STUDY ON HERB-DRUG INTERACTIONS

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Liping Yang

B.Sci.

School of Health Sciences
College of Science, Engineering and Health
RMIT University
March 2009

Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to quality for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Liping Yang _____

Date _____

Acknowledgements

I am most grateful to my supervisors, Associate Professor Chun Guang Li, Associate Professor Shufeng Zhou and my consultant Professor Charlie Changli Xue for their inspiration, outstanding intellect, encouragement, guidance, and support during my study for this degree. Sincere thanks also go to my consultant, Professor Hualiang Jiang and Professor Hong Liu, at Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China, for their invaluable support for my *in silico* studies. Without your support, the thesis would not have been possible.

I would like to greatly thank Dr. Mingyue Zheng, Dr. Cheng Luo and Professor Weiliang Zhu for their knowledge, friendship and support when I conducted my project in Shanghai. Special thanks go to Dr. Tony Lin Zhang for his support in my systematic review of relevant literatures. Many thanks also go to Dr. George Lenon for his help in ethnic application. I wish to thank Dr. Shujun Sheng for his valuable suggestion for my project. The help of the staff and students from the Discipline of Chinese Medicine, School of Health Sciences, RMIT University, Victoria, Australia, have also been most helpful. I would like particularly to acknowledge the contribution of Dr. Ye Shen, Mr. Michael Owen, and Dr. Thomas Cheung.

I wish to thank Professor Emilio Badoer and Dr Martin Stebbing, School of Medical Sciences, RMIT University, for their help and understanding since the first day of my study in RMIT University. Many thanks also go to Dr. Neil Owens and Dr. Ari Kantzides for their kind support for my study in RMIT University.

Most of all I would like to thank my parents, Yirao Yang and Shuqiu Li, who have been a constant source of support – emotional and moral– during my postgraduate years, and this thesis would not have existed without them. Finally, I would like to thank my son, Harry Xin, who is my spiritual source of support to go through all the hard years.

Summary of Thesis

Herbal medicines, such as St John's wort, garlic, gingko, and ginseng, are commonly used complementary therapies. These products are often available over the counter and self-administered along with conventional therapeutic drugs, which raise concerns of potential herb-drug interactions. Most reported herb-drug interactions are pharmacokinetic interactions, through modulation of the activities of cytochrome P450 (CYP), and/or drug transporters. The changes of CYP activities by herbal ingredients may lead to modifications of efficacies of prescribed drugs or result in adverse reactions. Hence, understanding of mechanism of interactions of herbal ingredients with human CYPs is important in evaluating and predicating potential herb-drug interactions and necessary for the safe practice of herbal and conventional medicines.

The human CYP enzymes are a superfamily which consists of at least 57 functional *CYP* genes. Among them, CYP1A2, 2C9, 2C19, 2D6 and 3A4/5 are the most important enzymes responsible for the Phase I metabolism of therapeutic drugs. There is a large variability in the expressions and activities of different CYPs, which are impacted by numerous factors, including genetic (e.g., mutation), host (e.g., diseases), and environmental (e.g., inducers and inhibitors), which makes the metabolism of drugs highly variable in individuals. Inhibition of CYP enzymes is one of the most common causes of harmful drug–drug interactions and some severe adverse reactions due to drug-CYP interactions, which has led to the recent withdrawal of several drugs from the market, such as the nonsedating antihistamine terfenadine.

When different compounds (e.g., a drug and herbal compound) are co-administered, they may compete at the same active site of CYPs, resulting in potential inhibition. We hypothesize that the atom-atom interactions between the ligands and the residues at the active site of CYPs determine the substrate and inhibitor specificity of individual CYPs. To test our hypothesis, we conducted a series of experiments including *in vitro* assays to determine inhibitory actions of a variety of natural compounds on human CYPs, pharmacokinetic-based predication of *in vivo* situation using the *in vitro* data; and *in silico* studies to explore the ligand-CYP interactions using docking and pharmacophore modeling methods.

We first determined the inhibitory effects (IC_{50}) of 56 herbal compounds on activities of five human drug metabolising CYPs (CYP1A2, 2C9, 2C19, 2D6 and 3A4) *in vitro* using a high

throughput approach. The tested herbal components included a variety of structurally distinct compounds such as triterpenoids of danshen (*Salvia miltiorrhiza*), flavonoids and their glycoside derivatives, saponine, other glucosides, lactones, alkaloids, and acids. A small number of them are found to significantly inhibit human CYP1A2, 2C9, 2C19, 2D6 and 3A4 with differential potency, including tanshinone I, tanshinone IIA, cryptotanshinone, baicalein, quercetin, silybin, osthole and γ-schisandrin.

Based on the *in vitro* data obtained, we predicted metabolic herb-drug interactions of these compounds *in vivo* with the application of appropriate pharmacokinetic principles. Some predicting results were consistent with published clinical reports. For example, the prediction of *S. miltiorrhiza* increasing the AUC value of warfarin is consistent with the results from clinical case reports. However, a marked disparity has been observed when some predictions are compared with results from clinical studies. For example, the prediction of *S. mariani* (containing silybin) increasing the AUC of indinavir (a CYP3A4 substrate) is not in agreement with the result of a clinical report where the plasma concentration of indinavir was not altered by co-administered silymarin in healthy volunteers.

Finally, we studied the interactions of a series of ligands including substrates and inhibitors with CYP1A2 using docking and pharmacophore modeling approaches. We have identified 6 residues at the active site of CYP1A2 which are essential for ligand recognition. Furthermore, the relative potency of potential inhibitors could be predicted through analysis of hydrophobic interactions between the ligand and the 6 essential residues at the active site of CYP1A2. Moreover, we developed a pharmacophore model on the basis of the common features of known CYP1A2 inhibitors. In combination with the docking results, the established pharmacophore model could be applied for screening novel CYP1A2 inhibitors.

In conclusion, our *in vitro* and *in silico* studies have provided further insights into the interactions of ligands including herbal components with the active site of CYP1A2, which may be useful for the future studies of herb-drug and herb-CYP interactions. Further studies are warranted to explore the mechanisms underlying herb-CYP and herb-drug interactions.

Table of Content

Table of Figures

Table of Tables

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Liu Y.H., Mo S.L., Di Y.M., Yang L.P., Zhou S.F., (2009). Multidrug Resistance Associated Proteins And Implications In Drug Development. *Clinical and Experimental Pharmacology and Physiology* (Accepted, Impact factor = 2.1).

Yang L.P., Li C.G., Zhou S.F., (2009). High-throughput Screening of Herbal Inhibitors for Human CYP Enzymes. *Combinatorial Chemistry & High Throughput Screening* (Submitted, Impact factor = 2.4)

Yang L.P., Li C.G., Zhou S.F., (2009). Predicting Pharmacokinetic Drug Interactions with Herbs (revision completed)

Published Conference Abstracts

Yang L.P., Li C.G. Danshen: a systematic review of adverse effects and drug interactions. ASCEPT-SEAWP Scientific Meeting November 2007, Adelaide, Australia.

Yang L.P., Xue C., Zhang L., and Li C.G. Adverse effects of Danshen agents: a systematic review. Third International Congress of Complementary Medicine Research, April 2008, Sydney, Australia

Abbreviations

[I]	Inhibitor (herbal components) unbound concentrations
AA	Aristolochic acid
AFB_1	Aflatoxin B_1
AhR	Aromatic hydrocarbon receptor
AhRR	AhR repressor
AIP1	AhR interacting protein 1
ANF	α-Naphthoflavone
ARA9	AhR-associated protein 9
AUC	Area under the concentration-time curve
B[a]P	Benzo[a]pyrene
bHLH	basic helix-loop-helix
CAR	Constitutive androstane receptor
CL	Clearance
CL_h	Hepatic clearance
CL_{tot}	Total clearance
CNV	Copy number variant
COX	Cyclooxygenase
CYP	Cytochrome P450
DAS	Diallyl sulfide
DBD	DNA binding domain
DMSO	Dimethyl sulfoxide
DRE	Dioxin response element
EM	Extensive metabolizer
FAD	Flavin adenine dinucleotide
f_h	Fraction of hepatic clearance (CL_h) in total clearance (CL_{tot})
f_m	Fraction in hepatic metabolism
GR	Glucocorticoid receptor
HBA	Fydrogen bond acceptors
HD	Hydrogen donor
HMG CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A
HNF	Hepatic nuclear factor
Hsp	Heat-shock proteins
5-HT	5-Hydroxytryptamine

HTP	High-throughput
IC_{50}	Concentration causing 50% inhibition
IM	Intermediate metabolizer
K_d	Dissociation constant
K_i	Inhibition constant
K_m	Michaelis-Menten constant
LBD	Ligand binding domain
LT	Leukotriene
MAMC	7-Methoxy-4-(aminomethyl)-coumarin 7-methoxy-4-(aminomethyl)-coumarin
MDMA	Methylenedioxymethamphetamine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
M_r	Molecular mass
NADPH	Nicotinamide adenine dinucleotide phosphate
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NR	Nuclear receptor
NSAID	Non-steroid antiinflammatory drug
PAH	Polycyclic aromatic hydrocarbon
PDB	Protein database
P-gp	P-glycoprotein
PM	Poor metabolizer
PPI	Proton pump inhibitor
PTP	4-Phenyl-1,2,3,6-tetrahydropyridine
PXR	Pregnane X receptor
QSAR	Quantitative structure-activity relationship
RH	Hydrocarbon substrate
ROH	Hydroxylated metabolite
RXR	Retinoic X receptor
SDM	Site-directed mutagenesis
SNP	Single nucleotide polymorphism
SRS	Substrate recognition site
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
UGT	Uridine diphosphate glucuronosyltransferase

UM	Ultra-rapid metabolizer
VKORC1	Vitamin K epoxide reductase complex subunit 1
V_{max}	Maximum velocity
WHO	World health organization
XAP2	X-associated protein 2
XRE	Xenobiotic response element

CHAPTER 1 GENERAL INTRODUCTION

1.1 An Introduction to Human Cytochrome P450s

The cytochrome P450 (CYP), an enzyme superfamily, has been found across all organisms in every kind of life forms but present in diverse shapes in prokaryotic and eukaryotic worlds. In prokaryotes, CYPs present as soluble proteins whereas in eukaryotes they are bound to the membranes of either mitochondrion or the endoplasmic reticulum (de Waziers et al., 1990). The name of CYP derived from its unique character, namely all the enzymes are bound to cell (cyto) membranes and compass a heme pigment (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide (Omura and Sato, 1964).

In general, CYPs are responsible for a vast number of oxidations in nature, resulting in biotransformation of endogenous (e.g. fatty acids and retinoic acid) and exogenous (e.g. drugs and carcinogens) compounds in living bodies. The oxidative reactions catalyzed by CYPs include hydroxylation, *N*-, *O*- and *S*-dealkylation, sulphoxidation, epoxidation, deamination, desulphuration, dehalogenation, peroxidation, and *N*-oxide reduction (Hannemann et al., 2007). Through these oxidation reactions, CYPs process a so-called Phase 1 metabolism for a number of therapeutic drugs, leading to biotransformations of the drugs from hydrophobic forms to hydrophilic forms that are generally less toxic and facilitate their elimination from the body. In some cases, CYPs may form toxic metabolites from drugs (Zhou et al., 2005a).

A typical CYPs reaction is presented by catalysing a reductive scission of molecular dioxygen (bound to the heme iron at the core of the CYP), and then introducing a single atom from oxygen into a hydrocarbon substrate (RH) to generate a hydroxylated metabolite (ROH) and a molecule of water (Guengerich, 2002). During the reaction, two electrons are transferred from nicotinamide adenine dinucleotide phosphate (NADPH) to CYP via electron transfer proteins (flavoproteins or ferredoxin-like proteins, see Eq. 1-1).

$$RH + O_2 + NADPH + 2e^- + H^+ \rightarrow ROH + H_2O + NADP^+ \qquad \text{Equation 1-1}$$

According to the methods of electron delivery from NADPH to catalytic site, CYPs can be divided into four classes (Werck-Reichhart and Feyereisen, 2000): class I CYPs need both a flavin adenine dinucleotide (FAD)-containing reductase and an iron sulphur redoxin, comprised by most prokaryotic bacterial CYPs and eukaryotic mitochondrial CYPs (Ewen et al., 2008); class II CYPs require only a FAD/FMN-containing CYP reductase for electron

transferring, including endoplasmic CYPs (the so-called microsomal CYPs) (Koymans et al., 1993a); class III CYPs require no electron donor and are self-sufficient; and class IV CYPs receive electrons directly from NADPH, which merely exist in fungal CYPs. The classification of the interactions with redux partners is unrelated to CYP evolutionary history. In mammals, the mitochondrial CYPs (class I) are essential for the biosynthesis of vitamin D, bile acids and cholesterol-derived steroid hormones, whereas the functions of microsomal CYPs (class II) are extremely diverse, from biosynthesis of steroid hormones to metabolism of therapeutic drugs. Meanwhile, class III CYPs catalyse the rearrangement or dehydration of alkylhydroperoxides or alkylperoxides initially generated by dioxygenases in both mammals and plants and class IV CYPs reduce nitric oxide (NO) generated by denitrigication nitrous oxide (N_2O) in fungi (Werck-Reichhart and Feyereisen, 2000).

Up to now, more than 7,000 named sequences in the CYP superfamily have been reported in animals, plants, bacteria and fungi (http://drnelson.utmem.edu/CytochromeP450.html, access date: 25 March 2009). In humans, there are 57 functional *CYP* genes (see Table 1-1) and 58 pseudogenes which are grouped into different classes or families. The nomenclature of CYPs employs a three-tiered classification based on amino acid sequence similarity determined through gene sequencing, indicated by an Arabic numeral (family, e.g. CYP1, > 40% similarity), a capital letter (subfamily, e.g. CYP1A, > 55% similarity) and another Arabic numeral (gene, e.g. CYP1A2, > 97% identity comprise alleles) (Brown et al., 2008).

Most of the human CYPs with much narrow substrate specificity are devoted mainly to the metabolism of endogenous substrates, such as sterols, fatty acids, eicosanoids, and vitamins (Guengerich, 2006). However, fifteen individual CYP enzymes in families 1 (1A1 and 1A2), 2 (2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1 and 2F1) and 3 (3A4, 3A5 and 3A7) with a wide-substrate binding profile are heavily involved in xenobiotics (including a number of therapeutic drugs) metabolism (Guengerich, 2006). Among them, CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4/5 are essential for most therapeutic drug oxidations and have been investigated extensively. CYP3A4 is responsible for metabolizing more than 50% of drugs that are CYP substrates (Zhou, 2008b). A typical feature of these drug-metabolizing CYPs is that they exhibit broad and overlapping substrate specificity (Guengerich et al., 2005).

Human CYP enzymes are the most important heme-thiolate enzyme system and are predominantly expressed in the liver, although they are found in practically all tissues, such as

small intestine, lung, kidney, brain, adrenal gland, gonads, heart, nasal and tracheal mucosa, and skin (Pelkonen et al., 2008). In human liver, all CYPs comprise approximately 2% of total microsomal proteins (0.3–0.6 nmol/mg, CYPs/microsomal protein). The relative abundance of individual CYPs in liver has been determined as CYP1A2 (>10%), 2A6 (~10%), 2B6 (<5%), 2C8 (~5%), 2C9 (>15%), 2C19 (<5%), 2D6 (~2-4%), 2E1 (~15%), and 3A4/5/7 (35%) (Guengerich, 2006; Pelkonen et al., 2008). The significance of the individual CYP enzyme in human drug metabolism varies, with CYP3A, CYP2D, and CYP2C being responsible for the metabolism of 50, 25, and 20% respectively of the currently known drugs (Guengerich, 2006). In addition to the liver, the CYPs are expressed appreciably in extrahepatic tissues including small intestinal mucosa, lung, kidney, brain, placenta, olfactory mucosa, and skin, with the intestinal mucosa probably being the most important extrahepatic site of drug biotransformation (Lin and Lu, 2001; Paine et al., 2006).

There is a large variability in the expression and activity of human CYPs. Large interindividual variation in the activity of CYPs is observed, ranging from 20- (CYP2E1 and 3A4) to >1,000-fold (CYP2D6) (Shimada et al., 1994a). The expression and activities of CYPs are impacted by numerous factors, including genetic (e.g., genetic mutation), host (e.g., diseases), and environmental factors (e.g., inducers and inhibitors), making drug metabolism highly variable (Meech and Mackenzie, 1997; Rendic and Di Carlo, 1997; Iyer, 1999; Snyder, 2000). For most CYPs, both environmental and genetic factors have important impact to their expression and activity.

The most common type of genetic variation in the human genome occurs as single nucleotide polymorphisms (SNPs) occurring at a frequency of ≧1% in a given population. Other genetic mutations, such as deletion, insertion and copy number variants (CNVs), have often been observed (Ingelman-Sundberg et al., 2007). Genetic mutations may lead to polymorphism, where two phenotypes, namely poor metabolisers (PMs) and extensive metabolisers (EMs), exist in the population. Poor metabolisers lack detectable activity of a certain enzyme as a result of an autosomal-recessively transmitted defect in its expression, which may lead to greater bioavailability, higher plasma concentrations, prolonged elimination half-life and possibly increased pharmacological response from standard doses of drugs (Ingelman-Sundberg et al., 2007; Zhou et al., 2008). A number of allelic variants have been identified in most human *CYP* genes (http://www.cypalleles.ki.se). The functional impact of these mutations on pharmacotherapy varies, depending on a number of factors. The

polymorphisms within CYP enzymes mainly affect the pharmacokinetics of drugs that are mainly metabolized by those enzymes. The genotype-induced pharmacokinetic changes might be particularly important for certain drugs that have narrow therapeutic windows and there is a high risk for developing adverse drug reactions, such as warfarin and theophylline (Ingelman-Sundberg et al., 2007).

Environmental factors, such as co-administration of two or more drugs/herbs, may significantly change the CYP expression or activity through induction or inhibition and subsequent impact on the pharmacokinetics of the drugs leading to clinically important drug-drug or herb-drug interactions (Lin and Lu, 2001; Zhou and Lai, 2008). The pharmacokinetic changes due to CYP induction and inhibition may occur with a large variety of therapeutic drugs that are extensively metabolized by CYPs (Lin and Lu, 2001).

Overall, almost 50% of the overall elimination of commonly used drugs can be attributed to one or more of the various CYP enzymes in humans (Wilkinson, 2005). CYP activity varies among individuals of a given population. Variability in CYP content and activities can have profound influence on the *in vivo* response of humans to drugs (Nebert and Russell, 2002). Most CYPs are subject to induction and inhibition, and genetic mutations play an important or dominant role in the enzyme activity variation of many CYPs, in particular CYP2A6, 2C9, 2C19, and 2D6 (Ingelman-Sundberg et al., 2007; Zhou et al., 2008). The major substrates, inhibitors and inducers of the principal drug metabolizing CYPs have been listed in Table 1-2.

1.2 Biology and Pharmacology of Human CYPs

1.2.1 Human CYP1A2 enzyme

There are three members in human CYP1 family, CYP1A1, 1A2 and 1B1. The expression of CYP1A2 is major in the liver (~13%) (Shimada et al., 1994a) and slightly in the lung (Wei et al., 2002; Liu et al., 2003), whereas CYP1A1 is mainly expressed in the extrahepatic tissues including intestine (Prueksaritanont et al., 1996; Paine et al., 1999), lung (Shimada et al., 1996b; Willey et al., 1997), placenta (Hakkola et al., 1996a), and lymphocytes (Vanden Heuvel et al., 1993; Dey et al., 2001; van Duursen et al., 2005). CYP1B1 is known to be expressed in almost every tissue, normally in fibroblasts, bone marrow stromal cells and steroidogenic tissues (Hakkola et al., 1997; Heidel et al., 1998) and in the liver at a low level but not in the lung (Hakkola et al., 1997). In humans, CYP1A2 shares 80% amino acid sequence identity with CYP1A1 and about 40% with 1B1, and the substrate specificities of these enzymes often

overlap. However, CYP1A1 as an extrahepatic enzyme is considered to play a minor role in the elimination of therapeutic drugs *in vivo*. Human CYP1 enzymes have demonstrated remarkably overlapping substrate specificities for which the molecular planarity of substrates and inhibitors is a determining factor.

1.2.1.1 Interindividual variability of the expression and activity of CYP1A2

CYP1A2 has been found to show approximately 40- to 130-fold interindividual variations in CYP1A2 expression and activity (Guengerich, 2006). Approximately 15- and 40-fold interindividual variations in CYP1A2 mRNA and protein expression levels have been observed in human livers (Ikeya et al., 1989). These findings may reflex a genetically-determined difference in constitutive and/or inducible *CYP1A2* gene expression. Environmental factors have been found to influence the interindividual differences in CYP1A2 activity and expression.

Unimodal, bimodal and trimodal distributions of CYP1A2 activity when measured by caffeine urinary metabolic ratios have been observed in different study populations (Butler et al., 1992; Nakajima et al., 1994; Catteau et al., 1995; Notarianni et al., 1995). The frequency of PMs in non-smokers was 5% in Australians (Ilett et al., 1993), 14% in Japanese (Nakajima et al., 1994) and 5% in Chinese (Ou-Yang et al., 2000). There is also marked racial difference in CYP1A2 activity. Swedes had a 1.54-fold higher CYP1A2 activity than Koreans (Kall and Clausen, 1995). A lower CYP1A2 activity has been found in Asian and African populations compared to Caucasians (Relling et al., 1992). Environmental factors have been thought to influence the interindividual differences in CYP1A2 activity and expression. Cigarette smoking and intake of oral contraceptive steroids are well established inducers of CYP1A2 activity (Rasmussen et al., 2002). However, it has been suggested that approximately 35 to 75% of the interindividual variability in CYP1A2 activity is due to genetic factors (Rasmussen et al., 2002).

1.2.1.2 Probe substrates of CYP1A2

Several compounds including phenacetin, caffeine and theophylline have been often used as probes for phenotyping CYP1A2 *in vivo*. The marker reactions include phenacetin *O*-deethylation, caffeine *N*-demethylation, and theophylline *N*-demethylation. Phenacetin undergoes oxidative *O*-deethylation to yield acetaminophen by CYP1A1/1A2 and has therefore been used to assess the catalytic activity of CYP1A2 *in vivo* and *in vitro* or to investigate its activity and regulation (Bartoli et al., 1996). Caffeine is predominantly (~95%) metabolised by

CYP1A2 to three metabolic dimethylxanthines and one hydroxylated metabolite and thus is usually used as a "gold standard" probe for determining CYP1A2 activity (Kalow and Tang, 1991; Tassaneeyakul et al., 1992; Tassaneeyakul et al., 1994; Carrillo et al., 2000a; Ryu et al., 2007). Theophylline *N*-demethylation to 3-methylxanthine is catalyzed by CYP1A2, while CYP2E1 and 3A4 catalyze the hydroxylation to 1,3-dimethyluric acid (Gu et al., 1992; Sarkar et al., 1992; Sarkar and Jackson, 1994; Ha et al., 1995; Zhang and Kaminsky, 1995; Tjia et al., 1996).

7-Ethoxycoumarin is a commonly used probe for determining CYP1A2 activity *in vitro* (Waxman and Chang, 2006). Oxidative deethylation of 7-ethoxycoumarin by CYP1A2 (low K_m component) and by CYP2E1 and 2B6 (high K_m components) (Yamazaki et al., 1996) produced 7-hydroxycoumarin (i.e. umbelliferone) which was subsequently metabolized by glucuronidation. 7-Ethoxyresorufin *O*-deethylation is often used as the marker reaction. Similarly, this method can be applied to assay CYP1A1/2-catalyzed formation of resorufin from other alkoxyresorufins, such as 7-methoxyresorufin, 7-benzyloxyresorufin, and 7-pentoxyresorufin (Chang and Waxman, 2006).

1.2.1.3 Therapeutic drugs as substrates of CYP1A2

CYP1A2 metabolises a variety of clinically important drugs, such as adenosine receptor inhibitors (e.g. paraxanthine (1,7-dimethylxanthine) (Tassaneeyakul et al., 1992), theophylline (Sarkar et al., 1992; Sarkar and Jackson, 1994; Ha et al., 1995; Zhang and Kaminsky, 1995), and caffeine (Kalow and Tang, 1991; Tassaneeyakul et al., 1992; Tassaneeyakul et al., 1994; Carrillo et al., 2000a; Ryu et al., 2007)); analgesics (e.g. phenacetin (Tassaneeyakul et al., 1993), paracetamol (Tassaneeyakul et al., 1993), and aminopyrine (Niwa et al., 1999)); antiarrhythmic agents (e.g. mexiletine (Nakajima et al., 1998), amiodarone (Ohyama et al., 2000a), and propafenone (Botsch et al., 1993; Zhou et al., 2003a)); anticancer drugs (e.g. tegafur (Ikeda et al., 2000; Komatsu et al., 2000b), flutamide (Shet et al., 1997; Goda et al., 2006), thalidomide (Miyata et al., 2003), bortezomib (Uttamsingh et al., 2005), and 5,6-dimethylxanthenone-4 acetic acid (Zhou et al., 2000; Zhou et al., 2002)); anticoagulants (e.g. *R*-acenocoumarol (Thijssen et al., 2000), and *R*-warfarin (Hermans and Thijssen, 1993; Kaminsky and Zhang, 1997)); antidepressants (e.g. amitriptyline (Mellstrom and von Bahr, 1981; Olesen and Linnet, 1997; Venkatakrishnan et al., 2000; Venkatakrishnan et al., 2001a), nortriptyline (Mellstrom and von Bahr, 1981; Olesen and Linnet, 1997; Venkatakrishnan et al., 2000; Venkatakrishnan et al., 2001a), imipramine (Koyama et al., 1997), clomipramine

(Nielsen et al., 1996; Wu et al., 1998), duloxetine (Lobo et al., 2008), maprotiline (Brachtendorf et al., 2002), mianserin (Koyama et al., 1996; Stormer et al., 2000), and mirtazapine (Stormer et al., 2000)); antihistamines (e.g. azelastine (Nakajima et al., 1999a), cinnarizine (Narimatsu et al., 1993; Kariya et al., 1996), flunarizine (Narimatsu et al., 1993; Kariya et al., 1996), and diphenhydramine (Akutsu et al., 2007)); antihypertensive drugs (e.g. verapamil (Kroemer et al., 1993), pranidipine (Kudo et al., 1999), and guanabenz (Clement and Demesmaeker, 1997)); anti-migraine drugs (e.g. almotriptan (Wild et al., 1999; McEnroe and Fleishaker, 2005), and zolmitriptan (Wild et al., 1999; McEnroe and Fleishaker, 2005)); antipsychotics (e.g. clozapine (Bertilsson et al., 1994), haloperidol (Fang et al., 2001), promazine (Wojcikowski et al., 2003), olanzapine (Ring et al., 1996), zotepine (Shiraga et al., 1999), and thioridazine (Wojcikowski et al., 2006)); β-blockers (e.g. propranolol (Masubuchi et al., 1994), and carvedilol (Oldham and Clarke, 1997)); cyclooxygenase-2 inhibitors (e.g. rofecoxib (Slaughter et al., 2003)); hypnotics (e.g. zolpidem (Pichard et al., 1995)); 5-lipoxygenase inhibitor (e.g. zileuton (Machinist et al., 1995)); local anaesthetics (e.g. and lidocaine (Orlando et al., 2004), and ropivacaine (Oda et al., 1995)); monoamine oxidase inhibitors (e.g. selegiline (Salonen et al., 2003)); reverse transcriptase inhibitors (e.g. efavirenz (Ward et al., 2003)); selective serotonin reuptake inhibitors (e.g. fluvoxamine (Carrillo et al., 1996)); and serotonin 5-HT_3 receptor antagonists (e.g. ondansetron (Dixon et al., 1995)).

CYP1A2 also plays a role in the metabolism of tacrine (Spaldin et al., 1994; Spaldin et al., 1995), triamterene (a potassium-sparing diuretic) (Fuhr et al., 2005), carbamazepine (Wolkenstein et al., 1998; Pelkonen et al., 2001; Pearce et al., 2002), tizanidine (Granfors et al., 2004a), terbinafine (Vickers et al., 1999), and aminoflavone (NSC686288) (Chen et al., 2006a). Tacrine is a centrally acting cholinesterase inhibitor for the treatment of Alzheimer's disease (Qizilbash et al., 1998); terbinafine is an orally active allylamine derivative that has antifungal activity against dermatophytes and many other pathogenic fungi (Gupta and Shear, 1997); and tizanidine is a centrally acting α_2 adrenergic agonist used as a muscle relaxant (Wagstaff and Bryson, 1997).

CYP1A2 also metabolizes cyclobenzaprine (a long-acting skeletal muscle relaxant) (Wang et al., 1996), naproxen (Miners et al., 1996), and leflunomide (Kalgutkar et al., 2003a). Leflunomide is an orally active disease-modifying anti-inflammatory agent for the treatment of advanced rheumatoid arthritis (Silverman et al., 2005). Propofol, a short-acting intravenous sedative agent used for the induction of general anesthesia, is partially metabolized by

CYP1A2 (Guitton et al., 1998). Riluzole, a drug used to slow the progress of amyotrophic lateral sclerosis (Lou Gehrig's disease) (Bryson et al., 1996; Wokke, 1996; Miller et al., 2007; Radunovic et al., 2007; Groeneveld et al., 2008), is substantially metabolized by CYP1A2 (Sanderink et al., 1997). In addition, the novel Janus kinase-3 inhibitor, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131/JANEX-1), was metabolized by CYP1A1 and 1A2 in a regio-selective manner to inactive 7-*O*-demethylation product 4-(4'-hydroxyphenyl)-amino-6-methoxy-7-hydroxyquinazoline (Uckun et al., 2002). It should be noted that most of above drugs are also metabolized by other CYPs and CYP1A2 plays a variable role in their metabolic clearance.

Overall, CYP1A2 is a major enzyme in the metabolism of a number of important therapeutic drugs, including theophylline, tacrine, acetaminophen, antipyrine, bufuralol, ondansetron, and phenacetin (Guengerich, 1995). With regard to the relative contribution, CYP1A2 is a major enzyme for the metabolism of theophylline, caffeine, phenacetin, and propranolol, with contributions from other CYPs. For other substrates, the contribution of CYP1A2 is often <30%.

1.2.1.4 Bioactivation of procarcinogens and environmental compounds by CYP1A2

CYP1A2 together with CYP1A1 and 1B1 is well known in the bioactivation for a variety of procarcinogens and mutagens, such as PAHs (e.g., benzo[a]pyrene (B[a]P)), heterocyclic aromatic amines/amides (e.g. 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) and mycotoxins (e.g. aflatoxin B_1 (AFB_1)) (Guengerich and Liebler, 1985). CYP1A1 generally metabolizes PAHs, whereas CYP1A2 activates aminofluorenes and nitrosamines. Recombinant CYP1A1 and 1A2 both catalyzed stereo-selective epoxidation of a series of PAHs (Buters et al., 1995; Shou et al., 1996). Oxidation of the chemicals by CYP1A1 and 1A2 serves as an initial step in the conversion of the substrates to more polar metabolites, resulting in increased excretion and thereby maintaining the chemical homeostasis in the body. However, the oxidation of carcinogenic PAHs and heterocyclic aromatic amines/amides gives rise to arene oxide, diolepoxide, and other electrophilic reactive species (ultimate carcinogen) that form DNA and protein adducts, leading to tumor formation and organ toxicity (Ma and Lu, 2007).

In the presence of epoxide hydrolase, CYP1A1/1A2 and 1B1 catalyze the conversion of B[*a*]P to its 7,8-epoxide and consequently to 7,8-dihydrodiol, and both enzymes can in turn metabolically activate this B[a]P metabolite to an ultimate mutagenic species, the dihydrodiol

epoxide (7*R*,8*S*)-dihydroxy-(9*S*,10*R*)-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (Figure 1-1) (Shimada et al., 1996a; Shimada et al., 1999). The first step for the bioactivation of B[a]P is the formation of B[a]P 7,8-oxide catalyzed by CYP1A1/1A2 and 1B1. The second step, catalyzed by epoxide hydrolase, is the hydrolytic conversion of 7,8-oxide to 7,8-diol. Finally, CYP1A1/2 and 1B1 catalyze the further oxidation of the 7,8-diol, producing four possible isomers of 7,8-diol-9,10-epoxide. Quantitatively, the most important of these metabolites is (*7R,8S*)-dihydroxy-(*9S,10R*)-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene which is the ultimate carcinogen binding DNA at the guanine residues and producing DNA adducts. The DNA binding will activate the *H-ras* and *K-ras* oncogenes (Marshall et al., 1984; Bizub et al., 1986; Stevens et al., 1988; Kerzee and Ramos, 2000) and other oncogenes such as *c-Jun*, *E6* and *E7* (Wu et al., 1992; Luch, 2005; Hockley et al., 2006; Jiao et al., 2008). B[a]P 7,8-dihydrodiol is a bay-region diolepoxide that can be reduced to catechol which is further oxidized to generate a reactive quinine metabolite. 7,8-Diol-9,10-epoxide can be conjugated by Phase II enzymes, resulting non-toxic glucuronides and sulfates. Alternatively, B[a]P undergoes oxidation to form 4,5-oxide. On the other hand, a one-electron oxidation pathway may be responsible for the formation of 3- and 6-hydroxy-B[a]P and subsequent metabolites 1,6-, 3,6-, and 6,12-quinones (Van Cantfort et al., 1979; Yun et al., 1992). The 3-hydroxylation of B[a]P is catalyzed by CYP1A2, 2C8, 2C9 and 3A4 (Yun et al., 1992). As such, CYP1A1/2 can convert PAHs to reactive electrophiles that can cause damage of macromolecules such as DNA and functional proteins, producing carcinogenic transformation of the cells.

The critically reactive metabolite of AFB_1 is the *exo* 7,8-epoxide formed by a two-electron oxidation mainly catalyzed by CYP3A4, with contribution from CYP1A1, 1B1, 1A2, 2A6 and 2B6 (Gillam et al., 1993; Penman et al., 1994; Sengstag et al., 1994; Ueng et al., 1995; Crespi et al., 1997). CYP3A4 catalyzes the formation of the genotoxic AFB_1 *exo* 8,9-epoxide only; while CYP1A2 forms both the *exo* and the non-genotoxic *endo* isomers. The *exo* 8,9-epoxide of AFB_1 can bind the N^7 atom of guanine in DNA, resulting in DNA adducts.

1.2.1.5 Metabolism of natural and herbal products by CYP1A2

CYP1A2 plays an important role in the metabolism of a number of natural and herbal compounds, which often results in toxic metabolites. Alkenylbenzenes include simple compounds like safrole, methyleugenol, and estragole, which are present in herbal medicines such as nutmeg, cinnamon, tarragon, basil, fennel, and anise. They are used as a constituent of various food flavours, aromatic oils, spices, perfumes, and detergents. The carcinogenicity of

estragole may be related to its metabolism, which involves the formation of several metabolites, some of which are carcinogenic. The metabolic bioactivation of estragole starts with its conversion into the putative proximate carcinogen 1'-hydroxyestragole by CYP1A2, 2A6, 2C19, 2D6, and 2E1, which is similar to the activation pathway of methyleugenol and safrole (Borchert et al., 1973; Jeurissen et al., 2004; Ueng et al., 2004; Jeurissen et al., 2007).

In 1991, a unique form of nephropathy associated with the long-term use of *Aristolochia fanchi* for slimming purpose was reported in Belgium. More than 100 young women suffered from kidney damage, developing in several patients into renal and urinary tract cancer (Kessler, 2000; Li, 2000; Nortier et al., 2000; Lampert and Xu, 2002). A line of evidence indicates that aristolochic acids (AAs) present in the herb are the compounds responsible for this renal toxicity (Cosyns et al., 1999; Lord et al., 2001; Debelle et al., 2002; Nortier et al., 2003). Thus, the Chinese herb-caused nephropathy is also called AA nephropathy (Stefanovic et al., 2006). AAs are known to be nephrotoxic, genotoxic and carcinogenic (Isnard Bagnis et al., 2004). AAs are a family of structurally related nitrophenanthrene carboxylic acids which are primarily from *Aristolochia* spp. (e.g. *A. fangchi* (Guang Fangji), *A. clematits*, and *A. manshuriensis*) (Ioset et al., 2003; Kumar et al., 2003). The predominant AAs are AAI (8-methoxy-6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid) and AAII (6-nitro-phenanthro-(3,4-*d*)-1,3-dioxolo-5-carboxylic acid) (Kumar et al., 2003). AA is demethylated to form a metabolite for phase II conjugation reactions. As alkaloids, both AAI and AAII underwent reduction of the nitro group catalyzed by oxidative enzymes to reactive cyclic nitrenium ions (Figure 1-2) (Schmeiser et al., 1997; Stiborova et al., 2001a; Stiborova et al., 2001b; Stiborova et al., 2002). Hepatic microsomal CYP1A1/2, NADPH:CYP reductase, DT-diaphorase, xanthine oxidase, cyclooxygenase-1 (COX1) and other peroxidases have been found to catalyze the oxidative reaction (Schmeiser et al., 1997; Stiborova et al., 2001a; Stiborova et al., 2001b; Stiborova et al., 2002). Addition of inhibitors or inducers of CYP1A1/2 was found to decrease or increase the formation of DNA adducts (Stiborova et al., 2001b). The primary route of AAI and II metabolism appears to be the nitro reduction pathway. Aristolactam nitrenium ion in turn can give rise to an isomeric carbonium ion that reacts covalently with DNA (in particular the amino groups of guanine and adenosine) and/or proteins, leading to adduct formation. For example, the DNA adducts (e.g. 7-(deoxyadenosin-N^6-yl)aristolactam I or II and 7(deoxyguanosin-N^2-yl)aristolactam I or II) have been detected in kidney and ureter tissues of patients taking herbs containing AAs, several months or even years after discontinuation of the herbal consumption (Pfau et al., 1990;

Schmeiser et al., 1996; Stiborova et al., 1999; Dong et al., 2006; Mei et al., 2006). An AT→TA mutation also was detected in the p53 gene of a urothelial tumor of a patient with AA nephropathy (Lord et al., 2004). AA-DNA adducts potentially could serve as useful biomarkers of exposure for monitoring of the mutagenic and/or carcinogenic potential of AAs.

CYP1A2 is involved in the oxidative metabolism of some natural flavonoids. Genistein is a soy-derived isoflavone that has been shown to be an effective chemopreventive agent of chemical-induced carcinogenesis *in vivo*. Biochanin A, a 4'-*O*-methyl derivative of genistein, is the major isoflavone in red clover (*Trifolium pratense*) but is not present in soy foods. This compound has also been shown to inhibit chemical-induced tumor carcinogenesis. The major metabolic routes of genistein and biocahnin A are sulfation and glucuronidation, however, several hydroxylated metabolites of genistein have been identified *in vitro* and *in vivo* (Kulling et al., 2002). The oxidative metabolites of genistein and biochanin A are mainly 3'-, 6-, and 8-hydroxylated products (Kulling et al., 2002). CYP1A2 is predominantly responsible for 3'-OH-genistein formation, with contribution from CYP2E1, 2C8 and 3A4 (Hu et al., 2003). Biochanin A can be regarded as a prodrug of genistein and is rapidly converted into the demethylated metabolite genistein *in vitro* and *in vivo* (Tolleson et al., 2002). Tangeretin was also mainly metabolized by CYP1A2 (Breinholt et al., 2003). CYP1A2 also rapidly catalyzed *O*-demethylation of prunetin to genistein, of formononetin (neochanin) to genistein and daidzein, and of 5,4'-dimethoxyisoflavone to formononetin and daidzein, respectively (Hu et al., 2003). Formononetic was also glucuronidated and hydroxylated at 2' and 5' positions. In addition, the flavonols galangin (3,5,7-trihydroxyflavone) and kempferide are metabolized by CYP1A1, 1A2 and 2C9 and (Otake and Walle, 2002). Galangin was oxidized at the 4'-position, whereas kaempferide was *O*-demethylated to 4'-OH-galangin. However, chrysin was not a substrate of CYP1A1 and 1A2.

1.2.1.6 Induction of CYP1A2

Many inducers for CYP1A1 such as 3-methylcholanthrene, 3-methylcholenthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can inducer CYP1A2. Rifampicin is only a weak inducer of CYP1A2 (Backman et al., 2006a). Tobacco smoking and dietary constituents such as cruciferous vegetables and charcoal-broiled meat can induce CYP1A2 activity (Wietholtz et al., 1981; Kalow and Tang, 1991; Fontana et al., 1999). Tobacco and marijuana smoking appears to increase the clearance of theophylline by induction of metabolic pathways (Zevin and Benowitz, 1999). Theophylline clearance has been shown to increase by ~50% in young

adult tobacco smokers and by ~80% in elderly tobacco smokers compared to non-smoking subjects (Grygiel and Birkett, 1981). Passive smoke exposure has also been shown to increase theophylline clearance by up to 50%. Abstinence from tobacco smoking for one week causes a reduction of ~40% in theophylline clearance.

Induction of CYP1A2 activity may also be influenced by coadministration with high-dose (120 mg/day) omeprazole (Han et al., 2002; Yoshinari et al., 2008). Omeprazole induced CYP1A2 in primary human hepatocytes at mRNA and protein levels (Diaz et al., 1990; Masubuchi et al., 1998). A 2- to 10-fold induction of the CYP1A2 protein and CYP1A-dependent activities as determined by the caffeine N^3-demethylation breath test was observed in liver biopsies from cancer patients before and after 4-day treatment with omeprazole at therapeutic doses (Rost et al., 1992). Similar induction was seen in cancer patients taking 20 mg/day for 4 days (Diaz et al., 1990). At a therapeutic dose (40 mg), omeprazole failed to induce CYP1A2 as measured by the caffeine N^3-demethylation breath test in individuals with extensive metabolizer phenotype for CYP2C19, but the induction was revealed at a higher dose (120 mg) in the same individuals (Rost et al., 1999). On the other hand, induction of CYP1A2 by omeprazole was observed in individuals with poor metabolizer phenotype for CYP2C19 at the dose of 40 mg. Clearly, individual variations in the metabolic rate of omeprazole by CYP2C19 affect the intracellular concentration of the inducer (omeprazole) contributing to variability of CYP1A induction (Rost et al., 1992; Rost et al., 1994; Han et al., 2002). However, several other studies did not observed remarkable induction of caffeine and phenacetin metabolism by omeprazole or pantoprazole (Andersson et al., 1991; Rizzo et al., 1996; Hartmann et al., 1999). Notably, omeprazole is a competitive inhibitor of CYP1A2 in vitro with a K_i of 150 μM (Rost et al., 1999).

All members of CYP1 subfamily are regulated by the aromatic hydrocarbon receptor (AhR) through AhR-mediated transactivation following ligand binding and nuclear translocation (see Figure 1-3). AhR is a ligand-activated transcription factor and a basic helix-loop-helix (bHLH) protein belonging to the Per-Arnt-Sim (PAS, where Per stands for *Drosophila* period clock protein, Arnt refers to AhR nuclear translocator and Sim is *Drosophila* single-minded protein) family of transcription factors (Ma, 2001; Ma and Lu, 2007). The bHLH motif is located in the *N*-terminal of the protein and is a common entity in a variety of transcription factors. Members of the bHLH superfamily have two functionally distinctive and highly conserved domains (Burbach et al., 1992). The first is the basic-region (b) which is involved in the binding of the

transcription factor to DNA; while the second is the helix-loop-helix (HLH) region which facilitates protein-protein interactions. AhR contains two PAS domains, PAS-A and PAS-B, which are stretches of 200-350 amino acids that exhibit a high sequence homology to the protein domains that were found in the *Drosophila* genes period (Per) and single minded (Sim) and in Arnt. The PAS domains support specific secondary interactions with other PAS domain-containing proteins, resulting in heterozygous and homozygous protein complexes. The ligand binding site of AhR is within the PAS-B domain that contains several conserved residues critical for ligand binding (Goryo et al., 2007). In addition, a Q-rich domain is located in the *C*-terminal region of AhR, which is involved in co-activator recruitment and transactivation (Kumar et al., 2001).

The mRNA of AhR is dominantly expressed in the placenta, lung, heart, pancreas, and liver (Dolwick et al., 1993). The AhR exists as cytoplasmic aggregates bound to two 90-kDa heat-shock proteins (Hsps), the cochaperone prostaglandin E synthase 3 (p23) and a 43-kDa immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2, also called AhR interacting protein 1, AIP1; or AhR-associated protein 9, ARA9) (Figure 1-3) (Carver et al., 1998; Petrulis et al., 2000; Petrulis et al., 2003; Ogiso et al., 2004; Hollingshead et al., 2006). These other proteins are involved in the correct folding and stabilization of AhR. For example, XAP2 interacts with the *C*-terminal of Hsp90 and binds to the AhR nuclear localization sequence and thus prevents the inappropriate trafficking of the receptor into the nucleus (Petrulis et al., 2000). The dimer of Hsp90 together with p23 protects the receptor from proteolysis, constrain the receptor in a conformation receptive to ligand binding and prevent the premature binding of Arnt (Carver et al., 1994; Carver et al., 1998).

Upon binding a ligand, after the replacement of its associated molecule with Arnt to form a heterodimer with release of 90 kDa HSPs, AhR translocates into the nucleus (Denison and Nagy, 2003). This heterodimer interacts with a 5'-GCGTG-3' DNA sequence, the core binding motif of the xenobiotic response element (XRE) or dioxin response element (DRE) of the target genes (Fujisawa-Sehara et al., 1987; Kubota et al., 1991), located and present in multiple copies in the upstream region of the *CYP1A1* gene promoter. The human *CYP1A1*, the mouse *Cyp1a2*, and the mouse *Cyp1b1* genes harbor 10, 12, and 11 dioxin response element motifs in their respective upstream regions (Zhang et al., 1998; Zhang et al., 2003). The AhR-regulated genes include *CYP1A1*, *1A2*, *1B1*, and *2S1*, *UGT1A1* and *1A6*, *GSTA1* (Yueh et al., 2003).

A Per-Arnt-Sim protein called AhR repressor (AhRR) inhibits AhR signal transduction by competing with AhR for Arnt and also by binding to XRE (Mimura et al., 1999; Baba et al., 2001; Watanabe et al., 2001; Haarmann-Stemmann et al., 2007; Evans et al., 2008). AhRR can be induced by AhR ligands, which represents an efficient negative feedback loop for the regulation of AhR signal transduction (Haarmann-Stemmann and Abel, 2006). The *AhR* knockout mice have been generated, which had decreased liver size and liver deformation, bile duct fibrosis, decreased accumulation of lymphocytes in the spleen and lymph nodes, loss of B[a]P carcinogenicity decreased constitutive expression of Cyp1a2, and resistance to TCDD-induced Cyp1a1 induction (Fernandez-Salguero et al., 1995b; Fernandez-Salguero et al., 1996; McDonnell et al., 1996; Shimizu et al., 2000; Harstad et al., 2006). Two strains of *Arnt*-null mice have also been generated, but these mice die *in utero* (Kozak et al., 1997). Deletion of Xap2 in mice results in cardiac malformation and embryonic lethality (Lin et al., 2007). However, *AhRR*$^{-/-}$ mice are normal and fertile (Hosoya et al., 2008). *AhRR*$^{-/-}$ mice expressed higher levels of Cyp1a1 mRNA induction in the skin, stomach and spleen than wild-type mice, while expression of Cyp1a1 mRNA was not significantly affected in the liver, lung, heart or other tissues, suggesting that the induction of Cyp1a1 mRNA in *AhRR*$^{-/-}$ mice takes place in a tissue-specific manner. *AhRR*$^{-/-}$ mice also displayed a delayed response to skin carcinogenesis caused by B[a]P (Hosoya et al., 2008).

Human AhR has been mapped to chromosome 7.17.3. Cloning of human AhR cDNA revealed that it encodes a protein of 848 amino acid residues (Ema et al., 1994). The human AhR is more similar to the DBA/2 (D2) mouse AhR than to the C57BL/6 (B6) mouse AhR, with two critical determinants reducing ligand-binding affinity observed in D2 AhR: a T to G mutation at the position equivalent to the termination codon (TGA) of the B6 AhR, causing an elongation of the carboxyl terminus, and a Val381 equivalent to the Val375 of D2 AhR replacing Ala375 of B6 AhR. Ligand-affinity differences range between 2- and 6-fold for the B6 and D2 AhRs when cDNA-expressed AHRs are studied. Recombinant human AhR gave a K_d value of 1.58 nM for TCDD in agreement with that of D2 AhR (1.66 nM), ~6-fold higher than that of B6 AhR (0.27 nM); the K_d values of the mouse AhRs are qualitatively similar to those reported earlier (16 nM for D2 and 1.8 nM for B6) (Okey et al., 1989). Human AhR protein consists of many functional domains, including bHLH-PAS (amino acid residues 13-81, 111-181, and 275-342), Hsp90-interacting (27-79 and 182-374), Arnt-interacting (40-79 and 182-374), nuclear localization (13-39), nuclear export (55-75), and transactivation (490-805) domains (Dolwick et al., 1993; Denison and Nagy, 2003). Typical ligands of human AhR are TCDD,

3-methylcholanthrene, and β-naphthoflavone. Several endogenous ligands have also been identified, such as tryptophan derivatives (e.g., indirubin) and arachidonic acid metabolites (e.g., lipoxin A4) (Song et al., 2002).

The regulation of expression of CYP1A1/1A2 is complex because gene transcription not only involves the AhR but also a number of transcription factors, and is potentially influenced by the actions of transcriptional coactivators and corepressors. AhR-mediated signalling pathways provide a first line of defense against potentially toxic environmental contaminants. However, induction of metabolic processes by the AhR can also produce highly carcinogenic metabolites, creating a link between AhR activation and chemical carcinogenesis. Induction of CYP1A1/1A2 is generally a means of maintaining the homeostasis of the chemical environment in cells by increasing the metabolic clearance of substrates. Since CYP1A1/1A2 catalyzes the metabolic activation of PAHs and heterocyclic aromatic amines/amides to ultimate carcinogens, it is expected that induction of the enzyme is detrimental in humans exposed to high levels of PAHs and heterocyclic aromatic amines/amides such as by cigarette smoking. Induction of the enzyme in humans exhibits large variations; high inducibility may impose additional risk for lung cancer to individuals who are smokers (Ma and Lu, 2007). Furthermore, CYP1A2 can metabolize a range of substrates; induction of the enzymes by one substrate may increase the metabolism of other chemicals (for instance, clinical drugs), resulting in unexpected drug-drug interactions.

In addition to the conventional AhR-mediated pathway for the induction of CYP1A1/1A2, omeprazole can trigger the induction of CYP1A1/1A2 not by binding to the AhR, but by activating the AhR via the signal transduction pathways (Backlund et al., 1997). Genistein, a tyrosine kinase inhibitor, and daidzein, an inhibitor of casein kinase II, efficiently inhibited omeprazole-mediated but not TCDD-mediated induction of CYP1A1, as monitored at the transcriptional, mRNA, and protein levels (Backlund et al., 1997). In addition, insulin pretreatment caused an almost complete inhibition of omeprazole-dependent CYP1A1 induction but only partially affected TCDD and B[a]P-mediated induction of CYP1A1. Staurosporine, an inhibitor of protein kinase C, impaired the induction by both omeprazole and B[a]P. In addition, omeprazole has been shown to induce several protein tyrosine kinase targets *in vitro* (Ishida et al., 2002).

Induction of CYP1A2 has important implication for clinical drug-drug interactions (Tang et al., 2005). CYP1A2-mediated caffeine metabolism, as determined by the caffeine breath test, was induced by omeprazole at 40 mg in subjects with a poor metabolizer phenotype for CYP2C19 (Rost et al., 1994). Potent inducers of CYP1A2 may reduce the clearance of drugs whose metabolism is mainly dependent on CYP1A2. On the other hand, the induction of CYP1A2 may increase the risk of carcinogenicity of certain chemicals and contribute to cancer risk. It had been reported that increasing activity of CYP1A2 may be associated with high risk for breast cancer (Hong et al., 2004), meanly due to metabolism modulation of estrogen, a CYP1A2 substrate.

1.2.1.7 Inhibitors of CYP1A2

Several drugs including carbamazepine (Masubuchi et al., 2001), dihydralazine (Masubuchi and Horie, 1998), furafylline (Kunze and Trager, 1993), isoniazid (Wen et al., 2002b), rofecoxib (withdrawn from the market due to its cardiovascular risk) (Karjalainen et al., 2006), and zileuton (Lu et al., 2003) are mechanism-based (suicide) inhibitors of CYP1A2 (Table 1-2). In addition, the deethylated metabolite of amiodarone, desethylamiodarone, can inactivate CYP1A2 (Ohyama et al., 2000b). Furafylline as a mechanism-based inhibitor of CYP1A2 (Kunze and Trager, 1993) is commonly used as a selective inhibitor for CYP1A2 in reaction phenotyping studies. Furafylline is a methylxanthine derivative that was introduced as a long-acting replacement for theophylline in the treatment of asthma (Segura et al., 1986).

Oltipraz, a chemo-protective agent, is a competitive and mechanism-based inhibitor of CYP1A2 (Langouet et al., 2000). *trans*-Resveratrol inactivates CYP1A2, but not CYP1A1 (Chang et al., 2001). Resveratrol selectively inhibits CYP1A1 in a concentration-dependent manner with an IC_{50} of 23 μM (Chun et al., 1999), through blocking of the activation of AhR (Ciolino et al., 1998a). Resveratrol showed 50-fold selectivity in its inhibition of CYP1A1 over 1A2. ε-Viniferin, the dimer of resveratrol, more potently inhibits CYP1A1, 1B1, and 2B6 *in vitro* (Piver et al., 2003). Several other hydroxystilbene compounds obtained from natural sources also showed inhibitory effect on CYP1A1/1A2 activity. Rhapontigenin is a potent mechanism-based inhibitor of CYP1A1 (Chun et al., 2001).

B[a]P and seven other PAH compounds tested inhibited CYP1A2 in a mechanism-based manner, but fluoranthene directly inhibited CYP1A2 (Shimada et al., 2007). All of the nine

PAHs examined were direct inhibitors of CYP1A1 and CYP1B1. Organophosphorothionate pesticides can inactive CYP1A2 and 3A4 (Di Consiglio et al., 2005).

Fluvoxamine, a selective serotonin reuptake inhibitor (SSRI), is a potent and relatively selective CYP1A2 inhibitor with IC_{50} of 0.12-0.30 μM (Brosen et al., 1993; Rasmussen et al., 1995; von Moltke et al., 1996; Becquemont et al., 1997). Other SSRIs, including fluoxetine, norfluoxetine, and sertraline also inhibited CYP1A2-mediated 7-ethoxyresorufin *O*-deethylase activity (Rasmussen et al., 1995). The K_i values for fluoxetine, norfluoxetine, sertraline, desmethylsertraline, and paroxetine were 4.4, 15.9, 8.8, 9.5 and 5.5 μM, respectively (von Moltke et al., 1996). The antidepressant nefazodone and four of its metabolites (*m*-chloro-phenylpiperazine, two hydroxylated derivatives, and a triazoledione) were very weak inhibitors of CYP1A2. Venlafaxine and its *O*- and *N*-desmethyl metabolites showed minimal inhibitory activity toward CYP1A2 (von Moltke et al., 1996). Isosafrole is a selective inhibitor of CYP1A2 (Pastrakuljic et al., 1997).

Many drugs, including oral contraceptives (Abernethy and Todd, 1985) and fluoroquinolones such as levofloxacin and ciprofloxacin (Parker et al., 1994; Granfors et al., 2004c), can inhibit CYP1A2 activity. Propafenone and mexiletine inhibited CYP1A2-mediated phenacetin *O*-deethylation with IC_{50} values of 29 and 37 μM, respectively (Kobayashi et al., 1998). Amiodarone, bepridil, aprindine, lidocaine, flecainide and quinidine inhibited CYP1A2-catalyzed phenacetin *O*-deethylation in a concentration-dependent manner, with IC_{50} values of 86 to 704 μM (Kobayashi et al., 1998). Cimetidine, ranitidine and ebrotidine all inhibited CYP1A2 *in vitro* (Martinez et al., 1999). Miconazole inhibited CYP1A2 with a K_i of 2.9 μM, but fluconazole, itraconazole, micafungin, and voriconazole did not inhibit this enzyme (Niwa et al., 2005). However, venlafaxine (Ball et al., 1997) did not inhibit CYP1A2-mediated ethoxyresorufin *O*-dealkylase and disopyramide, procainamide and pilsicainide (Kobayashi et al., 1998) did not inhibit CYP1A2-catalyzed phenacetin *O*-deethylation.

Some natural compounds can inhibit CYP1A2 and 1A1. Rutaecarpine, evodiamine, and dehydroevodiamine are quinazolinocarboline alkaloids isolated from *Evodia rutaecarpa*, which has been used in traditional Chinese medicine for the treatment of gastrointestinal disorder, headache, and hypertension. They are all inhibitors of CYP1A1 and 1A2, with rutaecarpine

being the most potent (Ueng et al., 2002). Phenethyl isothiocyanate is a competitive inhibitor of CYP1A2 (Nakajima et al., 2001).

Inhibition of CYP1A2 by drugs has important implications in drug-drug interactions. For example, coadministration of ciprofloxacin (a CYP1A2 inhibitor) and tizanidine (a CYP1A2 substrate) had demonstrated to increase the risk of hypotension, adverse effect of overdose of tizanidine (Granfors et al., 2004c).

1.2.2 Human CYP2C9 enzyme

The CYP2C subfamily comprises CYP2C8, 2C9, 2C18 and 2C19, metabolizing about 20% of clinical drugs (Totah and Rettie, 2005). CYP2C8, 2C9, and 2C19 proteins are primarily located in the liver where they account for approximately 20% of total CYP contents (Shimada et al., 1994a), whereas CYP2C18 protein seems to be primarily expressed in the skin (Zaphiropoulos, 1997). Low levels of CYP2C mRNAs and proteins have also been found in small intestine and other extra-hepatic tissues (Klose et al., 1999). A number of drugs are metabolized by CYP2C members, with CYP2C8 and 2C18 exhibiting a similar substrate specificity to that of 2C9 or 2C19 but with altered V_{max} and/or K_m. The human *CYP2C* genes are mapped to chromosome 10q24 in the following order: Cen-*CYP2C18*-*CYP2C19*-*CYPPP2C9*-*CYP2C8*-Tel (Gray et al., 1995).

