CLONING AND EXPRESSION OF CYP2D6*1 AND CYP2D6*10 AND ITS APPLICATION IN *IN VITRO* DRUG-HERBS INTERACTION STUDIES

by

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LIST OF ABBREVIATIONS

°C	Celsius centigrade
µl	Micro litre
µM	Micromole
bp	Base pairs
CL_{int}	Intrinsic clearance
CO	Carbon monoxide
CV	Coefficient variation
CYP	Cytochrome P450
δ-ALA	Delta-Alavilunic acid
ddH_2O	Deionised distilled water
DME	Drug metabolizing enzyme
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
EET	Epoxyeicosatrienoic acid
ELJ	*Eurycoma longifolia Jack*
EM	Extensive metabolizer
Fw	Forward
GC	Gas chromatography
IC_{50}	50% inhibitory concentration
IPTG	Isopropyl β-D-thiogalactopyranoside

H_2O	Water
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
k'	Retention factor (capacity factor)
kbp	kilo base pair
KCl	Potassium chloride
kDa	Kilo Dalton
K_i	Inhibition constant
K_m	Michaelis-Mentens constant
LOQ	Limit of quantification
$MgCl_2$	Magnesium chloride
min	Minute
MM	Michaelis-Menten
mmol	Millimole
NADP	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
OD	Optical density
OH	Hydroxyl
OxR	Oxidoreductase
PCR	Polymerase Chain Reaction
PM	Poor metabolizer
pmol	Piccomole
PMSF	Phenylmethanesulphonylfluoride

rpm	rounds per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
TB	Terrific buffer
TBE	Tris-borate buffer
Temed	N,N,N',N'-tetramethylethylenediamine
T_m	Melting temperature
UV	Ultraviolet
VLDL	Very low density lipoprotein
V_{max}	Maximum velocity
wt	Wild type
X-gal	5-Bromo-4-Chloro-3-indoyl-β-D-galactopyranoside

LIST OF PUBLICATIONS, PRESENTATIONS AND AWARDS

PRESENTATIONS AND POSTERS

1. WL Lee, FZ Zainul, LK Teh and R Ismail. Cloning, expression, characterization and inhibition of *CYP2D6*1 and *10* by *Eurycoma longifolia*, Jack (Tongkat Ali): An *in vitro* study. Presented at the 1st AMDI International Biohealth Science Conference (IBSC) 2010.

2. WL Lee, FZ Zainul, CE Ong, TH Tang, CY Choo, LK Teh and R Ismail. Cloning, expression, characterization and inhibition of CYP2D6*1 and *10 by Eurycoma longifolia, Jack (Tongkat Ali): An *in vitro* study. Presented at the 2nd National colloquium and workshop in Pharmcogenetics 2008.

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4. WL Lee, FZ Zainul, CE Ong, TH Tang, CY Choo, LK Teh and R Ismail. An approach to the *in vitro* evaluation of potential inhibition of *Eurycoma longifolia*, Jack (Tongkat Ali) on Cytochrome P450IID6. Presented at the First North-South Conference and workshop on pharmacogenetics 2005.

5. WL Lee, FZ Zainul, CE Ong, LK Teh, R Ismail. Cytochrome P450 IID6: Development of a heterologous expression system for drug metabolism study. Presented at the 1st Postgraduate Research Colloquium of Universiti Sains Malaysia, 2004.

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PENKLONAN DAN PENGEKSPRESAN CYP2D6*1 DAN CYP2D6*10 SERTA APLIKASINYA DALAM KAJIAN *IN VITRO* INTERAKSI UBAT-HERBAL

AKSTRAK

CYP2D6 merupakan salah satu enzim CYP utama dan ia bertanggungjawab dalam metabolisma kira-kira 20% daripada ubat-ubatan yang terdapat pada masa kini. CY2D6 adalah berpolymorfisma dan CYP2D6*10 merupakan alelle kebiasaan di kalangan populasi Malaysia. Data berkenaan interaksi herbal tempatan-ubatan adalah kekurangan. Objektif penyelidikan ini adalah menghasilkan assei *in vitro* berkenaan interaski ubatan-herbal dan mengunakannya untuk menganalisasi interaksi herbal tempatan. CYP2D6*1 rekombinan telah dihasilkan dan diekpreskan bersama CYP-reductase dalam bacteria *E. coli*. CYP2D6*10 dihasilkan dengan kaedah "site-directed mutagenesis" dan diekpreskan sebagai CYP2D6*1. Kaedah HPLC telah dibangunkan untuk mengukur aktiviti bufuralol 1'-hydroxylase dari CYP2D6. Dari segi pengukuran kinetik, Km dan Vmax bagi CYP2D6*1 merupakan 9.686 ± 0.674 µM untuk 6.867 ± 0.119 pmol/min/pmol CYP2D6*1. Manakala , Km dan Vmax CYP2D6*10 adalah 41.02 ± 0.674 µM dan 0.351 ± 0.0013 pmol/min/pmol CYP2D6*10. Parameter kinetik untuk analisis aktiviti catalistik CYP2D6 secara in vitro adalah bersamaan dengan kajian-kajian terbitan lain. Ekstrak daripada tongkat ali (ELJ) menghalang CYP2D6*1 melalui mekanisme tidak kompetitif dan Ki serta IC_{50} masing-masing adalah 302.03 ± 10.59 ng/ml dan 178.38 ± 12.29 ng/ml. Namun ELJ menghalang CYP2D6*10 untuk tahap yang lebih rendah melalui mekanisme yang kompetitif dengan Ki sebanyak 215.29 ± 11.76 ng/ml dan IC50 adalah 490.90 ± 13.88 ng/ml. ELJ tidak menunjukkan jenis penyekatan mekanisme. Perjumpaan ini

juga menunjukkan adanya interaksi ubat-herbal dan interaksi ubat-makanan ketika produk tertentu dimakan bersama-sama dengan ubat-ubatan konvensional. Penyelidikan ini telah berjaya mengekpresikan rekombinan protein CYP2D6*1 dan CYP2D6*10. Kaedah HPLC yang spesifik dan sensitif telah dikembangkan dan divalidasikan untuk analisis aktiviti in-vitro enzim katalitik dan kesan penyekatan ELJ. Penyelidikan selanjutnya diperlukan untuk menyiasat kesan penyekatan herbal tempatan lain-lain atas kegiatan CYP2D6 untuk peningkatan pemahaman interaksi ubat-herbal.

