docking
GA	Genetic algorithm
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
FDA	US Food and drug administration
EROD	Ethoxyresorufin O-deethylase
HepG2	Human hepatoma cell line
CEC	3-Cyano-7- ethoxycoumarin
MAMC	7-methoxy-4-(aminomethyl)-coumarin
BFC	7-benzyloxy-4-trifluoromethylcoumarin

MFC	7-methoxy-4-trifluoromethylcoumarin
HFC	7-hydroxy-4-trifluoromethylcoumarin
FLUO	Fluorescence
TCDD	2,3,7,8-Tetrachorodibenzo-*p*-dioxin
EDTA	Ethylenediaminetetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
α-OH	Alpha-hydroxy-
1´OH	1´hydroxy-
ODM	O-demethylation
4-OH	4-hydroxy-
m/z	mass-to-charge ratio
CL	Clearance
CL_{int}	Intrinsic clearance
V	Apparent volume of distribution
k	Elimination rate constant
$T_{1/2}$	half life
SRS	Substrate recognition site
NMR	Nuclear magnetic resonance
PEG	Polyethylene glycol
TRIS	Tris(hydroxymethy)aminomethane
DTT	Dithiothreitol
DMSO	Dimethylsulfoxide
BSA	Bovine serum albumin
PBS	Phosphate buffered saline
M	Molar
nmol	Nanomolar
µmol	Micromolar
µl	Microliter
µg	Microgram
***pmo*l**	Picomolar
nm	Nanometer
LCMS	Liquid chromatography mass spectrometry
RMSD	Root mean square distance
ANOVA	Analysis of variance
OD_{600}	Optical density at 600 nm

CHAPTER ONE

1.0 Introduction

The health of millions of people living in developing countries is threatened by a vast range of seemingly, endless problems. Of these, parasitic infections are among the most important with the bulk of mortality and morbidity attributable to them. The importance of parasitic diseases is exemplified by malaria, one of the most prevalent infectious diseases, and responsible for more than one million deaths each year (Roll Back Malaria fact sheets, http://www.who.int/inf-fs/en/1informationsheet03.pdf).

Several attempts at eradication of some parasitic diseases have been made, with results indicating the futility of such attempts, at least with the currently available methods. While considerable research effort is now directed towards finding more effective interventions, chemotherapy remains an important weapon in the fight against parasitic diseases. The fact that parasitic infections are "diseases of the poor", however, has resulted in the lagging behind of research and development of new antiparasitic drugs as the market-driven pharmaceutical companies shun this disease area. Of the 1 223 new drugs introduced onto the market between 1975 and 1997, only 11 were for tropical diseases and about half of these were initially for animals (Pécoul *et al*., 1999). The few new antiparasitic drugs including, eflornithine, ivermectin and more recently miltefosine (Ganguly, 2002), have been introduced through the efforts of the Special Programme for Research and Training in Tropical Diseases (TDR). Most of the few drugs available are old and toxic, and in addition, risk being lost to parasite-resistance.

1.1 Parasitic infections

Parasites

Parasites infectious to humans in the tropics, where most developing countries are located, may be protozoal or helminthic (Abdi *et al*., 1995). Protozoa infectious to humans are unicellular organisms found in the subphyla Mastigophora or Sarcodina and include leishmaniasis, Chaga's disease, trypanosomiasis, malaria and amoebiasis. Helminths that infect humans include those in the phyla annelida (segmented worms), nematoda (round worms) and platyhelminths or flatworms. Helminthic infections include hookworm disease, filariasis, schistosomiasis and onchocerciasis.

Treatment of parasitic infections

Drugs used for the treatment of parasitic infections have been reviewed by a number of authors (Abdi *et al*., 1995 and Bozdech and Mason, 1992, The Merck Manual of Diagnosis and Therapy, http://www.meck.com/pubs/mmanual/section13/chapter161/161a.htm). Of particular importance concerning the drugs is the fact that there are very few available with most of them being very old and toxic. This is due to the fact that the development of new drugs for use in the treatment of parasitic infections is generally slow compared to other disease areas (Rosenblatt, 1999). The situation is also made worse by the increase in resistance to the drugs, by the various parasites. One way suggested to tackle the problem of loss of drugs to resistance, is through the use of combination therapy (Bloland and Ettling, 1999). This involves use of a combination of drugs that do not share the same resistance mechanism (White, 1999). In addition to slowing down or preventing the loss of drugs to parasite resistance, combination therapy may also result in better treatment outcomes. Combination therapy, however, increases the risk for drug-drug interactions involving the antiparasitic drugs themselves and other non-antiparasitic therapies. Some of the

drug combinations in use for the treatment of the various parasitic infections are shown in Table 1.1. Knowledge of the metabolism of antiparasitic drugs, which, to a large extent, is lacking, is critical in order to increase their efficacy and rationalise observed toxicities (Abdi *et al*., 1995). It is against this background that the project's focus was directed towards understanding the interactions of antiparasitic drugs with the cytochrome P450 (CYP) superfamily of enzymes, which contributes to a large extent to the pharmacological and/or toxicological effects of drugs. In addition, the general paucity of data on enzyme kinetic profiles of CYP variants unique to black African populations that have implications on therapeutic outcomes of many drugs (including antiparasitic drugs), prompted the extensive characterisation of CYP2D6.17. The purpose of the following sections in this chapter is to demonstrate the central and critical role played by the CYP superfamily of enzymes in drug metabolism.

1.2 Drug metabolism

Exposure of humans to a variety of foreign compounds, which may be components of the diet, therapeutic drugs or environmental pollutants, is clearly an inevitable occurrence. A subset of the foreign compounds devoid of nutritional value, are referred to as xenobiotics. An important property of most xenobiotics is their lipophilicity, which while facilitating entry into cells makes their removal difficult. In order to prevent their subsequent accumulation to potentially toxic levels, the body has a complex system of drug metabolising enzymes that catalyse the introduction of more polar groups thus enabling excretion in urine or bile. Conversion of xenobiotics into less toxic and more excretable forms is referred to as biotransformation or metabolism, hence the name drug/xenobiotic metabolising/biotransforming enzymes (Parkinson, 1996). The drug metabolising enzymes are found in high concentrations in the liver albeit in different sub-cellular fractions and may be classified based on the reactions they catalyse into

Table 1.1 Combinations of drugs used for the treatment of some parasitic infections

Disease	Drug combinations in use
Malaria	sulfadoxine + pyrimethamine, artemisinin + quinine, pyrimethamine + dapsone, primaquine + chloroquine, proguanil + chloroquine, chloroquine + pyrimethamine.
African Trypanosomiasis	chloroquine + suramin, suramin + metronidazole, difluoromethylornithine + melarsoprol, suramin + difluoromethylornithine
Hookworm	pyrantel + praziquantel, albendazole + praziquantel
Intestinal amoebiasis	tinidazole + diloxanide, emetine + chloroquine, dehydroemetine + chloroquine, metronidazole + chloroquine
Visceral Leishmaniasis	allopurinol + meglumine antimoniate
Onchocerciasis	diethylcarbamazine + dexamethasone, levamisole + mebendazole
Pneumocystosis	clindamycin + primaquine, pyrimethamine + dapsone
Giardiasis	metronidazole + diloxanide
Cyclosporiasis/isosporiasis	trimethoprim + sulfamethoxazole, pyrimethamine + sulfadiazine

The drug combinations in the table are from the references (de Vries *et al*., 2000, Basco *et al*., 1998 and Goodman *et al*., 1999, Bozdech and Mason, 1992 and references therein, The Merck Manual of Diagnosis and Therapy, http://www.merck.com/pubs/mmanual/section13/chapter161/161a.htm).

phases I and II (Gibson and Skett, 1994). Of the phase I enzymes, the CYP superfamily of enzymes are the most important as far as drug metabolism is concerned.

1.3 Cytochrome P450 (CYP)

Cytochrome P450s (CYPs) are a superfamily of hemeproteins comprising a protein part (apoprotein) and an iron-protoporphyrin IX (heme moiety) as the prosthetic group (Omura and Sato, 1962, 1964). The protein part of the enzyme varies among members of the superfamily and forms the basis for their different properties such as substrate and product specificities. The prosthetic group that is critical for activation of molecular oxygen and subsequent oxidation of the vast range of lipophilic substrates is constant.

The iron-protoporphyrin IX consists of an iron cation, capable of existing in ferric and ferrous states, and liganded to four pyrrole nitrogens. The ferrous state allows for binding of a number of ligands to the iron cation. Binding of carbon monoxide gives rise to a peak in absorption around 450 nm, a property that enabled the discovery of CYPs, hence the name cytochrome P450 (Omura and Sato 1962, 1964). The fifth ligand to the iron, which is unique to CYPs, is the thiolate group from a cysteine located near the carboxyl end of the protein. The strength of the iron-sulphur bond results in considerable electron density around the iron, which is essential for the catalytic activity of CYPs.

The primary reaction catalysed by CYPs is the insertion of a single atom of oxygen into the substrate (RH), with concomitant production of a water molecule. NADPH serves as the source of the reducing equivalents as shown:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

The mechanism of transfer of electrons from NADPH is dependent on the location of the CYP. Microsomal CYPs obtain their electrons via NADPH-cytochrome P450 reductase while transfer to mitochondrial CYPs occurs via two proteins, ferredoxin and ferredoxin reductase. The detailed CYP catalytic cycle has been reviewed by several authors including Lewis and Pratt, (1998) and Guengerich, (2001).

1.3.1 Diversity of CYPs

CYPs are involved in the metabolism of a variety of lipophilic compounds of endogenous or exogenous origin. Some of the functions of CYPs include the formation of androgens, estrogens, gluco- and mineralocorticoids from cholesterol, synthesis and degradation of prostaglandins and other unsaturated fatty acids, metabolism of vitamins to their active forms and metabolism of a variety of xenobiotics (reviewed by Estabrook, 1999 and references therein). To date, at least 60 different CYPs have been identified in humans (http://drnelson.utmem.edu/human.P450.table.html). In order to avoid confusion associated with naming of CYPs according to different systems, a common nomenclature system has been adopted.

1.3.2 CYP nomenclature

The nomenclature system groups CYPs into families and subfamilies based on their amino acid sequence homologies (Nebert *et al*., 1987; Nebert *et al*., 1991; Nelson *et al*., 1993; Nelson *et al*., 1996). In the system, cytochrome P450 is named using the root CYP followed by an Arabic numeral denoting the gene family, a letter for the subfamily and another Arabic numeral for the individual protein. The protein and mRNA sequences are designated CYP in all species,

however, for the associated gene and cDNA, an italicised (*CYP)* is employed. CYPs in the same gene family exhibit sequence identities that are more than 40% (Nelson, 1996). CYPs within the same species, with sequences that are more than 55% identical are placed in the same subfamily. A web page that keeps an updated nomenclature system for the CYPs is available (http://drnelson.utmem.edu/human.P450.table.html).

1.3.3 Pharmacological/toxicological significance of CYPs

Considerable research effort has, for the past two decades, been directed at the xenobiotic or drug metabolising CYPs found in the first three families. This has seen the realisation of their importance in determining the pharmacokinetics and/or toxicological effects of drugs and other xenobiotics. The CYP isoforms 1A2, 2A6, 2C9, 2C19, 2D6 and 3A4 were shown to be responsible for the metabolism and disposition of more than 90% of therapeutics on the market (Bertz and Granneman, 1997). The clinical relevance of CYPs is exemplified by a study that has shown that adverse drug reactions are the 4^{th} to 6^{th} most common cause of death in hospitalised patients in the United States (Lazarou *et al*., 1998). In addition, a recent study at the Helsinki University Central Hospital during the year 2000 also showed adverse drug reactions to be a significant cause of death (Juntti-Patinen and Neuvonen, 2002). Most of the adverse drug reactions are pharmacokinetically based with those involving CYP-mediated metabolism being very important (Kedderis, 1997). In an effort to prevent or reduce fatalities associated with CYP-mediated adverse drug reactions, studies have, therefore, focused on factors affecting the activities of CYPs.

1.3.4 Regulation of CYP activity

The expression of CYP genes is under the control of several factors that may be environmental or endogenous in origin. Environmental factors modulate CYP activity in two ways: 1) through their interaction with foreign chemical receptors which activate gene expression by binding to xenobiotic responsive elements and 2) by interacting directly with the CYP protein resulting in inhibition or activation of enzyme activity.

The most important of the endogenous factors are genetic with much of the inter-individual and interethnic differences in CYP activities being attributable to genetic differences. Additional factors affecting CYP activity that will not be discussed include nutritional status and disease states, particularly during infection.

1.3.4.1 Inhibition of CYPs

The mechanisms involved in inhibition of CYP activity have been reviewed by several authors (Lin and Lu, 1998; Murray, 1999), and may be divided into reversible, quasi-irreversible and irreversible inhibition. Of the possible mechanisms of inhibition, reversible inhibition has been shown to be the most common cause of drug-drug interactions (reviewed by Hollenberg, 2002). Competition for the CYP active site is the underlying mechanism of reversible inhibition and, therefore, involves binding of substrate. The inhibitors may bind to the prosthetic heme iron or to the lipophilic part of the protein, with compounds capable of binding to both sites being potent inhibitors (reviewed by Murray, 1999). Classes of compounds that are potent reversible CYP inhibitors include nitrogen-containing drugs, imidazoles, pyridines and quinolones (Hollenberg, 2002 and references therein).

The literature abounds in examples of CYP inhibition-based drug-drug interactions. Most of the clinical consequences are attributable to increased plasma levels of the drugs with the type of effect being dependent on the particular drug involved (Figure 1.3). Most of the cases involve CYP3A4 by virtue of its involvement in metabolism of the greatest number of drugs (Smith *et al*., 1998). An effective illustration of the potential seriousness of adverse effects of CYP inhibition is Torsades de Pointes, a potentially fatal arrhythmia. Increased plasma concentrations, due to CYP3A4 inhibition, of several drugs including terfenadine, astemizole, cisapride and pimozide, are associated with increased risk of the arrhythmia (Honig *et al*., 1993; Woosley *et al*., 1993 and Dresser *et al*., 2000). Important interactions involving cholesterol-lowering medications such as simvastatin, lovastatin and cerivastatin have also been reported with effects such as rhabdomyolysis and acute renal failure being observed (reviewed by Dresser *et al*., 2000). In addition to potentiating pharmacological or toxic effects of drugs, inhibition may reduce the clinical effects of drugs. This occurs when a drug requires activation to an active metabolite and this is exemplified by codeine. For codeine to exert its analgesic effects, it requires conversion by CYP2D6 to morphine (Chen *et al*., 1988). Inhibition of CYP2D6 would, therefore, reduce the analgesic effect of codeine.

While most drug-drug interactions result in adverse effects, some may be beneficial. A good example involves cyclosporin, an expensive immunosuppressant. The use of cyclosporin together with ketoconazole, an inhibitor of CYP3A4 results in an increase in plasma concentrations of cyclosporine and that in turn necessitates a reduction in the dosage required for immunosuppression thus reducing the cost of the treatment (Gomez *et al*., 1995). Enhanced efficacy is another beneficial effect of drug-drug interactions and is exemplified by saquinavir, whose bioavailability is improved by coadministration with ritonavir (Merry *et al*., 1997).

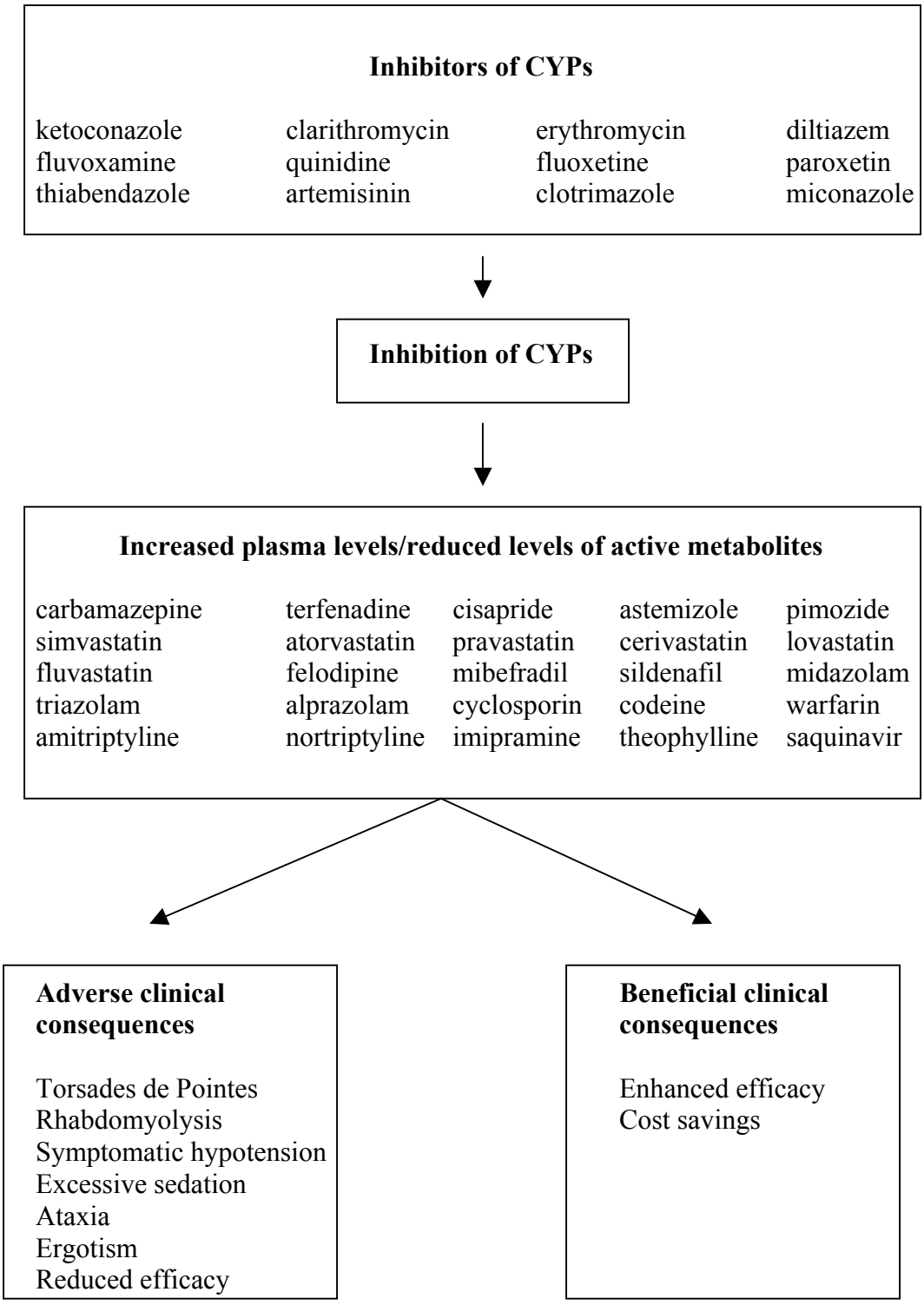


Figure 1.1. Drugs involved in CYP inhibition-based interactions and the possible clinical consequences

1.3.4.2 Induction of CYPs

Induction may be defined as the increase in enzyme activity through increase in the intracellular concentration of the enzyme. This may be achieved through increase in synthesis of the enzyme or decrease in rate of degradation. CYPs that have been shown to be inducible include CYPs 1A1/2, 2A6, 2C9, 2C19, 2E1 and 3A4 (Bresnick, 1993). Induction of most CYPs occurs at the transcriptional level, however, post-transcriptional processes may be involved and include stabilisation of CYP gene products such as mRNA or the protein itself. Of all the CYP induction mechanisms, induction of the CYP1A subfamily (CYP1A1 and CYP1A2) is perhaps the clearest, with considerable information now available.

The induction of CYP1A involves binding of an inducer (ligand) to an aryl hydrocarbon receptor (AhR) found in the cytoplasm of a cell (Gonzalez and Fernandez-Salguero, 1998; Whitlock, 1999). In the cytoplasm, the unliganded AhR interacts with Hsp90, a heat shock chaperone protein. Binding of an inducer to the AhR causes the dissociation of the Hsp90 resulting in translocation of the inducer-Ahr complex to the nucleus. In the nucleus, heterodimerisation of the AhR-ligand complex to the aryl hydrocarbon receptor nuclear translocator protein (Arnt) results in the formation of a transcription factor that binds to xenobiotic response elements (XREs). The XRE is found in multiple copies in the 5′-flanking region of the CYP1 genes where it functions as a transcriptional enhancer thus stimulating gene transcription. While binding to the AhR is the mechanism by which many compounds induce transcription of CYP1A, some studies (Kikuchi *et al*., 1996) have shown that other compounds may induce without binding to the AhR, a phenomenon not clearly understood.

The induction of CYPs in the families CYP2A, CYP2B, CYP2C and CYP3A has been clouded for a while, with the underlying mechanisms only becoming clear recently. The induction of these CYPs is mediated by members of the "orphan" nuclear receptor family of ligand-activated transcription factors namely the constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferator activated receptor (PPAR) (Kliewer *et al*., 1998; Pascussi *et al*., 2000; Goodwin *et al*., 1999; Savas *et al*., 1999). The activated transcription factors then bind to the respective DNA response elements: CAR (DR4), PXR (DR3, ER6), and PPAR (DR1) resulting in initiation of transcription. Additional post-transcriptional mechanisms may, however, be involved in induction of some of the CYPs such as CYP2E1 including, mRNA stabilisation, translational efficiency increase and post-translational protein stabilisation (Reviewed by Hollenberg, 2002).

The clinical implications of CYP induction are two-fold. Induction usually results in increased metabolism of drugs and, therefore, reduced pharmacological effects but in some cases, the metabolites formed are toxic and induction may cause increased toxicity. Rifampicin is a good example of the clinical relevance of induction. Rifampicin induces several CYPs including CYP2C, CYP3A and has been shown to increase the clearance of warfarin, resulting in reduced hypoprothrombinaemic response (Zhou *et al*., 1990; Combalbert *et al*., 1989; Harder and Thurmann, 1996 and references therein). The importance of induction in the formation of toxic intermediates is exemplified by the induction of CYP2E1-mediated formation of the toxic metabolite of paracetamol, *N*-acetyl-*p*-benzoquinone imine by alcohol (Slattery *et al*., 1996). Another illustration of the association between induction and toxicity, involves the CYP1A subfamily, which is thought to play a role in the aetiology of certain cancers, reviewed by Schrenk (1998).

1.3.4.3 Activation of CYPs

While the increase in enzyme activity following induction is accounted for by an increase in intracellular concentration of the enzyme, activation or stimulation enhances enzyme activity without affecting the concentration of the protein. An activator may act by increasing the rate-limiting step for the overall reaction and this could be achieved at one of the points in the CYP catalytic cycle. The result may be an increase in V_{max}, decrease in K_m or both. The underlying mechanisms of activation are not clear, but may involve allosteric effects on substrate binding, effects on the redox potential of the heme iron or alterations in interactions between the reductase and the CYP (Hollenberg, 2002).

Studies *in vitro* suggest that, activation may occur as a result of homotropic or heterotropic cooperativity (Ekins *et al*., 1998; Tang and Stearns, 2001; Huang *et al*., 1981). Homotropic cooperativity or substrate activation increases activity by the substrate itself while heterotropic activation describes the increase in metabolism of one compound by another referred to as the effector. While several examples of activation of CYPs *in vitro* have been reported, the occurrence of this phenomenon *in vivo* is rare and its clinical relevance is, at this point, not clear (Hollenberg, 2002).

1.3.4.4 Genetic determinants of CYP activity

Variation in CYP activity may be explained by genetic factors. The occurrence of mutations in the gene coding for a CYP may be associated with a marked deviation from the “normal” activity. The mutations may give rise to the absence of a protein, synthesis of a protein with lower or higher activity. In addition, mutations in the regulatory regions may affect the

expression of the respective *CYP* gene. The occurrence of the mutant allele in a normal population at a frequency of at least 1%, with a resultant different phenotype, constitutes a phenomenon referred to as a pharmacogenetic polymorphism (Meyer, 1994). To date, CYP isoforms shown to exhibit a genetic polymorphism include CYPs1A1, 1A2, 2A6, 2C9, 2C19, 2D6 and 2E1 (van der Weide and Steijns, 1999; Ingelman-Sundberg, 1998 and references therein). Considerable research effort has, however, been directed at the CYP2D6 isoform for a number of reasons.

1.3.5 CYP2D6 polymorphism

Originally known as the debrisoquine/sparteine hydroxylase (Tucker *et al*., 1977; Mahgoub *et al*., 1977; Eichelbaum *et al*., 1979), CYP2D6 is mainly found in the liver where it constitutes about 1-2% of total CYP protein (Shimada *et al*., 1994). Despite the relatively low expression, CYP2D6 metabolises up to 30% of clinically important drugs including antidepressants, antiarrythmics, β-adrenergic blockers and neuroleptics (Bertz and Granneman, 1997; Caraco, 1998 and references therein). The disposition of some of the drugs is predominantly CYP2D6-dependent with clinically significant differences apparent in response to CYP2D6 substrate drugs depending on genotype/phenotype, particularly for drugs with narrow therapeutic indices (Vandel *et al*., 1999).

The past two decades have seen extensive characterisation of the CYP2D6 polymorphism at both phenotype and genotype levels. To date, at least 75 allelic variants of CYP2D6, with varying effects on enzyme activity have been described (http://www.imm.ki.se/cypalleles/). Identification of these variants is through the use of restriction fragment length polymorphism (RFLP) and allele-specific polymerase chain reaction (PCR). Phenotyping on the other hand

involves administration of a probe drug with the index of enzyme activity expressed as a metabolic ratio (MR) obtained by dividing the concentration of unchanged drug recovered in biological fluids by the concentration of metabolite. Probe drugs that have been used for CYP2D6 phenotyping include dextromethorphan, debrisoquine, metoprolol and sparteine.

A clear ethnic-dependent pattern in distribution of MRs has been observed, with studies in Caucasians showing a bimodal distribution, and a 5-10% incidence of poor metabolisers (PMs) for all probe drugs (Alvan *et al*., 1990). The distribution in Orientals is almost unimodal with less than 1% PMs (Horai *et al*., 1989). Studies in black African populations have shown a wide range in the incidence of PMs of 0 –19% (Masimirembwa and Hasler, 1997, and references therein). While phenotyping studies in Caucasian and Oriental populations using different probe drugs, have yielded consistent results, a poor correlation of phenotype status when using different probe drugs was observed in African populations. Comparative studies of MR correlation of CYP2D6 probe drugs in different populations have shown generally poor correlations in black African populations. While Masimirembwa *et al*., 1996a showed a correlation of 0.67 between debrisoquine and metoprolol in a Zimbabwean population, a study in Caucasians showed a correlation of 0.81 for the same pair of drugs (McGourty *et al*., 1985). In another study, for each pair of the three probe drugs debrisoquine, dextromethorphan and sparteine, correlations in Ghanaians were lower compared to those in Caucasians or Orientals (Droll *et al*., 1998).

In addition to the variation in incidence of PMs in different populations, there is also variation in enzyme activity between extensive metabolisers (EMs) from different populations. The EM group is complex with a wide variation in enzyme activity of up to a thousand fold, hence its further subdivision into slow, intermediate and rapid metabolisers. Africans and Orientals show

generally higher MRs indicative of reduced CYP2D6 enzyme activity compared to their Caucasian counterparts. The molecular genetic basis for the reduced activity in Orientals and the possible clinical implications this might have for dose adjustment of prescribed CYP2D6 substrate drugs has been extensively characterised.

In Oriental populations, the molecular basis for the diminished CYP2D6 activity is a mutant variant allele, *CYP2D6*10* with low expression levels and coding for an unstable enzyme, with lower activity than CYP2D6.1 (wildtype) (Johansson *et al*., 1994; Fukuda *et al*., 2000). Pharmacokinetic studies with CYP2D6 substrate drugs in subjects with the *CYP2D6*10* variant compared to those with the *CYP2D6*1,* clearly demonstrate the clinical relevance of the reduced activity of CYP2D6.10 (Lai *et al*., 1995; Tseng *et al*., 1996; Yue *et al*., 1998). The high frequency of the *CYP2D6*10* variant in Orientals of up to 50% could, therefore, explain the prescription of CYP2D6 substrates such as propanolol, imipramine and amitriptyline at lower doses compared to those used for their Caucasian counterparts (Wood *et al*., 1991).

The basis for diminished activity in the EM group in black African populations is, at least in part, due to the presence of the *CYP2D6*17* and *CYP2D6*29* alleles, first discovered in Zimbabwean and Tanzanian populations respectively (Masimirembwa *et al*., 1996b; Wennerholm *et al*., 2001). The *CYP2D6*17* allele is, however, predominant and occurs at frequencies of 14 - 34% (Masimirembwa *et al*., 1996b; Dandara *et al*., 2000; Wennerholm *et al*., 1999). The *CYP2D6*17* variant bears three nucleotide exchanges 1111C>T, 2938C>T and 4268G>C associated with the amino acid changes T107I, R296C and S486T respectively. The implications of the *CYP2D6*17* allele for the use of clinically important CYP2D6 substrates or phenotyping studies, has not been investigated.

1.4 In perspective: CYPs *vis-à-vis* drug discovery and development (DDD)

Clearly, CYPs have far-reaching effects in medicine but where and how do they fit in the whole scheme of drug discovery and development (DDD)? Traditionally, the process of DDD placed emphasis on the pharmacological potency of a molecule (Gaviraghi *et al*., 2001). This resulted in production of molecules with high pharmacological potency but poor drug properties such as poor oral availability, high clearance and so on. In order to avoid or reduce this, it was necessary to address the factors affecting the ideal properties of drugs early in the DDD process. Pharmacokinetic properties (absorption, distribution, metabolism and excretion) of a compound have been shown to be a major factor. As a result, pharmacokinetic studies are now performed in the lead optimisation stage that precedes selection of a drug candidate. In this way, only compounds with both high pharmacological potency and good pharmacokinetic properties are allowed to go through to the next stage as shown in Figure 1.2. Given the critical role played by CYPs in metabolism, studies on metabolism of compounds focus heavily on their interactions with CYPs. Studies done at this stage include identification of the isoform involved in metabolism, identification of metabolites and screening for inhibition and induction (Lin and Rodrigues, 2001; Gonzalez, 1997). In addition, for drugs whose metabolism is mediated by polymorphic CYPs such as CYP2D6, the implications on the drug dosages of variants unique to particular populations (e.g. CYP2D6.17) are now being taken into consideration (Rodrigues and Rushmore, 2002). The numerous *in vitro* and "in silico" studies in use in the optimisation stage will be discussed in the following sections.