1.2.2.1 Substrates of CYP2C9

CYP2C9 is one of the most abundant CYP enzymes in the human liver (~20% of hepatic total CYP content), where it metabolizes approximately 15% clinical drugs (>100 drugs), including a number of drugs with narrow therapeutic ranges (Miners and Birkett, 1998). *S*-Flurbiprofen (4'-hydroxylation) (Yamazaki et al., 1998), *S*-warfarin (7-hydroxylation) (Yamazaki et al., 1998), tolbutamide (methylhydroxylation), phenytoin (4'-hydroxylation) (Giancarlo et al., 2001), losartan (oxidation) (Lee et al., 2003), and diclofenac (4'-hydroxylation) (Yamazaki et al., 1998) have been commonly used as probe substrates for CYP2C9 (Kumar et al., 2006). Diclofenac 4'-hydroxylase and tolbutamide methylhydroxylation have been well studied as marker reactions of CYP2C9 activity and are most commonly used in CYP2C9 phenotyping studies, although some activity of other CYP2C enzymes for these substrates has been observed (Wester et al., 2000). Flurbiprofen can be included into the 5-drug Pittsburgh cocktail without showing metabolic interactions (Zgheib et al., 2006).

The substrates of CYP2C9 include oral sulfonylurea hypoglycemics (e.g. tolbutamide, glyburide, glimepiride, gliclazide and glipizide), non-steroid antiinflammatory drugs (NSAIDs, e.g. diclofenac, ibuprofen, ketoprofen, suprofen, naproxen, flurbiprofen, indomethacin, meloxicam, piroxicam, tenoxicam, and lornoxicam); selective COX2 inhibitors (e.g. celecoxib, lumiracoxib, etoricoxib, and valdecoxib), diuretics (e.g. torasemide and sulfinpyrazone), antiepileptics (e.g. phenytoin and phenobarbital), angiotensin II receptor inhibitors (e.g. losartan, irbesartan, and candesartan), anticancer drugs (e.g. cyclophosphamide and tamoxifen), and anticoagulants (e.g. *S*-acenocumarol, phenprocoumon and *S*-warfarin) (Miners and Birkett, 1998; Rettie and Jones, 2005).

The non-sulfonylurea antidiabetic drug, nateglinide is extensively metabolized (~70%) by CYP2C9 and partially by CYP3A4 (McLeod, 2004). Ketobemidone, an opioid analgesic structurally related to pethidine, is mainly metabolized by CYP2C9 and 3A4 via *N*-demethylation to norketobemidone (Yasar et al., 2005). Methadone is partially metabolized by CYP2C9, although CYP2B6, 2C19 and 3A4 may play a more important role in its metabolism (Foster et al., 1999; Gerber et al., 2004). Sulfamethoxazole, a sulfonamide bacteriostatic antibiotic, is eliminated mainly by metabolism, and CYP2C9 plays an important role in its N^4-hydroxylation (Cribb et al., 1995). Terbinafine is mainly metabolized by CYP1A2, 2C9 and 3A4 (Vickers et al., 1999). Sildenafil is converted to its major circulating metabolite, UK-103,320, by CYP2C9 and 3A4, with contribution from CYP2C19 and 2D6 (Warrington et al., 2000). Vicriviroc (SCH 417690), a CCR5 receptor antagonist, is mainly metabolized by CYP2C9 (Ghosal et al., 2007). In addition, dapsone *N*-hydroxylation is mainly catalyzed by CYP2C9, with minor contribution from CYP2C8 and 2C19 (Winter et al., 2000).

CYP2C9 participates in the oxidation of several important endogenous compounds such as progesterone (Yamazaki and Shimada, 1997), testosterone (Yamazaki and Shimada, 1997), 17α-ethinylestradiol (Ball et al., 1990; Wang et al., 2004), all-*trans*-retinoic acid (Marill et al., 2000). CYP2C9 is also involved in the metabolism of arachidonic acid (Rifkind et al., 1995). This will result in biologically active epoxyeicosatrienoic fatty acids (e.g. 11,12- and 14,15-epoxyeicosatrienoic fatty acids) and hydroxyeicosatetraenoic fatty acids (e.g. 7-, 11-, 13-, or 15-hydroxyeicosatetraenoic fatty acids).

1.2.2.2 Induction of CYP2C9

Like CYP2C8, rifampicin and phenobarbital induced CYP2C9, and to a lesser extent CYP2C19 mRNAs and proteins in primary human hepatocytes (Gerbal-Chaloin et al., 2001). The concentration dependence of CYP2C8 and 2C9 mRNAs in response to rifampicin and phenobarbital paralleled that of CYP3A4 and 2B6, the maximum accumulation being reached with rifampicin at 10 μM or phenobarbital at 100 μM. Phenobarbital is not a potent inducer of *CYP2C8* and *2C9* genes. In contrast, dexamethasone resulted in maximum induction of CYP2C8 and 2C9 mRNAs at 0.1 μM while CYP3A4 and 2B6 were not induced. Dexamethasone, which has been recently shown to up-regulate pregnane X receptor (PXR) and constitutive androstane receptor (CAR) expression through the glucocorticoid receptor (GR/NR3C1), potentiated CYP2C8 and 2C9 mRNA induction in response to rifampicin and phenobarbital. Therefore, PXR/NR1I2, CAR/NR1I3, and GR/NR3C1 are all involved in the regulation of CYP2C9. In contrast to the other CYP2C messengers, CYP2C18 mRNA was not inducible in cultures human hepatocytes (Gerbal-Chaloin et al., 2001).

There are two DR1 elements at –152 and –185 bp of the promoter region of the *CYP2C9* gene, and hepatic nuclear factor-4α (HNF-4α/NR2A1) can activate the transcription of this gene via the DR1 element in HepG2 cells (Ibeanu and Goldstein, 1995; Chen et al., 2005b). HNF-4α/NR2A1 synergizes with CAR/NR1I3 and PXR/NR1I2 in HepG2 cells treated with rifampicin when the CAR/PXR binding site at –1839 bp is present (Chen et al., 2005b). Mutation of the two HNF-4α/NR2A1 binding sites differentially prevented up-regulation of *CYP2C9* promoter by both CAR/NR1I3 and HNF-4α/NR2A1; synergy between the two receptors essentially abolished induction by rifampicin in HepG2 cells transfected with PXR/NR1I2. These findings suggest that there is cross-talk between distal CAR/PXR sites and HNF-4α/NR2A1 binding sites in the *CYP2C9* promoter and that the HNF-4α/NR2A1 sites are required for maximal induction of the *CYP2C9* promoter.

Several clinical reports have focused on the changed pharmacokinetic parameters of drugs known as CYP2C substrates, in patients receiving rifampicin, dexamethasone, phenobarbital, or a high concentration of prednisone. For example, the systemic clearance of phenytoin, tolbutamide, and *S*-warfarin exhibited a 2- to 3-fold increase in patients receiving rifampicin, suggesting clinically significant CYP2C9 induction. Dexamethasone increased phenytoin clearance (McLelland and Jack, 1978; Wong et al., 1985), suggesting a clinically significant induction of CYP2C9. In addition, phenobarbital and prednisone decreased the half-life of

elimination of cyclophosphamide, a drug recently shown to be a low K_m substrate of CYP2C9 and 2C19, whereas dexamethasone produced an increase in the body clearance of this drug (Zhang et al., 2005; Zhang et al., 2006).

1.2.2.3 Inhibitors of CYP2C9

Sulfaphenazole is a commonly used selective inhibitor of CYP2C9 with K_i of 0.3 μM, but it has some inhibitory effects toward the other CYP2C8 (K_i = 63 μM) and 2C18 (K_i = 29 μM) (Mancy et al., 1996). Glyburide inhibited CYP2C9-catalyzed *S*-warfarin and phenytoin metabolism in a competitive manner, with K_i values of 2.4 and 3.1 μM, respectively (Kim and Park, 2003).

Sulfamethoxazole has been shown to inhibit CYP2C9-mediated tolbutamide hydroxylation with an apparent K_i value of ~250 μM (Komatsu et al., 2000a). It appears that trimethoprim and sulfamethoxazole are selective inhibitors of CYP2C8 and 2C9, respectively (Wen et al., 2002a). With concentrations ranging from 50 to 500 μM, sulfamethoxazole was a selective inhibitor of CYP2C9-mediated tolbutamide hydroxylation in human liver microsomes and recombinant CYP2C9, with apparent IC_{50} values of 544 and 456 μM, respectively (Wen et al., 2002a). Trimethoprim showed a selective inhibitory effect on CYP2C8-mediated paclitaxel 6α-hydroxylation in human liver microsomes and recombinant CYP2C8, with apparent IC_{50} values of 54 and 75 μM, respectively. Trimethoprim is frequently combined with sulfamethoxazole as cotrimoxazole, a broad-spectrum antibacterial agent, to treat a wide range of infections. Trimethoprim and sulfamethoxazole have increased the plasma concentrations or therapeutic effects of drugs such as tolbutamide (Wing and Miners, 1985), phenytoin (Hansen et al., 1979), warfarin (O'Reilly, 1980), and glipizide (Johnson and Dobmeier, 1990). Trimethoprim alone inhibited the metabolic clearance of tolbutamide by 14% and phenytoin by 30% in humans. Inhibition of CYP2C8/9 enzymes is considered the major mechanism for these drug interactions.

Kumar *et al*. (2006) investigated the inhibitory effects of 28 compounds which are mostly substrates of CYP2C9 on the oxidation of 5 probes of CYP2C9 (*S*-flurbiprofen, *S*-warfarin, tolbutamide, phenytoin, and diclofenac). They found that the estimated K_i value was ≤1.0 μM for 16 of the 28 inhibitors of *S*-warfarin hydroxylation in CYP2C9.1, including benzbromarone (0.001 μM); nicardipine (0.01 μM); miconazole (0.01 μM); ketoconazole (0.08 μM); dapsone (0.09 μM); sulfaphenadine (0.12 μM); quercetin (0.25 μM); α-naphthoflavone (0.29 μM);

nifedipine (0.34 μM); Vivid Green (0.53 μM); fluvoxamine (0.58 μM); omeprazole (0.64 μM); tamoxifen (0.66 μM); gemfibrozil (0.79 μM); piroxicam (0.92 μM); tolbutamide (1.0 μM). In contrast, only eight, six, nine, and nine of the inhibitors exhibited K_i values <1 μM against *S*-flurbiprofen hydroxylation, phenytoin hydroxylation, tolbutamide hydroxylation, and diclofenac hydroxylation, respectively. An additional eight compounds exhibited K_i values between 1 and 10 μM toward *S*-warfarin hydroxylation, resulting in 24 of 28 compounds exhibiting K_i values <10 μM toward this reaction (Kumar et al., 2006). For the other four probe substrates, the majority of inhibitors fell within this 1 to 10 μM range for the K_i values. Quinine was a relatively potent inhibitor of *S*-flurbiprofen hydroxylation with a K_i of 1.1 μM but was a very poor inhibitor of the oxidation of the other four probe substrates (K_i: 20 to >100 μM). Indomethacin was a very potent (K_i = 0.7 μM) inhibitor of *S*-warfarin hydroxylation but a relatively weak (K_i > 10 μM) inhibitor of all other probe substrates. Finally, *S*-ibuprofen was a poor (K_i > 40 μM) inhibitor of *S*-warfarin hydroxylation but a relatively potent (K_i ~4 μM) inhibitor for other four probe substrates.

1.2.3 Human CYP2D6 enzyme

CYP2D6 accounts for only a small percentage of all hepatic CYPs (~2%), however, it metabolises ~25% of all medications in the human liver (Cascorbi, 2003; Ingelman-Sundberg, 2005; Gardiner and Begg, 2006; Ingelman-Sundberg et al., 2007). The primarily hepatic expression of this enzyme governs first pass metabolism after oral drug administration, whereas the low level of its intestinal expression does not appear to be important. CYP2D6 has been identified in human kidney (Nishimura et al., 2003), intestine (Prueksaritanont et al., 1995; Madani et al., 1999; Nishimura et al., 2003), breast (Huang et al., 1997), lung (Guidice et al., 1997; Bernauer et al., 2006), placenta (Hakkola et al., 1996b) and brain (Siegle et al., 2001; Chinta et al., 2002; Miksys et al., 2002) at low to moderate levels. In fetal liver, CYP2D6 mRNA was undetectable (Hakkola et al., 1994). CYP2D6 protein and enzyme activity toward bufuralol have been detected at low levels in human intestine and are differentially expressed along the length of the gastrointestinal tract (de Waziers et al., 1990; Prueksaritanont et al., 1995; Madani et al., 1999). CYP2D6 expression is highest in the jejunum and decreased distally to the colon. However, CYP3A4/5 is the most expressed CYP enzyme in human small intestine (McKinnon et al., 1995), whereas CYP2D6 and 2C19 are less expressed enzymes. The expression level of CYP2D6 was 3-fold lower in bronchial mucosa and 6-fold lower in lung parenchyma compared to that in the liver (Guidice et al., 1997). CYP2D6 is expressed constitutively in neurons in human brain (Siegle et al., 2001). CYP2D6 protein was primarily

found in large principal neurons such as pyramidal cells of the cortex, pyramidal cells of the hippocampus, and Purkinje cells of the cerebellum (Siegle et al., 2001). In glial cells, CYP2D6 protein was absent. Higher expression of CYP2D6 was detected in brain regions of alcoholics compared to non-alcoholics (Miksys et al., 2002).

1.2.3.1 Substrates of CYP2D6

Sparteine and debrisoquine are two prototypical substrates of CYP2D6, which are widely used to determine the phenotype of CYP2D6. Debrisoquine was used as an anti-hypertensive agent and its 4-hydroxylation (so CYP2D6 is called debrisoquine 4-hydroxylase) is primarily mediated by the polymorphic CYP2D6 (Eiermann et al., 1998). Dextromethorphan, a synthetic analog of narcotic analgesics, is also a commonly used CYP2D6 probe *in vitro* and *in vivo*. In humans, it is primarily excreted as the unchanged parent drug and dextrorphan (Barnhart, 1980), which is pharmacologically active (Braga et al., 1994). In addition, bufuralol, a β-adrenoceptor blocker, has been extensively used as a probe substrate for the *in vitro* study of CYP2D6 activity.

CYP2D6 is a critical enzyme responsible for the metabolism of more than 100 therapeutic drugs although it only accounts for a small percentage (~2%) of all hepatic CYP enzymes. CYP2D6 can metabolize a number of drugs, including antidepressants (e.g. desipramine (Murphy et al., 2000), clomipramine and fluoxetine), neuroleptics (e.g. haloperidol), β-blockers (e.g. metoprolol (Yuan et al., 2008) and nebivolol (Lefebvre et al., 2007)), antiarrhythmics (e.g. debrisoquine (Eiermann et al., 1998)), analgesics (codeine (Kirchheiner et al., 2007) and oxycodone (Heiskanen et al., 1998)), antiemetics (ondansetron and tropisetron (Kaiser et al., 2002)) and anticancer drugs (cyclophosphamide) (Huang et al., 2000). Many of these drugs have narrow therapeutic index.

CYP2D6 also extensively metabolizes opioids (e.g. codeine, dihydrocodeine and tramadol), antiemetics (e.g. tropisetron, ondansetron, dolasetron, and metoclopramid), antihistamines (e.g. terfenadine (Jones et al., 1998), oxatomide (Goto et al., 2004), loratadine (Yumibe et al., 1995; Yumibe et al., 1996), astemizole (Matsumoto and Yamazoe, 2001), epinastine (Kishimoto et al., 1997), promethazine (Nakamura et al., 1996), mequitazine (Nakamura et al., 1998), azelastine (Imai et al., 1999; Nakajima et al., 1999a), diphenhydramine and chlorpheniramine), and antiarrhythmics (e.g. sparteine, propafenone, encainide, flecainide, cibenzoline, aprindine, lidocaine, procainamide and mexiletine).

CYP2D6 metabolizes drugs of abuse of amphetamine type such as methamphetamine ('meth', 'ice'), methylenedioxymethamphetamine (MDMA, 'ecstasy'), *N*-ethyl-3, 4-methylenedioxyamphetamine ('eve'), and 3, 4-methylenedioxyamphetamine (i.e. tenamfetamine, 'the love drug') (Lin et al., 1997; Wu et al., 1997; Kreth et al., 2000; Segura et al., 2005). CYP2D6 is the primary enzyme for the CYP2D6 in their aromatic 4-hydroxylation and *N*-demethylation (Lin et al., 1997). Similarly, MDMA is metabolized to methylenedioxyamphetamine via demethylation by CYP2D6 as a high-affinity enzyme, with low-affinity contributions from CYP1A2, 2B6, and 3A4 (Tucker et al., 1994; Lin et al., 1997; Kreth et al., 2000). However, CYP2D6 did not *N*-demethylated MDMA (Lin et al., 1997).

CYP2D6 has also been shown to metabolize carcinogens and neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Coleman et al., 1996; Gilham et al., 1997; Modi et al., 1997; Kalgutkar et al., 2003b), 1,2,3,4-tetrahydroquinoline (Ohta et al., 1990), and indolealkylamines (Yu et al., 2003b). MPTP is a neurotoxin and potent inducer of experimental Parkinson's disease in nonhuman primates (Barsoum et al., 1986; Jenner, 2003; Emborg, 2007). Besides MAO-B-mediated bioactivation of MPTP to the positively charged mitochondrial neurotoxin *N*-methyl-4-phenylpyridinium (MPP^+), CYP2D6, 1A2 and 3A4 metabolize MPTP to the corresponding non-neurotoxic *N*-4-(4'-hydroxyphenyl)-*N*-methyl-1,2,3,6-tetrahydropyridine and 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) metabolites via *N*-demethylation (Coleman et al., 1996; Modi et al., 1997). The high affinity activity toward MPTP was absent in liver microsomes from a PM subject (Coleman et al., 1996). Rat CYP2D and 2C can *N*-demethylate MPTP (Narimatsu et al., 1996) and female Dark Agouti rats are more sensitive to MPTP neutotoxicity than other strains (Jimenez-Jimenez et al., 1991). CYP2D6 efficiently hydroxylated various β-carbolines (Herraiz et al., 2006). N(2)-methyl-1,2,3,4-tetrahydro-β-carboline, a close MPTP analog, is extensively hydroxylated to 6-hydroxy-N(2)-methyl-1,2,3,4-tetrahydro-β-carboline and a corresponding 7-hydroxy-derivative (Herraiz et al., 2006). CYP2D6 is also involved in the metabolism of diuron, a widely used herbicide and antifouling biocide (Abass et al., 2007).

A study using the CYP2D6-humanized mouse line has established that CYP2D6 is a 5-methoxyindolethylamine *O*-demethylase (Yu et al., 2003b) and 5-methoxytryptamine, a metabolite and precursor of melatonin (*N*-acetyl-5-methoxytrytamine), is metabolized by

CYP2D6 to 5-hydroxytryptamine (5-HT/serotonin) with a high turnover of 51.7 min^{-1} and relatively low K_m of 19.5 μM (Yu et al., 2003a). Recombinant CYP2D6 exhibited remarkable ability to convert *p*-tyramine and *m*-tyramine to dopamine. Human CYP2D6 and rat CYP2D4 are the predominant CYP2Ds in the brain and exhibit 21-hydroxylation activity toward progesterone and its metabolite 17α-hydroxyprogesterone (Kishimoto et al., 2004).

1.2.3.2 Induction of CYP2D6

By employing cultured human hepatocytes, the induction of CYP1A, 2A,2B, 2C, 2E, and 3A subfamilies has been reported (Rodriguez-Antona et al., 2000; Gerbal-Chaloin et al., 2001). In contrast to these CYP enzymes, none of the model inducers examined increased levels of CYP2D6, 2E1, and 4A11 in 72-hr cultured human liver slices. For CYP2D6, previous studies have suggested that this P450 enzyme is refractory to induction by known inducers of other CYP subfamilies (Rodriguez-Antona et al., 2000). In cultured precision-cut human liver slices, treatment with 50 μM concentrations of β-naphthoflavone, lansoprazole, rifampicin, dexamethasone, and methylclofenapate or 500 μM sodium phenobarbital did not induce CYP2D6, with little effect on CYP2C8, 2C9, 2E1, and 4A1 (Edwards et al., 2003). Phenobarbital or rifampin failed to cause notable induction of CYP2D6 activity (Madan et al., 2003). Ritonavir and nelfinavir did no induce CYP2D6 in human hepatocytes, but significantly induced CYP1A2, 2B6, 2C9, 2C19 and 3A4 (Dixit et al., 2007). Using human enterocytes collected from 6 healthy subjects before and after 10 days of 600 mg/day oral rifampicin administration, CYP2D6 was not induced (Glaeser et al., 2005).

In contrast to other CYPs, CYP2D6 is generally not regulated by many known environmental agents and is not inducible by common known steroids (Bock et al., 1994). However, interindividual differences in response to drugs metabolized by CYP2D6 may also be influenced modestly by hormonal state, diet, and by xenobiotic regulation of expression of the enzyme in liver and extrahepatic organs such as brain, kidney, and intestine (Llerena et al., 1996; Miksys et al., 2002).

In vitro and *in vivo* studies indicate that the nuclear receptors (NRs) including PXR, CAR and GR do not appear to play a role in the regulation of CYP2D6. Prototypical inducers such as phenobarbital, rifampin and dexamethasone do not inducer CYP2D6 in cultured human hepatocytes (Edwards et al., 2003; Madan et al., 2003). Additionally, no or minor to moderate clinical drug interactions between P450 inducers and drug substrates that are mainly

metabolized by CYP2D6 have been reported (Branch et al., 2000). Therefore, the currently available data suggest that variability of CYP2D6 is largely governed by genetic factors, which is consistent with the large number of *CYP2D6* allelic variants that have been identified to date.

HNF-4α, a member of the nuclear receptor superfamily, is mainly expressed in a restricted manner in the liver, intestine, kidney, and pancreas (Mendel and Crabtree, 1991). It plays an important role in the regulation of many liver-specific genes, such as those encoding apolipoproteins, coagulation factors, and CYPs (Mendel and Crabtree, 1991; Erdmann and Heim, 1995). A direct-repeat element with a one-nucleotide spacer located in the proximal promoter region of the *CYP2D6* gene plays an important role in modulating CYP2D6 expression, and HNF-4α interacts with this binding element (Cairns et al., 1996). Cotransfection of the minimal *CYP2D6* promoter -CAT construct (-392 bp) with a mammalian *HNF-4α* expression vector resulted in a 30-fold induction of CAT activity in COS-7 cells. Although HNF-4α was originally identified as an orphan receptor, fatty acyl-CoA thioesters are identified to be endogenous ligands for HNF-4α (Hertz et al., 1998; Petrescu et al., 2002). The binding of ligand may shift the oligomeric-dimeric equilibrium of HNF-4α or may modulate the affinity of HNF-4α for its cognate promoter element, resulting in either activation or inhibition of HNF-4α transcriptional activity as a function of chain length and the degree of saturation of the fatty acyl-CoA ligands (Petrescu et al., 2002). The HNF-4α binding element is conserved in the proximal promoter regions of more than 20 *CYP2* genes (Chen et al., 1994; Ibeanu and Goldstein, 1995). Recently, Jover *et al*. (2001) demonstrated that HNF-4α plays a general role in the regulation of major P450 genes, including *CYP3A4*, *CYP3A5*, *CYP2A6*, *CYP2B6*, *CYP2C9*, and *CYP2D6*, in human hepatocytes using antisense technique. By using small interfering RNA technique, Kamiyama *et al*. (2007) found that suppression of HNF-4α caused decrease in the mRNA levels of CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, UGT1A1, UGT1A9, ABCB1, ABCB11, and ABCC2, as well as those of PXR and CAR. In addition, deletion of HNF-4α decreased debrisoquine 4-hydroxylase activity in *CYP2D6* humanized mice more than 50% (Corchero et al., 2001). These findings indicate that HNF-4α may act as a common regulator of the liver-specific transcription of many P450 genes.

1.2.3.3 Inhibitors of CYP2D6

A number of CYP2D6 substrates and other compounds have been found to inhibit CYP2D6 and this has important clinical implications when drugs are coadministered. Many antipsychotic drugs including chlorpromazine, fluphenazine, perphenazine, haloperidol, thioridazine, risperidone, clozapine, trifluperidol, and zuclopenthixol are metabolized by CYP2D6 and also significantly inhibit this enzyme (Shin et al., 1999). Metoclopramide, a gastroprokinetic and antiemetic agent, is a substrate and inhibitor of CYP2D6 (Desta et al., 2002a). Terfenadine, a nonsedating H_1 receptor antagonist, could interact with CYP2D6, either as a substrate or as an inhibitor (Smith and Jones, 1992; Jones et al., 1998).

Quinidine and fluoxetine are competitive inhibitors of CYP2D6, which did not exhibit a preincubation-dependent increase in inhibitory potency. Quinidine, pimozide and halofantrine compete for the substrate-binding site of CYP2D6 but are not metabolized by it (Otton et al., 1988). Terbinafine, used for the treatment of superficial dermatophytosis, inhibited dextromethorphan *O*-demethylation with an apparent K_i ranging from 28 to 44 nM in human hepatic microsomes and averaging 22.4 nM for the heterologously expressed enzymes (Abdel-Rahman et al., 1999). Terbinafine is not metabolized by any CYPs. A number of anti-HIV agents are CYP2D6 inhibitors. Ritonavir inhibits CYP2D6 *in vitro* (von Moltke et al., 1998a) and *in vivo* (Aarnoutse et al., 2005). Indinavir, saquinavir, nelfinavir, and delavirdine are all CYP2D6 inhibitors (von Moltke et al., 1998a; Voorman et al., 2001). Amobarbital, valproic acid, ethosuximide, caffeine, theophylline, disopyramide and phenytoin are not inhibitors of CYP2D6 (Broly et al., 1990). In addition, both bupropion and hydroxybupropion inhibited CYP2D6-mediated dextromethorphan *O*-demethylation, with IC_{50} values of 58 and 74 μM, respectively (Hesse et al., 2000).

Progesterone, testosterone, pregnanolone, pregnenolone, 17β-estradiol, and 17α-hydroxyprogesterone competitively inhibited CYP2D6 activity, whereas epiallopregnanolone and alfaxalone non-competitively inhibited the activity (Hiroi et al., 2001). Progesterone and testosterone inhibited bufuralol 1'-hydroxylation with K_i values of 33 and 63 μM, respectively. All these steroids lack the basic nitrogen atoms and are thus atypical substrates of CYP2D6.

Paroxetine (an SSRI) inhibits CYP2D6 activity at IC_{50} concentrations ranging from 150 nM to 2.0 μM, depending on the substrate (Fogelman et al., 1999). Paroxetine is also a mechanism-based inhibitor of CYP2D6, (Bertelsen et al., 2003), which has been shown to

reduce the clearance of desipramine (Alderman et al., 1997), perphenazine (Ozdemir et al., 1997), metoprolol (Hemeryck et al., 2000), risperidone (Spina et al., 2001), and atomoxetine (Belle et al., 2002), where the clearance of the victim drugs is impaired by 5- to 8-fold. MDMA is also a mechanism-based inhibitor of CYP2D6 (Heydari et al., 2004; Van et al., 2007).

1.2.4 Human CYP3A4 enzyme

CYP3A4 has the highest abundance in the human liver, representing about 40% of the total hepatic CYP content and CYPs in the gastrointestinal tract (Shimada et al., 1994b). There are three major proteins (CYP3A4, 3A5 and 3A7) and one additional protein (CYP3A34) in the CYP3A family. Among them, CYP3A7 is the predominant CYP form in embryonic, fetal, and newborn livers (Kitada and Kamataki, 1994; Hakkola et al., 2001) but a minor form in the adult liver (Schuetz et al., 1994), having less important for drug metabolism in general. CYP3A5, with minor polymorphism and relative weak catalytic capability, has the substrate and inhibitor specificity highly similar to CYP3A4 (Wrighton et al., 1990; Williams et al., 2002) and is consistently expressed in extrahepatic tissues, such as kidney, lung, colon, and esophagus (Ding and Kaminsky, 2003; Burk and Wojnowski, 2004). CYP3A4 is the most important one in the biotransformation of drugs, metabolizing more than 50% of all therapeutic drugs used in the clinical setting (Zhou, 2008a).

1.2.4.1 Substrate specificity of CYP3A4

The substrate specificity of the CYP3A4 enzymes is very broad, with an extremely large number of structurally divergent and weightly differential chemicals. A large variety of substrates of CYP3A4 varying in molecular weight from metyrapone (M_r 226 Dal) to cyclosporine (M_r 1,203 Dal), including macrolide antibiotics (e.g. clarithromycin and erythromycin), anti-arrhythmics (e.g. quinidine), benzodiazepines (e.g. alprazolam and midazolam1), immune modulators (e.g. cyclosporine and tacrolimus), HIV antivirals (e.g. indinavir and ritonavir), antihistamines (e.g. chlorpheniramine and terfenadine), calcium channel blockers (e.g. amlodipine, felodipine, nifedipine and verapamil) and 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG CoA) reductase inhibitors (e.g. atorvastatin, cerivastatin, lovastatin and simvastatin). CYP3A4 exhibits a relatively large substrate-binding cavity that is consistent with its capacity to oxidize bulky substrates such as cyclosporine, statins, taxanes, and erythromycin (Zhou, 2008a).

1.2.4.2 Inhibitors of CYP3A4

The relatively low degree of substrate selectivity makes CYP3A4 susceptible to inhibition by different chemicals. This is accordant with the fact that the inhibitors of CYP3A4 cover a broad variety of structurally unrelated substances. Many of CYP3A4 inhibitors are important therapeutic drugs and posses mechanism-based inhibitory property, including macrolide antibiotics (e.g., clarithromycin, and erythromycin), anti-HIV agents (e.g., ritonavir and delavirdine), antidepressants (e.g. fluoxetine and fluvoxamine), calcium channel blockers (e.g., verapamil and diltiazem), steroids and their modulators (e.g., gestodene and mifepristone), and several herbal and dietary components (Zhou, 2008a).

Chemicals used as selective inhibitors of CYP3A4 include a small number of compounds inhibiting CYP3A4 in an irreversible (e.g. triacetyloleandomycin, gestodene) and/or reversible (e.g. ketoconazole) manner (Zhou et al., 2005c). Ketoconazole is most widely used, probably because of advantages in potency, selectivity, commercial availability, and ease of use. However, selectivity of ketoconazole for CYP3A4 is often less than ideal. For example, CYP1B1, 2B6, and 2C8/9/19 enzymes are significantly inhibited (20-60%) at concentrations required to inhibit CYP3A4 by 95% (von Moltke et al., 1998b).

A number of drugs with widely differing structures and therapeutic targets have been reported to be mechanism-based inhibitors of CYP3A4 (Zhou, 2008a). These include macrolide antibiotics (e.g., clarithromycin, and erythromycin), anti-HIV agents (e.g., ritonavir and delavirdine), antidepressants (e.g. fluoxetine and fluvoxamine), calcium channel blockers (e.g., verapamil and diltiazem), steroids and their modulators (e.g., gestodene and mifepristone), and several herbal and dietary components (Zhou, 2008a). Large numbers of acetylenes, particularly those synthetic steroids such as gestodene, norethisterone, ethinylestradiol, and norgestrel, have been demonstrated to cause mechanism-based inactivation of CYPs (Guengerich, 1990). However, most of the alkynes that inactivate CYPs are terminal acetylenes. Studies have shown that internal acetylenes such as several different methyl-substituted aryl acetylenes (propynylaryl acetylenes) and 10-dodecynoic acid also cause mechanism-based inactivation of CYPs (Foroozesh et al., 1997; Helvig et al., 1997). Mifepristone, an internal acetylene that has a methyl group substituting for the hydrogen on the external carbon of the triple bond, is a potent and selective mechanism-based inactivator of CYP3A4 via irreversible modification of the apoprotein (He et al., 1999).

Most of these CYP3A4 inactivators are also substrates and reversible inhibitors of CYPs (in particular CYP3A4), and some of which are also inducers of CYP3A and other CYPs. Three glitazones, troglitazone, rosiglitazone and pioglitazone, are mechanism-based CYP3A4 inhibitors, and their order of potency for inactivation is troglitazone > rosiglitazone > pioglitazone (Lim et al., 2005a). Structurally, the three glitazones share a 2,4-thiazolidinedione functionality. Reactive metabolites from bioactivation of 2,4-thiazolidinedione moiety can inactivate CYP3A4. However, troglitazone is the only one containing a chromane moiety; instead, rosiglitazone has a dialkylamino-pyridine and pioglitazone has a dialkylpyridine group. Formation of quinone methide from chromane might contribute to the greater potency of troglitazone for inactivating CYP3A4. The less effective formation of covalent adducts in CYP3A4 by rosiglitazone and pioglitazone, combined with the much lower doses generally prescribed (<10 mg/day) may explain the lacking of idiosyncratic hepatotoxicity and pharmacokinetic drug-drug interactions of those drugs, compared with troglitazone, in clinical settings (Lim et al., 2005a).

1.2.4.3 Induction of CYP3A4

The PXR/NR1I2, also known as steroid and xenobiotic receptor and pregnane-activated receptor is a member of the NR family of ligand-dependent transcription factors (Synold et al., 2001; Moore et al., 2006; Stanley et al., 2006; Matic et al., 2007). PXR/NR1I2 has been identified as a key regulator for the expression of genes involved in all stages of drug metabolism and transport (Synold et al., 2001; Matic et al., 2007). Phase I drug metabolizing enzymes regulated by PXR/NR1I2 include CYP2B6, 2C8, 3A4, 3A5, and 3A7, carboxylesterases, and dehydrogenases (Synold et al., 2001; Moore et al., 2006; Stanley et al., 2006; Matic et al., 2007). The ligands of PXR/NR1I2 include a wide variety of structurally diverse, low-affinity exogenous and endogenous chemicals, e.g. steroid hormones and steroid metabolites, such as progesterone, estrogen, corticosterone, 5β-pregnane, and androstanol, and dietary and herbal compounds, such as coumestrol, carotenoids, and hyperforin, a constituent of the herbal antidepressant St John's wort (Blumberg et al., 1998; Moore et al., 2000a; Moore et al., 2000b). Therapeutic drugs that behave as PXR/NR1I2 activators include rifampicin, phenobarbital, nifedipine, clotrimazole, mifepristone, and metyrapone (Moore et al., 2000b). Many of the PXR ligands are also shared by CAR/NR1I3. Upon ligand binding, PXR/NR1I2 forms a heterodimer with RXRα/NR1B1 and transactivates ER6 (everted repeat with a 6 bp spacer) elements upstream of the *CYP* genes (Waxman, 1999). RXRα/NR1B1 serves as a common heterodimerization partner for many orphan nuclear receptors, including CAR/NR1I3.

The binding of PXR/RXRα to ER6 is followed by recruitment of coactivator proteins, e.g. steroid receptor coactivator-1 and transcriptional activation of the respective gene (Lanz et al., 1999). There is evidence for a second binding site for PXR/NR1I2 in the ~7,800 bp upstream 5'-flanking region of the *CYP3A4* gene having ER6-like binding sites (Goodwin et al., 1999). PXR/NR1I2 and RXRα/NR1B1 are induced by GR/NR3C1 (Pascussi et al., 2008). Thus, the activation of GR/NR3C1 by glucocorticoids, such as dexamethasone, leads to the induction of PXR/RXR and to the increase of CYP3A4 induction by endogenous and exogenous compounds. *Pxr* knockout mice showed no induction by typical mouse Cyp3a inducers. The loss of Pxr did not alter the basal Cyp3a expression in mice. Transgenic mice containing human PXR/NR1I2 were also generated showing induction by human specific inducers, such as rifampicin (Xie et al., 2000).

The most common clinical implication for the activation of PXR/NR1I2 is the occurrence of drug-drug interactions mediated by up-regulated CYP3A4. Therefore, altered function or expression of the *PXR/NR1I2* gene due to SNPs is considered an important additional source of inter-individual variation in the expression and activity of CYP3A4. To date, there are 401 reported SNPs for the human *PXR/NR1I2* gene in the SNP database at NCBI (http://www.ncbi.nlm.nih.gov/, access date: 25 March 2009). Multiple SNPs of *PXR/NR1I2* have functional effects on the expression of human PXR/NR1I2. Zhang *et al*. (2001) found that the -25385C>T was associated with a marked higher CYP3A4 induction ability by rifampin as determined by the erythromycin breath test, a marker of CYP3A4 hepatic activity. Individuals with the -25385C>T genotype had a 2-fold higher CYP3A4 activity after treatment with rifampin, as compared to subjects with the wild-type genotype. Out of nine SNPs reported in the 3'-UTR of *PXR/NR1I2*, four demonstrated association with the expression levels of target genes. Hustert *et al*. (2001) found 3 variants (V140M, D163G, and A370T) with significant functional defects in terms of CYP3A4 transcription. A Q158K mutation of *PXR/NR1I2* has been linked to decreased rifampin-mediated CYP3A4 induction. Koyano *et al*. (2004) have investigated the three variants [443G>A (R148Q), 1141C>T (R381W), 1207G>A (I403V)] of *PXR/NR1I2* and found their basal and rifampicin-induced transactivation of the *CYP3A4* enhancer/promoter was significantly reduced compared with the wild-type PXR/NR1I2 (Lim et al., 2005b). Our previous study showed that the activity of the recombinants with alleles containing the -24622A>T in the 5'-untranslated region (UTR) or -24446C>A in exon 1 was 30-40% higher than that in the reference genotype (Wang et al., 2007).

1.2.5 Other CYPs

In humans, there are three functional genes in the *CYP2A* subfamily: *CYP2A6*, *2A7* and *2A13* (Fernandez-Salguero and Gonzalez, 1995; Hoffman et al., 1995; Raunio et al., 1999). The *CYP2A6* and *2A7* genes have a 96 % similarity in the nucleotide sequence and a 94 % identity at the amino acid sequence (Miles et al., 1989). *CYP2A6* codes a functional enzyme that is polymorphically expressed in the human liver accounting for about 1-10% of total CYPs, and only trace amounts are found in extrahepatic tissues (Koskela et al., 1999), while the product of *CYP2A7* has been shown to not incorporate heme and is thus inactive (Yamano et al., 1990; Ding et al., 1995). *CYP2A13* is not expressed in the liver but expressed in the olfactory bulb and respiratory tract (Fernandez-Salguero and Gonzalez, 1995; Hoffman et al., 1995; Raunio et al., 1999). In addition, the *CYP2A* subfamily contains two identical copies of a pseudogene, *CYP2A7PT* and *CYP2A7PC* (or *CYP2A7P1*) which contain putative *CYP2A* coding sequences corresponding to exons 1 through 5 (Fernandez-Salguero et al., 1995a). CYP2A7 mRNA is expressed in liver at similar levels as CYP2A6.

CYP2A6 metabolizes about 1% of clinical drugs. CYP2A6 is involved in the metabolism of valproic acid, with substantial contribution from CYP2B6 and 2C9 (Sadeque et al., 1997). The reactive metabolite, 4-ene-valproic acid, is a hepatotoxin. Halothane is metabolized by CYP2A6 as well as 3A4 (Spracklin et al., 1996). CYP2A6 is responsible for the sulfoxidation and thiono-oxidation of diethyldithiocarbamate methyl ester to form *S*-methyl-*N,N*-diethylthiolcarbamate sulfoxide, the putative active metabolite responsible for the alcohol deterrent effects of disulfiram (Madan et al., 1998). CYP2A6, 2B6, and 3A4 are the high K_m components for cyclophosphamide and ifosfamide 4-hydroxylation, while CYP2C8 and 2C9 are the low K_m components (Chang et al., 1993). Pilocarpine is a cholinergic agonist that is metabolized to pilocarpic acid by serum esterase. Formation of 3-hydroxypilocarpine from pilocarpine, a cholinergic agonist, is mainly metabolised by CYP2A6 (Endo et al., 2007). Coumarin strongly inhibited the formation of 3-hydroxypilocarpine by >90%. 2n-Propylquinoline, a newly developed drug for the treatment of visceral leishmaniasis, is hydroxylated by CYP2A6, with contribution from CYP2E1 and 2C19 (Belliard et al., 2003).

Human CYP2A6 is the major catalyst in the metabolism of *R*-verbenone, a natural compound of the essential oil from rosemary species such as *Rosmarinus officinalis* L., *Verbena triphylla*, and *Eucalyptus globulus*, by liver microsomes (Miyazawa et al., 2003). Fenchol is a terpene and an isomer of borneol and the naturally occurring *S*-fenchol is used extensively in

perfumery. *S*-Fenchol is metabolized to fenchone by CYP2A6 (Miyazawa and Gyoubu, 2007b). Fenchone is further hydroxylated by CYP2A6 and 2B6 in human liver microsomes (Miyazawa and Gyoubu, 2007a). *R*-Camphor was oxidized to 5-exo-hydroxyfenchone by CYP2A6 (Gyoubu and Miyazawa, 2007). Camphor is found in wood of the camphor laurel (*Cinnamomum camphora*). Camphor is an active ingredient (along with menthol) in vapor-steam products, such as Vicks VapoRub, and it is effective as a cough suppressant.

CYP2A13 has similar substrate specificity to 2A6 with some marked differences. CYP2A13 is active in the metabolism of a number of procarcinogens. CYP2A13 is the most efficient enzyme in the metabolic activation of the tobacco-specific procarcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific lung carcinogen (Jalas et al., 2003; Smith et al., 2003; Brown et al., 2007). CYP2A13 is mainly expressed in the respiratory tract (Zhu et al., 2006) where it can convert NNK into carcinogenic species that crosslink DNA and consequently induce carcinogenesis (Su et al., 2000). Studies with recombinant enzymes have demonstrated that CYP2A13 is 30-215 times more efficient at activating NNK into its carcinogenic metabolites than CYP2A6 (Su et al., 2000; He et al., 2004a). CYP2E1, 2D6, and 3A4 have also been shown to metabolize NNK *in vitro*, but their K_m values are much higher than K_m values for the 2A enzymes (Patten et al., 1996). The level of lung CYP2A13, but not CYP2A6 which can also metabolize NNK, was correlated with human lung microsomal NNK metabolic activation activity (He et al., 2004a; He et al., 2004b; Zhang et al., 2007), suggesting a more important role of CYP2A13 in the activation of NNK in the lung.

CYP2B6 can metabolise ~8% of all pharmaceutical drugs to some extent. These include cyclophosphamide (Chang et al., 1993), ifosfamide (Chang et al., 1993; Granvil et al., 1999), tamoxifen (Crewe et al., 2002), ketamine (Yanagihara et al., 2001; Hijazi and Boulieu, 2002), artemisinin (Svensson and Ashton, 1999), nevirapine (Erickson et al., 1999; Ward et al., 2003), efavirenz (Erickson et al., 1999; Ward et al., 2003), bupropion (Faucette et al., 2000; Hesse et al., 2000), sibutramine (Bae et al., 2008), propofol (Court et al., 2001; Oda et al., 2001), *S*-mephenytoin (Heyn et al., 1996), selegiline (Hidestrand et al., 2001; Kamada et al., 2002; Salonen et al., 2003), *S*-mephobarbital (Kobayashi et al., 1999), triethylenethiophosphoramide (thioTEPA) (Jacobson et al., 2002), valproic acid (Kiang et al., 2006), pethidine (Turpeinen et al., 2006), perhexiline (Davies et al., 2007), and diazepam (Ono et al., 1996). Ketamine *N*-demethylation is catalysed by CYP3A4, 2B6 and 2C9 (Hijazi and Boulieu, 2002). CYP2B6,

2D6, and 3A4 catalyze the oxidation of perhexiline enantiomers (Davies et al., 2007). Meperidine is an opioid analgesic metabolized in the liver by CYP2B6, 3A4 and 2C19 via *N*-demethylation to normeperidine (Ramirez et al., 2004), a potent stimulant of the central nervous system. The novel uroprotective drug *N*-methyl,*N*-propargyl-2-phenylethylamine was converted by CYP2B6, 2C19 and 2D6 to *N*-methylphenylethylamine and *N*-propargylphenylethylamine (Rittenbach et al., 2007). In addition, human CYP2B6 preferentially metabolized benzyloxyresorufin and pentoxyresorufin, although other CYPs also metabolized these substrates in human liver microsomes (Gervot et al., 1999).

Some inhibitory agents against CYP2B6 have been characterized as to the potency and selectivity of inhibition toward CYP2B6. These include orphenadrine (Ekins et al., 1997; Guo et al., 1997), n-propylxanthate (Kent et al., 1999) and xanthates (Yanev et al., 1999), 2-phenyl-2-(1-piperidinyl)propane (Chun et al., 2000), ritonavir (Hesse et al., 2001), efavirenz (Hesse et al., 2001), and nelfinavir (Hesse et al., 2001). Xanthates have been reported to be selective mechanism-based inactivators of CYP2B6 (Kent et al., 1999; Yanev et al., 1999). Both clopidogrel and ticlopidine inhibited bupropion hydroxylation as mechanism-based inhibitors (Richter et al., 2004). Ticlopidine is also a selective mechanism-based inhibitor of CYP2C19 (Ko et al., 2000; Giancarlo et al., 2001; Ha-Duong et al., 2001). ε-Viniferin is a potent mechanism-based inhibitor of CYP2B6 using 7-benzoxyresorufin-*O*-debenzoyloxylation as a marker reaction (Piver et al., 2003). thioTEPA is a selective inhibitor of CYP2B6 catalyzed *S*-mephenytoin *N*-demethylation to nirvanol with an IC_{50} value of ~5 μM (Rae et al., 2002).

CYP2C8 accounts for about 7% of total hepatic CYP contents (Shimada et al., 1994a) and metabolizes ~5% of drugs cleared by Phase I reaction. The prototypical substrate for CYP2C8 is the potent antimicrotubule drug paclitaxel, and its 6α-hydroxylation has been widely used in *in vitro* reaction phenotyping (Rahman et al., 1994; Cresteil et al., 2002). CYP2C8 contributes substantially to the biotransformation of a variety of clinical drugs, including antimalarial agents (e.g. amodiaquine (Li et al., 2002) and chloroquine (Kim et al., 2003), thiazolidinedione antidiabetic drugs (e.g. troglitazone (Yamazaki et al., 1999), rosiglitazone (Baldwin et al., 1999), pioglitazone (also minor contribution from CYP2C9 and 3A4) (Jaakkola et al., 2006b)), statins (e.g. cerivastatin and fluvastatin (Wang et al., 2002a), atorvastatin (Jacobsen et al., 2000), and simvastatin (Prueksaritanont et al., 2003), also contribution from CYP3A4 and 2C9), opioids (e.g. morphine (Projean et al., 2003), methadone (Wang and DeVane, 2003),

buprenorphine (Picard et al., 2005), and loperamide (Kim et al., 2004)), repaglinide (a hypoglycaemic drug that stimulates insulin secretion) (Bidstrup et al., 2003; Kajosaari et al., 2005), and *R*-ibuprofen (Hamman et al., 1997).

Montelukast and zafirlukast, both leukotriene D_4 (LTD_4) receptor antagonists, seem to be a potent and relatively selective competitive inhibitor of CYP2C8 *in vitro* (Walsky et al., 2005a; Walsky et al., 2005b). Montelukast can also inhibit CYP2C9 with IC_{50} of 1.2 μM (50-fold higher than the interaction with CYP2C8) (Walsky et al., 2005b). However, montelukast and zafirlukast do not alter the pharmacokinetics of CYP2C8 substrates such as repaglinide (Kajosaari et al., 2006) and pioglitazone (Jaakkola et al., 2006a) *in vivo* in humans. This is likely to reflect the pharmacokinetic properties of montelukast that limit the *in vivo* concentration of montelukast available for CYP2C8 binding. The benzylic side chain of montelukast is known to be oxidized *in vivo* (Balani et al., 1997) and *in vitro* by CYP2C9 and 3A4, but not CYP2C8 (Chiba et al., 1997). Quercetin, trimethoprim and gemfibrozil are all inhibitors of CYP2C8 (Wang et al., 2002a; Wen et al., 2002a). Trimethoprim has been shown to increase the area under the plasma concentration-time curve (AUC) of repaglinide AUC 1.3- to 2.2-fold (Niemi et al., 2004). Cotreatment of gemfibrozil has been shown to increase the AUC of rosiglitazone 1.8- to 2.8-fold (Niemi et al., 2003a), AUC of pioglitazone 3.2-fold (Jaakkola et al., 2005), AUC of repaglinide 5.5- to 15-fold (Niemi et al., 2003b; Tornio et al., 2008), AUC of ibuprofen by 34% (Tornio et al., 2007), AUC of loperamide 2.2-fold (Niemi et al., 2006), and AUC of cerivastatin 1.3- to 10-fold (Backman et al., 2002).

CYP2C19 is primarily expressed in hepatic tissue, but a significant amount is also found in the gut wall, particularly the duodenum (Zhou et al., 2008). CYP2C19 is responsible for the metabolism of approximately 10% of therapeutic drugs, including proton pump inhibitors (e.g. omeprazole, lansoprazole and pantoprazole), antidepressants (e.g. imipramine, amitriptyline and escitalopram), benzodiazepines (e.g. diazepam and flunitrazepam), anticancer drugs (e.g. cyclophosphamide), anti-epileptics (e.g. phenytoin, mephenytoin, phenobarbital), clopidogrel and so on (Zhou et al., 2008). CYP2C19 is also contributes to the catabolism of endogenous substrates like estradiol (Justenhoven et al., 2008), progesterone and testosterone (Yamazaki and Shimada, 1997).

The expression level of *CYP2C9* in the human liver is about 20 times higher than that of *CYP2C19* (Romkes et al., 1991), indicating that there are some differences in the regulatory

mechanism of *CYP2C9* and *2C19*. It has been reported that PXR/NR1I2, CAR/NR1I3, GR/NR3C1, and HNF-3γ/NR2A2 and HNF-4α/NR2A1 are involved in the basal expression of *CYP2C9* and *2C19* (Gerbal-Chaloin et al., 2001; Raucy et al., 2002; Chen et al., 2003; Bort et al., 2004; Kawashima et al., 2006; Kojima et al., 2007; Wortham et al., 2007). Analysis of the *CYP2C19* promoter revealed a single CAR/NR1I3 binding site at -1891/-1876 bp which binds CAR/NR1I3 and PXR/NR1I2 and a glucocorticoid-responsive element at -1750/-1736 bp (Chen et al., 2003). Rifampicin induced a modest increase in promoter activity in cells cotransfected with PXR/NR1I2. Dexamethasone activated the -2.7-kb *CYP2C19* promoter in HepG2 cells only in the presence of cotransfected GR/NR3C1, whereas the GR/NR3C1 antagonist mifepristone inhibits this response and mutation of the glucocorticoid-responsive element abolishes Dexamethasone-induced activation (Chen et al., 2003).

CYP3A5 accounts for about 7-8% of total CYP3A content in only ~20% of the liver samples examined. CYP3A5 is polymorphically expressed in adults with detectable expression in about 10-20% in Caucasians, 33% in Japanese and 55% in African-Americans (Kuehl et al., 2001). The *CYP3A5* gene is localized in a cluster on chromosome 7q21-q22.1 and consists of 13 exons (Spurr et al., 1989; Schuetz and Guzelian, 1995; Finta and Zaphiropoulos, 2000). CYP3A4 and 3A5 are considered to have similar substrate specificity, but the contribution of CYP3A5 to the total metabolic clearance of CYP3A substrates in the liver *in vivo* has yet to be determined. The only human *CYP* gene induced directly by GR/NR3C1 is *CYP3A5*. There is no consensus glucocorticoid responsive element in the *CYP3A5* gene, but instead GR/NR3C1 binds to the glucocorticoid responsive element half-sites in the 5'-flanking region of *CYP3A5*.

1.3 Genetic Mutations of Human *CYP* Genes and the Functional Impact

In 1969, Alexanderson *et al*. (1969) provided the first direct evidence from a twin study that the metabolic clearance of nortriptyline was influenced by genetic factors. Mahgoub *et al.* (1977) and Eichelbaum *et al.* (1979) independently discovered that the metabolism of debrisoquine and sparteine, respectively, is polymorphic, and it was later shown that these drugs are metabolized by a common enzyme, i.e. CYP2D6 whose activity is determined by genetic trait. Phenotypically, a specific population are composed of ultra-rapid metabolizers (UMs), EMs, intermediate metabolizers (IMs), and PMs. The distribution of the genetic variations and the phenotypes is ethnicity-dependent (Chowbay et al., 2005). The PM phenotype is due to the presence of two non-functional (null) alleles or deletion of entire gene, while the EM phenotype is due to one or two alleles with normal function. An IM phenotype is

usually found in individuals carrying one null allele and another allele with reduced function, while UMs often carry more than one extra functional gene. Pharmacogenetics is the study of the influence of genetic factors in the individual variation in drug response, while pharmacogenomics is a more global definition and entails the study of the entire spectrum of genes and their contribution to variability in drug efficacy and toxicity using genome-wide approaches (Evans and Relling, 1999; McLeod, 2001; McLeod and Evans, 2001; Weinshilboum, 2003; Zhou et al., 2008). Genetic polymorphisms within CYPs mainly affect the metabolism of drugs that are substrates for those particular enzymes, probably leading to differences in drug response in addition to an altered risk for adverse drug reactions (Ingelman-Sundberg et al., 2007; Kirchheiner and Seeringer, 2007).

Genetic polymorphisms within CYPs mainly affect the metabolism of drugs that are substrates for those particular enzymes, probably leading to differences in drug response in addition to an altered risk for adverse drug reactions (Ingelman-Sundberg et al., 2007; Kirchheiner and Seeringer, 2007). Most members of the *CYP* families are polymorphic (see http://www.imm.ki.se/CYPalleles) and allelic variants resulting in altered protein expression and activity have significant effects on the disposition of drugs and may cause diseases as a phenotype.

1.3.1 The *CYP1A2* gene

To date, more than 15 variant alleles and a series of subvariants (**1B* to **16*) of the *CYP1A2* gene have been identified (see Table 1-3) (http://www.imm.ki.se/CYPalleles, access date: 25 March 2009), and 158 SNPs have been found in the *CYP1A2* upstream sequence, introns and exons in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/, access date: 25 March 2009). *CYP1A2*1A* is referred to as the wild-type. Among the SNPs located in seven exons, there are 22 non-synonymous that change amino acid sequence. These include 43C>T (L15F); 53C>G (S18C); 63C>G (F21L); 130G>A (E44K); 217G>A (G73R); 310G>A (D104N); 331C>T (L111F); 373T>A (F125I); 413G>A (R138H); 538A>G (M180V); 613T>G (F205V); 841C>T (R281W); 894C>A (S298R); 895G>A (G299S); 940A>G (I314V); 1042G>A (G348S); 1067G>A (R356Q); 1217G>A (C406Y); 1291C>T (R431W); 1313C>T (T438I); 1369C>T (R457W); 1434A>T (Q478H); 1543A>G (I515V). Synonymous SNPs of *CYP1A2* exons include 222C>T (D74D); 249G>T (T83T); 306C>T (G102G).

The most extensively studied polymorphisms are -3860G>A (*CYP1A2*1C*), -2467delT (*CYP1A2*1D*), -739T>G (*CYP1A2*1E*) and -163C>A located in intron 1 (*CYP1A2*1F*), which were first reported in a Japanese population (Chida et al., 1999a). *CYP1A2*1C* was reported to cause decreased inducibility of the enzyme in smokers of Japanese, probably due to decreased expression of the enzyme (Nakajima et al., 1999b). The -163C>A in intron 1 caused increased enzyme inducibility in the presence of an inducer (e.g. smoking) in white smokers (Sachse et al., 1999), although this association is controversial (Chida et al., 1999a; Nordmark et al., 2002; Shimoda et al., 2002; Aklillu et al., 2003; Larsen and Brosen, 2005). Smokers with the -163C/C genotype had 40% lower plasma 17X:137X ratios compared with those with the -163A/A genotype in smokers (Sachse et al., 1999).

*CYP1A2*1J* (-163C>A; -739T>G) and *CYP1A2*1K* (-163C>A; -739T>G; -729C>T, all located in intron 1) have been detected in Ethiopian non-smokers (Aklillu et al., 2003). The **1K* haplotype was associated with 40% lower inducibility *in vitro*, and non-smokers heterozygous for **1K* had significantly lower CYP1A2 activity compared with the wild-type (Aklillu et al., 2003). The -729C>T SNP abolishes a binding site for an Ets nuclear factor, resulting in highly decreased CYP1A2 expression and caffeine metabolism (Aklillu et al., 2003). *CYP1A2*1K* is relatively rare.

*CYP1A2*1G*, **1H*, **1L*, **1M*, **1N*, **1P*, **1Q*, **1R*, **1S*, **1T*, **1U*, **1V*, and **1W* are relatively rare and do not alter enzyme activity (Chevalier et al., 2001; Soyama et al., 2005; Ghotbi et al., 2007). More recently, the −3113A>G polymorphism, with a frequency of 10% in a Chinese population, has been reported to be associated with decreased CYP1A2 activity (Chen et al., 2005a). *CYP1A2*2* carries a 63C>G mutation that causes a F21L substitution, which was first detected from the direct sequencing of DNA from one of eight Chinese subjects (Huang et al., 1999), but its functional impact is unclear due to its rarity. The *CYP1A2*3* (2385G>A; 5347T>G), **4* (2499A>T), **5* (3497G>A) and **6* (5090C>T) variants all cause amino acid changes, which were first detected in a French population with very low frequencies (0.5%) (Chevalier et al., 2001). When expressed in *E. coli*, *CYP1A2*3*, **4*, and **5* had decreased enzyme expression and activity and altered substrate specificity for phenacetin and heterocyclic amines; whereas **6* did not express any enzyme (Zhou et al., 2004a). *CYP1A2*7* contains a 3534G>A mutation in intron 6, causing RNA splicing defect and leading to loss of CYP1A2 activity, which was found in a 70-year old patient who had very high plasma concentrations of clozapine when administered at normal dose (Allorge et al., 2003).

Other variants of *CYP1A2*, including **8* (5166G>A; 5347T>C), **9* (248C>T), **10* (502G>C), **11* (558C>A), **12* (634A>T), **13* (1514G>A), **14* (5112C>T), **15* (125C>G; 534T>C) and **16* (2473G>A; 5347T>C), have been detected in Japanese with very low frequencies (0.2-0.6%) (Murayama et al., 2004). The **11* variant (leading to F186L substitution) had a significantly decreased enzyme activity when expressed in V79 hamster cells, with 12% of the wild-type capacity for phenacetin *O*-deethylation and 28% for 7-ethoxyresorufin *O*-deethylation (Murayama et al., 2004). *CYP1A2*8*, **15*, and **16* alleles, leading to R456F, P42R, and R377Q changes, respectively, showed <1% of the 7-ethoxyresorufin *O*-deethylation capacity compared with the wild-type in transfected V79 hamster cells (Saito et al., 2005). It appears that the amino acids at residues 42, 186, 377 and 456 play an important role in enzyme-substrate interactions.