CLONING AND EXPRESSION OF CYP2D6*1 AND CYP2D6*10 AND ITS APPLICATION IN *IN VITRO* DRUG-HERBS INTERACTION STUDIES

ABSTRACT

CYP2D6 is one of the major CYPs enzymes and is responsible for the metabolism of about 20% of currently available therapeutic drugs. *CYP2D6* is polymorphic and *CYP2D6*10* is the common allele in the Malaysian population. There is lack of data on local herbal-drug interaction. The objective of this study was to generate an *in vitro* drug-herbs interaction assay and use it to analyse local herb interaction. Recombinant *CYP2D6*1* was generated and co-expressed with CYP-reductase in *E. coli*. Recombinant CYP2D6*10 was generated using site-directed mutagenesis and expressed as CYP2D6*1. The HPLC method was developed to measure bufuralol 1'-hydroxylase activity of CYP2D6. The kinetic parameters, K_m and V_{max} for CYP2D6*1 were 9.686 ± 0.674 µM and 6.867 ± 0.119 pmol/min/pmol of CYP2D6*1 respectively. For CYP2D6*10, the K_m and V_{max} were 41.02 ± 0.674 µM and 0.351 ± 0.0013 pmol/min/pmol of CYP2D6*10 respectively. Kinetic parameters from *in vitro CYP2D6* catalytic activity analysis were in accordance with others published studies. Extracts of ELJ inhibited CYP2D6*1 by non-competitive mechanism with Ki and IC_{50} of 302.03 ± 10.59 ng/ml and 178.38 ± 12.29 ng/ml, respectively. However ELJ inhibited CYP2D6*10 to a lesser extent by competitive mechanism with Ki of 215.29 ± 11.76 ng/ml and IC_{50} was 490.90 ± 13.88 ng/ml. ELJ did not show any mechanism-based inhibition. It also suggested the presence of drug-herb and drug-food interactions when certain natural products were consumed concurrently with conventional medicines. This study have successfully expressed recombinant CYP2D6*1 and CYP2D6*10 protein. A specific and sensitive HPLC

method was developed and validated to analysis the *in vitro* catalytic activity of enzymes and ELJ inhibition effect. Future studies are required to investigate the inhibitory effects of more local herbs on *CYP2D6* activity for an improved understanding of drug-herb interactions.

1 INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Introduction

Individual variations in response to drugs are a substantial clinical problem. In normal clinical practice, the same drug dosage is prescribed to different patients with the same disease without knowing their genetic backgrounds. Genetically determined variability in drug response defines the research area known as pharmacogenetics (Wolf *et al.*, 2000). Many drugs are metabolized by genetically polymorphic drug-metabolizing enzymes (DMEs), distributed and eliminated by genetically polymorphic drug transporters (Ingelman-Sundberg *et al.*, 1999). For activity, the drug needs to bind to another important genetically polymorphic protein, the receptor.

Cytochrome P450 (CYP) comprises a superfamily of haem-containing DMEs which catalyse the biotransformation of endogenous and foreign substances. This superfamily is divided into families and further subdivided into subfamilies according to their amino acid sequences (Nelson *et al.*, 1996). Families are defined as having more than 40% homology in their amino acid sequence and are designated by an Arabic numeral, and subfamilies have more than 55% analogy and are designated by a capital letter. An Arabic numeral after the letter denotes the individual enzyme and the gene associated with the enzyme is denoted in italics (Slaughter and Edwards, 1995). CYP2 is the largest family and among the member, CYP2D6 is of particular interest because it responsible for the metabolism of about 20-25% of currently available drugs including cardiovascular drugs, β-adrenergic

blocking agents, tricyclic antidepressants, analgesics and compounds such as methoxyamphetamine and dextromethorphan (Kroemer and Eichelbaum, 1995, Ingelman-Sundberg, 2005, Ingelman-Sundberg *et al.*, 2007). In addition, this enzyme is also genetically polymorphic leading to genetically polymorphic metabolism that can impact on medicine dosage (Desmeules *et al.*, 1991, Edeki *et al.*, 1995, Thuerauf and Lunkenheimer, 2006, Cascorbi, 2003, Gardiner and Begg, 2006).

1.2 Literature Review

1.2.1 Pharmacogenetics and Pharmacogenomics

An individual's response to medicine is dependent on factors that can be divided into physiological and environmental. Physiological factors include age, ethnicity, gender, body weight and gene. On the other hand, dietary intake, concomitant drug administration and exposure to chemicals are considered as environmental factors (Brazell *et al.*, 2002). Genetic variation is recognized as an important factor responsible for the variability in drug response and this defines the research area known as pharmacogenetics (Wolf *et al.*, 2000). Pharmacogenetics was born in the 1950s when researchers observed that some adverse drug reactions were caused by genetic variations in enzyme activity (Meyer, 2000). Individuals who inherit an enzyme deficiency and others who inherit enzyme abundance can benefit from pharmacogenetics with genetic tests (Koo and Lee, 2006) used to predict dose.