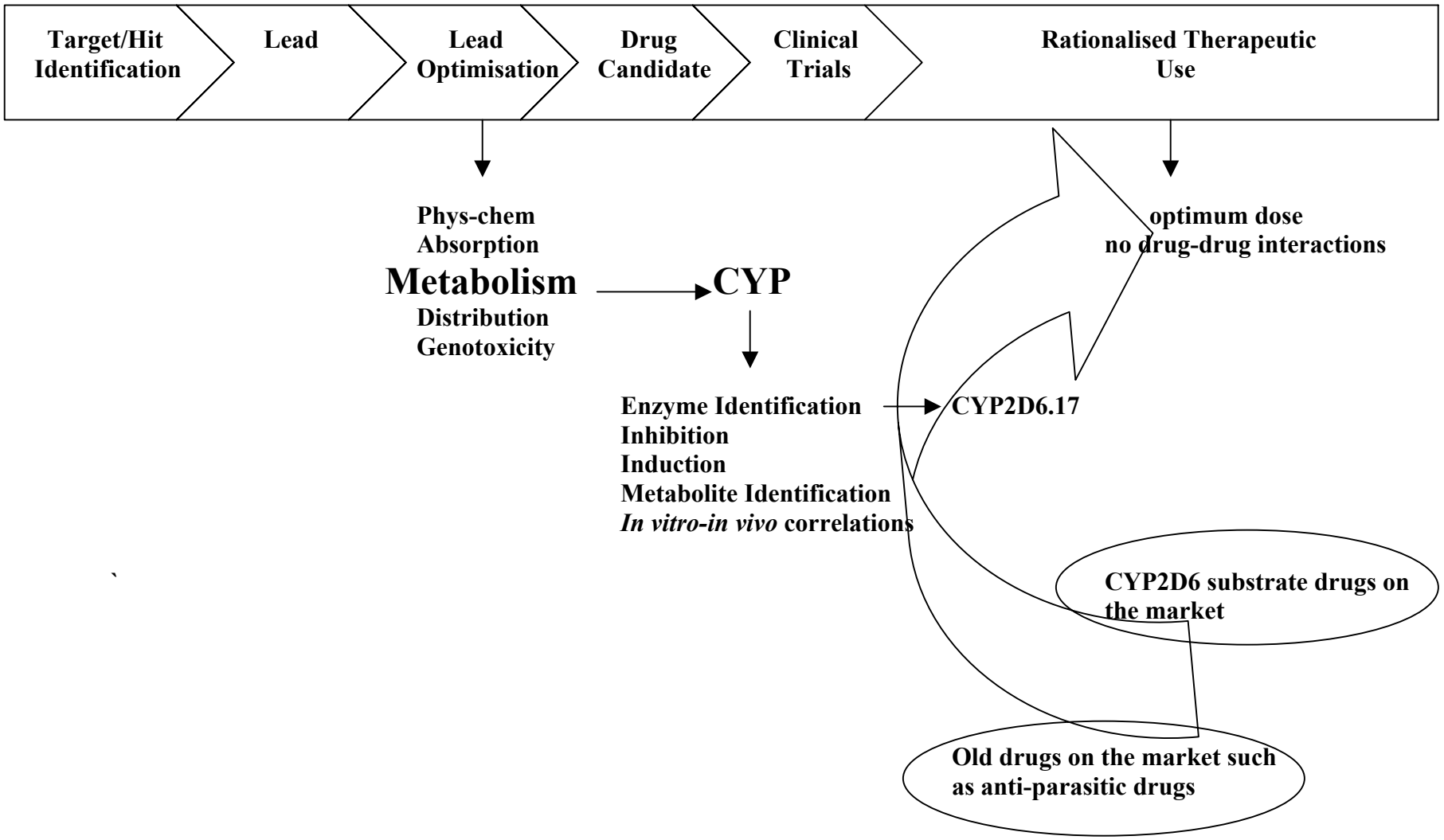


Figure 1.2. The importance of CYPs in the drug discovery and development process, their relevance in optimising use of old anti-parasitic drugs and relatively new drugs metabolised by CYP variants unique to African populations.

1.5 *In vitro* studies on CYPs

The past few years have seen the development of numerous *in vitro* methods directed at addressing the factors affecting CYP activity with current focus shifting towards production of faster or so-called high throughput screening (HTS) assays, excellently reviewed by Masimirembwa *et al*., (2001). In addition, numerous methods used to identify CYPs responsible for metabolism of a compound (reaction phenotyping) have also been developed with a lot of ongoing work aimed at validating them (Venkatakrishnan *et al*., 2001; Venkatakrishnan *et al*., 1998; Crespi, 1995; Rodrigues, 1999). Given the fundamental species differences in metabolism, any studies for humans would have to be done using human materials. This has the obvious drawback of lack of availability in sufficient quantities and the ethical issues involved. The major breakthrough in this area came with the advent of DNA recombinant technology that saw the heterologous expression of human CYPs in organisms such as yeast and *E. coli*.

1.5.1 Heterologous expression of CYPs

Heterologous expression systems in use today include yeast (*Saccharomyces cerevisiae* and to a lesser extent *Schizosaccharomyces pombe*), bacteria (*E. coli*), insect cells and mammalian cells. The procedure followed will differ depending on the system, however, all the systems have to address the basic requirements of CYPs, 1) the availability of non-covalently bound heme, which is added to the protein during synthesis, 2) the appropriate lipid milieu to anchor the protein and facilitate catalysis and 3) the presence of the accessory proteins such as NADPH-CYP reductase and in some cases cytochrome b_5 (Gonzalez *et al*., 1991). There are several advantages and disadvantages associated with the use of each of the expression systems mentioned and the choice somewhat depends on an individual.

Yeast expression system

Yeast was the first to be used as an expression system for CYPs (Oeda *et al*., 1985). The biggest advantage associated with yeast is that they are eukaryotes and, therefore, contain an endoplasmic reticulum and NADPH-CYP reductase. In addition, yeast cells have a low CYP background and are relatively easy to grow at low cost. The amount of CYP expressed is, however, not very high and the yeast reductase may not be sufficient to support enzyme function. To this end, genetically modified strains are now available that over-express the reductase and still some express the human NADPH-CYP reductase and cytochrome b_5 (Pompon *et al*., 1995).

While considerable research effort has been directed at optimisation of the various expression systems, a shift in focus to the application of the systems in answering pertinent questions in DDD is now apparent. Of particular importance is the identification (reaction phenotyping) and prediction of percentage contribution by a CYP isoform towards metabolism of a new drug as it determines the magnitude of an interaction due to inhibition or induction of the CYP (Rodrigues and Wong, 1997). The identification of the CYP isoform responsible for metabolism of a compound is simple. Predicting the relative contribution *in vivo,* of each of at least two isoforms involved in the metabolism of a particular compound is, however, not as simple.

Predicting relative contribution towards metabolism by a particular CYP

One method proposed by Becquemont *et al*., (1998), to estimate the relative contribution of a particular CYP, multiplies the rate of metabolism per unit enzyme for the recombinant enzyme by the average amount of that enzyme in human liver microsomes (HLM). The relative amounts of CYPs in human liver microsomes have been determined (Shimada *et al*., 1994; Code *et al*., 1997). One fundamental problem with the approach is that human liver microsomes and recombinant enzymes display different rates of substrate turnover per unit enzyme due to 1)

differences in amount of accessory proteins such as NADPH-CYP reductase and 2) the fact that recombinant enzymes are expressed singly while human liver microsomes have the whole complement of CYPs.

Another approach referred to as the relative activity factor (RAF) that takes into account the differences in activity per unit enzyme was proposed by Crespi (1995). The rate of metabolism of a probe drug by the recombinant enzyme is multiplied by a ratio (RAF) obtained by dividing the activity toward a probe substrate for HLMs by the activity of the recombinant enzyme as shown below:

$$\text{RAF} = \frac{\text{velocity for probe substrate with HLM (pmol/min/mg)}}{\text{velocity for probe substrate with recombinant enzyme (pmol/min/pmol CYP)}}$$

The contribution of a given isoform can then be estimated by multiplying the rate of metabolism of a probe drug by the recombinant enzyme by the RAF. The validity of the RAF approach depends on the selectivity in HLMs of the probe drug used, linearity with respect to the enzyme and time, and substrate given that requirements for accessory proteins are substrate-dependent (Crespi and Miller, 1999). Further validation of the RAF approach, has been carried out by several authors (Venkatakrishnan *et al*., 1998;Venkatakrishnan *et al*., 2001).

Assessment of inhibition

The important *in vitro* enzyme kinetic parameters used to evaluate inhibition are the IC_{50} (inhibitor concentration causing a reduction of enzyme activity by half) and K_i (inhibition constant). Until recently, these parameters were only generated using the laborious and

expensive HPLC-based CYP marker reaction assays (Pearce *et al*., 1996; Masimirembwa *et al*., 1999). Given the large numbers of compounds requiring screening for inhibition, high throughput screening (HTS) inhibition assays that make use of recombinant CYPs and substrates that produce fluorescent metabolites, have now been developed (Crespi *et al*., 1997). Clearly, the inhibition parameters obtained from experiments *in vitro* become useful only when they can successfully predict *in vivo* effects. Based on the mechanism of inhibition, it is possible to calculate the percentage inhibition *in vivo* as shown in Figure 1.3.

The major difficulty associated with predicting the percentage inhibition *in vivo* is estimating the actual concentration of inhibitor [I] available to the enzyme. Maximum plasma concentrations of drugs have been taken by many researchers to represent the concentration of drug available to an enzyme. It has been shown, however, that while plasma concentrations may reflect the concentration of drug available to interact with the enzyme, this is not always the case and could explain instances in which *in vitro* data fails to predict *in vivo* interactions (Davit *et al*., 1999). Some authors have suggested that protein binding be factored in *in vivo* predictions. Disregarding protein binding was, however, shown to give better predictions of *in vivo* clearance for basic and neutral compounds (Obach, 1999). For the acidic compounds, prediction was best when all binding was considered.

Others have proposed the use of the maximum unbound concentration of the drug at the inlet to the liver (meeting point of blood flow from the hepatic artery and portal vein), as the concentration of drug available to the enzyme (Ito *et al*., 2002). Using this approach, the authors were able to correctly predict the occurrence of *in vivo* drug-drug interactions for many drugs. In addition, their study demonstrated the increased possibility for underestimation of *in vivo* interactions associated with the use of the maximum plasma concentrations as being

representative of the amount of drug available to an enzyme. The other problem with *in vitro-in vivo* extrapolations involves the inhibitor constant (K_i). While initially thought to be characteristic for each particular inhibitor and enzyme, it is apparent that the K_i determined *in vitro* may be dependent on the substrate. This is exemplified in a study by (Kenworthy *et al*., 1999), in which they recommend the use of at least three substrates from different classes when assessing the inhibitory effects of a test compound on CYP3A4 activity.

1.6 "In silico" studies on CYPs

In addition to the various *in vitro* systems, several "in silico" methods are now available for use in studying various aspects of drug metabolism. These are essentially, computer-based programs that simulate possible pharmacokinetic or pharmacodynamic effects from various parameters such as physico-chemical properties of compounds and those of their respective receptors. In the same way, critical information on the mechanism of action of an enzyme and the determinants of substrate specificity can be obtained from studies on the active site structure of the enzyme. This is possible if crystal structures are available, however, the crystal structures for two human CYP isoforms (CYP2C9 and CYP3A4) have only recently been obtained (Astex Technology, http://www.astex-technology.com/one/29_37.html; Jhoti *et al*., 2002). The reason for the delay in obtaining a crystal structure of human CYPs lies in their membranous nature, which makes purification and subsequent crystallisation difficult. For the past two or so decades, structural studies of human CYPs have, therefore, relied heavily on homology modelling using bacterial CYPs as templates. The availability of the first mammalian microsomal CYP crystal structure, the rabbit CYP2C5 (Williams *et al*., 2000), resulted in a shift from bacterial CYPs to the CYP2C5 as a template for homology modelling of human CYPs.

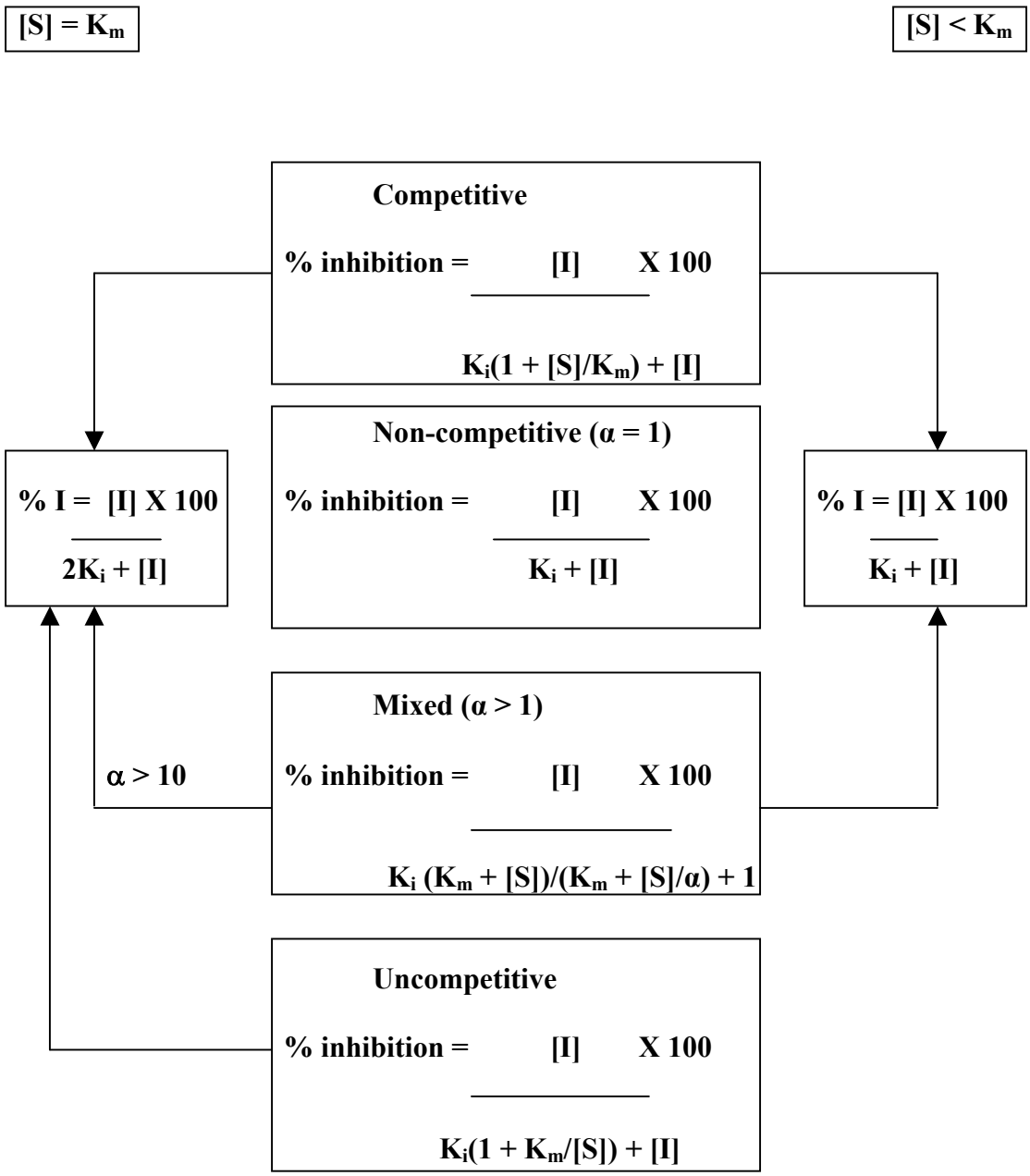


Figure 1.3 The relationship between the mechanism of inhibition and percent inhibition when the [S] = K_m or [S] < K_m. Adapted from Rodrigues, (1999). α is the factor by which Ki changes when a substrate is bound to the enzyme.

Homology modelling of CYPs

Homology modelling makes use of a closely related protein whose 3-dimensional structure is known, as a template to build a model of the protein of interest. Initially, an alignment of the primary structures of the two proteins is constructed using any of the numerous programs available. The overall 3-dimensional structure of the protein to be modelled is then copied from the known crystal structure and this is followed by energy minimisation of the model to enable docking of substrates into the active site (ter Laak and Vermeulen, 2001). CYP homology modelling has advantages and disadvantages, which have been reviewed by De Groot and Vermeulen (1997). The major drawback of homology modelling lies in the fact that the model created will somewhat, closely resemble the crystal structure used as the template. Both proteins should, therefore, share high amino acid sequence homology. In addition, force fields used will affect the geometry of the final model and the appropriate force fields should, therefore, be applied to specific proteins (De Groot and Vermelen, 1997).

Crystal structures of CYPs that have been resolved include the bacterial CYP101 (cam), CYP102 (BM3) and CYP108 (terp). Although the sequence similarity between mammalian microsomal CYPs and the bacterial crystal structures is low, these have been used to model human CYPs (Lewis, 1998; De Groot *et al*., 1996). This is due to the fact that CYPs seem to possess important structural similarities regardless of their amino acid sequence homologies. This is supported by a comparison of structures of CYP101 (cam) and CYP102 (BM3) that shows important structural similarities even though they share only about 20% sequence identity (Peterson and Graham, 1998). A conserved structural core consisting of a 4-helix bundle made up of helices D, E, I and L, and helices J and K is apparent. Structurally conserved β-sheets include β-sheet 1 with five strands and β-sheet 2 with two strands, which are important for the formation of the substrate access channel. Another structure that is conserved is the consensus sequence on the proximal

face of the heme containing the absolutely conserved cysteine residue involved in formation of the fifth ligand with the heme iron. All CYPs also have a highly conserved structure found on the proximal face referred to as the “meander”.

Comparison of the mammalian CYP2C5 with the structures of the bacterial CYPs available is also in support of a conserved folding pattern across all CYPs, however significant differences in some aspects are apparent (Williams *et al*., 2000). The overall structure of CYP2C5 is shown in Figure 1.4. The spatial arrangement of the heme is highly conserved in bacterial CYPs and CYP2C5 with the helices L and I sandwiching the heme. Residues thought to be involved in important interactions with substrates, however, differ significantly in their location relative to the heme (Williams *et al*., 2000).

With respect to membrane binding, while the N-terminus is the only trans-membrane structure, additional membrane-binding domains are present and these are shown in purple in Figure 1.4 (Williams *et al*., 2000 and references therein). This gives rise to a stronger orientation and results in burial of the substrate access channel in the hydrophobic core of the membrane. This would allow hydrophobic substrates to access the active site straight from the membrane. The substrate access channel is thought to be in contact with the membrane and the F-G loop, A helix and β strands 1-1 and 1-2. An alternative access channel along helix I, allows access to the cytoplasm and could provide a way out for the more hydrophilic products. The orientation also places the region of the enzyme proximal to the heme that is thought to interact with the cytochrome P450 reductase, perpendicular to the surface of the membrane. This arrangement is thought to facilitate electron transfer from the reductase. Some of the differences in structure of the CYP2C5 and bacterial ones stem from the differences in redox partners and membranous nature of mammalian CYPs. The CYP2C5 crystal structure should, therefore, serve as a much better model for human CYPs.

Available protein models of CYP2D6 have been based on the crystal structures of bacterial CYPs 101, 102 and 108 (Lewis *et al*., 1997; Lewis, 1998; Ellis *et al*., 1996; de Groot *et al*., 1999; Modi *et al*., 1996). Important active-site residues identified by these models include Thr 107, Val 119, Glu 216, Asp 301, Ser 304, Val 374 and Phe 481. The Phe 481 is thought to interact with aromatic regions of substrates. Recent homology models based on the rabbit CYP2C5 have also identified Asp 301, Val 374 and Glu 216 as important active site residues with the Glu216 being important in interactions with the basic nitrogen of substrates (Lewis, 2002).

Pharmacophore models

Critical information on the active site architecture of an enzyme may also be obtained from small molecule (pharmacophore) models (De Groot and Vermeulen, 1997). Information on the structure of the enzyme is deduced from the physical/chemical properties of numerous substrates, inhibitors or metabolites. Data from numerous such pharmacophore models has shown that CYP2D6 substrates generally have a basic nitrogen atom that is charged at physiological pH and a flat hydrophobic region extending from the nitrogen. The positively charged nitrogen interacts with a negatively charged residue such as glutamate or aspartate on the protein, an interaction thought to be optimal for oxidation at a site usually 5 to 10 Å (from the nitrogen), depending on the size of the substrate (Koymans *et al*., 1992; Strobl *et al*., 1993; De Groot *et al*., 1999).

Molecular docking

Docking basically refers to the computational process of searching for a geometrically and energetically favourable orientation of a ligand to enable it to fit in the binding site of a protein (http://www.biochem.wisc.edu/phillips/lab/pdfs/108-docking.pdf). This is thought to be

representative of the natural processes involved in recognition and interaction of a ligand and receptor in biological systems. The program used must be able to take into account the conformational changes that occur during the binding process for both the ligand and receptor (protein).

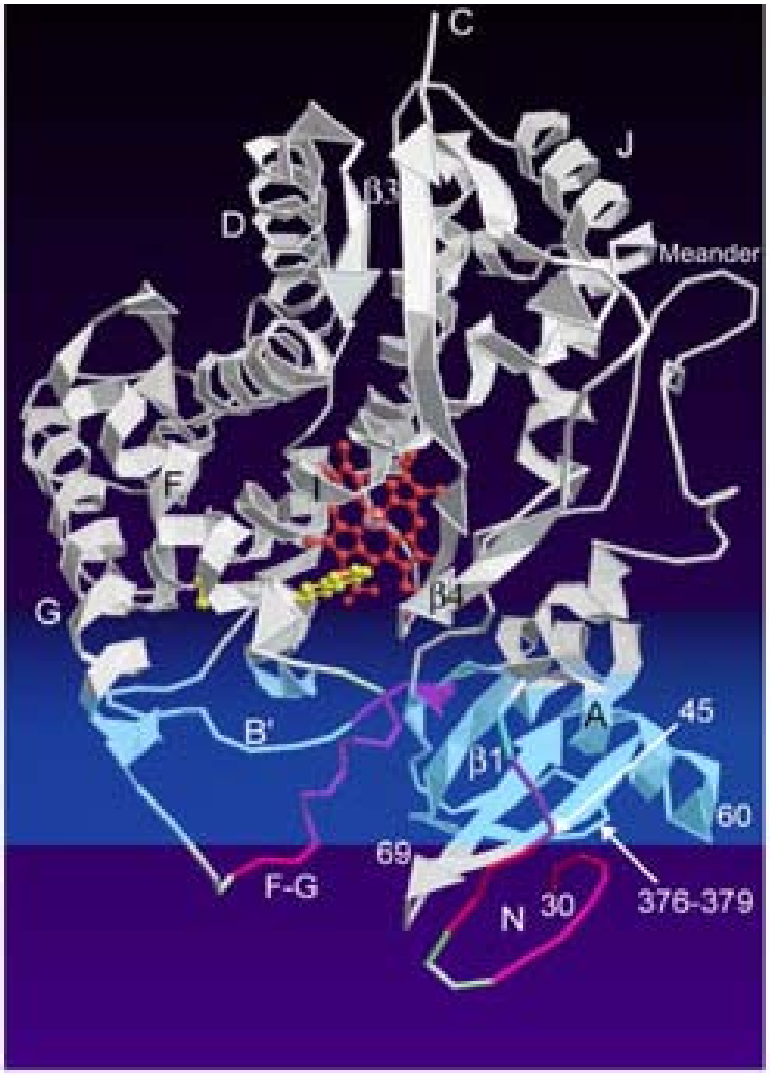


Figure 1.4 Ribbon diagram of the CY2C5 crystal structure showing its possible association with the membrane (shown in purple), the trans-membrane amino terminal that was removed to facilitate crystalisation is not shown. The heme is shown in red and a substrate (yellow) is docked in the active site (Figure adapted from Werck-Reichhart and Feyereisen, 2000).

While programs available can consider the changes in the ligand through incorporating ligand flexibility, the same is not true for the receptors. This is due to the fact that the degree of freedom is determined largely, by the rotatable bonds – ligands have 3-15 of these while receptors have up to 2000 (Molecular docking: a problem with thousands of degrees of freedom, http://www.biochem.wisc.edu/phillips/lab/pdfs/108-docking.pdf). Taking into account the complete flexibilities of the ligand and receptor is, therefore, computationally difficult to solve. Programs available can only take into account partial flexibilities of receptors and an example is the program GOLD (Genetic Optimisation for Ligand Docking).

GOLD assumes full acyclic and partial cyclic flexibilities of ligands. As far as the receptor is concerned, the program assumes partial flexibility and it achieves this by taking into account potential hydrogen bonding between the protein and the ligand (Jones *et al*., 1997). Hydrogen bonds are an important component or determinant in ligand recognition and binding. The program then searches for binding modes that give good solutions using a genetic algorithm (GA) that contains hydrogen bond motifs. In addition, potential hydrophobic interactions are also taken into account (Jones *et al*., 1997).

A GA is a model of machine learning that imitates some of the natural mechanisms of evolution (http://www.faqs.org/faqs/ai-faq/genetic/part2/section-2.html). In other words, it is a program that enables a computer to solve a given problem in a way that is analogous to the process of evolution in living organisms – the solution is therefore, evolved. The program GOLD makes use of a collection of data structures that are analogous to chromosomes. Each of the chromosomes code for a solution (possible orientation within a receptor) that is given a fitness score based on its relative merit. Implementation of the GA initiates a cycle involving evaluation of fitness of all individuals in the population, creation of a new population through cross-over, reproduction,

mutations on the off-spring and discarding the old population and this is repeated on the new population. Termination of the GA will give the ligand and protein conformations associated with the fittest chromosome in the population as the output (Jones *et al*., 1997).

1.7 Justification for study

While the various *in vitro* and "in silico" technologies reviewed, have no doubt contributed significantly towards development of more effective medicines and improved risk assessment, very little of it has found applications in tropical medicine. Knowledge of antiparasitic drug-CYP interactions, which could aid in understanding any observed interactions and rationalisation of their clinical use, is lacking. In addition, there is also a general paucity of data on the enzyme kinetic profiles of CYP variants unique to black African populations and important in drug metabolism (Figure 1.4). This led us to come up with the following hypotheses:

- The basis for some of the observed adverse effects of antiparasitic drugs could involve interactions with the drug metabolising CYPs.

- The amino acid exchanges in CYP2D6.17 result in an altered active-site structure that could explain its unique kinetic properties.

- The enzyme kinetic properties of the CYP2D6.17 variant might explain the poor correlation of phenotype status observed in African populations in which the variant is common. In addition and more importantly, it may warrant appropriate dosage adjustment of substrate drugs in the populations.

Work in this thesis was, therefore, aimed at testing the hypotheses stated by focusing on the inductive/inhibitory effects of antiparasitic drugs on CYPs and enzyme kinetic characterisation of CYP2D6.17.

1.7.1 Inhibitory effects of antiparasitic drugs on CYPs involved in drug metabolism

The use of multiple drugs at any one time is particularly common in tropical medicine with several factors promoting the practice such as the use of combination therapy for treatment of a single parasitic infection. Individuals infected by one parasite, may still be exposed to other parasites, resulting in polyparasitism, which is common in the tropics (Bozdech and Mason, 1992). In addition, for better treatment outcomes to be achieved, some diseases such as AIDS, which are prevalent in the tropics, require the use of at least 3 drugs at one time. While multiple drug use has several advantages, it brings with it an increased risk for drug-drug interactions (Stockely, 1996).

Manyclinically significant drug-drug interactions involving CYPs are due to enzyme inhibition (Lin and Lu, 1998). As a result many pharmaceutical companies are now using *in vitro* systems to evaluate the potential of test compounds to cause clinically significant inhibition-based drug-drug interactions. This information forms an important part of data submitted to drug regulatory agencies by companies seeking approval for their new drugs. The various drug regulatory agencies such as The U.S. Food and Drug Administration (FDA) have in turn, acknowledged the utility of such information (Davit *et al*., 1999 and references therein). Most of the antiparasitic drugs are old and did not go through such characterisation for CYP inhibition potential (Figure 1.2). As a result, the inhibitory effects of 29 clinically important antiparasitic drugs on the CYP isoforms involved in drug metabolism (CYP1A2, 2C9, 2C19, 2D6 and 3A4), were, therefore, investigated. In addition, one of the numerous and relatively fast *in vitro* methods used to assess CYP inhibition using fluorescent substrates and recombinant enzymes, was evaluated against the traditional HPLC-based methods with human liver microsomes.

1.7.2 CYP1A induction by antiparasitic drugs

The CYP1A subfamily is involved in the metabolism of several pro-carcinogens and a number of clinically important drugs (Gonzalez and Gelboin, 1994 and references therein). The induction of the CYP1A subfamily, therefore, has implications for the aetiology of certain cancers and alteration of therapeutic efficacy of some drugs. Identification of compounds capable of inducing the CYP1A subfamily is, therefore, critical in order to avoid any CYP1A-mediated adverse effects. Clinically significant interactions due to induction may occur following long-term exposure to inducers, a scenario found in populations treated for parasitic infections, given the prolonged periods of time required for treatment and prophylaxis. The inductive effects of antiparasitic drugs on CYPs 1A1 and 1A2 were, therefore, investigated in HepG2 cells by measuring the ethoxyresorufin O-deethylase (EROD) activity indicative of CYP1A enzyme activity, and mRNA levels.

1.7.3 Molecular and enzyme kinetic basis for the reduced activity of CYP2D6.17 and clinical implications for the use of CYP2D6 substrate drugs

While studies done thus far, as evident from the literature review, have contributed significantly towards the current knowledge of the CYP2D6 polymorphism and its implications in black African populations, the picture is, however, still not clear with perhaps three important issues outstanding:

1. The poor correlation of metabolic ratios (MRs) between different probe drugs in black African populations compared to their Caucasian or Oriental counterparts.
2. The basis for the generally lower CYP2D6 activities causing a “right shift” of MRs in African populations compared to Caucasians and Orientals.

3. The need for dose adjustment of CYP2D6 substrate drugs in African populations particularly those with narrow therapeutic indices.

Work in this thesis, therefore, sought to investigate the possible contribution of the *CYP2D6*17* allele in these areas. This was accomplished by a) studying the enzyme kinetics towards metabolism of commonly used phenotyping probe drugs, debrisoquine, metoprolol, dextromethorphan and the *in vitro* marker substrate bufuralol, using recombinant CYP2D6.1 (valine variant), CYP2D6.2, CYP2D6.17 and CYP2D6.T107I, b) investigating the capacity of CYP2D6.17 compared to CYP2D6.1 to clear clinically used substrates of CYP2D6 and the clinical significance after determining the contribution of CYP2D6 towards clearance of the drugs using human liver microsomes, and c) making a homology model of CYP2D6 and investigating the effects of CYP2D6.17 amino acid exchanges on the 3-dimensional structure of the enzyme using the recently available CYP2C5 crystal structure as a template.