There are significant ethnic differences in the distribution of common and rare *CYP1A2* SNPs and alleles. The −3860G>A and the −2467delT mutations are lower in Caucasians compared with Asians, while -739G was frequent in Ethiopians and Saudi Arabians (Chida et al., 1999a; Sachse et al., 1999; Nordmark et al., 2002; Larsen and Brosen, 2005). The -163C>A SNP has similar frequencies in all populations studied, with highest frequency in Africans. The *CYP1A2*1F* allele is more frequent in Caucasians and Africans, while **1D*, **1L*, **1M* and **1N* are more common in Asians (Chida et al., 1999a; Sachse et al., 1999; Nordmark et al., 2002; Larsen and Brosen, 2005). *CYP1A2*1J*, **1K* and **1W* are rare in all populations studied.

Resistance to clozapine therapy due to low plasma drug levels has been reported in smokers harbouring the -163A/A genotype (Ozdemir et al., 2001; Eap et al., 2004). Higher plasma concentrations of clozapine and its metabolite *N*-desmethylclozapine have been observed in patients carrying two *CYP1A2* variants associated with reduced enzyme activity (-3860A, -2467del, -163C, -739G and/or -729T) compared with those with one or none (Melkersson et al., 2007).

Since CYP1A2 can bioactivate procarcinogens, epidemiological studies have been conducted to explore the relationship of *CYP1A2* polymorphisms and cancer risk. Chinese smokers homozygous for the *CYP1A2* haplotype -3860G/-3113G/5347C have increased hepatocellular carcinoma risk (Chen et al., 2006b). A 2-fold increased risk for squamous lung cancer has been observed in patients carrying -2467del mutation (Pavanello et al., 2007). Increased lung cancer

risk was also found in Japanese non-smokers carrying the -163A/A genotype (Osawa et al., 2007). Increased gastric cancer risk was shown in non-smokers carrying the -3860 mutation (Agudo et al., 2006), while -163C and -2467delT alleles were associated with pancreatic cancer in heavy smokers (Li et al., 2006a). On the other hand, lower risk of breast cancer has been found in *CYP1A2* -163C/C carriers (Le Marchand et al., 2005), but this allele is associated with endometrial and ovarian cancers (Mikhailova et al., 2006). Lower circulating estradiol levels have been detected in premenopausal women with the -163C/C genotype compared with -163A/A and -163A/C carriers (Lurie et al., 2005). High estradiol levels are known to increase breast cancer risk.

1.3.2 The *CYP2C9* gene

To date, 33 variants and a series of subvariants of *CYP2C9* (**1B* through to **34*) have been identified (Table 1-4) (http://www.imm.ki.se/CYPalleles, access date: 25 March 2009). *CYP2C9*1A* is referred to the wild-type. There have been 520 SNPs found in the *CYP2C9* upstream sequence, introns and 9 exons in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/, access date: 25 March 2009). Among these SNPs, there are 19 non-synonymous SNPs found in exons 3, 5, 7, 8, and 9. These include 334A>C (I112L); 371G>A (R124Q); 430C>T (R144C); 448C>T (R150C); 449G>A (R150H); 752A>G (H251R); 815A>G (E272G); 817insA (273frameshift); 980T>C (I327T); 1003C>T (R335W); 1010C>T (P337R); 1073A>G (Y358C); 1075A>C (I359L); 1076T>C (I359T); 1080C>G (D360E); 1238T>C (L413P); 1341A>C (L447F); 1465C>T (P489S). Eleven SNPs in exons of *CYP2C9* are synonymous: 96C>G (G32G); 228G>A (V76V); 390G>T (T130T); 837A>C (P279P); 840T>A (S280S); 936C>A (L312L); 1026G>A (R342R); 1140C>A (L380L); 1185A>T (L395); 1323C>T (A441A); 1425A>T (G475G).

One of the first identified and most common allelic variants is *CYP2C9*2*, a missense mutation of 430T>C causing the substitution of R144C (Rettie et al., 1994). Typically, this mutation causes a decrease in enzyme activity toward CYP2C9 substrates such as *S*-warfarin and tolbutamide. *CYP2C9*3* is a missense mutation of 1075A>C on exon 7 that leads to an I359L substitution (Sullivan-Klose et al., 1996). *CYP2C9*2* causes ~20-30% loss of enzyme activity toward *S*-naproxen, whereas the **3* mutation may reduce V_{max} activity by as much as 70%. It is possible this loss is due to enzyme conformational changes that reduce the enzyme's ability to bind to substrates. *CYP2C9*4* is an extremely rare missense mutation of 1076T>C originally identified in a Japanese epilepsy patient with an adverse reaction to phenytoin (Imai et al.,

2000). It is believed the lack of activity is due to an I359T substitution. The *CYP2C9*5* allele contains the 1080C>G transversion in exon 7 causing a D360E change, which has been found almost exclusively in African-Americans (Dickmann et al., 2001; Allabi et al., 2004; Allabi et al., 2005). Approximately 3% of this population carries the *CYP2C9*5* allele. Unlike *CYP2C9*2* and **3*, **5* appears to affect the Michaelis-Menten (K_m) constant of various drugs, substantially reducing the efficiency of the enzyme and increasing the K_m values (Dickmann et al., 2001; Allabi et al., 2004).

*CYP2C9*6* is a null allele because of deletion of A at 818 nucleotide on exon 5 originally identified in an African American patient with a high sensitivity to phenytoin, which results in a shortened protein (Kidd et al., 2001; Allabi et al., 2005). *CYP2C9*13* has been identified in a Chinese poor metabolizer of lornoxicam and the allele has a T269C transversion in exon 2 of *CYP2C9* that leads to an L90P substitution (Si et al., 2004). Frequency analysis shows that approximately 2% of the Chinese populations carry this variant allele. The half-life of lornoxicam was about 105 hr in this carrier which was markedly longer than that of other *CYP2C9*1/*3* and *CYP2C9*1/*1* carriers (half-lives of 5.8–8.1 and 3.2–6.3 hr, respectively), suggesting that the *CYP2C9*13* allele has a larger effect on CYP2C9-mediated drug metabolism.

There are significant ethnic differences in the frequency of *CYP2C9* variants (Table 1-5). *CYP2C9*2* is reasonably frequent among Caucasians with ~1% of the population being homozygous carriers and a significant 22% are heterozygous (Sullivan-Klose et al., 1996). The corresponding figures for the *CYP2C9*3* allele are 0.4% and 15%; with another 1.4% being compound heterozygotes – *CYP2C9*2/*3* (Kamali and Pirohamed, 2006). *CYP2C9*5* is estimated to be inherited in ~3% of the African-American population as a single allele mutation of 1080C>G (Allabi et al., 2004; Allabi et al., 2005). In addition, African-Americans have a significantly lower rate of *CYP2C9*2* and **3* inheritance than Caucasians, with 2.5% and 1.25% frequency, respectively.

There are a number of clinical studies that address the impact of *CYP2C9* polymorphisms on the clearance and/or therapeutic response of drugs that are substrates of CYP2C9. The drugs most extensively studied include coumarin anticoagulants, sulfonylurea drugs, angiotensin II inhibitors, phenytoin, and NSAIDs. Mutant alleles of the *CYP2C9* gene have been associated with slow hydroxylation of *S*-warfarin (Lal et al., 2006). There are two common allelic

polymorphisms in the *CYP2C9* gene, including *CYP2C9*2* and **3* that encode enzymes that are approximately 12% and 5% as efficient as the wild-type, respectively, and both have a substantial effect on the intrinsic clearance of warfarin (Gage and Lesko, 2008). Subjects who were homozygous for the *CYP2C9*3* allele showed a 90% reduction in the elimination of *S*-warfarin in comparison to subjects who were homozygous for the wild-type allele (Takahashi and Echizen, 2001). Impaired metabolism of a low therapeutic index drug such as warfarin has important clinical implications. Carriers of such polymorphisms require both smaller loading and maintenance doses and have a 4-fold increase in risk of bleeding complications, particularly at the beginning of therapy (Gage and Lesko, 2008). An individual that requires a low dose of warfarin is 6-fold more likely to be positive for one or more of the variant alleles compared with the general population. Patients who are *CYP2C9*3* homozygous require the lowest doses (Kamali and Pirohamed, 2006). Pharmacogenetic testing of *CYP2C9* would be useful to identify this subgroup of patients who have difficulty at the initiation of warfarin therapy, and are potentially at a higher risk of haemorrhage. These findings clearly demonstrate the need for clinical assessment of *CYP2C9* genotype when establishing optimal warfarin therapy (Bussey et al., 2008).

There are case reports describing 4- to 5-fold increase in phenytoin AUCs in patients with *CYP2C9*3/*3* or *6** (Kidd et al., 1999; Kidd et al., 2001). In Caucasian patients receiving a stable dose of phenytoin who had plasma concentrations within the therapeutic range, the presence of at least one *CYP2C9*2* or **3* allele correlated with one-third lower mean dose requirements (199 vs 314 mg/day, respectively) (van der Weide et al., 2001). The dose requirements for individuals carrying the *CYP2C9*1/*1, *1/*2, *1/*3, *2/*2,* and **2/*3* genotypes needed 314, 193, 202, 217, and 150 mg/day for phenytoin, respectively (van der Weide et al., 2001). Similar results have been observed in Japanese (Odani et al., 1997; Mamiya et al., 1998) and Taiwanese (Hung et al., 2004) patients. A single-dose study in healthy volunteers also revealed that there was a 30% lower concentrations in wild-type individuals compared with carriers of *CYP2C9*2* or **3* alleles (Aynacioglu et al., 1999). In another study, the AUCs of phenytoin were 1.5- and 2.7-fold higher in healthy individuals with one or two *CYP2C9*2* and **3* variant alleles, respectively, compared with those with the *CYP2C9*1/*1* genotype (Caraco et al., 2001). Several studies examined whether *CYP2C9* genotype affects the toxicity of phenytoin. There were more individuals with the *CYP2C9*1/*3* genotype among Korean patients with skin reactions to phenytoin compared with non-exposed controls (Lee et al.,

2004). However, no association between *CYP2C9* variants and gingival overgrowth was observed in patients (Soga et al., 2004).

1.3.3 The *CYP2D6* gene

The genetic variation contributes largely to the interindividual variation in the activity of CYP2D6. Presently, 71 different human *CYP2D6* variant alleles (**1B* to **72*) and a series of subvariants have been identified (Table 1-6) and designated by the human cytochrome P450 allele nomenclature committee (http://www.imm.ki.se/CYPalleles, access date: 25 March 2009). There have been 134 SNPs of *CYP2D6* described at NBCI dbSNP, with 32 non-synonymous SNPs reported (http://www.ncbi.nlm.nih.gov/SNP/, access date: 25 March 2009). These include 31G>A (V11M); 77G>A (R26H); 100C>T (P34S); 124G>A (G42R); 271C>A (L91M); 281A>G (H94R); 320C>T (T107I); 358T>A (F120I); 364G>T (G122S); 454delT (152frameshift); 463G>A (E155K); 496A>G (N166D); 501C>A (H167Q); 502T>G (S168A); 505G>T (G169C); 635G>A (G212E); 692T>C (L231P); 709G>T (A237S); 775delA (259frameshift); 886A>G (N285S); 886T>C (C296R); 899C>G (A300G); 901G>A (D301); 931delA (281frameshift); 932C>T (S311L); 971A>C (H324P); 986G>A (G329V); 1012G>A (V338M); 1094G>A (R365H); 1117G>A (G373S); 1405C>G (P469A); 1408A>G (T470A); 1432C>T (H478Y); 1435G>C (G479R); 1441T>G (F481V); 1457C>G (T486S). Synonymous SNPs of *CYP2D6* exons include 84C>A (R28R); 294C>G (T98T); 333T>C (G111G); 336C>T (F112F); 408C>G (V136V); 657T>C (F219F); 801C>A (P267P); 828G>T (L276L); 935_937delAAG> (K281K); 972T>C (H324H); 1083T>C (H361H); 1203G>A (S401S); 1401G>C (S467S); 1410T>C (T470T); 1443T>C (F481F); 1449C>T (F483F); 1457C>G (T486S).

Null alleles of *CYP2D6* do not encode a functional protein and there is no detectable residual enzymatic activity. They are responsible for the PM phenotype when present in homozygous or compound heterozygous constellations. The mechanism by which leading to a total loss of function includes: a) single base mutations or small insertions/deletions that interrupt the reading frame or interfere with correct splicing leading to prematurely terminated protein/stop codon (e.g. *CYP2D6*3*, **4*, **5*, **6*, **7*, **8*, **11*, **12*, **13*, **14*, **15*, **16*, **18*, **19*, **20*, **21*, **38*, **40*, **42*, **44*, **56* and **62*); b) non-functional full length coded alleles (e.g. *CYP2D6*12, *14* and **18*); c) deletion of entire *CYP2D6* gene as a result of large sequence deletions (e.g. *CYP2D6*5*); and formation of hybrid genes (e.g. *CYP2D6*13* and **16*). There is a large

deletion of sequence in **13,* and **16* and as a result both contain a *CYP2D7-2D6* hybrid gene (Gaedigk et al., 1991).

The alleles *CYP2D6*10*, **14*, **17*, **18*, **36*, **41*, **47*, **49*, **50*, **51*, **54*, **55*, and **57* give rise to significantly decreased activity. The enzyme activity change may be substrate-dependent for some alleles such as **17*. Individuals harboring either of these alleles are PMs or IMs.

Functional studies did not demonstrate altered enzyme activity with several alleles of *CYP2D6*, including *2A, **17×2*, **35*, **41×2*, and **48*. The *CYP2D6*27*, **39* and **48* alleles encode enzymes with largely normal activity compared to the wild-type protein (Sakuyama et al., 2008). CYP2D6.27 (E410K), CYP2D6.39 (S486T) and CYP2D6.48 (A90V) expressed in COS-7 cells showed a slightly higher intrinsic clearance than the wild-type enzyme. A90, E410 and S486 are located in β-sheet 3, between the K' and K'' helices and in B helix, respectively (Rowland et al., 2006). It appears that these residues are not important for the function of CYP2D6.

On the other hand, extremely high CYP2D6 activity results from gene duplication/multiduplication of functional alleles (e.g. **1* and **2*) fused in a head to tail orientation, as a result of unequal crossover events and other mechanisms. This was noted by a molecular characterization of the *CYP2D6* locus in patients with extremely rapid metabolisms (Bertilsson et al., 1993). Initially, alleles with 0, 1, 2, 3, 4, 5, and 13 gene copies were reported by Johanson *et al*. (1993) and Aklillu *et al*. (1996) In a Swedish family (father, daughter, and son) as many as 13 copies of a functional allele of *CYP2D6* have been identified (Johansson et al., 1993). Carriers of *CYP2D6*2×N* (*N* = 2, 3, 4, 5, or 13) with extremely high CYP2D6 activity were identified in a Swedish population (Dahl et al., 1995) and an Ethiopian population (Aklillu et al., 1996). The gene duplication/multiduplication results from unequal crossover events and other mechanisms. Gene duplication and multiduplication of *CYP2D6* can result enzymes which are functional, partly functional and non-functional. Gaedigk *et al*. (2007b) found gene duplication events in**1*, **2*, *4, **6*, **10*, **17*, **29*, **35*, **43*, and **45*. Duplications occurred at 1.3, 5.75, and 2.0% in Caucasian, African American, and racially mixed populations, respectively. Most of the variant duplications except **35×N* were found in African Americans. The **4×N* was as frequent as **2×N* in African Americans (Gaedigk et al.,

2007b). Extremely high CYP2D6 activity can result from gene duplication or multiduplication of functional allele **1* and **2* fused in a head to tail orientation (Gaedigk et al., 2007b).

1.3.4 Other *CYP* genes

The *CYP2A6* gene spans a region of approximately 6 kb pairs consisting of 9 exons and has been mapped to the long arm of chromosome 19 (between 19q12 and 19q13.2) (Miles et al., 1989). It is located within a 350-kb pair gene cluster together with the *CYP2A7* and *2A13* genes, two *CYP2A7* pseudogenes, as well as genes in the *CYP2B* and *2F* subfamilies (Hoffman et al., 1995). To date, more than 33 variant alleles (**1B* to **34*) of the *CYP2A6* gene have been identified (http://www.imm.ki.se/CYPalleles, access date: 25 March 2009). There have been 227 SNPs found in the *CYP2A6* upstream sequence, 8 introns and 9 exons in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/, access date: 25 March 2009). There are 28 non-synonymous SNPs in exons 1-9. These include 13G>A (G5R); 86G>A (S29N); 352T>C (F118L); 361G>C (G121R); 383G>A (R128Q); 391T>G (S131A); 451G>A (E151K); 457T>C (S153P); 474C>G (D158E); 478C>A (L160I); 479T>A (L160H); 607C>A (R203S); 773C>A (T258K); 835G>C (E279Q); 874G>A (V292M); 881C>G (T294S); 902G>C (G301A); 931C>T (R311C); 997A>T (R333*); 1093G>A (V365M); 1175T>A (F392Y); 1226A>G (Q409R); 1252A>G (N418D); 1257G>C (E419D); 1412T>C (I471T); 1427A>G (K476R); 1436G>T (G479V); 1454G>T (R485L).

Because of the substantial involvement of CYP2A6 in nicotine elimination, it has been proposed that the *CYP2A6* polymorphism is a major determinant of an individual's nicotine metabolic clearance and smoking behavior. Individuals homozygous for a *CYP2A6* gene deletion displayed only 15% of urinary cotinine levels compared with individuals carrying at least one active *CYP2A6* gene after smoking the same number of cigarettes (Kitagawa et al., 1999). Subjects with *CYP2A6*7/*7*, **7/*10* (1.8), and **7/*19* showed prominently lower cotinine/nicotine ratios compared with that of subjects with *CYP2A6*1A/*1A* (Fukami et al., 2005). Benowitz *et al*. (2006) revealed that individuals harboring *CYP2A6*1/*9* or **1/*12* showed 17.6% lower nicotine clearance than individuals with the wild-type (15.5 vs 18.8 ml/min/kg). Healthy subjects with the *CYP2A6*1/*2*, **1/*4*, **9/*12*, **9/*4*, or **9/*9* showed 37.8% lower nicotine clearance than the wild-type (11.7 vs 18.8 ml/min/kg). Overall, individuals carrying either of above variant alleles of *CYP2A6* showed lower clearance of cotinine, longer half-lives for nicotine and cotinine, and greater fraction of the nicotine dose as

unchanged nicotine and nicotine glucuronide in the urine compared with the wild-type (Benowitz et al., 2006).

The *CYP2C19* gene is mapped to the long arm of chromosome 10, located in a densely packed region also containing genes encoding CYP2C8, 2C9 and 2C18 (Romkes et al., 1991). The CYP2C19 enzyme is a protein of 490 amino acids. It is encoded by the *CYP2C19* gene consisting of 9 exons which is mapped to chromosome 10 (10q24.1-q24.3) (Romkes et al., 1991). CYP2C19 is primarily present in hepatic tissue, but a significant amount is also found in the gut wall, particularly the duodenum. To date, at least 24 (**1B* to **25*) variants and a series of subvariants of *CYP2C19* have been identified (http://www.imm.ki.se/CYPalleles, access date: 25 March 2009). *CYP2C19*1A* represents the wild-type. There have been 553 SNPs found in the *CYP2C9* upstream sequence, introns and 9 exons in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/, access date: 25 March 2009). Among these SNPs, there are 26 non-synonymous SNPs found in exons 3, 5, 7, 8, and 9. These include 1A>G (M1V); 50T>C (L17P); 55A>C (I19L); 221T>C (M74T); 276G>C (E92D); 358T>C (W120R); 365A>C (E122A); 431G>A (R144H); 449G>A (R150H); 502T>C (F168L); 518C>T (A173V); 527A>G (N176S); 636G>A (W212*); 680C>T (P227L); 836A>C (Q279P); 839C>A (S280Y); 905C>G (T302R); 985C>T (R329C); 991G>A (V331I); 1030C>T (H344Y); 1180G>A (V394M); 1228C>T (R410C); 1297C>T (R433W); 1316delG (439frameshift); 1390C>A (P464T); 1473A>C(*491C). Synonymous SNPs in exons of *CYP2C19* include 99T>C (P33P); 390G>T (T130T); 681G>A/C (P227P); 903A>G (T301T); 990C>T (V330V); 993T>G (V331V); 1059C>T (H353H); 1062G>A (E354E); 1251A>C (G417G); 1440G>A (P480P).

The first *CYP2C19* variant allele discovered was *CYP2C19*2A* containing 681G>A on exon 5 that causes splicing defect (de Morais et al., 1994b). **2B* and **2C* also carry this mutation and additional SNPs (99C>T; 990C>T; 991A>G) (Ibeanu et al., 1998). *CYP2C19*3A* and **3B* share the 636G>A SNP resulting in a premature stop codon in exon 4 together with 991A>G and 1251A>C (**3B* also contains 1078G>A) (Fukushima-Uesaka et al., 2005). *CYP2C19*2A*, **2B*, **2C*, **3A*, and **3B* are null alleles, resulting in complete loss of enzyme activity (De Morais et al., 1994a). The majority of PMs of CYP2C19 are due to these variant alleles (Desta et al., 2002b). *CYP2C19*4* is an initiation codon variant of 1A>G, resulting in GTG initiation codon and also carries 99C>T and 991A>G (Ferguson et al., 1998).

The majority of enzyme deficiency associated with PMs of *S*-mephenytoin has been found to be the responsibility of various variant alleles of *CYP2C19*. As with other CYPs, the frequency of PMs varies across races, with 13 to 23% of Asians and 1 to 8% of Caucasians and black Africans lacking functioning enzyme (Desta et al., 2002b). Three common types of *CYP2C19* genotypes of the PM phenotype exist, including two homozygous genotypes, **2/*2* and **3/*3*, and one heterozygous genotype, **2/*3*. The homozygous *CYP2C19*2/*2* genotype is by far the most frequent of the three defective PM genotypes (Desta et al., 2002b). For EMs, there are two genotypes that are heterozygous for the *CYP2C19* wild-type, **1/*2* and **1/*3*, and one genotype that is homozygous for the wild-type allele, **1/*1*.

The distribution of common variant alleles of *CYP2C19* has been found to vary among different ethnic groups. The allelic frequency of *CYP2C19*2* has been shown to be ~17% in African-Americans, 30% in Chinese and ~15% in Caucasians (Desta et al., 2002b). *CYP2C19*3* has been shown to be more frequent in Chinese (5%) and less frequent in African-Americans (0.4%) and Caucasians (0.04%). *CYP2C19*2* is the dominant defective allele and accounts for around 75-85% of PM phenotype in Chinese and Caucasian populations (Desta et al., 2002b). Almost all PMs in the Asians and Africans can be attributed to *CYP2C19*2* and *CYP2C19*3*.

The AUCs of both omeprazole and lansoprazole in PMs are 4- to 15-fold higher compared to homozygous EMs, whereas the values in heterozygous EMs are only 2- to 3-fold higher than homozygous EMs (Furuta et al., 1999a; Furuta et al., 1999b; Furuta et al., 2001; Ieiri et al., 2001; Shirai et al., 2001; Cho et al., 2002; Kim et al., 2002; Shirai et al., 2002). With multiple dosing, the increase in the AUC of omeprazole, but not of lansoprazole or pantoprazole, decreases to ~2-fold in EMs, due to inhibition of its own metabolism (Andersson et al., 1998; Shirai et al., 2001). Such auto-inhibition does not occur in PMs who lack functional CYP2C19 or has very low enzyme activity. There is a 6-fold higher AUC of lansoprazole in PMs than in heterozygous and homozygous EMs (Tanaka et al., 1997; Andersson et al., 1998). The AUC of rabeprazole are also increased 3.0- to 5.3-fold in PMs compared to homozygous EMs (Horai et al., 2001; Ieiri et al., 2001; Shirai et al., 2001; Lin et al., 2003).

1.4 Structural Features of Major Human Drug Metabolizing CYPs

The structural information of CYPs was first obtained from bacterial CYPs, such as CYPBM3 simply because they are all soluble proteins and much easy for crystallization. The crystal

structure of rabbit CYP2C5 is the first reported mammalian microsomal CYP. The crystallinity of the CYP2C5 (protein database (PDB) ID: 1DT6) (Williams et al., 2000) is a milestone for the relevant research since then the crystal structures of human CYPs could be gradually revealed based on the first mammalian membrane-binding CYP work. The structures of CYP2C5 in complex with diclofenac (1NR6) or a dimethyl derivative of sulfaphenazole (1N6B) (Wester et al., 2003) have been reported. The structure of rabbit CYP2B4 in a free form (1PO5) (Scott et al., 2004) or in complex with 1-(4-cholorophenyl)imidazole (2Q6N) (Zhao et al., 2006), bifonazole (2BDM) (Zhao et al., 2006), or 4-(4-chlorophenyl)imidazole (1SUO) (Scott et al., 2004) has also been determined.

To date, the crystal structures of at least twelve human CYPs, including human CYP1A2 (Sansen et al., 2007), 2A6 (Yano et al., 2005), 2A13 (Smith et al., 2007), 2C8 (Schoch et al., 2004; Schoch et al., 2008), 2C9 (Williams et al., 2003), 2D6 (Rowland et al., 2006), 2E1, 2R1 (Strushkevich et al., 2008), 3A4 (Williams et al., 2004; Yano et al., 2004), 7A1, 8A1 (prostacyclin synthase) (Li et al., 2008), and 46A1 (Mast et al., 2008), have been solved by X-ray crystallography (also see http://www.rcsb.org/pdb/, access date: 25 March 2009). The general information of these structures is summarized in Table 1-7.

1.4.1 Common structural features of CYPs

Comparisons to the bacterial soluble CYPs, the mammalian CYP conserves the general aspects of the overall folding pattern of these proteins. However, the substrate binding cavity is poorly conserved (Williams et al., 2000). The sequence variation of substrate recognition sites (SRS) in the active site is partially responsible for the change and furthermore for the catalytic diversity displayed by the CYP2C5 and subsequently human CYPs.

In general, human CYP enzymes share a conserved overall fold and topology, although sequence conservation within CYPs family is relatively low at less than 20% sequence identity (Brown et al., 2008). The conserved core is formed by a four-helix bundle, helices J and K, a coil termed the 'meander' and two sets of β-sheets. The four-helix bundle composed of three parallel helices labelled D, L, and I and one antiparallel helix E and the heme group is bound between distal I helix and proximal L helix (a heme-binding loop) through an absolutely conserved cysteine that serves as the fifth ligand for the heme iron (see Figure 1-4). The most characteristic CYP consensus sequence (Phe-X-X-Gly-X-Arg-X-Cys-X-Gly) is located in the heme-binding loop on the proximal face of the heme just before the L helix, while another

CYP consensus sequence (Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser) accommodated at the central part of the long I helix which forms a wall above the heme (Werck-Reichhart and Feyereisen, 2000).

Despite the highly conserved fold, there is plenty structural discrimination due to distinct amino acid sequences in diverse CYP enzymes, especially the difference of certain key amino acid residues in the CYP active sites. These key residues in the active sites usually govern certain metabolisms occur in specific positions through binding certain rather than any substrates. A number of studies suggest that the topography and the character of certain key amino acid residues at the CYP active site are the major determinants of substrate specificity. Therefore, a single amino acid difference may affect substrate reactivity, representing by the change of binding affinity, reaction regioselectivity and velocity. This kind of alterations was actually observed in individuals with SNPs of the *CYP2D6* gene (Zhou et al., 2006), although they were classified into poor or normal metabolism groups without aware of genetic polymorphisms.

1.4.2 CYP1A2

CYP1A2 substrates generally contain planar ring that can fit the narrow and planar active site of the enzyme (Sansen et al., 2007). Before the crystal structure of human CYP1A2 was resolved, the knowledge of the active site of CYP1A2 enzyme was obtained mainly from homology models that were built up on the basis of the structure of either bacteria CYPBM3 (Lozano et al., 1997; Lozano et al., 2000) or rabbit CYP2C5 (Kim and Guengerich, 2004). These homology models did provide valuable information for understanding the structure-activity relationship of CYP1A2. However, the gap between the models and real structure of CYP1A2 had always existed until 2007 when the first X-ray structure of a human CYP1 family enzyme, CYP1A2, was determined (Sansen et al., 2007).

Several pharmacophore models have been established for a number of structurally diverse inhibitors of CYP1A2 previously (Korhonen et al., 2005; Roy and Roy, 2008). Based on the inhibitory potencies on CYP1A2, a group of naphthalene, lactone and quinoline derivatives (n = 52) have been analysed (Korhonen et al., 2005). The results indicated that electronegative substitutions at position 1 of naphthalene (dibenzene) increased the inhibitory potency whereas other substitutions and heterocyclic nitrogen atom (e.g. quinoline) decreased the effect. In addition, a long side chain decreased the inhibition of five-ring lactones (Korhonen et al.,

2005). Another study of 21 naturally occurring flavonoids has demonstrated that a non-substituted phenyl ring at position 2 and a double bond between position 2 and 3 of the 1,4-benzopyrone nucleus are essential for the inhibitory effects of the flavonoids (Roy and Roy, 2008). Namely, any substitutions on these positions will lead to poor inhibitory activity of the flavonoids. On the other hand, hydroxyl groups present at position 5 and 7 of the benzopyran nucleus should not be glycosylated for the potent inhibitory activity of CYP1A2 enzyme (Roy and Roy, 2008). The two group chemicals, flavonoids and the derivatives of naphthalene, lactone and quinoline, had been used for quantitative structure-activity relationship (QSAR) analysis to extract novel structural information related to the interaction between inhibitory molecules and the CYP1A2 active site. However, these models are mainly based on particular core structures and may be useful to screen potential inhibitors with similar structures instead of distinct structure chemicals.

The structure of human CYP1A2 was crystallized in a complex with ANF, an inhibitor of CYP1A2, which has been refined to 1.95 Å (PDB ID: 2HI4) (Sansen et al., 2007). In the 2HI4 structure, both helix F' and G' are 3^{10} helical fragments instead of typical α-helices. In addition, the CYP1A2 structure is different from those of CYP2 and 3 members in the length and local structure of loop regions. CYP1A2 also contains an additional β3'-sheet between helices H and I and a small α-helix (K") residing at the proximal surface (see Figure 1-4). Furthermore, the region connecting helices C and D possesses a Ser-rich insertion, which forms a loop extending into the solvent.

In the 2HI4 structure in complex with ANF, the compact active site is closed with a relatively small volume of the cavity of 375 $Å^3$ (Sansen et al., 2007), which is 44.2% larger than that of CYP2A6 (260 $Å^3$) (Yano et al., 2005), but smaller than that of CYP2D6 (~540 $Å^3$) (Rowland et al., 2006) and CYP3A4 (1386 $Å^3$) (Yano et al., 2004). The substrate binding cavity of CYP1A2 is narrow, formed by residues on helices F and I that define a relatively planar binding platform for the substrate on either side. It is clear that the narrow and flat active site cavity of CYP1A2 can fit well with planar compounds such as ANF and typical CYP1A2 substrates such as theophylline, caffeine, melatonin, tacrine, clozapine. ANF binds CYP1A2 in a single preferred orientation, which places the phenyl ring close to the heme iron and makes it an inhibitor rather than a substrate for CYP1A2.

The relatively narrow, planar substrate binding cavity of CYP1A2 is of great importance in drug metabolism as well as in procarcinogen activation. The unique active site architecture defines a distinctive substrate binding site that is different from the structures of CYP2 and CYP3 members. The residues lining along the helices I and F contribute to the narrow binding pocket (see Figure 1-4) and fit the structural properties of its substrates and inhibitors. Especially the side chain of Phe226 on helix F (SRS2) makes a great contribution to the π–π stacking with ANF. Amino acid substitutions for Phe226 (F226I or F226Y) showed a reduced catalytic efficiency (Yun et al., 2000), indicating the prominent role of the Phe226 at the active site both for binding and catalysing substrates. This result is consistent with the fact that most of the CYP1A2 substrates and inhibitors are small, planar and lipophilic molecules (Korhonen et al., 2005). The crystal structure of CYP1A2 improves the understanding of drug recognition on the basis of molecular level and provides a rational platform for exploring CYP1A2-ligand interactions.

1.4.3 CYP2C9

Before the release of CYP2C9 structure in 2003, many structure-activity relationship studies were conducted through homology model of CYP2C9 on the basis of rabbit CYP2C5 (Oda et al., 2004). De Groot *et al*. (2002) have constructed diverse pharmacophores for CYP2C9 inhibitors and substrates, respectively. They had built up a pharmacophore model for CYP2C9 ligand using 16 structurally diverse substrates and pointed out that a hydrophobic region and a hydrogen bond acceptor are common features for these CYP2C9 ligands. Differentially, Ekins *et al*. (2000a) reported three pharmacophore models based on three groups of inhibitors and extracted additional features, such as an acceptor and a donor of hydrogen bond plus two hydrophobic zones in 2 of 3 models whereas two hydrogen bond acceptors in the third model. However, these models either were with very basic common features that are too wild to screen specific inhibitors for CYP2C9, or were built by structurally similar inhibitors of CYP2C9 that obstruct these models for a wide application.

To date, three crystal structures of human CYP2C9 enzyme have been resolved by X-ray analysis: one in ligand-free form (PDB ID: 1OG2) and two in complex with warfarin (PDB ID: 1OG5) and flurbiprofen (PDB ID: 1R9O), respectively (Williams et al., 2003; Wester et al., 2004). The structure of 1OG5 enzyme is significantly different from that of 1R9O. There were extensively altered or mutated amino acids in the 1OG5 enzyme that encompass residues 30–53, 97–121, 196–233, and 467–478. Seven amino acids had been substituted in specific

regions (K206E, I215V, C216Y, S220P, P221A, I223L, and I224L) and 4 histidine tags had been added to the 1OG5 structure (Williams et al., 2003). There were no mutations introduced into the catalytic domain of the protein for 1R9O construct beside two terminal modifications: one on *C*-terminus extended by a 4-residue histidine tag and another on *N*-terminal transmembrane domain which has been removed (Wester et al., 2004).

In the 1OG5 structure, the seven residue substitutions (whether bound to warfarin or not) are similar to that of rabbit CYP2C5 (PDB ID: LVdH) in the region, which may result in warfarin binding to the 1OG5 enzyme in the distal end of the active site cavity, more than 10 Å from the heme iron (Williams et al., 2003). In this orientation, the *S*-warfarin molecule is believed to be too distant for the hydroxylation to occur. Furthermore, a relative large pocket (~470 $Å^3$) and no basic residue had been identified in the active site from the 1OG5 structure, which rendered the selectivity of CYP2C9 for small lipophilic anions difficult to understand.

In the 1R9O structure, however, the two terminal modifications merely facilitated purification and structural determination of the catalytic domains of the truncated enzyme without impact on the active site. The flurbiprofen in the active site of CYP2C9 was positioned at a reasonable distance (4.9 Å) from the iron to facilitate hydroxylation. A relative small structure encompassed the active site cavity in the 1R9O construct. Most importantly, the 1R9O structure reveals that the basic Arg108 residue points into the active site and is able to interact with the negatively charged lipophilic substrate, such as naproxen, ibuprofen, diclofenac, indomethacin, and gemfibrozil (Wester et al., 2004). This structure is in accordance with several experimental observations that were difficult to rationalize based on the 1OG5 structure.

The crystal structures of the CYP2C9 provide insight into the ligand-CYP interaction. However, the differences in the active sites of the two crystal structures of CYP2C9 may not only reflect conformational flexibility of the protein but also present challenge to understand the structure determinants of substrate oxidation. Most likely, the structure of CYP2C9-flurbiprofen complex is more reasonable than that of CYP2C9-warfarin complex for the explanation of substrate specificity of CYP2C9.

1.4.4 CYP2D6

Although the first modelling study of CYP2D6 published as early as 1993 based on the crystal structure of bacterial CYP101 (Koymans et al., 1993b), the human crystal structure of CYP2D6 was disclosed until 2006, a decade late (Rowland et al., 2006). A number of homologic models had appeared during the decade, on the basis of either bacterial enzymes (Koymans et al., 1993b; Lewis et al., 1997) or more recently the rabbit CYP2C5 enzyme (Kirton et al., 2002; Venhorst et al., 2003). These models provided some important information, such as the implication of Asp301 as a residue necessary for catalytic activity (Koymans et al., 1993b). However, a lot of difference among the models gives rise to some challenging questions regarding the explanations for experimental results from site-directed mutagenesis (SDM) studies. The structure of human CYP2D6, indeed, is able to explain many reported data of SDM and to help understand the metabolism of some substrates.

The crystal structure of CYP2D6 shows a fold similar to other recently solved human structures, especially to CYP2C9. Although the lengths and orientations of the individual secondary structural elements in CYP2D6 are very similar to those seen in CYP2C9, there are several notable differences existing at the helix C-D connection, the G-H loop, the turn number of F helix, the location of F-G loop related to B' helix. These differences are considered to be essential for CYP2D6 to shape the active site cavity that stands above the heme like a "right foot" with the volume of ~540 $Å^3$ (Rowland et al., 2006). The cavity is bordered by the heme group and residues from the B, F, G and I helixes, the B -C loop, the loop between helix K and β-sheet 1 strand 4, and the loop between the strands of β-sheet 4 (Rowland et al., 2006).

There are two negatively charged residues, Asp301 on I helix and Glu216 on F helix, identified as binding residues for substrates and inhibitors of CYP2D6. Between them, Asp301 played a key role in the binding of substrates to CYP2D6 as well as a structural role in hydrogen bonding to a backbone NH of the B-C loop, whereas Glu216 is more likely responsible for residue recognition and an intermediate binding form (Rowland et al., 2006). Mutation of either Asp301 or Glu216 to a neutral amino acid results in loss of CYP2D6 activity (Lennard, 1990), which implicate the importance of the two residues.

Two phenylalanine residues, Phe481 and Phe483, in the loop between two strands of β-sheet 4 region (SRS6) attracted attention and also gave rise to some debate around the Phe481 positioning (de Groot et al., 1999a). Early homology modelling studies suggested that Phe481

is an important aromatic residue associated with ligand binding (de Groot et al., 1999b). This residue appears to interact with ligands via a π-π interaction between its phenyl ring and the planar hydrophobic aromatic moiety common to many CYP2D6 substrates. Substitution of Phe481 by Leu or Gly reduced the affinity of several typical CYP2D6 substrates, including debrisoquine, metoprolol and dextromethorphan, with 3-16-fold higher K_m values compared to the wild-type (Hayhurst et al., 2001). However, replacement of Phe481 with Thr did not alter the K_m and V_{max} values for *S*-metoprolol, debrisoquine and dextromethorphan. Homology models based on rabbit CYP2C5 suggest that, however, Phe481 is positioned outside the binding pocket, but in close contact with the active site residue Phe483 (Smith et al., 1998; Venhorst et al., 2003). The crystal structure clearly shows that Phe483 is oriented into the cavity, whereas Phe481 is located remotely rather than pointing directly toward the heme group (Rowland et al., 2006).

Another critical Phe residue is Phe120 located in the B-C loop, and its importance has been recognised in SDM studies (Flanagan et al., 2004; McLaughlin et al., 2005). Keizers *et al.* (2004) revealed that the F120A mutant abolished the *O*-demethylation activity toward 7-methoxy-4-(aminomethyl)-coumarin 7-methoxy-4-(aminomethyl)-coumarin (MAMC, used as an *in vitro* probe for CYP2D6), whereas bufuralol 1'-hydroxylation was not affected. Surprisingly, the mutant protein carrying the F120A mutation can metabolize quinidine via *O*-demethylation and 3-hydroxylation (McLaughlin et al., 2005), unlike the wild-type CYP2D6. The mutation F120I (358T>A; rs1135822) can be found in a small percentage of the Southeast Asian population (Solus et al., 2004). All of these findings indicate that residue Phe120 in the active site is important in substrate binding and catalysis in CYP2D6. The position of Phe120 is confirmed in the active site of CYP2D6 by the crystal structure and the role of the Phe120 is suggested to orient substrates with respect to the heme and to form π-π stacking interactions with CYP2D6 substrates that contain aromatic rings.

1.4.5 CYP3A4

Two similar ligand-free structures of human CYP3A4 were published independently in 2004 (Williams et al., 2004; Yano et al., 2004). In contrast to the structures of CYP2 family, the most prominent features of the CYP3A4 structure are the short F and G helixes that do not pass over the active site cavity, as well as a large, highly ordered hydrophobic core of phenyl alanine residues above the active site (Yano et al., 2004). However, the volumes (~670 $Å^3$ and ~950 $Å^3$) of the active sites in the published both ligand-free structures seem to be too small to

metabolize large substrates such as bromocriptine (M_r 655 Dal) and cyclosporine (M_r 1,203 Dal). It is speculated that conformational changes may occur upon ligand binding.

Yano *et al*. (2004) reported an active-site volume of 1,386 $Å^3$ while Williams *et al*. (2004) found a small volume ~520 $Å^3$. Although the active site volume of CYP3A4 is similar to that of CYP2C8, the shape of the active site cavity differs considerably due to differences in the folding and packing of portions of the protein that form the cavity (Yano et al., 2004). Compared with CYP2C8, the active site cavity of CYP3A4 is much larger near the heme iron (Yano et al., 2004). CYP3A4 contains an unexpected peripheral binding site located above a 7-Phe residue cluster, which may be involved in the initial recognition of substrates or allosteric effectors (Williams et al., 2004). The progesterone molecule resides in the peripheral "nest" formed by loops between the F and F′ helices and the G′ and G helices, i.e. in the F/G-loop region. This resembles that of palmitate binding in the CYP2C8 (1PQ2) structure (Schoch et al., 2004).

There is a remarkable difference between the two ligand-free CYP3A4 structures in the position of the Arg212 residue located within the linker between F and F0 and lining the active site. In the structure reported by Yano *et al*. (Yano et al., 2004) this side chain was directed towards the heme iron and hydrogen bonds to surrounding residues in a conformation that could disable the proton transfer pathway required for catalytic cycle. In the structure reported by Williams et al. (Williams et al., 2004), however, this side chain was rotated by ~120^o and oriented away from the putative proton transfer pathway. This discrepancy between the two structures indicates that this might be a flexible element of the structure.

To date, there are four crystal structures of CYP3A4–ligand complex with metyrapone, progesterone, ketoconazole and erythromycin respectively (1TQN, 1WOE, 1WOF and 1WOG). Surprisingly, the protein conformational change upon ligand binding failed to be observed in two CYP3A4–ligand complex structures with metyrapone and progesterone (Williams et al., 2004). However, dramatic conformational changes were observed in the structures of CYP3A4–ketoconazole and CYP3A4–erythromycin complexes, with the increase in the active site volume by >80%. The volume of the active site is increased to 1,650 $Å^3$ in the ketoconazole-complexed structure and to ~2,000 $Å^3$ in erythromycin-complexed structure (Ekroos and Sjogren, 2006), although these are less pronounced than those seen in the structures of rabbit CYP2B4 (Scott et al., 2003). Interestingly, four ketoconazole molecules

have been identified simultaneously binding in the active site of CYP3A4 (Ekroos and Sjogren, 2006). One of the four ketoconazoles bound to the heme iron with its imidazole nitrogen. If the ligand-induced conformational changes can reflect the flexibility of CYP3A4, the simultaneous binding of multiple ligands may partially explain the atypical Michaelis–Menten kinetics and drug–drug interactions displayed by CYP3A4.

1.4.6 Other CYPs

To date, there are 10 structures of CYP2A6 available in PDB. These include the structures of 2A6 in complex with coumarin (PDB: 1Z10) (Yano et al., 2005), methoxsalen (1Z11) (Yano et al., 2005) and synthetic 3-heteroaromatic analogues of nicotine as inhibitors (2FDY, 2FDW, 2FDV, and 2FDU) (Yano et al., 2006). Several structures of the CYP2A6 N297Q (2PG5), L240C/N297Q (2PG6) and N297Q/I300V (2PG7) mutants have also been solved to a resolution of 1.95, 2.50 and 2.80 Å, respectively (Sansen et al., 2007). Recently, Devore *et al*. (2008) reported the structure of 2A6 I208S/I300F/G301A/S369G mutant in complex with phenacetin (3EBS).

All X-ray structures of CYP2A6 show the common typical CYP fold as other CYP members. The identities of the residues that contact the ligand molecules are identical in different CYP2A6 complex structures, and changes in the contacting amino acids are generally restricted to slight rearrangements of 107Phe to maximize orthogonal aromatic interactions.

CYP2A6 has the smallest active site cavity with a volume of 260 $Å^3$ among all human CYPs whose structures have been determined. This is about 4-fold smaller than those of CYP2C8, 2C9 or 3A4 (Yano et al., 2005). The CYP2A6 structure shows a clearly well-adapted enzyme for the oxidation of small, planar substrates that can fit into the compact, small, and hydrophobic active site with one hydrogen bond donor, Asn297, which orients ligands such as coumarin for regio-selective oxidation. The small active site volume of 2A6 may be associated with rather tight packing of the secondary structural units. In contrast to other mammalian CYP structures, helix I of CYP2A6 has an ideal secondary structural fold with no remarkable kink in the vicinity of heme. In addition, there was no water molecule found between the heme cofactor and helix I. The proximity of the substrate hydroxylation site (~3.3 Å) to the heme Fe atom may explain the displacement of a water molecule from the sixth coordination of the heme iron, leading to conversion of the heme ion from a low to a high spin state.

The structure of CYP2C8 was first determined in the absence of substrates or inhibitors by Schoch *et al*. (2004). Consistent with the large size of several substrates and inhibitors such as paclitaxel and montelukast, the enzyme exhibits a relatively large substrate-binding cavity compared with the ones evident for structures of most other human CYPs (Schoch et al., 2004). Computer-simulated docking indicated that the large active-site cavity is likely to accommodate substrates in several possible binding poses that do not necessarily conform closely to the proposed pharmacophore. Additionally, the docking simulations suggested that anionic groups might be accommodated in a large substrate access channel located between the helix B-C loop and β1 sheet with the potential for polar interactions with protein side chains as well as residual water molecules (Melet et al., 2004). Further computer simulations also indicated that all-*trans*-retinoic acid might bind in either a proximal site or an alternative distal site near helix B' that places the retinoid carboxylate close to Arg241. The latter suggested that conformational changes could allow Arg241 to neutralize the charge of the retinoid in the distal site.

Recently, Schoch *et al*. (2008) further determined the crystal structures of CYP2C8 complexed with montelukast (2.8 Å, 2NNI), troglitazone (2.7 Å, 2VN0), felodipine (2.3 Å, 2NNJ), and 2 molecules of 9-*cis*-retinoic acid (2.6 Å, 2NNH) (Schoch et al., 2008). Montelukast is a relatively large anionic (M_r = 586) inhibitor that exhibits a tripartite structure and complements the size and shape of the active-site cavity; while the inhibitor troglitazone (M_r = 442) occupies the upper portion of the active-site cavity, leaving a substantial part of the cavity unoccupied. The smaller neutral felodipine molecule (M_r = 384), a high affinity inhibitor of CYP2C8 with a K_i of 90 nM (Marill et al., 2000), is sequestered with its dichlorophenyl group positioned close to the heme iron, and water molecules fill the distal portion of the cavity. The structure of the 9-*cis*-retinoic acid (M_r = 300) complex reveals that two molecules bind simultaneously in the active site of CYP2C8. A second molecule of 9-*cis*-retinoic acid is located above the proximal molecule and can restrain the position of the latter for more efficient oxygenation (Schoch et al., 2008). Solution binding studies do not discriminate between cooperative and noncooperative models for multiple substrate binding. The complexes of CYP2C8 with structurally distinct ligands further demonstrate the conformational adaptability of active site-constituting residues, especially Arg241, which can reorient in the active-site cavity to stabilize a negatively charged functional group and define two spatially distinct binding sites for anionic moieties of substrates.

1.5 Drug-drug, herb-drug and herb-CYP interactions

1.5.1 Clinical significance of drug interactions

Drug interactions may occur during recent or concurrent use of another drug or drugs or ingestion of food. It was defined as the action of a drug that may affect the activity, metabolism, or toxicity of another drug. A drug interaction is any pharmacological modification of an exogenous substance (in drug, herb and food) in a body caused by another exogenous compound (in drug, herb and food) during a diagnostic or therapeutic period (MacLennan et al., 2006). This relates to so-called drug-drug interactions (interactions between drugs), herb-herb interactions (interactions between herbs) or drug-food interactions (interactions between drug and food).

Most drug interactions are involved in pharmacodynamic and/or pharmacokinetic mechanisms. Pharmacodynamic interactions involve synergistic or antagonistic interactions on drug targets, e.g. receptors, which can often be predicted and avoided. For example, Ma Huang contains ephedrine-like alkaloids which exhibit sympathomimetic activities. Thus, Ma Huang may interact with other sympathomimetic agents and then increase the actions of monamine oxidase inhibitors and adrenergic agonists such as clonidine, and decrease the actions of bethanidine and guanethidine (Wooltorton and Sibbald, 2002) On the other hand, pharmacokinetic interactions are much more difficult to anticipate, which occur through multiple mechanisms, including alterations of compounds' absorption, distribution, metabolism and excretion. Most reported drug interactions are pharmacokinetic interactions. Coadministration of two or more drugs or herbs may give rise to drug interactions due to an alteration of CYPs activity (Lynch and Price, 2007) if the drugs or herbs are metabolized by the same enzyme system(s). The drug interactions may potentially result in altered pharmacokinetics for one or all of the coadministered compounds due to either inhibition or induction of a specific CYP enzyme. If these effects of the drug interaction occur to certain extent, clinical efficacy of those drugs may be lost and furthermore adverse drug interactions, including some fatal interactions may overcome their therapeutic anticipation (Li, 2001; Lin and Lu, 2001).

1.5.2 Drug interactions due to inhibition of CYP enzymes

Inhibition of CYP enzymes is one of the most common causes of harmful drug–drug interactions and has led to the withdrawn of several marketed drugs during the past decades. The nonsedating antihistamines terfenadine and astemizole, for instance, and the gastrointestinal motility agent cisapride, were all withdrawn from the U.S. market because

metabolic inhibition by other drugs led to life-threatening arrhythmias (Dresser et al., 2000). The calcium channel blocker mibefradil was withdrawn from the U.S. market in 1998 because it was a potent mechanism-based enzyme inhibitor that increased the plasm concentration of other cardiovascular drugs to toxic levels (Mullins et al., 1998).

Inhibition of CYPs activity can reduce metabolism and elimination of the parent compounds that are subject to first-pass metabolism and lead to increased bioavailability even toxicity of these compounds, especial for those extensively metabolized mainly by CYP enzymes. For example, a clinical trial had indicated that fluconazole, a potent inhibitor of CYP2C9, reduced approximately 70% of metabolic clearance of *S*-warfarin, leading to significant bleeding at clinical setting (Black et al., 1996). With regard to prodrugs, inhibition may result in a decrease in the amount of the active drug form, leading to therapeutic failure due to lack of efficacy of the drug. Tamoxifen, a selective estrogen receptor modulator, could significantly reduce the conversion of prodrug losartan to its active form by inhibiting CYP2C9 activity in breast cancer patients (Boruban et al., 2006).

The type of CYP inhibition can be either reversible (competitive or non-competitive) or irreversible (mechanism-based). Reversible inhibition is the most common type of enzyme inhibition and takes place directly, while irreversible inhibition requires biotransformation of the inhibitor. Reversible inhibition can be further divided into competitive, noncompetitive, uncompetitive, and mixed-type inhibition (Lin and Lu, 1998; Hollenberg, 2002). In competitive situation, substrate and inhibitor are competitory to bind to the same position at the active site of an enzyme with hydrophobic, electrostatic or hydrogen-bond interactions, which are both formed and broken down easily (Lin and Lu, 1998; Hollenberg, 2002). In a noncompetitive inhibition, however, the binding site of the inhibitor is different from that of the substrate. As for mixed-type inhibition, both competitive and noncompetitive inhibitions are frequently observed. For example, *in vitro* studies have demonstrated that glyburide strongly inhibited CYP2C9-catalyzed *S*-warfarin and phenytoin metabolism in a competitive manner (Kim and Park, 2003).

Irreversible inhibition, on the other hand, usually occurs by forming metabolite intermediate complexes, which bind to the residues or heme of the CYP with strong covalent bond leading to a long lasting inactivation (Zhou et al., 2004c; Zhou et al., 2005c). This process is called ‘mechanism based inhibition’ or ‘suicide inhibition’ — the metabolic product inactivates the

enzyme completely. Classical mechanism-based inhibitors include the CYP1A2 inhibitor furafylline (Sesardic et al., 1990; Kunze and Trager, 1993), the CYP3A4 inhibitor gestodene (Guengerich, 1990; Back et al., 1991), and the CYP2E1 inhibitor disulfiram (Kharasch et al., 1993). The typical feature of mechanism-based inhibition is the time-, concentration- and NADPH-dependent and is terminated by enzyme re-synthesis (Halpert, 1995; Ito et al., 1998c; Kent et al., 2001).

1.5.3 Drug interaction due to induction of CYP enzymes

In contrast to inhibition, induction of CYP enzymes usually occurs through two general mechanisms: stabilizing the mRNA or enzyme (e.g., CYP2E1) (Gonzalez, 2007) and increasing gene transcription. Increase in gene transcription of CYP enzyme is more common than stabilization of the mRNA or enzyme and is mediated by nuclear receptors, such as AhR, CAR, and PXR (Moore et al., 2002; Honkakoski et al., 2003; Wang and LeCluyse, 2003; Mandal, 2005; Qatanani and Moore, 2005; Tirona and Kim, 2005). Induction of gene transcription is usually triggered by ligand (drug) binding to the ligand binding domain (LBD) of the nuclear receptors. Subsequently, the ligand-activated transcription factors conduct conformational changes of the receptors leading to the release of co-repressors and recruitment of co-activators and a dimerization partner (RXR, for CAR/PXR and the AhR nuclear translocator, ARNT, for AhR) to form the actual DNA-binding complex. Finally, the DNA binding domain (DBD) on the nuclear receptor is exposed and binds to respective DNA response elements present in the promoter region of target genes (CYP enzymes) leading to gene transcription (Wang and LeCluyse, 2003; Lemaire et al., 2004).

This inductive process produces more CYP enzyme than that present normally in a biological system. The increased CYP enzyme along with increased activity elevates metabolic clearance of certain drugs, substrates of relative CYP enzyme. Consequently, pharmacokinetics of these drugs is influenced, reflecting by reduced AUC, maximum plasma concentration (C_{max}), and half-life. A typical example is the herbal antidepressant St John's Wort (a potent CYP3A4 inducer) that had been reported to increase CYP3A4 expression and consequently decrease the AUC and C_{max} of midazolam (a CYP3A4 substrate) by 79 and 65%, respectively (Mueller et al., 2006). The induction of CYP3A4 by St John's wort resulted in reduced ethinylestradiol levels from oral contraceptives, leading to unexpected pregnancies (Gordon, 1998; Barbenel et al., 2000). Furthermore, St John's Wort has been reported to reduce cyclosporine concentrations in transplant patients and lead to organ rejection (Breidenbach et al., 2000a;

Breidenbach et al., 2000b). Both ethinylestradiol and cyclosporine are predominately metabolized by CYP3A4 (Zhou, 2008a).

1.5.4 Herb-drug interactions

1.5.4.1 Clinically reported herb-drug interactions

Herbal medicines, such as St John's wort, garlic, gingko, and ginseng, are freely available over the counter and very often self-administered complements along with therapeutic drugs (De Smet, 2002; De Smet, 2005). This has given rise to potential adverse herb-drug interactions in clinical settings when co-administered with prescribed medicines. A number of adverse herb-drug interactions have been identified in humans and frequently impacted medications are those with a narrow therapeutic window and extensively metabolized by CYP enzymes, such as warfarin, digoxin and cyclosporine (Fugh-Berman, 2000; Hu et al., 2005; Li et al., 2007; Zhou et al., 2007). One of the most commonly reported herbs is St John's wort which interacts with a broad therapeutic drugs, including cyclosporine, digoxin, theophylline, oral contraceptives, methadone, fluoxetine, and buspirone (Table 1-8) (Barone et al., 2000; Karliova et al., 2000; Mai et al., 2000; Ruschitzka et al., 2000; Ahmed et al., 2001; Beer and Ostermann, 2001; Moschella and Jaber, 2001; Turton-Weeks et al., 2001; Alscher and Klotz, 2003). *Gingko biloba* was also reported to interact with ibuprofen, trazodone, fluoxetine, buspirone and phenytoin (Table 1-8). It should be noted that both warfarin and cyclosporine are well-known substrates of CYP2C9 and 3A4, respectively, while St John's wort is a potent inducer of CYP3A4 and P-glycoprotein (P-gp). An additional example is licorice (*Glycyrrhiza glabra*) which was reported to increase the plasma concentrations of prednisolone (Chen et al., 1990; Chen et al., 1991) by inhibiting the metabolism of prednisolone, and also potentiated the skin vasoconstrictive action of hydrocortisone (Teelucksingh et al., 1990).

The use of multiple medicines will significantly increase the risk of potential herb-drug interactions, especially in the elderly or certain group of consumers such as cancer patients. The risk for drug interactions increases with the number of products consumed. For example, the risk for potential interactions for consuming two products is 6%; five products, 50%, and the risk increases to 100% for consuming eight or more products. In this regards, the likelihood of herb-drug interactions is theoretically higher than drug-drug interactions since most therapeutic drugs usually contain a single chemical entity.

It should be pointed out, however, that our understanding about the interactions between herbs and drugs is still limited (Zhou et al., 2007). It is difficult to characterise and identify definitely an herb-drug interaction. The knowledge of herb-drug interaction is largely based on case reports or case series reports. Considering that a significant number of patients or herbal consumers failed to disclose the use of herbal products to their physicians (Klepser et al., 2000), and most physicians have limited knowledge on various herbal products, the risk of potential herb-drug interactions is increased. Thus, there have been efforts for implementation of coordinated toxicity monitoring systems by the World Health Organization (WHO), eg WHO Collaborating Centre for International Drug Monitoring (http://www.who-umc.org/), and by various governments including Australia, UK, USA, Singapore and China, aimed at improving monitoring and timely reporting of potential herb-drug interactions. To date, a number of herb-drug interactions have been identified in humans and these have been summarized in Table 1-8.

1.5.4.2 Mechanisms for herb-drug interactions

In general, a single herb contains multiple phytochemicals that may be biologically active and capable of modulating physiological actions, similar to therapeutic drugs, through complex synergistic and antagonistic effects. Therefore, it is not hard to understand that most herb-drug interactions are mediated by pharmacodynamic and/or pharmacokinetic mechanisms. Pharmacodynamic interactions involve synergistic or antagonistic interactions on the same drug targets, e.g. receptors, which can often be predicted and avoided. For example, Ma Huang contains ephedrine-like alkaloids which exhibit sympathomimetic activities. Thus, Ma Huang may interact with other sympathomimetic agents and thus increase the actions of monamine oxidase inhibitors and adrenergic agonists such as clonidine, and decrease the actions of bethanidine and guanethidine (Wooltorton and Sibbald, 2002) On the other hand, pharmacokinetic interactions are much more difficult to anticipate, which occur through multiple mechanisms, including alterations of drug's absorption, distribution, metabolism and excretion. Most common reported herb-drug interactions are pharmacokinetic interactions, especial those resulting from the modulation of the activities of CYPs and/or drug transporters.