Pharmacogenetics is the study of the genetic factors influencing variation in drug metabolism and response (Palmer *et al.*, 2005). The completion of the human

genome project facilitated the study of variable drug effects based on individual genetic make-up (Ma and Lu, 2011). The human genome project has identified numerous types of genetic variations among individuals that may or may not alter drugs effects. The genetic variations are either classified as polymorphisms or mutations (Goodman *et al.*, 2011). A polymorphism is defined as variation in a DNA sequence that occurs at a frequency of at least 1% in the human population, whereas a mutation occurs in less than 1 % (Wyatt *et al.*, 2012). It results from single nucleotide polymorphisms (SNP), gene deletion or gene duplication at the same locus that encodes an enzyme or a protein that exists in the normal population (Nebert *et al.*, 1996). Indeed SNP occurs throughout the human genome at a frequency of about 1 per 300-500 DNA base pair (Hinds *et al.*, 2005, Reich *et al.*, 2003, Brazell *et al.*, 2002). Individual functional variants caused by SNPs are associated with inter-individual and interethnic variation in drug response (Koo and Lee, 2006). SNP mapping technology is a promising approach in pharmacogenetics. It enables the entire human genome to be interrogated to identify SNP profiles that are associated with medicine response. Previous approaches involved the identification a particular 'candidate gene' that influence drug action (Brazell *et al.*, 2002).

Cost effectiveness is a priority for healthcare providers and governments (Brazell *et al.*, 2002). Pharmacogenetics has the potential to maximize the value of medicines. The rapid accumulation of knowledge on genome-disease and genome-drug interactions has also impelled the transformation of pharmacogenetics into a new entity of human genetics – pharmacogenomics – and, at the same time, provided a rationale for the hope that individualized medicine can be achieved in the near

future (Ma and Lu, 2011). It will enable more informed prescribing decisions to be made so that patients receive treatments based on a predetermined efficacy and safety profile (Brazell *et al.*, 2002). The ability to identify individuals who are exquisitely sensitive to medicine either before drug treatment or after an adverse drug response would also be of economic importance. It would avoid the empiricism associated with matching the most appropriate drug at its optimal dose for each patient (Wolf *et al.*, 2000). It may also substantially reduce the need for hospitalization with its associated costs, because of adverse drug reactions (Wolf, 2003). Pharmacogenetics is also able to provide information about genetic characteristics of a disease which could be used to improve drug design and improve efficacy and safety of existing drugs (Brazell *et al.*, 2002). In future, pharmacogenetics may help in the determination of risk of disease based on the identification of susceptibility gene early in life so that measures can be taken to avoid the disease. Pharmacogenetics is especially important when the drugs prescribed have narrow therapeutic indexes and its metabolism polymorphic (Wolf *et al.*, 2000). It is also important that when new drugs are developed, relevant pharmacogenetic variations that can alter drug disposition and/or pharmacodynamic are known early in the developmental stage so as to avoid unnecessary costs of drug misadventures due to genetic traits (Wolf *et al.*, 2000).

In drug development, the identification of new drug targets and the understanding of genetic factors that determine patient response to drugs may allow the researchers venture into new paths in designing drugs that are specifically targeted towards particular populations or that avoid genetic variability in therapeutic response. For example, polymorphism of the β2 adrenoceptor gene has produced

responders and non responder phenotypes (Durham *et al.*, 2004). This can lead to inconsistent results in the preclinical and clinical studies that would follow if such a drug compound is pursued. Such targets can be avoided for drug compounds. Thus, at an early stage, the targets can be characterised based on pharmacogenetic studies combined with proteomics and suitable drug compounds selected for further investment. The extent of genetic polymorphism in the human population indicates that pharmacogenetic variability will probably be an issue for most new drugs (Wolf, 2003).

In most cases, variation in drug response in a disease is attributed to many genes rather than a single gene mutation. The results of pharmacogenetic studies do not apply when used clinically, as only single gene mutations are studied when in fact multiple genes are involved. In such cases, it would be appropriate to do pharmacogenomic studies comparing single nucleotide polymorphism (SNP) maps and gene expression between normal and affected individuals (Surendiran *et al.*, 2008). This can identify the genetic factors associated with the disease and thus provide newer targets to characterise and evaluate, for the purpose of drug development. Those that could be potential future drug targets can be called as "tractable" or "drugable" targets (McCarthy *et al.*, 2005). With the availability of advanced human genome sequences, the genes can now be analyzed *in silico* for coding regions of the tractable targets. Polymorphisms of $P2Y_{12}$ receptors in platelets have been identified to be associated with increased risk of coronary artery disease by haplotype analysis (Cavallari *et al.*, 2007). In the future, this can be a potential target for a drug compound produced against coronary artery disease.

1.2.2 Cytochrome P450 2D6

The cytochrome P450 (CYP) superfamily represents the most important phase I drug-metabolizing enzymes. They oxidize a large number of endogenous substances (e.g. eicosanoids and steroids) and xenobiotics (e.g. therapeutic drugs and environmental compounds). The products from the these metabolism are usually more hydrophilic compounds that will facilitate elimination (Nebert and Russell, 2002). There are as many as 57 functional CYP genes and 58 pseudogenes within the 18 families (i.e. CYPs 1–5, 7, 8, 11, 17, 19–21, 24, 26, 27, 39, 46, and 51) in humans (http://drnelson.uthsc.edu/cytochromeP450.html). More than 90% of human drug oxidation can be attributed to the following CYPs: 1A2 (4%), 2A6 (2%), 2C9 (10%), 2C19 (2%), 2E1 (2%), 2D6 (30%) and 3A4 (50%) (Rendic, 2002). Genetic variability in CYP content and activities can have a profound influence on the *in vivo* response of humans to drugs.