CHAPTER TWO

2.0 Materials and methods

2.1 Materials

2.1.1 Chemicals

3-Cyano-7-ethoxycoumarin was obtained from Molecular Probes (Eugene, OR). 7-Methoxy-4-(aminomethyl)-coumarin, and 7-benzyloxy-4-trifluoromethylcoumarin were from GENTEST Corporation (Woburn, MA). Ticlopidine was obtained from ICN Biomedicals Inc. (Aurora, OH, USA). Glucose, galactose, L-histidine, L-leucine, Glucose 6-phosphate, β-nicotinamide adenine dinucleotide phosphate, reduced NADPH, diclofenac, 7-methoxy-4-trifluoromethylcoumarin (MFC), α-naphthoflavone, sodium dithionite, cytochrome *c*, quinidine and sulfaphenazole were purchased from Sigma Chemical Co. (St Louis, MO). 4-OH-diclofenac, bufuralol, 1´OH-bufuralol, S-mephenytoin, and 4-OH-*S*-mephenytoin were from Ultrafine (Manchester, UK). 4-Acetamidophenol and phenacetin were purchased from Sigma-Aldrich (Steinheim, Germany). Sorbitol and polyethylene glycol 4000 were from Merck (Darmstadt, Germany). Pefabloc SC and dithiothreitol were from Boehringer Mannheim (Mannheim, Germany). Wizard Plus SV Minipreps DNA Purification System Kits were purchased from Promega (Madison, WI, USA). The QuikChange Site-Directed Mutagenesis Kit was obtained from Stratagene (Sweden). The DNA sequencing kit, BigDye Terminator Cycle Sequencing Ready Reaction kit was obtained from Applied Biosystems (La Jolla, CA, USA). Minimum essential medium, fetal bovine serum albumin, non-essential amino acids, sodium pyruvate, penicillin/streptomycin solution, versene, trypsin and Dulbecco's phosphate buffered saline were from GIBCOBRL, Life technologies. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Larodan Fine Chemicals AB (Sweden). All other chemicals used were, of analytical or HPLC grade.

2.1.2. Antiparasitic drugs and metabolites

Praziquantel, tinidazole, metrifonate, pentamidine, primaquine, niclosamide, ivermectin, quinine, pyrimethamine, albendazole, thiabendazole, suramin, pyrantel, and diethylcarbamazine were purchased from Sigma Chemical Co. (St. Louis, MO). 4-Aminophenyl sulfone (dapsone) and artemisinin were obtained from Aldrich Chemical Co. (Milwaukee, WI). Amodiaquine, atovaquone, desethylamodiaquine, (+)-chloroquine, melarsoprol, (-)-chloroquine, desethylchloroquine, 4-chlorophenylbiguanide, proguanil, and cycloguanil were generous gifts from Prof. Anders Björkman and Prof. Lars Gustafsson (Karolinska Institute, Stockholm, Sweden). Dr. Michael Ashton (Uppsala University, Uppsala, Sweden) kindly provided dihydroartemisinin and artesunate.

2.1.3 CYP2D6 substrates and metabolites

Metoprolol, the α-hydroxy and demethylated metabolites of metoprolol were from AstraZeneca (Mölndal, Sweden). Bufuralol, 1´-hydroxybufuralol and 4-hydroxy-debrisoquine were obtained from Ultrafine (Manchester, UK). Dextromethorphan, debrisoquine, propafenone, timolol, sparteine, clomipramine, thioridazine and fluphenazine were purchased from Sigma Chemical Co. (St. Louis, MO). Dextrorphan was obtained from ICN Biomedicals Inc (Aurora, OH).

2.1.4 Recombinant CYPs

The recombinant enzymes used for screening for inhibition were obtained from AstraZeneca R & D Mölndal, Sweden. Those used for kinetic characterisation of CYP2D6.17, CYP2D6.1, CYP2D6.2 and CYP2D6.T107I, were prepared by the author.

2.1.5 Human liver microsomes

Human liver microsomes were prepared from a pooled set of liver pieces obtained from patients of Caucasian origin, undergoing liver resections. Approval for use of the liver pieces was obtained from the Local Ethics Committee, at Salgreska Hospital, Gothenburg, Sweden.

2.1.6 *Saccharomyces cerevisiae*

The *Saccharomyces cerevisiae* strain INVSc1-HR containing the human reductase gene was a gift from the LINK Project (a program of the University of Dundee/Biotechnology and Biology Research Council/Department of Trade and Industry/Pharmaceutical Industry). The galactose-inducible expression vector and cDNA for *CYP2D6*1* (with valine in position 374) were generous gifts from Dr Magnus Ingelman-Sundberg (Karolinska Institute, Stockholm, Sweden).

2.2 Methods

2.2.1 Assessment of the inhibitory effects of antiparasitic drugs

2.2.1.1 Preparation of recombinant enzymes

Microsomes from yeast expressing human CYP isoforms were prepared as previously described (Masimirembwa *et al*., 1999). Briefly, transformed yeast cells were grown in 75 ml selective medium (yeast nitrogen base, 0.05 mg/ml histidine and leucine) for 16 hrs with shaking at 180 rpm and 30^{o}C. 50 ml of the culture was used to inoculate 500 ml YPGE medium (20 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and grown for 10 hrs before addition of ethanol (final concentration 2%). After growing the cells for another 16 hrs, galactose (final concentration 2%) was added to induce transcription. A further incubation of 10 hrs was required before harvesting

the cells by centrifugation at 6600 g. The cells were washed in deionised water followed by 50 mM Tris buffer pH 7.4 containing 1 mM EDTA and 0.1 M potassium choride before resuspension in 10 mM Tris buffer pH 7.5 containing 2 M sorbitol, 0.1 mM DTT, 0.1 mM EDTA (TES) and 5mg/ml yeast lytic enzyme and incubation at 30^{o}C for 1 hr with gentle shaking. Subsequent procedures were carried out on ice. The cells were disrupted by hand-shaking using glass beads in 50 mM Tris buffer pH 7.4 containing 1 mM EDTA, 0.6 M sorbitol, 4 mM pefabloc SC and 10% (v/v) glycerol. The resultant supernatant after addition of TES buffer containing 0.4 mM pefabloc was centrifuged for 10 min at 3500 g followed by another centrifugation step at 23 000 g for 10 min. The microsomes were precipitated by addition of sodium chloride (0.1 M final concentration) and PEG 4000 (final concentration 10%) and incubation on ice for 15 min. The microsomes were obtained by centrifugation at 12 400 g and homogenised in Tris buffer pH 7.4 containing 1 mM EDTA and 20% glycerol.

2.2.1.2 Preparation of human liver microsomes

Liver pieces from five different individuals stored at –80^{o}C were pooled and weighed. They were allowed to thaw on ice in Tris-HCl buffer pH 7.6 containing 250 mM sucrose. The liver pieces were cut into smaller pieces using a pair of scissors and then homogenised. The resultant mixture was centrifuged at 10 000 g for 20 min and the pellet was discarded. The supernatant was centrifuged at 105 000 g for 60 min to pellet the microsomes which were re-suspended in Tris-HCl pH 7.6 containing 250 mM sucrose and stored at –80^{o}C.

2.2.1.3 Determination of protein

The protein concentration was measured by the modified method of Lowry (Markwell *et al*., 1978). A sample of the microsomes was diluted 100 times in 1 ml of water. 3 ml of reagent A, containing (4% Na_2CO_3, 0.2 M NaOH, 0.32% K-Na-tartrate), 2% SDS and 4% $CuSO_4.5H_2O$ in the ratio of 50:50:1 respectively, was added to the sample of microsomes and vortex-mixed. The mixture was allowed to stand for 60 min at room temperature. 0.3 ml of Folin-Ciocalteu reagent, diluted with an equal volume of water, was added and vortex-mixed. The resultant mixture was allowed to stand in the dark for 45 min and the absorbance was measured at 660 nm. The protein concentration in the microsomes was calculated from a standard curve of bovine serum albumin whose concentrations ranged from 0 – 250 µg/ml.

2.2.1.4 Determination of CYP concentration

The CYP content was determined from the reduced carbon monoxide difference spectrum (Omura and Sato, 1964). In brief, the microsomes were suspended in buffer (50 mM sodium phosphate pH 7.4 containing 1 mM EDTA and 20% glycerol for recombinant enzyme and 50 mM Tris-HCl pH 7.4 containing 15 mM KCl in human liver microsomes) to give a total volume of 3 ml and protein concentration of 2.0 mg/ml. Carbon monoxide gas was bubbled at the rate of approximately 1 bubble per second for 1 min. The mixture was mixed and divided between two cuvettes. The cuvettes were placed in a spectrophotometer and a baseline was run between 400 and 500 nm. Sodium dithionite was added to the sample cuvette and the absorbance spectrum between 400 and 500 nm was recorded. The concentration of CYP was calculated using the molar extinction of 91 $mM^{-1}cm^{-1}$ i.e. $(Ab_{450} – Ab_{490})$ X 1000/ 91 $mM^{-1}cm^{-1}$

2.2.1.5 Determination of NADPH-CYP-reductase

NADPH-CYP reductase was determined by the reduction of cytochrome *c* (Pearce *et al*., 1996). In brief, microsomes were diluted to a concentration giving a linear reaction rate in 1790 µl of 0.5 M potassium phosphate buffer pH 7.4, 200 µl of cytochrome c (3.7 mg/ml) to a final volume of 2 ml. The mixture was divided between two cuvettes. The reaction was started by addition of 10 µl of NADPH (32 mM) and followed at 550 nm. The linear range was used to calculate the NADPH-CYP reductase activity using the extinction coefficient for reduced cytochrome *c* at 550 nm of 19.6 $mM^{-1}cm^{-1}$.

2.2.1.6 Fluorometric assays

Fluorometric assays (Crespi *et al*., 1997) were done in black Costar 96-well plates (Corning Incorporated, NY, USA) under experimental conditions shown in Table 2.1. Addition of reagents to the 96-well plates was done by hand pipetting. Each reaction mixture consisted of the appropriate concentration of enzyme, 1mM NADPH, substrate and inhibitor in the appropriate concentration of potassium phosphate buffer (pH 7.4) as described by Gentest (http://www.gentest.com.). 12 data points were used to generate the K_m and V_{max}. Test compounds and positive control inhibitors were dissolved in water, methanol, acetonitrile, or dimethylformamide to give 10 mM stock solutions. The final solvent concentrations in incubation mixtures were between 1 and 3%. Due to problems of solubility, only 1 mM solutions of niclosamide, primaquine, and albendazole could be made. DMSO was avoided as a solvent due to its high capacity to inactivate CYPs. All compounds were tested for fluorescence or metabolism to fluorescent metabolites at the different excitation and emission wavelengths for

the assays. Primaquine was very fluorescent under all the assay conditions and so could not be studied with these assays. The inhibitors were serially diluted to give final concentrations, in duplicate, ranging from 0.09 – 200 μM for 10 mM stock solutions and 0.009 – 20 μM for 1mM stock solutions. The following were used as positive controls: α-naphthoflavone for CYP1A2, sulfaphenazole for CYP2C9, ticlopidine for CYP2C19, quinidine for CYP2D6 and ketoconazole for CYP3A4. The reaction mixtures were pre-warmed for 10 min at 35^{o}C and NADPH was added to start the reactions. The incubation times used are shown in Table 2.1 and 75 μl of 20% 0.5 M TRIS and 80% acetonitrile was used to stop the reactions. For the CYP2D6 assay an NADPH-regenerating system (0.4 mM glucose 6-phosphate, 0.4 mM $MgCl_2$, 0.5 M sodium citrate and 0.4 U/ml glucose 6-phosphate dehydrogenase) was used. The fluorescence was measured using a Wallac 1420 Victor$^{2}_{TM}$ (WallacSverige AB, Upplands Väsby, Sweden). The excitation and emission wavelengths are shown in Table 2.1.

2.2.1.7 HPLC assays to assess the inhibitory effects of antiparasitic drugs

In order to compare fluorescence and HPLC-based assays, the effect of 20 μM solutions of all the compounds on enzyme activities was determined using HPLC-based assays. Complete IC_{50} curves were done for compounds identified as potent inhibitors. Incubation conditions for HPLC assays were done as described by Masimirembwa *et al.,* (1999) and the phenacetin O-deethylation for CYP1A2 as described by Kobayashi *et al*., (1998). Briefly, all the incubations were performed in the same way as those for fluorescence-based assays mentioned in the previous section. The stop solutions used were 10 μl of 60 % perchlorate for bufuralol 1'-hydroxylation, 50 μl of a 94% acetonitrile, 6% glacial acetic acid for diclofenac 4-hydroxylation, 100 μl methanol for testosterone 6β-hydroxylation and phenacetin O-deethylation and 100 μl of

6% perchlorate for *S*-mephenytoin 4-hydroxylation. After centrifugation at 10 000 g for 10 min, the supernatant was analysed by reversed phase chromatography on a Hewlett Packard HPLC (Waldbronn, Germany) coupled with a Diode Array detector (Hewlett-Packard) and fluorescence detector (JASCO, Tokyo, Japan) using a Zorbax C_{18} 4.6 mm X 15 cm column and C_{18}, 4.6 mm X 1.25 cm guard column. A Zorbax C_{18} 4.6 mm X 30 cm column was used for *S*-mephenytoin 4-hydroxylation and phenacetin O-deethylation assays. The metabolites of all the reactions were quantified by external standardisation using authentic metabolites and the details of the experimental conditions are shown in Table 2.2.

2.2.1.8 Data analysis

The parameters K_m, V_{max} and IC_{50} were determined by nonlinear least-squares regression analysis using Grafit Version 3.0 (Erithacus Software Limited, Middlesex, UK). The inhibitor constant (K_i) values were calculated from IC_{50} values, assuming competitive inhibition, according to the following relationship:

$IC_{50} = K_i(1 + S/K_m)$; thus when $S = K_m$, $K_i = IC_{50}/2$

Table 2.3 shows the substrate concentrations equal to K_m used in the determination of IC_{50} values. For compounds identified as potent inhibitors, the mechanism of inhibition was determined by fitting the data to different inhibition models by non-linear regression using GraFit Version 4.0.

Table 2.1: Experimental conditions and Michaelis-Menten kinetics for different rCYP fluorescence assays

CYP	Enzyme amount [linearity range]	Incubation time [linearity range]	Substrate	Excitation wavelength	Emission wavelength	Enzyme kinetic parameters	
						K_m	V_{max}
	pmol	*min*		*nm*		*μM*	*nmol/min/nmolCYP*
1A2	1 [0.5-2]	20 [10-35]	CEC	405	460	3	14
2C9	3 [0.5-3]	50 [10-60]	MFC	405	535	47	0.4
2C19	5 [0.5-10]	40 [10-45]	MFC	405	535	75	0.4
2D6	2 [0.5-2]	35 [10-45]	MAMC	390	460	100	132
3A4	2 [0.5-2]	30 [10-30]	BFC	405	535	13	1.8

Values in brackets represent linearity range.

The predicted percentage inhibition of a specific CYP *in vivo* was then calculated using the K_i values generated from human liver microsomes and the reported C_{max} of the drugs using the following relationship:

For competitive inhibition, % inhibition $= \dfrac{[I]}{[I] + K_i(1 + [S]/K_m)} \times 100$

For mixed inhibition, % inhibition $= \dfrac{[I] \times 100}{K_i[(K_m + [S])/(K_m + [S]/\alpha)] + [I]}$

Thus, when $[S] << K_m$, for both mixed and competitive inhibition, percentage inhibition is given by $[I]/ ([I] + K_i)$ (Rodrigues and Wong, 1997)

2.2.2 Assessment of the inductive effects of antiparasitic drugs on CYPs1A1/2

2.2.2.1 Culturing of cells

The human hepatoma (HepG2) cells, were cultured in minimum essential medium containing fetal bovine serum albumin 10% (v/v), non-essential amino acids 1% (v/v), sodium pyruvate (final concentration 1 mM) and penicillin/streptomycin solution (100 μg/ml), and incubated in a humidified atmosphere of 5% CO_2/95% air at 37^{o}C. Cells were routinely passaged by trypsinization when confluent. Trypsinization was done to remove cells from the surface of the culturing vessel to enable counting and splitting of the cells. Briefly, the culturing media was removed by aspiration. 2 ml of versene (0.2g/L EDTA.4Na in phosphate buffered saline)enough to cover the cell monolayer were added and were left for 5 min in the incubator. The versene was removed by aspiration and 1 ml of trypsin was added and left to stand for 5 min. Culturing media (10 ml) was added and the mixture shaken gently to remove the cells from the surface of the culturing vessel.

Table 2.2. Methods for the HPLC-based assays

Reaction	Analytical Method	Wavelength (nm)	Mobile phase and method
Testosterone 6β-hydroxylation	UV	254	60% methanol in 50 mM KH_2PO_4 Isocratic elution. Flow rate 1 ml/min.
Bufuralol 1´-hydroxylation	fluorescence	excitation: 252 emission: 302	A: 20 mM perchlorate B: acetonitrile Isocratic: 70% A & 30% B. Flow rate 1 ml/min
Diclofenac 4-hydroxylation	UV	280	A: 20% acetonitrile in 1 mM perchlorate B: methanol Gradient: 0-3 (40%) B, 3-8 (40-100%) B, 8-10 (100-40%) B. Flow rate: 1 ml/min.
S-Mephenytoin 4-hydroxylation	UV	214	A: 50 mM potassium phosphate pH 6.0 B: Methanol. Isocratic 65%A, 45%B. Flow rate 1 ml/mi
Phenacetin O-deethylation	UV	245	A: 50 mM KH_2PO_4 B: 100% acetontrile Gradient: 0-6 min (15% B), 7-17 min (30% B), 18 min (15% B). Flow rate: 1 ml/min

Table 2.3: Michaelis-Menten kinetics for different CYP marker reactions.

		Human liver microsomes		**Recombinant CYPs**	
CYP	Reaction	Enzyme kinetic parameters			
		K_m (μM)	V_{max} (nmols/min/mg protein)	K_m (μM)	V_{max} (nmol/min/nmol)
1A2	Phenacetin O-deethylation	43	2.5	107	7.8
2C9	Diclofenac 4-hydroxylation	1.5	1.7	8.3	15
2C19	S-Mephenytoin 4-hydroxylation	ND[a]	ND[a]	54	2.1
2D6	Bufuralol 1′-hydroxylation	9.3	0.12	15	8
3A4	Testosterone 6β-hydroxylation	71	6.8	55	8

[a]ND: not determined
(Determined by Masimirembwa *et al*., 1999)

2.2.2.2 Treatment of cells

Cells (15 000 cells/well in 200 µl media) were seeded in 96-well plates and incubated for 48 hr. The test compounds dissolved in DMSO (final concentration 1%) were added together with fresh media at 0.3 µM, 30 µM and 300 µM with each concentration done in duplicate. The cells were exposed to the compounds for 24 hr and cell viability was assessed by the trypan blue exclusion test. The viability of cells was assessed by counting the number of stained and unstained cells using a microscope and hemacytometer.

The percentage cell viability was obtained from the following expression:

$$\frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \text{ X } 100$$

The cells were washed twice with PBS and stored at –70^{o}C.

2.2.2.3 Assay for ethoxyresorufin O-deethylase activity (EROD) in 96-well plates

In brief, the reaction mixture consisting of BSA (1.0 mg/ml), magnesium sulphate (8.3 mM), NADPH (1.2 mM) and NADH (1.4 mM) in HEPES buffer pH 7.8 was added to the 96-well plates containing the cells. The mixture was pre-incubated for 15 min at 37^{o}C with shaking. The reaction was started by addition of ethoxyresorufin (final concentration of 3.5 µM) dissolved in HEPES buffer pH 7.8. The reaction was stopped by addition of 150 µl of methanol (100%) and the plate was allowed to stand for 15 min in the dark before reading at excitation and emission wavelengths of 544 nm and 590 nm respectively.

2.2.2.4 Treatment of cells for determination of CYP1A1/2 mRNA expression

The general procedure and the principle, behind the method used to determine the effect of the anti-parasitic drugs on mRNA expression of CYP1A1/2 is shown in Figure 2.2. HepG2 cells (1.5 x 10^5 cells/well in 2 ml media) were seeded in 6-well plates and incubated for 48 hr. The test compounds were added together with fresh media at six concentrations (within the range found in plasma after administration of therapeutic dose of drug) for each of the three compounds (quinine, primaquine and albendazole). The cells were exposed to the compounds for 24 hr after which the cells were washed twice with PBS before storage at –80^{o}C. The EROD activity of the cells treated with the six concentrations of compounds was also determined as described in the previous section.

2.2.2.5 Preparation of RNA from HepG2 cells and cDNA synthesis

The total RNA was prepared using the RNeasy Mini Kit from Qiagen Inc (CA, USA) according to the manufacturer's instructions. The total RNA samples were treated with Dnase RQ1 (Promega) and 2 μg of the RNA was used for the synthesis of the first strand and amplification of the target cDNA using the Superscript First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer's instructions.

2.2.2.6 Quantification of mRNA

The following primers and probe were used to quantitate the amount of CYP1A1 mRNA:

- Forward primer: 5' GTT CTA CAG CTT CAT GCA GAA GAT G 3'
- Reverse primer: 5' TTG GCG TTC TCA TCC AGC T 3'
- Probe: 5' AAA ACC TTT GAG AAG GGC CAC ATC CG 3'

The following primers and probe were used to quantify the amount of CYP1A2 mRNA:

- Forward primer: 5' CTG TGG TTC CTG CAG AAA ACA G 3'
- Reverse primer: 5' CCC TTC TTG CTG TGC TTG AAC 3'
- Probe: 5' CCG GAC ACT GTT CTT GTC AAA GTC CTG A 3'

Additional primers and probe were used to amplify the housekeeping gene h36B4 that served as the internal standard:

- Forward primer: 5' CCA TTC TAT CAT CAA CGC GTA CAA 3'
- Reverse primer: 5' AGC AAG TGG GAA GGT GTA ATC C 3'
- Probe: 5' TCT CCA CAG ACA AGG CCA GGA CTC GT 3'

The reaction mixture in a total volume of 25 µl, consisted of the appropriate cDNA preparation, forward and reverse primers (final concentrations of 0.9 µM and 0.3 µM for CYPs 1A1 and 1A2 respectively), probe (final concentration of 0.25 µM for both CYP1A1 and 1A2) and 12.5 µl of Taqman Universal Master Mix (Applied Biosystems). The thermal cycle conditions had initial steps of 50^{o}C for 2 min and a 10 min step at 95^{o}C followed by 40 PCR cycles of 95^{o}C for 15 s, 60^{o}C for 1 min. The starting mRNA copy number in each sample (done in duplicate for each concentration) was measured in triplicate by comparing, the number of cycles required to reach a set threshold level of fluorescence, of the samples and controls treated with the solvent alone (Figure 2.1). TCDD was used as a positive control.

2.2.3 Characterisation of CYP2D6.17 and implications for use of CYP2D6 substrates

2.2.3.1 Mutagenesis

The three enzyme variants with their characteristic amino acid exchanges, CYP2D6.17 (T107I, R296C and S486T), CYP2D6.2 (R296C and S486T) and CYP2D6.T107I, were

produced by site-directed mutagenesis of the *CYP2D6*1* cDNA coding for valine instead of methionine at position 374, using the QuikChange Site-directed mutagenesis Kit (Stratagene). The site-directed mutagenesis procedure is illustrated in Figure 2.2. In the first step, *E. coli* cells containing plasmid with cDNA coding for the CYP2D6.1 valine variant (valine instead of methionine at position 374) were cultured at 37^{o}C in liquid broth media (2 ml) and containing carbenicillin (50 µg/ml). The plasmid DNA was isolated and purified using a kit (Promega). The mutagenic primers (5′- CCT GTG CCC ATC A**T**C CAG ATC CTG GGT – 3′, 5′ – GAT GAG AAC CTG **T**GC ATA GTG GTC GCT – 3′ and 5′ – GCT TTC CTG GTG A**C**C CCA TCC CCC TAT – 3′) containing the required base changes in bold, were designed for use in the mutagenic PCR. The PCR reaction mixture consisted of reaction buffer (1x final concentration), 5-50 ng DNA (5 µl of plasmid DNA), 62.5 ng/µl forward/reverse primers (2 µl of each), 2.5 mM of each dNTP (4 µl of dNTP mix) and 2.5 U/µl of PfuTurbo DNA polymerase in a final volume of 50 µl. The mutagenic PCR conditions consisted of an initial denaturation step at 95^{o}C for 1 min. This was followed by 16 cycles of 95^{o}C for 30 s, 55^{o}C for 4 min and 68^{o}C for 25 min. The last step was a 10 min period at 68^{o}C. The mutagenic PCR product was digested using Dpn1 that removes the methylated parental DNA used as template leaving the mutated strands containing the base changes of interest. The products of Dpn1 digestion were used to transform competent *E. coli* cells. The preparation of competent *E. coli* cells involved growing an overnight culture of the cells in 2 ml LB media at 37^{o}C. The following day, 200 µl of the overnight culture was used to inoculate 100 ml of LB media and grown at 37^{o}C to an OD_{600} of 0.5. The cells were harvested by centrifugation at 6 000 g for 5 min. The cells were re-suspended in 10 ml of cold 50 mM $CaCl_2$ and incubated on ice for 30 min. The cells were spun down at 6 000 g for 5 min and were re-suspended in 7 ml of cold 50 mM $CaCl_2$ to which 3 ml of 60% (v/v) glycerol was added. 2 µl of the Dpn1 digested PCR product was added to 200 µl of competent cells and the mixture was incubated on ice for 30 min.

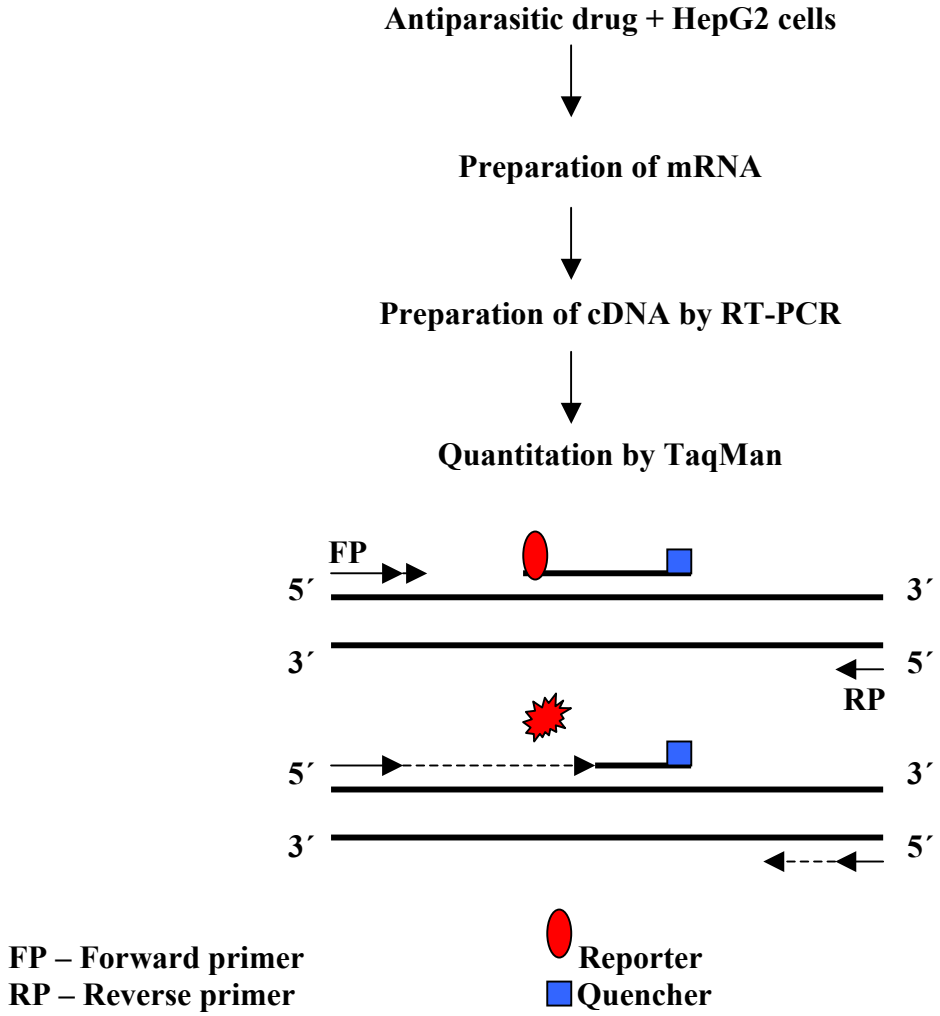


Figure 2.1 The general procedure used to assess the effects of anti-parasitic drugs on CYP1A1/2 mRNA expression and the principle behind the TaqMan technology. TaqMan technology makes use of a fluorogenic probe which is an oligonucleotide carrying a reporter and a quencher. The fluorogenic probe hybridises to a region flanked by the forward and reverse primers. Cleavage of the fluorescent reporter by the 5′ ⟶ 3′ exonuclease activity of Taq polymerase occurs only if the oligonucleotide probe becomes part of the replication complex. The intensity of the fluorescence will, therefore, depend on the initial mRNA copy number (Figure adapted from Masimirembwa *et al*., 2001).

The cells were heat-shocked at 42^{o}C for 90 s after which they were put on ice. The cells were plated on LB-plates containing 50 µg/ml carbenicillin and incubated overnight at 37^{o}C. Colonies of transformed cells were grown at 37^{o}C in LB media containing carbenicilllin (50 µg/ml) and plasmid DNA was prepared from the transformed cells.

Confirmation of the base changes introduced was by sequencing using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Briefly, primers were designed in a way that ensured that the whole CYP2D6 sequence was covered as shown in Figure 2.3. The sequences of the primers used for sequencing are shown below:

- 5′ - CGC CGC TTC GAG TAC GAC GA - 3′
- 5′ - TAC TTC GAT GTC ACG GGA TGT CA - 3′
- 5′ - CCT GTG CCC ATC ATC CAG ATC CTG GGT - 3′
- 5′ - AGC CAC CAC TAT GCA CAG GTT CTC ATC - 3′

Preparation of reaction mixtures for sequencing, were performed on ice. The reaction mixture consisted of buffer 4 µl (200 mM Tris-HCl pH 9.0, 5 mM $MgCl_2$), 4 µl (Big Dye Terminator Cycle Sequencing Ready Reaction Kit), 1 µl (10 µM primer) and 5 µl (about 0.5 µg DNA from mini-preparation in a final volume of 20 µl. The sequencing PCR conditions consisted of 25 cycles, each of 96^{o}C for 30 s, 50^{o}C for 15 s and 60^{o}C for 4 min. After the 25 cycles, the PCR products were kept at 4^{o}C. To the PCR product, 18 µl of water were added followed by 62 µl of 99.5% ethanol. The mixture was allowed to stand at room temperature to precipitate the DNA. The DNA was pelleted by centrifugation at 3000 g for 30 min. The pelleted DNA was washed using 150 µl of 70% ethanol and spun for 2000 g for 10 min. The ethanol was carefully poured out leaving the DNA, which was dried by inverting the tubes on paper towels. An hour before running on the sequencer, the DNA was dissolved in 20 µl of a mixture of formamide:water (1:1).