The activity of CYPs may be changed by herbal ingredients through enzyme induction and inhibition. Like therapeutic drugs, the induction of CYPs by herbal product usually requires several days, which may lead to decreased drug plasma levels (through increased drug metabolism), and subsequently to reduced drug effects (Zhou et al., 2003b). Conversely, the

inhibition of CYPs is often immediate and may lead to increased drug plasma levels (through decreased drug metabolism), and thus increased drug effect which may result in significant adverse reactions or toxicities. Many clinical adverse events induced by herbal produces have been associated with CYP inhibitions (Hu et al., 2005; Li et al., 2007; Zhou et al., 2007).

Herbs may inhibit CYPs by three mechanisms: competitive inhibition, non-competitive inhibition, and mechanism-based inhibition. Mutual competitive inhibition may occur between a herbal constituent and a drug, as both are often metabolised by the same CYP isoform. For example, diallyl sulfide from garlic is a competitive inhibitor of CYP2E1 (Teyssier et al., 1999). Non-competitive inhibition is caused by the binding of herbal constituents containing electrophilic groups (e.g. imidazole or hydrazine group) to the heme portion of CYP. For example, piperine inhibited arylhydrocarbon hydroxylase (CYP1A) and 7-ethoxycourmarin deethylase (CYP2A) by non-competitive mechanism (Dalvi and Dalvi, 1991). Hyperforin present in St John's wort is also a potent noncompetitive inhibitor of CYP2D6 activity *in vitro* (Obach, 2000a). The mechanism-based inhibition of CYP is due to the formation of a complex between herbal metabolite with CYP. For example, diallyl sulfone derived from diallyl sulfide is a suicide inhibitor of CYP2E1 by forming a complex via an epoxide metabolite (Premdas et al., 2000), leading to autocatalytic destruction of CYP2E1 (Jin and Baillie, 1997a).

1.5.4.3 Prediction of metabolic herb-drug interactions

Herb-drug interactions may be harmful or even fatal. For example, feverfew, garlic, ginkgo, ginger, and ginseng may potentiate the effect of warfarin, resulting in longer bleeding time (Fugh-Berman, 2000; Fugh-Berman and Ernst, 2001). Kava has resulted in coma when used with alprazolam (Miller, 1998). Therefore, it is important to be able to extrapolate both *in vitro* and *in vivo* data of herb-drug interactions to humans. Some successes have occurred in the prediction of drug-drug interactions from *in vitro* metabolic inhibition data based on *in vitro* models such as hepatic microsomes and hepatocytes, if the following criteria can be met: a) drug clearance must be primarily by metabolism; b) drug is not subject to substantial conjugation or other non-CYP metabolism; c) the liver is the primary organ of metabolic clearance; and d) the compound does not possess physico-chemical properties that are associated with absorption problems (i.e. limited solubility, low gastroenteral permeability) (Houston, 1994; Obach, 2000b). The prediction of the alteration in plasma concentration or the area of the plasma AUC by a coadministered compound involves the determination of inhibition constant (K_i), and the unbound concentration of inhibitor ([I]).

However, the prediction of metabolic drug interactions from *in vitro* systems is limited due to several problems including inappropriate design of *in vitro* experiments; presence of extra-hepatic metabolism; and active transport in liver. In addition, the in vitro scaling of kinetic and inhibition data from human tissues is more complex, particularly as the metabolism of many drugs by CYP3A4 is inconsistent with a classical Michaelis-Menten kinetic model (Lin, 1998; Houston and Kenworthy, 2000). Despite these difficulties, quantitative *in vitro* metabolic inhibition data can be extrapolated reasonably well to *in vivo* situations with the application of appropriate pharmacokinetic principles (Ito et al., 1998a; Ito et al., 1998b). Thus, the prediction of metabolic herb-drug interactions could provide a useful tool to offer the opportunity to use *in vitro* inhibition data as a criterion for monitoring herb-drug metabolic interactions involving human drug metabolising enzymes (in particular CYPs).

1.6 Tools to study drug interactions

A combination of *in silico*, *in vitro* and i*n vivo* models are often used in drug-drug, herb-drug and herb-CYP interaction studies. *In silico* is a term used for experiments done using a high-performance computer (i.e. on a silicon chip), while *in vitro* and *in vivo* refer to experiments done outside of living organisms and in living organisms, respectively.

1.6.1 *In silico* methods

There is an increasing use of *in silico* methods to study CYPs and their interactions with xenobiotics (Hutter, 2009). The major *in silico* methods include simple rule-based modelling, structure-activity relationships, three-dimensional quantitative structure-activity relationships (QSAR), and pharmacophores (Krejsa et al., 2003; Hutter, 2009). All represent useful tools for understanding reactions catalyzed by CYPs, predicting possible herb-drug metabolism interactions, pharmacokinetic parameters such as clearance, and toxicity (Harris, 2004). The resulting data based on *in silico* approaches may be of clinical relevance (Norinder, 2005). For example, knowledge of the substrate specificity and regulation of the CYP is essential, as this will provide information on the possible herb-drug interaction.

In silico approaches have also been used to study herb-CYP interactions (Wilson et al., 2003; de Groot et al., 2004; de Graaf et al., 2005). A structure-activity relationship analysis was used to investigate the effect of structural modifications of piperine (pentadienyl or piperidine) on the inhibition of the CYP-catalyzed reactions, arylhydrocarbon hydroxylation (CYP1A) and

7-methoxycoumarin-O-demethylation (CYP2) in microsomes prepared from untreated, 3-methylcholanthrene- and phenobarbital-treated rat liver (Koul et al., 2000). This study has indicated that saturation of the side chain resulted in a marked increase in the inhibition of CYPs; while modifications in the phenyl and basic moieties in a few analogues led to maximum selectivity in inhibiting either constitutive or inducible CYP activities (Koul et al., 2000). QSAR studies have been used to analyze the inhibitory effects on caffeine N^3-demethylation (a marker activity of CYP1A2) in human liver microsomes of naturally occurring flavonoids that exist in many herbs (Lee et al., 1998). This study demonstrated that the number of hydroxyl groups and their glycosylation had an important influence on the inhibitory effect of various flavonoids. QSAR analysis has indicated that the volume to surface area ratio was the most effective factor for producing the inhibition of caffeine N^3-demethylation by these flavonoids, and the electron densities on the C3 and C4' atoms exercised significant influence on the inhibitory effect. The suppression of 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline-induced *umu* gene expression by flavonoids was well correlated with their calculated CYP1A2 inhibitory potencies (Lee et al., 1998).

The use of computational techniques will add chemical knowledge to the empirical data obtained with *in vitro* systems and enable the prediction of substrates or inhibitors of specific enzymes through computer-based models. The predictions by means of a rapid *in silico* screen might be useful as a drug interaction screen, and assist medicinal chemists understanding potential inhibitors for certain enzymes in the use of therapeutics and making necessary chemical modifications in the drug discovery process. There is an increasing use of *in silico* methods to study CYPs and their interactions with xenobiotics (Ekins and Wrighton, 2001; Lewis and Dickins, 2001; Vedani et al., 2006) since they are an important family of drug-metabolizing enzymes.

In silico screening can be performed in two fundamental ways: ligand-based and protein-based manner. Combinations of protein- and ligand-based methods have often been used. For the ligand-based method, molecular descriptors extract information from a set of CYP ligands to build a model (e.g. QSAR or pharmacophore model) that provides rules to classify other chemicals as potential CYP ligands and is validated by another set of known CYP ligands. Alleged pharmacophore is a hypothesis representing generalized molecular features including 3D (hydrophobic groups, charged/ionizable groups, hydrogen bond donor/acceptors), 2D

(substructures), and 1D (physical and biological properties) aspects that are considered to be responsible for a desired activity (Purushottamachar et al., 2007).

There are often two different approaches applied in the hypothesis generation: Catalyst Hypogen and HipHop programs. Hypogen is an activity-based alignment derived from a training set that collects conformational models of compounds spanning activities of 4–5 orders of magnitude. At least 16 molecules are necessary to ensure statistical significance of pharmacophores computed in the Catalyst Hypogen algorithm. HipHop, on the other hand, is a common feature alignment of highly potent compounds based on 3D feature information without consideration of the activity in the set molecules. In addition, two separated sets of compounds (training and validating sets) are critical for the applicability and predictivity of both Hypogen and HipHop models. If the training set for the building of both models are narrowed at a single core structure, the produced model may be of limited value to other research programs, unless the same structure type is used. Once the training set is comprised by a number of distinct core structures, a good generalizable hypothesis is possible to be produced (Ekins, 2003). A retrieved pharmacophore model is expected to discriminate between active and inactive compounds.

In the protein-based approach, candidate ligands are docked into the crystal structure or a homology model of certain CYPs, and the estimated low free energy of binding and inhibition constant (K_i) will be calculated for the evaluation of CYP inhibitory potency.

Although it is a virtual screening system, *in silico* study could provide some early prediction of the possible involvement of CYPs in the metabolism of drugs or drug candidates, not only improving drug safety but also contributing to make drug design more effective and less cost. Available crystal structures of human CYPs have provided important functional information of these proteins and are very useful for further *in silico* studies.

1.6.2 *In vitro* methods

A number of *in vitro* systems have established to investigate drug-CYP interactions, including cDNA expressed recombinant human CYP enzymes (from baculovirus-infected insect cells and *E. coli*), subcellular fractions (liver microsomes, cytosols, and homogenates), B lymphoblastoid cells, isolated and cultured hepatocytes or liver cell lines and precision-cut liver slices (Eddershaw and Dickins, 1999; Ekins et al., 2000b; Streetman et al., 2000a;

LeCluyse, 2001; Venkatakrishnan et al., 2001b; Baranczewski et al., 2006). Each of these systems has advantages and limitations, and it is most likely that a combination of methods will provide the most accurate information on drug-CYP interactions.

A number of cDNA expressed recombinant human CYP enzymes are available, and offer a great chance for interaction study between drug candidates and CYP enzymes by a rapid manner, which make high throughput screening *in vitro* available. There are several CYP screening kits aimed to offer a simple "mix-and-read" fluorescent assay that is designed for high-throughput (HTP) screening in multi-well plates. Test compounds are analyzed by their capacity to inhibit the production of a fluorescent signal in reactions using recombinant CYP enzymes and specific CYP substrates along with appropriate positive and negative controls.

To date, 26 human CYP enzymes have had commercial screening kits containing recombinant cDNA-expressed CYP enzymes prepared from the baculovirus-infected insect cell system, including CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9.1 (Arg144), 2C9.1 (Arg144), 2C9.2 (Cys144), 2C9.3 (Leu359), 2C18, 2C19, 2D6.1 (Val374), 2D6.10, 2E1, 2J2, 3A4, 3A5, 3A7, 4A11, 4F12, 4F2, 4F3A, 4F3B, CYP19 (aromatase) and CYP oxidoreductase (www.bdbiosciences.com/product_families). These enzyme systems provide high level of catalytic activity (six-fold higher than an average human live microsomes sample) and are used for screening study of diverse compounds related to metabolism *in vitro*. All of the major drug metabolism enzymes are available in this expressed enzyme system.

For inhibition studies, IC_{50} values obtained from cDNA-expressed enzyme system can be compared with that of known inhibitors detected by the same enzyme without the complication of competing pathways of metabolism. With the cDNA-expressed enzyme system, Phase II reaction can also be investigated through different kits with different enzyme system (not in the scale of present project). However, induction effect of test compounds on CYP enzymes could not be investigated by these systems (Crespi and Penman, 1997). Even though, the use of cDNA-expressed CYP system is also a reasonable starting point for the preliminary determination of the principal CYPs involved in a drug candidate in a drug discovery setting.

Liver microsomes systems sound like an ideal for the production of most major metabolites from both Phase I and Phase II reactions. However, cofactors (nicotinamide adenine dinucleotide phosphate–NADPH or urindine diphosphate glucuronic acid) need to be add

artificially since CYP- or uridine diphosphate glucuronotransferase (UGT)-catalyzed reactions to replace those lost due to the destruction of cell integrity. In addition, because of the latter, no coupled metabolism is present, and Phase II reactions following a Phase I reaction cannot be studied.

In contrast, hepatocytes provide cellular integrity with respect to enzyme architecture and allow the study of Phase II reactions following Phase I metabolism. In addition, hepatocytes allow for any concentration gradients mediated by transporters that may affect exposure of substrate/inhibitor to enzymes. However, some transporters are rapidly down-regulated after isolation of hepatocytes (Li et al., 1997), and support matrices (sandwich cultures) may introduce artefacts (e.g., additional collagen diffusion barrier; and loss of enzyme activity) (LeCluyse, 2001). Precision-cut liver slices probably best simulate the *in vivo* situation as they retain the physiological environment for the enzymes and cofactors of both Phase I and Phase II reactions and partially retain the architecture of the liver (Parrish et al., 1995; Ekins, 1996; Ferrero and Brendel, 1997; Olinga et al., 1998). However, both uptake and/or metabolism in liver slices are often lower than in hepatocytes, which limit their utility as a predictive model for pharmacokinetic scaling.

Obviously, *in vitro* models may provide fundamental information of drug-CYP interactions by a quick screening manner but is impossible to draw a comprehensive picture for the interactions. However, high throughput screening with cDNA-expressed enzyme system can provide relative accurate inhibitory potency (e.g. IC_{50} or K_i values) of tested compounds on a specific CYP (Carlson and Fisher, 2008).

1.6.3 *In vivo* methods

Although *in silico* and *in vitro* models may provide quick screening methods for the herb-CYP interactions, *in vivo* interaction studies are usually necessary to provide evidence of their clinical importance. Animal studies may give important information on herb-CYP interactions, but inter-species variations in the substrate specificity, catalytic features and amino acid sequences of CYPs may cause difficulty in extrapolating animal data to humans (Boobis et al., 1990; Lewis et al., 1998). For example, chlorzoxazone 6-hydroxylation is extensively catalyzed by CYP2E1 in humans (de Vries et al., 1994), but by CYP1A2 and 3A1 in rats (Kobayashi et al., 2002). It may be difficult to predict accurately the effects of tested

compounds in humans based on animal data. Therefore, clinical trials of human studies are usually required to confirm herb-CYP interactions.

A common approach for estimation of *in vivo* drug interactions in animals and humans is through the administration of a specific probe compound, which is predominately or exclusively metabolized by an individual CYP enzyme. Probe substrates and selective inhibitors (see Table 1-2) can be used to explore the effects of herbs on the activity of specific CYP enzyme *in vivo*, e.g. caffeine for CYP1A2 (Carrillo et al., 2000b), tolbutamide or warfarin for CYP2C9 (Bourrie et al., 1996; Chainuvati et al., 2003), mephenytoin or omeprazole for CYP2C19 (Streetman et al., 2000a; Chainuvati et al., 2003), dextromethorphan, or debrisoquin for CYP2D6 (Wieling et al., 2000), chlorzoxazone for CYP2E1 (Lucas et al., 1999), and midazolam (Rivory et al., 2001) or erythromycin (Rivory et al., 2001) for CYP3A4 (Brockmoller and Roots, 1994; Streetman et al., 2000a). In clinical trial, there are two basic strategies to handle probe drugs, individual administration of a specific probe targeting one CYP enzyme and simultaneous administration of multiple probes targeting multiple enzymes at one trial session. The later method is so-called "cocktail" strategy.

The cocktail of probe drugs have been used to explore the activities of multiple CYPs (Frye et al., 1997; Adedoyin et al., 1998; Dierks et al., 2001) and could provide information on several metabolism pathways in a single session of clinical trial, which minimizes the complicating influence of intra-individual variability over time. For example, alprazolam and caffeine can be administered simultaneously for the assessment of in vivo CYP3A4 and 1A2 activity, respectively (Schmider et al., 1999). A cocktail, including probe drugs caffeine, chlorzoxazone, mephenytoin, metoprolol, and midazolam administered simultaneously has effectively phenotyped CYP1A2, 2E1, 2C19, 2D6, and 3A4, respectively, in humans (Zhu et al., 2001). Similarly, a cocktail containing tolbutamide (CYP2C9), caffeine (CYP1A2), dextromethorphan (CYP2D6), oral midazolam (intestinal wall and hepatic CYP3A), and intravenous midazolam (hepatic CYP3A) have been used to investigate the effects of St John's wort on the activities of various CYPs in humans (Wang et al., 2001).

However, the value of the "cocktail" approach may be limited due to marked intrasubject variability and the possibility of interaction between the coadministered probes. Palmer *et al*. (2001) (Palmer et al., 2001) reported that chlorzoxazone significantly altered the pharmacokinetics of oral midazolam, perhaps through inhibition of first-pass metabolism by

CYP3A in the intestine. However, since Streetman *et al*. (Streetman et al., 2000b) have validated a 4-drug cocktail (caffeine, dextromethorphan, omeprazole and midazolam), a modified "Cooperstown 5+1" cocktail (add warfarin plus vitamin K1) (Chainuvati et al., 2003) have been wildly applied in clinical trials for drug interaction studies, which minimized the interactions among the probe drugs and succeeded to study drug interactions with CYP1A2, 2C9, 2C19, 2D6 and 3A4, e.g. the evaluation of the drug interaction potential of a triphasic oral contraceptive and aplaviroc (a novel human immunodeficiency virus entry inhibitor) (Shelepova et al., 2005; Johnson et al., 2006).

1.7 Hypothesis and General Aims

There is increasing evidence that modulation of CYPs is the major cause of a number of drug-drug and herb-drug interactions. However, little is known about the interaction of ligands (substrates and inhibitors) with CYPs at molecular levels. It is still unclear how activities of specific CYPs are influenced by the presence of most herb medicines in body system. We hypothesize that the atom-atom interactions in the residues of the ligand and CYPs are the basis on which the substrate and inhibitor specificity is determined for individual CYPs.

To test our hypothesis, we attempted to: a) conduct *in vitro* inhibitory studies of human CYPs by herbal compounds; b) to extrapolate the *in vitro* data to *in vivo* situations; and c) to explore the ligand-CYP1A2 interactions using docking and pharmacophore modelling studies. The data arising from this project have important clinical and toxicological implications.

Table 1-1. List of human *CYP* genes and their non-synonymous SNPs.

Gene	Chromosomal location	Substrates	Number of amino acids	Number of exons	Number of nsSNPs
Family 1					
CYP1A1	15q22-q24	Xenobiotics	512	7	25
CYP1A2	15q24	Xenobiotics	516	7	31
CYP1B1	2p21	Xenobiotics, sterols	543	3	30
Family 2					
CYP2A6	19q13.2	Xenobiotics	494	9	37
CYP2A7	19q13.2	Unknown	494	9	18
CYP2A13	19q13.2	Xenobiotics	494	9	11
CYP2B6	19q13.2	Xenobiotics	491	9	32
CYP2C8	10q23.33	Xenobiotics	490	9	14
CYP2C9	10q24	Xenobiotics	490	9	28
CYP2C18	10q24	Xenobiotics	490	9	9
CYP2C19	10q24.1-q24.3	Xenobiotics	490	9	31
CYP2D6	22q13.1	Xenobiotics	497	9	52
CYP2E1	10q24.3-qter	Xenobiotics	493	9	19
CYP2F1	19q13.2	Xenobiotics	491	10	7
CYP2J2	1p31.3-p31.2	Fatty acids	502	9	10
CYP2R1	11p15.2	Vitamins	501	2	1
CYP2S1	19q13.1	Unknown	504	9	5
CYP2W1	7p22.3	Unknown	490	9	2
CYP2U1	4q25	Unknown	544	5	0
Family 3					
CYP3A4	7q21.1	Xenobiotics	503	13	32
CYP3A5	7q21.1	Xenobiotics	502	13	15
CYP3A7	7q21-q22.1	Xenobiotics	503	13	5
CYP3A43	7q21.1	Unknown	503	13	5
Family 4					
CYP4A11	1p33	Fatty acids	519	12	7
CYP4A22	1p33	Unknown	519	12	15
CYP4B1	1p34-p12	Fatty acids	511	12	18
CYP4F11	19p13.1	Unknown	524	12	5
CYP4F12	19p13.1	Fatty acids	524	13	11
CYP4F2	19pter-p13.11	Eicosanoids	520	13	11
CYP4F22	19p13.12	Unknown	531	14	2
CYP4F3	19p13.2	Eicosanoids	520	13	6
CYP4F8	19p13.1	Eicosanoids	520	13	3
CYP4V2	4q35.2	Unknown	525	11	15
CYP4X1	1p33	Unknown	509	12	1
CYP4Z1	1p33	Unknown	505	12	0
Family 5					
CYP5A1	7q34-q35	Eicosanoids	534	13	23
Family 7					
CYP7A1	8q11-q12	Sterols	504	6	2
CYP7B1	8q21.3	Sterols	506	6	1
Family 8					
CYP8A1	20q13.13	Eicosanoids	500	10	14
CYP8B1	3p22-p21.3	Sterols	501	1	5
Family 11					
CYP11A1	15q23-q24	Sterols	521	6	10
CYP11B1	8q21	Sterols	503	9	26
CYP11B2	8q21-q22	Sterols	503	9	20

Family 17					
CYP17A1	10q24.3	Sterols	508	8	31
Family 19					
CYP19A1	15q21.1	Sterols	503	10	13
Family 20					
CYP20A1	2q33.2	Unknown	462	13	4
Family 21					
CYP21A2	6p21.3	Sterols	495	10	68
Family 24					
CYP24A1	20q13	Vitamins	514	12	4
Family 26					
CYP26A1	10q23-q24	Vitamins	497	7	3
CYP26B1	2p13.3	Vitamins	512	6	3
CYP26C1	10q23.33	Vitamins	522	4	3
Family 27					
CYP27A1	2q33-qter	Sterols	531	8	15
CYP27B1	12q13.1-q13.3	Vitamins	508	9	22
CYP27C1	2q14.3	Unknown	372	8	1
Family 39					
CYP39A1	6p21.1-p11.2	Sterols	469	12	7
Family 46					
CYP46A1	14q32.1	Sterols	500	15	0
Family 51					
CYP51A1	7q21.2-q21.3	Sterols	509	10	3

Data are from Wang *et al*. (2009).

Table 1-2. The major substrates, inhibitors and inducers for the major drug metabolizing CYPs.

CYP1A2				
	Substrates		**Inhibitors**	**Inducers**
1	Acetaminophen	Olanzapine	Amiodarone	Broccoli
2	Amitriptyline	Ondansetron	Cimetidine	Brussel sprouts
3	Caffeine*	Phenacetin*	Ciprofloxacin**	Char-grilled meat
4	Clomipramine	Propranolol	Fluvoxamine**	Insulin
5	Clozapine	Riluzole	Furafylline**	Methylcholanthrene
6	Cyclobenzaprine	Ropivacaine	Interferon	Modafinil
7	Estradiol	Tacrine	Methoxsalen	Nafcillin
8	Fluvoxamine	Theophylline	Mibefradil	β-Naphthoflavone
9	Haloperidol	Tizanidine		Omeprazole
10	Imipramine	*R*-Warfarin		Tobacco
11	Melatonin*	Verapamil		
12	Mexiletine	Zileuton		
13	Naproxen	Zolmitriptan		
CYP2C9				
	Substrates		**Inhibitors**	**Inducers**
1	Amitriptyline	Losartan	Amiodarone	Rifampin
2	Celecoxib	Lornoxicam	Fenofibrate	Secobarbital
3	Diclofenac*	Meloxicam	Fluconazole	
4	Fluoxetine	Nateglinide	Fluvastatin	
5	Flurbiprofen*	Phenytoin (4'-OH)	Fluvoxamine	
6	Fluvastatin	Piroxicam	Isoniazid	
7	Glimepiride	Rosiglitazone	Lovastatin	
8	Glipizide	*S*-Naproxen	Phenylbutazone	
9	Glipizide	Suprofen	Probenicid	
10	Glyburide	*S*-Warfarin*	Sertraline	
11	Glyburide/Glibenclamide	Tamoxifen	Sulfamethoxazole	
12	Ibuprofen	Tolbutamide*	Sulfaphenazole**	
13	Irbesartan	Torsemide	Teniposide	
14			Voriconazole	
15			Zafirlukast	
CYP2C19				
	Substrates		**Inhibitors**	**Inducers**
1	Amitriptyline	Nelfinavir	Chloramphenicol	Carbamazepine
2	Carisoprodol	Nilutamide	Cimetidine	Norethindrone
3	Chloramphenicol	Omeprazole*	Felbamate	Prednisone
4	Citalopram	Pantoprazole	Fluoxetine	Rifampin
5	Clomipramine	Phenobarbitone	Fluvoxamine	
6	Clopidogrel	Phenytoin	Indomethacin	
7	Cyclophosphamide	Primidone	Ketoconazole	
8	Diazepam	Progesterone	Modafinil	
9	E-3810	Proguanil	Oxcarbazepine	
10	Hexobarbital	Propranolol	Probenicid	
11	Imipramine *N*-demethylation	Rabeprazole	Ticlopidine	
12	Indomethacin	*R*-Mephobarbital	Topiramate	
13	Lansoprazole	*R*-Warfarin (8-OH)	Lansoprazole	
14	Moclobemide	*S*-Mephenytoin*	Omeprazole	
15		Teniposide	Pantoprazole	
16			Rabeprazole	
CYP2D6				
	Substrates		**Inhibitors**	**Inducers**

1	Alprenolol	Metoclopramide	Amiodarone	Dexamethasone
2	Amitriptyline	Mexilletine	Bupropion	Rifampin
3	Amphetamine	Minaprine	Celecoxib	
4	Aripiprazole	Nebivolol	Chlorpheniramine	
5	Atomoxetine	Nortriptyline	Chlorpromazine	
6	Bufuralol*	Ondansetron	Cimetidine	
7	Carvedilol	Oxycodone	Cinacalcet	
8	Chlorpheniramine	Paroxetine	Citalopram	
9	Chlorpromazine	Perhexiline	Clemastine	
10	Clomipramine (Antidepres)	Perphenazine	Clomipramine	
11	Codeine (*O*-demethylation)	Phenacetin	Cocaine	
12	Debrisoquine*	Phenformin	Diphenhydramine	
13	Desipramine	Promethazine	Doxepin	
14	Dexfenfluramine	Propafenone	Doxorubicin	
15	Dextromethorphan*	Propranolol	Duloxetine	
16	Duloxetine	Risperidone	Escitalopram	
17	Encainide	*S*-Metoprolol	Fluoxetine	
18	Flecainide	Sparteine	Goldenseal	
19	Fluoxetine	Tamoxifen	Halofantrine	
20	Fluvoxamine	Thioridazine	Hydroxyzine	
21	Haloperidol	Timolol	Levomepromazine	
22	Imipramine	Tramadol	Methadone	
23	Lidocaine	Venlafaxine	Metoclopramide	
24	Methoxyamphetamine	Zuclopenthixol	Mibefradil	
25			Midodrine	
26			Moclobemide	
27			Paroxetine	
28			Perphenazine	
29			Quinidine**	
30			Ranitidine	
31			Red-Haloperidol	
32			Ritonavir	
33			Sertraline	
34			Terbinafine	
35			Ticlopidine	
36			Tripelennamine	

CYP2E1

	Substrates		Inhibitors	Inducers
1	Acetaminophen	Halothane	Diethyl-Dithiocarbamate	Ethanol
2	Aniline	Isoflurane	Disulfiram	Isoniazid
3	Benzene	Methoxyflurane		
4	Chlorzoxazone	*N, N*-Dimethylformamide		
5	Enflurane	Sevoflurane		
6	Ethanol	Theophylline		

CYP3A4

	Substrates		Inhibitors	Inducers
1	Alfentanyl	Lidocaine	Amiodarone	Barbiturates
2	Alprazolam	Lovastatin	Aprepitant	Carbamazepine
3	Amlodipine	Methadone	Chloramphenicol	Efavirenz
4	Aprepitant	Midazolam*	Cimetidine	Efavirenz
5	Aripiprazole	Nateglinide	Clarithromycin	Glucocorticoids
6	Astemizole	Nelfinavir	Delaviridine	Modafinil

7	Atorvastatin	Nifedipine*	Diethyl-dithiocarbamate	Nevirapine
8	Buspirone	Nisoldipine	Diltiazem	Nevirapine
9	Cafergot	Nitrendipine	Erythromycin	Oxcarbazepine
10	Caffeine	NOT Azithromycin	Fluconazole	Phenobarbital
11	Cerivastatin	NOT Pravastatin	Fluvoxamine	Phenytoin
12	Chlorpheniramine	NOT Rosuvastatin	Gestodene	Pioglitazone
13	Cilostazol	Ondansetron	Grapefruit Juice	Rifabutin
14	Cinacalcet	Pimozide	Imatinib	Rifampin
15	Cisapride	Progesterone (Steroid 6β-OH)	Indinavir**	St. John's Wort
16	Clarithromycin	Propranolol	Itraconazole**	Troglitazone1
17	Cocaine	Quetiapine	Ketoconazole	
18	Codeine-(*N*-Demethylation)	Quinidine 3-OH (Not 3A5)	Mibefradil	
19	Cyclosporine	Quinine	Mifepristone	
20	Dapsone	Risperidone	Nefazodone	
21	Dexamethasone	Ritonavir	Nelfinavir	
22	Dextromethorphan	Salmeterol	Norfloxacin	
23	Diazepam (3-OH)	Saquinavir	Norfluoxetine	
24	Diltiazem	Sildenafil	Ritonavir	
25	Docetaxel	Simvastatin	Saquinavir	
26	Domperidone	Sirolimus	Telithromycin**	
27	Eplerenone	Tacrolimus (FK506)	Verapamil	
28	Erythromycin (Not 3A5)	Tamoxifen	Voriconazole	
29	Estradiol (Steroid 6β-OH)	Taxol		
30	Felodipine	Telithromycin (Macr-Antibio)		
31	Fentanyl	Terfenadine		
32	Finasteride	Terfenadine (Antihistamines)		
33	Gleevec	Testosterone* (Steroid 6β-OH)		
34	Haloperidol	Trazodone		
35	Hydrocortisone (Steroid 6β-OH)	Triazolam		
36	Indinavir	Verapamil		
37	Irinotecan	Vincristine		
38	LAAM	Zaleplon		
39	Lapatinib	Ziprasidone		
40	Lercanidipine	Zolpidem		

*: model substrate; **: highly selective inhibitor. Data are also extracted from the Drug-Interaction website http://medicine.iupui.edu/flockhart/table.htm.

Table 1-3. Reported variants of human *CYP1A2*.

CYP1A2	Nucleotide change	Effect	Enzyme activity		Reference
			In vivo	*In vitro*	
**1A*	Wild-type		Normal	Normal	(Ikeya et al., 1989; Quattrochi and Tukey, 1989)
**1B*	5347T>C				(Nakajima et al., 1999b; Welfare et al., 1999)
**1C*	**-3860G>A**[a]		↓		(Nakajima et al., 1999b)
**1D*	-2467delT				(Chida et al., 1999a)
**1E*	-739T>G				(Chida et al., 1999a)
**1F*	**-163C>A**		↑Inducibility		(Chida et al., 1999a; Sachse et al., 1999; Han et al., 2002)
**1G*	-739T>G; 5347T>C				(Chevalier et al., 2001)
**1H*	2025A>C; 5347T>C				(Chevalier et al., 2001)
**1J*	-739T>G ; **-163C>A**				(Aklillu et al., 2003)
**1K*	-739T>G; **-729C>T**; **-163C>A**		↓		(Aklillu et al., 2003)
**1L*[b]	-3860G>A; -2467delT; **-163C>A**; 5347T>C				(Soyama et al., 2005)
**1M*[b]	**-163C>A**; 2159G>A				(Soyama et al., 2005)
**1N*[b]	-3594T>G; -2467delT; **-163C>A**; 2321G>C; 5521A>G; 5347T>C				(Soyama et al., 2005)
**1P*[b]	-3594T>G; -2467delT; -733G>C; **-163C>A**; 2321G>C; 5521A>G; 5347T>C				(Soyama et al., 2005)
**1Q*[b]	-2808A>C; **-163C>A**; 2159G>A				(Soyama et al., 2005)
**1R*[b]	-3594T>G; -2467delT; -367C>T; **-163C>A**; 2321G>C; 5521A>G; 5347T>C				(Soyama et al., 2005)
**1S*[b]	-3053A>G; 5347T>C				(Soyama et al., 2005)
**1T*[b]	-2667T>G; 5347T>C				(Soyama et al., 2005)
**1U*[b]	678C>T; 5347T>C				(Soyama et al., 2005)
**1V*[b]	-2467delT; **-163C>A**				(Ghotbi et al., 2007)
**1W*[b]	-3113A>G; -2467delT; -739T>G; **-163C>A**				(Ghotbi et al., 2007)
**2*	63C>G	F21L			(Huang et al., 1999)
**3*	**2385G>A**; 5347T>C	**D348N**	↓Expression		(Chevalier et al., 2001; Zhou et al., 2004a)
**4*	**2499A>T**	**I386F**			(Chevalier et al., 2001; Zhou et al., 2004a)
**5*	3497G>A	C406Y	↓Expression		(Chevalier et al., 2001)
**6*	**5090C>T**	**R431W**	↓Expression		(Chevalier et al., 2001; Zhou et al., 2004a)
**7*	**3533G>A**	**Splicing defect**	↓		(Allorge et al., 2003)
**8*	**5166G>A**; 5347T>C	**R456H**		↓	(Saito et al., 2005; Soyama et al., 2005)
**9*	248C>T	T83M			(Murayama et al., 2004)
**10*	502G>C	E168Q			(Murayama et al., 2004)
**11*	**558C>A**	**F186L**		↓	(Murayama et al., 2004)
**12*	634A>T	S212C			(Murayama et al., 2004)
**13*	1514G>A	G299S			(Murayama et al., 2004)
**14*	5112C>T	T438I			(Murayama et al., 2004)
**15*	**125C>G; 5347T>C**	**P42R**		↓	(Saito et al., 2005; Soyama et al., 2005)

**16*	**2473G>A; 5347T>C**	**R377Q**		↓	(Saito et al., 2005; Soyama et al., 2005)
-	-1051T>C; -733G>C; 1590C>T; 2570G>A; 2646C>T; 2694A>C; 5010C>T; 5521A>G				(Solus et al., 2004)
-	53C>G	S18C			(Solus et al., 2004)
-	1513C>A	S298R			(Solus et al., 2004)
-	1559A>G	I314V			(Solus et al., 2004)

Data are extracted from http://www.imm.ki.se/CYPalleles (access date: 25 March 2009).

[a]Nucleotide variations in bold are the major SNPs responsible for the phenotype of the corresponding allele.

[b]Predicted.

Table 1-4. Reported variants of the human *CYP2C9* gene.

CYP2C9	**Nucleotide change**		**Amino acid change**	**Reference**
	cDNA	**Gene**		
**1A*	Wild-type			(Romkes et al., 1991)
**1B*[a]		-2665_-2664delTG; -1188T>C		(King et al., 2004)
**1C*[a]		-1188T>C		(Shintani et al., 2001; King et al., 2004)
**1D*[a]		-2665_-2664delTG		(King et al., 2004)
**2A*[a]	**430C>T**[b]	-1188T>C, -1096A>G; -620G>T; -485T>A; -484C>A; **3608C>T**	**R144C**	(Rettie et al., 1994)
**2B*[a]	**430C>T**	-2665_-2664delTG, -1188T>C; -1096A>G; -620G>T; -485T>A; -484C>A; **3608C>T**	**R144C**	(King et al., 2004)
**2C*[a]	**430C>T**	-1096A>G; -620G>T; -485T>A; -484C>A; **3608C>T**	**R144C**	(King et al., 2004)
**3A*[a]	**1075A>C**	-1911T>C; -1885C>G; -1537G>A; -981G>A; **42614A>C**	**I359L**	(Haining et al., 1996)
**3B*[a]	**1075A>C**	-1911T>C; -1885C>G; -1537G>A; -1188T>C; -981G>A; **42614A>C**	**I359L**	(Shintani et al., 2001; King et al., 2004)
**4*	1076T>C	42615T>C	I359T	(Imai et al., 2000)
**5*	**1080C>G**	**42619C>G**	**D360E**	(Dickmann et al., 2001)
**6*	**818delA**	**10601delA**	**273Frame shift**	(Kidd et al., 2001)
**7*	55C>A	55C>A	L19I	(Blaisdell et al., 2004)
**8*	**449G>A**	**3627G>A**	**R150H**	(Blaisdell et al., 2004)
**9*	752A>G	10535A>G	H251R	(Blaisdell et al., 2004)
**10*	815A>G	10598A>G	E272G	(Blaisdell et al., 2004)
**11A*[a]	**1003C>T**	**42542C>T**	**R335W**	(Higashi et al., 2002)
**11B*[a]	**1003C>T**	-2665_-2664delTG; -1188T>C; **42542C>T**	**R335W**	(King et al., 2004)
**12*	**1465C>T**	**50338C>T**	**P489S**	(Blaisdell et al., 2004)
**13*	269T>C	3276T>C	L90P	(Si et al., 2004)
**14*	**374G>A**	**3552G>A**	**R125H**	(Zhao et al., 2004)
**15*	485C>A	9100C>A (linkage with -1188T>C can not be excluded)	S162X	(Zhao et al., 2004)
**16*	**895A>G**	-1188T>C; **33497A>G**	**T299A**	(Zhao et al., 2004)
**17*	1144C>T	42683C>T	P382S	(Zhao et al., 2004)

**18*	1075A>C; 1190A>C; 1425A>T	-1911T>C; -1885C>G; -1537G>A; -1188T>C; -981G>A; 42614A>C; 47391A>C; 50298A>T	I359L; D397A	(Zhao et al., 2004)
**19*	1362G>C	-1188T>C; 50235G>C	Q454H	(Zhao et al., 2004)
**20*	208G>C	-1188T>C; 3215G>C	G70R	(Zhao et al., 2004)
**21*	89C>T	89C>T	P30L	(Veenstra et al., 2005)
**22*	121A>G	121A>G	N41D	(Veenstra et al., 2005)
**23*	226G>A	3233G>A	V76M	(Veenstra et al., 2005)
**24*	1060G>A[c]	42599G>A	E354K	(Herman et al., 2006)
**25*	353_362del[d]	3531_3540del (AGAAATGGAA)	118Frameshift	(Maekawa et al., 2006)
**26*[a]	389C>G	1565C>T; -1188T>C; 3567C>G; 3856G>A; 8763C>T; 9032G>C; 10311A>G; 33349A>G; 50056A>T	T130R	(Maekawa et al., 2006)
**27*[a]	449G>T	-3089G>A; -2665_-2664delTG; -1188T>C; 3627G>T; 3898C>T; 47639C>T; 50056A>T	R150L	(Maekawa et al., 2006)
**28*	641A>T	9256A>T	Q214L	(Maekawa et al., 2006)
**29*[a]	835C>A	251T>C; 3411T>C; 33437C>A; 33658A>G; 50056A>T	P279T	(Maekawa et al., 2006)
**30*	1429G>A	50302G>A	A477T	(Maekawa et al., 2006)
-	-	96C>G; 251T>C; 2191T>A; 2340G>A; 2638G>T; 2737T>C; 3162G>C; 3235G>A; 3898C>T; 3924T>C; 4033A>G; 4157C>T; 4309A>G; 4628T>A; 4670G>T; 9032G>C; 9069G>A; 10682T>C; 10787G>A; 10814G>T; 33349A>G; 33658A>G; 42469T>C; 42726C>T; 47545A>T; 47593T>C; 50053G>A; 50066G>A; 50081G>C; 50434C>T; 50454C>G; 50566A>G; 50658A>G; 50742T>A; 52104C>A; 52175T>C; 52236C>T; 52319G>C; 53194insTGACAT; 53403C>T; 53498delT; 53538G>C; 53557T>C		(Solus et al., 2004)
-	-	49C>A	L17I	(Maekawa et al., 2006)
-	-	47439T>C	L413P	(Solus et al., 2004)
-	-	42612A>G	Y358C	NCBI dbSNP
-	-	-8897C>A; -8553C>A; -8422A>G; -8416T>G; -7419A>G; -7336G>A; -5813A>G; -5661C>A; -5146G>C; -5143A>C; -5140A>T; -4877G>A; -4302C>T; -3597A>G; -3579G>A; -3360T>C	-	(Kramer et al., 2008)

[a]Predicted.
[b]Nucleotide variations in bold are the major SNPs responsible for the phenotype of the corresponding allele.
[c]Existence of the *CYP2C9*2* polymorphism 430C>T on the same allele can not be excluded.
[d]AGAAATGGAA (deleted).

Table 1-5. Frequencies of *CYP2C9* alleles and genotypes in different ethnic groups.

Ethnic group	**No. of subject (*n*)**	**Allele frequency (%)**			**Genotype frequency (%)**					**Reference**
		1	***2***	***3***	***1/*2***	***1/*3***	***2/*2***	***2/*3***	***3/*3***	
Caucasian										
American-Caucasian	100	86.0	8.0	6.0	16.0	12.0	0	0	0	(Sullivan-Klose et al., 1996)
American-Caucasian	461	90.3	9.7	0						(London et al., 1996)
American-Caucasian	140	82.5	13.2	4.3	22.1	8.6	2.1	0	0	(Dickmann et al., 2001)
American-Caucasian	200	82.5	9.8	7.8	15.1	9.7	2.2	1.6	2.7	(Higashi et al., 2002)
Belgian[a]	121	82.2	10.0	7.4	18.2	11.6	0	1.6	0.8	(Allabi et al., 2003)
Brazilian	103	83.0	9.7	7.3	16.5	11.6	1.0	1.0	1.0	(Lima et al., 2008)
Brazilian[b]	331	84.9	8.6	6.5	14.5	10.9	0.9	0.9	0.6	(Scordo et al., 2002)
British	561	84.1	10.6	5.3	19.1	9.4	0.5	1.1	0	(Taube et al., 2000)
British	94	80.9	19.1	0						(Furuya et al., 1995)
British	100	79.0	12.5	8.5	19.0	15.0	3.0	0	1	(Stubbins et al., 1996)
Canadian	325	78.0	15.0	7.0	20.3	15.7	1.2	1.6	0	(Gaedigk et al., 2001)
Egyptian	247	82.0	12.0	6.0	19.0	11.7	2.4	0	0	(Hamdy et al., 2002)
Faroese	311	81.2	18.8	0	17.7	10.6	0	1.6	0	(Halling et al., 2005)
German	127	86.6	13.4	0						(Xie et al., 2002)
German	108	81.0	14.0	5.0						(Burian et al., 2002)
German	367	81.5	10.7	7.8						(Xie et al., 2002)
Israeli	156	84.0	10.0	6.0	17.9	12.8	0	1.3	0	(Loebstein et al., 2001)
Italian	157	77.8	12.5	0.97	16.8	14.0	2.5	1.9	1.3	(Scordo et al., 2001)
Italian	93	74.7	12.4	12.9	16.1	17.2	2.2	4.3	2.2	(Scordo et al., 2002)
Portuguese	135	78.8	13.2	8.0						(Oliveira et al., 2007)
Russian	290	83.9	9.1	7.0	18.3	11.3	0.7	1.4	0.3	(Gaikovitch et al., 2003)
Spanish	157	69.4	14.3	16.2	15.9	23.5	1.9	8.9	0	(Garcia-Martin et al., 2001)
Spanish	102	74.5	15.6	9.8	19.6	13.7	3.9	3.9	1.0	(Dorado et al., 2003)
Spanish	70	70.0	10.7	19.3	17.1	25.7	1.4	1.4	5.7	(Llerena et al., 2003)
Spanish	89	77.9	14.1	8.0	23.6	11.2	2.3	1.1	1.1	(Llerena et al., 2003)
Spanish	138	78.1	14.6	7.3	19.6	11.6	2.9	2.9	0.7	(Llerena et al., 2003)
Spanish	64	72.7	14.8	12.5	21.9	17.2	3.1	1.5	3.1	(Llerena et al., 2004a)

Spanish	355	71.0	19.0	10.0	28.2	15.5	3.1	2.8	1.1	(Martinez et al., 2005)
Spanish	200	81.8	12.0	6.2	22.0	12.5	1.0	0	0	(Mas et al., 2005)
Spanish	142	78.5	13.7	7.7	19.0	9.6	2.8	2.8	1.4	(Dorado et al., 2008)
Swedish	430	81.9	10.7	7.4	18.6	11.6	0.5	1.9	0.7	(Yasar et al., 1999)
Swedish	201	82.3	11.2	6.5						(Wadelius et al., 2004)
Turkish	499	79.4	10.6	10.0	18.0	17.2	1.0	1.1	0.8	(Aynacioglu et al., 1999)
Turkish	85	-	-	-	11.8	14.1	3.5	1.2	1.2	(Babaoglu et al., 2004)
Turkish[c]	205	76.8	12.7	9.8	18.5	13.7	1.5	3.9	1.0	(Oner Ozgon et al., 2008)
African										
African American	100	98.5	1.0	0.5	2.0	1.0	0	0	0	(Sullivan-Klose et al., 1996)
African American	239	96.4	3.6	0						
African American	123	-	-	-	5.0	2.5	0	0	0	(Dickmann et al., 2001)
African American[d]	110	96.2	0	1.5						(Xie et al., 2002)
African American[e]	115	85.0	5.0	5.0						(Momary et al., 2007)
Belgian Beninese	111	-	-	-	0	0	0	0	0	(Allabi et al., 2003)
Ethiopian	150	94.0	4.0	2.0	8.7	4.6	0	0	0	(Scordo et al., 2001)
Asian										
Chinese	135	100	0	0						(Wang et al., 1995)
Chinese	115	98.3	0	1.7	0	3.0	0	0	0	(Wang et al., 1995)
Chinese	102	95.0	0	5.0	0	10.8	0	0	0	(Gaedigk et al., 2001)
Chinese	711	96.2	0	3.8	0	7.6	0	0	0	(Hong et al., 2005)
Chinese	376	96.7	0	3.3	0	6.6	0	0	0	(Hong et al., 2005)
Chinese	178	95.5	0	4.5	0	8.9	0	0	0	(Miao et al., 2007)
Japanese	218	97.9	0	2.1	0	4.1	0	0	0	(Nasu et al., 1997)
Japanese	86	98.3	0	1.7						
Japanese	140	98.2	0	1.8	0	3.6	0	0	0	(Kimura et al., 1998)
Korean	574	98.9	0	1.1	0	2.3	0	0	0	(Yoon et al., 2001)
Koran[f]	358	93.4	0	6.0	0	12.0	0	0	0	(Bae et al., 2005)
Malasian	191	93.2	0	6.8						(Ngow et al., 2008)
Taiwanese	98	97.4	0	2.6	0	8.2	0	0	0	(Sullivan-Klose et al., 1996)
Others										
Bolivian	778	92.2	4.8	3.0	9.3	5.7	0	0.4	0	(Bravo-Villalta et al., 2005)

Canadian native Indian	114	91.0	3.0	6.0	6.1	11.4	0	0	0	(Gaedigk et al., 2001)
Canadian Inuit	151	100	0	0	0	0	0	0	0	(Gaedigk et al., 2001)
Iranian	160	79.3	11.0	9.7	17.5	13.7	2.5	0	1.9	(Peyvandi et al., 2002)
Iranian	200	87.2	12.8	0	10.5	0	7.5	0	0	(Zand et al., 2007)
Mexican-American	98	86.0	8.0	6.0	15.0	10.0	0	1.0	0	(Llerena et al., 2004b)
Omani	189	89.7	7.4	2.9	12.7	5.8	1.1	0	0	(Tanira et al., 2007)
Tamilian	135	90.7	2.6	6.7	4.4	12.7	0	0.7	0	(Adithan et al., 2003)

[a]The frequency of *CYP2C9*11* was 0.4%. (Allabi et al., 2003)
[b]The population contained a mixture of white (*n* = 136), black (*n* = 77), and intermediate (*n* = 118). (Scordo et al., 2002)
[c]The frequency of *CYP2C9*4* was 0.7%. (Oner Ozgon et al., 2008)
[d]The frequency of *CYP2C9*5* was 2.3%. (Xie et al., 2002)
[e]The frequency of *CYP2C9*5* was 5.0%. (Momary et al., 2007)
[f]The frequency of *CYP2C9*13* was 0.6%. (Bae et al., 2005)

Table 1-6. Reported variants of human *CYP2D6*.

CYP2D6	Nucleotide change	Amino acid change	Impact on enzyme activity	Reference
**1A*	None		Normal	(Kimura et al., 1989)
**1B*	3828G>A		Normal	(Marez et al., 1997)
**1C*	1978C>T		Normal	(Marez et al., 1997)
**1D*	2575C>A			(Marez et al., 1997)
**1E*	1869T>C			(Sachse et al., 1997)
**1XN*		***N* active genes**	↑	(Dahl et al., 1995; Sachse et al., 1997)
**2A*	-1584C>G; -1235A>G; -740C>T; -678G>A; *CYP2D7* gene conversion in intron 1; 1661G>C; 2850C>T; 4180G>C	R296C; S486T	Normal	(Johansson et al., 1993; Panserat et al., 1994; Raimundo et al., 2000; Sakuyama et al., 2008)
**2B*	1039C>T; 1661G>C; 2850C>T; 4180G>C	R296C; S486T		(Marez et al., 1997)
**2C*	1661G>C; 2470T>C; 2850C>T; 4180G>C	R296C; S486T		(Marez et al., 1997; Sachse et al., 1997)
**2D*	2850C>T; 4180G>C	R296C; S486T		(Marez et al., 1997)
**2E*	997C>G; 1661G>C; 2850C>T; 4180GC	R296C; S486T		(Marez et al., 1997)
**2F*	1661G>C; 1724C>T; 2850C>T; 4180G>C	R296C; S486T		(Marez et al., 1997)
**2G*	1661G>C; 2470T>C; 2575C>A; 2850C>T; 4180G>C	R296C; S486T		(Marez et al., 1997)
**2H*	1661G>C; 2480C>T; 2850C>T; 4180G>C	R296C; S486T		(Marez et al., 1997)
**2J*	See *CYP2D6*59*			
**2K*	1661G>C; 2850C>T; 4115C>T; 4180G>C	R296C; S486T		(Marez et al., 1997)
**2L* (formerly **41B*)	-1584C; -1298G>A; -1235A>G; -740C>T; 310G>T; 746C>G; 843T>G; 1513C>T; 1661G>C; 1757C>T; 2850C>T; 3384A>C; 3584G>A; 3790C>T; 4180G>C	R296C; S486T		(Gaedigk et al., 2005a)
**2M*	-1584C; -1237_-1236insAA; -1235A>G; -750_-749delGA; -740C>T; -678G>A; *CYP2D7* gene conversion in intron 1; 310G>T; 746C>G; 843T>G; 1661G>C; 2850C>T; 2988G; 3384A>C; 3584G>A; 3790C>T; 4180G>C; 4481G>A	R296C; S486T		(Gaedigk et al., 2005b)
**2XN* (N=2, 3, 4, 5 or 13)	1661G>C; 2850C>T; 4180G>C	R296C; S486T; ***N* active genes**	↑	(Johansson et al., 1993; Dahl et al., 1995; Aklillu et al., 1996)
**3A*	**2549delA**[a]	**259Frameshift**	None	(Kagimoto et al., 1990)
**3B*	1749A>G; 2549delA	N166D; 259frameshift		(Marez et al., 1997)
**4A*	100C>T; 974C>A; 984A>G; 997C>G; 1661G>C; **1846G>A**; 4180G>C	P34S; L91M; H94R; **splicing defect**; S486T	None	(Gough et al., 1990; Hanioka et al., 1990; Kagimoto et al., 1990)
**4B*	100C>T; 974C>A; 984A>G; 997C>G; **1846G>A**; 4180G>C	P34S; L91M; H94R; **splicing defect**; S486T	None	(Kagimoto et al., 1990)
**4C*	100C>T; 1661G>C; **1846G>A**; 3887T>C; 4180G>C	P34S; **splicing defect**; L421P; S486T	None	(Yokota et al., 1993)

**4D*	100C>T; 1039C>T; 1661G>C; 1846G>A; 4180G>C	P34S; **splicing defect**; S486T	None	(Marez et al., 1997)
**4E*	100C>T; 1661G>C; **1846G>A**; 4180G>C	P34S; **splicing defect**; S486T		(Marez et al., 1997)
**4F*	100C>T; 974C>A; 984A>G; 997C>G; 1661G>C; **1846G>A**; 1858C>T; 4180G>C	P34S; L91M; H94R; **splicing defect**; R173C; S486T		(Marez et al., 1997)
**4G*	100C>T; 974C>A; 984A>G; 997C>G; 1661G>C; **1846G>A**; 2938C>T; 4180G>C	P34S; L91M; H94R; **splicing defect**; P325L; S486T		(Marez et al., 1997)
**4H*	100C>T; 974C>A; 984A>G; 997C>G; 1661G>C; **1846G>A**; 3877G>C; 4180G>C	P34S; L91M; H94R; **splicing defect**; E418Q; S486T		(Marez et al., 1997)
**4J*	100C>T; 974C>A; 984A>G; 997C>G; 1661G>C; **1846G>A**	P34S; L91M; H94R; **splicing defect**		(Marez et al., 1997)
**4K*	100C>T; 1661G>C; **1846G>A**; 2850C>T; 4180G>C	P34S; **splicing defect**; R296C; S486T	None	(Sachse et al., 1997)
**4L*	100C>T; 997C>G; 1661G>C; **1846G>A**; 4180G>C	P34S; **splicing defect**; S486T		(Shimada et al., 2001)
**4M*	-1235A>G; 746C>G; 843T>G 974C>A; 984A>G; 997C>G; 1661G>C; **1846G>A**; 2097A>G; 3384A>C; 3582A>G; 4401C>T	L91M; H94R; **splicing defect**		(Agundez et al., 1997; Fuselli et al., 2004; Gaedigk et al., 2006)
**4N (Found in a gene duplication)*	-1426C>T; -1235A>G; -1000G>A; 100C>T; 310G>T; 746C>G; 843T>G; 974C>A; 984A>G; 997C>G; 1661G>C; **1846G>A**; 2097A>G; 3384A>C; 3582A>G; gene conversion to CYP2D7 in exon 9; 4180G>C; 4401C>T	P34S; L91M; H94R; **splicing defect**; P469A; T470A; H478S; G479A; F481V; A482S; S486T	None	(Gaedigk et al., 2006)
**4X2*			None	(Lovlie et al., 1997; Sachse et al., 1998)
**5*	***CYP2D6* deleted**	***CYP2D6* deleted**	None	(Gaedigk et al., 1991; Steen et al., 1995)
**6A*	**1707delT**	**118Frameshift**	None	(Saxena et al., 1994)
**6B*	**1707delT**; 1976G>A	**118Frameshift**	None	(Evert et al., 1994a; Daly et al., 1995)
**6C*	**1707delT**; 1976G>A; 4180G>C	**118Frameshift**	None	(Marez et al., 1997)
**6D*	**1707delT**; 3288G>A	**118Frameshift**		(Marez et al., 1997)
**7*	**2935A>C**	**H324P**	None	(Evert et al., 1994b)
**8*	1661G>C; 1758G>T; 2850C>T; 4180G>C	G169X	None	(Broly et al., 1995)
**9*	**2615_2617delAAG**	**K281del**	↓	(Tyndale et al., 1991; Broly and Meyer, 1993)
**10A*	**100C>T**; 1661G>C; 4180G>C	**P34S**; S486T	↓	(Yokota et al., 1993; Sakuyama et al., 2008)
**10B*	-1426C>T; -1237_-1236insAA; -1235A>G; -1000G>A; **100C>T**; 1039C>T; 1661G>C; 4180G>C	**P34S**; S486T	↓	(Johansson et al., 1994)
**10C*	See **36*			
**10D*	**100C>T**; 1039C>T; 1661G>C; 4180G>C, *CYP2D7*-like 3'-flanking region	**P34S**; S486T		(Ishiguro et al., 2004b)
**10X2*			↓	(Garcia-Barcelo et al., 2000; Ji et al., 2002;

				Mitsunaga et al., 2002; Ishiguro et al., 2004a)
**11*	**883G>C**; 1661G>C; 2850C>T; 4180G>C	**Splicing defect**; R296C; S486T	None	(Marez et al., 1995)
**12*	**124G>A**; 1661G>C; 2850C>T; 4180G>C	**G42R**; R296C; S486T	None	(Marez et al., 1996)
**13*	*CYP2D7P/CYP2D6* hybrid: Exon 1 *CYP2D7*, exons 2-9 *CYP2D6*	Frameshift	None	(Panserat et al., 1995)
**14A*	100C>T; **1758G>A**; 2850C>T; 4180G>C	P34S; **G169R**; R296C; S486T	None	(Wang et al., 1999; Sakuyama et al., 2008)
**14B*	intron 1 conversion with CYP2D7 (214-245); 1661G>C; **1758G>A**; 2850C>T; 4180G>C	**G169R**; R296C; S486T	↓	(Ji et al., 2002; Sakuyama et al., 2008)
**15*	**137_138insT**	**46Frameshift**	None	(Sachse et al., 1996)
**16*	*CYP2D7P/CYP2D6* hybrid: Exons 1-7 *CYP2D7P*-related, exons 8-9 *CYP2D6*	Frameshift	None	(Daly et al., 1996)
**17*	**1023C>T**; 1661G>C; **2850C>T**; 4180G>C	**T107I**; **R296C**; S486T	↓	(Masimirembwa et al., 1996; Oscarson et al., 1997)
**17XN*			Normal (if $N = 2$)	(Cai et al., 2006)
**18*	**4125_4133dupGTGCCCACT**	**468_470dupVPT**	None	(Yokoi et al., 1996; Sakuyama et al., 2008)
**19*	1661G>C; **2539_2542delAACT**; 2850C>T; 4180G>C	**255Frameshift**	None	(Marez et al., 1997)
**20*	1661G>C; **1973_1974insG**; 1978C>T; 1979T>C; 2850C>T; 4180G>C	**211Frameshift**	None	(Marez-Allorge et al., 1999)
**21A*	-1584C>G; -1426C>T; -1258_-1257insAAAAA; -1235A>G; -740C>T; -678G>A; -629A>G; 214G>C; 221C>A; 223C>G; 227T>C; 310G>T; 601delC; 1661G>C; 2573_2574insC; 2850C>T; 3584G>A; 4180G>C	267Frameshift	None	(Chida et al., 1999b)
**21B*	-1584C>G; -1235A>G; -740C>T; -678G>A; intron 1 conversion with *CYP2D7* (214-245); 1661G>C; **2573_2574insC**; 2850C>T; 4180G>C	**267Frameshift**	None	(Yamazaki et al., 2003)
**22*	82C>T	R28C		(Marez et al., 1997)
**23*	957C>T	A85V		(Marez et al., 1997)
**24*	2853A>C	I297L		(Marez et al., 1997)
**25*	3198C>G	R343G		(Marez et al., 1997)
**26*	3277T>C	I369T		(Marez et al., 1997)
**27*	3853G>A	E410K	Normal	(Marez et al., 1997; Sakuyama et al., 2008)
**28*	19G>A; 1661G>C; 1704C>G; 2850C>T; 4180G>C	V7M; Q151E; R296C; S486T		(Marez et al., 1997)
**29*	1659G>A; 1661G>C; 2850C>T; 3183G>A; 4180G>C	V136M; R296C; V338M; S486T	↓	(Marez et al., 1997; Wennerholm et al., 2001; Wennerholm et al., 2002)
**30*	1661G>C; 1863_1864insTTTCGCCCC; 2850C>T; 4180G>C	174_175insFRP; R296C; S486T		(Marez et al., 1997)
**31*	1661G>C; 2850C>T; 4042G>A; 4180G>C	R296C; R440H; S486T		(Marez et al., 1997)