Genetic mutations indeed play an important role in the enzyme activity variations of many CYPs, in particular CYP2A6, 2C9, 2C19 and 2D6 (Ingelman-Sundberg *et al.*, 2007). Genetic polymorphisms of CYPs mainly affect the metabolism of drugs that are their substrates, probably leading to the differences in drug response and an altered risk for adverse drug effects (Ingelman-Sundberg *et al.*, 2007, Kirchheiner and Seeringer, 2007, Tomalik-Scharte *et al.*, 2008, Zhou *et al.*, 2009). Most members of the CYP families are polymorphic, (http://www.cypalleles.ki.se/cyp2d6.htm). Allelic variants resulting in altered protein expression or activity have significant effects on the disposition of drugs (Zhou, 2009).

CYP2D6 has been one of the most intensively investigated CYPs in relation to genetic polymorphism. It however accounts for only a small percentage of the total hepatic CYPs content (2–4%). Nonetheless it metabolizes about 25% of currently used drugs in the human liver (Ingelman-Sundberg *et al.*, 2007, Cascorbi, 2003, Gardiner and Begg, 2006, Ingelman-Sundberg, 2005, Zhou *et al.*, 2008). Typical substrates for CYP2D6 are largely lipophilic bases and include some antidepressants, antipsychotics, antiarrhythmics, antiemetics, β-adrenoceptor antagonists (β-blockers) and opioids. Most CYP2D6 substrates are bases containing a basic nitrogen atom 5–10 Å from the site of metabolism (Marechal *et al.*, 2008). Cytochrome P450 2D6 appears to have a high affinity and a low capacity for its substrates. It becomes saturated at relatively low concentrations.

The primarily hepatic expression of this enzyme governs first-pass metabolism after oral drug administration. The low level of its intestinal expression does not appear to be important. In contrast to other CYPs, CYP2D6 is generally not regulated by many known environmental agents and is not inducible by common known enzyme inducers such as steroids (Bock *et al.*, 1994). However CYP2D6 is subject to inhibition by a number of drugs, resulting in clinically significant drug interactions.

1.2.3 Alleles of the *CYP2D6* Gene

CYP2D6 is highly polymorphic, to date, 74 allelic variants and a series of subvariants of the *CYP2D6* gene have been reported (http://www.cypalleles.ki.se/cyp2d6.htm). Its number of alleles is still increasing. Among these alleles, there are fully functional alleles, alleles with reduced function and null (non-functional) alleles (Zhou *et al.*, 2008, Zanger *et al.*, 2004). Depending

on the specific *CYP2D6* genotype, individuals can be divided into poor CYP2D6 metabolizers (PM), intermediate CYP2D6 metabolizers (IM), extensive CYP2D6 metabolizers (EM) or an ultrarapid CYP2D6 metabolizers (Daly, 2003).

Null alleles of *CYP2D6* are alleles that do not encode a functional protein and there is no detectable residual enzymatic activity. Alleles *3, *4, *5, *6, *7, *8, *11, *12, *13, *14, *15, *16, *18, *19, *20, *21, *38, *40, *42, *44, *56 and *62 are null alleles and have no enzyme activity (Zhou, 2009). They are responsible for the poor metabolizer (PM) phenotype when present in homozygous or compound heterozygous constellations. These alleles are of clinical significance as they often cause altered drug clearance and drug response.

Alleles *CYP2D6**10, *14, *17, *18, *36, *41, *47, *49, *50, *51, *54, *55 and *57 on the other hand give rise to a significantly decreased CYP2D6 activity. The reduced activity is often due to decreased protein stability, disrupted substrate recognition or reduced substrate-enzyme affinity. The enzyme activity change may be substrate-dependent for some alleles such as *17. Individuals harbouring either of these alleles are PMs or IMs. CYP2D6*10 occurs in 33 – 43% of Asians, including Malaysians, Japanese, Korean and Chinese, and in Pacific Islanders (Bradford, 2002, Ji *et al.*, 2002, Johansson *et al.*, 1994, Ishiguro *et al.*, 2004, Teh *et al.*, 2001).

1.2.4 Substrates and Inhibitors of Human Cytochrome P450 (CYP) 2D6

Apart from its genetic polymorphism, *CYP2D6* is subject to environmental factors including inhibition by various drugs, such as cimetidine and quinidine (Knodell *et al.*, 1991, Madeira *et al.*, 2004). Consequently, the extent of CYP2D6-mediated drug clearance may be influenced by other, co-administered therapeutic

agents. The competitive inhibition by quinidine is both potent, with a K_i of 3-30nM, and selective for CYP2D6. Quinidine is thus frequently used as an inhibitory chemical probe for *in vitro* CYP2D6 enzyme kinetic study. Other probe drugs used as substrate for *in vitro* CYP2D6 enzyme kinetic study include debrisoquine (4-hydroxylation), dextromethorphan (*O*-demethylation) and bufuralol (1'-hydroxylation). In this study, bufuralol was used. The advantages of using bufuralol as a CYP2D6 selective substrate include the high sensitivity of the assay to study its kinetics owing to the highly fluorescent 1'-hydroxybufuralol metabolite. It also avoids the use of radio-labelled substrate as is required by some other substrates.

1.2.4.1 Bufuralol, the Substrate Probe

Bufuralol is a non-cardioselective β-adrenoceptor antagonist (Fothergill *et al.*, 1975). It possesses partial agonist activity at the β-adrenoceptor (Hamilton and Parkes, 1977). Although, partial agonist is of questionable therapeutic importance (McDevitt, 1983) bufuralol has been found to have vasodilator properties in man (Magometschnigg *et al.*, 1978, Magometschnigg *et al.*, 1979a, Magometschnigg *et al.*, 1979b) and bronchodilator activity in animals (Blaber, 1982) which have been attributed to a β-adrenoceptor agonist effect.