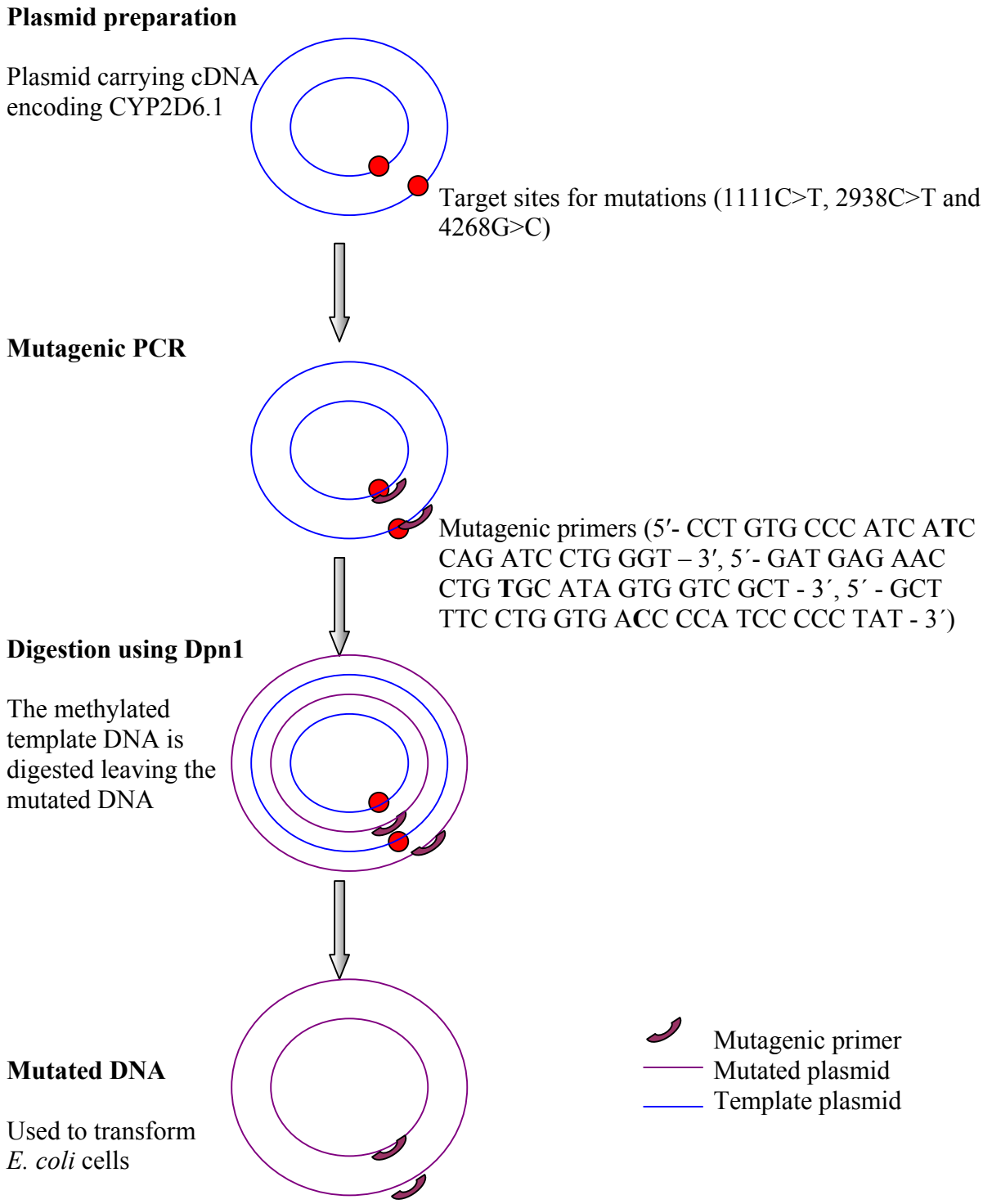


Figure 2.2 Showing the general steps in site-directed mutagenesis (Figure based on the overview given in the QuikChange Site-directed Mutagenesis kit instruction manual)

After confirmation of the sequences, the yeast strain INVSc1-HR, bearing the human reductase was transformed with the appropriate cDNA, by the lithium acetate method (Gietz *et al*., 1992). In brief, the yeast cells grown to an OD_{600} of between 0.5 – 0.3 were harvested by centrifugation at 5000 g for 5 min. The cells were washed with 10 mM Tris-HCl pH 7.8 containing 1 mM EDTA and spun for 5 min at 5000 g. The cells were re-suspended in 0.5 ml Tris-HCl pH 7.8 containing 0.1 M lithium acetate and incubated for 12 h. To 100 µl of the cells, 80 µg single stranded sperm carrier DNA and 5 µg plasmid DNA were added. The mixture was incubated at 30^{o}C for 30 min. 0.7 ml of PEG 4000 (40% w/v) in 10 mM Tris-HCl buffer pH 7.8 containing 0.1M lithium acetate, was added and the mixture was further incubated at 30^{o}C for 2.5 h. The cells were heat-shocked at 42^{o}C for 15 min. The cells were harvested by centrifugation at 10 000 g for 1 min. The cells were washed in 10mM Tris-HCl buffer pH 7.8 and spun down at 10 000 g for 5 min. The cells were re-suspended in 10 mM Tris-HCl buffer pH 7.8 and plated onto selective media (lacking uracil). The transformed yeast cells were grown and used for preparation of microsomes as described in section 2.2.1.1.

2.2.3.2 Determination of CYP, NADPH-CYP reductase and protein concentration

The determination of CYP, NADPH-CYP reductase and protein concentration were done as described in sections 2.2.1.3-5.

2.2.3.3 Enzyme kinetics

Each reaction mixture consisted of the appropriate concentration of enzyme, substrate (dissolved in 0.1 M phosphate buffer pH 7.4) and 1 mM NADPH, in 0.1 M potassium phosphate buffer pH 7.4 to a final volume of 200 µl. The reactions were started by addition of NADPH after a pre-incubation of 3 min at 35^{o}C and optimised for linearity with respect to

time and CYP concentration. All reactions were stopped by the addition of 10 µl of perchloric acid (60%). The supernatant (5-20 µl) obtained after centrifugation at 4 000 g for 10 min was analysed by reversed-phase chromatography. The metabolites formed were quantified by external standardisation using authentic metabolites.

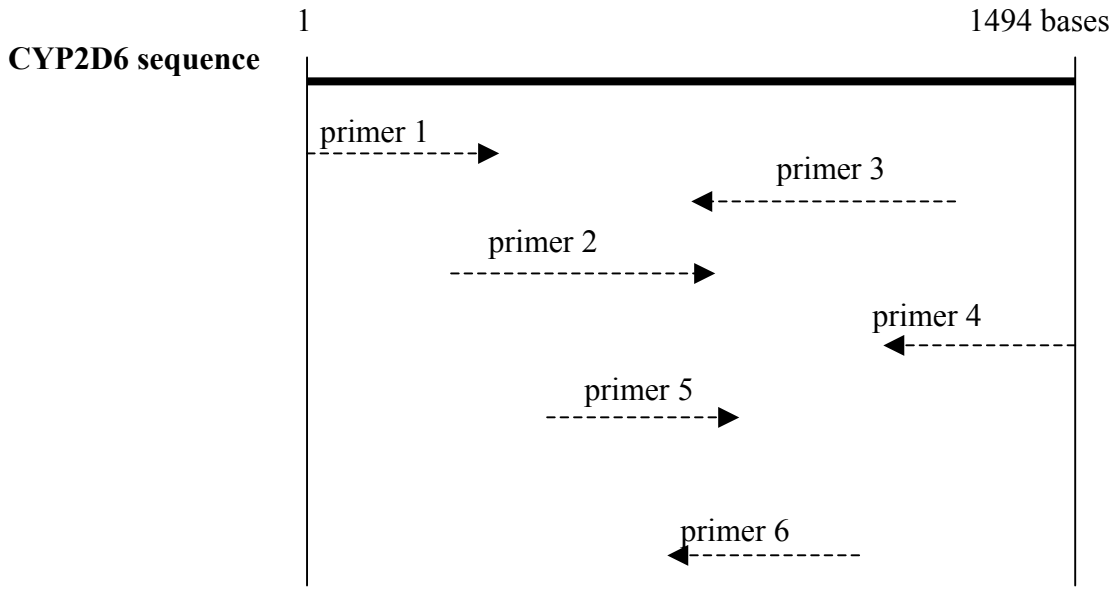


Figure 2.3 The sequencing of the CYP2D6 and the importance of designing primers that ensured that the whole CYP2D6 sequence was covered.

Bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase and dextromethorphan O-demethylase activities were measured according to the method of (Kronbach *et al*., 1987). All metabolites of metoprolol were assayed by the method of (Chiu *et al*., 1997). In brief, metoprolol and its metabolites were separated on a Zorbax C_{18} 4.6 X 15 cm column and detected using excitation and emission wavelengths of 229 and 309 nm respectively. A gradient elution was used with the mobile phases consisting of A: 0.5% triethylamine adjusted to pH 3.0 using orthophosphoric acid and containing 1% (final concentration) methanol and 4% (final concentration) acetonitrile and B: 100% acetonitrile. The gradient consisted of 5%B for the first 6 min, 10% B for the following 7 min and the last 2 min at 5% all at a flow rate of 1.4 ml/min.

Dextromethorphan and its metabolite dextrorphan, were separated on a Zorbax C_{18} 4.6 X 15 cm column and detected using excitation and emission wavelengths of 270 and 312 nm. An isocratic elution was used with the mobile phase consisting of A: 20 mM perchlorate containing 20% acetonitrile and B: 100% acetonitrile pumped at 85% A, 15% B and flow rate of 1 ml/min.

2.2.3.4 Data analysis

Eight data points were used to generate K_m and V_{max} values. These values were determined by non-linear least-squares regression analysis using GraFit version 3.0 (Erithacus Software Limited).

2.2.3.5 Determination of intrinsic clearance (CL_{int})

For ten known substrates of CYP2D6 dissolved in water or acetonitrile (1% final concentration), intrinsic clearance studies were performed with human liver microsomes

(HLM), recombinant CYP2D6.1, and CYP2D6.17 using the substrate disappearance approach (Obach, 1999). The typical incubation mixture consisted of 10 pmol of enzyme, 1 µM substrate and 1 mM NADPH, in 0.1 M potassium phosphate buffer pH 7.4 to a final volume of 200 µl. The reactions were pre-incubated for 3 min, started by the addition of NADPH, and incubated for 5, 10, 20, 30 and 40 min. The reactions were stopped by addition of 100 µl ice-cold methanol containing 0.8% formic acid and 0.4 µM verapamil (internal standard). After centrifugation at 4 000 g, the supernatant was transferred and diluted 1:1 with water and analysed by LC-MS as described below. The intrinsic clearance was determined using the expression: CL_{int} = Volume x *k*,where the volume is the total incubation volume (200 µl in this study) and *k* is the elimination rate constant.

Using debrisoquine 4-hydroxylation as a marker reaction, the relative contribution of CYP2D6 in the clearance of the substrate drugs by HLM was evaluated using the relative activity factor (RAF) approach (Crespi, 1995). The RAF was calculated using the expression:

$$RAF_{isoform} = \frac{\text{velocity for probe substrate with HLM (pmol/min/mg)}}{\text{velocity for probe substrate with recombinant enzyme (pmol/min/pmol)}}$$

The units for RAF are, therefore, pmol CYP/mg protein. The percent contribution towards the clearance of a drug was calculated as follows:

$$\% \text{ contribution} = \frac{\text{clearance by recombinant enzyme x RAF x 100}}{\text{clearance by HLM}}$$

2.2.3.6 Liquid chromatography-mass spectrometry

Supernatant (10 µl) was injected using a CTC HTS autosampler (CTC Analytics) and chromatography was performed on a Zorbax Extend C_{18} column (2.1 x 50 mm, 3.5 micron, Agilent) employing an Agilent 1100 pump with gradient elution at 300 µl/min. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The organic modifier content was 5% for B for 0.6 min, then increased linearly from 5% to 95% B over 2 min, kept at 95% B for 1 min, and followed by a step gradient to 5% B for 1.4 min. The total run-time including equilibration amounted to 5.5 min. The pump's mixing chamber was omitted to reduce the system's dwell volume. The front was sent to waste by means of a VICI 6-port two position-switching valve. After 0.6 min the column effluent was directed towards the mass spectrometer without splitting. Detection was performed with a triple quadrupole mass spectrometer from MDS Sciex, API 3000 equipped with a Turbo-Ionspray source. The MS operated at turbogas at 300^{o}C, nebuliser gas 11, curtain gas 10, CAD gas 6, dwell time 200 ms, electrospray voltage 4.8 kV. Instrument control, data acquisition and data evaluation were performed with Analyst 1.1 software (Applied Biosystems). Mass spectrometric optimisation for the various analytes and batch acquisition were performed automatically employing Automation Software (Applied Biosystems).

2.2.3.7 Homology modelling and docking

The general steps used to construct the homology model are shown in Figure 2.4. The rabbit CYP2C5 and human CYP2D6.1 sequences were obtained from Swissprot database (accesion numbers P00179 and P10635 respectively). The first 33 amino acids in the CYP2D6.1 sequence were removed to allow for the absence of the first 29 N-teminal amino acids from the CYP2C5 crystal structure (Williams *et al*., 2000). In addition, the amino acid residue

methionine 374 was changed to valine that is found in the 'wildtype' CYP2D6 protein. The programs: GCG BestFit and Swiss-PdbViewer v 3.7b2 (Glaxo Wellcome Experimental Research) were used to align the CYP2D6.1 sequence to the CYP2C5 sequence with manual adjustments being made to the gap regions. Subsequent work was done on a Silicon Graphics workstation. The alignment was introduced into the program INSIGHT II version 2000 (Molecular Simulations. Inc. 2000) for modelling. Analysis of the model was done in SYBYL 6.7 (Tripos Associates Inc.) using the Protable module.

The structures of the CYP2D6 substrates were drawn in SYBYL. Atom charges (MMFF94 charges) were added to the structures and energy minimised using the MMFF94s forcefield. The docking experiments were done as previously described (Afzelius *et al*., 2001). In brief, dockings of the substrates were performed using the program GOLD (Genetic Optimisation for Ligand Docking, Dr Gareth Jones) with active site radii of 10, 12 and 15 Å defined from the heme-bound oxygen, requesting 10 solutions, however, termination was allowed if the root mean square distances (RMSD) were within 1.5 Å. GOLD is an automated ligand docking program that uses a genetic algorithm which explores possible hydrogen-bonding and hydrophobic interactions between the ligand and protein. It indicates the strong interactions it picks during the docking runs (Jones *et al*., 1997). The distances from the heme-bound oxygen to the carbon at the likely sites of oxidation on each substrate were measured.

2.2.4 Statistical analyses

All statistical analyses were performed using the program GraphPad InStat. Analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test, were used to compare differences in: 1) inductive effects, between controls and the varying concentrations

of drugs, 2) enzyme kinetic parameters between CYP2D6.1 and the three variants (CYP2D6.2, CYP2D6.17 and CYP2D6.T107I). Intra- and inter-day variability in the methods used to assess CYP1A1/2 induction, was expressed as coefficient of variation (% CV).

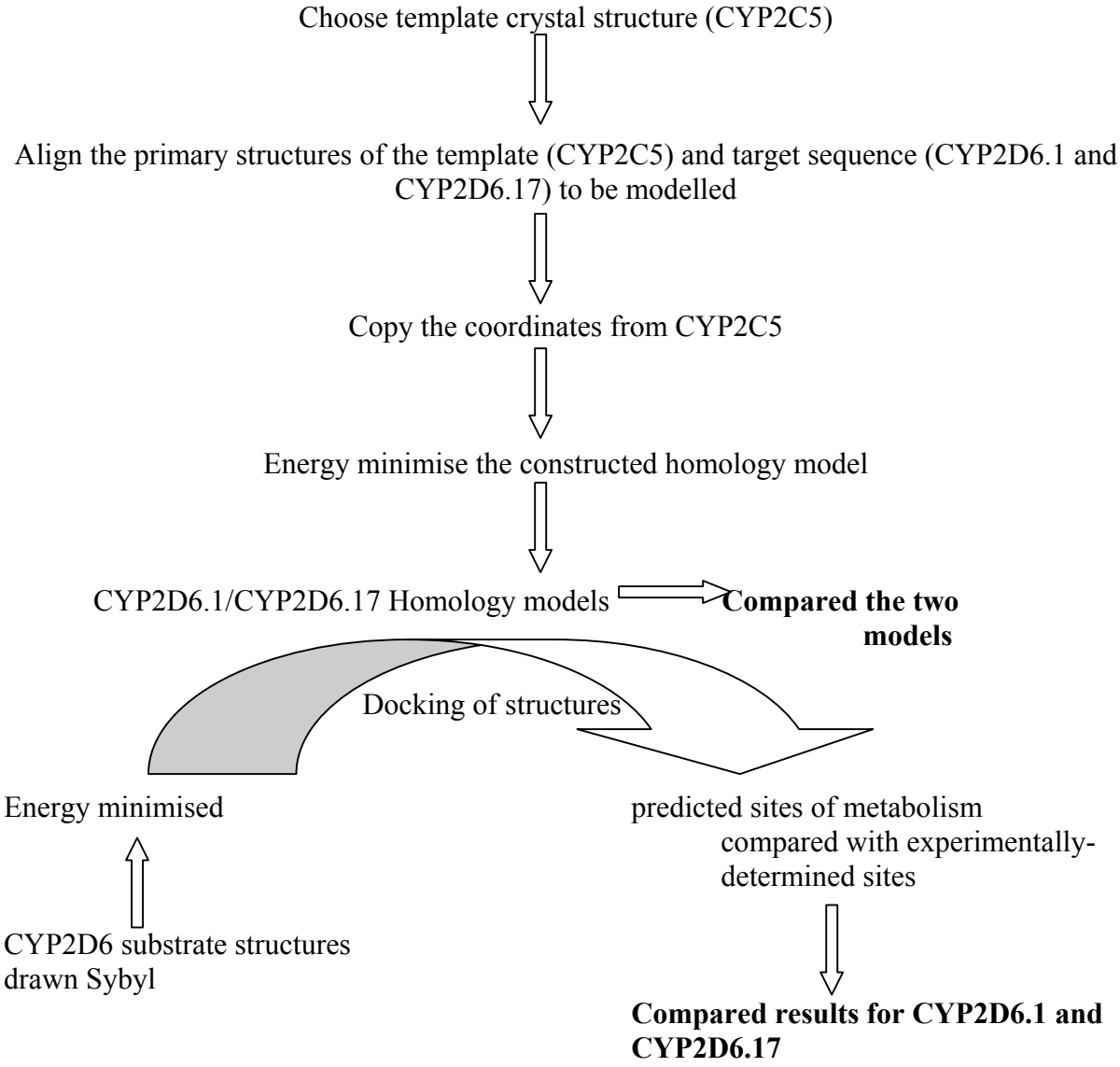


Figure 2.4. The general steps involved in homology modelling of CYP2D6.1 and CYP2D6.17 and subsequent docking of known CYP2D6 substrates into the models. The effect of amino acid exchanges in CYP2D6.17 on the structure of the enzyme was investigated at the two points shown in bold. Figure adapted and modified from ter Laak and Vermeulen, (2001).

CHAPTER THREE

3.0 Results

3.1 Inhibitory effects of antiparasitic drugs on CYPs

Initial work in the project focused on assessing the inhibitory effects of antiparasitic drugs on members of the CYP superfamily important in drug metabolism, namely CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Fluorometric assays based on the work by Crespi *et al*., (1997), were used to screen for inhibition as described in detail in section 2.2.1.6. Increasing concentrations of the antiparasitic drug were incubated with the enzyme at a substrate concentration equal to K_m. In each experiment, known potent inhibitors of the respective CYPs were used as positive controls. From the data, the enzyme activity, expressed as a percentage of control (enzyme activity in absence of inhibitor), was plotted against the inhibitor concentration as illustrated in Figure 3.1. The IC_{50} value for each drug was determined from the plot and this enabled the calculation of the inhibitor constant (K_i) from the relationship: $IC_{50} = K_i(1 + S/K_m)$, thus when $S = K_m$, $K_i = IC_{50}/2$.

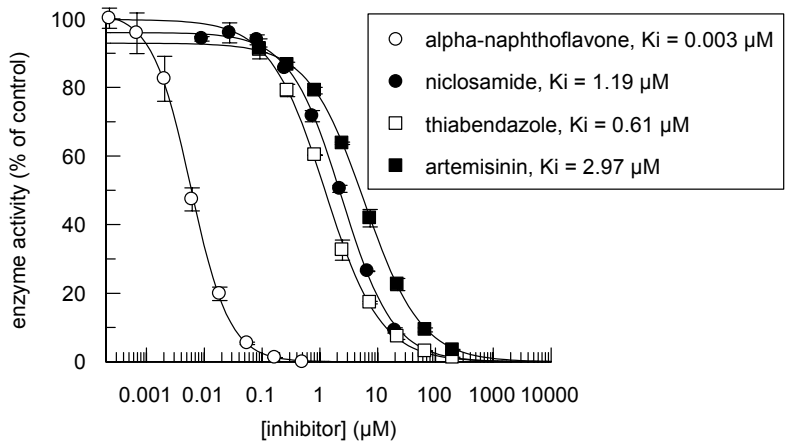


Figure 3.1. A typical plot used to determine IC_{50} and K_i values showing inhibition of the recombinant CYP1A2-catalysed fluorometric reaction by antiparasitic drugs.

The K_i values obtained for the positive controls were in the same range as those reported in the literature: α-naphthoflavone for CYP1A2 (K_i = 0.003 μM), sulfaphenazole for CYP2C9 (K_i = 0.67 μM), ticlopidine for CYP2C19 (K_i = 0.77 μM), quinidine for CYP2D6 (K_i = 0.009 μM) and ketoconazole for CYP3A4 (K_i = 0.008 μM) (http://www.gentest.com). The inhibitory effects of the antiparasitic drugs and some of their metabolites are shown in Table 3.1. The results show that CYP1A2 and CYP2D6 were affected most by the drugs. Potent inhibitors of CYP1A2 included artemisinin, thiabendazole, niclosamide and dihydroartemisinin. Proguanil, quinine, chloroquine, pyrimethamine, amodiaquine, desethylamodiaquine and cycloguanil were potent inhibitors of CYP2D6.

3.1.1 Assessment of the reliability of fluorescence-based assays

The reliability of the fluorescence-based assays was evaluated by comparison of the results to those obtained from the traditional HPLC-based assays. Given the large number of compounds used, the effect of a single concentration of drug (20 μM) was used to assess the inhibitory effects of the drugs using HPLC-based assays. A concentration of 20 μM enables a simple comparison based on the fact that inhibition of at least 50% would be expected for a compound with a *Ki* of 10 μM or less. A compound with a Ki value above 10 μM would, therefore, be expected to cause less than 50% inhibition at a concentration of 20 μM. The percentage inhibition caused by 20 μM of drug was determined by comparison of the peak areas of metabolites formed in the presence and absence of drug. The 1′-hydroxylation of bufuralol was used to assess the effects of the compounds on CYP2D6 and Figure 3.2 shows a typical chromatogram.

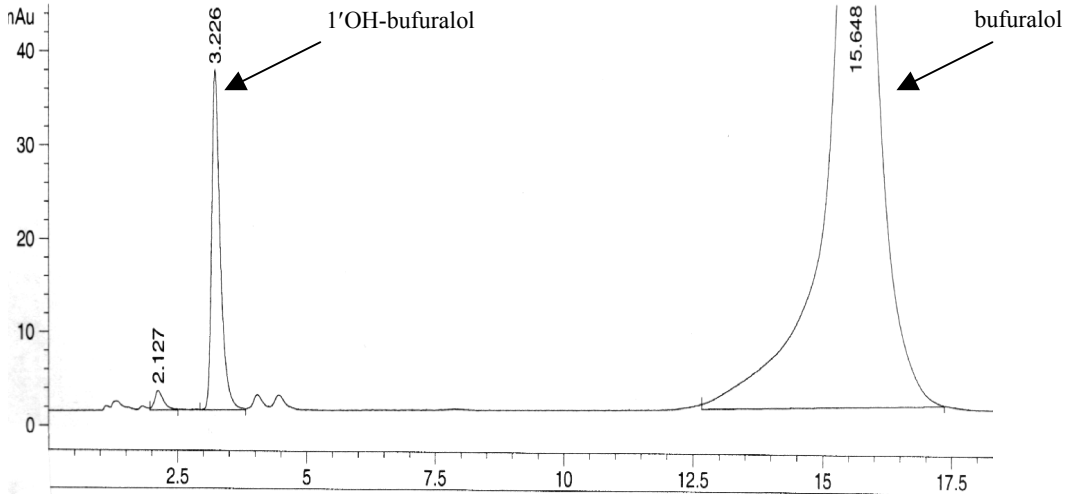


Figure 3.2 A typical chromatogram of bufuralol and its metabolite 1′-hydroxybufuralol detected by fluorescence at excitation and emission wavelengths of 252 and 302 nm respectively.

CYP3A4 catalyses the 6β-hydroxylation of testosterone and this was used to assess the effect of the antiparasitic drugs on its activity. A typical chromatogram is shown in Figure 3.3.

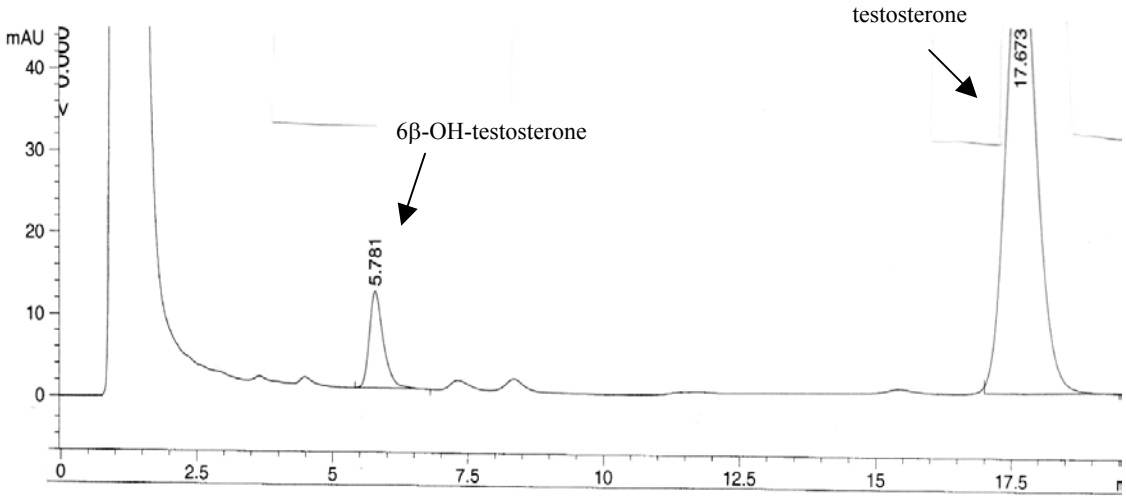


Figure 3.3 A typical chromatogram of testosterone and its metabolite 6β-hydroxy-testosterone detected using UV at a wavelength of 254 nm.

The O-deethylation of phenacetin to form paracetamol is catalysed by CYP1A2 and was used to assess the effects of antiparasitic drugs on the enzyme. Figure 3.4 shows a typical chromatogram of phenacetin and paracetamol after separation on a C_{18} 4.6 X 30 cm column.

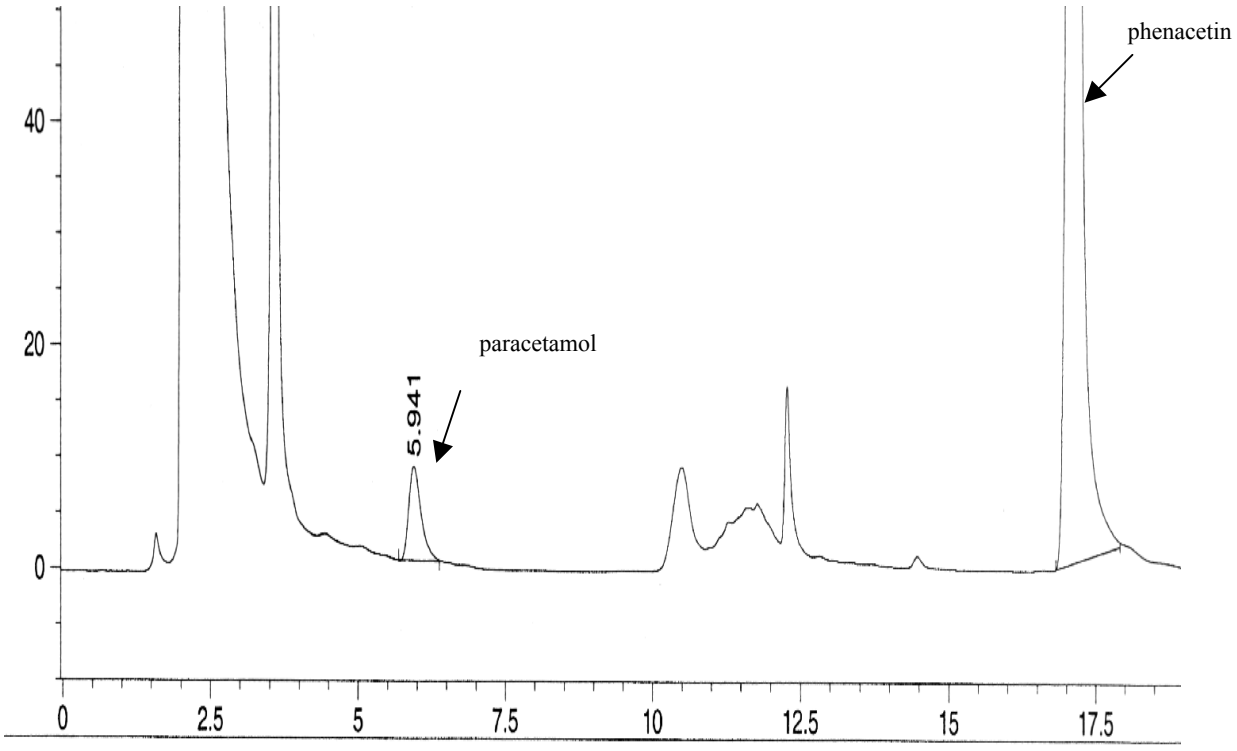


Figure 3.4 A typical chromatogram of phenacetin and its metabolite paracetamol detected using UV at a wavelength of 245 nm.

The 4-hydroxylation of *S*-mephenytoin was used to assess the effects of the antiparasitic drugs on CYP2C19 activity. Figures 3.5 shows a typical chromatogram for the probe drug and its metabolite.