**32*	1661G>C; 2850C>T; 3853G>A; 4180G>C	R296C; E410K; S486T		(Marez et al., 1997)
**33*	2483G>T	A237S	Normal	(Marez et al., 1997)
**34*	2850C>T	R296C		(Marez et al., 1997)
**35*	-1584C>G; 31G>A; 1661G>C; 2850C>T; 4180G>C	V11M; R296C; S486T	Normal	(Marez et al., 1997; Gaedigk et al., 2003b)
**35X2*	31G>A; 1661G>C; 2850C>T; 4180G>C	V11M; R296C; S486T	↑	(Griese et al., 1998)
**36 (Duplication or tandem)*	-1426C>T; -1237_-1236insA; -1235A>G; -1000G>A; **100C>T**; 1039C>T; 1661G>C; gene conversion to *CYP2D7* in exon 9; 4180G>C	**P34S**; P469A; T470A; H478S; G479A; F481V; A482S; S486T	Negligible	(Johansson et al., 1994; Leathart et al., 1998)
**36 (Single)*	-1426C>T; -1235A>G; -1000G>A; **100C>T**; 310G>T; 843T>G; 1039C>T; 1661G>C; 2097A>G; 3384A>C; 3582A>G; gene conversion to *CYP2D7* in exon 9	**P34S**; P469A; T470A; H478S; G479A; F481V; A482S; S486T	Negligible	(Gaedigk et al., 2006; Sakuyama et al., 2008)
**37*	100C>T; 1039C>T; 1661G>C; 1943G>A; 4180G>C;	P34S; R201H; S486T		(Marez et al., 1997)
**38*	**2587_2590delGACT**	**271Frameshift**	None	(Leathart et al., 1998)
**39*	1661G>C; 4180G>C	S486T	Normal	(Shimada et al., 2001; Sakuyama et al., 2008)
**40*	1023C>T; 1661G>C; **1863_1864ins(TTT CGC CCC)2**; 2850C>T; 4180G>C	T107I; **174_175ins(FR**P)2; R296C; S486T	None	(Gaedigk et al., 2002)
**41*	-1584C; -1235A>G; -740C>T; -678G>A; *CYP2D7* gene conversion in intron 1; 1661G>C; 2850C>T; **2988G>A**; 4180G>C	R296C; **splicing defect**; S486T	↓	(Raimundo et al., 2000; Raimundo et al., 2004; Rau et al., 2006; Toscano et al., 2006a)
**42*	-1584C; 1661G>C; 2850C>T; **3259_3260insGT**; 4180G>C	R296C; **365Frameshift**	None	(Gaedigk et al., 2003a)
**43*	77G>A	R26H		(Marez et al., 1997)
**44*	82C>T; **2950G>C**	**Splicing defect**	None	(Yamazaki et al., 2003)
**45A*	-1601_-1600GA>TT; -1584C; -1238_-1237delAA; -1094_-1093insA; -1011T>C; 310G>T; 746C>G; 843T>G; 1661G>C; 1716G>A; 2129A>C; 2575C>A; 2661G>A; 2850C>T; 3254T>C; 3384A>C; 3584G>A; 3790C>T; 4180G>C	E155K; R296C; S486T		(Gaedigk et al., 2005a)
**45B*	-1584C; -1543G>A; -1298G>A; -1235A>G; -1094_-1093insA; -740C>T; -695_-692delTGTG; 310G>T; 746C>G; 843T>G; 1661G>C; 1716G>A; 2575C>A; 2661G>A; 2850C>T; 3254T>C; 3384A>C; 3584G>A; 3790C>T; 4180G>C	E155K; R296C; S486T		(Gaedigk et al., 2005a)
**46*	-1584C; -1543G>A; -1298G>A; -1235A>G; -740C>T; 77G>A; 310G>T; 746C>G; 843T>G; 1661G>C; 1716G>A; 2575C>A; 2661G>A; 2850C>T; 3030G>G/A*; 3254T>C; 3384A>C; 3491G>A; 3584G>A; 3790C>T; 4180G>C	R26H; E155K; R296C; S486T		(Gaedigk et al., 2005a)
**47*	-1426C>T; -1235A>G; -1000G>A; 73C<T; **100C>T**; 1039C>T; 1661G>C; 4180G>C	R25W; **P34S**; S486T	Negligible	(Soyama et al., 2004; Sakuyama et al., 2008)

**48*	972C>T	A90V	Normal	(Soyama et al., 2004; Sakuyama et al., 2008)
**49*	-1426C>T; -1235A>G; -1000G>A; **100C>T**; 1039C>T; 1611T>A; 1661G>C; 4180G>C	**P34S**; F120I; S486T	↓	(Soyama et al., 2004; Sakuyama et al., 2008)
**50*	1720A>C	E156A	↓	(Soyama et al., 2004; Sakuyama et al., 2008)
**51*	-1584C>G; -1235A>G; -740C>T; -678G>A; *CYP2D7* gene conversion in intron 1; 1661G>C; 2850C>T; 3172A>C; 4180G>C	R296C; E334A; S486T	Negligible	(Soyama et al., 2004; Sakuyama et al., 2008)
**52*	-1426C>T; -1245_-1244insGA; -1235A>G; -1028T>C; -1000G>A; -377A>G; 100C>T; 1039C>T; 1661G>C; 3877G>A; 4180G>C; 4388C>T; 4401C>T	P34S; E418K		http://www.cypalleles.ki.se
**53*	1598A>G; 1611T>A; 1617G>T	F120I; A122S	↑	(Ebisawa et al., 2005; Sakuyama et al., 2008)
**54*	100C>T; 1039C>T; 1661G>C; 2556C>T; 4180G>C	P34S; T261I; S486T	↓	(Ebisawa et al., 2005; Sakuyama et al., 2008)
**55*	1661G>C; 2850C>T; 3790C>T; 3835A>C; 4180G>C	R296C; K404Q; S486T	↓	(Ebisawa et al., 2005; Sakuyama et al., 2008)
**56A*	-1584C>G; -1235A>G; -740C>T; -678G>A; CYP2D7 gene conversion in intron 1; 1661G>C; 2850C>T; **3201C>T**; 3384A>C; 3584G>A; 3790C>T; 4180G>C	R296C; **R344X**	None	(Li et al., 2006b)
**56B*	-1426C>T; -1235A>G; -1000G>A; 100C>; 310G>T; 843T>G; 1039C>T; 1661G>C; 2097A>G; **3201C>T**; 3384A>C; 3582A>G, 4180G>C	P34S; **R344X**		(Gaedigk et al., 2007a)
**57 (In tandem with *10)*	100C>T; 310G>T; 843T>G; 887C>T; 1039C>T; 1661G>C; 3384A>C; 3582A>G; gene conversion to *CYP2D7* in exon 9; 4180G>C	P34S; R62W; P469A; T470A; H478S; G479A; F481V; A482S; S486T	Negligible	(Soyama et al., 2006; Sakuyama et al., 2008)
**58*	-1426C>T; -1235A>G; -740C>T; CYP2D7 gene conversion in intron 1; 310G>T; 843T>G; 1023C>T; 1661G>T; 1863_1864insTTTCGCCCC; 2850C>T; 3384A>C; 3584G>A; 3790C>T; 4180G>C	T107I; 174_175insFRP; R296C; S486T		http://www.cypalleles.ki.se
**59*	1661G>C; **2291G>A**; 2850C>T; 2939G>A; 4180G>C	R296C; S486T	↓	(Marez et al., 1997; Toscano et al., 2006b)
**60*				
**61*	gene conversion to CYP2D7 in exon 9	P469A; T470A; H478S; G479A; F481V; A482S; S486T		http://www.cypalleles.ki.se
**62*	**4044C>T**	**R441C**	None	(Klein et al., 2007)
**63*	2850C>T; gene conversion to CYP2D7 in exon 9	R296C; P469A; T470A; H478S; G479A; F481V; A482S; S486T		http://www.cypalleles.ki.se
**64*	-1426C>T; -1235A>G; -1000G>A; 100C>T; 310G>T; 843T>G; 1023C>T; 1661G>C; 2097A>G;	P34S; T107I; S486T		(Gaedigk and Coetsee, 2008)

	3384A>C; 3582A>G; 4180G>C; 4401C>T; 4722T>G			
**65*	100C>T; 310G>T; 843T>G; 1661G>C; 2850C>T; 3384A>C; 3584G>A; 3790C>T; 4180G>C; 4481G>A	P34S; R296C; S486T		(Gaedigk and Coetsee, 2008)
**66*	*CYP2D7P/CYP2D6* hybrid: Exons 1-6 *CYP2D7*, exons 7-9 *CYP2D6*	Frameshift		(Gaedigk and Coetsee, 2008)
**67*	*CYP2D7P/CYP2D6* hybrid: Exons 1-5 *CYP2D7*, exons 6-9 *CYP2D6*	Frameshift		http://www.cypalleles.ki.se
	-98C>T; -43insG; 1923C>T; 1998T>C; 2303C>T; 2663G>A; 2760T>A; 3408T>C; 3435C>A; 4172C>T			(Solus et al., 2004)
	4155C>	H478Y		(Solus et al., 2004)
	1707T>G/C/A	W152G/R/R		NCBI dbSNP
	1847G>A	G169E		NCBI dbSNP
	CYP2D7 gene conversion in intron 4 (2050-2392)			http://www.cypalleles.ki.se
	2466T>C	L231P		http://www.cypalleles.ki.se
	2606G>A	E278K		http://www.cypalleles.ki.se
	2610A>T	M279K		http://www.cypalleles.ki.se
**68*	To be released			
**69*	-1426C>T; -1235A>G; -1000G>A; 100C>T; 310G>T; 746C>G; 843T>G; 1062A>G; 1661G>C; 2850C>T; 2988G>A; 3384A>C; 3584G>A; 3790C>T; 4180G>C; 4401C>T; 4481G>A	P34S; R296C; splicing defect; S486T	↓	(Gaedigk et al., 2009)
**70*	-175G>A; 310G>T; 843T>G; 1608G>A; 1659G>A; 1661G>C; 3183G>A; 3384A>C; 4180G>C; 4722T>G	V119M; V136M; V338M; S486T		http://www.cypalleles.ki.se
**71*	-1584C>G; 125G>A; 1494 T>C	G42E		http://www.cypalleles.ki.se
**72*	-1426C>T; -1235A>G; -1000G>A; 100C>T; 310G>T; 843T>G; 1039C>T; 1661G>C; 2097A>G; 3318G>A; 3384A>C; 3582A>G; 4180G>C; 4401C>T	P34S; E383K; S486T	↓	(Matsunaga et al., 2009)
-	-98C>T; -43insG; 1923C>T; 1998T>C; 2303C>T; 2663G>A; 2760T>A; 3408T>C; 3435C>A; 4172C>T			(Solus et al., 2004)
-	4155C>T	H478I		(Solus et al., 2004)
	1707T>G/C/A	W152G/R/R		NCBI dbSNP
	1847G>A	G169E		NCBI dbSNP
	CYP2D7 gene conversion in intron 4 (2050-2392)			http://www.cypalleles.ki.se
	2466T>C	L231P		http://www.cypalleles.ki.se
	2606G>A[b]	E278K		http://www.cypalleles.ki.se
	2610A>T[b]	M279K		http://www.cypalleles.ki.se
	1621G>T	R123L		http://www.cypalleles.ki.se
	4057G>A	G445E		http://www.cypalleles.ki.se

Data are extracted from http://www.imm.ki.se/CYPalleles (access date: 25 March 2009).
[a]Nucleotide variations in bold are the major SNPs responsible for the phenotype of the corresponding allele.
[b]Part of novel *CYP2D7* gene conversion in exon 5 (2470-2610) that includes 2470T>C and 2575C>A.

Table 1-7. Overview of published structures of human CYPs.

	CYP	PDB ID	Ligand	Mean resolution (Å)	Publishing year	Reference
1	1A2	2HI4	α-Naphthoflavone (ANF)	1.95	2007	(Sansen et al., 2007)
2	2E1	3E4E	4- Methylpyrazole		2008	(Porubsky et al., 2008)
3	2E1	3E6I	Indazole		2008	(Porubsky et al., 2008)
4	2A13	2P85	Indole	2.35	2007	(Smith et al., 2007)
5	2A6	1Z10	Coumarin	1.9	2005	(Yano et al., 2005)
6	2A6	1Z11	Methoxsalen	2.05	2005	(Yano et al., 2005)
7	2A6	2FDU	*N*,*N*-Dimethyl(5-(Pyridin-3-Yl)furan-2-Yl) methanamine		2006	(Yano et al., 2006)
8	2A6	2FDV	N-Methyl(5-(Pyridin-3-Yl)furan-2-Yl)methanamine		2006	(Yano et al., 2006)
9	2A6	2FDW	(5-(Pyridin-3-Yl)furan-2-Yl) methanamine		2006	(Yano et al., 2006)
10	2A6	2FDY	Adrithiol		2006	(Yano et al., 2006)
11	2A6	2PG5	Free	1.95	2007	(Sansen et al., 2007)
12	2A6	2PG6	Free	2.5	2007	(Sansen et al., 2007)
13	2A6	2PG7	Free	2.8	2007	(Sansen et al., 2007)
14	2A6	3EBS	Phenacetin		2008	(Sansen et al., 2007)
15	2C8	1PQ2	Free	2.7	2004	(Schoch et al., 2004)
16	2C8dh	2NNH	2 × 9-cis-retinoic acid	2.6	2008	(Schoch et al., 2008)
17	2C8dh	2NNI	Montelukast	2.8	2008	(Schoch et al., 2008)
18	2C8dh	2NNJ	Felodipine	2.28	2008	(Schoch et al., 2008)
19	2C8dh	2VN0	Troglitazone	2.7	2008	(Schoch et al., 2008)
20	2C9	1OG2	Free	2.6	2003	(Williams et al., 2003)
21	2C9	1OG5	Warfarin	2.55	2003	(Williams et al., 2003)
22	2C9	1R9O	Flurbiprofen	2	2004	(Wester et al., 2004)
23	2D6	2F9Q	Free	3	2006	(Rowland et al., 2006)
24	2R1	3C6G	Vitamin D3		2008	(Strushkevich et al., 2008)
25	2R1	3CZH	Vitamin D2		2008	
26	2R1	3DL9	1α-hydroxy-vitamin D2		2008	
27	3A4	1TQN	Free	2.05	2004	(Yano et al., 2004)
28	3A4	1W0E	Free	2.8	2004	(Williams et al., 2004)
29	3A4	1W0F	Metyrapone	2.65	2004	(Williams et al., 2004)
30	3A4	1W0G	Progesterone	2.74	2004	(Williams et al., 2004)
31	3A4	2J0D	Erythromycin		2006	(Ekroos and Sjogren, 2006)
32	3A4	2V0M	Ketoconazole	2.8	2006	(Ekroos and Sjogren, 2006)
33	46A1	2Q9F	Cholesterol-3-sulphate	1.9	2008	(Mast et al., 2008)
34	46A1	2Q9G	Free	2.4	2008	(Mast et al., 2008)
35	7A1	3DAX	Free		2008	
	PGIS	3B6H	Minoxidil		2008	

Data are from the PDB at http://www.rcsb.org.

Table 1-8. Case reports and clinical trials of herb-drug interactions in humans.

Herb	Drug	Evidence	Reference
St John's wort [*Hypericum perforatum*]	Cyclosporine	Case reports	(Gordon, 1998; Rey and Walter, 1998; Bon S et al., 1999; Barone et al., 2000; Karliova et al., 2000; Mai et al., 2000; Ruschitzka et al., 2000; Yue et al., 2000b; Ahmed et al., 2001; Barone et al., 2001; Beer and Ostermann, 2001; Moschella and Jaber, 2001; Turton-Weeks et al., 2001; Alscher and Klotz, 2003)
		Case series	(Breidenbach et al., 2000a; Breidenbach et al., 2000b)
	Cyclosporine	Clinical trial	(Bauer et al., 2003)
	Sertraline	Case reports	(Lantz et al., 1999; Barbenel et al., 2000) (Lantz et al., 1999)
	Oral contraceptives	Case series	(Gordon, 1998; Barbenel et al., 2000)
	Paroxetine	Case reports	(Waksman JC et al., 2000)
	Theophylline	Case report	(Nebel et al., 1999)
	Loperamide	Case report	(Khawaja et al., 1999)
	Nefazodone	Case report	(Lantz et al., 1999)
	Phenprocoumon	Case report	(Gordon, 1998)
	Venlaxafine	Case report	(Prost et al., 2000)
	Amitriptylin	Clinical trial	(Johne et al., 2002)
	Tacrolimus	Clinical trial	(Hebert et al., 2004) (Mai et al., 2003)
	Simvastatin	Clinical trial	(Sugimoto et al., 2001)
	Imatinib	Clinical trial	(Frye et al., 2004)
	Indinavir	Clinical trial	(Piscitelli et al., 2000)
	Irenotecan	Clinical trial	(Mathijssen et al., 2002)
	R- and *S*-verapamil	Clinical trial	(Tannergren et al., 2004)
	Midazolam	Clinical trial	(Mueller et al., 2006)
	Digoxin	Clinical trials	(Johne et al., 1999; Durr et al., 2000; Mueller et al., 2004)
	Fexofenadine	Clinical trial	(Wang et al., 2002b)
	Fexofenadine	Clinical trial	(Dresser et al., 2003)
	Oral contraceptives	Clinical trials	(Hall et al., 2003; Murphy et al., 2005)
	Warfarin	Clinical trial	(Jiang et al., 2004)
	Warfarin	Case series	(Yue et al., 2000b)
Ginseng	Phenelzine	Case report	(Shader and Greenblatt, 1985; Jones and Runikis, 1987)
	Warfarin	Case report	(Janetzky and Morreale, 1997; Rosado, 2003)
	Warfarin	Clinical trials	(Jiang et al., 2004; Jiang et al., 2006)
American Ginseng	Warfarin	Clinical trial	(Yuan et al., 2004)
Danshen [*Salvia miltiorrhiza*]	Warfarin	Case report	(Izzat et al., 1998)
		Case reports	(Tam et al., 1995; Yu et al., 1997)
Dong quai [*Angelica sinensis*]		Case report	(Page and Lawrence, 1999)

			Case report	(Ellis GR and MR., 1999)
Papaya extract [*Papaya carica*]			Case report	(McRae, 1996)
Devil's claw [Harpargo-phytumprocumbens]			Case report	(Shaw et al., 1997)
Garlic [*Allium sativum*]			Case report	(WH, 1991)
Garlic [*Allium sativum*]		Saquinavir	Clinical trial	(Piscitelli et al., 2000)
		Alprazolam level	Clinical trial	(Markowitz et al., 2003)
Ginkgo [*Ginkgo biloba*]		Warfarin	Clinical trials *	(Egashira et al., 2003; Jiang et al., 2005; Jiang et al., 2006)
		Warfarin	Case report	(Matthews, 1998)
		Trazodone	Case report	(Galluzzi et al., 2000)
		Valerian	Case report	(Chen et al., 2002)
		Thiazide diuretic	Case report	(McRae, 1996)
		Aspirin	Case report	(Rosenblatt and Mindel, 1997)
		Ibuprofen	Case report	(Meisel et al., 2003)
		Phenytoin	Case report	(Kupiec and Raj, 2005)
		Omeprazole	Clinical trial	(Yin et al., 2004)
Evening primrose oil [*Oenethera biennis*]		Anaesthetics	Case report	(McRae, 1996)
Kava [*Piper methysticum*]		Alprazolam	Case report	(Almeida and Grimsley, 1996)
		Levodopa	Case report	(Schelosky et al., 1995)
Betel nut [*Areca catechu*]		Flupenthixol	Case report	(Deahl, 1989)
		Fluphenazine	Case report	(Deahl, 1989)
Eleuthero [*Eleutherococcus senticosis*]		Digoxin	Case report	(McRae, 1996)
Gan Cao (Licorice)		Digitalis	Case report	(Harada et al., 2002)
Gan Cao (Licorice)		Enalapril	Case report	(Iida et al., 2006)
Chili pepper [*Capsicum species*]		ACE inhibitor	Case report	(Hakas, 1990)
Formula	Xiao Chai Hu Tang (sho-saiko-to)	Caffeine	Clinical trial	(Saruwatari et al., 2003)
	Xiao Chai Hu Tang (sho-saiko-to) Saiboku-To Sairei-To	Prednisolone	Clinical trial	(Homma et al., 1995)

Data are also from Zhou *et al*. (2007), Hu *et al*. (2005) and Li *et al*. (2007). Note: All clinical trials included in the Table demonstrated significant interactions between the concerned herbs and drugs, except for those marked with "*" which showed no significant interaction between warfarin and Ginkgo.

Figure 1-1. Metabolic activation of benzo[a]pyrene (B[a]P) by CYP1A1, 1A2 and 1B1. B[a]P is a polycyclic aromatic hydrocarbon that is mutagenic and highly carcinogenic. The first step of B[a]P activation includes the formation of B[a]P 7,8-oxide catalyzed by CYP1A1, 1A2 and 1B1.

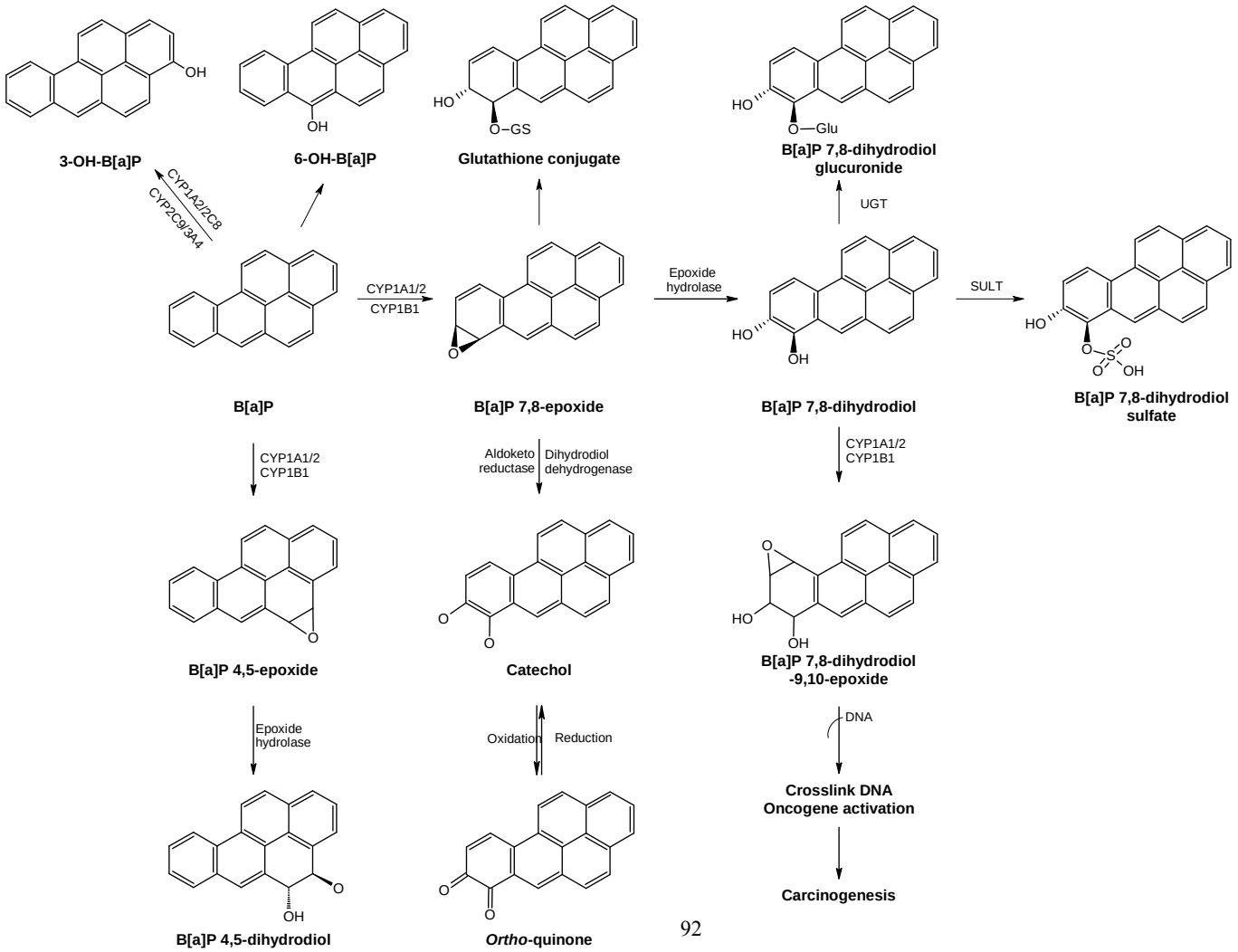


Figure 1-2. Metabolic activation of aristolochic acids (AAs). Both AAI and AAII undergo reduction of the nitro group catalyzed by enzymes to reactive cyclic nitrenium ions.

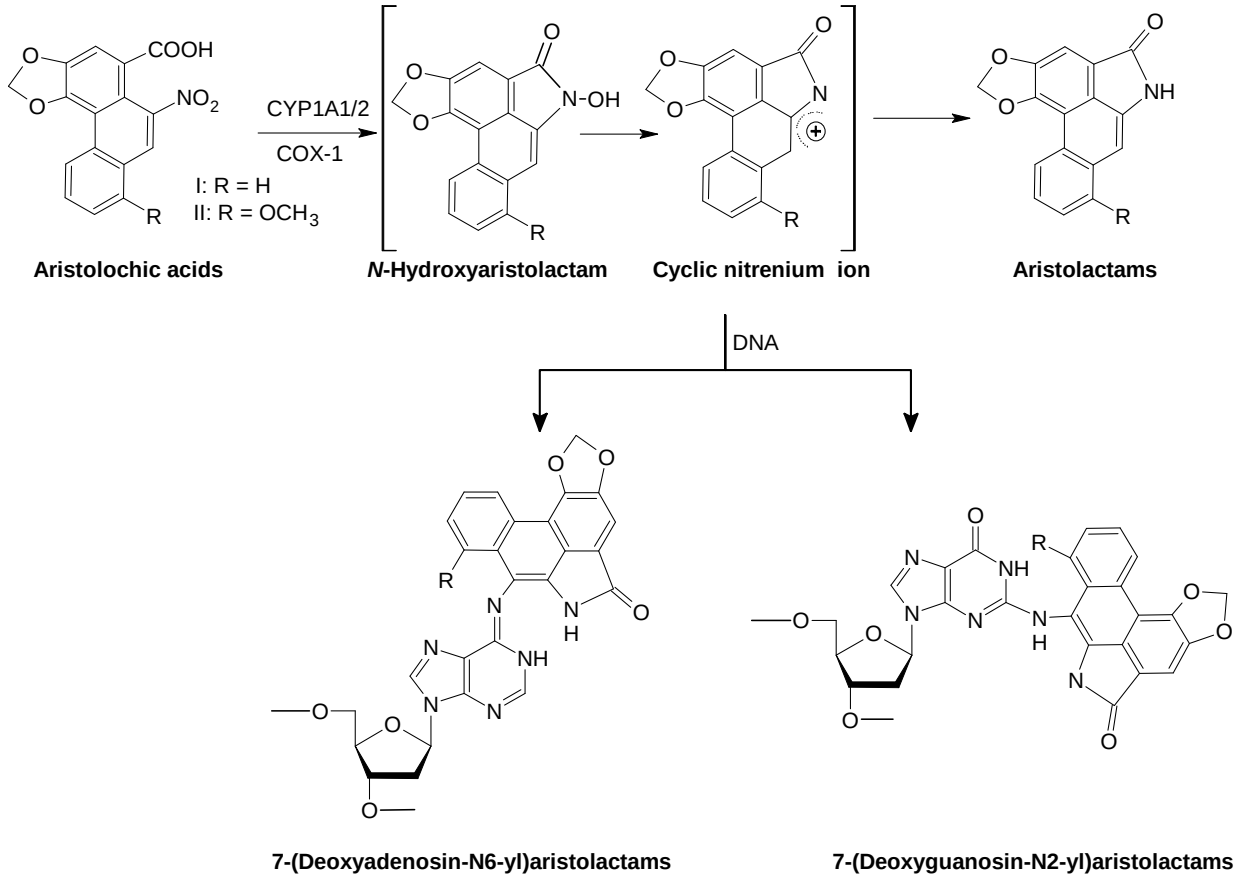


Figure 1-3. A schematic illustration of the aromatic hydrocarbon receptor (AhR)-mediated induction of Phase I and Phase II drug metabolizing enzymes and drug transporters such as human CYP1A1, 1B1, 1A2, and 2S1, UGT1A1 and 1A6, and MDR1/ABCB1. The AhR exists as cytoplasmic aggregates bound to two 90-kDa heat-shock proteins (HSPs), the cochaperone p23 and a 43-kDa immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2).

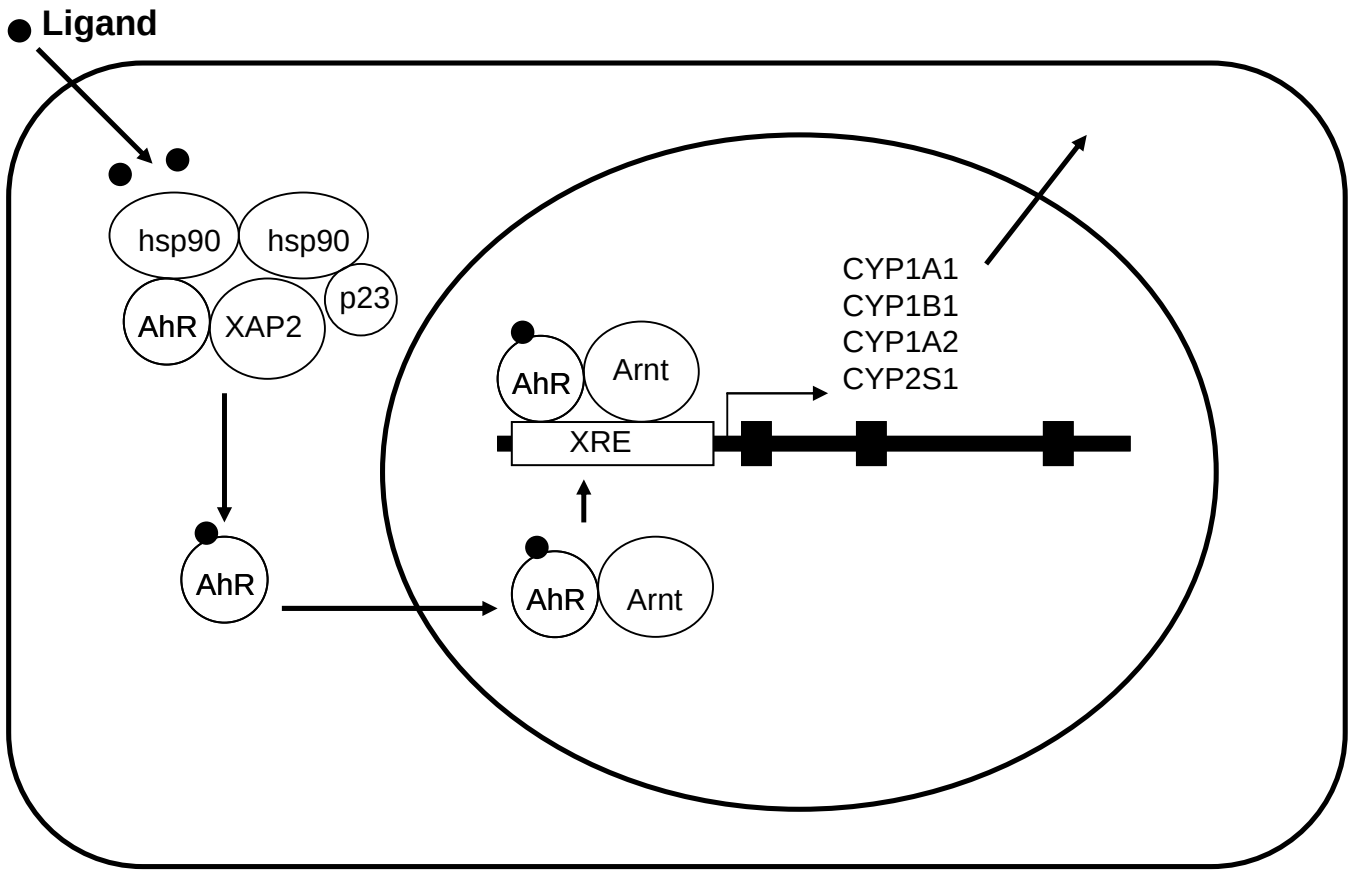
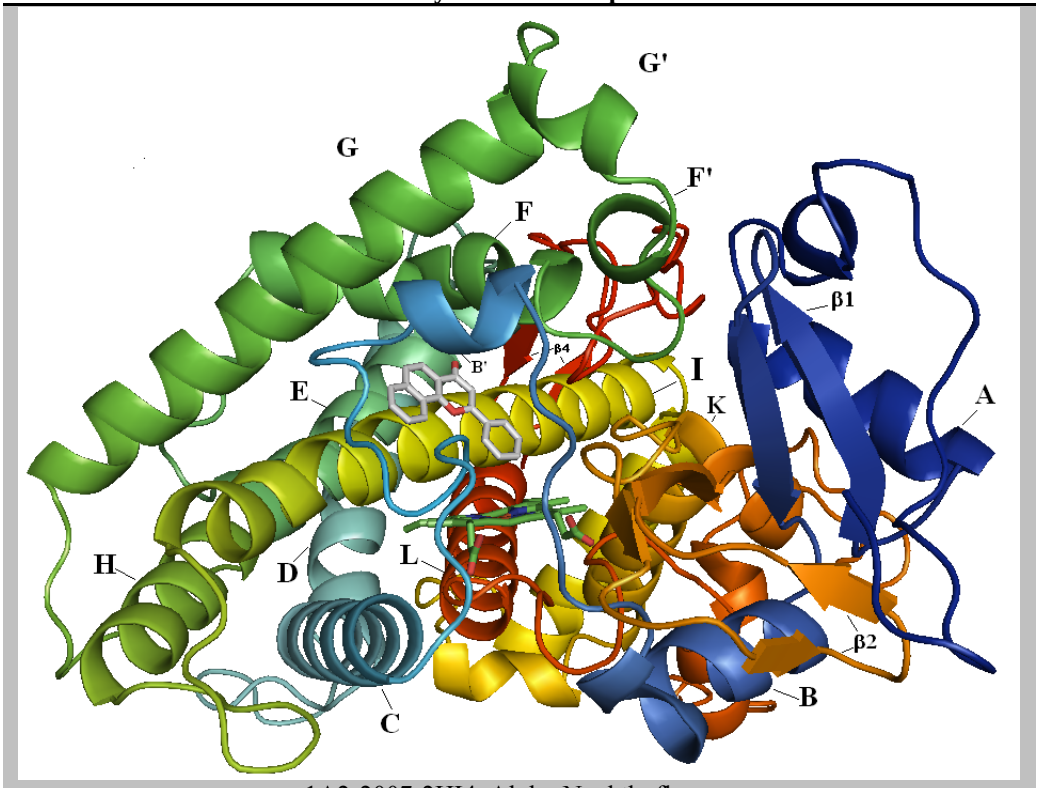


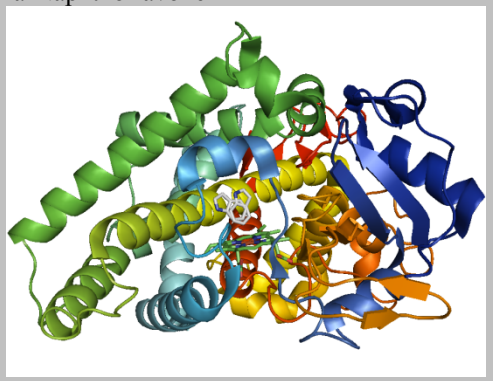
Figure 1-4. Crystal structures of published human CYPs with or without complexed ligands.

CYP-year-PDB: complex*

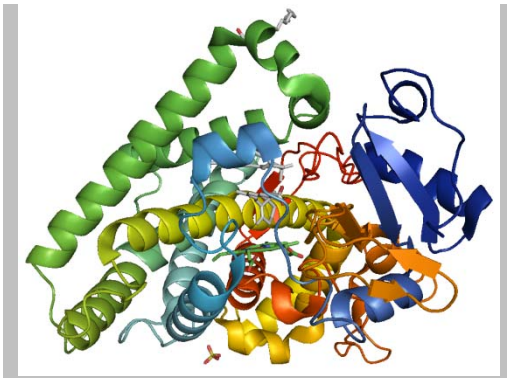


1A2-2007-2HI4: Alpha-Naphthoflavone

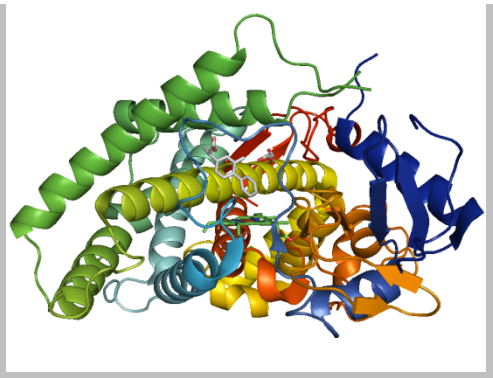
2A6-2005-1Z10: Coumarin



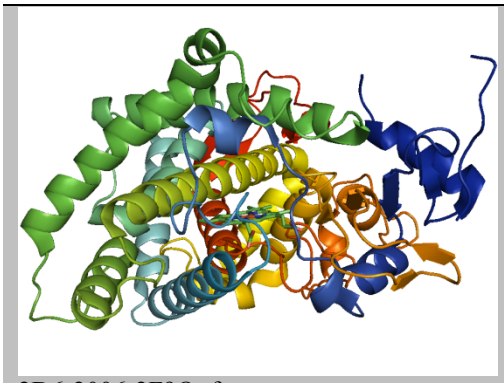
2A13-2007-2P85: Indole



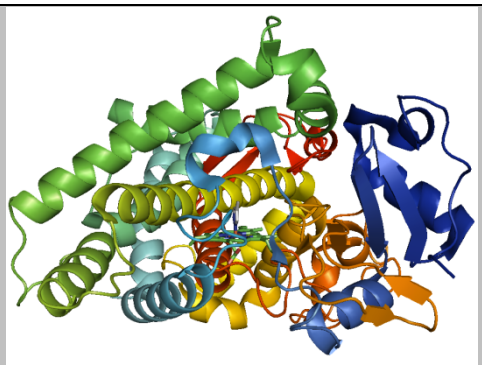
2C8-2008-2NNH: 9-cis-Retinoic Acid



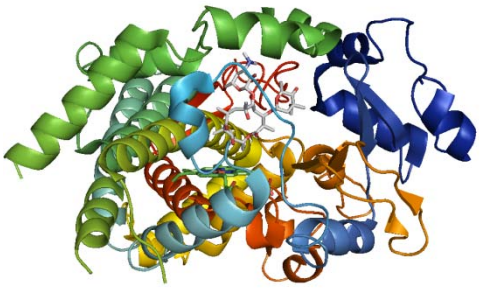
2C9-2004-1R9O: flurbiprofen



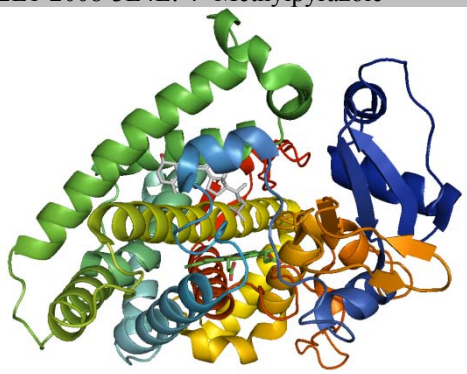
2D6-2006-2F9Q: free



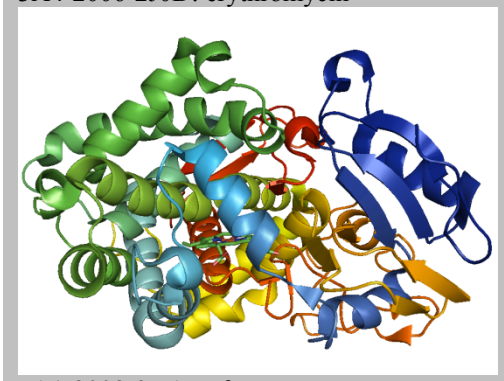
2E1-2008-3E4E: 4- Methylpyrazole



3A4-2006-2J0D: erythromycin



2R1-2008-3C6G: vitamin D3



7A1-2008-3DAX: free

46A1-2008-2Q9F: Cholesterol-3-Sulphate

*CYP-year-PDB: complex: the name of CYP enzymes with the publication year of their crystal structure; PDB codes; crystal state or complex name.

CHAPTER 2 HIGH-THROUGHPUT SCREENING OF HERBAL INHIBITORS FOR HUMAN CYP ENZYMES

2.1 Introduction

Herbs and herbal products are more like to be used as botanical supplements in Australia and many other Western countries but many herbs and herbal preparations are used as medications to treat diseases in China (Qiu, 2007). No matter as supplements or as medications, the active components in herbs can significantly affect the outcome of medical treatment if herbal supplements are used in combination with conventional medications. In another words, herb-drug interactions may happen at any time when the efficacy or toxicity of a conventional medication is changed by the administration of herbal supplements (Zhou et al., 2007). The well-known clinical case is St. John's wort that had been reported to reduce the blood concentration of a variety of clinical drugs such as cyclosporine and indinavir (Mills et al., 2004; Zhou et al., 2004d; Hu et al., 2005). Theoretically, the likelihood of herb-drug interactions is higher than drug-drug interactions because drugs usually contain a single and well-known chemical entity while almost all herbal products contain multiple constitutes. Potential herb-drug interactions are safety concern, especially for drugs with narrow therapeutic range (e.g. warfarin and theophylline) and for high-risk groups, such as the elderly or patients with renal or hepatic diseases. A number of clinically important herb-drug interactions have been reported based on case reports and randomized clinical studies (Hu et al., 2005). For most of these interactions, the underlying mechanism is yet to be determined, although both pharmacokinetic and pharmacodynamic components are considered to play an important role.

For pharmacokinetic herb-drug interaction, altered drug metabolism is probably the most important mechanism. Like many drug-drug interactions, modulation of CYPs is the major mechanism for some herb-drug interactions (Zhou et al., 2003b). CYPs are a superfamily of membrane-bound, heme-containing and mixed function oxygenases, with at least 57 members in humans (Rendic, 2002). Among them, CYP1A2, 2C9, 2C19, 2D6, and 3A4 are responsible for the metabolism of more than 90% of currently known drugs (Rendic, 2002). Most CYPs are subject to inhibition and induction by a variety of structurally distinct compounds including herbal medicines. Herbal components such as St John’s wort are well-known CYP3A4 and 2C9 inducer while most other herbal ingredients exhibit inhibitory effect on various CYPs (Zhou et al., 2003b). Thus, it is important to explore the effect of herbal components on CYPs.

Recently, high throughput (HTP) screening methods have been applied to examine the effect of natural compounds on CYPs, which represent a useful and efficient strategy for the study of herb-CYP interactions (Ansede and Thakker, 2004). They are capable of handling a great number of herbal constituents, and have the ability to provide *in vitro* inhibition data as a criterion for monitoring herb-drug metabolic interactions involving human drug metabolizing enzymes (in particular the CYPs). For example, an HTP screening procedure has been validated to assess the effects of various dietary and herbal flavonoids on human CYP1A1 expression using HepG2 cells expressing this enzyme (Allen et al., 2001) and Zou *et al*. (2002) have examined the effect of selected herbal components on human CYPs. In this study, we investigated the effect of 60 purified herbal compounds and 7 crude herbal products from commonly used herbs on human CYPs using a HTP screening method.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Fifty-seven purified herbal compounds tested in this study (Figure 2-1) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). These compounds mainly include triterpenoids, flavonoids, saponins, lactones, and alkaloids. 18α-Glycyrrhetinic acid, 18β-glycyrrhetinic acid and glycyrrhizic acid ammonium were obtained from Sigma-Aldrich Chemicals Co. (St Louis, MO). Compound danshen dropping pills were from Tianjin Tasly Pharmaceutical Co. ltd., Tianjin, China; Tanshinone capsule were from Hebei Xinglong Xili Pharmaceuticals Co. Ltd., Xinglong, China; Diammonium Glycyrrhizinate Enteric-coated Capsules were from Chia-tai Tianqing Pharmaceutical Co., Ltd., Lianyungang, China; and Compound Yiganling Tablets were from Beijing Double-crane Pharmaceutical Co., Ltd. Beijing, China. Concentrated wuweizi granules (*Schizandra chinensis* fruit extract) were obtained from Cathay Herbal Laboratories Pty Ltd. (Surry hills, NSW, Australia); concentrated licorice granules (*Glycyrrhiza uralensis*) were obtained from Koda International Pty. Ltd. (Sydney, Australia); Dried danshen roots were obtained from Chinese Medicine Research Group, RMIT University (Melbourne, Australia). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Whitehouse Station, NJ). All other reagents were of analytical or HPLC grade.

2.2.2 Source of recombinant human CYP enzymes

The inhibition of human CYP1A2, 2C9, 2C19, 2D6 and 3A4 enzymes was assessed using commercial kits containing recombinant CYP expressed in insect cells using BD Supersomes, NADPH-generating system ($NADP^+$ and glucose-6-phosphate dehydrogenase), and corresponding fluorescent substrates (Table 2-1) (http://www.bdbiosciences.com/discovery_labware) (Favreau et al., 1999; Crespi and Stresser, 2000; Crespi et al., 2002).

2.2.3 Enzyme inhibition assays

The CYP inhibition assays were conducted in 96-well microplates in duplicate as described previously (Crespi and Stresser, 2000). Briefly, all purified herbal compounds for this study were dissolved in acetonitrile (24 compounds), methanol (17 compounds), or dimethyl sulfoxide (DMSO, 19 compounds). For crude herbal products, further extraction was conducted using 100% methanol. The final concentration of acetonitrile, methanol, and DMSO in the reaction system was 2% acetonitrile, 1% methanol, and 0.2% DMSO, respectively (v/v). To each well, the test compound at various concentrations and NADPH were added and pre-incubated for 10 min at 37°C without shaking. The reaction was initiated by addition of enzyme/substrate mixture. The final concentration of each CYP enyzme is as following: 0.0018 μM for CYP1A2, 0.0036 μM for CYP2C9, 0.0036 μM for CYP2C19, 0.0055 μM for CYP2D6 and 0.0036 μM for CYP3A4. The incubation time was 15 min for CYP1A2, 45 min for CYP2C9 and 30 min for CYP2D6, 2C19 and 3A4. The reaction was terminated by addition of 75 μl acetonitrile-0.5 M Tris (4:1, pH 7.5) base solution to each well. The fluorescence was measured using a PolarStar Microplate Reader (BMG LABTECH Pty. Ltd., Offenburg, Germany).

The excitation wavelengths were 390 nm for CYP1A2, 2D6, 2C9 and 3A4; and a 405-nm absorption filter for CYP2C19; while the emission wavelengths were 460 nm for CYP1A2, 2D6 and 2C19, and 530 nm for CYP2C9 and 3A4. For each assay, the positive control and control vehicle were included and a standard curve was constructed. The positive control inhibitor of each CYP enzyme is as following: furafylline for CYP1A2, sulfaphenazole for CYP2C9, tranylcypromine for CYP2C19, quinidine for CYP2D6 and ketoconazole for CYP3A4. Duplicate samples were used for each test compound in each assay. For each compound**,** the assay was conducted once following the manufacture introduction. Repeat assay was conducted for selected samples to confirm the results.

2.2.4 IC_{50} determination

The IC_{50} value was determinate by a linear interpolation method according to the following equation:

$$IC_{50} = \frac{(50\% - Low\%Inhibition)}{(High\%\ Inhibition - Low\%Inhibition)} \times (HighCon. - LowCon.) + LowCon. \quad \text{Equation 2-1}$$

2.3 Results

2.3.1 Inhibitory effects on CYP1A2

There were three herbal compounds exhibiting remarkable inhibitory effects on CYP1A2, with the IC_{50} values <1.0 μM (Figure 2-2). These included tanshinone I, tanshinone IIA and cryptotanshinone with the IC_{50} value of 0.027, 0.187and 0.910 μM, respectively. In addition, baicalein, osthole, quercetin, cordycepin, sodium tanshinone IIA sulfonate and hyperoside showed moderate inhibition on the CYP1A2, with the IC_{50} value of 1.22, 1.49, 3.97, 6.69, 7.08 and 14.46 μM, respectively. Quercitrin, icariin, alloin, baicalin and triptolide had minor inhibitory effects on CYP1A2 with the IC_{50} value of 33.76, 43.00, 66.00, 70.03 and 98.22 μM, respectively. The other 42 herbal compounds showed little or negligible inhibition (IC_{50} > 100 μM) on CYP1A2. Notably, four herbal compounds including rutaecarpine, scopoletin, puerarin and andrographolide produced fluorescence and thus interfered with the determination for CYP1A2 (Table 2-2).

2.3.2 Inhibitory effects on CYP2C9

Three herbal compounds exhibited remarkable inhibitory effects on CYP2C9. They were tanshinone I, tanshinone IIA and γ-schisandrin with the IC_{50} of 0.106, 0.209 and 0.520 μM, respectively (Figure 2-3). Ten herbal compounds showed moderate inhibition on the CYP2C9, including cryptotanshinone, sodium tanshinone IIA sulfonate, baicalein, quercetin, silybin, osthole, icariin, hyperoside, baicalin and quercitrin with the IC_{50} value of 1.23, 1.36, 2.52, 3.01, 3.14, 8.30, 14.34, 14.37, 20.42 and 21.76 μM, respectively. Eight herbal compounds including gallic acid, dehydroandrographolide, 18β-glycyrrhetinic acid, ginsenoside Rg3, andrographolide, sodium danshensu, schisandrin, protocatechuicaldehyde and ursolic acid only had minor inhibitory effect on the CYP2C9, with the IC_{50} of 30.64, 39.76, 43.37, 61.53, 69.22, 73.12, 85.2, 90.66 and 100.75 μM, respectively. Other twenty five herbal had weak or no

inhibit CYP2C9. Notably, fourteen herbal compounds generated fluorescence and could not be detected by this approach for CYP2C9. These included salvianolic acid B, rutaecarpine, scopoletin, puerarin, alloin, liquiritin, jujuboside B, asperosaponin VI, saikosaponin D, astragaloside, amygdalin, gastrodin, trigonelline and polydatin (Table 2-2).

2.3.3 Inhibitory effects on CYP2C19

Only two of the sixty herbal compounds, γ-schisandrin and osthole, exhibited remarkable inhibitory effects on CYP2C19 with the IC_{50} value of 0.072 and 0.920 μM, respectively (Figure 2-4). Eight herbal compounds showed moderate inhibition to CYP2C19, including baicalein, quercetin, dehydroandrographolide, cryptotanshinone, sodium tanshinone IIA sulfonate, silybin, tanshinone I and protocatechuicaldehyde with the IC_{50} of 2.12, 7.23 8.87, 13.65, 19.44, 20.26, 21.09 and 25.7μM, respectively. Other eight herbal compounds, gallic acid, schisandrin, hyperoside, baicalin, icariin, andrographolide, 18β-glycyrrhetinic acid and quercitrin, only had minor inhibitory effect on CYP2C19 with the IC_{50} value of 31.53, 36.81, 37.08, 46.11, 72.17, 79.03, 96.67 and 98.77 μM, respectively. Other thirty-one herbal compounds showed weak (IC_{50} > 100 μM) or no inhibitory effect on CYP2C19. Notably, eleven herbal compounds produced fluorescence and interfered with the detection for CYP2C19, including rutaecarpine, tanshinone IIA, scopoletin, jujuboside B, asperosaponin VI, saikosaponin D, astragaloside, amygdalin, gastrodin, trigonelline and puerarin (Table 2-2).

2.3.4 Inhibitory effects on CYP2D6

None of the sixty herbal compounds exhibited remarkably inhibitory effects on CYP2D6. Only three herbal compounds, sodium tanshinone IIA sulfonate, γ-schisandrin and matrine, showed moderate inhibition to CYP2D6 with the IC_{50} value of 11.55, 16.97 and 24.96 μM, respectively (Figure 2-5). Baicalein, osthole, hyperoside, quercetin and quercitrin had minor inhibition to CYP2D6, with the IC_{50} of 36.78, 51.37, 53.22, 54.59 and 90 μM, respectively. Other 41 herbal compounds had little (IC_{50} > 100 μM) or negligible inhibition to CYP2D6. Notably, eleven herbal compounds produced fluorescence which interfere the detection for CYP2D6. These are salvianolic acid B, puerarin, protocatechuic acid, polydatin, ferulic acid, protocatechuicaldehyde, bilobalide, ginkgolide B, ginkgolide C, rutaecarpine and scopoletin (Table 2-2).

2.3.5 Inhibitory effects on CYP3A4

There were only two herbal compounds, γ-schisandrin and tanshinone I, exhibiting remarkably inhibitory effects on CYP3A4 with the IC_{50} of 0.009 and 0.220 μM, respectively (Figure 2-6). Thirteen herbal compounds including baicalein, evodin, sodium tanshinone IIA sulfonate, silybin, cryptotanshinone, paclitaxol, osthole, ursolic acid, polydatin, schisandrin, quercetin, ferulic acid and dehydroandrographolide showed moderate inhibition to CYP3A4, with the IC_{50} value of 1.24, 1.33, 1.78, 2.85, 2.96, 9.66, 12.01, 16.24, 16.78, 19.4, 19.8, 21.7 and 24.12 μM, respectively. Hyperoside, gallic acid, quercitrin, 18β-glycyrrhetinic acid and protocatechuicaldehyde had minor inhibition on CYP3A4, with the IC_{50} value of 47.49, 64.44, 71.01, 73.18 and 81.19 μM, respectively. In addition, Sanqi saponin and total notoginsenosides also showed inhibitory effects on CYP3A4 and the IC_{50} value was 40.85 and 60.91 μg/ml, respectively. Other 31 herbal compounds had weak (IC_{50} > 100 μM) or no inhibitory effect on CYP3A4. Seven herbal compounds, amygdalin, salvianolic acid B, puerarin, ginkgolide B, ginkgolide C, rutaecarpine and scopoletin, produced fluorescence which interfered with the detection for CYP3A4 (Table 2-2).

2.4 Conclusions and Discussion

HTP method based on fluorometric assay for screening of potential inhibitors of CYPs is available since 1997 (Crespi and Stresser, 2000). In the present study, we examined the effect of a number of herbal components in five human CYPs using a validated HTP approach. The herbal components tested include a variety of structurally distinct compounds such as triterpenoids of danshen (*Salvia miltiorrhiza*), flavonoids and their glycoside derivatives, saponine, other glucosides, lactones, alkaloids, and acids. As all the 57 compounds are purified herbal components, three organic solvents (acetonitrile, methanol and DMSO) had been used to prepare stock solutions at high concentrations. For the subsequent reaction, the test compounds were diluted with water at very low concentrations. These organic solvents were only used as vehicles to get a proper amount of test compounds dissolving into water for final reaction.

Tanshinone I, tanshinone IIA and cryptotanshinone, all from danshen, significantly inhibited the activities of CYP1A2 and 2C9; tanshinone I also considerably inhibited CYP3A4. In contrast, the hydrophilic constituents of danshen (sodium danshensu, protocatechuic acid, salvianolic acid B and protocatechuicaldehyde) showed weak or negligible inhibitory effects on the five CYP enzymes. Notably, the derivative of tanshinone IIA, sodium tanshinone IIA

sulfonate, had a remarkable inhibition to CYP2C9 and 3A4. Sodium tanshinone IIA sulfonate has been commonly used for patients with unstable angina pectoris in China.

In the present study, we found that the activities of CYP2C9, 2C19 and 3A4 were remarkably inhibited by γ-schisandrin. Silybin, a major component in milk thistle (*Silybi mariani*), significantly inhibited the activities of CYP2C9 and 3A4, which is in line with the report by Sridar *et al*. (Sridar et al., 2004). It is evident that free flavonoids such as baicalein and quercetin (rich in Ginkgo leaves and many other herbs), have significant inhibitory effects on CYP1A2, 2C9, 2C19 and 3A4. However, the flavonoid glucosides (baicalin, hyperoside, quercitrin and icariin) have much lower inhibitory effects on CYP1A2, 2C9, 2C19 and 3A4 than their free flavonoids.

Osthole, a coumarin derivative, had remarkably inhibitory effects on CYP2C19 and moderate inhibitory effects on CYP1A2, 2C9 and 3A4. Alkaloids are one of the largest groups of natural products. We also tested eight alkaloids (stachydrine chloride, trigonelline, rutaecarpine, oxymatrine, sophoridine and matrine) and only found matrine having moderate inhibition to CYP2D6. Among the seven natural acids including salvianolic acid B, protocatechuic acid, 18α/β-glycyrrhetinic acid, gallic acid, ferulic acid and ursolic acid tested in this study, only ferulic acid and ursolic acid moderately inhibited CYP3A4. All lactones tested except scopoletin had little inhibitory effect on CYP enzymes. In contrast, dehydroandrographolide (from *Andrographis paniculata*) inhibited CYP2C19 significantly and 3A4 to a moderate level, while evodin (from *Evodia rutaecarpa*) significantly inhibited CYP3A4.