Bufuralol has been extensively used as a probe substrate for the *in vitro* study of CYP2D6 (Zanger *et al.*, 2004, Yuan *et al.*, 2002). It is metabolized to three metabolites, namely 1'-hydroxybufuralol, 1'-oxobufuralol, and 1'2'-ethenylbufuralol (Hiroi *et al.*, 2002). The level of 1'-hydroxybufuralol, a major metabolite of

bufuralol, is often measured as an index of CYP2D6 activity and/or levels, and the amount of 1'-hydroxybufuralol formed from bufuralol is known to be small in PMs of CYP2D6 (Carcillo *et al.*, 2003).

1.2.4.2 Quinidine, the Reversible Competitive Inhibitor

Studies in the early 20th century identified quinidine, a diastereomer of the antimalarial quinine, as the most potent of the antiarrhythmic substances extracted from the cinchona plant, and by the 1920s, quinidine was used as an antiarrhythmic agent. Quinidine is used to maintain sinus rhythm in patients with atrial flutter or atrial fibrillation and to prevent recurrence of ventricular tachycardia or ventricular fibrillation (Grace and Camm, 1998). Quinidine is not metabolized by CYP2D6 but has long been established as a potent competitive inhibitor of the enzyme (Branch *et al.*, 2000, Guengerich *et al.*, 2002, Guengerich *et al.*, 1986, Otton *et al.*, 1988, von Bahr *et al.*, 1985).

1.2.4.3 Paroxetine, Irreversible Mechanism Based Inhibitor

Paroxetine is a selective serotonin reuptake inhibitor with nonlinear kinetics that is both a substrate for and an inhibitor of CYP2D6 (Belpaire *et al.*, 1998, Bloomer *et al.*, 1992, Greenblatt *et al.*, 1999, Otton *et al.*, 1996, Sindrup *et al.*, 1992a, Sindrup *et al.*, 1992b). Paroxetine is metabolized by CYP2D6 via demethylenation of the methylenedioxy group, yielding a catechol metabolite and formic acid (Bloomer *et al.*, 1992, Haddock *et al.*, 1989). Paroxetine inhibits CYP2D6 activity at IC_{50} concentrations ranging from 150 nM to 2.0 μM, depending on the substrate (Crewe

et al., 1992, von Moltke *et al.*, 1995, Fogelman *et al.*, 1999). Paroxetine has been shown to be a mechanism-based inhibitor of CYP2D6 (Bertelsen *et al.*, 2003).

1.2.5 *Eurycoma longifolia*, Jack

Tongkat Ali is a popular medicinal plant in Malaysia. There is four different species of the Tongkat Ali plant, namely *Eurycoma longifolia*, *Eurycoma apiculata*, *Polyathia bulata* and *Goniothala, mus sp* (Aziz *et al.*, 2003). *Eurycoma longifolia* is the most commonly used species (Athimulam *et al.*, 2006). Its belongs to Simaroubacea family (Shafiqul Islam *et al.*, 2006) and is indigenous to Southeast Asia, in the lowland forests of up to 500 m above sea level (Kuo *et al.*, 2003). It grows as a single-stemmed slow growing tree in the rainforest (Rahman *et al.*, 2004) jungle slopes in Malaysia. This plant bears fruit after 2 ½ years of cultivation while the root is usually taken to be processed after 4 years of cultivation.

Eurycoma longifolia is known as 'Pasak Bumi' in Indonesia, 'Cay ba binh' in Vietnam and 'Ian-don' in Thailand. It is popularly sought after as an herbal remedy and is frequently prescribed either as a single ingredient preparation or as a mixture with other herbs (Kuo *et al.*, 2003). In Malaysia it is also known as Payung Ali, Penawar Pahit, Setunjang Bumi, Bedara Pahit, Tongkat Baginda, Pokok Syurga, Tongkat Ali Hitam, Pokok Jelas and Jelaih (Athimulam *et al.*, 2006). Its most popular name however is 'Tongkat Ali'.

Tongkat Ali is a popular folk medicine for the treatment of many diseases. It is used throughout the region as essential components in herbal medicines for a variety of illnesses such as aches, persistent fever, tertient malaria, sex insufficiency

and granular swelling (Kuo *et al.*, 2003) and also as a health supplement. Tongkat Ali has been reported to also possess antimalarial, antiulcer, anti-pyretic, cytotoxic and aphrodisiac properties (Rahman *et al.*, 2004). The nomadic aborigines (Orang Asli) in Malaysia consume *Eurycoma longifolia,* Jack daily when they have febrifuge and malaria (Ang *et al.*, 1995). Nowadays, extracts of Tongkat Ali is popular in commercial drinks and in tonics such as Tongkat Ali Power Root, Long Jack Tongkat Ali and many others. These drinks are marketed as being effective to restore energy and to increase sexual qualities for men.

The most popularly used part of Tongkat Ali is the root. Root extracts of Tongkat Ali contain quassinoids (Chan *et al.*, 2004) such as eurycomanone, 14, 15 β-dihydroxyklaineanone, eurycomanol and eurycomalactones (Shafiqul Islam *et al.*, 2006) giving it a bitter taste (Ang *et al.*, 2002).

1.2.6 *In vitro* Drug Interaction Study Models

The selection of correct human models is necessary for the study and characterization of drug metabolism (Masimirembwa *et al.*, 1999). *In vivo* animal studies are both time-consuming and expensive and will not necessarily yield metabolic or pharmacokinetic information similar to that obtained in humans (Masimirembwa *et al.*, 1999). Ethical issue is another factor that needs to be considered when dealing with *in vivo* animal studies. Indeed human models are now widely accepted and utilized (Masimirembwa *et al.*, 1999). Researches on enzyme kinetics in in vitro systems with CYPs have been extensively reported, acting as human models (Ekins *et al.*, 1997). The following details some of the *in vitro* models applied to metabolism and how their use have been changing as we begin to

understand the enzymes and underlying cellular processes involved (Ekins *et al.*, 2000).