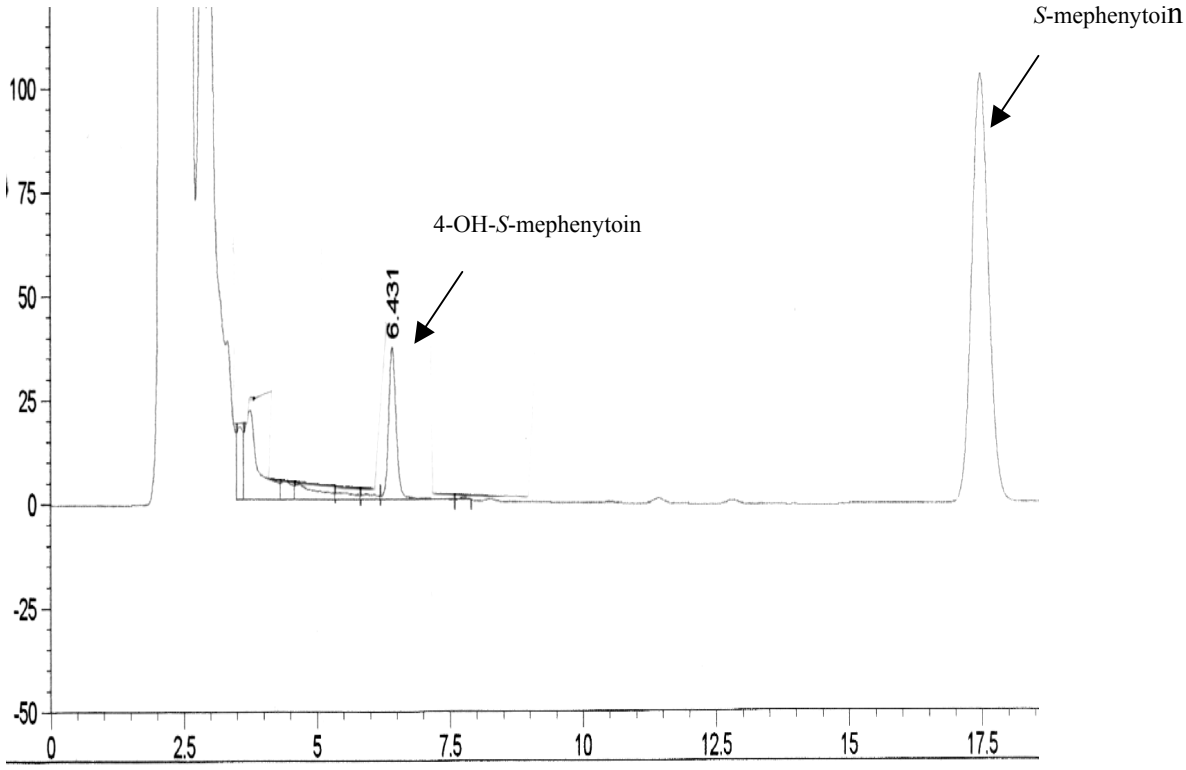


Figure 3.5 A typical chromatogram of *S*-mephenytoin and its metabolite 4-hydroxy-S-mephenytoin detected using UV and a wavelength of 214 nm.

The results from such experiments for the other CYP isoforms were compared with the fluorescence-based assays as shown in Table 3.1. Results from the fluorescence-based assays were in general agreement with those from the HPLC-based ones in that compounds that were not inhibitors with the fluorescence assays showed reduced or no effect on enzyme activity using the HPLC-based assays. The compounds with low K_i values in the fluorescence-based assays caused, as expected, a great reduction in activity at 20 μM using HPLC-based assays. Artemisinin, for example, inhibited CYP1A2 activity with a K_i of 2.97 μM as assessed by the fluorescence-based assays. Given such a low K_i value, 20 μM of the drug would be expected to inhibit a high percentage of enzyme activity and this is consistent with the value obtained using the HPLC-based assays of 69%. The few discordant cases involved apparent activation observed using the fluorescence-based assays.

Table 3.1. Comparison of FLUO assays and HPLC assays in determination of the inhibitory effects of antiparasitic drugs on the five major drug metabolising CYPs

Compound	**Cytochrome P450 inhibition: Ki[a] (% inhibition)[b]**				
	1A2	**2C9**	**2C19**	**3A4**	**2D6**
Artemisinin	3.0 (69)	*ni*[c] (0)	16 (17)	*ni*[c] (11)	*ni*[c] (0)
Proguanil	*ni*[c] (0)	*ni*[c] (14)	33 (0)	100 (0)	7.7 (40)
Quinine	*ni*[c] (0)	27 (0)	44 (9)	*ni*[c] (0)	4.8 (70)
Chloroquine	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (2)	*ni*[c] (0)	12 (37)
Pyrimethamine	*ni*[c] (0)	48 (0)	*ni*[c] (12)	*ni*[c] (0)	9.0 (26)
Primaquine	*nd*[d] (100)	*nd*[d] (0)	*nd*[d] (6)	*nd*[d] (*nd*[d])	*ni*[c] (30)
Amodiaquine	47 (16)	24 (25)	39 (14)	*ni*[c] (17)	1.8 (80)
Dapsone	*ni*[c] (0)	37 (0)	*ni*[c] (0)	*ni*[c] (9)	*ni*[c] (4)
Atovaquone	Act[e]~160%(0)	*ni*[c] (2)	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (0)
Artesunate	*ni*[c] (2)	*ni*[c] (2)	*ni*[c] (0)	*ni*[c] (4)	*ni*[c] (0)
Metrifonate	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (4)	*ni*[c] (0)	*ni*[c] (0)
Praziquantel	*ni*[c] (10)	*ni*[c] (0)	*ni*[c] (25)	*ni*[c] (9)	*ni*[c] (1)
Suramin	15 (8)	*ni*[c] (0)	69 (2)	*ni*[c] (8)	45 (0)
Melarsoprol	*ni*[c] (6)	29 (4)	41 (0)	36 (35)	46 (8)
Pentamidine	*ni*[c] (0)	52 (0)	53 (14)	30 (20)	55 (0)
Ivermectin	*ni*[c] (8)	*ni*[c] (0)	*ni*[c] (11)	27.2 (0)	*ni*[c] (10)
Diethylcarbamazine	*ni*[c] (0)	*ni*[c] (0)	Act[e]~130% (3)	*ni*[c] (0)	*ni*[c] (0)

Table 3.1 (continued from previous page)

Compound	1A2	2C9	2C19	3A4	2D6
Niclosamide	1.2 (71)	Act[e]~400% (36)	*ni*[c] (12)	5.3 (32)	*ni*[c] (9)
Tinidazole	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (0)
Pyrantel	*ni*[c] (12)	*ni*[c] (0)	*ni*[c] (3)	*ni*[c] (0)	39 (14)
Albendazole	*ni*[c] (8)	*ni*[c] (0)	*ni*[c] (5)	*ni*[c] (5)	*ni*[c] (1)
Thiabendazole	0.61 (87)	69 (0)	*ni*[c] (0)	*ni*[c] *(nd*[d]*)*	*ni*[c] (0
Desethylamodiaquine	56 (11)	54 (0)	34 (6)	*ni*[c] *(nd*[d]*)*	6.5 (54)
Cycloguanil	*ni*[c] (0)	*ni*[c] (5)	*ni*[c] (0)	*ni*[c] (0)	11 (71)
4-Chlorophenylbiguanide	*ni*[c] (10)	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (0)	23 (19)
Desethylchloroquine	*ni*[c] (8)	*ni*[c](0)	*ni*[c] (0)	18 (11)	15 (21)
Dihydroartemisinin	7.8 (42)	*ni*[c] (6)	65 (0)	*ni*[c] (6)	*ni*[c] (0)
(+) Chloroquine	*ni*[c] (0)	*ni*[c] (0)	*ni*[c] (2)	*ni*[c] (0)	6.2 (38)
(-) Chloroquine	*ni*[c] (0)	*ni*[c] (9)	*ni*[c] (3)	*ni*[c] (11)	20 (19)

[a] K_i(μM) calculated from IC_{50} determined at Km using fluorescence assays
[b] percentage inhibition by 20 μM on HPLC CYP marker reactions given in parentheses
[c] *ni*: no inhibition, an IC_{50} could not be determined with the highest concentration.
[d] *nd*: not determined
[e] activation

3.1.1.1 Apparent activation of CYP activity

Some compounds showed an apparent activation of the activity of some of the CYPs (Table 3.1). An increase in activity of more than 20% compared to the control activity was considered to be apparent activation. Activation was observed with atovaquone and CYP1A2, niclosamide and CYP2C9, and diethylcarbamazine and CYP2C19. This apparent activation of enzyme activities observed from fluorescence-based assays was, however, not seen with the HPLC-based assays using recombinant enzymes (Table 3.1). Instead, niclosamide inhibited CYP2C9 catalysed diclofenac 4-hydroxylation (K_i = 6.00 μM). The apparent activation of CYP2C9 by niclosamide was further studied (Figure. 3.6). In the presence of niclosamide, the CYP2C9 activity was increased by about 400%. Interestingly, co-incubation of niclosamide with sulfaphenazole, a potent inhibitor of CYP2C9 resulted in inhibition of enzyme activity by 30 % (i.e. abolishing the activation by niclosamide and reducing the inhibitory effect of sulfaphenazole). Further experiments showed that the apparent activation was not due to fluorescence by a complex of niclosamide and the fluorescent metabolite or a complex of an unknown metabolite of niclosamide and the substrate or a complex of an unknown metabolite of niclosamide and the fluorescent metabolite (HFC).

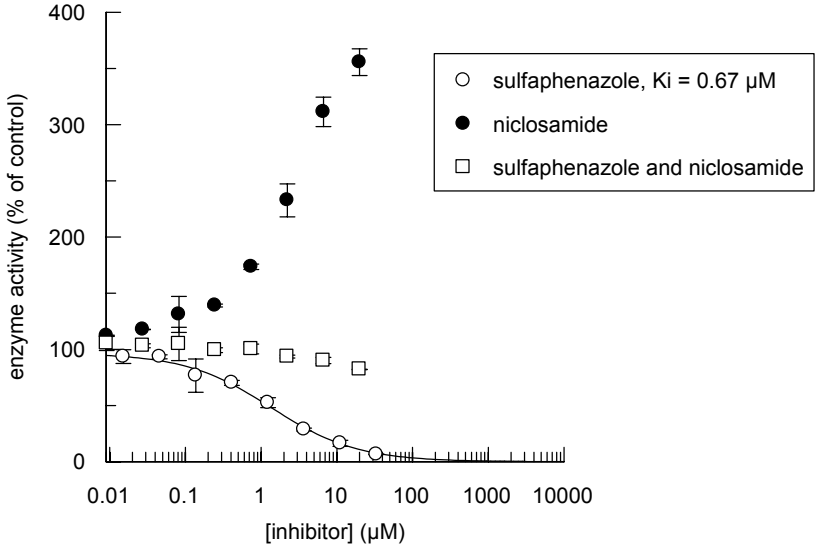
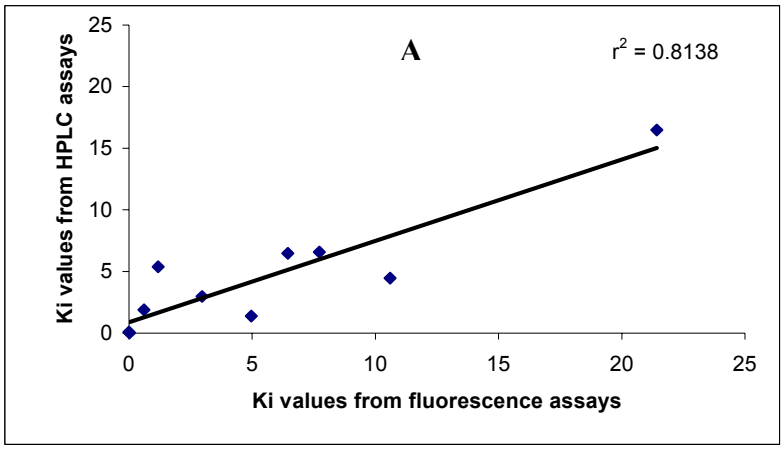


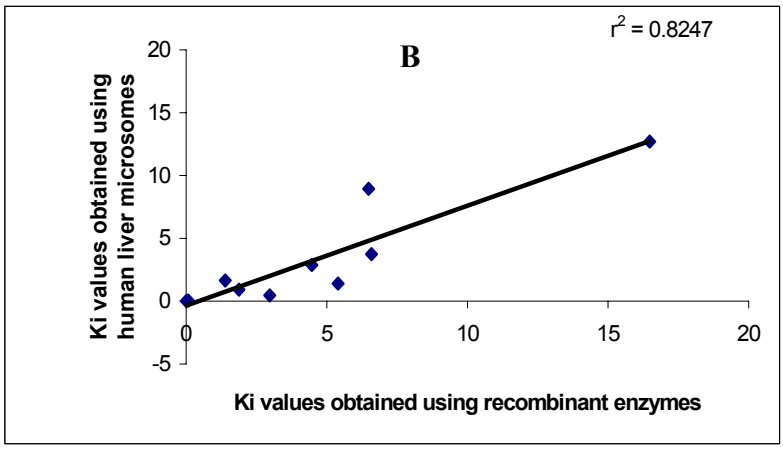
Figure 3.6. Apparent activation of recombinant CYP2C9 activity by niclosamide

3.1.1.2 Comparison of inhibition data in the different systems

A comparison of the actual K_i values obtained using both methods would be a better way to evaluate the fluorescence-based assays. For nine compounds that were potent inhibitors of CYP1A2 and CYP2D6, K_i values were, therefore, determined using the HPLC-based assays. Phenacetin O-deethylation and bufuralol 1′-hydroxylation were used to assess the inhibitory effects of antiparasitic drugs on CYP1A2 and CYP2D6 respectively. The incubations were carried out in the same way as for the fluorescence-based assays. Increasing concentrations of drug were incubated with enzyme at a substrate concentration equal to K_m. The enzyme activity expressed as a percentage of enzyme activity in the absence of drug/inhibitor was also plotted against concentration of drug. The plot was also used to determine the IC_{50} and K_i values as described for the fluorescence-based assays. In addition, results obtained using human recombinant enzymes were compared with those from human liver microsomes.

For the potent inhibitors of CYP1A2 and 2D6, the K_i values obtained using the fluorescence assays and those obtained using the HPLC assays showed a fairly good agreement; $r^2 = 0.81$ (Figure. 3.7a) using recombinant CYPs. There was also a good agreement between K_i values obtained using recombinant CYPs and using human liver microsomes on the same HPLC marker reactions, $r^2 = 0.82$ (Figure 3.7b). Importantly, there was a good correlation, $r^2 = 0.72$, between K_i values obtained using the fluorescence assays with recombinant CYPs and those obtained using HPLC-based assays with human liver microsomes (Figure. 3.7c). Discordances involving the drugs quinine and dihydroartemisinin were, however, observed. Quinine and dihydroartemisinin had K_i values of 2.4 μM and 16 μM using recombinant CYPs 2D6 and 1A2 but had relatively different values, 16 μM, and 2.7 μM using human liver microsomes for the same CYP marker reactions respectively.





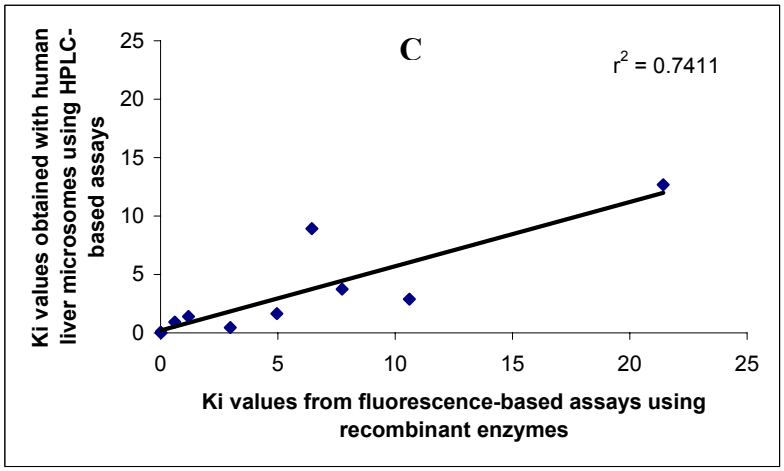


Figure 3.7. Correlations of K_i values obtained for 9 inhibitors of CYP1A2 and CYP2D6 using different systems. A, data using the fluorescence assays with recombinant CYPs against data using HPLC assays with recombinant enzymes. B, data using HPLC assays with recombinant CYPs against data using HPLC assays with human liver microsomes. C, data using fluorescence assays with recombinant CYPs against data using HPLC assays with human liver microsomes.

3.1.2 Prediction of *in vivo* inhibitory effects from *in vitro* data

The inhibition parameters IC_{50} and K_i are useful if they can predict *in vivo* effects. Drugs found to be potent inhibitors of CYP1A2 and CYP2D6 are commonly used for treatment of various parasitic infections. It was, therefore, important to study the *in vivo* relevance of the observed inhibition. In calculating the percentage inhibition *in vivo* from the *in vitro* data, use was made of the K_i value and plasma concentration achieved *in vivo* after administration of a therapeutic dose. The type of inhibition was important as it determined the expression used for calculating the percentage inhibition *in vivo*. The actual mechanism of inhibition was determined by analysing the effect of four different concentrations of inhibitor, on K_m and V_{max} using GraFit 4.0.12, as shown in Figure 3.8.

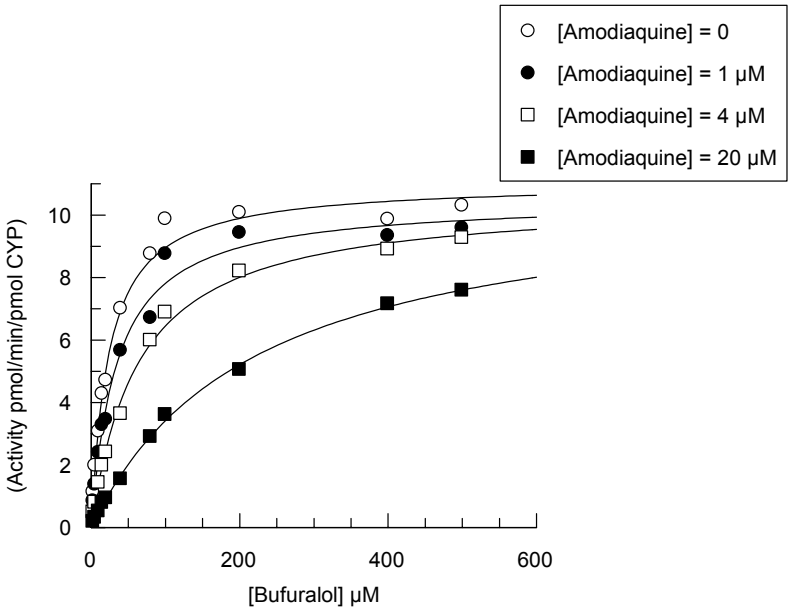


Figure 3.8 A typical curve used for the determination of the mechanism of inhibition, showing competitive inhibition of CYP2D6-catalysed bufuralol 1´hydroxylation by amodiaquine in human liver microsomes.

The mechanism of inhibition was either competitive or mixed type for all the compounds studied. The percentage inhibition was, therefore, calculated using the same expression:

$$\% \text{ Inhibition} = \{[I]/([I] + K_i)\} \times 100$$

The predicted percentage inhibition of CYP1A2 and CYP2D6 by antiparasitic drugs is shown in Table 3.2. Thiabendazole, primaquine and artemisinin are expected to significantly inhibit CYP1A2 *in vivo* by 99%, 80% and 75% respectively. Amodiaquine and its metabolite desethylamodiaquine are expected to inhibit CYP2D6 activity by about 3% and 9% respectively.

3.2 Inductive effects of antiparasitic drugs on CYP1A1 and CYP1A2

The CYP1A subfamily has been shown to be inducible and the HepG2 cell line has been used successfully to assess induction of the subfamily. In this project, the HepG2 cell line was also used to investigate the inductive effects of antiparasitic drugs on CYP1A. Apparent lack of induction may be due to antiparasitic drug-induced loss of viability. Initial work, therefore, sought to establish the effect of the antiparasitic drugs on the viability of the cells. This was done using the Trypan Blue exclusion test. Viable cells do not take up the dye while non-viable cells do and are stained blue. The cells were counted using a microscope and hemacytometer as described in detail in section 2.2.2.2.

3.2.1 Viability of cells

Thiabendazole affected the viability of HepG2 cells under the concentrations used as shown by the Trypan Blue exclusion test with a cell viability of only 38%. The other compounds did

not affect the viability of cells appreciably at the concentrations used (the cell viability was at least 78%).

3.2.2 Identification of inducers of EROD activity

Initial screening of the antiparasitic drugs for induction of CYP1A was done using the EROD activity assay in HepG2 cells exposed to the antiparasitic drugs at three concentrations of 0.3 μM, 30 μM and 300 μM. The intensity of the fluorescence was proportional to the amount of product (resorufin) formed, which in turn was dependent on the amount of CYP1A enzyme present. This identified the compounds albendazole, primaquine, quinine and niclosamide as inducers. Figure 3.9 shows the results of the initial screen after subtracting the contribution of the solvent DMSO. The inductive effects of six concentrations, taking into account those found in plasma after administration of therapeutic doses of the drugs quinine, albendazole and primaquine were investigated. Niclosamide was left out because of the negligible absorption of the drug (Abdi *et al*., 1995). The dose-dependent increase in EROD activity caused by quinine is shown in Figure 3.10A. Albendazole (Figure 3.10B) caused a dose-dependent increase in EROD activity up to 1.0 μM, with the activity levelling off at 5.0 μM and dropping at 10 μM. An increase in EROD activity was also observed with increasing concentrations of primaquine (Figure 3.10C).

Table 3.2: Inhibition of human liver microsomal CYP1A2 phenacetin O-deethylation and CYP2D6 bufuralol 1´-hydroxylation by antiparasitic drugs

CYP Compound	K_i (μM)	Type of inhibition	Plasma C_{max} (μM)	inhibitory potency $[I]/K_i$	Predicted % inhibition $\{[I] / ([I] + K_i)\}x100$
CYP1A2					
Artemisinin	0.43	competitive	1.38	3.20	76
Niclosamide	2.70	mixed	negligible	-[a]	neglible
Thiabendazole	1.54	mixed	89	57.8	98
Primaquine	0.22	competitive	0.44	2.00	67
Dihydroartemisinin	3.67	competitive	2.50	0.68	41
CYP2D6					
Quinine	15.5	competitive[b]	15.4	0.99	50
Chloroquine	12.7	competitive[c]	0.39	0.03	3
Amodiaquine	2.1	competitive	0.07	0.03	3
Desethylamodiaquine	4.1	mixed	0.44	0.11	10
Proguanil	6.8	mixed	0.76	0.11	10
Cycloguanil	6.0	competitive	0.21	0.04	3

[a] – cannot calculate since [I] is negligible. Mechanisms for [b] and [c] are from (Kobayashi *et al*., 1998; Masimirembwa *et al*., 1995)

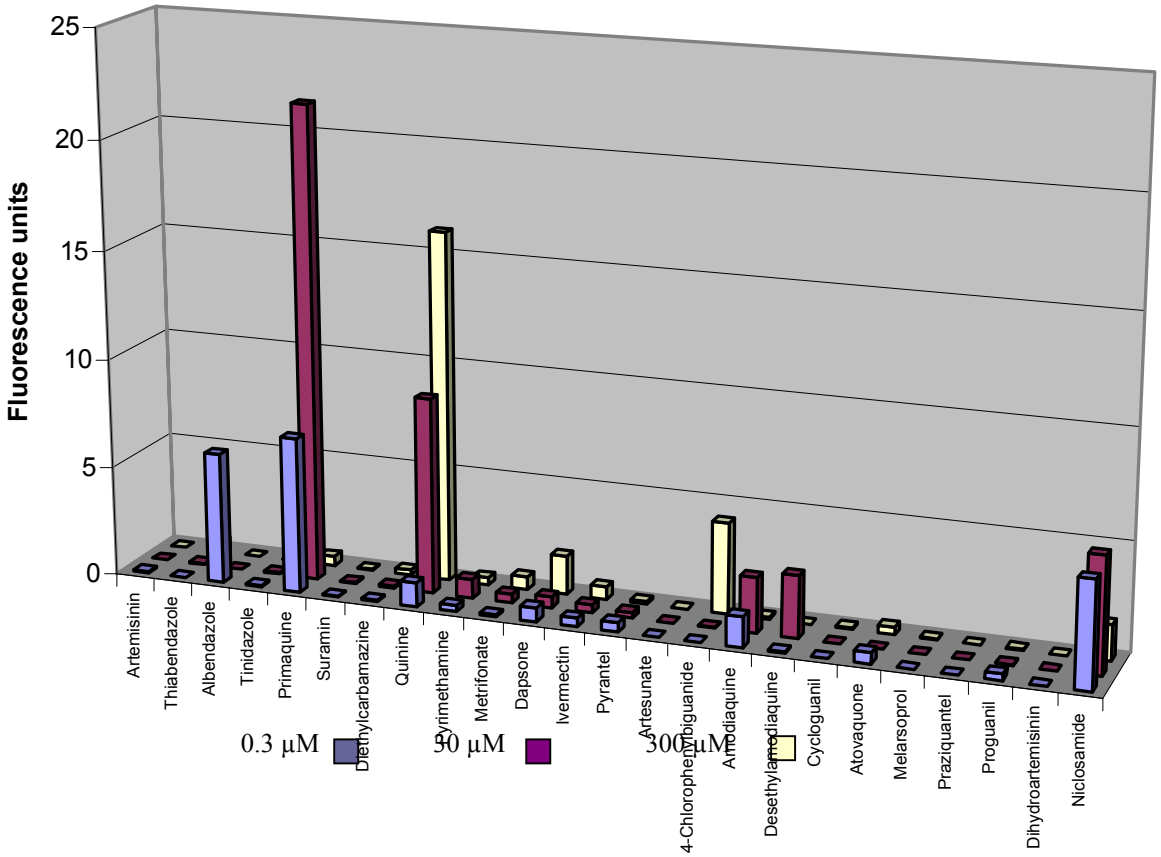


Figure 3.9. Results of the initial screen for EROD induction by antiparasitic drugs in HepG2 cells. Values represent the mean of quadruplicate determinations after subtracting the fluorescence from the control containing DMSO alone.

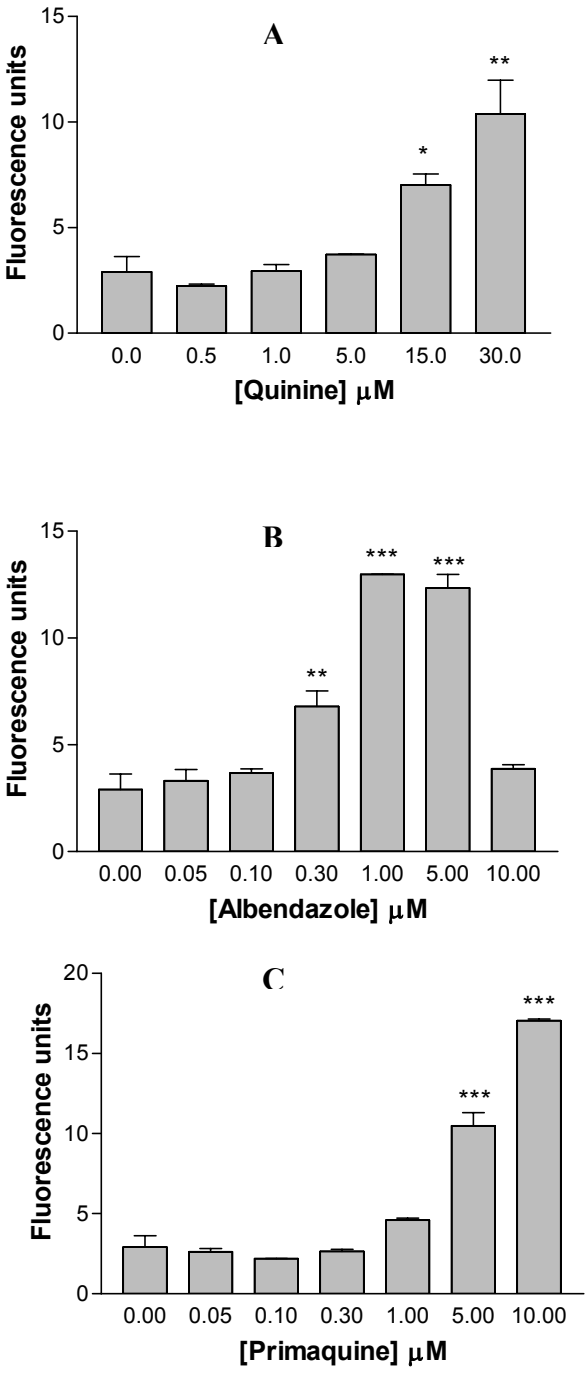


Figure 3.10. The inductive effects of A) quinine, B) albendazole and C) primaquine on EROD activity in HepG2 cells. * significantly different ($P<0.05$), ** significantly different ($P<0.01$) and *** significantly different ($P<0.001$) from DMSO controls.

3.2.3 Effect of antiparasitic drugs on CYP1A1/2 mRNA expression

The O-deethylation of ethoxyresorufin is catalysed by both CYP1A1 and CYP1A2 as a result an increase in EROD activity could be due to induction of either or both isoforms. Induction of CYP1A has been shown to occur at the transcriptional level and measuring the mRNA levels of the individual CYPs would enable the identification of the specific CYP1A isoform induced. After exposing the HepG2 cells to antiparasitic drugs, mRNA was prepared and from it cDNA was prepared by RT-PCR. The cDNA was quantitated using TaqMan technology as described in detail in section 2.2.2.6. This was done using a fluorogenic probe carrying a reporter and quencher, and specific for either CYP1A1 or CYP1A2 cDNA. If the probe became part of the replication complex, the reporter was cleaved and would fluoresce. The starting mRNA copy number was established by determining the number of PCR cycles required to reach a pre-set threshold level of fluorescence. A large initial mRNA copy number requires less PCR cycles to reach the pre-set threshold level compared to a low mRNA copy number. Figure 3.11 shows a typical amplification plot obtained from the TaqMan. The number of cycles from such a plot was used to determine the amount of mRNA in cells exposed to drug relative to the control containing DMSO only. The intra- and inter-day variabilities in quantification of 1A2 and 1A1 mRNA levels were less than 5% and 22% respectively.

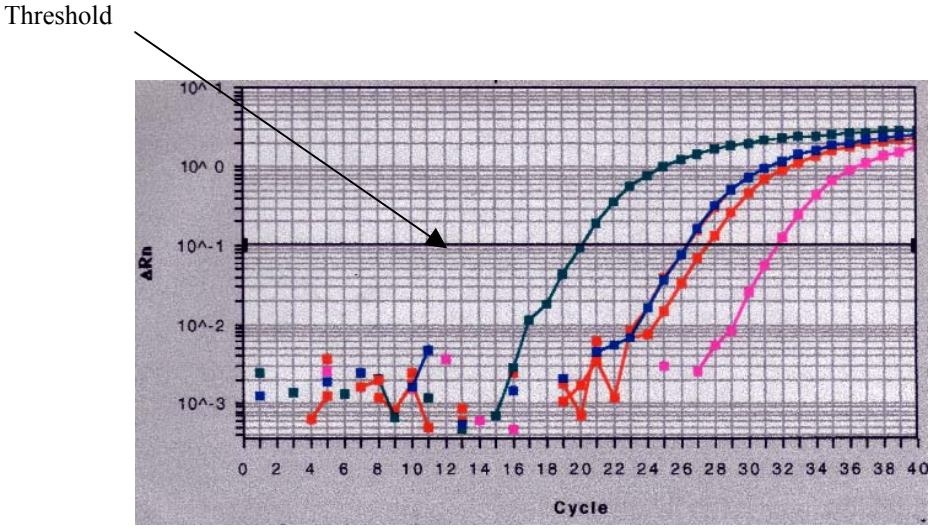


Figure 3.11 Showing a typical amplification plot of the effect of albendazole on CYP1A2 mRNA levels. The curves from left to right (at the set threshold) TCDD the most potent inducer, required less PCR cycles to reach the threshold followed by albendazole (5.0 µM), (1.0 µM), (10 µM), with DMSO requiring the most number of cycles to reach the threshold.