Saponins, a heterogeneous group of sterol and triterpene glucosides, are found in a large number of plants and some animals (e.g. the sea cucumber) (Skene and Sutton, 2006). Most of the tested saponin compounds failed to show any inhibitory effects on the five CYP enzymes. Only ginsenoside Rg3 (*panax Ginseng*) exhibited minor inhibitory effect on CYP2C9, while sanqi saponin and total notoginsenosides (both from *panax notoginseng*) exhibited weak inhibition to CYP3A4. All other glucosides including alloin, amygdalin, arctiin, forsythin, gastrodin, liquiritin, polydatin and puerarin exhibited little inhibition to CYPs. Notably, cordycepin, an analogue of adenosine, merely inhibited CYP1A2, but not CYP2C9, 2C19, 2D6 and 3A4.

In conclusion, a variety of structurally distinct herbal compounds have been examined with their ability to inhibit major human CYPs using HTP approach and a small number of them are found to significantly inhibit human CYP1A2, 2C9, 2C19 and CYP3A4. Given that these enzymes play a key role in the metabolism of many important clinical drugs, further investigations in humans are needed to explore the clinical impact.

Table 2-1. The reaction systems consisting of the CYP enzymes, positive inhibitors and probe substrates.

Enzyme	**Enzyme Assay**	**Positive Inhibitor**	**Fluorescent Substrate**
CYP1A2	Phenacetin *O*-deethylase	Furafylline	3-Cyano-7-ethoxycoumarin
CYP2C9	Diclofenac 4'-hydroxylase	Sulfaphenazole	7-Methoxy-4-trifluoromethylcoumarin
CYP2C19	*S*-Mephenytoin 4'-hydroxylase	Tranylcypromine	3-Cyano-7-ethoxycoumarin
CYP2D6	Bufuralol 1'-hydroxylase	Quinidine	3-[2-(*N*,*N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin
CYP3A4	Midazolam 1'-hydroxylase	Ketoconazole	7-Benzyloxy-4-trifluoromethylcoumarin

Table 2-2. IC_{50} value of the sixty test compounds and seven herbal products.

Test compound	Number of compound	Herbal source	Highest concentration (μM)	IC_{50} (μM)				
				CYP1A2	CYP2C9	CYP2D6	CYP3A4	CYP2C19
Alloin	1	*Rheum palmatum* *Aloe vera*	128.35	66.00	#	-	-	-
Amygdalin	2	*Prunus armeniaca* *Prunus persica*	110.62	-	#	-	#	#
Andrographolide	3	*Andrographis paniculata*	101.18	#	69.22	-	-	79.03
Arctiin	4	*Arctium lappa*	100.00	-	-	-	-	-
Asperosaponin VI	5	*Dipsacus asperoides*	57.04	-	#	-	-	#
Astragaloside	6	*Astragalus membranaceus*	66.20	-	#	-	-	#
Baicalein	7	*Scutellaria baicalensis*	101.40	1.22	2.52	36.78	1.24	2.12
Baicalin	8	*Scutellaria baicalensis* *Lonicera japonica*	101.88	70.03	20.42	-	-	46.11
Bilobalide	9	*Ginkgo biloba*	100.16	-	-	#	-	-
Borneol	10	*Dryobalanops aromatic* *Chrysanthemum morifolium*	100.20	-	-	-	-	-
Canthridin	11	*Mylabris*	99.82	-	-	-	-	-
Cordycepin	12	*Cordyceps sinensis*	100.10	6.69	-	-	-	-
Cryptotanshinone	13	*Salvia miltiorrhiza*	100.22	0.91	1.23	-	2.96	13.65
Dehydroandrographolide	14	*Andrographis paniculata*	100.20	-	39.76	-	24.12	8.87
Evodin	15	*Evodia rutaecarpa*	100.49	-	-		1.33	-
Ferulic Acid	16	*Ligusticum chuanxiong* *Angelica sinensis*	100.22	-	-	#	21.7	-
Forsythin	17	*Forsythia suspensa*	99.93	-	-	-	-	-
Gallic acid	18	*Rheum palmatum* *Cornus officinalis*	104.13	-	30.64	-	64.44	31.53
Gastrodin	19	*Gastrodia elata*	100.22	-	#	-	-	#
Ginkgolide A	20	*Ginkgo biloba*	100.06	-	-	-	-	-
Ginkgolide B	21	*Ginkgo biloba*	100.18	-	-	#	#	-
Ginkgolide C	22	*Ginkgo biloba*	100.18	-	-	#	#	-
Ginsenoside Rg3	23	*Panax ginseng*	68.07	-	61.53	-	-	-

18α-Glycyrrhetinic acid	24	*Glycyrrhiza uralensis*	99.77	-	-	-	-	-
18β-Glycyrrhetinic acid	25	*Glycyrrhiza uralensis*	98.89	-	43.37	-	73.18	96.67
Glycyrrhizic acid ammonium	26	*Glycyrrhiza uralensis*	97.11	-	-	-	-	-
Hyperoside	27	*Epimedium brevicornum* *Crataegus pinnatifida* *Apocynum venetum*	101.03	14.46	14.37	53.22	47.49	37.08
Icariin	28	*Epimedium brevicornum*	102.86	43.00	14.34	-	-	72.17
Jujuboside B	29	*Ziziphus jujuba*	50.00	-	#	-	-	#
Liquiritin	30	*Glycyrrhiza uralensis*	134.09	-	#	-	-	-
Matrine	31	*Sophora flavescens* *Sophorae alopecuroidis* *Sophora tonkinensis*	100.38	-	-	24.96	-	-
Notoginsenosides (total)	32	*Panax notoginseng*	81.10 μg/ml	-	-	-	60.91 μg/ml	-
Osthole	33	*Angelica pubescens* *Cnidium monnieri*	100.42	1.49	8.3	51.37	12.01	0.92
Oxymatrine	34	*Sophora flavescens* *Sophorae alopecuroidis*	100.12	-	-	-	-	-
Paclitaxol	35	*Ramulus et folium taxi chinensis*	115.47	-	-	-	9.66	-
Polydatin	36	*Polygonum cuspidatum*	155.64	-	#	#	16.78	-
Protocatechuic Acid	37	*Salvia miltiorrhiza* *Ilicis pubescentis* *Petiolus trachycarpi*	100.56	-	-	#	-	-
Protocatechuicaldehyde	38	*Salvia miltiorrhiza* *Ilicis pubescentis* *Petiolus trachycarpi*	100.70	-	90.66	#	81.19	25.7
Puerarin	39	*Scutellaria baicalensis* *Pueraria lobata*	111.92	#	#	#	#	#
Quercetin	40	*Ginkgo biloba* *Bupleurum chinensis*	101.08	3.97	3.01	54.59	19.8	7.23
Quercitrin	41	*Hypericum japonicum* *Viscum coloratum*	108.83	33.76	21.76	90	71.01	98.77
Rutaecarpine	42	*Evodia rutaecarpa*	100.78	#	#	#	#	#

Saikosaponin A	43	*Bupleurum chinensis*	67.35	-	-	-	-	-
Saikosaponin D	44	*Bupleurum chinensis*	62.23	-	#	-	-	#
Salvianolic acid B	45	*Salvia miltiorrhiza*	100.14	-	#	#	#	-
Sanqi saponin	46	*Panax notoginseng*	59.90 μg/ml	-	-	-	40.85 μg/ml	-
γ-Schisandrin	47	*Schisandra chinensis*	100.02	-	0.52	16.97	0.009	0.072
Schisandrin	48	*Schisandra chinensis*	100.08	-	85.2	-	19.4	36.81
Scopoletin	49	*Morus alba*	100.54	#	#	#	#	#
Silybin	50	*Silybi Mariani*	100.10	-	3.14	-	2.85	20.26
Sodium danshensu (Salt of salvianolic acid A)	51	*Salvia miltiorrhiza*	99.76	-	73.12	-	-	-
Sodium tanshinone IIA sulfonate	52	*Salvia miltiorrhiza*	101.67	7.08	1.36	11.55	1.78	19.44
Sophoridine	53	*Sophora tonkinensis Sophorae alopecuroidis*	100	-	-	-	-	-
Stachydrine chloride	54	*Leonurus heterophyllus*	101.12	-	-	-	-	-
Tanshinone I	55	*Salvia miltiorrhiza*	27.75	0.027	0.106	-	0.220	21.09
Tanshinone IIA	56	*Salvia miltiorrhiza*	51.05	0.187	0.209	-	-	#
Tetramethylpyrazine Hydrochloride	57	*Ligusticum chuanxiong*	100.3	-	-	-	-	-
Trigonelline	58	*Trigonella foenum-graecum*	99.90	-	#	-	-	#
Triptolide	59	*Tripterygium wilfordii*	100.22	98.22	-	-	-	-
Ursolic acid	60	*Forsythia suspense Cornus officinalis*	104.01	-	100.75	-	16.24	-
Dried danshen roots (equal to raw herb mg/ml)		*Salvia miltiorrhiza*	0.4*	0.0012*	0.0065*	0.106*	0.013*	0.08*
Tanshinone capsule (equal to cryptotanshinone)		*Salvia miltiorrhiza*	100.05	0.077	0.061	0.899	0.122	1.56

Compound danshen dropping pills (equal to salvianolic acid A)	*Salvia miltiorrhiza*	100.09	27.82	-	-	19.96	26.55
Concentrated licorice granules (equal to raw herb mg/ml)	*Glycyrrhiza uralensis*	9.962*	1.04*	0.0045*	2.325*	0.055*	0.0645*
Diammonium glycyrrhizinate Enteric-coated Capsules	*Glycyrrhiza uralensis*	58.34	-	-	-	-	-
Concentrated wuweizi granules (equal to raw herb mg/ml)	*Schisandra chinensis*	12.6*	3.17*	-	0.99*	0.82*	1.49*
Compound Yiganling tablet (equal to a tablet weight)	*Schisandra chinensis* + *Silybi Mariani*	1.12*	1.09*	0.01*	0.392*	0.004*	0.012*

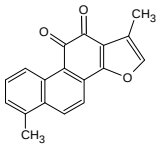
Inhibition percentage was presented at the highest concentration tested if IC_{50} could not be calculated. For concentration and IC_{50} values, μM was used as the unit except those crude herbal products with a symbol of "*".

"-" No effect at the highest concentration tested.

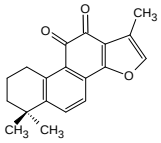
"#:" IC_{50} value may not be estimated; compound exhibited native fluorescence at concentrations tested.

Figure 2-1. Chemical structures of the natural compounds tested in this study.

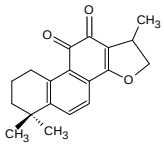
Triterpenoids



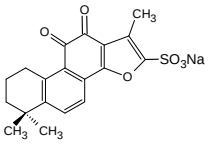
Tanshinone I



Tanshinone IIA

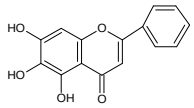


Cryptotanshinone

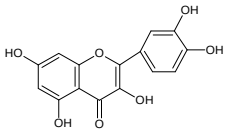


Sodium tanshinone IIA sulfonate

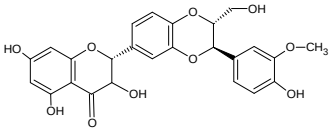
Flavonoids



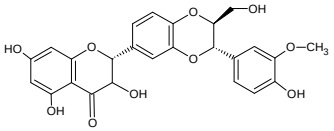
Balcalein



Quercetin

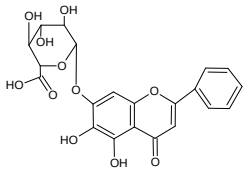


Silybin A

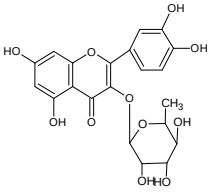


Silybin B

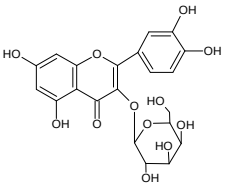
Flavonoid glucosides



Balcalin



Quercitrin



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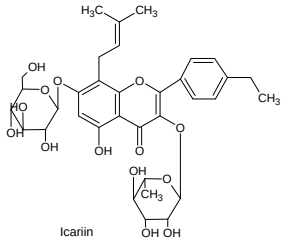
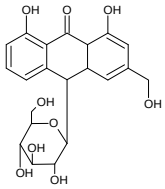


Icariin

Other glucosides



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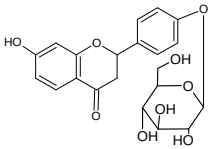
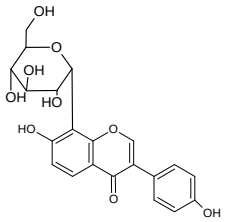


Liquiritin



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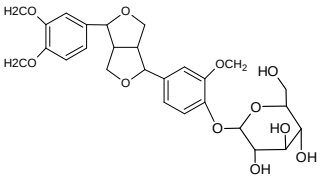
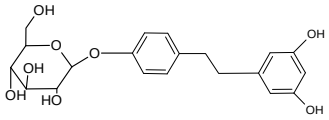
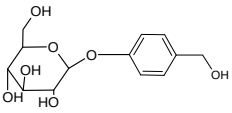


Forsythin



Polydatin



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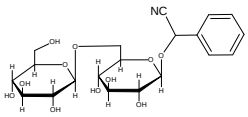

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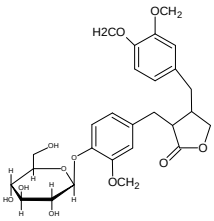

Arctiin

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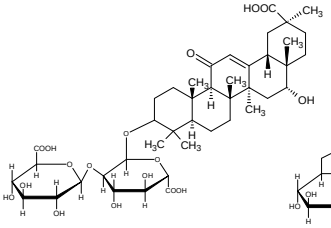
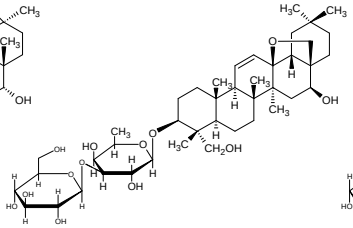
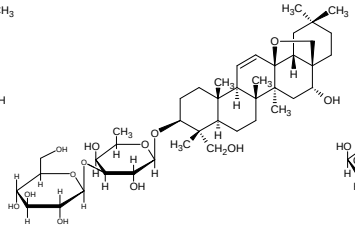
Saponine



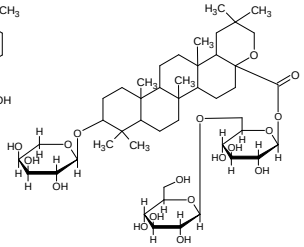
Glycyrrhizin



Saikosaponin A



Saikosaponin D



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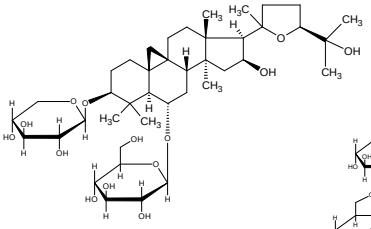
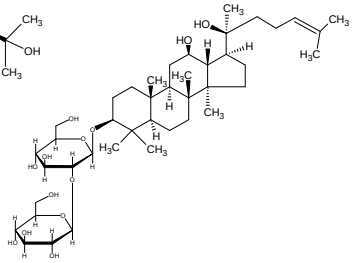
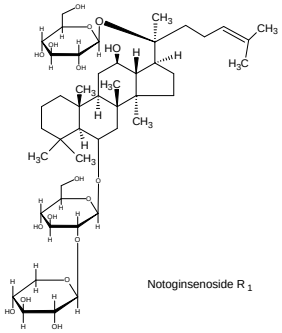


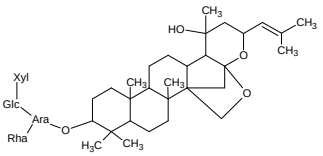
Astragaloside IV



Ginsenoside Rg3

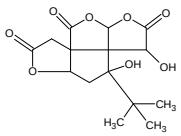


Notoginsenoside R 1

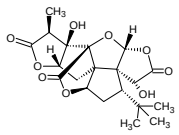


Jujuboside B

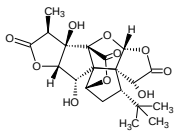
Lactones



Bilobalide



Ginkgolide A



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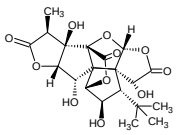
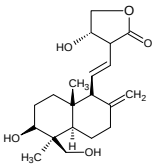
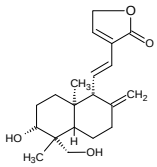


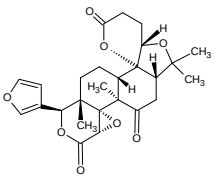
Ginkgolide C



Andrographolide



Dehydroandrographolide



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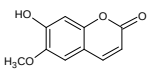

Scopoletin

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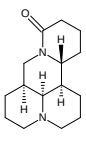
Alkaloids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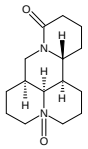
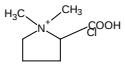
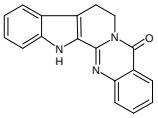

Oxymatrine


Trigonelline


Stachydrine chloride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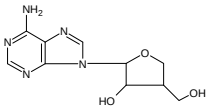
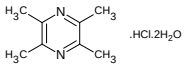
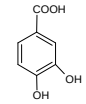
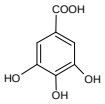

Cordycepin


Tetramethylpyrazine Hydrochloride

Acids


Protocatechuic acid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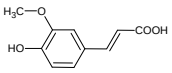
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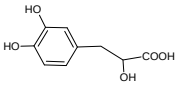
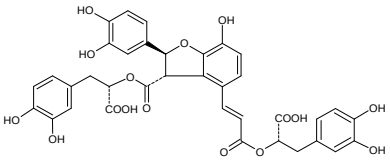
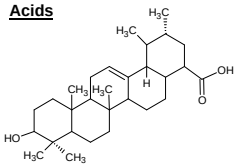
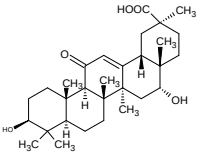

Danshensu (Salvianolic acid A)

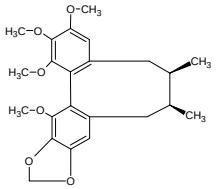

Salvianolic acid B

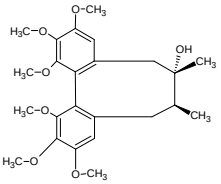
Acids


Ursolic acid

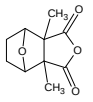

Glycyrrhetinic acid

Others


Γ-Schisandrin


Schisandrin

Others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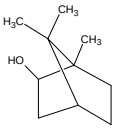
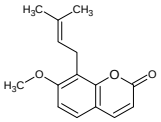
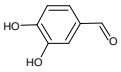
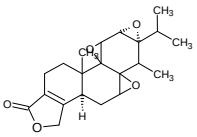
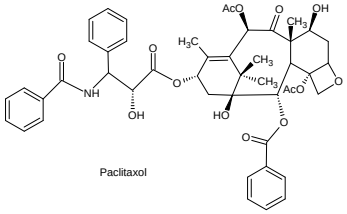

Borneol

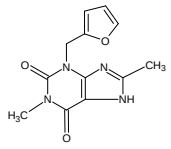

Osthole

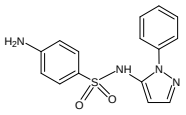

Protocatechuicaldehyde

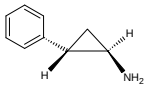

Triptolide

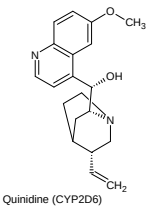

Paclitaxol

Positive inhibitors


Furafylline (CYP1A2)


Sulfaphenazole (CYP2C9)


Tranylcypromine (CYP2C19)


Quinidine (CYP2D6)

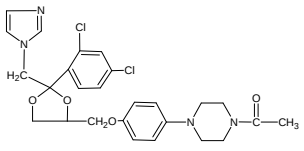

Ketoconazole (CYP3A4)

Figure 2-2. Inhibitory effects of herbal compounds on human CYP1A2.

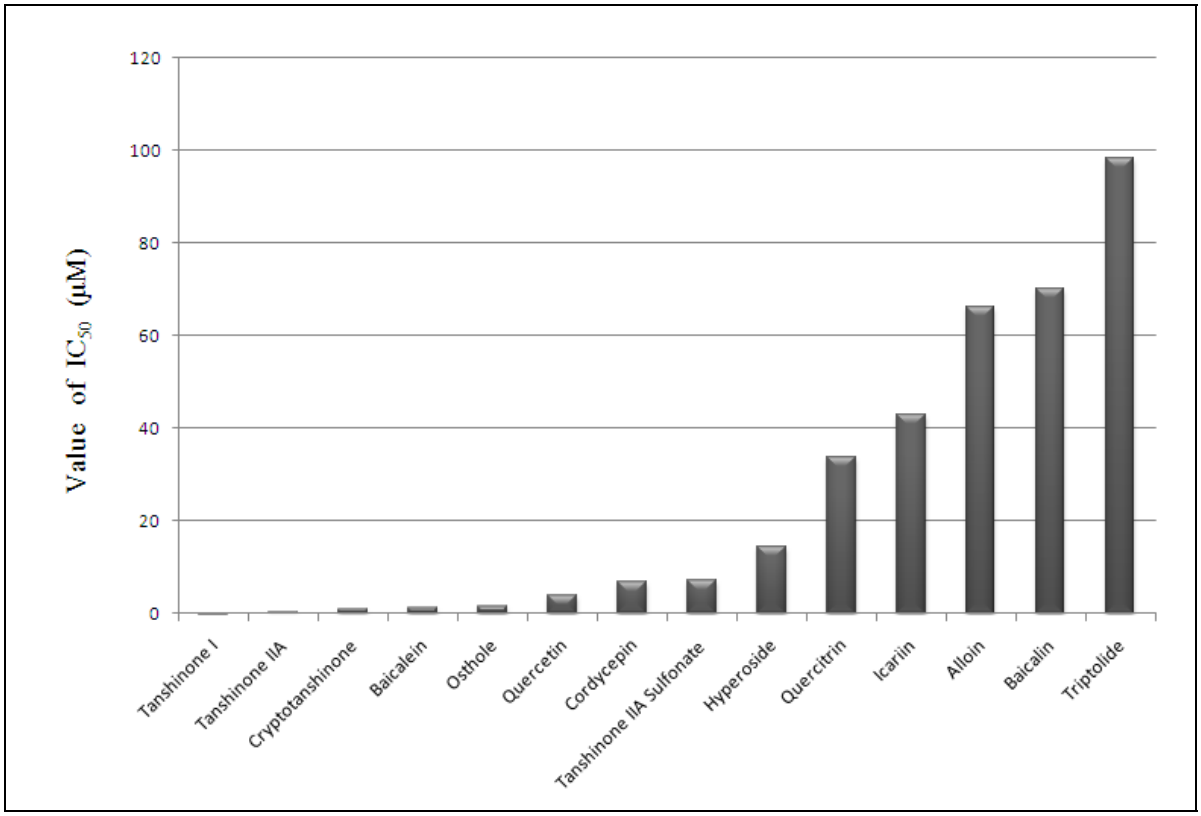


Figure 2-3. Inhibitory effects of herbal compounds on human CYP2C9.

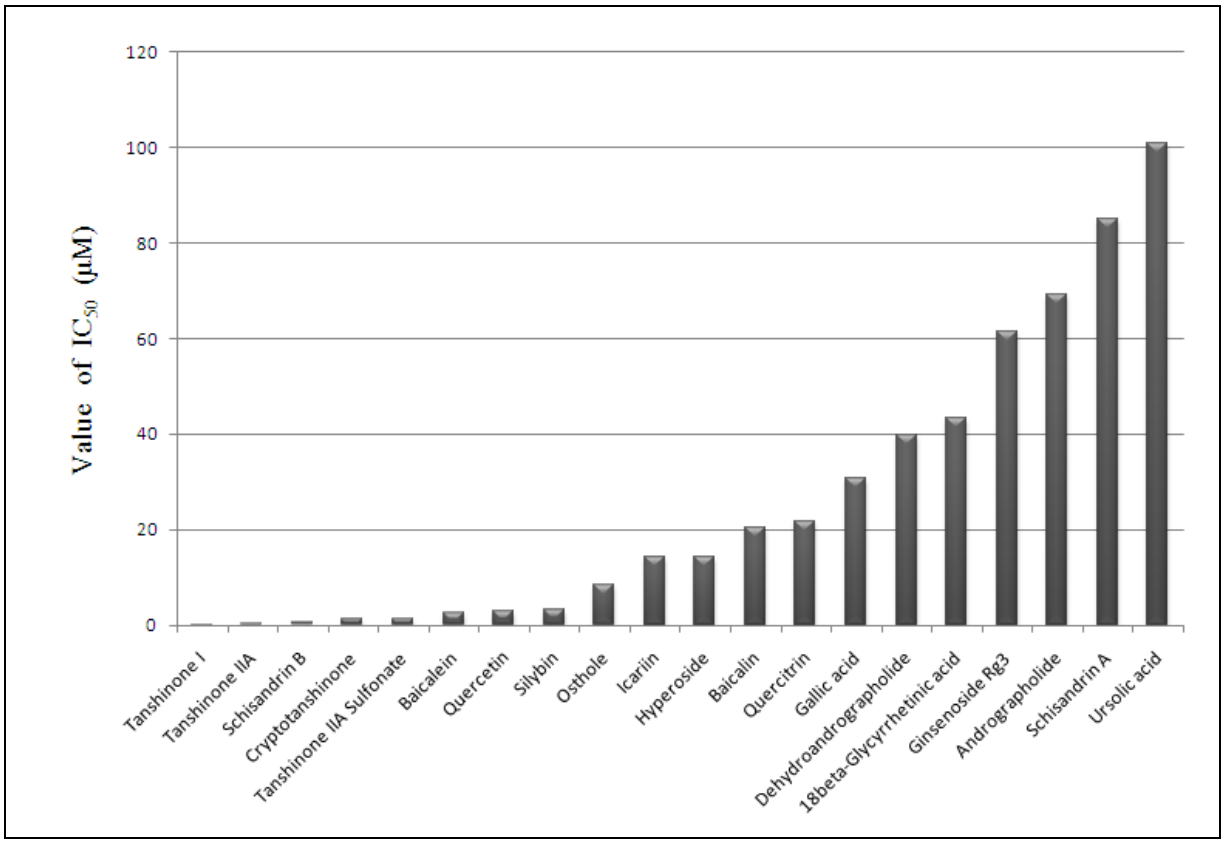


Figure 2-4. Inhibitory effects of herbal compounds on human CYP2C19.

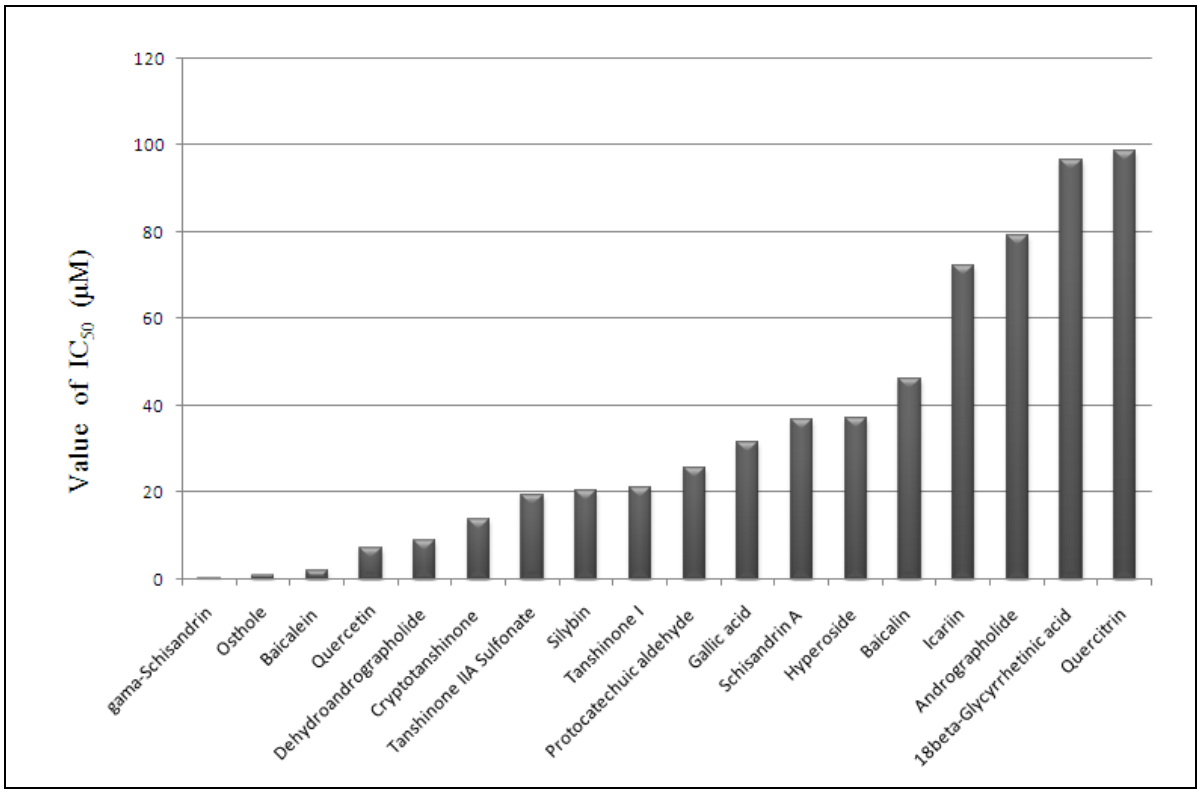


Figure 2-5. Inhibitory effects of herbal compounds on human CYP2D6.

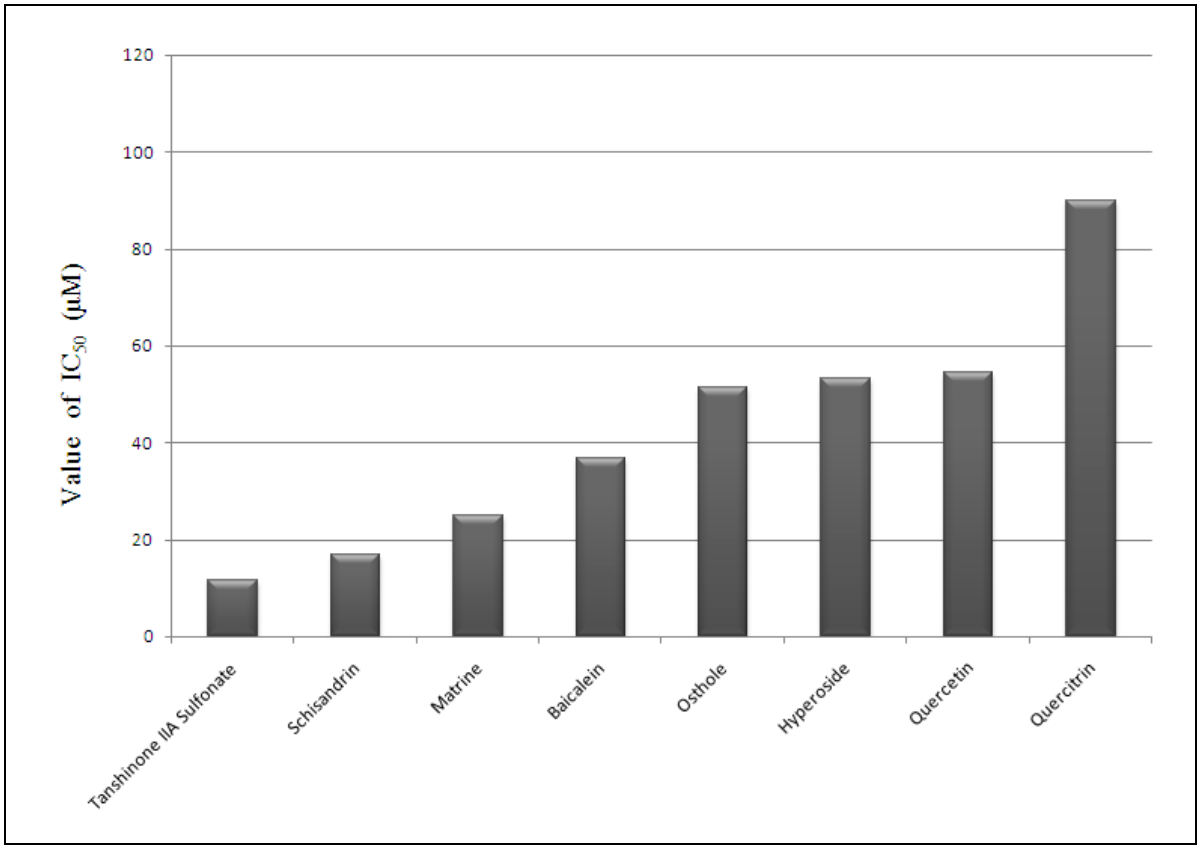
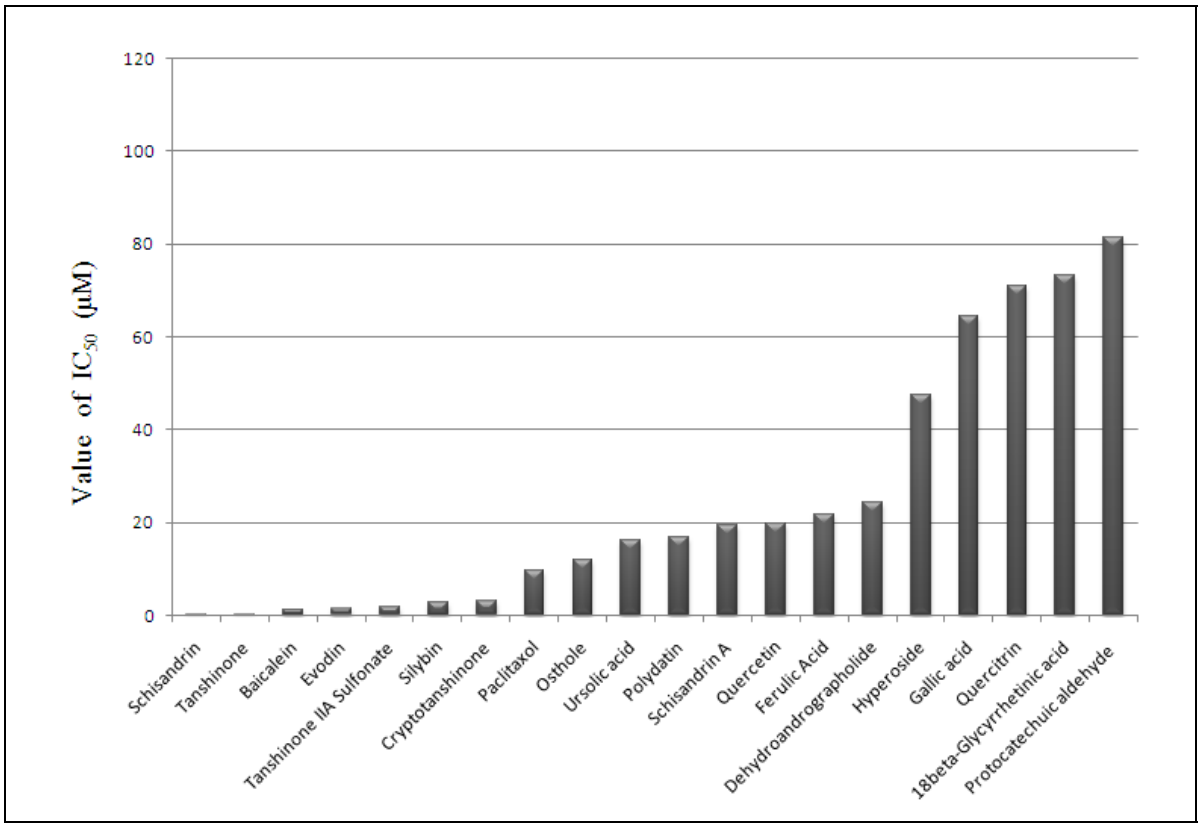


Figure 2-6. Inhibitory effects of herbal compounds on human CYP3A4.



CHAPTER 3 PREDICTING PHARMACOKINETIC HERB-DRUG INTERACTIONS

3.1 Introduction

Many commonly used herbal products have been reported to modulate the pharmacokinetics of important prescribed drugs, leading to altered absorption, distribution, metabolism and excretion. The well-known clinical case is St. John's wort that had shown to reduce the AUC of a variety of clinical drugs, including cyclosporine (Breidenbach et al., 2000b), amitriptyline (Johne et al., 2002), digoxin (Johne et al., 1999), indinavir (Piscitelli et al., 2000), nevirapine (de Maat et al., 2001), oral contraceptives (Yue et al., 2000a), warfarin (Yue et al., 2000a), phenprocoumon (Maurer et al., 1999), theophylline (Nebel et al., 1999), and simivastatin(Sugimoto et al., 2001). The outcomes due to certain herb-drug interactions may be fatal threaten, such as St John's wort decreasing cyclosporine's plasma concentration and then causing tissue rejection in transplant patients. Therefore, combining use of certain herbs with certain therapeutic drugs is on the risk, especially for drugs with narrow therapeutic range (e.g. warfarin and theophylline) and for high-risk groups, such as the elderly or patients with renal or hepatic diseases. Few severe herb-drug interactions have been reported based on case reports (Hu et al., 2005) but the clinical study on herb-drug interactions are still limited, despite many opportunities of combining use of herbs with interventional drugs. Efforts to identify all potential herb-drug interactions will lead to limitless investigations. However, efforts to predict pharmacokinetic drug interactions with certain herbs may provide a perspective view to avoid toxic or fatal herb-drug interactions, if properly using *in vitro* herb-drug interaction data.

Prediction of herb-drug interactions from *in vitro* data is commonly obtained using estimates of enzyme inhibition constant (K_i), inhibitor (herbal components) unbound concentrations ([I]), fraction (f_h) of hepatic clearance (CL_h) in total clearance (CL_{tot}) for the potentially inhibited drug and its fraction (f_m) of the metabolic process subject to inhibition in CL_h. Therefore, the clearance of co-administered drugs must be primarily through metabolism but not subject to substantial conjugation or other non-CYP metabolism. Furthermore, the liver is the primary organ of metabolic clearance and the drug does not possess physiochemical properties that are associated with absorption problems (i.e. limited solubility, low intestinal permeability). We have conducted an *in vitro-in vivo* extrapolation for herb-drug interactions based on our *in vitro* inhibitory data following pharmacokinetic principles.

3.2 Pharmacokinetic principles for inhibitory drug interactions

Herbs may inhibit CYPs by three mechanisms (Zhou et al., 2005b): competitive inhibition, non-competitive inhibition, and mechanism-based inhibition. Mutual competitive inhibition may occur between herbal constituent and drug, which are often metabolised by the same CYP enzyme. For example, diallyl sulfide from garlic is a competitive inhibitor of CYP2E1 (Teyssier et al., 1999). Non-competitive inhibition is caused by the binding of herbal constituents containing electrophilic groups (e.g. imidazole or hydrazine group) to the heme portion of CYP. For example, piperine inhibited arylhydrocarbon hydroxylase (CYP1A) and 7-ethoxycourmarin deethylase (CYP2A) by non-competitive mechanism (Dalvi and Dalvi, 1991). Hyperforin present in St John's wort is a potent non-competitive inhibitor of CYP2D6 activity (Obach, 2000c). The mechanism-based inhibition of CYP is due to the formation of a complex between herbal metabolite with CYP. Diallyl sulfone is a suicide inhibitor of CYP2E1 by forming complex, leading to autocatalytic destruction of CYP2E1 (Jin and Baillie, 1997b).

Generally, the extent of inhibition (R, %) of drug metabolism by herbal constituents depends on the inhibition mechanism when the substrate concentration [S] is high. For example, the R value of a particular metabolic pathway by a competitive inhibitor from coadministered herb can be calculated by Eq. 3-1 (Lin, 1998; von Moltke et al., 1998b):

$$\mathrm{R}\,(\%) = \frac{\mathrm{CL_{int}}'}{\mathrm{CL_{int}}} \times 100 = \frac{[\mathrm{I}]}{[\mathrm{I}] + K_{\mathrm{i}} \times \left(1 + [\mathrm{S}]/K_{\mathrm{m}}\right)} \times 100 \qquad \text{(Equation 3-1)}$$

where [S] and [I] are the maximal unbound substrate and inhibitor concentration respectively; K_{i}, the inhibitory constant; and $\mathrm{K_m}$, Michaelis-Menten constant.

When multiple inhibitory herbal constituents are involved, R is calculated by Eq. 3-2:

$$\mathrm{R}\,(\%) = \sum_{i=1}^{n} \left[\frac{[\mathrm{I_i}]}{[\mathrm{I_i}] + \mathrm{K_{i(i)}} \times \left(1 + [\mathrm{S}]/\mathrm{K}_m\right)} \times 100 \right] \qquad \text{(Equation 3-2)}$$

However, in clinical situations, [S] is often much lower than K_{m}, then R is expressed by Eq. 3-3, independent of the inhibition nature, except for the non-competitive inhibition (Tucker, 1992):

$$\mathrm{R}\,(\%) = \frac{\mathrm{CL_{int}}'}{\mathrm{CL_{int}}} \times 100 = \frac{1}{1 + \mathrm{K}_i\,/[\mathrm{I}]} \times 100 \qquad \text{(Equation 3-3)}$$

In addition, the expected increase (AUC ratio) in the AUC or steady-state concentration by an inhibiting constituent is dependent on the route of administration, as this will determine if the drug undergoes first pass in the liver and/or the gut (Ito et al., 1998c). If drugs are administered by i.v. bolus, the AUC ratio ($\frac{\text{AUC}'}{\text{AUC}}$, the ratio of AUC in the presence of inhibitor over that in the absence of inhibitor) can be calculated by Eq. 3-4:

$$\text{AUC ratio} = \frac{AUC'}{AUC} = \frac{CL_{\text{int}}}{CL_{\text{int}}'} = 1 + [\text{I}]/\text{K}_\text{i} \qquad \text{(Equation 3-4)}$$

where CL_{int} is the intrinsic clearance inhibited by the inhibiting constituent; ' represents the value after alteration by herb-drug interaction. Since herbs usually contain multiple inhibitory constituents, an herb-drug interaction *in vivo* is considered likely if the following is true:

$$\text{AUC ratio} = 1 + \sum_{i=1}^{n} \left[[\text{I}_\text{i}]/\text{K}_{\text{i(i)}}\right] \qquad \text{(Equation 3-5)}$$

where $[I_i]$ is the maximal unbound inhibitor concentration of each inhibitory constituent, $K_{i(i)}$, the inhibition constant for each constituent, n, the number of inhibitory constituents in the herb.

Given consideration of the fraction (f_h) of hepatic clearance (CL_h) in total clearance (CL_{tot}), the expected AUC ratio in the AUC or steady-state concentration by an inhibiting constituent can also be calculated by Eq. 3-6:

$$\text{AUCratio} = \frac{AUC'}{AUC} = \frac{\text{C}_\text{ss}'}{\text{C}_\text{ss}} = \frac{\text{CL}_\text{tot}}{\text{CL}_\text{tot}'} = \frac{\text{CL}_\text{h}/\text{f}_\text{h}}{\text{CL}_\text{h}'+\text{CL}_\text{h}/\text{f}_\text{h}-\text{CL}_\text{h}} = \frac{1}{f_h \times CL_h'/CL_h + 1 - f_h} \qquad \text{(Equation 3-6)}$$

where f_h is the fraction of hepatic clearance in total clearance; CL_h is the hepatic clearance; and ' represents the value after alteration by drug interaction.

For high clearance drugs administered by i.v. bolus, CL_h is rate-limited by the flow rate. When the altered CL_h remains rate-limited by the flow rate, then $CL_h = CL_h'$, i.e. AUC ratio = 1, AUC is not altered.

However, for a low clearance drug administered by i.v., it is necessary to consider the fraction (f_m) of the metabolic process subject to inhibition in CL_h. Therefore, the AUC ratio is given by Eq. 3-7.

$$\text{AUC ratio} = \frac{1}{f_h \times f_m \times CL_{int}'/CL_{int} + 1 - f_h \times f_m} \quad \text{(Equation 3-7)}$$

where CL_{int} is the intrinsic clearance inhibited by the inhibiting constituent; ' represents the value after alteration by herb-drug interaction; and f_m is the fraction of the specific metabolic pathway in hepatic clearance.

In the clinical settings, [S] is often much lower than K_m, then AUC ratio is given by the following equation:

$$\text{AUC ratio} = \frac{1}{f_h \times f_m \times \left\{\frac{1}{(1+[I]/K_i)}\right\} + 1 - f_h \times f_m} \quad \text{(Equation 3-8)}$$

Obviously, the AUC ratio is determined by K_i, [I], f_h, and f_m, but not by K_m or [S]. It should be noted that multiple inhibitory herbal constituents are always involved in the inhibition of the same metabolic pathway of a drug, thus AUC ratio is calculated by Eq. 3-9.

$$\text{AUC ratio} = \frac{1}{\sum_{i=1}^{n}\left[f_h \times f_m \times \left\{\frac{1}{(1+[I]/K_i)}\right\} + 1 - f_h \times f_m\right]} \quad \text{(Equation 3-9)}$$

The values of f_h and f_m can be determined from the urinary recovery of the parent molecule and each metabolite. K_i can be estimated by *in vitro* inhibition studies using liver microsomes, hepatocytes and cDNA-expressed microsomes. However, the determination of these parameters is difficult for herbs that often contain multiple components and low plasma levels are reached when administered.

3.3 Predicting metabolic herb-drug interactions based on *in vitro* data

We had examined the effect of a number of herbal components in five human CYPs using a validated high throughput approach. The herbal components tested include a variety of structurally distinct compounds such as triterpenoids, flavonoids, saponine, lactones, alkaloids, and acids. We found that a small number of herbal compounds exhibited remarkable inhibitory effect ($IC_{50} < 1.0$ μM) on CYP1A2, 2C9, 2C19, or 3A4, including γ-schisandrin, tanshinone I, tanshinone IIA, cryptotanshinone, osthole and silybin (Table 3-1).

Following above pharmacokinetic principles, we conducted an exercise to predict pharmacokinetic herb-drug interactions using these *in vitro* results, with a focus on the constituents purified from *Schizandra chinensis* (Wuweizi), *Salvia miltiorrhiza* (Danshen), *Angelica pubescens* (osthole) and *Silybi Mariani* (Shuifeiji).

The expected AUC ratio was mainly dependent on [I], K_i, f_h, number of inhibitory herbal constituents (n) and f_m. As shown in Equation 3-10 , herb-drug interactions would be with low risk if $\sum_{i=1}^{n} \left[[I_i] / K_{i(i)} \right]$ is less than 0.1, medium risk if it is between 0.1-1.0, and high risk if it is greater than 1 (Zhou et al., 2004b). Furthermore, a K_i value of a competitive inhibitor can be estimated by its IC_{50} as it equal to half IC_{50} value (Zhou et al., 2004b). In present study, we hypothesize that all the herbal compounds used here are competitive inhibitors of CYP1A2.

3.4 Results

Table 3-2 shows the predicted risk of pharmacokinetic changes by various herbal medicines. Table 3-3 shows the estimated AUC ratio (based on Eq. 5) with regard to CYP isoform inhibited by individual herbal constituents using *S. chinensis, S. miltiorrhiza, S. Mariani* (milk thistle) and *A. pubescens/Cnidium monnieri* as examples. It appeared that the *S. chinensis* might cause high risk for metabolic interactions with drugs that are primarily metabolised by CYP2C9, 2C19 or 3A4; *S. miltiorrhiza* would cause high risk for metabolic interactions with drugs that are mainly eliminated by CYP1A2 at low blood concentration and might also cause high risk for metabolic interactions with drugs that are metabolised by CYP2C9 or 3A4 at high blood concentration; Sodium tanshinone IIA sulfonate, the artificial derivate of tanshinone II A (*S. miltiorrhiza*), would cause medium to high risk for metabolic interaction with drugs that are primarily metabolized by CYP1A2, 2C9, 2C19, 2D6 or 3A4, whereas *S. Mariani* (milk thistle) might cause medium to high risk for metabolic interaction with drugs that are mainly eliminated by CYP2C9, 2C19 or 3A4. The other five herbal constituents, baicalein, baicalin, quercitrin, quercetin and icariin, would just cause low to moderate risk for metabolic interactions with drugs that are mainly eliminated by these enzymes.

As shown in Table 3-3, the AUC ratio due to herb-drug combination can be estimated using Eq. 3-9. Coadministration of *S. chinensis* was expected to significantly increase the AUC values of warfarin (a CYP2C9 substrate) and most CYP3A4 substrates, such as carbamazepine,

cyclosporine A, indinavir, midazolam and tacrolimus. It was also expected to significantly increase the AUC of omeprazole (a CYP2C19 substrate).

Coadministration of the herb *S. miltiorrhiza* (Danshen) was expected to increase the AUC values of CYP1A2 and 2C9 substrates, such as caffeine and theophylline (both CYP1A2 substrates), and CYP2C9 substrate warfarin, but it would not remarkably change the AUC of CYP3A4 substrates (including carbamazepine, cyclosporine, indinavir, midazolam and tacrolimus). However, sodium tanshinone IIA sulfonate, the artificial derivate of tanshinone IIA (*S. miltiorrhiza*), was expected to remarkably increase the AUC of warfarin (a CYP2C9 substrate) and the AUC of carbamazepine, cyclosporine, indinavir, midazolam and tacrolimus (all CYP3A4 substrates), but it would not significantly change the AUC of CYP1A2 (e.g. theophylline) and 2C19 substrates (e.g .omeprazole).

The *S. Mariani* (milk thistle) was expected to remarkably increase the AUC values of warfarin and most CYP3A4 substrates such as carbamazepine, cyclosporine, indinavir, midazolam and tacrolimus, but it would not remarkably change the AUC of omeprazole (CYP2C19 substrates).

Concurrent use of *A. pubescens* or *C. monnieri* (including osthole) might significantly increase the AUC values of caffeine/theophylline (CYP1A2 substrates), warfarin (CYP2C9 substrate) and omeprazole (CYP2C19 substrate), while it would not remarkably change the AUC of most CYP3A4 substrates such as carbamazepine, cyclosporine A, indinavir, midazolam and tacrolimus.

3.5 Conclusions and Discussion

For low clearance drug by i.v. injection, the AUC ratio was generally determined by inhibition constant (K_i), unbound inhibitor concentration ([I]), hepatic fraction (f_h), number of inhibitory herbal constituents (n) and metabolic pathway fraction in hepatic metabolism (f_m), while the AUC ratio for a high clearance drug by oral route, the AUC ratio was determined by K_i, [I], n and f_m. By varying these parameters, the AUC ratio changed accordingly. It appeared likely to predict an herb-drug metabolic interaction, if the inhibiting herbal constituents could be qualitatively and quantitatively determined. High throughput screening assays provide a useful strategy for the qualitative and quantitative study of herb-CYP interactions. High throughput screening assays are capable of handling the great number of herbal constituents (if using

purified herbal components), thus offer the opportunity to use the resulting *in vitro* inhibition data as a criterion for monitoring herb-drug metabolic interactions involving human drug metabolising enzymes (in particular CYPs) (Masimirembwa CM et al., 2001).

Based on our previous high throughput results, we conducted predictions for *S. chinensis* (γ-schisandrin and schisandrin), *S. miltiorrhiza* (tanshinone I, tanshinone IIA, and cryptotanshinone), sodium tanshinone IIA sulfonate, *S. Mariani* (silybin A and B) and *A. pubescens/Cnidium monnieri* (osthole). Some predicting results were consisting with clinical reports. For example, it was expected that *S. chinensis* (γ-schisandrin and schisandrin) would increase the AUC value of tacrolimus, which is consistent with the report (Xin et al., 2007) where *Schisandra sphenanthera* extracts increase the oral bioavailability of tacrolimus. Furthermore, the prediction of *S. miltiorrhiza* increasing the AUC value of warfarin (CYP2C9 substrate) is consistent with the report by Chan (2001). It is also in agreement with the case reports that *S. miltiorrhiza* products increased 2-fold in prothrombin time of warfarin and induced over-anticoagulation in patients.

However, some predictions were opposite or different to the clinical studies. For example, clinical studies of *S. miltiorrhiza* interaction with caffeine/theophylline (CYP1A2 substrates) showed a different picture. An early study reported that compound danshen tablets (mainly contained *S. miltiorrhiza*) increased the metabolism of caffeine in healthy subjects, implicating that compound danshen tablets induced the activity of CYP1A2. However, a recent study by Qiu *et al*. (2008a) reported that *S. miltiorrhiza* extracts did not influence the metabolism of theophylline in healthy volunteers. The *in vitro* study showed that human CYP1A2 is inhibited by the ethyl acetate extract of danshen and danshen products (Ueng et al., 2003; Qiu et al., 2008b). Another example is indinavir (a CYP3A4 substrate), whose plasma concentration was not altered by co-administered silymarin (containing silybin) in healthy volunteers (DiCenzo et al., 2003). However, the prediction was that *S. Mariani* would increase the AUC of indinavir.

These findings reflect the difficulties and complexity when predicting herb-drug interactions. The reasons are as following: a) using above pharmacokinetic principles plus *in vitro* data to predict herb-drug interactions *in vivo* can only be used for the herbs with inhibitory effects. If herbal compounds act as inductors *in vivo*, like St John Wort, current predicting procedure is not proper. b) herbal preparations may contain multiple CYP-modulating constituents, with unknown amounts and inhibition/induction potency for CYPs. Therefore the total effects *in*

vivo are balance results of individual effects of the multiple constituents; c) the inhibitor/induction of CYP by herbs may by temporally distinguishable, depending on the herb's dosing, administration route and tissues; d) marked variability in the contents of herbal constituents (Bergonzi et al., 2001); e) presence of extra-hepatic metabolism; and active transport in liver; and f) many herbs are used chronically.

In conclusion, the prediction of metabolic herb-drug interactions based on *in vitro* inhibition data involving human drug metabolising enzymes (in particular CYPs) is possible, but the prediction is uncertain and complex when multiple factors are involved.

Table 3-1. The IC_{50} of potential herbal inhibitors.

Herbal compound	**IC_{50} (μM)**				
	CYP1A2	**CYP2C9**	**CYP2D6**	**CYP3A4**	**CYP2C19**
γ-Schisandrin	-	0.52	16.97	0.009	0.07
Schisandrin	-	85.20	-	19.40	36.81
Tanshinone I	0.027	0.11	-	0.22	21.09
Tanshinone IIA	0.19	0.21	-	-	-
Cryptotanshinone	0.91	1.23	-	2.96	13.65
Sodium tanshinone IIA sulfonate	7.08	1.36	11.55	1.78	19.44
Sodium Danshensu	-	73.12	-	-	-
Salvianolic acid B	-	-	-	-	-
Protocatechuicaldehyde	-	90.66	-	81.19	25.70
Protocatechuic Acid	-	-	-	-	-
Osthole	1.49	8.30	51.37	12.01	0.92
Silybin	-	3.14	-	2.85	20.26
Baicalein	1.22	2.52	36.78	1.24	2.12
Baicalin	70.03	20.42	-	-	46.11
Quercetin	3.97	3.01	54.59	19.80	7.23
Quercitrin	33.76	21.76	90.00	71.01	98.77
Icariin	43.00	14.34	-	-	72.17

Table 3-2. Prediction for the risk of herb-drug interaction.

		[I] μM	K_i μM	[I]/K_i	Estimated AUC ratio (R)	Risk of herb-drug interaction
***Schisandra chinensis* (Wuweizi)**						
CYP1A2	γ-Schisandrin	-	-	-	-	NA
	Schisandrin	-	-	-		
CYP2C9	γ-Schisandrin	0.689	0.26	2.65	3.75	High
	Schisandrin	4.31	42.6	0.101		
CYP2C19	γ-Schisandrin	0.689	0.036	19.15	20.38	High
	Schisandrin	4.31	18.41	0.234		
CYP2D6	γ-Schisandrin	0.689	8.49	0.081	1.08	Low
	Schisandrin	4.31	-	-		
CYP3A4	γ-Schisandrin	0.689	0.0045	153.16	154.61	High
	Schisandrin	4.31	9.70	0.445		
***Salvia miltiorrhiza* (Danshen)**		Low Con.				
CYP1A2	Tanshinone I	0.006	0.014	0.437		
	Tanshinone IIA	0.009	0.094	0.101	2.0	High
	Cryptotanshinone	0.209	0.455	0.460		
CYP2C9	Tanshinone I	0.006	0.053	0.111		
	Tanshinone IIA	0.009	0.105	0.090	1.56	Medium
	Cryptotanshinone	0.209	0.615	0.340		
CYP2C19	Tanshinone I	0.006	10.55	0.0006		
	Tanshinone IIA	0.009	-	-	1.03	Medium
	Cryptotanshinone	0.209	6.83	0.031		
CYP2D6	Tanshinone I	0.006	-	-		
	Tanshinone IIA	0.009	-	-	-	NA
	Cryptotanshinone	0.209	-	-		
CYP3A4	Tanshinone I	0.006	0.11	0.054		
	Tanshinone IIA	0.009	-	-	1.19	Medium
	Cryptotanshinone	0.209	1.48	0.141		
***Salvia miltiorrhiza* (Danshen)**		High Con.				
CYP1A2	Tanshinone IIA	5.44*	0.094	58.14	59.14	High
	Cryptotanshinone	2.09	0.455	4.60	5.60	High
CYP2C9	Tanshinone IIA	5.44*	0.105	52.02	53.02	High
	Cryptotanshinone	2.09	0.615	3.40	4.40	High
CYP2C19	Tanshinone IIA	5.44*	-	-	-	NA
	Cryptotanshinone	2.09	6.83	0.307	1.31	Medium
CYP2D6	Tanshinone IIA	5.44*	-	-	-	NA
	Cryptotanshinone	2.09	-	-	-	NA
CYP3A4	Tanshinone IIA	5.44*	-	-	-	NA
	Cryptotanshinone	2.09	1.48	1.414	2.41	High
Sodium tanshinone IIA sulfonate						
CYP1A2	Sodium tanshinone IIA sulfonate	1.26	3.54	0.357	1.36	Medium
CYP2C9		1.26	0.679	1.86	2.86	High
CYP2C19		1.26	9.72	0.130	1.13	Medium
CYP2D6		1.26	5.78	0.219	1.22	Medium
CYP3A4		1.26	0.890	1.42	2.42	High

Angelica pubescens / Cnidium monnieri **(Duhuo / Shechuangzi)**						
CYP1A2	Osthole	2.75	0.75	3.69	4.69	High
CYP2C9		2.75	4.15	0.66	1.66	Medium
CYP2C19		2.75	0.46	5.98	6.98	High
CYP2D6		2.75	25.69	0.107	1.11	Medium
CYP3A4		2.75	6.01	0.458	1.46	Medium
Silybi Mariani **(Shuifengji, milk thistle)**						
CYP1A2	Silybin A	4.84	-	-	-	NA
	Silybin B	1.21	-	-		
CYP2C9	Silybin A	4.84	1.57	3.08	4.85	High
	Silybin B	1.21	1.57	0.768		
CYP2C19	Silybin A	4.84	10.13	0.478	1.60	Medium
	Silybin B	1.21	10.13	0.119		
CYP2D6	Silybin A	4.84	-	-	-	NA
	Silybin B	1.21	-	-		
CYP3A4	Silybin A	4.84	1.43	3.40	5.24	High
	Silybin B	1.21	1.43	0.847		
Scutellaria baicalensis **(Huangqin)**						
CYP1A2	Baicalein	0.207	0.61	0.340	1.34	Medium
CYP2C9		0.207	1.26	0.164	1.16	Medium
CYP2C19		0.207	1.06	0.196	1.20	Medium
CYP2D6		0.207	18.39	0.011	1.01	Low
CYP3A4		0.207	0.620	0.334	1.33	Medium
Scutellaria baicalensis /Lonicera japonica **(Huangqin/Jinyinhua)**						
CYP1A2	Baicalin	0.067	35.02	0.002	1.002	Low
CYP2C9		0.067	10.21	0.007	1.007	Low
CYP2C19		0.067	23.06	0.003	1.003	Low
CYP2D6		0.067	-	-	-	NA
CYP3A4		0.067	-	-	-	NA
Hypericum japonicum / Viscum coloratum **(Diercao/Hujisheng)**						
CYP1A2	Quercitrin	8.19	16.88	0.49	1.49	Medium
CYP2C9		8.19	10.88	0.75	1.75	Medium
CYP2C19		8.19	49.39	0.166	1.17	Medium
CYP2D6		8.19	45	0.182	1.18	Medium
CYP3A4		8.19	35.51	0.231	1.23	Medium
Ginkgo biloba / Bupleurum chinensis **(Yinxinye/Caihu)**						
CYP1A2	Quercetin	0.051*	1.99	0.026	1.03	Low
CYP2C9		0.051*	1.51	0.034	1.03	Low
CYP2C19		0.051*	3.62	0.014	1.01	Low
CYP2D6		0.051*	27.30	0.002	1.002	Low
CYP3A4		0.051*	9.90	0.005	1.005	Low
Epimedium brevicornum **(Yinyanghe)**						
CYP1A2	Icariin	0.151	21.50	0.007	1.007	Low
CYP2C9		0.151	7.17	0.021	1.02	Low
CYP2C19		0.151	36.09	0.004	1.004	Low
CYP2D6		0.151	-	-	-	NA
CYP3A4		0.151	-	-	-	NA

Table 3-3. Prediction of AUC ratio.