1.2.6.1 Recombinant Enzymes

Advances in molecular biology have opened the path to the stable expression of catalytically active CYPs in a variety of expression systems including bacteria (Waterman, 1993), yeast (Oeda *et al.*, 1985), insects (Buters *et al.*, 1994) and mammalian cells (Crespi and Penman, 1997). These allowing the study of enzyme function and structure, leading to a greater understanding of the individual isoforms. Additionally site directed mutagenesis and expression of these mutants for *in vitro* investigation has become a powerful tool in providing useful information with regards to substrate-enzyme interactions (Domanski *et al.*, 1999).

Recombinant CYPs are also useful in high-throughput screening assays, isoform-selective metabolism, metabolite formation as well as in small scale bioreactors to generate useable amount of metabolic product (Friedberg *et al.*, 1999).

Isolated heterogenous human CYP enzymes, expressed as single enzyme from complimentary DNA (cDNA) for *in vitro* models are commercially available. The availability of so many CYPs can be directly related to the initial isolation and reconstitution of CYP and its enzymatic cofactor, cytochrome P450 reductase (Lu and Coon, 1968). By the early 1990s, over 100 different CYPs have been cloned and sequenced, including the major human CYP isoforms (Nebert *et al.*, 1991).

1.2.6.2 Subcellular Fractions

Subcellular fractions which include microsomes, other cellular organelles and liver homogenates continue to be the most widely used *in vitro* system for drug metabolism. Microsomes however remain the most widely used subcellular fraction (Ekins *et al.*, 2000). Furthermore, liver homogenates also contain all phase I and phase II drug metabolizing enzymes (Ekins *et al.*, 2000).

The advantages of subcellular fractions are that they are easy to prepare, are reproducible, are capable for long term storage and can provide ample characterization of optimal incubation conditions (Ekins and Wrighton, 1999). Their disadvantages include the loss of some enzyme activity during preparation. However this can be satisfactorily recreated by the addition of appropriate cofactors. Liver homogenates fortified with appropriate cofactors exhibit the same enzyme activities as intact tissues do (Pelkonen *et al.*, 2005). Fresh tissues which are properly handled and frozen immediately after excision, are shown to have stable enzymatic activities even during prolonged storage (Pearce *et al.*, 1996).

1.2.6.3 Hepatocytes

Hepatocytes contain the full complement of both phase I and phase II drug metabolizing enzymes. They are therefore valuable tools for multiple uses including for metabolite identification, for transportation studies, and for the elucidation of possible toxic effects of drugs (Holme, 1985). Good *in vitro-in vivo* correlations in the metabolic activity of a number of drugs have been demonstrated and consequently, cultured human hepatocytes are currently the most recommended tools

to study CYP-mediated metabolism and induction (Guillouzo *et al.*, 1993). Widespread use of primary hepatocytes is however limited by the limited availability of liver tissues. Hepatocyte cultures can be prepared from whole livers and surgical wedge biopsies. The maintenance of normal cellular physiology and intercellular contacts in hepatocytes requires special matrix configurations demanding technical capabiliies (Kohira and Nakamura, 1989).

Although several cryopreservation applications have been developed, the time for efficient use of a single hepatocyte batch is still quite short (Cross and Bayliss, 2000); (Ekins *et al.*, 2000). The use of hepatocytes was also briefly challenged by precision-cut liver slices but interest on hepatocytes rose again as hepatocytes techniques such as sandwich culture (Ferrini *et al.*, 1997) and gel immobilized hepatocytes (Guyomard *et al.*, 1996) became available.

1.2.6.4 Liver Slices

Precision-cut liver slices closely resemble the liver at the organ level. They possess a wide range of enzymatic activities there are suitable for enzyme characterisation. Liver slices are also suitable for induction studies (Edwards *et al.*, 2003). The system is moreover a valuable tool when whole cell metabolism needs to be studied in a short period (Ekins *et al.*, 2000).

Liver slices system became popular with the development of a slicer which was capable of producing slices of consistent dimensions with minimal cellular trauma (Krumdieck *et al.*, 1980). This allows for the incubation of the liver or other tissue slices for many hours with appropriate oxygen and nutrient conditions

(Vickers *et al.*, 1995). The time for use and to prepare a single slice is short; however, cryopreservation methods have overcome some of these problems (Ekins *et al.*, 1996). Many studies have however shown lower cellular uptake, clearance, and metabolic capacity of liver slices compared to hepatocytes (Worboys *et al.*, 1996).

1.2.7 Mechanism of Inhibition of CYP

Two major mechanisms are responsible for cytochrome P450 mediated drug interactions: induction and potent inhibition. Induction refers to increased synthesis or decreased degradation of Cytochrome P450 enzymes. Induction promotes enzyme activity. Induction results in decreased plasma concentrations of the substrate. This can either cause a decrease or increase in its pharmacodynamic effect (Lin and Lu, 1998) depending on whether the drug is pharmacologically active or a pro-drug.

Inhibition refers to either enzyme inactivation or competition of substrates for a catalytic site. Both responses have the net effect of decreasing the rate of drug metabolism and thereby prolonging the half-life of the affected drug or active metabolite and modifying its pharmacodynamic effect (Lin and Lu, 1998).

In terms of metabolic drug interactions, inhibition of enzyme activity seems more relevant for study than induction. Inhibition often occurs immediately whereas induction usually takes time to bring about its effect. Inhibition occurs rapidly as soon as the inhibitor is present whereas induction takes some time to start (3 to 10 days) or stop (5 to 12 days) since protein synthesis must first occur and then cease.

Inhibition of CYP by drugs, chemicals or natural sources such as herbs can be either reversible or irreversible. Irreversible inhibition entails a biotransformation of the inhibitor, while reversible inhibition can take place directly, without metabolism. Reversible inhibition is the most common type of enzyme inhibition and can be further divided into competitive, non-competitive, uncompetitive, and mixed-type inhibitions (Lin and Lu, 1998).