The effect of varying concentrations of the three drugs on CYP1A1/2 mRNA levels, were assessed as described above. Quinine caused an increase in levels of CYP1A1 mRNA with increase in concentration of the drug as shown in Figure 3.12A. Its effect on CYP1A2 mRNA expression was, however, less pronounced (Figure 3.13A). For albendazole, an increase in CYP1A1 and CYP1A2 mRNA was observed from a concentration of 1.0 µM (Figures 3.12B, 3.13B). Primaquine caused an increase in CYP1A1 mRNA expression from a concentration of 5.0 µM (Figure 3.12C). A significant increase in CYP1A2 mRNA level was observed at a concentration of 10 µM (Figure 3.13C).

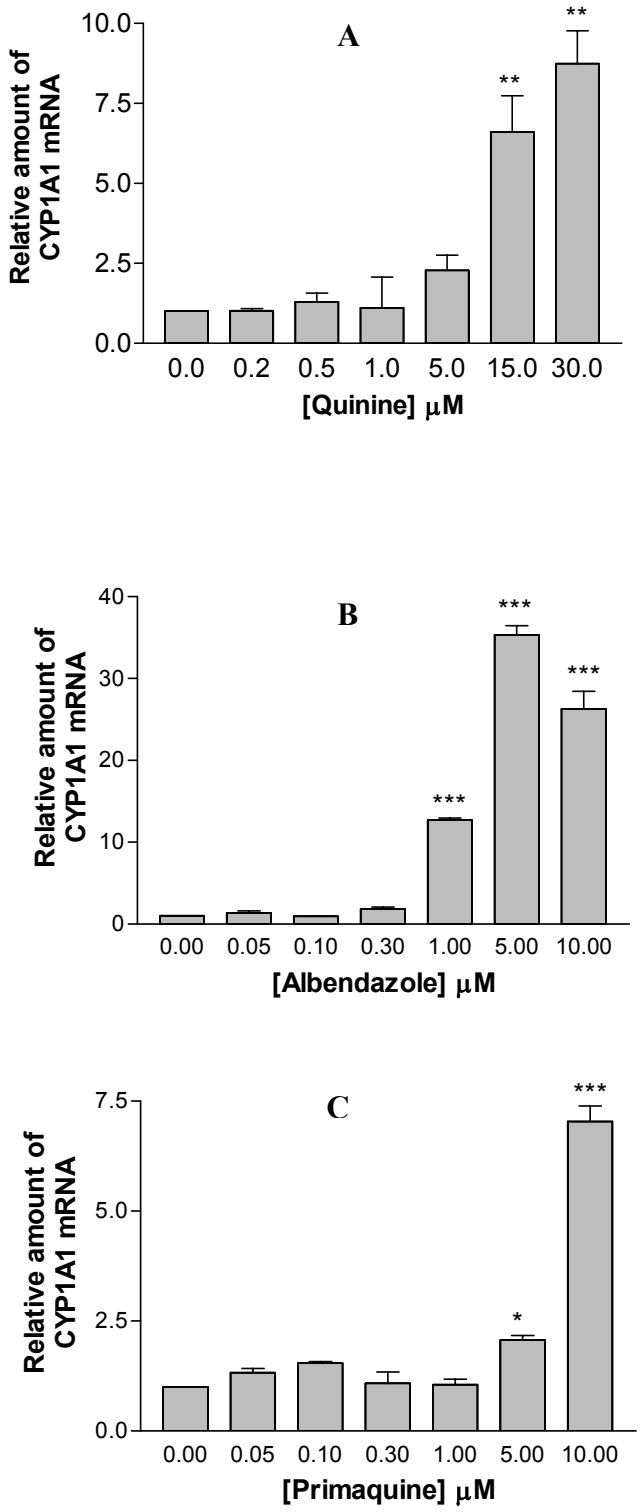


Figure 3.12. The effect of A) quinine B) albendazole and C) primaquine on CYP1A1 mRNA expression n HepG2 cells. * significantly different ($P<0.05$), ** significantly different ($P<0.01$) and *** significantly different ($P<0.001$) from DMSO controls.

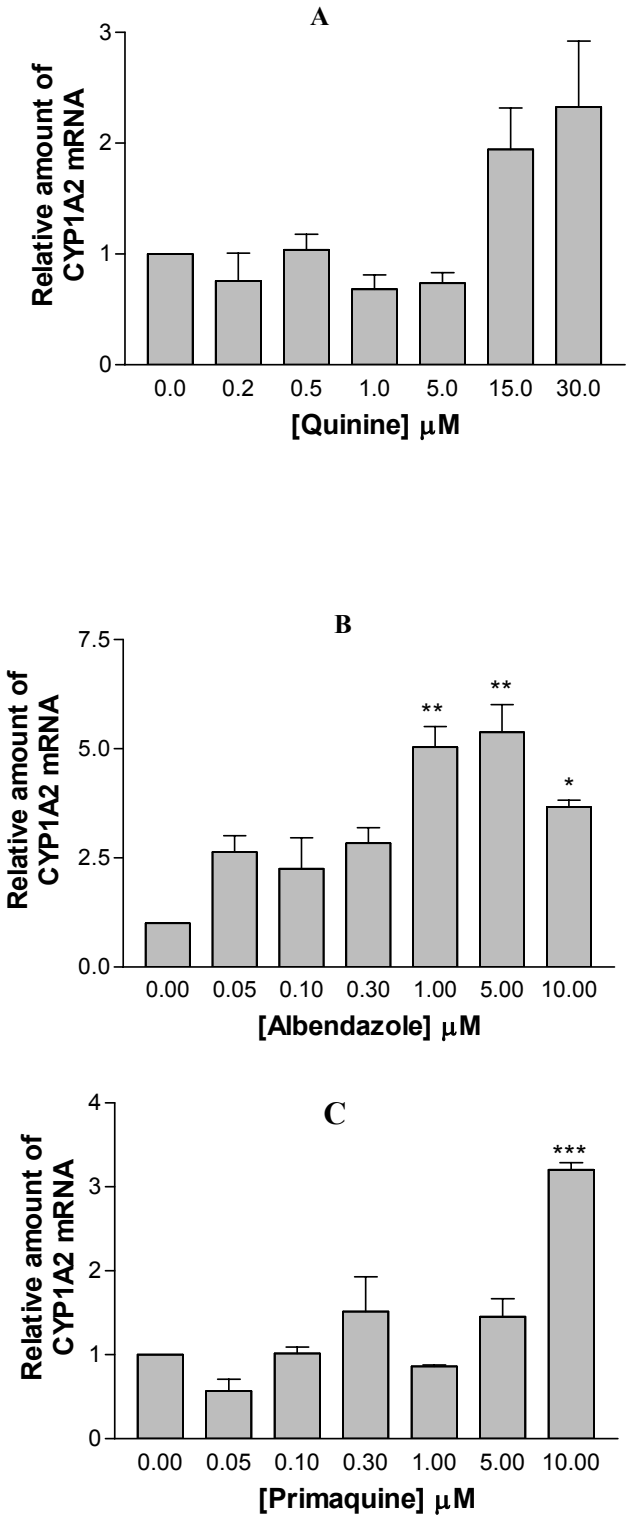


Figure 3.13. Effect of A) quinine, B) albendazole and C) primaquine on CYP1A2 mRNA expression in HepG2 cells. * Significantly different ($P<0.05$), ** significantly different ($P<0.01$), significantly different ($P<0.001$).

3.3 Characterisation of CYP2D6.17

Initial work on the characterisation of CYP2D6.17 focused on investigating the kinetics of metabolism of commonly used phenotyping probe drugs debrisoquine, metoprolol and dextromethorphan and the *in vitro* marker substrate bufuralol. In order to determine the effect on kinetic properties of each amino acid exchange in CYP2D6.17, three CYP2D6 variants, CYP2D6.1 (without any amino acid changes), CYP2D6.2 (with two amino acid exchanges R296C and S486T) and CYP2D6T107I (with one amino acid exchange T107I) were also studied. A CYP2D6.1 cDNA was used to synthesize the other CYP2D6 variants as described in detail in section 2.2.3.1 and were expressed in a *Saccharomyces cerevisiae* strain expressing the human reductase.

3.3.1 CYP, protein and NADPH-CYP reductase concentrations of microsomal preparations

The CYP content of each preparation of microsomes was determined using the reduced carbon monoxide difference spectrum as shown in Figure 3.14. In addition, the protein concentration and NADPH-CYP reductase activities of the microsomal preparations were measured as described in detail in sections 2.2.1.3 and 2.2.1.4. The protein and NADPH-CYP reductase concentrations in each microsomal preparation are shown in Table 3.3. Microsomes containing CYP2D6.1 and CYP2D6.17 proteins had similar CYP concentrations that were higher than those for CYP2D6.2 and CYP2D6.T107I, which also had similar CYP concentrations. The activities of NADPH-CYP reductase in the microsomes were comparable although CYP2D6.T107I-containing microsomes had a tendency for lower activities.

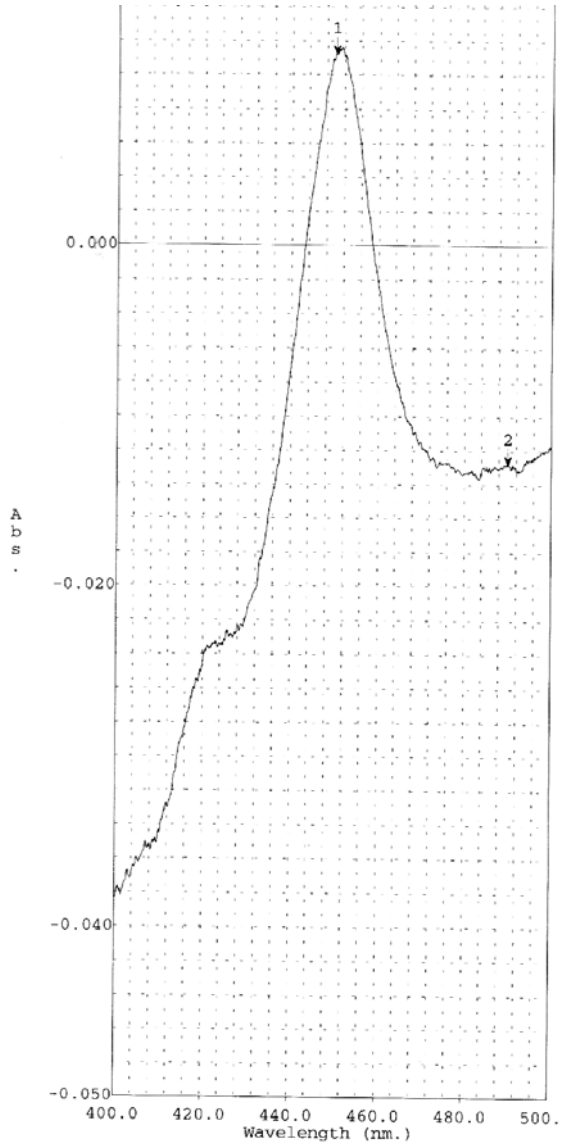


Figure 3.14. A typical difference spectrum used to determine the concentration of CYP in yeast microsomes. The absorbance at 450 nm (1 in the Figure), 490 nm (2 in the Figure) and the molar extinction of 91 $mM^{-1}cm^{-1}$ were used to calculate the CYP concentration.

Table 3.3. CYP, protein concentration and NADPH-CYP reductase activity in yeast microsomes

CYP2D6	CYP concentration (pmol/µl)	Protein concentration (mg/ml)	CYP concentration (pmol/mg)	NADPH-CYP reductase activity (µmol reduced cyt. *c*/min/mg)
*1	2.7	29.6	93	0.113
*17	3.0	33.2	95	0.118
*2	1.3	36.6	84	0.108
T107I	1.3	24.9	86	0.094

The results are an average of two separate expressions for each CYP2D6 variant

3.3.2 Enzyme kinetic characterisation

The enzyme kinetic characterisation involved determination of the kinetic parameters K_m and V_{max} for each probe drug. The reactions were first optimised for linearity with respect to CYP concentration and incubation time. The enzyme was incubated with increasing concentrations of substrate. The enzyme activity at each substrate concentration was calculated from the amount of product formed (metabolite) determined by HPLC as described in detail in section 2.2.3.3. Figure 3.15 shows a typical chromatogram for debrisoquine 4-hydroxylation. The O-demethylation of dextromethorphan and hydroxylation/demethylation of metoprolol were analysed by HPLC with fluorescence detection and a typical chromatograms are shown in Figures 3.16 and 3.17 respectively.

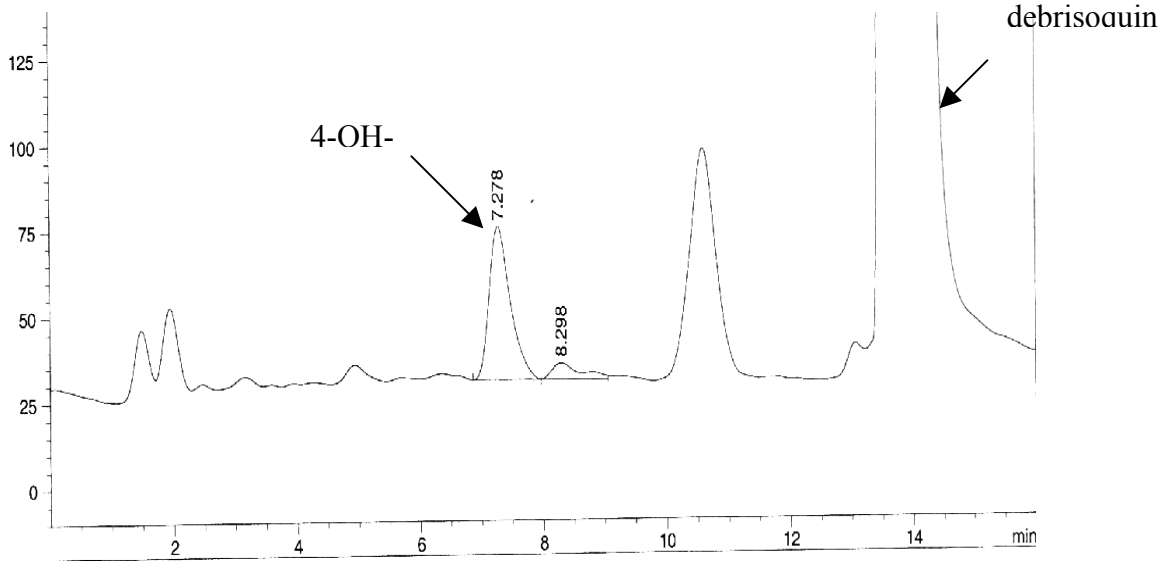


Figure 3.15. Showing a typical chromatogram of debrisoquine and its metabolite 4-hydroxy-debrisoquine.

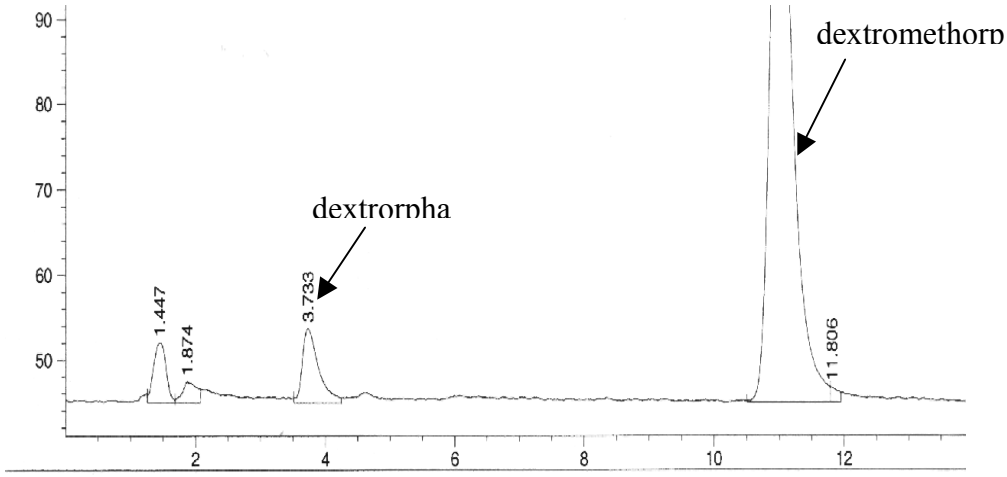


Figure 3.16. A typical chromatogram of dextromethorphan and its metabolite dextrorphan detected by fluorescence at excitation and emission wavelengths of 270 and 312 nm respectively.

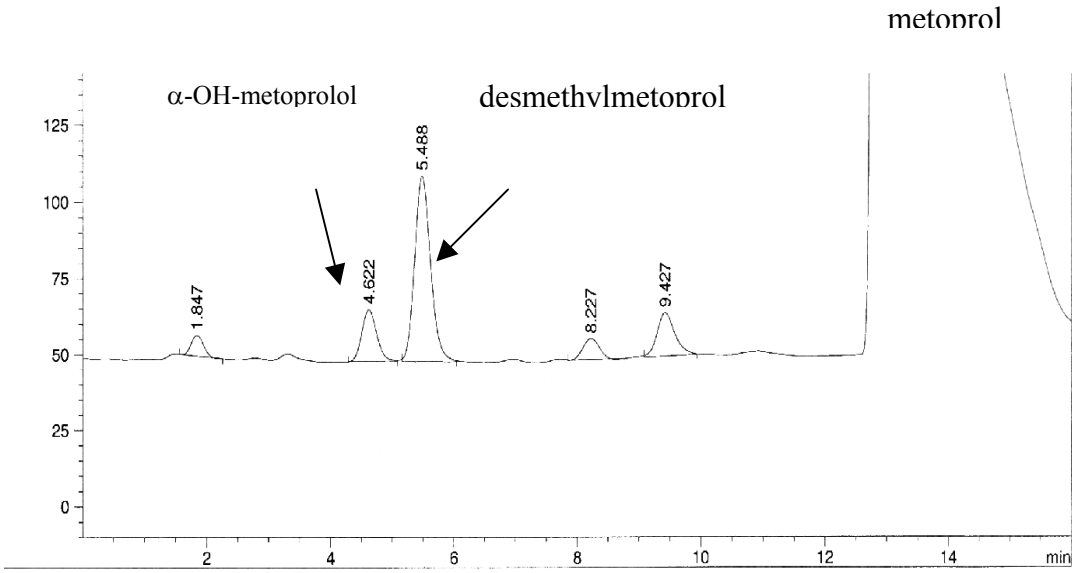


Figure 3.17 Showing a typical chromatogram of metoprolol and its metabolites α-hydroxy-metoprolol and desmethylmetoprolol separated on a C_{18} 4.6 X 15 cm column and detected using fluorescence at excitation and emission wavelengths of 229 and 309 nm respectively.

The enzyme activities at eight different substrate concentrations were used to generate K_m and V_{max} values. These values were determined by non-linear least-squares regression analysis using GraFit version 3.0 (Erithacus Software Limited). A typical plot of enzyme activity against substrate concentration is shown in Figure 3.18.

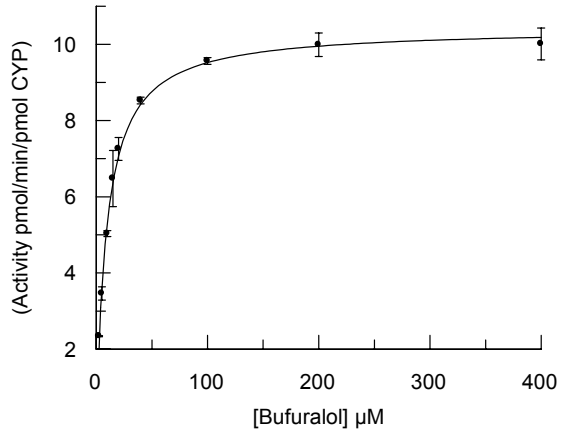


Figure 3.18. A typical Michaelis-Menten curve used to determine the K_m and V_{max} values of CYP2D6 variants and shows the kinetic properties of CYP2D6.1 towards bufuralol with K_m and V_{max} values of 9.65 μM and 8.64 pmol/min/pmol CYP respectively.

The enzyme kinetic parameters (K_m, V_{max}, and V_{max}/K_m) for the metabolism of bufuralol, dextromethorphan, debrisoquine and metoprolol by the CYP2D6 variants compared to CYP2D6.1 are shown in Table 3.4. CYP2D6.17 exhibited generally higher K_m values compared to CYP2D6.1. The V_{max} values were generally not different except for metoprolol α-hydroxylation with the V_{max} value for CYP2D6.17 being about half that of CYP2D6.1. The V_{max}/K_m values for CYP2D6.17 were lower than those of CYP2D6.1 with the largest difference apparent with dextromethorphan. The V_{max}/K_m value was affected to a much lesser extent by CYP2D6.17 when debrisoquine was used as substrate. CYP2D6.1 and CYP2D6.2 displayed similar kinetics with all probe drugs except for dextromethorphan O-demethylation.

The V_{max}/K_m value of CYP2D6.2 was about half that of CYP2D6.1. The enzyme kinetic profile of the CYP2D6.T107I variant differed from that displayed by CYP2D6.1 for all probe drugs. The effects of the T107I amino acid exchange are, however, of academic interest only, as the amino acid change has not been observed by itself in any subject analysed so far.

In order to investigate the possible clinical implications of the CYP2D6*17 genotype, the ability of CYP2D6.17 *vis-à-vis* CYP2D6.1, to clear clinically important CYP2D6 substrate drugs, was studied. This was done using the substrate disappearance approach in which the drug was incubated with enzyme and the remaining drug measured using LCMS. The LCMS analysis was done at AstraZeneca, Mölndal, Sweden by Dr Mareike Lutz as explained in detail in section 2.2.3.6. Initially each compound was injected to determine the ions to monitor as shown by the mass spectrum for thioridazine (*m/z* 371) in Figure 3.19. This was used to monitor the concentration of thioridazine in the experimental samples as shown in Figure 3.20. This was done for the other compounds and Table 3.5 shows the ions monitored and their retention times.

Table 3.4. Kinetic properties of the four variants towards CYP2D6 probe drugs

CYP2D6		Bufuralol 1′OH	Dextromethorphan ODM	Debrisoquine 4OH	Metoprolol ODM	Metoprolol αOH
1	K_m [a]	9.65 ± 0.3	1.30 ± 0.2	55.2 ± 17	51 ± 3.2	41 ± 5.3
	V_{max} [b]	8.64 ± 2.1	2.21 ± 0.5	0.55 ± 0.1	4.81 ± 1.1	1.47 ± 0.40
	V_{max}/K_m [c]	0.89 ± 0.2	1.77 ± 0.6	0.01 ± 0.002	0.095 ± 0.02	0.036 ± 0.009
17	K_m	21.0 ± 3.6***	7.16 ± 1.7***	59.2 ± 10	75 ± 6.5***	76 ± 7.31***
	V_{max}	4.74 ± 2.5	1.68 ± 0.7	0.40 ± 0.1	3.73 ± 0.06	0.90 ± 0.04*
	V_{max}/K_m	0.22 ± 0.1***	0.27 ± 0.2***	0.007 ± 0.002	0.05 ± 0.004**	0.012 ± 0.001
2	K_m	13.0 ± 0.5	2.42 ± 0.5	112 ± 41	57 ± 4.9	47 ± 6.82
	V_{max}	8.48 ± 4.1	1.72 ± 0.5	0.79 ± 0.08	5.25 ± 0.37	1.98 ± 0.16*
	V_{max}/K_m	0.66 ± 0.3	0.77 ± 0.4*	0.008 ± 0.002	0.093 ± 0.006	0.042 ± 0.003
T107I	K_m	12.4 ± 2.5	4.08 ± 1.1*	90 ± 51	37 ± 0.73**	42 ± 4.00
	V_{max}	6.08 ± 1.4	2.40 ± 0.4	0.51 ± 0.13	3.82 ± 0.31	0.71 ± 0.05**
	V_{max}/K_m	0.49 ± 0.1*	0.61 ± 0.2**	0.007 ± 0.002	0.103 ± 0.009	0.017 ± 0.003

ODM, O-demethylation; 4OH, 4-hydroxylation; α-OH, alpha-hydroxylation.
[a] K_m (μM)
[b] V_{max} (pmol product/min/pmol CYP)
[c] V_{max}/K_m (μl/min/pmol CYP)Values represent mean ± S.D. of four determinations (twice for each of two separate expressions).* significantly different (P<0.05), ** significantly different (P<0.01) and *** significantly different (P<0.001) from values for CYP2D6 1.

Table 3.5 The ions of the compounds monitored by LCMS and their retention times

Compound	***m/z***	**retention time**
Dextromethorphan	272.2	2.9
Fluphenazine	438.3	2.8
Bufuralol	262.1	3.0
Propafenone	342.2	3.0
Metoprolol	268.1	2.8
Debrisoquine	176.3	4.3
Timolol	317.2	2.8
Clomipramine	315.0	3.1
Thioridazine	371.0	3.2
Sparteine	235.1	3.7

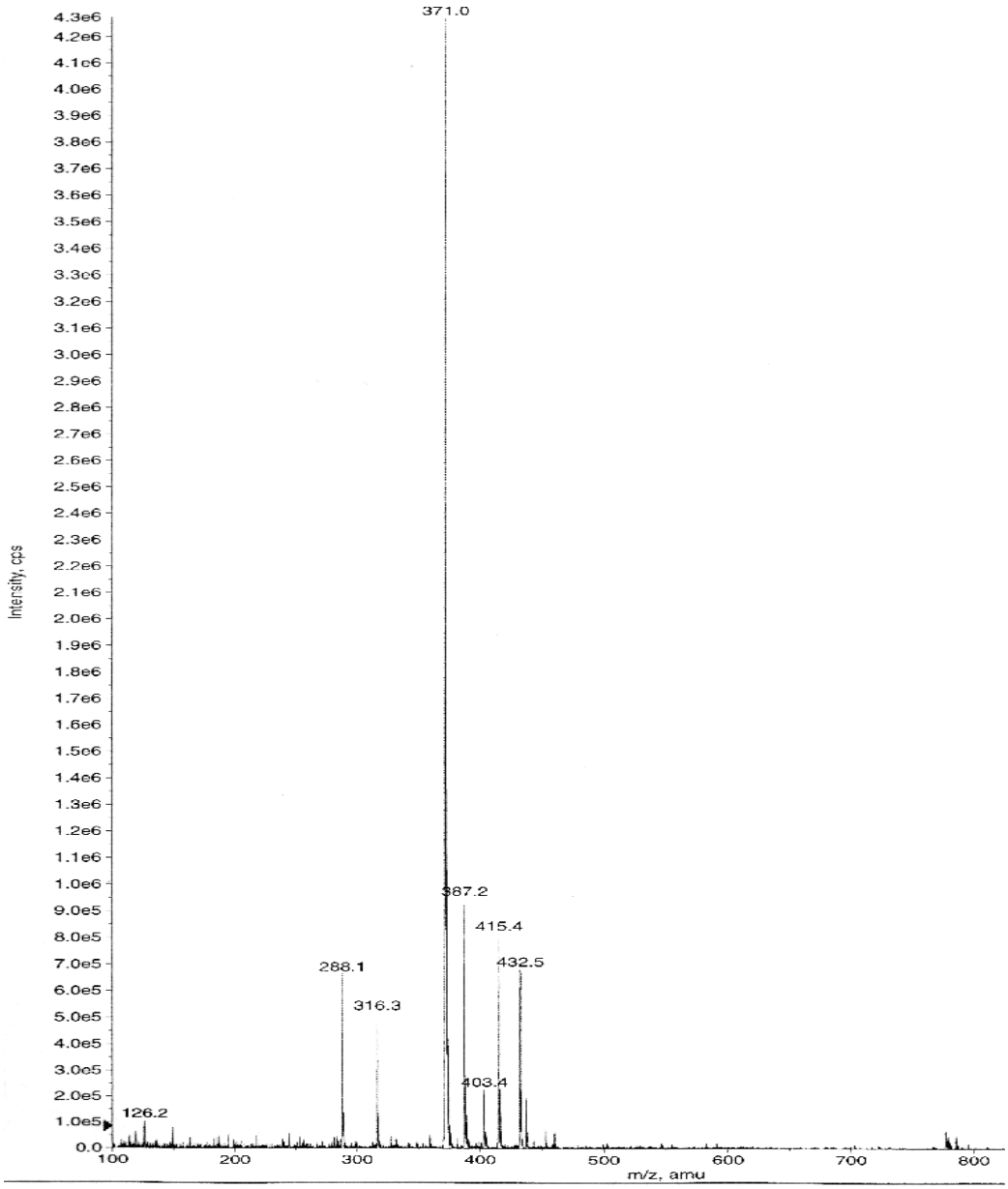


Figure 3.19 Mass spectrum showing the detection of thioridazine (*m/z* 371) in the positive ion mode.

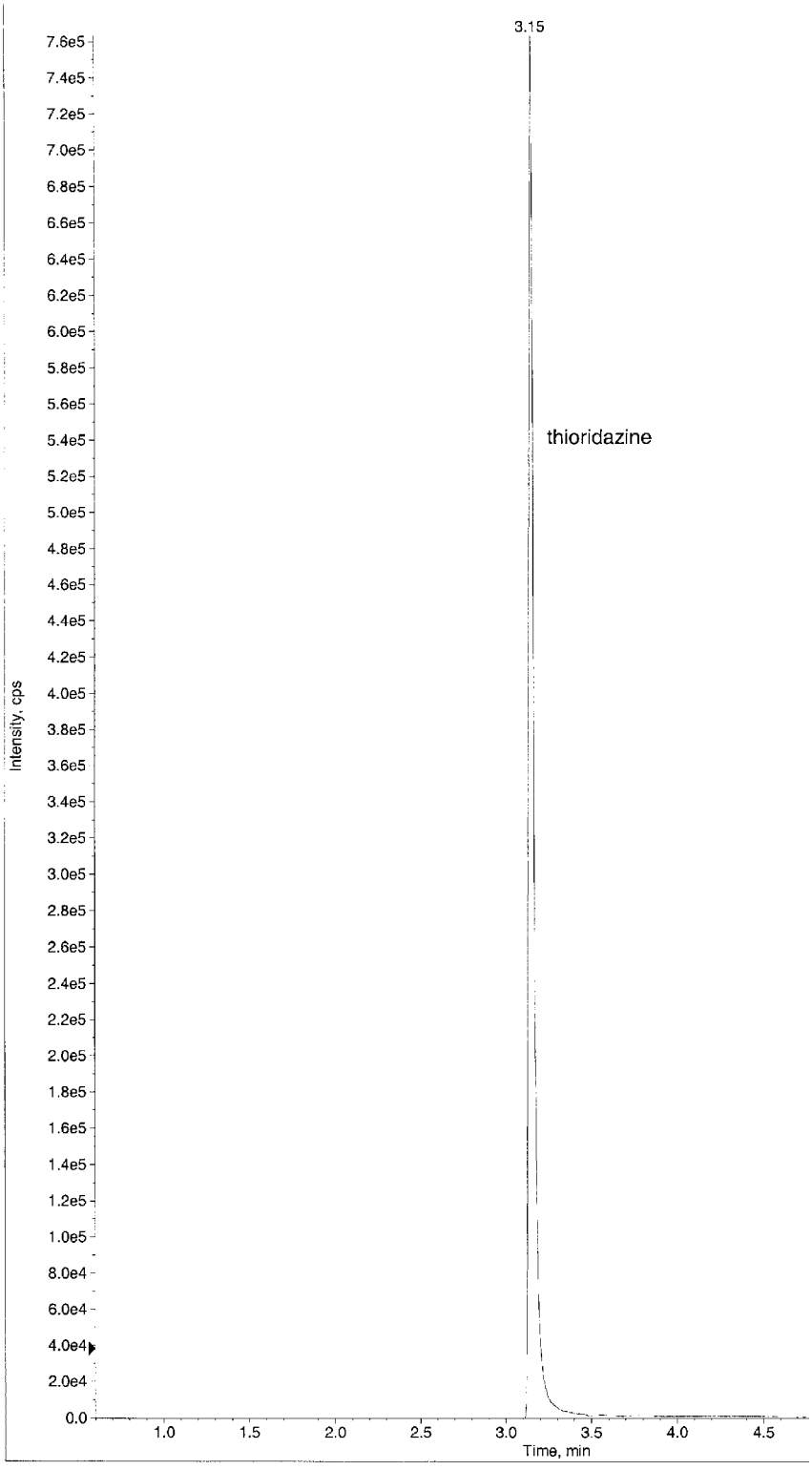


Figure 3.20. The concentration of drug remaining in the incubation mixtures was calculated from the peak area such as the one for thioridazine shown in the figure.