Major CYP		$\sum_{i=1}^{n} [I_i]/K_{i(i)}$	f_h	f_m	Estimated AUC ratio	Observed AUC ratio	Ref.
***Schisandra chinensis* (γ-schisandrin + schisandrin)**							
CYP2C9	Warfarin	2.75	1	0.85	2.66		
CYP2C19	Omeprazole	19.38	0.75	0.56	1.66		
CYP3A4	Carbamazepine	153.61	0.8	0.65	2.07		
CYP3A4	Cyclosporine A	153.61	0.94	0.76	3.45		
CYP3A4	Indinavir	153.61	0.85	0.7	2.45		
CYP3A4	Midazolam	153.61	0.88	0.75	2.90		
CYP3A4	Tacrolimus	153.61	0.86	0.7	2.49	1.64	(Xin et al., 2007)
***Salvia miltiorrhiza* (tanshinone I + tanshinone IIA + cryptotanshinone)**							
CYP1A2	Caffeine	0.998	0.95	0.79	1.60		
CYP1A2	Theophylline	0.998	0.84	0.69	1.41		
CYP2C9	Warfarin	0.559	1	0.85	1.44	Increased	(Chan, 2001)
CYP2C19	Omeprazole	0.031	0.75	0.56	1.01		
CYP3A4	Carbamazepine	0.195	0.8	0.65	1.09		
CYP3A4	Cyclosporine A	0.195	0.94	0.76	1.13		
CYP3A4	Indinavir	0.195	0.85	0.7	1.11		
CYP3A4	Midazolam	0.195	0.88	0.75	1.12		
CYP3A4	Tacrolimus	0.195	0.86	0.7	1.11		
Sodium tanshinone IIA sulfonate							
CYP1A2	Caffeine	0.357	0.95	0.79	1.25		
CYP1A2	Theophylline	0.357	0.84	0.69	1.18		
CYP2C9	Warfarin	1.86	1	0.85	2.24		
CYP2C19	Omeprazole	0.130	0.75	0.56	1.05		
CYP3A4	Carbamazepine	1.42	0.8	0.65	1.44		
CYP3A4	Cyclosporine A	1.42	0.94	0.76	1.72		
CYP3A4	Indinavir	1.42	0.85	0.7	1.54		
CYP3A4	Midazolam	1.42	0.88	0.75	1.63		
CYP3A4	Tacrolimus	1.42	0.86	0.7	1.55		
***Silybi Mariani* (silybin A + silybin B)**							
CYP2C9	Warfarin	3.85	1	0.85	3.07		
CYP2C19	Omeprazole	0.597	0.75	0.56	1.19		
CYP3A4	Carbamazepine	4.24	0.8	0.65	1.73		
CYP3A4	Cyclosporine A	4.24	0.94	0.76	2.37		
CYP3A4	Indinavir	4.24	0.85	0.7	1.93	0.91	(Piscitelli et al., 2002)
CYP3A4	Midazolam	4.24	0.88	0.75	2.15		
CYP3A4	Tacrolimus	4.24	0.86	0.7	1.95		
***Angelica pubescens/Cnidium monnieri* (Osthole)**							
CYP1A2	Caffeine	3.69	0.95	0.79	2.44		
CYP1A2	Theophylline	3.69	0.84	0.69	1.84		
CYP2C9	Warfarin	0.663	1	0.85	1.51		
CYP2C19	Omeprazole	5.98	0.75	0.56	1.56		
CYP3A4	Carbamazepine	0.458	0.8	0.65	1.20		
CYP3A4	Cyclosporine A	0.458	0.94	0.76	1.29		
CYP3A4	Indinavir	0.458	0.85	0.7	1.23		
CYP3A4	Midazolam	0.458	0.88	0.75	1.26		
CYP3A4	Tacrolimus	0.458	0.86	0.7	1.23		

CHAPTER 4 A COMPUTERIZED MODELING STUDY FOR THE INTERACTION OF LIGANDS WITH HUMAN CYP1A2 ENZYME

4.1 Introduction

In the superfamily of the human CYP enzymes, family 1 contains three well characterized monooxygenases, namely CYP1A1, 1A2 and 1B1. These CYPs participates in over 10% of all Phase 1 oxidative reactions. Among them, CYP1A2 is the most important one for the oxidative metabolism of exogenous compounds in human liver, including a variety of procarcinogens such as PAHs and therapeutic drugs (Brosen, 1995; Eaton et al., 1995; Hammons et al., 1997; Rendic and Di Carlo, 1997). CYP1A1 is not expressed in the liver, but inducible by smoking and some compounds. CYP1A2 is one of the enzymes responsible for activating aromatic heterocyclic amines and PAHs to highly reactive metabolites that crosslink DNA and ultimately cause carcinogenesis (Eaton et al., 1995; Guengerich et al., 1999; Zhou et al., 2005a). The activation of procarcinogens such as heterocyclic amines and PAHs by CYP1A2 makes this enzyme particularly important in carcinogenesis. The amino radical ($-NH_2$) in these amines, rich in cooked meat and fish, is converted by CYP1A2 into a hydroxyamino group (*N*-OH-) which is further activated to form esters that ultimately produce DNA adducts (Yamashita et al., 1988). In general, aromatic amines are bioactivated in two steps, *N*-oxidation by CYP1A2, followed by a conjugation (usually acetylation or sulphonation) (Yamashita et al., 1988). These conjugation reactions introduce good leaving groups, resulting in a highly reactive resonance-stabilized nitrenium/carbonium ion (Yamashita et al., 1988). Therefore, induction of CYP1A2 enzyme may enhance individual susceptibility to carcinogenesis, whereas inhibition of the CYP1A2 enzyme might have important implications for cancer chemoprevention. In fact, some natural compounds with potent inhibitory effect on CYP1A2 have been shown to reduce chemical-induced carcinogenesis in preclinical studies (Zhou et al., 2005a). On the other hand, since CYP1A2 is involved in the metabolic clearance of a number of clinical drugs such as theophylline, tacrine and propranolol, inhibition or induction of CYP1A2 activity is associated with a number of pharmacokinetic drug interactions when drugs are administered concomitantly (Zhou et al., 2009). The drug interactions are more clinically important when the victim drug has a narrow therapeutic index (e.g. theophylline).

The crystal structure of human CYP1A2 (Sansen et al., 2007) has been recently solved, which provides us a firm basis for further investigation of the mechanism of ligand-CYP1A2 interaction at molecular level. A narrow, planar ligand binding cavity in the active site of

CYP1A2 is observed in the structure (PDB ID: 2HI4, see Figure 1-4), which is consistent with the fact that most of the CYP1A2 substrates and inhibitors are planar, small molecules with high log P values (highly lipophilic) (Korhonen et al., 2005; Zhou et al., 2009). CYP1A2 contributes significantly to the hepatic metabolism of many hydrophobic drugs such as amitriptyline, haloperidol, olanzapine, tacrine, theophylline, zileuton, and zolmitriptan, as well as its probe substrate caffeine (Zhou et al., 2009).

The known inhibitors of human CYP1A2 include amiodarone, ciprofloxacin, cimetidine, fluvoxamine, furafylline, mibefradil, ANF, propafenone, rofecoxib and rofecoxib (Zhou et al., 2009). Fluvoxamine, for instance, a selective serotonin reuptake inhibitor, is a potent mechanism-based inhibitor of CYP1A2 and has been shown to significantly increase the plasma levels of tizanidine (a substrate of CYP1A2), leading to tizanidine intoxication when coadministered (Granfors et al., 2004b). Drugs behaving as potent mechanism-based inhibitors of CYP1A2 may explain some drug-drug interactions observed in clinical practice. For example, zileuton is a mechanism-based inhibitor of CYP1A2 (Lu et al., 2003) and this may explain why it decreased the oral clearance of antipyrine (St Peter et al., 1995), propranolol (Lau, 1997), *R*-warfarin (Awni et al., 1995e), and theophylline (Granneman et al., 1995), at doses that have a minimal effect on the pharmacokinetics of *S*-warfarin (Awni et al., 1995e), phenytoin (Samara et al., 1995), digoxin (Awni et al., 1995d), naproxen (Awni et al., 1995a), prednisone (Awni et al., 1995c), sulfasalazine (Awni et al., 1995b), and terfenadine (Awni et al., 1997). Rofecoxib moderately increases the plasma level and effects of theophylline (Bachmann et al., 2003) and the *R*-warfarin (Schwartz et al., 2000). Like fluvoxamine, rofecoxib at therapeutic doses of 25 mg per day increased more than 10-fold the plasma concentrations and adverse effects of the CYP1A2 substrate tizanidine in humans (Backman et al., 2006b).

Previous studies have established several pharmacophore models to explore the interaction of ligands with CYP1A2 (Lozano et al., 1997; Lewis et al., 2003). However, these models have intrinsic limitations as they are all based on homology models arising from bacterial and rabbit CYP structures. The structural information obtained from these models is limited. This has prompted us to conduct docking studies for known CYP1A2 substrates and inhibitors, and then established pharmacophore models using a set of known CYP1A2 inhibitors. We then validated the models with a set of other known CYP1A2 inhibitors and compared with our *in vitro* inhibitory data (see **Section 2.3** of **Chapter 2**).

4.2 Modelling Methods

The AutoDock program and the Ligplot program were used to establish models for CYP1A2 and its ligands including substrates and inhibitors. The HipHop module in Catalyst (Accelrys, Inc., installed in Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China) was used to generate pharmacophore hypotheses with qualitative common features utilizing a series of ligands.

4.2.1 Docking study

The binding mode of 25 known substrates of CYP1A2 with diverse structures in the active site of CYP1A2 was estimated by docking simulation using the AutoDock 4.0 program. These substrates included acetaminophen, amitriptyline, caffeine, estradiol, tacrine, naproxen, fluvoxamine, phenacetin, tizanidine and zileuton (Table 4-1). The AutoDock program can calculate the binding energy of a ligand when it binds to a protein and, in present study, was used to determine the energy when a ligand was docked into the active site of CYP1A2.

Ligands were added polar hydrogens; Gasteiger charges computed; non-polar hydrogens merged; and energy minimization was performed as entries before docking as described previously (Paxton et al., 2005). Docking was carried out in a standard grid-based mode within the active site of CYP1A2 structure, derived from 2HI4 (PDB) where water and ligand (α-naphthoflavone) were removed. Default values for van der Waals scaling, electrostatics, and ligand minimization were used. A modified genetic search algorithm employing a local minimum refinement was used to identify low energy binding sites and orientations of the probe molecule. A grid of 54 × 54 × 54-point with a spacing of 0.375 Å centred at 2.674 × 18.041 × 19.672 Å that fully encompassed the active site was employed. The top ten scoring conformations of each ligand were saved. The other parameters were set as follows: number of genetic algorithm evals, 700,000; number of genetic algorithm popular size, 120; number of genetic algorithm runs, 10; maximum number of generations, 30,000; maximum number of top individuals, 1; rate of gene mutation, 0.02; rate of crossover, 0.8; GA crossover mode, twopt. Only the substrate/inhibitor and amino acid residues within 4.5 Å were allowed during the determination. The protein was frozen when the docking was performed automatically.

4.2.2 Ligplot study

The Ligplot program was used to analyse the docking results of ligands at the active site of CYP1A2 by generating the schematic diagrams for the protein–ligand interactions (Wallace et al., 1995). These diagrams indicate which residue atoms in the CYP1A2 protein interact with which ligand atoms. The atom-atom interaction carried out by the Ligplot program was presented as hydrogen bond (O-H) and hydrophobic (C-C) interactions. After obtaining the diagrams from substrate and inhibitor analysis, the binding residues involved in these interactions were selected.

4.2.3 Pharmacophore hypotheses generation for CYP1A2 inhibitors

The HipHop module in Catalyst was routinely used to generate pharmacophore hypotheses with qualitative common features utilizing a series of ligands. To identify the common features of CYP1A2 inhibitors, we employed a series of ligands with distinct core structures to generate a pharmacophore model.

The training set of ligands (n = 5) included fluvoxamine, galangin, miconazole, α-naphthoflavone (used as initial template), and rutaecarpine which are all CYP1A2 inhibitors (Fig. 4-1). To identify pharmacophore features necessary for potent CYP1A2 inhibitors, the qualitative HipHop model was generated based on these five compounds in training set. Besides to study common features of CYP1A2 inhibitors, we also explored the common features shared by substrates and inhibitors of CYP1A2, on the assumption that the ligands with high affinity bind in a similar manner at the enzyme active site. Therefore, we used CYP1A2 inhibitors as the training set and utilized both substrates and inhibitors of CYP1A2 as validating sets.

The validating set of ligands encompassed 9 well-known CYP1A2 inhibitors, namely, amiodarone, cimetidine, ciprofloxacin, enoxacin, furafylline, methoxsalen, mibefradil, propafenone and rofecoxib. We further examined the usefulness of the established pharmacophore models employing a series of ligands, including 56 herbal compounds and 18 well-known CYP1A2 substrates.

4.2.4 Training set selection and conformational analysis

All structural models were built and minimized within the Catalyst (Accelrys Inc.). Before starting the pharmacophore generation process, conformational models for the molecules were

calculated using the best conformer generation method. When the lowest energy was more than 20 kcal/mol, the conformers were excluded. The poling algorithm was used, which sought to provide a broad coverage of conformational space. The number of conformers generated for each compound was limited to a maximum number of 255 which was set as a default value.

4.2.5 Generation of pharmacophore models

In the HipHop model generation process using above five CYP1A2 inhibitors, the highest weight was assigned to the most active ligand with the highest binding affinity, α-naphthoflavone, in the training set. α-Naphthoflavone was considered as a 'reference compound' specifying a 'principal' value of 2 and a 'MaxOmitFeat' value of 0. A 'principle' value of 2 ensures that all of the chemical features in the ligand will be considered in building hypothesis space, while a 0 of 'MaxOmitFeat' value forces mapping of all features of the ligand. For the remaining four inhibitors, the 'principle' value was set at 1 and 'MaxOmitFeat' value at 1 since these ligands show lower binding affinity compared to α-naphthoflavone. Maximum pharmacophore hypotheses were set to 10 and minimum interfeature distance to 2, while all other parameters were set at default values.

4.3 Results

4.3.1 AutoDock study of known CYP1A2 substrates

Most substrates of CYP1A2 are known as small, planar, hydrophobic, and either weakly basic or neutral molecules (Sansen et al., 2007). For perspective of substrate-CYP1A2 interaction at molecular level, an AutoDock program was used to dock the ligands into the active site of the ligand-free CYP1A2 crystal structure (PDB ID: 2HI4) (Sansen et al., 2007). Our docking experiments resulted in a maximum of 10 docking poses that needed to be analyzed manually. To reasonably analyze the docking results of CYP1A2 substrates, the known metabolic pathway of each substrate catalyzed by CYP1A2 was employed to select their unique conformation based on the fact that only such unique poses produce metabolites (see Figure 4-2).

After docking, a total of 77 conformers had been generated from 25 known substrates for CYP1A2 (Figure 4-3), with 3.1 conformers per substrate molecule. According to the known metabolic pathway of these substrates, a unique pose of each substrate was identified from their multi-conformations, resulting in unique poses for 18 substrates only (Figure 4-2). For the remaining 7 substrates, their unique poses could not be identified even when we conducted

further docking using their conformers at the lowest energy. These compounds included acetaminophen, clozapine, estradiol, mexiletine, olanzapine, riluzole, and theophylline. It is unknown why these known substrates of CYP1A2 could not give rise to unique poses. Therefore, a total of 18 poses for the 18 substrates were used for further Ligplot analysis.

4.3.2 Ligplot study for substrate-CYP1A2 interaction

The Ligplot program was used to analyze the docking results of ligands at the active site of CYP1A2. Substrate-CYP1A2 interaction occurred in the active site of CYP1A2 between the atoms on substrates and atoms in residues of CYP1A2, including hydrogen bonds and hydrophobic contacts. This program can identify specific atom-atom interactions including both hydrogen bond (O-H) and hydrophobic (C-C) interactions between the ligand and protein. Therefore, the interactions between the atoms on 18 substrates of CYP1A2 with 18 identified unique conformers resulting in metabolite production and the atoms in residues at the active site of CYP1A2 were analyzed using the Ligplot algorithm. Since the unique conformers of other seven substrates had not been successfully gained, they were not included in the Ligplot study.

As for hydrogen bond (O-H) interactions, a total of 11 hydrogen bonds, including 5 hydrogen bond donors (HBDs) and 6 hydrogen bond acceptors (HBAs) on CYP1A2 residues were found from the interactions between the 18 conformers and CYP1A2. Three residues, Ala317, Thr124 and Thr118 of the CYP1A2 were identified as hydrogen donors, while other four residues (Asn257, Asn312, Asp320 and Thr124) were found as hydrogen acceptors. As hydrogen donors, residues Ala317 and Thr124 were involved in the generation of two HBDs for each, while Thr118 formed one HBD only. For hydrogen acceptors, Asn312 and Asp320 were involved in the formation of two HBAs for each, and Asn257 and Thr124 generated one HBA for each (see Table 4-1).

With regard to hydrophobic (C-C) interactions, a total of 21 residues at the active site of CYP1A2 were identified to participate in the hydrophobic interactions between the substrates and the enzyme. These residues included Ala317, Asn312, Asp313, Asp320, Gly316, Ile117, Ile386, Leu382, Leu497, Phe125, Phe226, Phe256, Phe260, Ser122, Thr118, Thr124, Thr223, Thr321, Thr498 and Val227 (Table 4-2). Hem900 was also involved in the substrate-enzyme interactions and thus was included in the Ligplot study.

As expected, the residue Phe226 interacted with most CYP1A2 substrates tested in this study. According to the sum of interacting C-C pairs on each residue with 18 conformers, it was identified that interactive C-C pairs of Phe226 were over 150 (Group 1). Ala317, Hem900, Gly316, Phe125, Phe260 and Thr124 were between 50 and 100 C-C pairs (Group 2); Asp313, Asp320 and Leu497 were between 25 and 50 C-C pairs (Group 3); and the remaining residues only had less than 25 C-C pairs (Group 4, please see Table 4-2). Obviously, Phe226 was the most essential residue for binding interaction between the 18 substrates and CYP1A2, followed by the 6 residues (with 50~100 C-C pairs) acting as the second essential group and then the third group of residues (with 25~50 C-C pairs). On average, each substrate interacted with the enzyme via 3.0 C-C pairs.

Among the 21 residues, 13 of which were involved in the interaction of more than half of the 18 substrates with CYP1A2. These residues included Ala317 (18 substrates), Phe226 (18), Hem900 (17), Gly316 (16), Phe125 (16), Thr124 (15), Ile386 (14), Leu497 (14), Asp313 (12), Phe260 (12), Thr321 (12), Thr118 (10), and Asp320 (10) (see Table 4-2 and Figure 4-4). There were six residues including Phe226, Ala317, Gly316, Phe125, Thr124 and Hem900 that participated in the interactions of 15-18 substrates with CYP1A2, indicting their essential role in the substrate-CYP1A2 binding.

Phe226 in helix F, which has been found to interact with all 18 substrate tested in our study, appears to play a critical role in substrate recognition, acting as a sensor for aromatic hydrocarbon substrates. Another Phe, Phe260 in helix G, was located on the other side of Phe226, forming an aromatic platform together with Phe226 which can accommodate aromatic hydrocarbons (Figure 4-4). Since the distance between the two benzol rings of Phe226 and Phe260 is slightly different, it may facilitate recognition of some substrates containing polycyclic aromatic hydrocarbons. SDM studies have confirmed the important role of Phe226 in substrate recognition (Parikh et al., 1999)

The active site of CYP1A2 contains three important residues, Asp313, Ala317, and Thr321 positioning side by side in helix I above the heme. These three residues are well reserved in other CYPs which are also found in CYP2C9 (PDB ID: 1R9O) and 2D6 (PDB ID: 2F9Q) with identical positioning (see Figure 4-5). These three residues together with the heme constitute a conservative core of the active site for these enzymes. Furthermore, in CYP1A2, 2C9 and 2C19 (a homology model), a Gly is conserved sequentially before the Ala, while a Ser instead

of Gly was found neighboring the Ala in CYP2D6. This conserved structure at active sites could explain why CYP1A2 and 2C9 share certain substrates (e.g. amitriptyline and naproxen) and inhibitor (fluvoxamine). However, diverse residue constitution and 3rd-grade structure beyond the active core makes most CYP1A2 substrates different from those of CYP2C9.

Overall, there are six hydrophobic residues (Phe226, Ala317, Gly316, Phe125, Thr124 and Hem900) and two acidic residues (Asp313 and Asp320 on helix I located besides Ala317) forming the core of CYP1A2 active site cavity. These residues play a critical role in substrate recognition. At position Phe125, there is a natural mutation (373T>A) identified in humans.

4.3.3 Pharmacophore study for CYP1A2 inhibitors

Previous pharmacophore studies of CYP1A2 inhibitors have demonstrated that typical CYP1A2 inhibitors are aromatic, lipophilic, neutral, and acidic compounds (Lewis et al., 2004; Gleeson et al., 2007). However, these studies have only provided limited structural information for CYP1A2 inhibitors. To further explore the common configuration features of CYP1A2 inhibitors, we conduct pharmacophore modelling studies using Catalyst. The models were further validated using a series of well-known CYP1A2 inhibitors using Ligand Pharmacophore Mapping module of the Catalyst.

For the first step, we have set up the preliminary pharmacophores representing the common chemical features such as HBA, HBD, and hydrophobic area. The best pharmacophore (Hopyo-1) based on the five typical CYP1A2 inhibitors (i.e. fluvoxamine, galangin, miconazole, naphthoflavone-template, and rutaecarpine) indicated that two hydrophobic areas, one aromatic ring and one HBA were common (see Figure 4-6).

We further validated these pharmacophores derived from five known CYP1A2 inhibitors, using another 9 well-known CYP1A2 inhibitors (see Figure 4-7) through Ligand Pharmacophore Mapping program. During validation, the four essential features including two distinct hydrophobic areas, one aromatic ring and one HBA were all tested. The mapping result showed that 8 out of 9 inhibitors (88.9%) were hit and 6 of them (67%) showed a good agreement (Fit value > 2.77/4) with the four features, one with reasonable agreement (Fit value ~2.4/4), and only one with a poor Fit value (1.8/4) (see Table 4-3).

Furthermore, we conducted a small-scale screening of 56 herbal compounds based on the validated pharmacophores. Except four compounds without optimized conformations, the Hopyo-1 hit 21 of remaining 52 herbal compounds and 19 of the 21 hits have Fit value over 2.7/4, including 9 potential inhibitors shown in our *in vitro* studies (Table 2-2). If one hydrophobic region was excluded from the pharmacophore model, 26 of the 52 herbal compounds could be hit including 12 potential inhibitors observed in our *in vitro* studies (Table 4-4).

Moreover, we mapped the Hopyo-1 to 25 known CYP1A2 substrates to check how many of the common features were shared between inhibitors and substrates of CYP1A2. It hit 13 substrates with 8 of them showing good agreement (Fit value > 2.9/4) and 5 exhibiting poor agreement (Fit value < 2.3/4) (Table 4-3). The 8 substrates showing good agreement with pharmacophore features arising from CYP1A2 inhibitors included fluvoxamine, haloperidol, olanzapine, ondansetron, ropivacaine, tizanidine, verapamil and *R*-warfarin. Four more substrates (baicalein, hyperoside, polydatin and quercetin) were hit if one hydrophobic region of the four common features was omitted. These results implicate that there are certain common structural features between substrates and inhibitors of CYP1A2. However, some CYP1A2 inhibitors may not be as hydrophobic as most CYP1A2 substrates.

4.3.4 AutoDock study for herbal components

Following pharmacophore screening of 56 purified herbal compounds, we conducted a series docking of the 56 herbal compounds into the active site of CYP1A2 using AutoDock 4.0 program. After docking, a total of 180 conformers had been generated from the 56 herbal compounds, with an average of 3.2 conformers per compound. Only the optimal pose of each compound with the lowest free energy of binding were used for docking analysis.

These dockings yielded values of estimated free energy of binding ranging from -11.09 to +2,870 kcal/mol (note that the more negative the value is, the tighter the predicted binding is). Thirty seven of 56 herbal compounds showed high values of binding energy ranging from -6.51 to +2,870 kcal/mol and were predicted to be poor inhibitors of CYP1A2. In contrast, other remaining 19 herbal compounds had relatively low binding energy ranging from -11.09 to -6.73 kcal/mol and were estimated to be potential inhibitors of CYP1A2. These 19 compounds included amygdalin, andrographolide, baicalein, baicalin, cordycepin, cryptotanshinone, dehydroandrographolide, matrine, osthole, oxymatrine, polydatin, quercetin,

quercitrin, rutaecarpine, scopoletin, sophoridine, tanshinone I, tanshinone IIA, and tanshinone IIA sulfonate sodium.

If these results were combined with those from the pharmacophore studies, 8 of the 19 predicted CYP1A2 inhibitors were hit by the hypothesis (Hopyo-1) (see Table 4-4), and 11 of the 19 predicted inhibitors could be hit if one hydrophobic region was omitted from the hypothesis 1 (Hopyo-1m) (Table 4-4). Indeed, 8 of the 11 predicted inhibitors which were also hit the Hopyo-1 were found to be moderate to potent CYP1A2 inhibitors in our *in vitro* studies. There were other three weak inhibitors of herbal compounds (alloin, hyperoside and icariin) hit by the Hopyo-1 but scored with high values of binding energy (-5.64, -6.10 and +50.81 kcal/mol, respectively). In fact, these three compounds were weak to moderate inhibitors of CYP1A2 with IC_{50} of 66.00, 14.46 and 43.00 μM, respectively.

Our bench work identified 14 of the 56 herbal compounds as inhibitors *in vitro* and 13 of the 14 potential inhibitors (92.9%) were successfully predicted by pharmacophore model in combination with the data from docking results. Only cordycepin, as an exception, was not included in the correct prediction list because it failed to form proper conformation to map the Hopyo-1. However, cordycepin had a low estimated binding energy and indeed it was a moderate inhibitor of CYP1A2 *in vitro*.

4.3.5 Ligplot study for herb-CYP1A2 interaction

Using the Ligplot program, we further performed analysis of ligand-enzyme binding studies with the 19 herbal compounds showing a low binding energy (see Table 4-5). We counted the C-C pair number interacting between each of the 19 compounds and the 6 essential residues of CYP1A2 (Phe226, Ala317, Gly316, Phe125, Thr124 and Hem900). Rutaecarpine and quercitrin had the most and same C-C pairs (42) with the 6 residues, followed by andrographolide (40), dehydroandrographolide (40), baicalin (36), polydatin (36), tanshinone IIA sulfonate sodium (36), tanshinone I (31), oxymatrine (30), sophoridine (29), cryptotanshinone (28), osthole (26), amygdalin (25), tanshinone IIA (25), matrine (24), cordycepin (21), baicalein (15), quercetin (15) and scopoletin (12) (Table 4-5).

Since molecules with M_r >310 Dal are often complicated with steric and electrochemical characteristics that make the compound difficult to enter the active site of CYP1A2, we analysed the impact of molecular weight on the atom-atom pairing. There are 7 of the 19

molecules with M_r >310 Dal, i.e. alloin, baicalin, hyperoside, icariin, quercitrin, triptolide and tanshinone IIA sulfonate sodium. It was found that the 7 relatively large molecules had more C-C pairs than other 13 ones with M_r < 310, except rutaecarpine. However, only 3 of the 7 compounds had shown weak to moderate inhibition on CYP1A2 in our *in vitro* study, i.e. tanshinone IIA sulfonate sodium (IC_{50}, 7.1 μM), quercitrin (IC_{50}, 33.8 μM) and hyperoside (IC_{50}, 14.5 μM). Among the relatively small 13 compounds, the order of C-C pair numbers were rutaecarpine > tanshinone I > oxymatrine > sophoridine > cryptotanshinone > osthole > amygdalin > tanshinone IIA > matrine > cordycepin > baicalein > quercetin > scopoletin (Table 4-5). Among these 13 compounds, seven had shown moderate to potent inhibitory effect on CYP1A2 in our *in vitro* results, in an order of inhibitory potency as follow: tanshinone I > tanshinone IIA > cryptotanshinone > baicalein > osthole > quercetin > cordycepin. Table 4-6 and Figure 4-8 show the details of C-C interactions between tanshinone I and CYP1A2. The inhibitory potency of these smaller compounds was higher than those with M_r > 310 Dal (Figure 2-2).

It was worthy to note that rutaecarpine, andrographolide and scopoletin produced fluorescence and thus interfered with the determination for CYP1A2 activity. However, rutaecarpine had been reported to have potent inhibition on CYP1A2 with IC_{50} of 22 nM by other group (Don et al., 2003) and had have the most C-C pair number in our Ligplot analysis. The remaining compounds, amygdalin, dehydroandrographolide, polydatin, matrine, oxymatrine and sophoridine, did not hit by Hopyo-1 in pharmacophore analysis and also did not exhibit any inhibitory effect on CYP1A2 in our *in vitro* study, although these compounds had hydrophobic interaction with the functionally essential residues in the active site of CYP1A2 with a low binding energy.

4.4 Conclusions

These results indicate that hydrophobic contact of ligand and certain residues in the active site of CYP1A2 are important for substrate-CYP1A2 and inhibitor-CYP1A2 interactions, which may partially explain why most CYP1A2 substrates and inhibitors are small, planar with aromatic ring and hydrophobic molecules. The common features of these ligands are of one to two hydrophobic regions, an aromatic ring and a hydrogen bond acceptor. Phe226, Ala317, Gly316, Phe125, Thr124 and Hem900 were identified as the most important residues to influence inhibitory potency of CYP1A2 inhibitors. Furthermore, three essential residues (Asp, Ala and Thr) standing side by side in helix I immediately above the heme were identified as

conservative residues in the active sites of CYP1A2, which are also found in CYP2C9 and 2D6.

Using a combined *in silico* approach of estimating binding energy, pharmacophore modelling and hydrophobic atom-atom interaction analysis between the ligand and the 6 functionally essential residues in the active site of CYP1A2, it is likely to screen potential inhibitors for CYP1A2 from herbal sources and synthetic compound library. The data from the *in silico* screening can also be used to predict relative inhibitory potency of potential CYP1A2 inhibitors.

Table 4-1. Known CYP1A2 substrates and the data relevant to metabolic pathways catalyzed by CYP1A2 and conformations in the active site of CYP1A2.

Number	Substrate	Total number of metabolite	Total number of conformer based on known metabolic pathway	Total number of pose[a]	Distance of reactive atom to Fe (X, Å)	Residue as hydrogen bond donor	Residue as hydrogen bond acceptor
1	Acetaminophen	1	0	4	ND		
2	Amitriptyline	1	1	2	4.34		
3	Caffeine	1	1	2	4.34		
4	Clomipramine	1	1	3	3.36		
5	Clozapine	1	0	1	ND		
6	Cyclobenzaprine	1	1	3	3.29		
7	Estradiol	1	0	2	ND		
8	Fluvoxamine	1	1	7	3.94		
9	Haloperidol	1	1	5	5.42		
10	Imipramine	2	1	2	5.02		
11	Mexiletine	3	0	3	ND		
12	Naproxen	1	1	3	ND	Ala317	
13	Olanzapine	3	0	1	ND		
14	Ondansetron	4	1	2	4.94		Asn257
15	Phenacetin	1	1	2	4.00		Asn312
16	Propranolol	2	1	5	4.1	Thr124	
17	Riluzole	1	0	3	ND		
18	Ropivacaine	2	1	2	4.00		
19	Tacrine	4	1	1	5.27		Asp320
20	Theophylline	2	0	1	ND		
21	Tizanidine	1	1	4	6.66		Thr124
22	Verapamil	1	1	7	7.20	Thr118	
23	Warfarin	3	1	2	4.46	Thr124	
24	Zileuton	1	1	3	4.86	Ala317	
25	Zolmitriptan	1	1	7	3.35		Asn312 Asp320
Total	25	41	18	77		5	6
Mean ± SD	-	1.64 ± 0.99	0.72 ± 0.46	3.08 ± 1.85	4.62 ± 1.09 (range: 3.29 to 6.66)	-	-

[a]Total pose number refers to docking generated conformation number. ND = Not determined.

Table 4-2. The total amino acid residues and C-C pairs involved in the binding of known substrates (n = 18) to the active site of CYP1A2 as analyzed by Ligplot.

Number	Residue	Sum of number of interaction atom pairs (n = 18)	Number of substrates involved	Average number of C-C pairs per substrate	Group[a]
1	Phe226	173	18	9.6	1
2	Ala317	94	18	5.2	2
3	Hem900	91	17	5.4	2
4	Gly316	71	16	4.4	2
5	Phe125	59	16	3.7	2
6	Thr124	55	15	3.7	2
7	Phe260	52	12	4.3	2
8	Asp313	34	12	2.8	3
9	Asp320	31	10	3.1	3
10	Leu497	27	14	1.9	3
11	Phe256	24	8	3.0	3
12	Ile386	22	14	1.6	3
13	Thr223	19	7	2.7	4
14	Thr118	18	10	1.8	4
15	Thr321	17	12	1.4	4
16	Ser122	14	9	1.6	4
17	Asn312	12	6	2.0	4
18	Leu382	8	6	1.3	4
19	Thr498	8	7	1.1	4
20	Ile117	7	6	1.2	4
21	Val227	7	6	1.2	4
Mean ± SD	-	-	-	3.0 ± 2.0	-

[a]Grouping was based on the number of interaction atom pairs.

Table 4-3. Ligand-pharmacophore (Hopyo-1 & Hopyo-1m) mapping results for known substrates and inhibitors of CYP1A2. The mapping extent was determined by the Fit value out of 4 features.

Compounds	**Fit value (Hopyo-1)**	**Fit value (Hopyo-1m)**	**LogP**	**M_r (Dal)**
CYP1A2 substrates	***(13/25)***	***(17/25)***		
Caffeine	NM	1.153	-0.131	194.191
Estradiol	1.279	1.971	4.131	272.382
Fluvoxamine	2.909	2.742	3.113	318.335
Haloperidol	3.873	2.995	3.014	375.864
Naproxen	1.983	1.996	2.998	230.259
Olanzapine	2.950	2.651	1.507	312.432
Ondansetron	3.934	2.949	2.074	293.363
Phenacetin	NM	1.861	1.626	179.216
Propranolol	0.109	0.683	3.097	259.343
Riluzole	0.400	1.908	2.843	234.198
Ropivacaine	2.994	2.863	3.105	274.401
Tacrine	NM	1.121	3.316	198.264
Tizanidine	3.093	2.793	0.653	253.711
Verapamil	3.607	2.884	3.899	454.602
Warfarin	3.195	2.868	3.417	308.328
Zileuton	2.337	2.079	3.74	236.290
Zolmitriptan	NM	1.819	1.644	287.357
Mean ± SD	2.51 ± 1.24	2.20 ± 0.73	2.591 ± 1.179	275.443 ± 68.360
CYP1A2 inhibitors	***(8/9)***	***(9/9)***		
Amiodarone	3.824	2.994	8.891	645.312
Cimetidine	3.434	2.96	0.19	252.339
Ciprofloxacin	2.412	2.387	0.654	331.342
Enoxacin	2.770	2.583	0.552	320.319
Furafylline	3.491	2.839	-0.244	260.249
Methoxsalen	1.840	1.545	1.93	216.19
Mibefradil	3.262	2.828	6.294	495.629
Propafenone	3.455	2.991	3.934	341.444
Rofecoxib	NM	2.472	1.342	314.356
Mean ± SD	3.061 ± 0.665	2.622 ± 0.463	2.616 ± 3.141	353.020 ± 135.280

Table 4-4. Ligand-pharmacophore (Hopyo-1 and Hopyo-1m) mapping results for herbal compounds tested in this study. The mapping extent was determined by the Fit value out of 4 features for Hypyo-1 and 3 for Hypro-1m, respectively.

Herb compound (21/56)	**Fit value (Hopyo-1)**	**Fit value (Hopyo-1m)**	**IC_{50} (μM)**
Alloin	2.869	2.767	66
Arctiin	3.691	2.931	ND
Baicalein	NM	2.999	1.22
Baicalin	3.078	3.000	70.03
Cryptotanshinone	2.876	2.675	0.91
Evodin	2.467	2.392	ND
Ferulic Acid	0.126	1.424	ND
Forsythin	3.823	2.929	ND
Hyperoside	NM	2.999	14.46
Icariin	3.490	2.999	42.998
Liquiritin	2.913	2.999	ND
Osthole	3.179	2.989	1.49
Paclitaxol	3.228	2.707	ND
Polydatin	NM	2.848	ND
Puerarin	3.319	2.888	ND
Quercetin	NM	2.998	3.97
Quercitrin	3.127	3.000	33.76
Rutaecarpine	2.765	2.880	ND
Salvianolic acid B	3.413	2.929	ND
Schisandrin A	3.411	2.717	ND
Schisandrin B-γ	3.025	2.499	ND
Scopolein	NM	1.455	ND
Silybin	3.279	2.996	ND
Tanshinone I	3.719	2.979	0.027
Tanshinone IIA	3.085	2.986	0.187
Tanshinone IIA sulfonate	3.157	2.988	7.077
Mean ± SD	**3.05 ± 0.75**	2.77 ± 0.42	-

ND = Not determined due to lack of inhibitory effect at the highest concentrations tested or interfering fluorescence.

Table 4-5. The results for tested herbal compounds: IC50, fit value for pharmacophore (Hopyo-1) mapping, free binding energy for the conformations in the active site of CYP1A2, C-C pairs of the first and second pose for each compound and CYP1A2 interaction. The first half table list the herbal compounds that the free energy of binding lower than -6.60 kcal/mol; the second half table list the herbal compounds that the free energy of binding higher than -6.60 kcal/mol.

Test herbal compound	**M_r (Dal)**	**IC_{50}** (μM)	**Total number of pose[a]**	**Hopyo-1 Fit value**	**Binding energy[b]** (kcal/mol)	**C-C pairs[c]**
Amygdalin	457.42	ND	5	NM	-7.98	25
Andrographolide	350.46	ND	2	NM	-8.07	40
Baicalein	270.23	1.22	2	2.999*	-9.06	15
Baicalin	446.36	70.03	4	3.078	-6.73	36
Cordycepin	251.24	6.69	4	Nm	-6.76	21
Cryptotanshinone	296.35	0.91	1	2.876	-10.89	28
Dehydroandrographolide	332.42	ND	2	NM	-9.53	40
Matrine	248.37	ND	1	NM	-8.05	24
Osthole	244.28	1.49	2	3.179	-8.28	26
Oxymatrine	264.36	ND	9	Nm	-8.28	30
Polydatin	390.00	ND	5	2.848*	-7.81	36
Quercetin	302.24	3.97	2	2.998*	-8.67	15
Quercitrin	448.39	33.76	2	3.127	-8.24	42
Rutaecarpine	287.31	ND	1	2.765	-10.39	42
Scopoletin	192.16	ND	2	1.455*	-6.80	12
Sophoridine	248.37	ND	1	NM	-8.86	29
Tanshinone I	276.28	0.027	1	3.719	-10.82	31
Tanshinone IIA	294.33	0.187	1	3.085	-11.09	25
Tanshinone IIA Sulfonate	396.00	7.077	2	3.157	-10.72	36
Mean ± SD	315.61 ± 78.13		2.58 ± 2.04		-8.79 ± 1.43	29.11 ± 9.26
18β-Glycyrrhetinic acid 2	470.70	ND	1	NM	+12.34	
Alloin	418.39	66	5	2.869	-5.64	
Arctiin	534.54	ND	7	3.691	+6.03	
AsperosaponinVI	929.10	ND	10	NM	+530.35	
Astragaloside	784.00	ND	6	NM	+223.59	
Bilobalide	326.30	ND	2	NM	-4.42	
Borneol	154.20	ND	2	NM	-5.92	
Canthridin	196.21	ND	2	NM	-6.27	
Sodium Danshensu	185.13	ND	7	NM	-5.87	
Evodin	470.50	ND	3	2.467	+20.55	
Ferulic Acid	194.18	ND	2	0.126	-6.20	
Forsythin	534.54	ND	6	3.823	+31.25	
Gallic acid	170.12	ND	2	NM	-5.21	
Gastrodin	286.27	ND	2	NM	-6.51	
Ginkgolide A	408.41	ND	1	NM	-0.21	
Ginkgolide B	424.41	ND	2	NM	+9.50	
Ginkgolide C	440.41	ND	1	NM	+11.13	
Ginsenoside Rg3	785.03	ND	8	NM	+473.18	
Glycyrrhetinic acid	839.99	ND	1	NM	+19.42	

Hyperoside	464.37	14.46	4	2.999*	-6.10	
Icariin	676.65	42.998	5	3.490	+50.81	
Liquiritin	418.39	ND	6	2.913	-1.42	
Paclitaxol	853.92	ND	4	3.228	+415.88	
Protocatechuic Acid	154.12	ND	2	NM	-5.59	
Protocatechuic aldehyde	138.12	ND	2	NM	-5.24	
Puerarin	416.37	ND	5	3.319	-4.12	
Saikosaponin A	780.96	ND	6	NM	+295.57	
Saikosaponin D	780.96	ND	3	NM	+284.80	
Salvianolic acid B	718.60	ND	10	3.413	+130.71	
Schisandrin A	432.50	ND	1	3.411	-3.16	
γ−Schisandrin	400.45	ND	2	3.025	-4.25	
Silybin	482.43	ND	5	3.279	+22.48	
Stachydrine	179.64	ND	2	NM	-4.41	
Tetramethylpyrazine	136.20	ND	1	NM	-5.27	
Trigonelline	137.13	ND	2	NM	-4.58	
Triptolide	360.39	98.22	1	NM	-6.48	
Ursolic acid	456.68	ND	1	NM	+13.81	
Mean ± SD	447.18 ± 242.81	-	3.58 ± 2.59	-	66.34 ± 144.99	-

[a]Docking generated conformation number.
[b]Estimated free energy of binding for the optimized pose.
[c]The hydrophobic interaction between herbal compounds with their optimized poses and the 6 essential residues of the CYP1A2 active site.
*The mapping extent was determined by the Fit value out of 3 for Hypro-1m, instead of 4 features for Hypyo-1.

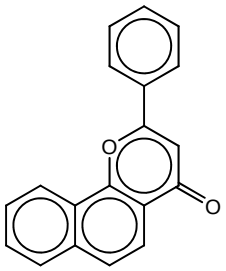
Table 4-6. Hydrophobic interaction between tanshinone I and the residues in the active site of CYP1A2.

Residue	Atom (from residue)	Atom (from ligand)	Distance (Å)
Ala317	C (B)	C21	3.59
Ala317	C (A)	C21	3.76
Ile386	C (D1)	C20	3.70
Ala317	C (B)	C20	3.75
Thr124	C (G2)	C20	3.43
Hem900	C (4D)	C19	3.65
Hem900	C (HA)	C19	3.75
Ile386	C (D1)	C19	3.27
Thr124	C (G2)	C19	3.26
Hem900	C (4D)	C18	3.60
Hem900	C (2A)	C18	3.82
Hem900	C (1A)	C18	3.31
Hem900	C (HA)	C18	3.46
Ile386	C (D1)	C18	3.81
Hem900	C (4A)	C17	3.63
Hem900	C (HB)	C17	3.58
Leu382	C (D2)	C17	3.20
Thr321	C (G2)	C17	3.21
Leu497	C (D2)	C15	3.85
Thr498	C (G2)	C14	3.66
Leu497	C (D2)	C14	3.81
Thr321	C (G2)	C14	3.88
Asp320	C (G)	C13	3.87
Asp320	C (B)	C13	3.77
Ala317	C (A)	C12	3.85
Ala317	C (B)	C11	3.84
Ala317	C (A)	C11	3.49
Ala317	C (A)	C9	3.71
Gly316	C	C9	3.70
Gly316	C	C7	3.51
Gly316	C (A)	C7	3.56
Phe226	C (E2)	C7	3.75
Gly316	C	C6	3.53
Gly316	C (A)	C6	3.76
Phe226	C (E2)	C6	3.66
Phe226	C (D2)	C6	3.64
Asp320	C (B)	C5	3.87
Gly316	C	C5	3.79
Phe226	C (E2)	C5	3.84
Phe226	C (D2)	C5	3.66
Asp320	C (B)	C3	3.84
Phe226	C (D2)	C3	3.78
Thr223	C (G2)	C3	3.58
Thr223	C (B)	C3	3.48
Phe226	C (D2)	C2	3.72
Phe226	C (G)	C2	3.77
Phe260	C (E1)	C1	3.55
Phe260	C (D1)	C1	3.79
Phe256	C (Z)	C1	3.35

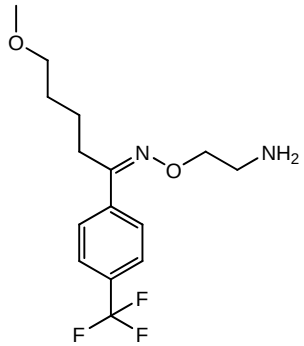
Phe256	C(E2)	C1	3.20
Mean ± SD	-	-	3.64 ± 0.20

The alphabet letter in the brackets of column 2 is the position of the C-atom of the amino acid residue.

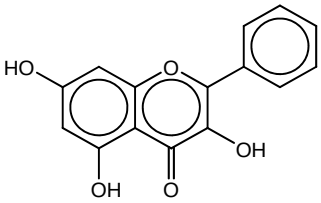
Figure 4-1. Chemical structures of fluvoxamine, galangin, miconazole, α-naphthoflavone (used as initial template), and rutaecarpine which are all CYP1A2 inhibitors. These compounds were used as the training set.



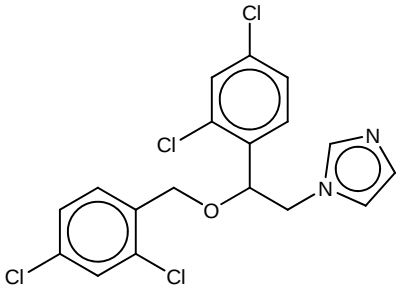
α-Naphthoflavone IC_{50} = 26 nM



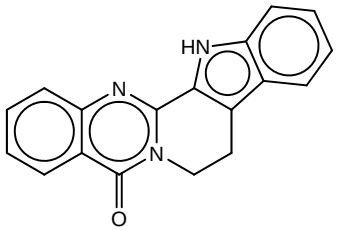
Fluvoxamine IC_{50} = 1.24 μM



Galangin K*i* = 8 nM

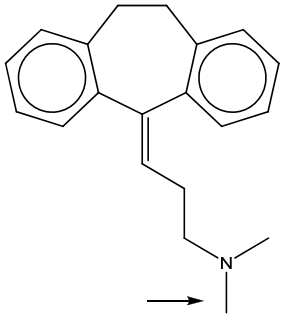
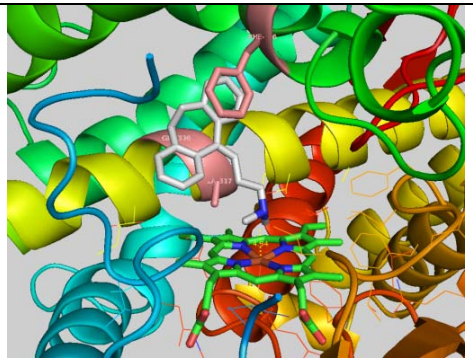
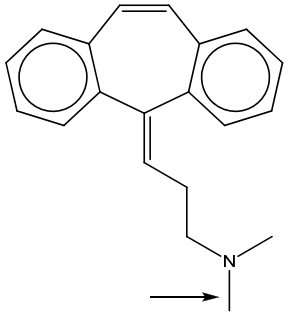
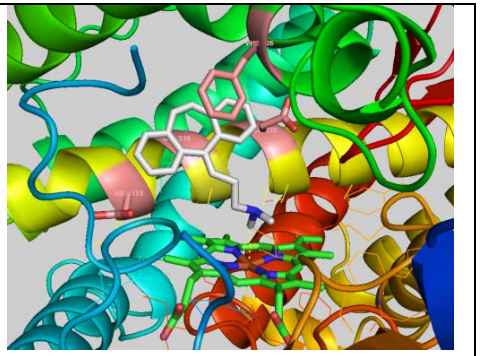
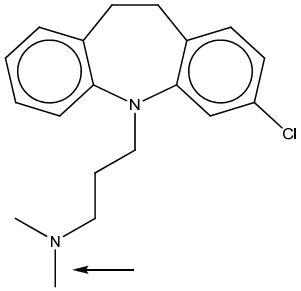
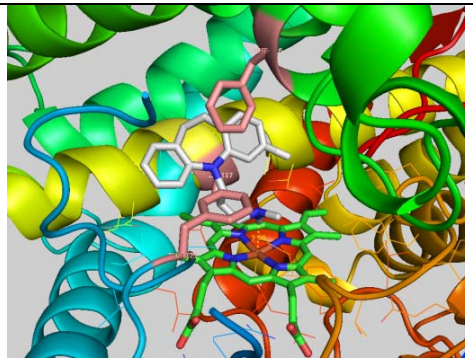
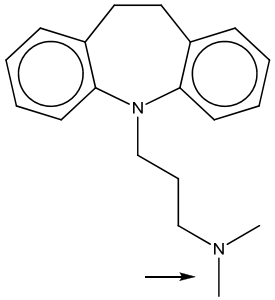
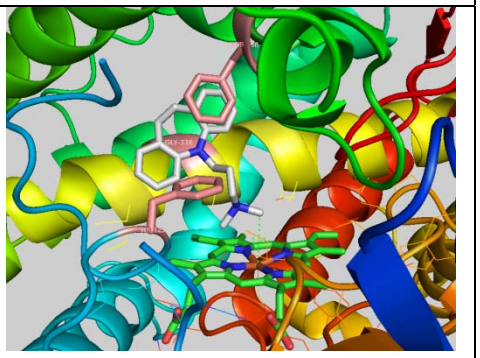
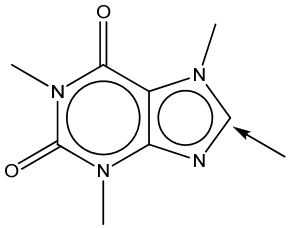
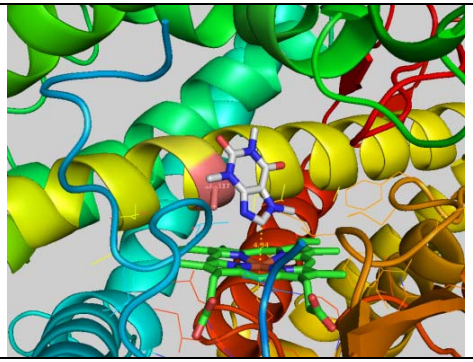
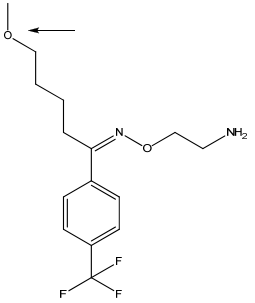
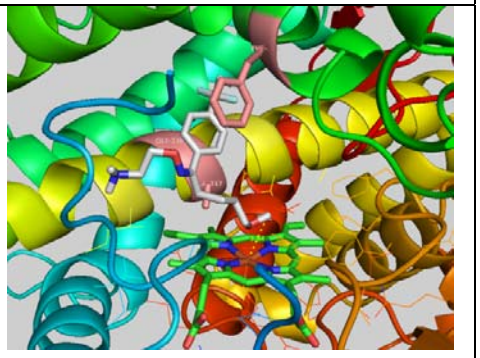


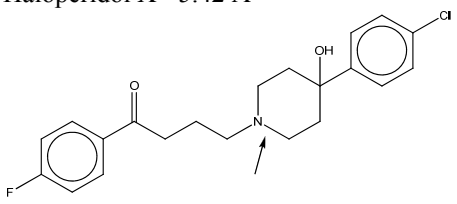
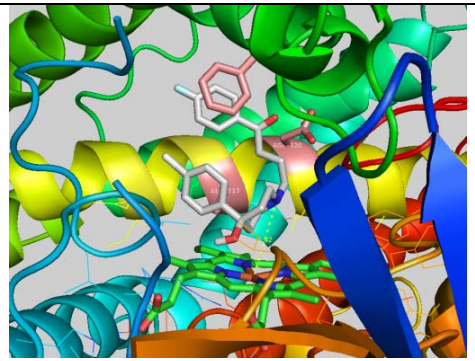
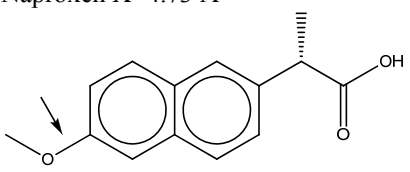
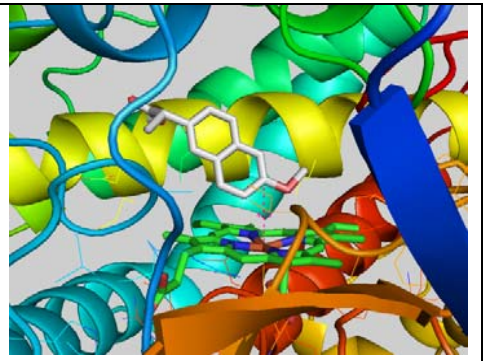
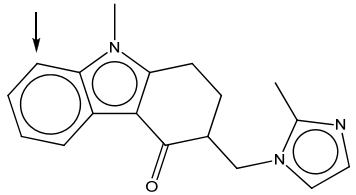
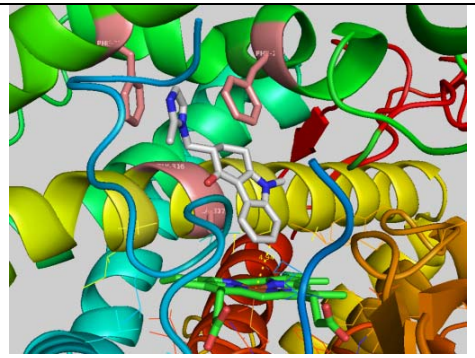
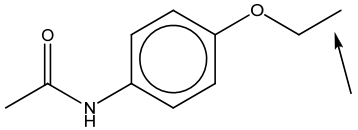
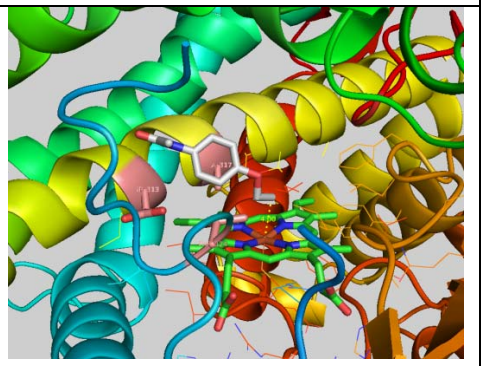
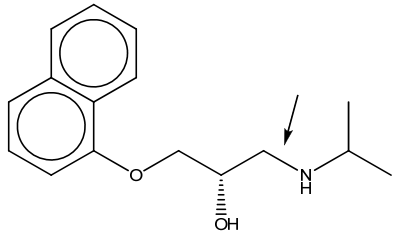
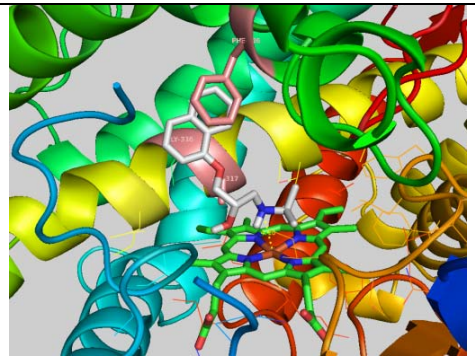
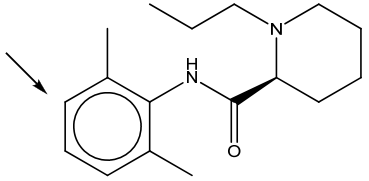
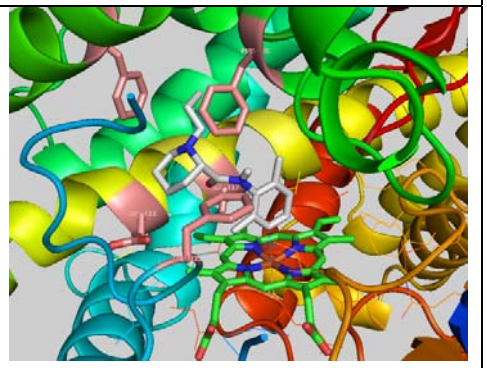
Miconazole IC_{50} = 2.9 μM



Rutaecarpine IC_{50} = 22 nM

Figure 4-2. Known CYP1A2 substrates and their structures relevant to metabolic pathways and conformations in the active site of CYP1A2.