Irreversible inhibition is also known as mechanism-based inhibition or suicidal inhibition. It is characterized by a time-dependent loss of enzyme activity and irreversible modification of the enzyme (Flockhart and Tanus-Santos, 2002). Suicide inhibition involves the formation of CYP-mediated formation of a reactive inhibitor. This covalently binds to the protein or heme of the CYP enzyme leading to its inactivation. Mechanism-based inhibition is potentially harmful, since re-synthesis of the new enzyme is the only approach to restore the enzyme activity, and the inhibitory effects of mechanism-based inhibitors remain after the elimination of the inhibitor from blood and tissue (Ohyama *et al.*, 2000). The most important phenomenon of mechanism-based inhibition is the time-, concentration-, and NADPH-dependent enzyme inactivation (Halpert, 1995). Classical mechanism-based inhibitors used as *in vitro* tools for selective inhibition of CYPs include furafylline (CYP1A2) (Sesardic *et al.*, 1990), paroxetine (CYP2D6) (Blobaum, 2006, Hollenberg *et al.*, 2008) and gestodene (CYP3A4) (Guengerich, 1990).

A clinically important interaction can occur when a drug or herb inhibits a drug-metabolizing enzyme (DME). Reversible inhibition is probably the most common for the many documented drug-drug or drug-herb interactions. Examples

include HMG-CoA reductase inhibitors (Lin and Lu, 1998). Different kinds of enzyme inhibition that can impair drug metabolism have however been described:

i) Competitive inhibition:

This is the most common kind of inhibition but it is unlikely to be the most clinically relevant. This competition occurs between the substrate (S) and inhibitor (I) for binding at the same position on the active site of the enzyme (E). The inhibitor can bind to E but not enzyme-substrate (ES) complex, therefore increases the binding efficiency, K_m (the inhibitor interferes with substrate binding) but does not affect maximum velocity, V_{max} (the inhibitor does not hamper the catalysis in ES because it does not bind to ES complex).

ii) Uncompetitive inhibition:

In uncompetitive inhibition the inhibitor binds only to the enzyme-substrate complex, but not to the free enzyme entity. The enzyme-substrate-inhibitor complex is nonproductive (Segel 1975; Lin and Lu, 1998). Under this mechanism, both V_{max} and K_m are decreased.

iii) Mixed-type inhibition:

In practice, with two substrates this type of inhibition displaying elements of both competitive and noncompetitive inhibition is the most frequently observed form of inhibition (Madan *et al.*, 2002). The inhibitor can bind both to the free enzyme and to the enzyme-substrate complex but their affinities for these two forms are different. This type interferes with substrate binding (increase K_m) and hampers catalysis in the ES complex (decrease V_{max}).

iv) Non-competitive inhibition:

This is a special case of mixed-type inhibition. The active binding site for the substrate and inhibitor is different from each other. The inhibitor has no effect on binding of substrate, but the enzyme-substrate-inhibitor complex is nonproductive. This type has identical affinities for both E and ES complex, therefore it does not change K_m (does not affect substrate binding) but it decreases V_{max} (inhibitor binding hampers catalysis).

1.2.8 Characterization of *in vitro* Potency of Inhibition

Inhibitory activity of selective chemical inhibitors of specific CYP enzymes can be expressed either as an inhibition constant (K_i) or the 50% inhibitory concentration (IC_{50}) (Bertz and Granneman, 1997) . The IC_{50} is an estimate of the concentration of the drug inhibiting the maximum rate of metabolism of a fixed concentration of substrate by 50%. It is a measure of the inhibitory potency (http://www.fda.gov/cder/guidance/clin3.pdf).

The IC_{50} values are useful when comparing the relative inhibitory potency of different candidate inhibitors. However, an IC_{50} value is unsuitable to be used in the *in vitro-in vivo* scaling, because it only equals the enzyme inhibition constant (K_i) in the case of noncompetitive inhibition (von Moltke *et al.*, 1998). For any other types of inhibition, the IC_{50} value is generally higher than the respective K_i value, and therefore this may result in an underestimation of the *in vivo* inhibitory effect of an inhibitor. For competitive inhibitors, the IC_{50} will approximate the K_i only if the substrate concentration studied is considerably below the K_m (the substrate concentration at which the rate of metabolism is half-maximal).

In vitro inhibition constant (K_i) of a drug or herb for a particular CYP isoform is useful in the investigation of drug interactions. The K_i is the measure of the affinity of the inhibitor for the enzyme. K_i require the study of inhibition at a range of concentrations for both the inhibitor and substrate. K_i values, while being theoretically dependent on the binding characteristics of the inhibitor, may vary with different substrates (Prakash *et al.*, 2000).

1.2.9 Study Hypothesis and Objectives

1.2.9.1 General Objective

The objective of this study is to generate an *in vitro* drug-herbs interaction assay for CYP2D6*1 and CYP2D6*10 and to use it to investigate the influence of *Eurycoma longifolia*, Jack on CYP2D6.

1.2.9.2 Specific Objectives

The specific objectives of this study are:

1. to clone and express the recombinant *CYP2D6*1* protein for *in vitro* catalytic study.
2. to clone and express the recombinant CYP2D6*10 for *in vitro* catalytic study.
3. to develop a high performance liquid chromatography (HPLC) method for the *in vitro* interaction study of CYP2D6.
4. to study the kinetic parameters of CYP2D6*1 and CYP2D6*10
5. to study *in vitro* effect of *Eurycoma longifolia*, Jack (ELJ) on CYP2D6*1 and CYP2D6*10 activity.