Clearance (CL) can be expressed in terms of the elimination rate constant (*k*) and volume of distribution (V):

$CL = k \times V$

An expression for intrinsic clearance (CL_{int}) can be derived for an *in vitro* incubation with the total volume of incubation as the volume of distribution and elimination rate constant:

$CL_{int} = \text{Volume} \times k$ $\qquad (T_{1/2} = 0.693/k)$

k is the elimination rate constant and was determined from a plot of time against the log of remaining drug concentration as shown in Figure 3.21. The remaining concentration (c) of a drug at time (t) can be expressed as:

$\log c(t) = \log c(0) - kt/2.303$

k can, therefore, be obtained from the slope, $-k/2.303$

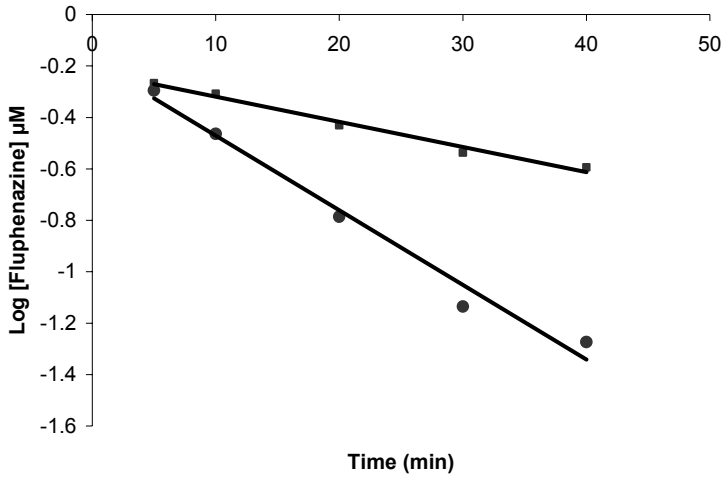


Figure 3.21 A typical plot used to calculate the elimination rate constant *k* and shows the clearance of fluphenazine by CYP2D6.1 (●) and CYP2D6.17 (▪).

The CL_{int} calculated from *in vitro* half-lives for ten drugs which are known to be substrates of CYP2D6 are shown in Table 3.6. CYP2D6.17 generally displayed reduced clearance of the CYP2D6 substrates, with the magnitude of the reduction being dependent on the compound.

Large differences in clearance between CYP2D6.1 and CYP2D6.17 were observed with bufuralol, dextromethorphan, clomipramine and fluphenazine. Clearance values that were affected to a much lesser extent were observed with metoprolol, debrisoquine, sparteine and thioridazine.

The relative contribution of CYP2D6 towards metabolism and disposition of the drugs *in vivo* would determine the clinical relevance of administering the same dose of drug to individuals carrying the CYP2D6*17 or CYP2D6*1 genotypes. A drug whose clearance is predominantly CYP2D6 dependent and is affected most by CYP2D6.17 would most likely result in clinically significant differences in individuals with the CYP2D6*17 or CYP2D6*1 genotypes. The relative contribution of CYP2D6 towards clearance of the drugs was, therefore, determined using the RAF determined as described in detail in section 2.2.3.5. The relative contribution (using a RAF of 47 pmol/mg) of CYP2D6 towards clearance of the CYP2D6 substrate drugs by HLM is shown in Table 3.6. For six of the drugs, including bufuralol, propafenone, timolol and fluphenazine, CYP2D6 is predicted to be the most important liver microsomal enzyme for clearance of the drugs with percentage contributions of over 65%. For the remaining drugs dextromethorphan, metoprolol and thioridazine, CYP2D6 is predicted to contribute significantly towards their clearance with percentage contributions of at least 43%.

Table 3.6. A comparison of the capacity of CYP2D6.1 and CYP2D6.17 to clear CYP2D6 substrates *in vitro*

Compound	CL by HLM (µl/min/mg)	CL by CYP2D6.1 (µl/min/pmol CYP)[c]	CL by CYP2D6.17	% Reduction in CL	% Contribution by CYP2D6[b]
Bufuralol	37	0.813 ± 0.07	0.306 + 0.020	62	103
Dextromethorphan	41	0.421 ± 0.02	0.081 + 0.02	81	48
Metoprolol	6.9	0.069 ± 0.007	0.040 + 0.004	42	47
Debrisoquine	4.4	0.062 ± 0.003	0.044 + 0.010	29	66
Propafenone	140	2.771 ± 0.060	1.712 + 0.020	38	93
Timolol	3.2	0.115 ± 0.050	0.053 + 0.020	54	169
(-)-Sparteine	ND	0.111	0.097	13	ND
Clomipramine	29	0.422 ± 0.080	0.065 + 0.006	85	68
Thioridazine	71	0.654 ± 0.140	0.459 + 0.020	30	43
Fluphenazine	48	1.336	0.451	66	131

[a]Calculated as follows: [(CL by CYP2D6.1 – CL by CYP2D6.17)/CL by CYP2D6.1] x 100

[b] relative contribution of CYP2D6.1 towards clearance of the drug calculated by multiplying the CL_{int} for recombinant CYP2D6.1 by the RAF (47 pmol/mg) and expressing as a percentage of the CL_{int} by HLM.

[c] Values represent mean ± S.D. of duplicate determinations.HLM, human liver microsomes; RAF, relative activity factor; CL, clearance; ND, not determined.

3.3.3 CYP2D6 homology models

The rabbit CYP2C5 crystal structure was used as a template for homology modelling of CYP2D6.1 and CYP2D6.17. The first step in homology modelling of the two proteins was the alignment of their primary structures with that of the CYP2C5 used as the template. The alignments were done using the programs GCG BestFit and Swiss-Pdb Viewer v 3.7b2 with manual adjustments being made to the gaps. The alignment between CYP2D6.1 and CYP2C5 is shown in Figure 3.22. The two proteins have amino acid sequence similarity and identity of 51 and 41%, respectively. The alignment also shows the amino acid residues involved in the formation of the helices, β-sheets and loops. In addition, the substrate recognition sites that were proposed by Gotoh (1992) are also shown. The amino acid exchanges in CYP2D6.17 are all located in the SRS regions: T107I in SRS1, R296C in SRS4 and S486T in SRS6. 1.

The alignments were imported into the program SYBYL 6.7 (Tripos Associates Inc.) for modelling. The models were analysed using the Protable module. The root mean square distance for the polypetide backbone of the CYP2D6.1 model was 0.92 Å compared to the CYP2C5 crystal structure. The Matchmaker average score was -0.09 kT and –0.12 kT for the CYP2D6 1 model and CYP2C5 template, respectively. Energy scores below zero are advantageous and values above zero could indicate incorrectly modelled regions. Visual inspection of the CYP2D6.1 and CYP2D6.17 models showed different arrangement of amino acid residues in their active sites. Important active site residues in CYP2D6.1 are shown in Figure 3.23 and include Glu 216, Phe 219, Leu 213, Ser 304, Phe 483, Val 374, Thr 309, Ala 305, Ile 369, Leu 121 and Val 370. The arrangements of these active site residues were altered in the CYP2D6.17 active site. In addition, the overall structures of the CYP2D6.1 and CYP2D6.17 models differed in the B-C loop region.

3.3.4 Docking of CYP2D6 substrate drugs into homology models

Dockings were done using radii of 10, 12 and 15 Å to define the CYP2D6.1 active site. The distance between site of oxidation and the iron-bound oxygen for other CYPs/substrates has been shown to be around 3-6Å from NMR and crystallography studies (Schlichting *et al*., 2000; Poli-Scaife *et al*., 1997). The interpretation of Table 3.7, therefore, took into account the number of solutions favouring known sites of oxidation, and the distance of the closest possible site of oxidation to the heme-bound oxygen. Based on this approach, the results of the docking experiments showed that (1) active site volumes described by 10 or 12Å best approximated the experimental data (2) the hydrogen bond interactions of the basic nitrogen in half of the compounds, were with Glu 216.

Figure 3.23 shows bufuralol docked in the CYP2D6.1 active site with a hydrogen bond between the basic nitrogen of bufuralol and Glu 216. In this orientation, bufuralol is well positioned for hydroxylation at the 1´position that is the experimentally preferred position by CYP2D6.1. Other compounds involved in interactions with the Glu 216 included debrisoquine, metoprolol, timolol and propafenone. For these compounds, the site of oxidation was consistent with the experimentally determined sites except for propafenone. For the other compounds not involved in hydrogen bond interactions with the Glu 216, the program was able to correctly predict the sites of oxidation for dextromethorphan and sparteine. Docking of the substrates into the CYP2D6.17 model generally gave different results from those for CYP2D6.1. Bufuralol for example docked in at an orientation favouring oxidation at site II (in Table 3.7). Sparteine, however, adopted the same orientation in the CYP2D6.17 and CYP2D6.1 active sites.

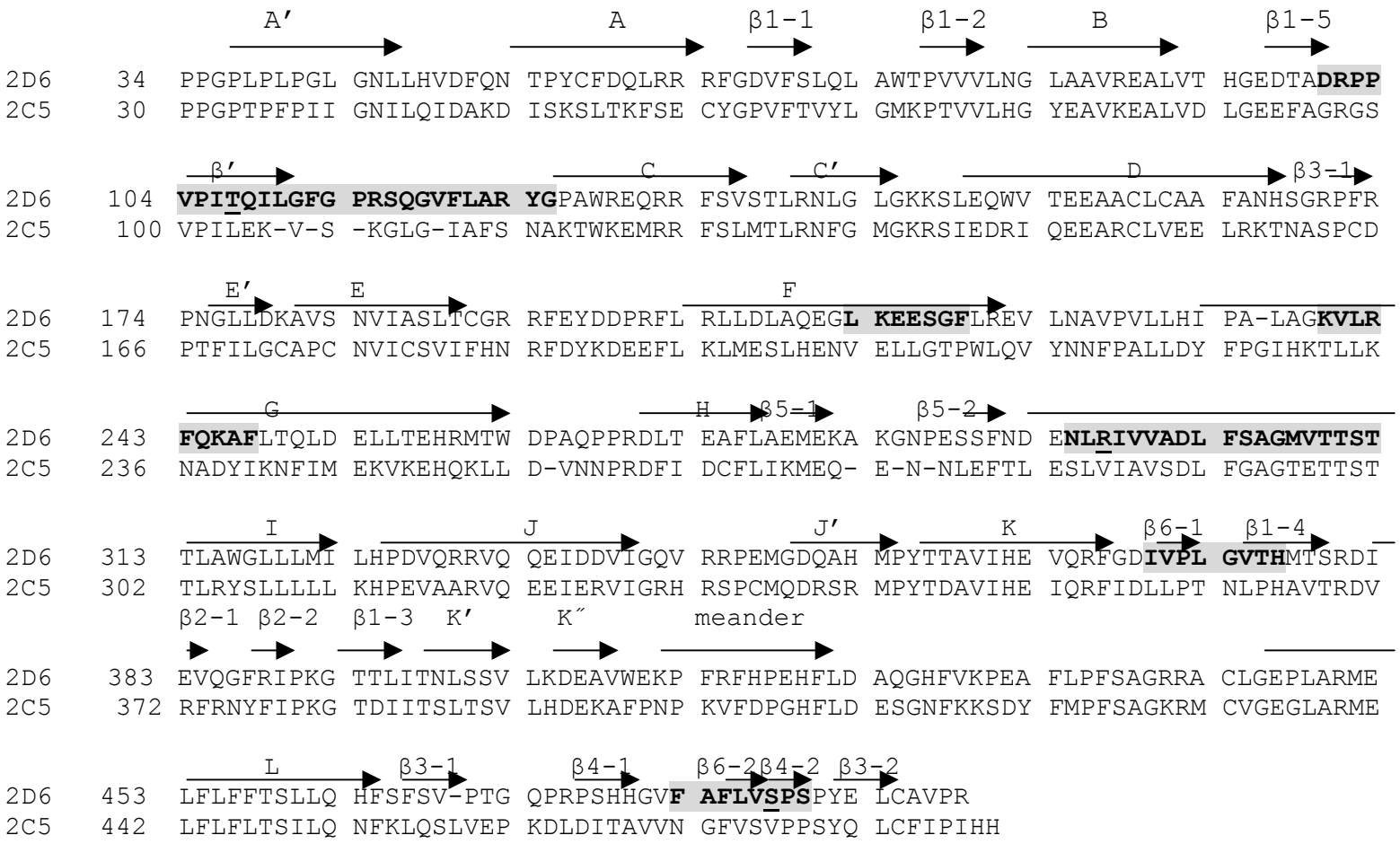


Figure 3.22. Alignment between CYP2C5 and CYP2D6.1 showing the amino acids (shaded and bold) in the substrate recognition sites (SRS) proposed by Gotoh (1992) from an alignment by de Groot *et al*., (1996). The amino acid exchanges in CYP2D6.17 (underlined) are all located in the SRS regions.

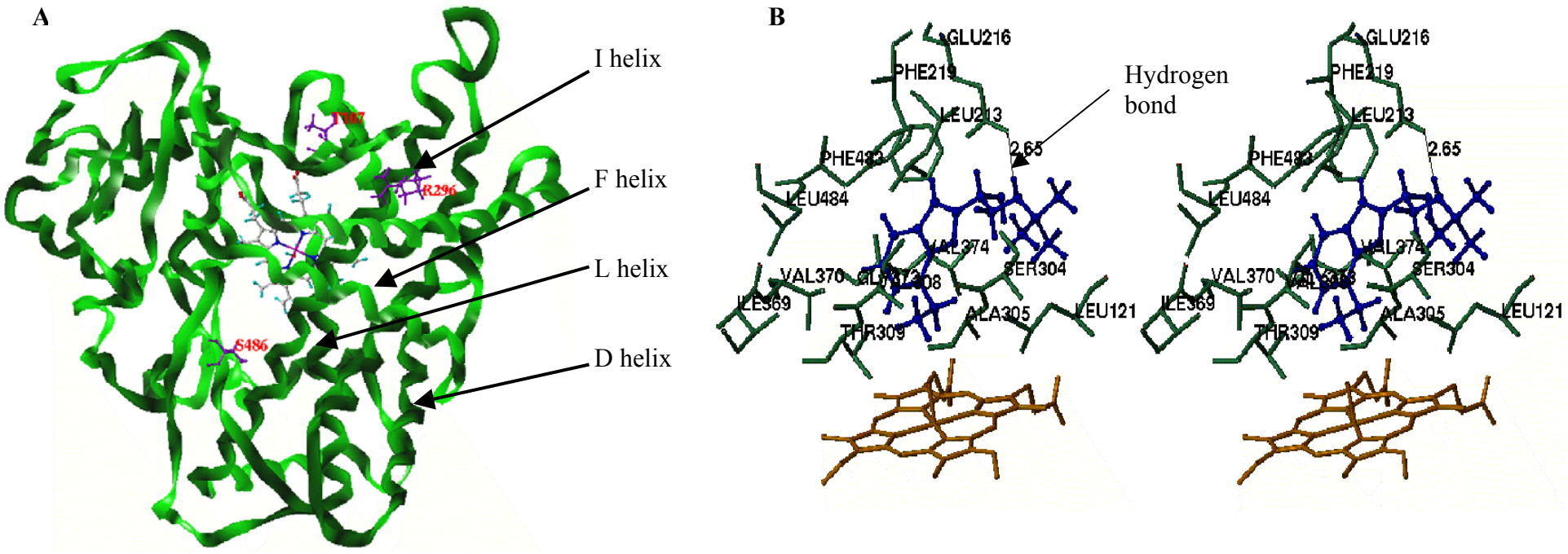
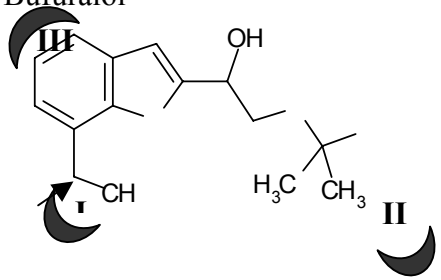
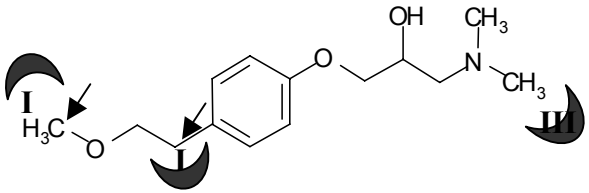
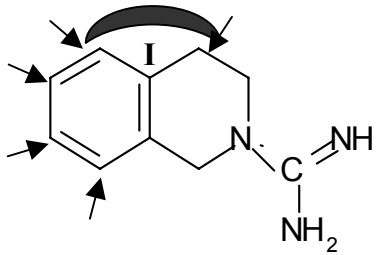
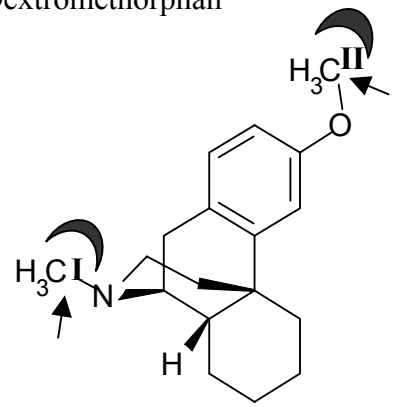


Figure 3.23. **A**) Ribbon diagram showing the CYP2D6.1 protein model and location of the amino acid exchanges in CYP2D6.17. **B**) Bufuralol (in blue) docked in the active site of CYP2D6.1 and the interaction between Glu 216 and the nitrogen on bufuralol.

Table 3.7. Results from the docking experiments with CYP2D6.1

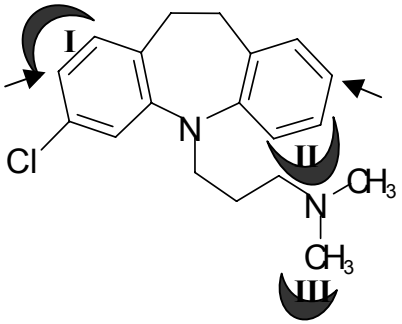
Compound	Active site radius (Å)	No. of solutions	Hits on specified region	Distance (Å)[a]
Bufuralol 	10	5	5 (**I**)	3.20
	12	3	3 (**I**)	2.78
	15	10	3(**I**), 5(**II**), 1(**III**)	4.85, 2.81, 3.01
Metoprolol 	10	10	6(**I**), 4(**II**)	2.32, 3.68
	12	10	5(**I**), 4(**II**), 1(**III**)	3.36, 4.08, 1.89
	15	10	7(**I**), 1(**II**),2(**III**)	3.36, 2.20, 2.91
Debrisoquine 	10	4	4(**I**)	4.77
	12	3	3(**I**)	4.33
	15	5	5(**I**)	4.88

Dextromethorphan



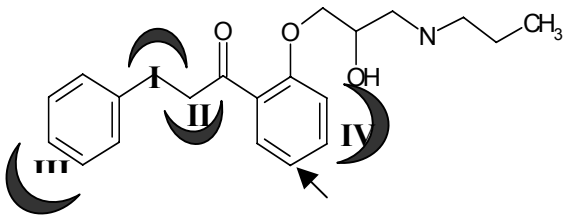
10	3	3(**I**)	2.82
12	7	6(**I**), 1(**II**)	3.32, 7.38
15	3	3(**I**)	2.81

Clomipramine



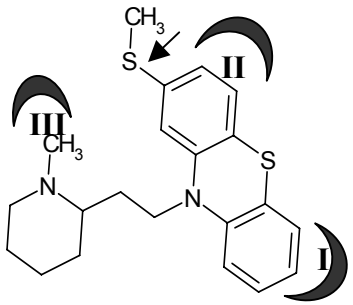
10	8	7(**II**), 1(**I**)	2.91, 4.11
12	6	2(**II**), 1(**I**), 3(**III**)	2.88, 3.50, 6.47
15	3	3(**III**)	6.64

Propafenone



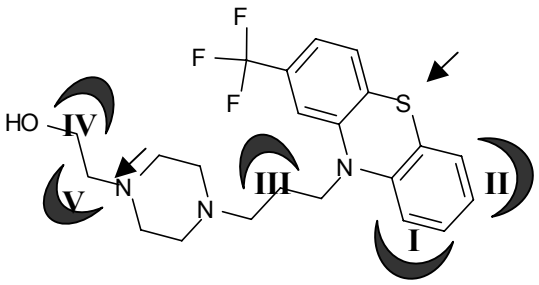
10	10	5(**I**), 1(**II**), 3(**III**), 1(**IV**)	2.14, 2.90, 2.29,2.20
12	8	3(**I**), 5(**IV**)	2.52, 3.00
15	9	1(**I**), 2(**III**), 3(**IV**),	3.59, 2.78, 4.10

Thioridazine



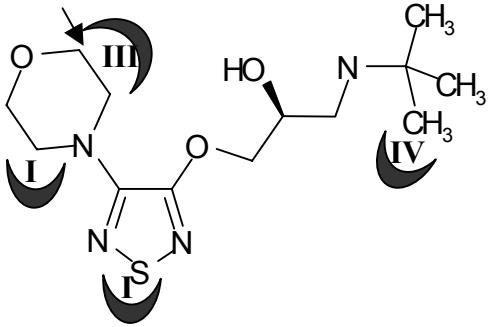
10	10	5(**I**), 2(**II**),3(**III**)	3.21, 3.73, 3.98
12	10	2(**I**), 7(**II**), 1(**III**)	3.95, 3.60, 5.36
15	6	2(**I**), 4(**II**)	3.31, 3.73

Fluphenazine[b]



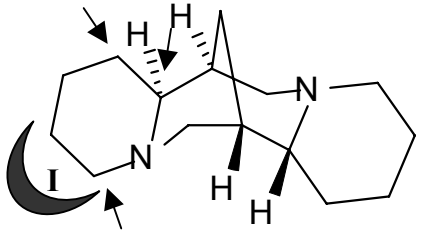
10	10	3(**I**), 2(**II**), 2(**III**), 1(**IV**), 2(**V**)	3.89, 3.50, 4.53, 3.20, 2.70
12	4	1(**I**), 3(**IV**)	3.00, 3.59
15	5	5(**I**)	3.04

Timolol



10	8	3(**I**), 3(**II**), 2(**III**)	2.07, 3.94, 3.80
12	5	3(**I**), 2(**III**)	1.85, 3.53
15	3	3(**IV**)	3.02

Sparteine



10	3	3(**I**)	3.01
12	5	5(**I**)	3.21
15	9	9(**I**)	3.08

The sites of oxidation indicated by arrows are from the references: (von Bahr *et al*., 1991; Lightfoot *et al*., 2000; Otton *et al*., 1988; Yamazaki *et al*., 1994; von Moltke *et al*., 1998; Ebner *et al*., 1995).

[a] Distance from heme-bound oxygen to the carbon on the site of oxidation.

[b] The site of oxidation is from a similar compound perphenazine (Olesen and Linnet, 2000) as that for fluphenazine is not known

CHAPTER FOUR

4.0 Discussion

Modern drug discovery and development (DDD) programs now place emphasis, in addition to pharmacological potency, on other critical aspects such as safety, occurrence of drug-drug interactions and pharmacokinetics (Li and Jurima-Romet, 1997; Gaviraghi *et al*., 2001). This has seen studies aimed at elucidating the metabolic properties of compounds becoming an integral part of such programs. The critical role played by CYPs in metabolism has in turn, resulted in potential drug-CYP interaction studies predominating. The studies focus on identification of CYP isoforms involved in metabolism and screening for possible inhibitory or inductive effects on CYP activity, of the compounds under development (Ajayi *et al*., 2000). Additionally, variant CYP genes whose expression patterns exhibit an ethnic dependency and are important determinants of the outcome of drug therapy are considered (Rodrigues and Rushmore, 2002 and references therein). Such efforts have seen a significant improvement in the production of safer and more effective drug therapies. These advances in DDD have, however, not found applications in tropical medicine with respect to antiparasitic drugs in use and CYP variants unique to the native populations, circumstances that constituted the primary driving force of work in this thesis.

Most antiparasitic drugs in use are toxic and were discovered through the traditional drug discovery approach more than 35 years ago. Knowledge of antiparasitic drug-CYP interactions that could contribute towards understanding any toxicities and rationalisation of their use is, therefore, lacking. The adverse effects associated with the use of antiparasitic drugs may be complicated by poly-pharmacy, a phenomenon common in the tropics and promoted by poly-parasitism, the high prevalence of HIV and related infections in the region. This led to the hypothesis that some of the observed adverse effects involving antiparasitic

drugs could be due to their interactions (inhibition or induction) with CYPs. Concerning CYP variants unique to populations in the tropics, attention was directed at the African-specific CYP2D6.17 that exhibits reduced activity. The involvement of CYP2D6 in metabolism of clinically important drugs, some of which have narrow therapeutic windows, formed the basis for the supposition that CYP2D6.17 might have implications for use of CYP2D6 substrate drugs in these populations. Studies were, therefore, designed in line with these hypotheses.

4.1 Methodological issues

4.1.1 Screening for CYP inhibition

Standard methods for assessing the inhibitory effects of test compounds make use of human liver microsomes and probe drugs (substrates) specific for a particular CYP isoform. The effect of a test compound on the activity of an enzyme is usually determined, by monitoring the formation of a product using HPLC and UV or fluorescence detection. The need to screen many compounds and overcome drawbacks associated with the use of human tissues has, however, given rise to several new higher throughput *in vitro* methods. The literature abounds in descriptions of methods for evaluating CYP inhibition and one such method which makes use of recombinant CYPs and substrates that produce fluorescent metabolites was developed by Crespi, (1995).

4.1.1.1 Evaluation of fluorescence-based assays utilising recombinant CYPs

The fluorescence-based assays utilising recombinant CYPs (rCYPs) have a number of advantages over the traditional HPLC-based assays using human liver microsomes (HLM) that include reduced costs in terms of reagents and equipment. In addition, the unreliable availability in sufficient quantities of HLM is overcome by rCYPs, which represent a

virtually unlimited source of enzyme. The fluorescence-based assays utilising rCYPs were, therefore, appropriate to use in this study given the large number of antiparasitic drugs to be investigated for their inhibitory effects on the drug metabolising CYPs. The use of different methods may, however, give different results and this is exemplified by a study, which showed that, K_i values may be dependent on the substrate used (Kenworthy *et al*., 1999). It was, therefore, important to ascertain any loss in data quality associated with the use of fluorescence-based assays and rCYPs.

A comparison of K_i values obtained from HPLC-based assays utilising rCYPs and fluorescence assays also using rCYPs showed a good correlation that is in support of the fluorescence-based assays. This means that there will not be much loss in data quality in the move to the higher throughput fluorescence assays. Distinct disparities between the two methods were, however, observed with three compounds atovaquone, diethylcarbamazine and niclosamide. While with fluorometric assays, the three compounds exhibited apparent activation of CYP activity (Table 3.1), the HPLC marker reactions, showed the compounds to be either non-inhibitors (atovaquone and diethylcarbamazine) or potent inhibitors (niclosamide). Diethylcarbamazine and atovaquone activated CYP2C19 and CYP1A2 respectively with the activation of CYP2C9 by niclosamide being the most dramatic at 400%.

The apparent activation of CYP2C9-catalysed 7-methoxy-4-trifluoromethylcoumarin (MFC) dealkylation to 7-hydroxy-4-trifluoromethylcoumarin (HFC) by niclosamide, was further studied to try and establish its authenticity. In the presence of a potent CYP2C9 inhibitor sulfaphenazole, niclosamide's activation was abolished (Figure 3.6) indicating a role of the enzyme in the increase in fluorescence. Further experiments showed that the activation was not due to the fluorescence of niclosamide, its possible metabolite or complexes of niclosamide or its metabolite with MFC or HFC thus supporting a role of the enzyme in the

increase in fluorescence. These observations are consistent with reports of apparent activation of CYP2C9 activity by other researchers. The *in vitro* activation of CYP2C9 activity was shown to be effected by dapsone and N-hydroxydapsone on flurbiprofen 4'-hydroxylation (Hutzler *et al*., 2003). The underlying mechanism of the activation by dapsone was shown to be an increase in affinity of flurbiprofen for the enzyme resulting in a decrease in K_m while that by N-hydroxydapsone is not clear. Acetonitrile was also shown to cause substrate-dependent effects on CYP2C9 activity (Tang *et al*., 2000). The solvent increased CYP2C9 catalysed diclofenac 4-hydroxylation and tolbutamide methyl hydroxylation, but decreased celecoxib methyl hydroxylation with changes in K_m and /or V_{max} accompanying the observations. Effects on affinity of the substrates for the enzyme and/or other unknown mechanisms may, therefore, also explain the activation of CYP2C9 by niclosamide.

The use of rCYPs in place of HLM immediately brings the question of their suitability given differences in the lipid milieu and concentrations of accessory proteins such as cytochrome b_5 and NADPH-CYP-reductase. In addition, the whole complement of CYPs is present in HLM whereas only a single CYP is available in experiments utilising rCYPs. In this study, the use of rCYPs was, therefore, evaluated against HLM using the same HPLC-based marker reactions and a good correlation was observed between the inhibition data. This validates the use of rCYPs and is consistent with results obtained by others (Masimirembwa *et al*., 1999). A comparison of data obtained from HPLC assays utilising HLM and fluorescence assays using rCYPs also gave a good correlation. The correlation was, to a large extent, affected by two compounds quinine and dihydroartemisinin that showed very different K_i values using the two systems. We currently do not have an explanation for this observation but it could be due, at least in part, to differences in the extent of non-specific protein binding in the two systems. This notion is supported by Obach, (1996), who suggested protein concentration to be an underlying factor of discrepancies in values of K_m and K_i reported in literature from

different systems (HLM *vis-à-vis* rCYPs). Additionally, non-specific binding was also shown to be dependent on the compound in question, with some compounds being affected more than others. The benefits of the higher throughput fluorescence assays, however, clearly far out-weigh the few discordant cases and the data is comparable with that from the standard HPLC-based assays utilising HLM.

4.1.2 Screening for CYP induction

The potential for compounds to induce CYPs has been studied in animal models such as rats, however, fundamental differences in response to inducers compared to humans has limited their use. Omeprazole and rifampicin were shown to be potent inducers of CYP1A2 and CYP3A in humans respectively, but not in rats (Lu and Li, 2001). As a result an effort was made to study induction in human derived tissues such as human hepatocytes and cell lines.