Amitriptyline X =4.34 Å 		Cyclobenzaprine X =3.29 Å 	
Clomipramine X =3.36 Å 		Imipramine X= 5.02 Å 	
Caffeine X =4.34 Å 		Fluvoxamine X =3.94 Å 	

Haloperidol X= 5.42 Å 		Naproxen X=4.73 Å 	
Ondansetron X= 4.94 Å 		Phenacetin X= 4.00 Å 	
Propranolol X= 4.10 Å 		Ropivacaine X= 4.00 Å 	

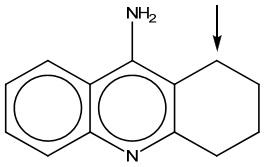
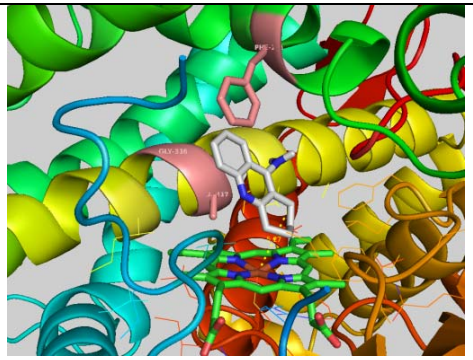
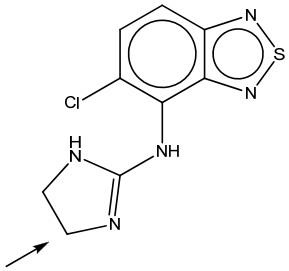
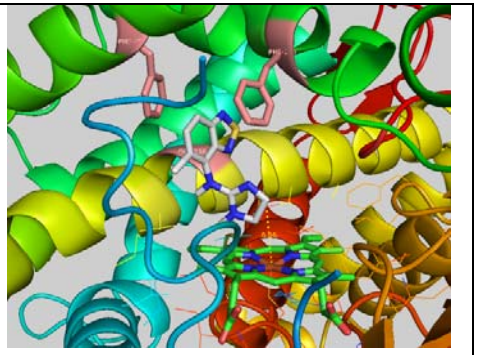
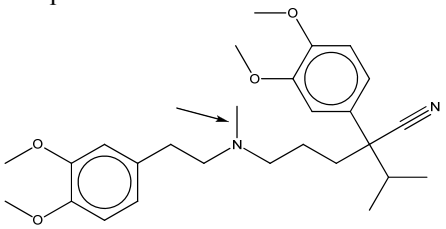
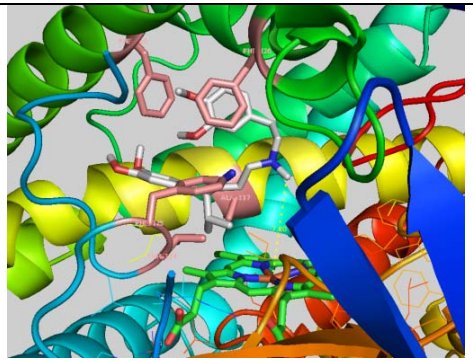
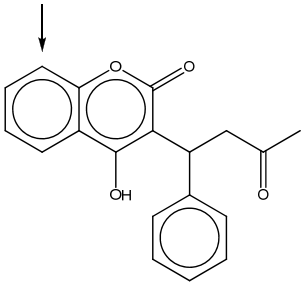
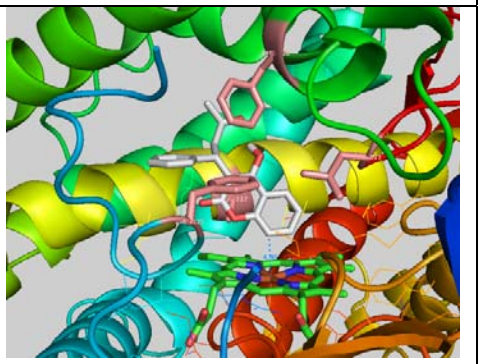
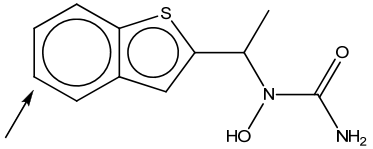
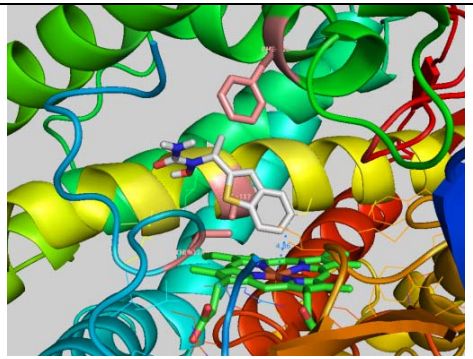
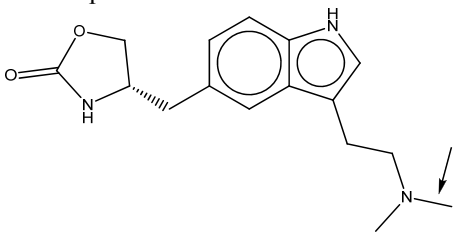
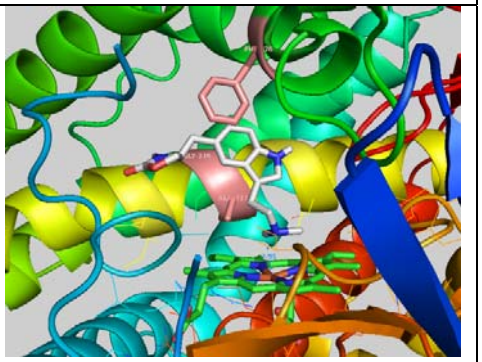
Tacrine X= 5.27 Å 		Tizanidine X= 6.66 Å 	
Verapamil X= 7.20 Å 		*R*-warfarin X =4.46 Å 	
Zileuton X =4.86 Å 		Zolmitriptan X =3.35 Å 	

Figure 4-3. Chemical structures of 25 substrates of CYP1A2.

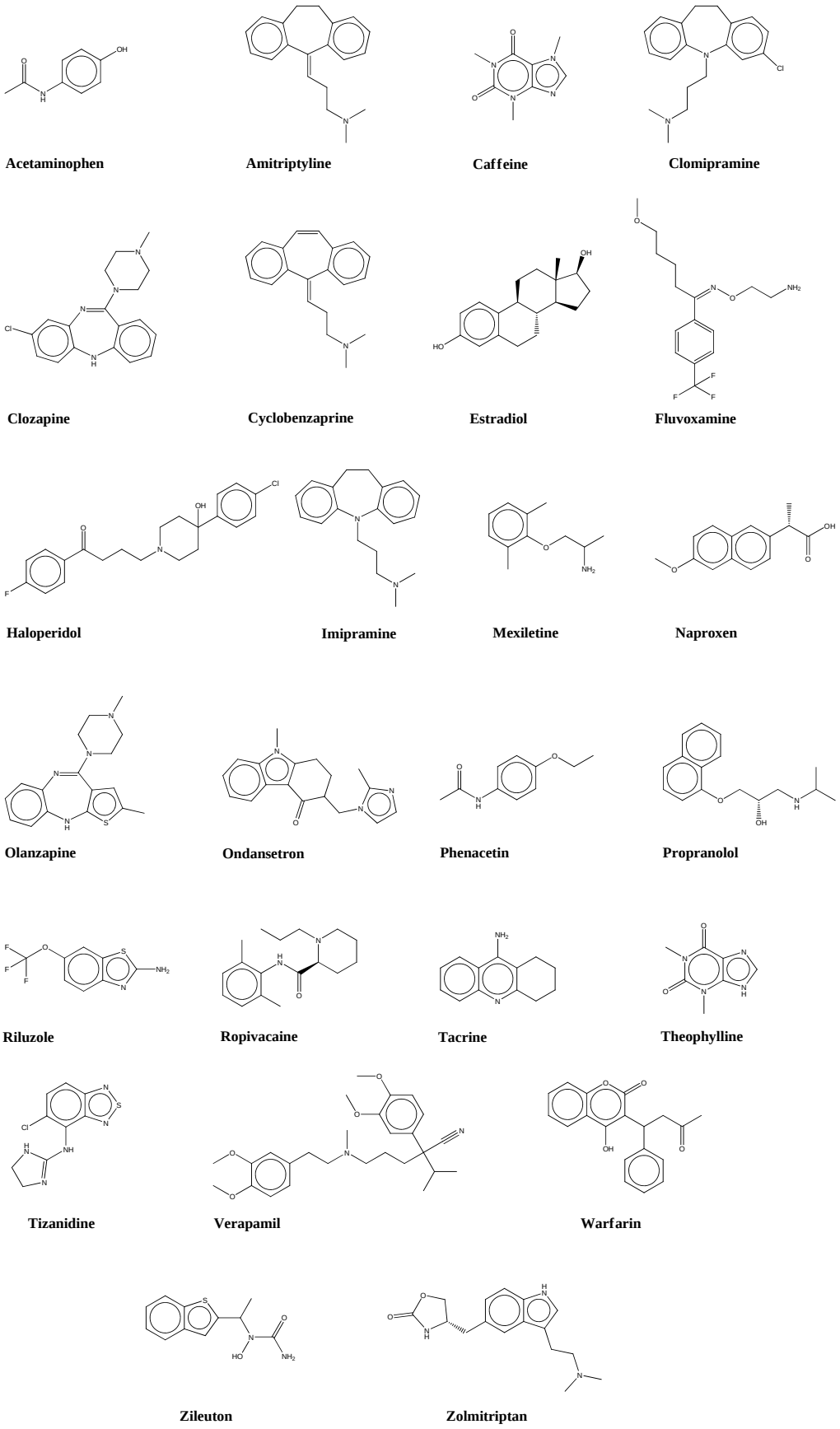


Figure 4-4. The active site of CYP1A2 and the key residues responsible for substrate binding.

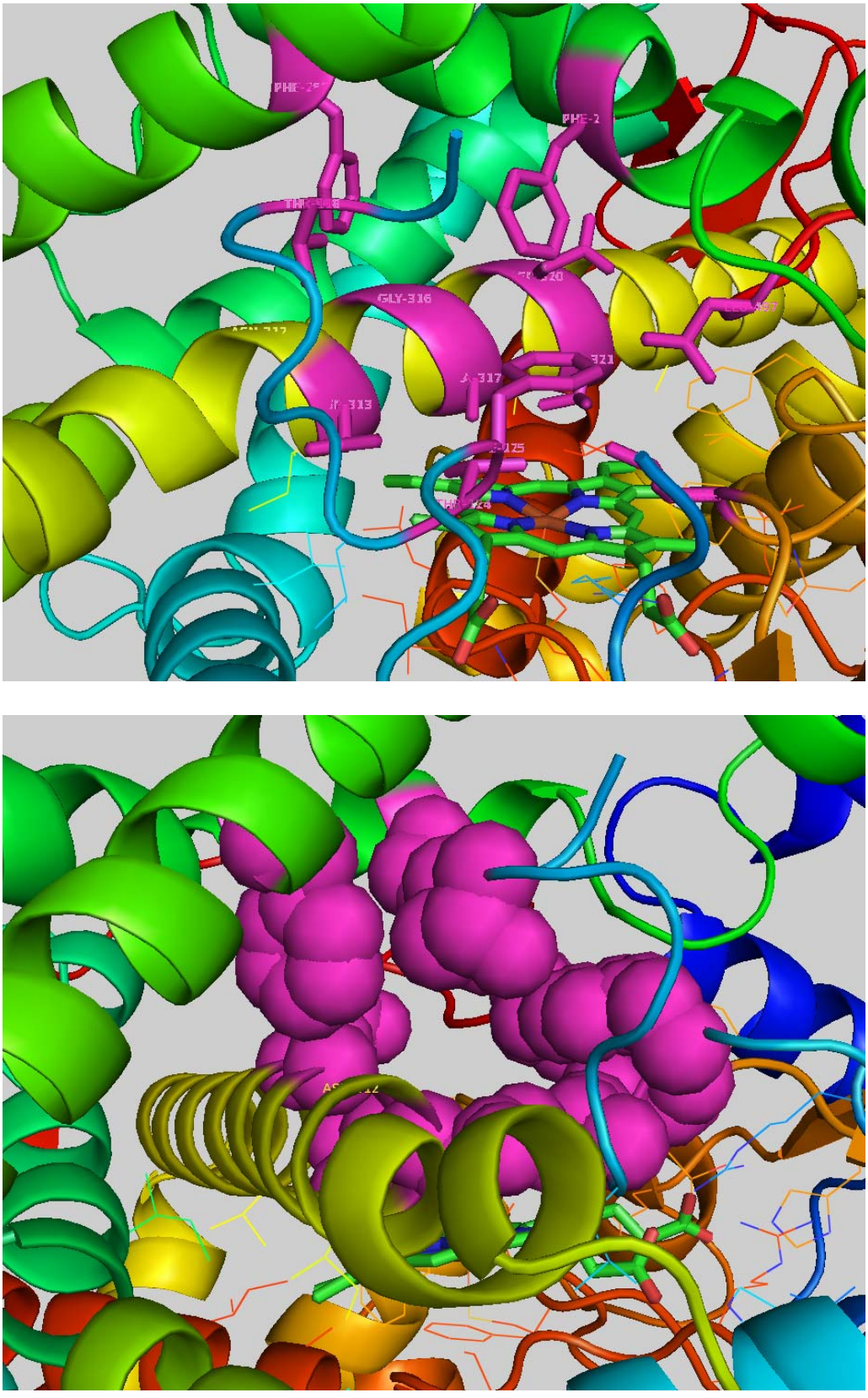
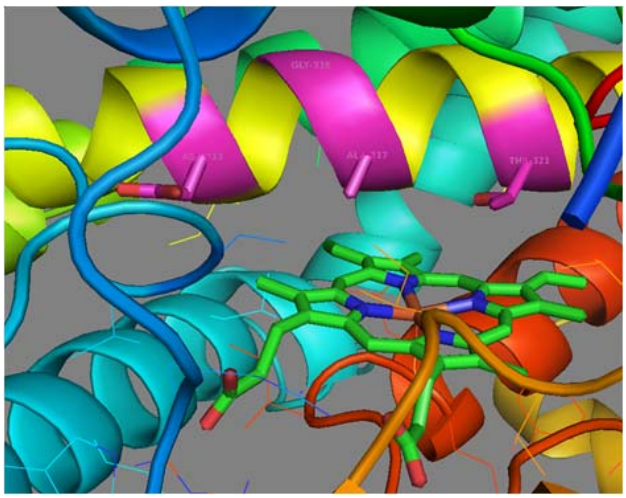
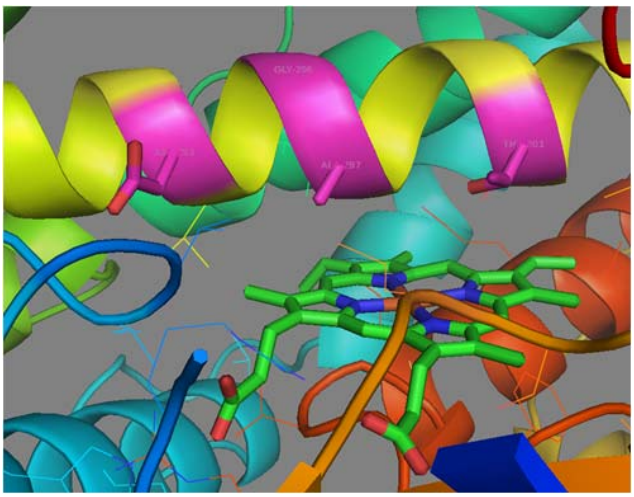


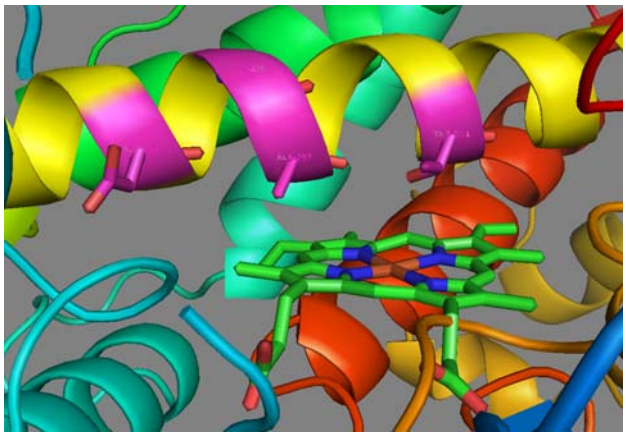
Figure 4-5. The three conserved residues (Asp, Gly/Ser, Ala and Thr) in the active sites of CYP1A2, 2C9, 2C19 and 2D6.



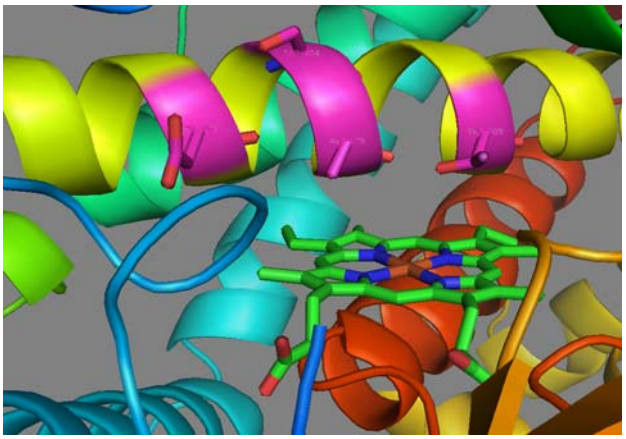
Active site of CYP1A2



Active site of CYP2C9



Active site of CYP2C19



Active site of CYP2D6

Figure 4-6. Pharmacophore models generated by five potent inhibitors of CYP1A2 with the HipHop module in Catalyst. Hopyo-1: the intact model with all the four features (two distinct hydrophobic areas, one aromatic ring and one HBA); Hopyo-1m: a model modified by excluding a hydrophobic area.

Hopyo-1 **Hopyo-1m**

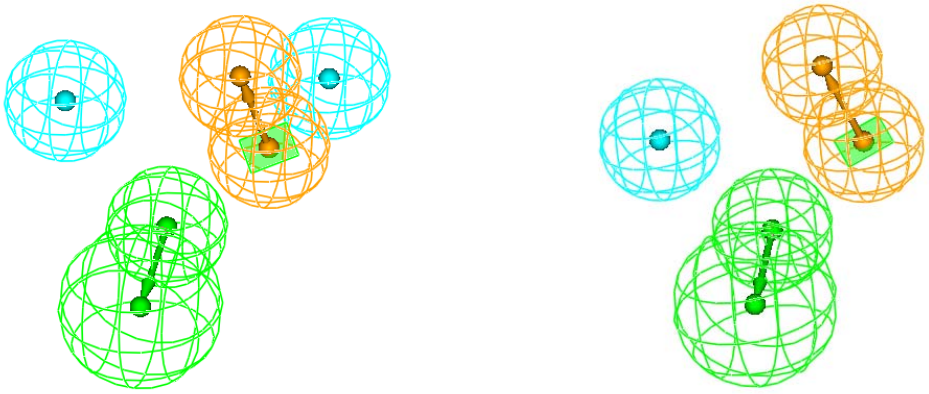
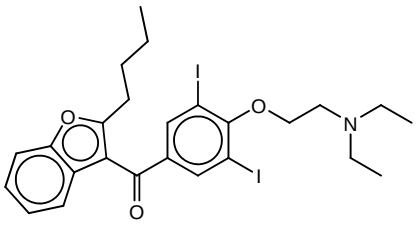
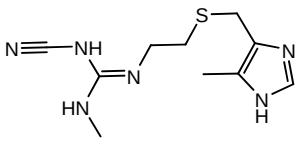


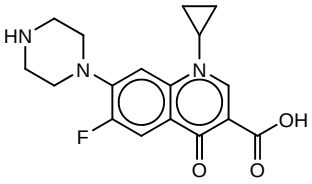
Figure 4-7. Chemical structures of 9 known CYP1A2 inhibitors, which were used as the validating set.



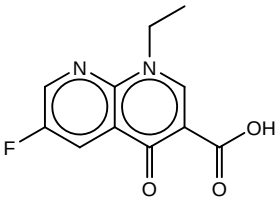
Amiodarone



Cimetidine



Ciprofloxacin



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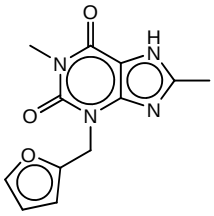
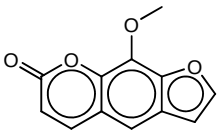
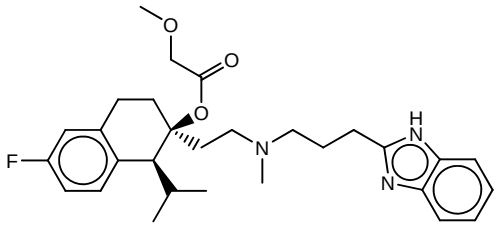


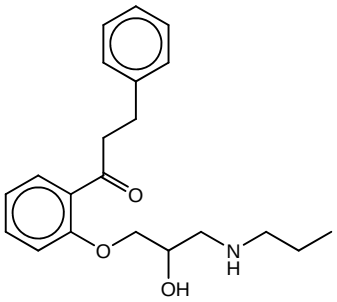
Furafylline



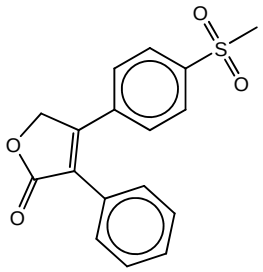
Methoxsalen



Mibefradil

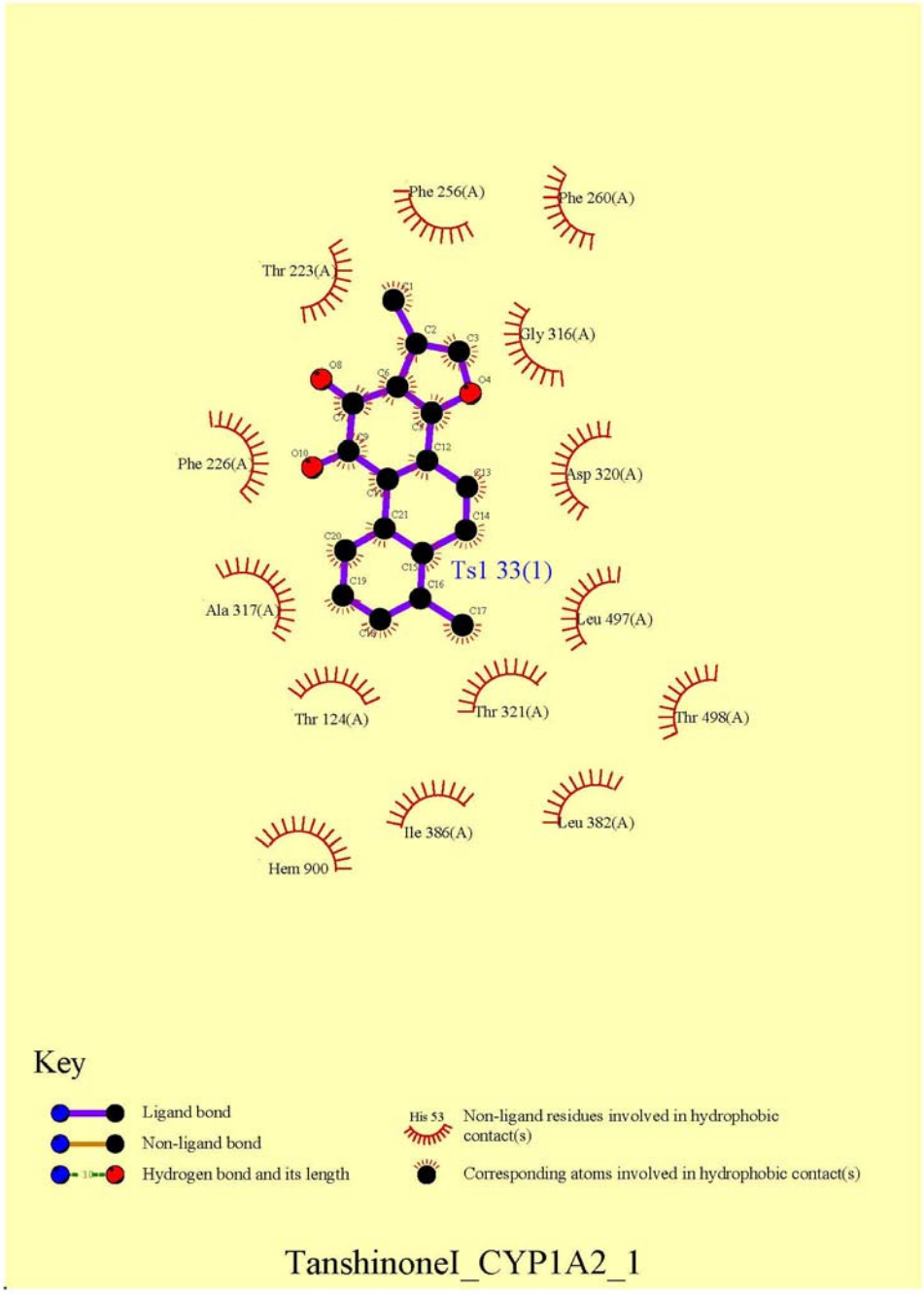


Propafenone



Rofecoxib

Figure 4-8. The interaction between tanshinone I and the residues in the active site of CYP1A2.



CHAPTER 5 GENERAL DISCUSSION

5.1 A Summary of Objectives Achieved

In this project, we have hypothesized that the substrate and inhibitor specificity of individual human CYPs is based on the atom-atom interactions between the ligand and the residues in the active site of the particular CYP. In order to test the hypothesis, we first employed an HTP approach to examine the inhibitory effect of a number of herbal components on five important drug-metabolising CYPs (1A2, 2C9, 2C19, 2D3, and 3A4, Table 2-1). The tested herbal components include a variety of structurally distinct compounds such as triterpenoids of danshen (*S. miltiorrhiza*), flavonoids and their glycoside derivatives, saponine, other glucosides, lactones, alkaloids, and acids (Table 3-1). A small number of them are found to significantly inhibit human CYP1A2, 2C9, 2C19, 2D6 and 3A4 with differential potency, including tanshinone I, tanshinone IIA, cryptotanshinone, baicalein, quercetin, silybin, osthole and γ-schisandrin. Thereafter, we predicted potential herb-drug interactions of these compounds *in vivo* based on the *in vitro* inhibition data. Some predicting results are consistent with the data observed in clinical reports, but some predictions are wrong. Finally, we have conducted docking studies for a series of known CYP1A2 substrates and inhibitors and established pharmacophore models using a set of CYP1A2 inhibitors. We have identified 6 residues in the active site of CYP1A2 being essential for ligand recognition through the analysis of docking results. Furthermore, we set up and validated the pharmacophore model for virtual screening of CYP1A2 inhibitors. In combination with docking results, the pharmacophore hypothesis and hydrophobic contact between ligand and the 6 essential residues in the active site of CYP1A2, it is likely to screen potential CYP1A2 inhibitors and to predict their inhibitory potency for the CYP1A2 enzyme. Our results provide insights into the mechanisms for ligand-CYP1A2 interactions and partial explanation for the substrate and inhibitor specificity of CYP1A2, an important enzyme that metabolizes a number of therapeutic drugs and activate a variety of procarcinogens.

5.2 Herb-Drug and Herb-CYP Interactions

Botanical products are increasingly becoming popular as alternative medicines, and an estimated one third of adults in the developed countries use alternative therapies, including herbs. Herbs are often administered in combination with therapeutic drugs, raising the potential of pharmacokinetic and/or pharmacodynamic herb-drug interactions.

There are an increased number of reports on herb-drug interactions, although many of them are from case reports and limited clinical observations. Thus, herb-drug interactions may be significantly under-reported and underestimated, and more frequently than drug-drug interactions, since most patients (up to 70%) do not reveal their herbal use to their allopathic practitioners (Eisenberg et al., 1993).

Despite the widespread use of herbal medicines, documented herb-drug interactions are sparse and many of the observed herb-drug interactions are based on individual case and case series reports (Table 1-8). Although some herb-drug interactions may be beneficial by enhancing the efficacy and reducing the toxicities of the coadministered drugs, in many cases, the herb-drug interactions may increase drug toxicity, or even be fatal. Thus, more studies are needed to confirm and assess the clinical significance of these potential herb-drug interactions.

A number of *in vitro* systems can be used to investigate herb-CYP interactions (e.g. liver microsomes, precision-cut liver slices, cultured hepatocytes, and cDNA-expressed enzymes). We have adopted an HTP approach to screen the inhibitory effect of a number of herbal compounds on five major drug-metabolizing CYP enzymes. From our *in vitro* study, it was found that all three lipophilic components of Danshen (e.g. tanshinone IIA) had significantly inhibition on both CYP1A2 and 2C9 activity, whereas the hydrophilic constituents of Danshen (e.g. danshensu) only showed poor to weak inhibitory effects on all the five CYP enzymes.

We have found that the activities of CYP2C9, 2C19 and 3A4 were remarkably inhibited by γ-schisandrin, a major active compound present in *S. chinensis* (Wuweizi). Wuweizi is traditionally used to protect the liver and treatment of chronic liver diseases. The total CYP content and the metabolic rate of antipyrine were enhanced significantly in the liver microsomes obtained from the rats pretreated with Wuweizi (*S. chinensis*) (Zhu et al., 2000). Treatment with extracts of Wuweizi induced the expression of drug-metabolizing enzymes and transporters in reporter gene assays and in cultured human hepatocytes (Mu et al., 2006). The affected enzymes and transporters included CYP3A and 2C enzymes and the multidrug resistance-associated protein 2. In rats, the administration of Wuweizi enhanced the clearance of warfarin (Mu et al., 2006). These results demonstrate a potent inducing effect of Wuweizi *in vivo* and have important implications in drug-herb interactions.

We found that two free flavonoids (baicalein and quercetin) had significant inhibitory effects on CYP1A2, 2C9, 2C19 and 3A4, but their flavonoid glucosides (baicalin, hyperoside, quercitrin and icariin) only showed minor to moderate inhibitory effects on these enzymes. Flavonoids are a diverse group of phytochemicals that are produced by various plants including medicinal herbs (e.g. *Silybum marianum*, *Alpinia officinarum*, and *H. perforatum*) (Dixon and Steele, 1999). Flavonoids are structurally classified into eight groups: flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidines, chalcones and flavonolignans. Flavonoids exhibit a wide range of biological activities arising mainly from their antioxidant properties and ability to modulate several enzymes or cell receptors. These include anti-bacterial and antiviral activity, antiinflammatory, antiangionic, analgesic, antiallergic effects, hepatoprotective, cytostatic, apoptotic, estrogenic and antiestrogenic properties (Dwyer, 1995; Gordon et al., 1995; Nagai et al., 1995; Galati et al., 2000; Rice-Evans, 2001). As the chemical structure and activities of some flavonoids are similar to those of naturally occurring estrogens, they are assigned as phytoestrogens.

Flavonoids can also directly modulate the activities of various CYPs (Chan et al., 1998; Zhai et al., 1998; Doostdar et al., 2000; Henderson et al., 2000; Boek-Dohalska et al., 2001; Ho et al., 2001; Piver et al., 2001; Hodek et al., 2002; Kent et al., 2002). Some naturally occurring flavonoids are potent inhibitors of CYP1A1, 1A2, 1B1, 3A4, 3A6, and CYP19. In contrast, some flavonoids enhanced/stimulated the activities of CYP3A4 and 1A2 (Tsyrlov et al., 1994; Ueng et al., 1997; Boek-Dohalska et al., 2001). The different effects of various flavonoids on CYP3A4 may be partly explained by the presence of distinct ligand binding sites on CYP3A4 (Hosea et al., 2000). Structure-activity analysis indicated that flavonoids containing hydroxyl groups inhibited CYP activity, whereas those lacking hydroxyl groups stimulated the enzyme activity. For example, non-substituted 7,8-benzoflavone increased CYP3A4 activity (Ueng et al., 1997; Boek-Dohalska et al., 2001). In another study, quercetin inhibited the activity of aryl hydrocarbon hydroxylase (CYP1A), but enhanced the activity of cDNA-expressed human CYP1A2 (Tsyrlov et al., 1994). Likewise, 7,8-benzoflavone was an inhibitor of human CYP1A1 and 1A2, but an activator of CYP3A4 (Tassaneeyakul et al., 1993).

Flavonoids of oral herbal products or food may be metabolized by microflora in the gut, where flavonoid glycosides are usually cleaved into free flavonoids (aglycones), and both glycosides and aglycones are absorbed (Hollman and Katan, 1997). The degradation of a flavonoid skeleton occurs mainly in the gut, resulting in degradation products including various phenolic

acids, some of which still exhibit a radical-scavenging activity. These metabolites can be absorbed and consequently found in urine (Hollman and Katan, 1997; Rice-Evans, 2001). Some flavonoids have been identified as substrates of CYPs (Silva et al., 1997a; Silva et al., 1997b; Roberts-Kirchhoff et al., 1999; Doostdar et al., 2000; Rice-Evans, 2001). In the liver, flavonoids are hydroxylated and/or *O*-demethylated by various CYPs and then subjected to conjugation reactions (glucuronidation, sulfation, *O*-methylation) catalyzed by phase II enzymes. For example, genistein (5,7,4'-trihydroxyisoflavone) is converted into orobol (5,7,3',4'-tetrahydroxyisoflavone) by CYPs 1A1, 1A2, 1B1 and 2E1, while CYP 3A4 metabolizes genistein into two other undefined metabolites (Roberts-Kirchhoff et al., 1999).

Notably, many flavonoids have been reported to be potent inducers of various CYPs (Canivenc-Lavier et al., 1996; Ciolino et al., 1998b; Ciolino and Yeh, 1999; Hodek et al., 2002). For example, galangin, quercetin, diosmin and its aglycone form, diosmetin, increased the expression of CYP1A1, while other flavonoids such as flavone, tangeretin and synthetic β-naphthoflavone stimulated the expression of CYP1A1/2 and CYP2B1/2 (Ciolino et al., 1998b; Ciolino and Yeh, 1999). Flavanone appears to be specific inducer of CYP2B1/2 (Canivenc-Lavier et al., 1996). However, other CYPs such as CYP2E1 and 3A4 which are responsible for the metabolism of a number of therapeutic drugs and the activation of many procarcinogens, appeared not to be inducible by flavonoids. Similarly, some flavonoids such as genistein, equol or hop prenylflavanones and prenylchalcones did not modulate CYP (Helsby et al., 1997).

An additional free flavonoid, silybin with a relatively large molecular mass (M_r 482.44), was found to significantly inhibit the activities of CYP2C9 and 3A4 in our study. Silibin, also known as silybinin, is the major active constituent of silymarin, the mixture of flavonolignans extracted from milk thistle (*S. marianum*). Extracts of milk thistle are well-known to prevent or reverse hepatotoxicity of reactive drug metabolites or naturally occurring toxins (Kroll et al., 2007). Silibinin has hepatoprotective properties that protect liver cells against toxins (Vogel et al., 1984; Das and Vasudevan, 2006; Pradhan and Girish, 2006). Silibinin has also demonstrated anti-cancer effects against human prostate adenocarcinoma cells, estrogen-dependent and -independent human breast carcinoma cells, human ectocervical carcinoma cells, human colon cancer cells, and both small and nonsmall human lung carcinoma cells both *in vitro* and in mouse models (Raina et al., 2008; Singh et al., 2008a; Singh et al., 2008b; Singh et al., 2008c; Garcia-Maceira and Mateo, 2009; Singh et al., 2009).

Silybin inhibited CYP3A4, 2D6 and 2E1 in human liver microsomes (Zuber et al., 2002). Silybin and its β-glycosides did not induce the expression of CYP1A2 and 3A4 (Kosina et al., 2005). Silybin did not affect the activity of P-gp (Patel et al., 2004). Co-administration of silymarin does not considerably change the extent of absorption or metabolism of nifedipine but may decrease the absorption rate in healthy subjects (Fuhr et al., 2007). This finding indicates that silymarin is not a potent CYP3A4 inhibitor *in vivo*. Another flavonoid, tangeretin, did not alter the CYP3A4 activity in human volunteers (Backman et al., 2000). It appears that silybin has limited effects on the pharmacokinetics of drugs *in vivo* (Wu et al., 2009).

From our *in vitro* inhibition results, it can be expected that lipophilic and small herbal components show greater inhibition on human CYP1A2. Most known inhibitors of CYP1A2 are lipophilic and small. Since CYP1A2 contains a small active site cavity, it can readily accommodate small molecules.

Herbal compounds can inhibit human CYPs to variable extent in vitro, but many of them induce these enzymes through nuclear receptor-mediated pathways. Flavonoids modulated most CYPs, in particular CYP3A4, the predominant human hepatic and intestinal CYP, which is responsible for the metabolism of approximately 50% of therapeutic agents. Concomitant administration of herbs and drugs may alter the pharmacokinetics of the latter, which may result in an altered therapeutic effect or cause toxicity.

5.3 Prediction of Pharmacokinetics Herb-Drug Interactions Based on *in vitro* Data

Pharmacokinetic herb-drug interactions are caused due to altered absorption, metabolism, distribution and excretion of drugs. The underlying mechanisms for the altered drug concentrations by concomitant herbal medicines are always to be determined, but the induction or inhibition of hepatic and intestinal CYPs and/or drug transporters such as P-gp (Walter-Sack and Klotz, 1996; Wilkinson, 1997; Evans, 2000; Ioannides, 2002; Zhou et al., 2003c) have been suggested. Herbs are often given orally and thus herbal constituents may modulate gastrointestinal pH and motility. Due to high concentrations in the gut lumen, herbal constituents are likely to exert a major effect on intestinal enterocytes. These cells represent the first cell lining limiting entry of orally administered drugs into the body. Both P-gp and CYP3A4 are expressed at high levels in the villus tip of enterocytes, the primary site of absorption for orally administered drugs. The interplay of both intestinal P-gp and CYP3A4 determines bioavailability of many drugs such as cyclosporine (Kolars et al., 1991), midazolam

(Paine et al., 1996), HIV protease inhibitors (Kim et al., 1998), verapamil (Fromm et al., 1996), digoxin (Greiner et al., 1999), and talinolol (Westphal et al., 2000). Thus, the modulation of intestinal P-gp and CYP3A represents an important mechanism for the enhanced or reduced bioavailability of coadministered drugs.

Based on the *in vitro* results, we predicted the pharmacokinetic herb-drug interactions following pharmacokinetic principles, with a focus on purified constituents from *S. chinensis* (γ-schisandrin), *S. miltiorrhiza* (tanshinone I and II A), *A. pubescens* (osthole) and *S. Mariani* (silybin). We predicted that the *S. chinensis* (γ-schisandrin and schisandrin) might increase the AUC of drugs that are primarily metabolised by CYP2C9, 2C19 or 3A4 in humans (Table 3-2 and Table 3-3). The oral bioavailability of tacrolimus (a CYP3A4 substrate) was increased in humans when *S. sphenanthera* extracts were co-administrated (Xin et al., 2007). Our prediction of *S. miltiorrhiza* causing pharmacokinetic drug interactions is also consistent with results from the clinical study (Chan, 2001) where *S. miltiorrhiza* products increased the prothrombin time of warfarin 2-fold and induced over-anticoagulation in patients. Thus, using *in vitro* inhibition data, it is possible to predict some pharmacokinetic herb-drug interactions with certain herbs and to provide a perspective view on how potential for the herb would interact with the drug coadministered. The prediction data can be used to avoid toxic or fatal herb-drug interactions.

The clinical importance of herb-drug interactions depends on factors that are related to coadministered drugs (dose, dosing regimen, administration route, pharmacokinetic and therapeutic range), herbs (species, dose, dosing regimen, and administration route) and patients (genetic polymorphism, age, gender and pathological conditions) (Dresser et al., 2000). Generally, a doubling or more in drug plasma concentration/AUC has the potential for enhanced adverse effects. However, less marked changes may still be clinically important for drugs with a steep concentration-response relationship or a narrow therapeutic index. In most cases, the extent of herb-drug interaction varies markedly among individuals, depending on interindividual differences in drug metabolizing enzymes (in particular CYP3A4) and transporters (e.g. P-gp), existing medical condition, age and other factors (Zhou et al., 2003b; Zhou et al., 2004e). Due to the difficulties in determining the specific constituents responsible for the inhibition of CYPs and/or P-gp, it appears to be difficult to predict herb-drug interactions (Zhou et al., 2004b).

5.4 Docking and Pharmacophore Modeling Studies for CYP1A2

CYP1A2 accounts for ~13% of the total CYP content of the human liver and is the major enzyme involved in the metabolism of a number of drugs including acetaminophen, caffeine, imipramine, propranolol, tacrine and theophylline as well as the metabolism of endogenous substances such as 17*â*-estradiol, melatonin and uroporphyrinogen III (Table 1-2). Many clinical drugs and some herbal medicines are known to inhibit the activity of CYP1A2, which may provide an explanation of some clinical drug interactions observed.

To assess the molecular factors affecting the inhibitory effect of herbal compounds on CYP1A2, we have conducted ligand-based analysis in the basis of Catalyst/HipHop programs to evaluate the common features for structurally diverse inhibitors and to develop pharmacophore models. The corresponding results offered better understanding of the structural features that are important for selective binding in the CYP1A2 active site and also provide us with clues towards novel selective inhibitors of the CYP1A2. Meanwhile, we have employed AutoDock 4.0 programs for protein-based analysis to explore the binding mode and binding energy in the active site of substrates, inhibitors and tested herbal compounds of CYP1A2. The Ligplot program has also been used to analyse the docking results of the substrates, inhibitors and tested herbal compounds for CYP1A2 and for the ligand-protein interactions.

5.4.1 Residues in CYP1A2 active site involved in substrate recognition

In the 2HI4 structure in complex with ANF (Figure 1-4), the rather compact active site is closed without clear solvent or substrate access channels with a relatively small volume of the cavity of 375 $Å^3$ (Sansen et al., 2007). Sansen *et al*. (2007) have found that the substrate binding cavity of CYP1A2 is narrow, which is lined by residues on helices F and I that define a relatively planar binding platform for the substrate on either side. Helix I bends as it crosses the heme prosthetic group and its residues form one flat side of the substrate binding cavity, resulting in a coplanarity through the Ala317 side chain, the Gly316-Ala317 peptide bond, and the Asp320-Thr321 peptide bond. On the other side of the cavity, the side chain of Phe226 of helix F forms a parallel substrate binding surface.

The active site cavity of CYP1A2 is stabilized through a strong hydrogen-bonding interaction between the side chain of Thr223 on helix F and the side chain of Asp320 on helix I. Both Thr223 and Asp320 play a role in forming an extensive network of hydrogen-bonded water

molecules and side chains, including Tyr189, Val220, Thr498, and Lys500. It is clear that the narrow and flat active site cavity of CYP1A2 can fit well with planar compounds such as ANF and typical CYP1A2 substrates such as theophylline, caffeine, melatonin, tacrine, clozapine. ANF is a potent, competitive inhibitor of CYP1A2 with K_i values of 1-50 nM (Shimada et al., 1998; Cho et al., 2003). ANF binds CYP1A2 in a single preferred orientation, which places the phenyl ring close to the heme iron and makes it an inhibitor rather than a substrate for CYP1A2. Similarly, CYP2A6 contains a narrow and flat active site cavity and this protein preferentially oxidizes small planar compounds such as nicotine, coumarin and naphthalene (Yano et al., 2005).

We have employed computerized programs to analyze the ligand-CYP1A2 interaction based on the crystal structure of CYP1A2 (PDB ID: 2HI4). Our substrate-CYP1A2 interaction study identified 12 residues at the active site of CYP1A2 as important residues for ligand binding. These residues define the substrate specificity of CYP1A2 as small, planar aromatic-ring containing and hydrophobic ligands. In particular, there are 6 residues in the active site of CYP1A2 identified as essential residues for substrate recognition, including Thr124, Phe125, Phe226, Gly316, Ala317, and Hem900. The 6 residues are also identified as the most important residues for the binding of CYP1A2 inhibitors and the extent of ligand and the 6 residue interaction determines the extent of inhibitory potency. This is consistent with the results from the study by Sansen *et al*. (2007).

Interestingly, a recent study of CYP1A1 homology models based on the rabbit CYP2C5 and a composite of CYP2C5, 2C8, and 2C9 X-ray crystal structures has revealed several residues in its active site that are potentially involved in binding of the prototypic CYP1A1 substrate 7-ethoxyresorufin (Lewis et al., 2007). These include Ser122, Phe123, Phe224, Ala317, Thr321, and Ile386. SDM studies have confirmed their importance in 7-ethoxyresorufin binding and turnover and aromatic interactions over hydrogen bonding in orientating 7-ethoxyresrufin play a critical role in a catalytically favorable manner (Lewis et al., 2007).

Our data demonstrated that Phe226 is the most significant residue in the hydrophobic active site of CYP1A2 for most substrate binding and this is supported by an SDM study (Parikh et al., 1999). The SDM study has indicated that three mutants at Phe226 position (F226I, F226T, and F226Y) of human CYP1A2 displayed very low k_{cat} values for 7-ethoxyresorufin and phenacetin oxidations (Parikh et al., 1999).

Other two acidic residues (Asp313 and Asp320) at the active site of CYP1A2 are found to be essential for the hydrogen bond formation between a ligand and CYP1A2, and also determine the basic preference of CYP1A2 ligands. This is partially supported by an SDM study at the Asp320 position of human CYP1A2. One of the Asp320 mutants, D320A, was found to substantially decrease the activity of CYP1A2 (Parikh et al., 1999).

Notably, we found that there are three conservative residues (Asp, Ala and Thr) located at the same positioning in the active sites of CYP1A2, 2C9 and 2D6. The conservation of the three residues implies the fundamental function of these three CYPs in substrate recognition and catalytic reactions.

Human CYP1 enzymes have demonstrated remarkably overlapping substrate specificities for which the molecular planarity of substrates and inhibitors is a determining factor. The planar active site architecture in the CYP1A2 structure, which is well adapted for the oxidation of relatively large aromatic compounds, is likely to be conserved among the CYP1 enzymes. Relatively small changes in the enzyme active site residues can provide an explanation for CYP1A specificities for the *O*-dealkylation of alkoxyresorufins. Although wild-type CYP1A1 shows a clear preference for 7-ethoxyresorufin *versus* 7-methoxyresorufin *O*-dealkylation compared to CYP1A2, the reciprocal CYP1A1 V382L and CYP1A2 L382V mutants display interchanged specificities (Liu et al., 2004). In the 2HI4 structure, the distance between Leu382 C^{δ} and C'3 and C'4 of ANF is only 3.9 and 4.1 Å, respectively, which demonstrates the restricted architecture at the base of the CYP1A2 active site cavity and explains the preference of CYP1A2 for shorter alkoxyresorufins. The unique active site topology of CYP1A2 demonstrates how CYP1 enzymes have evolved to catalyze efficiently polycyclic aromatic hydrocarbon oxidation and delineates structural properties that define a distinctive substrate binding site.

5.4.2 Common features of CYP1A2 ligands

Our pharmacophore modelling studies showed the common features of CYP1A2 inhibitors as one to two hydrophobic regions, an aromatic ring and a HBA. The model presents the common features of CYP1A2 inhibitors, which could hit 88.9% known inhibitors of CYP1A2 and 64% herbal inhibitors tested. Interestingly, this model could hit 56% known CYP1A2 substrates as well. It is worthy to note that excluding one hydrophobic feature of the model could improve

the hitting rate of known inhibitors to 100%; known CYP1A2 substrates to 68% and herbal inhibitors tested to 86%. The model is efficient to screen most inhibitors and a number of substrates of CYP1A2. Since the modified model (a hydrophobic region, an aromatic ring and a HBA) hit more substrates and inhibitors of CYP1A2, it is suggested to be fundamental common features for CYP1A2 ligands.

Since both substrates and competitive inhibitors interact with the active side of CYP1A2, there must be some common features shared by substrates and inhibitors. Therefore, using five potent inhibitors, we developed pharmacophore Hopyo-1 that can effective distinguish CYP1A2 inhibitors from a set of herbal compounds in combination with docking analysis. This pharmacophore model may not be specific for certain category inhibitors with certain core structures but represent common features shared by most CYP1A2 inhibitors. The three to four identified common features (one to two hydrophobic regions, an aromatic ring and a hydrogen bond acceptor) may help us to conduct initial screening for searching work of novel CYP1A2 inhibitor. The pharmacophore Hopyo-1 represents the fundamental 3D structure features of most CYP1A2 inhibitors that may appear different 2D structures. This model is useful for early stage of screening and additional docking study is necessary to exclude the molecules with high binding energy in the active site of CYP1A2. In combination with docking program, the pharmacophore model may serve for database searching to hit potential new lead inhibitors for CYP1A2.

In addition to hydrophobic and hydrophilic interaction, rutaecarpine and tanshinone I have a planar polycyclic structure, which is critical element for inhibitory potency of CYP1A2 inhibitors. A slight break of the polycyclic and planar structure shapely decreases the inhibitory extent. Tanshinone IIA and cryptotanshinone, two close analogues of tanshinone I, possess similar polycyclic with a slight different on steric structure leading their IC_{50} increasing to 187 nM and 910 nM, respectively. Further analysis indicated that both Tanshinone IIA and cryptotanshinone have the same number of the polycyclic but loss a double bond at cycle D that breaks the planarity of the two molecules in 3D structures. Moreover, the methyl on cycle A of cryptotanshinone breaks the planar structure at another head of the polycyclic, which is a possible reason for the inhibitory potency of cryptotanshinone is lower than that of Tanshinone IIA.

These results can be supported by the residue constitute in the active site of CYP1A2. There are three Phe residues in the active site of CYP1A2. The Phe residues implicate that aromatic rings are involved heavily in ligand-CYP1A2 interactions. Further analysis showed that the Phe226 participates all researched interactions and most π–π stacking interplays while the Phe260 participates most of the two interactions. Additionally, the Phe125 locates at the pocket entry of the active side and is supposed to be responsible for ligand recognition. These results may partially interpret the favourite of CYP1A2 for aromatic polycyclic chemicals.

In addition, the pharmacophore model derived from five potent inhibitors of CYP1A2 also support this finding. Aromatic ring is one of the four common features of the hypothesis (Hopyo 1) and another two hydrophobic areas may also be possible to hit aromatic rings. Only one hydrogen bond donor implicates most CYP1A2 ligands should be quite lipophilic molecule that has at least one aromatic ring. Most importantly, our lab data and literature reports fully support the finding. The aromatic polycyclic compounds, such as tanshinone I, tanshinone IIA and cryptotanshinone, show the most potent inhibition on CYP1A2. Alternatively, the finding gives rise of a good explanation for the characters of CYP1A2 ligands with multiple aromatic rings.

An additional analysis of oxymatrine, sophoridine and matrine further emphasizes the importation of the planar polycyclic structure for CYP1A2 inhibitory potency. The three compounds are analogues with four cycles linking together constructed merely by single bonds, which leads to an inflated instead of planar structure in space. Although three of them accommodated in the active site with low binding energy by docking and interacted with the six essential residues with a number of C-C pairs, none of them were hit by the pharmacophore and also detected any inhibitory effect on CYP1A2 in our *in vitro* study.

The *in silico* approaches provide useful tools for understanding ligand-CYP interactions and for predicting possible drug interactions (Ekins and Wrighton, 2001). The resulting data based on *in silico* approaches may be of clinical and toxicological relevance. For example, it is possible to identify or design very potent CYP1A2 inhibitor which can be used to block procarcinogen bioactivation.

In combination with our *in vitro* study and the Ligplot analysis of the interaction between herbal compounds and CYP1A2, we identified that the C-C number of hydrophobic

interactions between small ligand and the six residues are able to predict relatively inhibitory potency of potential inhibitors. Our screening results showed that rutaecarpine and tanshinone I (M_r < 310 Del) hold the most C-C pairs with the 6 residues of CYP1A2 and predicted to be the strongest herbal inhibitors, which is in accordance with our *in vitro* study and literature reports. The inhibitory effect on CYP1A2 of tanshinone I was detected as IC_{50} of 27 nM, while rutaecarpine was reported (Don et al., 2003) as a selective and potent inhibitor of CYP1A2 with IC_{50} of 22 nM. Rutaecarpine, in docking result, showed an additional hydrogen bond and π–π stacking interactions with CYP1A2, which may let rutaecarpine bind in the active site tighter than other inhibitors that have only hydrophobic interactions, like tanshinone I.

5.5 Limitations of the Present Project

Although the present study has conducted *in silico* and *in vitro* experiments to investigate the herb and drug interaction, there are several limitations for this project. With the high throughput approach, we have assessed the inhibitory effects of the 56 herbal compounds on five principal CYP enzymes (CYP1A2, 2C9, 2C19, 2D6 and 3A4). However, the inhibitory effect on the remaining important CYPs (e.g. CYP2E1, 2B6, 2C8 and 2A6) is not determined. We can only screen a small number of natural compounds given that there are more than 22,000 compounds isolated from natural medicinal products so far. We have only determined the IC_{50} values, rather than the K_i values. As such, we can only assume that the nature of inhibition was competitive and estimate the K_i values based on IC_{50} values when extrapolating the *in vitro* data to *in vivo* situations. Since only microsomes are used, the inducing effects of these herbal compounds on CYPs are not determined. Further studies are warranted to explore the potential effects of herbal components on drug-metabolizing enzymes and drug transporters using cultured human hepatocytes and precision-cut liver slices.

Although the inhibition test is an HTP approach, the inhibition potency cannot be determined for a proportion of herbal components due to interfering fluorescence or very low levels of metabolite formation in the enzyme reaction systems. Thus, the reaction system should be optimized and alternative probes should be used to avoid signal interference.

In our *in silico* study, the pharmacophore models built for CYP1A2 ligands are based merely on certain structural information without relevant activity values. Therefore, the models can only be applied for initial screening to identify potential CYP1A2 inhibitors but could not predict the inhibitory potency of the potential inhibitors. To make a relatively accurate

prediction, QSAR analysis is a proper approach to address this issue. Although the *in silico* results were validated by *in vitro* data, it is still necessary to explore these herb-drug interactions *in vivo* (both animal and human studies). In our ligand-CYP1A2 interaction studies, the dynamics is not considered. Molecular dynamic studies are needed to explore the ligand-enzyme interactions at molecular levels.

In addition, the computer-based docking and pharmacophore studies are only conducted for CYP1A2. These models may fail as disappointing results can be linked to the key aspects of the model and modelling procedure, and many of these related to the original data and its interpretation (Stouch et al., 2003). Further work is ongoing to analyze the interactions of ligand with other important CYPs such as CYP2A6, 2C9, 2C19, 2D6 and 3A4. Furthermore, the essential amino acid residues identified for binding at the active site of CYP1A2 require further validation by SDM studies.

Finally, we did not conduct any animal and human studies in this project. Although *in vitro* and *in silico* studies can provide useful information for CYPs and potential herb-CYP interactions, animal studies can offer valuable data on potential inducing and inhibitory effect of herbs on important CYPs, although caution is often needed when extrapolating the data from animal studies to humans due to marked interspecies variations. For any potential herb-drug interactions, well-designed clinical studies with reasonable sample size are certainly required to confirm the interaction, but these studies are always time-consuming and expensive.

5.6 Conclusions and Future Directions

Evidence from *in vitro* and *in vivo* studies has indicated that the constituents of herbal preparations interact with various CYP enzymes extensively, either as substrates, inhibitors and/or inducers, and it is apparent that the modulation of CYPs by herbs is complex, depending on the type of source of herb, their administration dose, regimen and route, the target organ and the species. These interactions will not be confined to the liver, but may also occur in other tissues where the CYPs are considerably expressed, in particular in the gastrointestinal site, as medicinal herbs are most often given orally. In addition, the multiple ingredients in herbs may modify the intestinal pH and motility, and inhibit and/or induce intestinal drug transporters such as P-gp, and thus change the rate and extent of concomitant drug absorption.

High throughput screening assays may represent a useful strategy for the study of herb-CYP interactions. They are capable of handling the great number of herbal constituents (e.g. a single herb usually contains dozens of constituents), and have the ability to provide in vitro inhibition data as a criterion for monitoring herb-drug metabolic interactions involving human drug metabolizing enzymes (in particular the CYPs).

In silico approaches represent a useful tool for the study of herb-CYP interactions as demonstrated by our studies and studies by other researchers. Our established pharmacophore model could readily distinguish the most potent inhibitor if CYP1A2. Thus, this model could be used as a high throughput-screening tool to identify natural constituents of herbal preparations that inhibit CYP1A2, before undertaking *in vitro* determinations. This will help avoid coadministration of drugs that are extensively metabolized by CYP1A2 with herbal products that showed potent inhibitory effects on this enzyme.

Herb-CYP interactions may have important clinical and toxicological implications, and rigorous testing for possible drug interactions with widely used herbs is needed. It is perhaps time to consider herbs not as alternative medicine based on tradition and experience, but as phytotherapy, and an integrated part of modern medical treatment. Regulations on medicinal herbs would be desirable, but this would be a matter of considerable debate. However, safety (e.g. herb-drug interactions), quality and efficacy should be proved, based on an objective and appropriate standard as for modern medicines.

However, herb-drug interactions are difficult to characterize and resolve, because of the lack of comprehensive federal regulations regarding safety, efficacy, and manufacturing standards for herbal medicines. It has been proposed that herbs are appropriately labelled to alert consumers to possible interactions with other concomitantly used drugs and to recommend a consultation with their general practitioners, pharmacists, and/or other medical carers. It is time to consider herbs not as alternative medicine based on tradition and experience, but as phytotherapy, an integrated part of medical treatment (Qiu, 2007). Regulations with regard to safety (e.g. herb-drug interactions), quality and efficacy of herbs would be highly desirable. Thus, monitoring of adverse events when herbal medicines are coadministered with drugs can be systematically carried out and potential herb-drug interactions be identified. This would enable more accurate product labelling and a body of useful information on potential herb-drug interactions to medical professionals.

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