The induction of CYP1A has been investigated in several *in vitro* models including cell lines, hepatocytes and humans. Differences in occurrence and/or extent of induction depending on the model used have been reported, with differences in regulation of enzyme expression as a possible explanation (Kashfi *et al*., 1995). Additionally, cell-specific differences in toxicities of test compounds accounted for some of the disparities when using different cell lines (Kleeberg *et al*., 1999). In this study, the HepG2 cell line was used and only thiabendazole caused appreciable toxicity to the cells as assessed by the trypan blue exclusion test. The HepG2 cell line has been used to evaluate CYP induction in humans, with results from some of the studies correlating well with *in vivo* induction in humans and other *in vitro* models (Sumida *et al*., 2000). There is, however, conflicting data from literature on the suitability of HepG2 cells as a model for CYP1A2 induction (Berglund *et al*., 2002; Sumida *et al*., 2000; Vakharia *et al*., 2001).

4.1.3 Prediction of *in vivo* relevance of CYP inhibition or induction by antiparasitic drugs

The induction data and inhibition parameters such as, K_i , IC_{50} ,and EC50 generated *in vitro* only become useful when they can successfully predict *in vivo* effects. For *in vitro-to-in vivo* extrapolations, one of the major difficulties is estimating the actual concentration of the inhibitor/inducer available to the enzyme or regulatory site. In a study to evaluate the ability of *in vitro* data to predict *in vivo* results by the US Food and Drug Administration (FDA), use of plasma concentrations in predicting *in vivo* effects was one of the reasons for failure (Davit *et al*., 1999). In the predictive cases, however, plasma concentrations were also assumed to represent the concentration of drug available to the enzyme. Consequently, while plasma concentrations may reflect the concentration of drug available to interact with the enzyme, this is not always the case. Some authors have suggested that protein binding be factored in *in vivo* predictions, however, disregarding protein binding was shown to give better predictions of *in vivo* clearance for basic and neutral compounds (Obach *et al*., 1999 and references therein). For the acidic compounds, all binding would have to be taken into account, that is, binding to the *in vitro* incubation matrix and blood proteins.

With respect to antiparasitic drugs, some of which accumulate in specific tissues (e.g. chloroquine in the liver and melanin containing tissues), calculation of the concentration of drug reaching the enzyme is even more difficult (Abdi *et al*., 1995). In addition, transporters (efflux and influx) may also determine the concentration of a drug in a cell and are a topic of ongoing research (Kusuhara and Sugiyama, 2002 and references therein). In this study, we have used the maximum plasma concentrations (C_{max}) as the concentration of drug available to the enzyme or regulatory site and no attempt was made to estimate the concentration of inhibitor/inducer in liver or to correct for protein binding. Additionally, in an effort to

increase chances of approximating the actual concentration of drug *in vivo* for the induction studies, several concentrations around the C_{max} were used.

Most of the antiparasitic drugs are predicted not to pose any risk for important drug-CYP interactions. Some of the drugs, however, could have clinically important interactions with CYP1A1, CYP1A2 and CYP2D6. Potent inhibitors of CYP1A2 included artemisinin, dihydroartemisinin, thiabendazole, niclosamide and primaquine. Cycloguanil, proguanil, amodiaquine, desethylamodiaquine, chloroquine and quinine were potent inhibitors of CYP2D6 while niclosamide was a potent inhibitor CYP3A4. Primaquine, quinine and albendazole induced the activities and mRNA expression levels of CYP1A1 and CYP1A2.

4.2. Inductive effects of antiparasitic drugs on CYP1A subfamily

Clearly from the literature review, the net effect of induction, which may be beneficial or harmful to the organism, is dependent on several factors. In cases where accumulation of a compound might result in toxicity, induction enables a fast removal of the compound and is, therefore, protective. In some instances, however, induction may give rise to increased concentrations of a toxic intermediate through metabolic activation of the parent compound, by an induced enzyme. The involvement of the CYP1A subfamily in activation of several compounds to potentially toxic intermediates, in metabolism of clinically important drugs and in general detoxication reactions, therefore, imparts a pharmacological and/or toxicological significance to CYP1A induction.

Quinine, an arylaminoalcohol is used extensively for the treatment of malaria. Maximum plasma concentrations of around 18 μM are achieved in healthy individuals (Wanwimolruk *et al*., 1995) but concentrations may be as high as 50 μM in malaria patients (van Hensbroek *et al*., 1996). The drug undergoes extensive CYP-mediated metabolism with formation of the major metabolite, 3-hydroxyquinine, catalysed by CYP3A4 (Zhao *et al*., 1996). Smoking has been shown to increase the clearance of quinine thus implicating CYP1A in the metabolism of the drug (Wanwimolruk *et al*., 1995). In this study, quinine caused a dose-dependent increase in EROD activity that was accompanied by an increase in CYP1A1 mRNA expression levels, showing that the induction is transcriptional. The induction could be of clinical significance as concentrations at which it occurs fall within the range found *in vivo* after administration of a therapeutic dose and particularly given the multiple dosing of up to 14 days (Bozdech and Mason, 1992). The decrease in quinine plasma levels by 36 % from day two to day seven might be explained by induction of CYP1A observed in a study of malaria patients (Karbwang *et al*., 1991), and should be investigated further. The effect of quinine on CYP1A2 mRNA levels at 15 and 30 μM was low and on CYP1A2 enzyme activity not obvious since EROD is catalysed by both CYP1A1 and 1A2.

Albendazole is a benzimidazole carbamate used for the treatment of a wide spectrum of intestinal infections (Abdi *et al*., 1995). Studies in rats showed the drug to be an inducer of CYPs 1A1, 1A2, 2B1, 2B2 and 2E1 (Asteinza *et al*., 2000; Souhaili-El Amri *et al*., 1988). In this study, albendazole caused maximal induction of CYP1A1/1A2 mRNA and EROD activity at concentrations between 1 and 5 μM. The induction could, therefore, be of clinical significance given the peak plasma concentrations attained by the drug of up to 2 μM (Marriner *et al*., 1986). These results are consistent with observations made *in vivo* in which prolonged use of albendazole during treatment of echinococcosis was shown to induce the CYP1A1-mediated metabolism of albendazole sulphoxide in humans (Steiger *et al*., 1990).

The decrease in induction of both EROD activity and CYP1A mRNA levels at concentrations of 10 μM and above could be due to the inhibitory effects of the drug rather than toxicity because the trypan-blue exclusion test did not show any appreciable toxicity.

The treatment of malaria due to *P. vivax* or *P. ovale* infections requires a radical approach using a drug capable of clearing the hypnozoites which may later develop into liver schizonts and cause relapse. Primaquine is the drug of choice at a dose of 0.5 mg/kg for up to 14 days and it is usually used in combination with chloroquine (Bozdech and Mason, 1992). The increase in CYP1A1 mRNA levels and EROD activity caused by primaquine is in agreement with observations made by others (Fontaine *et al*., 1999). In this study, however, primaquine does not cause appreciable induction at the concentrations found in plasma of around 0.4 μM. We do not, therefore, expect the induction of CYP1A1 by primaquine to be of any pharmacological or toxicological significance. The increase in CYP1A2 mRNA observed at a concentration of 10 μM primaquine is also not expected to be of any significance *in vivo*.

The possible pharmacological implications of CYP1A induction by albendazole and quinine such as reduced efficacy, are fairly straightforward. They may occur due to increased metabolism of co-administered CYP1A substrate drugs resulting in sub-therapeutic levels of the drug or increased concentrations of an active metabolite. Any pharmacological adaptations that may be required to counter the effects of the induction such as reduction in dosage of an affected drug are also fairly simple. For clinicians, the knowledge that quinine and albendazole may induce the CYP1A subfamily is important as it helps them to apply the appropriate interventions when they encounter interactions involving the two drugs. The possible roles of the drugs in carcinogenesis, given the involvement of the CYP1A subfamily in activation of several procarcinogens are, however, not simple.

Exposure to aflatoxin B_1, for example through the diet, is associated with hepatocarcinogenesis due to the CYP1A2-mediated formation and accumulation of the carcinogen aflatoxin-8,9-epoxide (Wang *et al*., 2001; Gallagher *et al*., 1996). The importance of CYP1A2 in hepatocarcinogenesis is supported by a study in which inhibition of CYP1A2 by oltipraz is thought, at least in part, to be the mechanism for the drug's cancer chemopreventative activity (Sofowora *et al*., 2001). It is very likely that people taking the drugs quinine or albendazole may be exposed to procarcinogens such as aflatoxin B_1 which has been detected in ground corn samples, cooking peanut oil and rice samples (Wang *et al*., 2001). Whether the simultaneous exposure to the drugs and aflatoxin B_1 would increase an individual's risk of hepatocarcinoma, requires further studies.

4.3 Inhibitory effects of antiparasitic drugs on CYPs

The majority of documented drug-drug interactions with serious clinical consequences are due to inhibition of the metabolism of one drug by another. The clinical consequences are, to a large extent, attributable to increased plasma levels of a drug, with the type and severity dependent on the drug involved. In some cases, inhibition may result in reduction in clinical effects of a drug due to reduced levels of an active metabolite. Of the different mechanisms of inhibition, reversible inhibition of CYPs has been shown to be the most common cause of drug-drug interactions (reviewed by Hollenburg, 2002), as a result, the experimental design utilised in this study, was aimed at identifying reversible inhibitors of the drug metabolising CYPs.

Thiabendazole, a broad-spectrum anthelminthic drug (Edwards and Breckenridge, 1988), has been shown to increase theophylline serum levels resulting in serious side effects (Schneider *et al*., 1990; Lew *et al*., 1989). The K_i for thiabendazole's inhibition of CYP1A2 obtained

with human liver microsomes (0.93 μM), is far below the plasma concentration it achieves (89 μM). Thiabendazole is, therefore, predicted to inhibit 98 % of CYP1A2 activity *in vivo* (Table 3.2). Given that theophylline is metabolised by CYP1A2 (Tjia *et al*., 1996) and has a narrow therapeutic index, our results suggest that the interaction between thiabendazole and theophylline may be due to the inhibition of CYP1A2 by thiabendazole.

In our study, primaquine is predicted to inhibit 67 % of CYP1A2 activity *in vivo* (Table 3.2). Primaquine has been shown to decrease the clearance of antipyrine resulting in an increase in its half-life (Back *et al*., 1983). Antipyrine has been shown to be metabolised by many CYPs with CYP1A2 significantly contributing to its metabolism (Sharer and Wrighton, 1996). Our results, therefore, show that the interaction between antipyrine and primaquine may be due to inhibition of CYP1A2 by primaquine. Primaquine has been recommended for use as prophylactic for malaria (Schwartz and Regev-Yochay, 1999). There is, therefore, a high risk of it interacting with drugs that are substrates of CYP1A2 since the drug will be taken for up to three weeks.

Artemisinin and dihydroatemisinin are very important antimalarials as they are effective against multi-drug resistant strains of malaria parasites (Dhingra *et al*., 2000). The drugs are usually used in combination with other antimalarials (Na-Bangchang *et al*., 1999). Careful drug and patient monitoring should be done when artemisinin is co-administered with drugs that are CYP1A2 substrates as it is predicted to inhibit about 76 % of CYP1A2 activity *in vivo*. Although niclosamide is a potent inhibitor of CYPs1A2 and 3A4, it is not significantly absorbed (Abdi *et al*., 1998) and is, therefore, not expected to inhibit these enzymes *in vivo.*

Amodiaquine, an antimalarial is converted in the body, to the active metabolite desethylamodiaquine, which has a much longer elimination half-life than the parent drug.

Although both amodiaquine and desethylamodiaquine are potent inhibitors of CYP2D6 with K_i values of 2.1 and 4.1 μM respectively, they are not expected to cause any appreciable inhibition *in vivo*. Concomitant use of probe drugs of CYP2D6 with inhibitors of the enzyme has been shown to contribute towards erroneous assignment of metaboliser status or phenocopying (Siddoway *et al*., 1987). Knowledge of these potential interactions with CYP2D6 is important when doing phenotyping studies (Masimirembwa and Hasler, 1997). We, therefore, do not expect amodiaquine or its metabolite to interfere with phenotyping for CYP2D6 activity.

Interventions to address the effects of inhibition are also fairly straightforward. In cases where thiabendazole, primaquine or artemisinin are co-administered with drugs whose clearance is predominantly CYP1A2 dependent, the appropriate adjustments in dosage should be made. One such intervention, is illustrated by Lew *et al*., (1989), who recommend a reduction by 50%, of the dose of theophylline when it is administered together with thiabendazole. For the other antiparasitic drugs that are not potent inhibitors, caution should still be exercised as they may inhibit through the time dependent mechanisms (irreversible and quasi-irreversible inhibition) that were not investigated in this study.

An important use of selective inhibitors of CYPs is in the identification of individual CYP isoforms responsible for the metabolism of drugs (Lu *et al*., 2003). Of the possible approaches utilised in CYP identification, the use of chemical inhibitors is the simplest, however, lack of selectivity of some inhibitors presents a major problem (Lu *et al*., 2003; Sai *et al*., 2000). In this study, thiabendazole and primaquine showed selective inhibition of CYP1A2 with respect to the five CYP isoforms in this study. They could, therefore, be used as selective positive control competitive inhibitors instead of the commonly used mechanism-

based inhibitor, furafylline (Clarke *et al*., 1994), the toxic α-naphthoflavone (Liehr *et al*., 1991), or the unselective fluvoxamine (Venkatakrishnan *et al*., 1999).

4.4 Characterisation of CYP2D6.17

A number of studies done thus far have clearly shown the *CYP2D6*17* allele to be associated with reduced enzyme activity (Panserat *et al*., 1999; Masimirembwa *et al*., 1996b, Wennerholm *et al*., 2002). Studies on the enzyme kinetic or molecular basis for the diminished activity of CYP2D6.17 and its clinical significance are, however, limited. In one *in vitro* study, the kinetic effects of amino acid changes associated with CYP2D6.17, introduced in a CYP2D6-methionine variant, were investigated using bufuralol and codeine (Oscarson *et al*., 1997). However, the valine and not the methionine variant, has been found in human populations (Crespi *et al*., 1995). In this study, in order to obtain a clearer picture concerning the effects of the amino acid exchanges found in the CYP2D6.17 protein, the kinetics of three other variants bearing none or some of the amino acid exchanges were also studied. The variants included CYP2D6.1 lacking any amino acid exchanges, CYP2D6.2 bearing two of the amino acid exchanges (R296C and S486T) and CYP2D6.T107I. While the CYP2D6.1 and CYP2D6.2 proteins are found in human populations, the CYP2D6.T107I has not been found occurring alone in human populations and is, therefore, of academic interest.

4.4.1 *In vitro* kinetics of mutant variants

The kinetic profiles of CYP2D6.1 and CYP2D6.17 differed in a substrate dependent manner with CYP2D6.17 displaying a generally reduced capacity to clear the compounds (Tables 3.4 and 3.6). The structure of the substrate seemed to be critical, and the largest difference in V_{max}/K_m apparent with dextromethorphan. The V_{max}/K_m data (Table 3.4) was consistent with

CL_{int} data (Table 3.6), which show that dextromethorphan clearance was affected most by CYP2D6.17, whilst debrisoquine was the least affected. In some cases, the enzyme variants exhibited similar V_{max} values, suggesting a reduction in affinity as a possible explanation for the lower CL_{int} (V_{max}/K_m) values of CYP2D6.17. While CYP2D6.2 displayed similar kinetics to CYP2D6.1 for all probe substrates, except for dextromethorphan, the CYP2D6.T107I variant displayed a different profile for all probe drugs. The kinetic profile exhibited by CYP2D6.17, therefore, probably cannot be attributed to any one single amino acid exchange *per se,* but to the presence of all three amino acid changes. The combination of the amino acid exchanges might change the geometry and/or chemistry of the active site by either being part of the active-site architecture themselves or through long-distance structural effects. In order to investigate whether the observed kinetic properties of CYP2D6.17 could be rationalised in terms of an altered 3-dimensional structure of the enzyme *vis-à-vis* that of CYP2D6.1, homology models of the two enzymes were constructed using the CYP2C5 crystal structure as a template.

4.4.2 The CYP2D6.1 homology model and effects of amino acid changes in CYP2D6.17

Available protein models of CYP2D6 have been based on the crystal structures of bacterial CYPs 101, 102 and 108 (Modi *et al*., 1996; Lewis *et al*., 1997; Lewis, 1998; Ellis *et al*., 1996; de Groot *et al*., 1999). Important active-site residues identified by these models include Thr 107, Val 119, Glu 216, Asp 301, Ser 304, Val 374 and Phe 481. The Phe 481 was postulated to make hydrophobic interactions with the aromatic regions of substrates, and the amino acids Asp 301 and Glu 216, predicted to be critical for interactions with the basic nitrogen atoms of substrates. Given the significant structural differences between mammalian CYPs and bacterial ones, the CYP2C5 crystal structure may, therefore, be a better template. In addition, in some of the previous studies, the fitting of compounds into the active site was done taking

into account the positions of oxidation obtained from experimental data and the amino acid residues postulated to interact with the substrates. In this study, we have used the program GOLD to explore the best docking orientations without any constraints on the positions of oxidation or interactions with particular amino acid residues being made.

The docking results from our study (Table 3.7) show orientations of substrates favouring oxidation in the known positions for most of the compounds. Our homology model was, therefore, able to predict sites of oxidation for some of the compounds, in particular bufuralol, metoprolol, debrisoquine, dextromethorphan and sparteine. In half of the compounds (bufuralol, debrisoquine, metoprolol, timolol and propafenone), the docking program identified strong hydrogen bond interactions of the basic nitrogen of the substrates with the carboxylate group of Glu 216. This is, however, inconsistent with some predictions in which smaller substrates, such as debrisoquine, would interact with Asp 301, whilst the larger ones (e.g. metoprolol) interact with Glu 216 (de Groot *et al*., 1999). The Asp 301 residue in our model, although close to the heme is not well positioned for interactions optimal for oxidation in the known sites. Recent studies have also supported the non-involvement of Asp 301 in direct binding to the basic nitrogen of substrates but have instead identified Glu 216 as the critical residue interacting with the basic nitrogen of substrates (Kirton *et al*., 2002). Interestingly, the disparity in identification of the key amino acid residues involved in interactions with the basic moieties of the substrates by the different CYP2D6 protein models seem to be explained by the crystal structure used for construction of the models. While the models based on the bacterial CYPs seem to identify Asp 301 as the key residue, recent models utilising the mammalian CYP2C5 crystal structure point towards Glu 216. This highlights the major drawback of modelling – that the accuracy of the model strongly depends on the crystal structure used as template. The protein to be modelled must, therefore, resemble closely, the structure of the protein used as the template. At this point, the

CYP2C5 crystal structure is, perhaps, the best available in terms of homology to the human CYP2D6, and may, therefore, give the most accurate information concerning the structure of this important enzyme.

The orientations adopted by compounds in the active site are also determined by the shape and hydrophobic interactions between substituents of substrates and corresponding regions on the protein (Modi *et al*., 1996) thus explaining the orientations assumed by the other half of compounds not involved in interactions with Glu 216. The aromatic rings of substrates are oriented towards a hydrophobic region in our model consisting of Phe 219, Val 370, Phe 483, and Leu 484. The Thr 107 residue was predicted to be involved in interactions with some substrates (Modi *et al*., 1996). In our model the Thr 107 is, however, not close to the active site and does not interact with any of the substrates.

Homology modelling has a number of limitations and one of them is the difficulty encountered when modelling loop regions due to insertions and deletions (Figure 3.22) that are usually present in these regions (Poulos *et al*., 1991). The amino acid exchanges in CYP2D6.17 (T107I, R296C and S486T), are located in the B-C loop, I-helix and β4-2 sheet, respectively. An attempt was made to model the CYP2D6.17 protein and comparison of the overall shapes of CYP2D6.1 and CYP2D6.17 models revealed a difference in the B-C loop, which is close to the T107I and R296C amino acid changes. A closer inspection of the active site residues revealed a drastic change in their positions, with some residues being totally absent from the CYP2D6.17, which could explain the *in vitro* kinetic results (Tables 3.4 and 3.6). It was, however, difficult to attribute the drastic changes in the CYP2D6.17 active site to the amino acid changes *per se* as the changes could, in addition, be due to differences in modelling the B-C loop that are magnified by the proximity of T107I and R296C.

4.4.3 Clinical implications of the *CYP2D6*17* allele in metabolism of CYP2D6 substrate drugs in African populations

The location of pharmaceutical companies in America, Europe and other developed countries has meant that the bulk of studies to determine the dosage of drugs have been carried out in Caucasian populations. The prescription of drugs at the same dosages in different populations has, however, been questioned with the discovery of ethnic dependent differences in pharmacological response to many drugs, particularly antidepressants and neuroleptics (reviewed by Kalow, 2002). Extensive research effort during the last two decades has shown the CYP2D6 polymorphism to be an important factor in explaining some of the observed ethnic-dependent differences in response to CYP2D6 substrate drugs and this has been excellently reviewed by (Bradford and Kirlin, 1998; Poolsup *et al*., 2000). The clinical relevance of a polymorphism is determined by four factors namely the therapeutic index of a drug, its pharmacokinetics and metabolism and the frequency of the polymorphism. An allele occurring at a frequency of at least 10% and responsible for metabolising a drug with a narrow therapeutic index would be expected to be clinically significant and appropriate adjustment of dosage in individuals carrying the allele may be necessary (Rodrigues and Rushmore, 2002).

The clinical relevance of the CYP2D6 polymorphism has been investigated in several populations. Asians and Orientals generally have lower CYP2D6 activity compared to Caucasians which is explained by the occurrence of the *CYP2D6*10* allele at high frequency in Orientals. Studies on the implications of the *CYP2D6*10* allele for the use of clinically important CYP2D6 substrate drugs were, therefore, done and are still ongoing with results pointing towards a general reduction of dosages compared to those used in Caucasians

(Poolsup *et al*., 2000 and references therein; Tseng *et al*., 1996; Yue *et al*., 1998; Lai *et al*., 1995). There is, however, very little clinical data on Africans.

In this study, drugs whose clearances were CYP2D6 dependent and affected the most by CYP2D6.17 (table 3.6) may require dose optimising in black Africans. The predicted percentage contribution towards clearance by CYP2D6 of at least 43 %, is generally in agreement with *in vivo* studies, which show differences in plasma concentrations, and in some cases, pharmacodynamics between EMs and PMs (Schadel *et al*., 1995; Jazwinska-Tarnawska *et al*., 2001; von Bahr *et al*., 1991; Dayer *et al*., 1982; Lennard *et al*., 1982; Lewis *et al*., 1984; Nielsen *et al*.1992). We are unable to explain the predicted percentage contribution of more than 100 % for fluphenazine, timolol and bufuralol. This could, however, be associated with the RAF, which has been shown to be dependent on the choice of index substrate (Venkatakrisnan *et al*., 2000).

A study in depressed Tanzanian patients showed that they required lower doses of clomipramine compared to those recommended for Caucasians (Kilonzo *et al*., 1994). The *CYP2D6*17* allele has been found at high frequencies (15-30%) in Tanzanians (Dandara *et al*., 2000; Wennerholm *et al*., 1999). Our study shows a 7-fold difference in CL_{int} values of clomipramine for CYP2D6.1 and CYP2D6.17, with CYP2D6 predicted to contribute significantly (68 %) to its clearance (Table 3.6). The decreased clearance of the drug by CYP2D6.17 could, therefore, explain the observations *in vivo* in which Tanzanian patients respond to lower than recommended doses of clomipramine.

Studies in African-Americans showed higher plasma concentrations of nortriptyline than in Caucasians after the administration of the same drug dose (Ziegler *et al*., 1977). The *CYP2D6*17* allele was shown to occur at a frequency of 14.6 % in African-Americans (Wan

et al., 2001) and could, therefore, explain these clinical observations. In our study, CYP2D6 is also predicted to contribute significantly to the metabolism of propafenone and timolol. There is a 2-3 fold difference in the clearance of these drugs by CYP2D6.1 and CYP2D6.17 that could be of clinical significance. Our study has, therefore, set the stage for a guided choice of drugs requiring dose optimising studies (clomipramine, propafenone, timolol and fluphenazine) in African populations in which the *CYP2D6*17* allele is common.

4.4.4 Implications of the *CYP2D6*17 allele* for CYP2D6 phenotyping studies in African populations

Studies in Caucasians and Orientals have provided almost conclusive genetic explanations for the different phenotypes in the populations. The picture is, however, still unfolding for African populations that show unresolved issues of EM/PM discordance with different probe drugs, no known genetic or environmental explanation for this discordance, and a poor correlation of MRs when using different probe drugs in the same populations.

The extent of the decrease in CL_{int} of the probe drugs by CYP2D6.17 compared to CYP2D6.1 is dependent on the drug (Tables 3.4 and 3.6). Dextromethorphan shows the largest difference, followed by metoprolol, with debrisoquine showing the smallest difference. We would, therefore, expect the increase in MRs (right shift) caused by CYP2D6.17 to be different depending on the probe drug, with dextromethorphan having the largest "right shift". This is consistent with observations made *in vivo* (Wennerholm *et al*., 2002) in which the increase in MRs associated with the *CYP2D6*17*allele was substrate dependent and greatest with dextromethorphan. The *CYP2D6*17* allele, therefore, probably contributes to the poor correlation of MRs between different probe drugs observed in African populations. Data from phenotyping studies (Droll *et al*., 1998; Masimirembwa *et al*., 1996b; Wennerholm

et al., 1999) suggest additional factors such as the *CYP2D6*29* allele, tropical diseases, diet, or unknown mutations. The presence of other yet unknown population-specific mutations seems to be an important factor. This is supported by a recent study in which a non-functional allele previously genotyped as *CYP2D6*2* but exhibiting a discordant phenotype, was shown to be *CYP2D6*42* in an African American (Gaedigk *et al*., 2002).

The CL_{int} of debrisoquine and sparteine by CYP2D6.17, although lower compared to that by CYP2D6.1, was not significantly different and this was in contrast to the *in vivo* studies (Griese *et al*., 1999; Wennerholm *et al*., 2002; Panserat *et al*., 1999; Leathart *et al*., 1998), in which individuals homozygous for the *CYP2D6*17* allele had a 4-10 fold difference in MRs when compared to those carrying the *CYP2D6*1* or *CYP2D6*2* alleles. The lack of a significant difference in our study was also in contrast to recently published *in vitro* studies, in which the CYP2D6 variants were expressed in COS-7 and baculovirus-insect cell systems, with the CL_{int} (V_{max}/K_m) for debrisoquine 4-hydroxylation by CYP2D6.17 being 20% that by CYP2D6.1 (Marcucci *et al*., 2002). We currently do not have an explanation for these disparities. Although CYP2D6.17 consistently showed lower Cl_{int} (V_{max}/K_m) for CYP2D6 substrates in the study by Marcucci *et al*., (2002), it can, however, be noted that kinetic changes (K_m and/or V_{max}) in the metabolism of dextromethorphan, bufuralol and debrisoquine depended on the system used to express the CYP2D6 variants. The use of a yeast expression system in our study could, therefore, in part, explain the above contrasting observations.

Enzyme kinetic results (Table 3.4) show that dextromethorphan is the only probe drug able to distinguish the three allelic variants. CYP2D6.1 has the highest clearance, followed by CYP2D6.2 whilst CYP2D6.17 presents the lowest clearance. This is consistent with *in vivo* observations showing that the *CYP2D6*1* and *CYP2D6*2* alleles were associated with similar activities for debrisoquine (Johansson *et al*., 1993), but *CYP2D6*2* had a lower

activity than *CYP2D6*1* when dextromethorphan was employed (Panserat *et al*., 1999). In a comparative study of the probe drugs in three populations, dextromethorphan was also shown to be the most sensitive in identifying outlying CYP2D6 activity (Droll *et al*., 1998). Dextromethorphan is, therefore, probably the best probe drug for use in resolving individuals carrying the *CYP2D6*1* or *CYP2D6*17* alleles.

CHAPTER FIVE

5.0 Conclusions

The past two decades have been characterised by an explosion of knowledge of determinants of pharmacological and/or toxicological effects of drugs. This has been accompanied by an increase in novel technologies geared towards implementation of this knowledge in designing safe and effective drugs. The CYP superfamily of enzymes represents one such factor of the outcome of drug therapy whose importance has also seen the discovery and development of several *in vitro* and *in silico* technologies for use in assessing drug-CYP interactions. Nowhere is the lack of application of such knowledge, however, more apparent than in tropical medicine, where the antiparasitic drugs in use are toxic and CYP variants unique to the populations in these areas are not well characterised. Work in this thesis, therefore, sought to contribute towards redressing this situation.

The majority of antiparasitic drugs are not expected to pose any risk for clinically significant effects based on their interactions with CYPs, although some of them, however, could result in undesirable interactions with CYPs1A1, 1A2 and 2D6. Potent inhibitors of CYP1A2 included artemisinin, dihydroartemisinin, thiabendazole, niclosamide and primaquine. Cycloguanil, proguanil, amodiaquine, desethylamodiaquine, chloroquine and quinine were potent inhibitors of CYP2D6 while niclosamide was a potent inhibitor of CYP3A4. Of these inhibitors, only artemisinin, thiabendazole and primaquine are predicted to be of clinical significance. The results suggest inhibition of CYP1A2 as the mechanism behind the observed thiabendazole/theophylline and primaquine/antipyrine interactions *in vivo*. Primaquine, quinine and albendazole induced the activities and mRNA levels of CYP1A1 and CYP1A2 with the induction by quinine and albendazole likely to be of significance *in vivo*. Knowledge of these possible interactions with CYPs is critical as it will enable

clinicians to avoid combinations of drugs likely to interact and to also apply the correct interventions when they encounter them. The possible implications of induction of CYP1A by the drugs for carcinogenesis, given the involvement of CYP1A in activation of procarcinogens is, however, not clear. Clearly, the antiparasitic drug-CYP interactions (inhibition or induction) do not explain all the observed toxicities of the drugs, which may be due to other factors such as interactions with membrane receptors or ion channels.

Our study has shown that the amino acid changes in CYP2D6.17 could alter the positions of active site residues causing the reduced activity of the enzyme, with the degree of reduction dependent on the substrate. In addition to other possible factors such as unknown mutations, diet or unreported medications, the *CYP2D6*17* allele might contribute to the poor correlation of phenotyping results when using different probe drugs in African populations. Our study has also highlighted the need for dose optimising studies of some CYP2D6 substrate drugs in African populations.

Future studies

While results of work in this thesis are important, studies are necessary to confirm some of the observations made. The importance of the *CYP2D6*17* allele in disposition of drugs such as clomipramine, propafenone and timolol can be assessed *in vivo* by measuring plasma levels of the drugs in patients or volunteers carrying the allele. Given that the reduced activity exhibited by CYP2D6.17 is substrate dependent, the homology model could be used to identify compounds that could possibly require dose-optimising. With respect to the clinical significance of the inhibitory effects of drugs such as thiabendazole, simple *in vivo* studies can be done in which the inhibitory effects are assessed using probe drugs such as caffeine whose disposition is dependent on the activity of CYP1A2.

CHAPTER SIX

6.0 References

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