2 MATERIALS AND METHODS

The overall methodology of this study is shown in Figure 2.1. This study consists of 5 main components namely:

1. Cloning, expression, and characterization of CYP2D6*1 and NADPH-Cytochrome P450 reductase (CYP-reductase);
2. Generation, expression, and characterization of CYP2D6*10 and NADPH-Cytochrome P450 reductase;
3. Development of HPLC assay for quantification of bufuralol and 1'-hydroxy-bufuralol;
4. *In vitro* kinetic characterization of CYP2D6*1 and CYP2D6*10 using bufuralol as substrate;
5. *In vitro* interaction study of Eurycoma longifolia, Jack on CYP2D6*1 and CYP2D6*10.

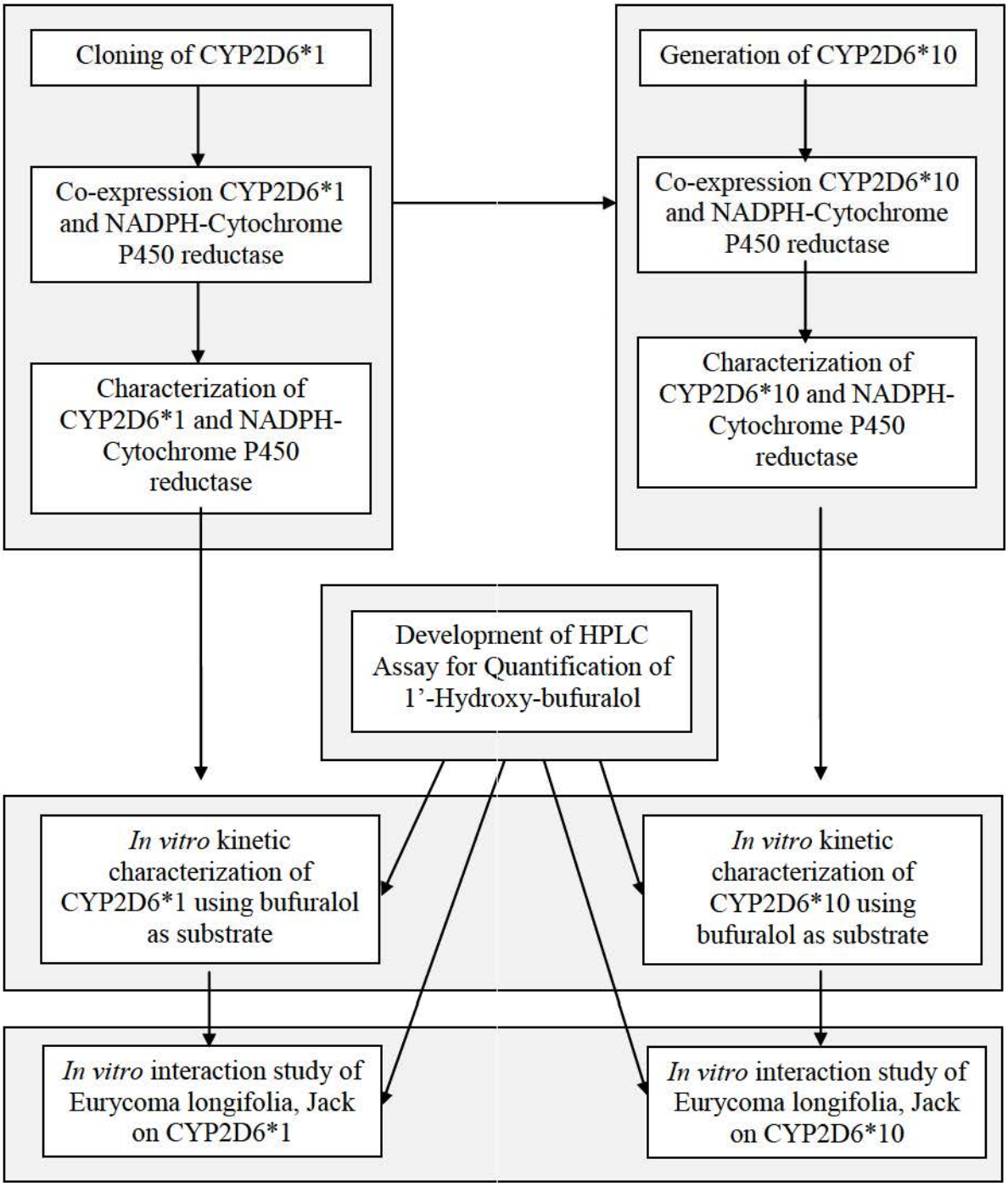

Figure 2.1 Overall study methodology

2.1 Cloning, Expression and Characterization of CYP2D6*1 and NADPH-Cytochrome P450 Reductase

Cytochrome P450 2D6 was cloned expressed and characterized together with NADPH-Cytochrome P450 reductase using method described below.

2.1.1 Chemicals, Reagents and Instruments

Reagents used are listed in Table 2.1 and the instruments are listed in Table 2.2.

Table 2.1 Reagent for cloning, expression and characterisation of CYP2D6*1 and NADPH-Cytochrome P450 reductase

No.	Reagent	Supplier
1.	Cytochrome *c*	Sigma. Poole, UK
2.	δ-aminolevulinic acid (δ-ALA)	Sigma. Poole, UK
3.	IPTG	Sigma. Poole, UK
4.	Lysozyme	Sigma. Poole, UK
5.	NADPH	Sigma. Poole, UK
6.	Sodium hydrosulfite	Sigma. Poole, UK
7.	Restriction enzymes	New England Biolabs, Beverly, US
8.	*Pfu*Turbo DNA polymerase	Stratagene, La Jolla, US
9.	*E. coli* strain DH5α F'IQ	Stratagene, La Jolla, US

Table 2.1 Continued

No	Reagent	Supplier
10.	QiAquick® PCR clean kit, Gel extraction kit and mini-preparation kit	Qiagen, Crawley, UK
11.	Primary and secondary Antibody	Chemicon, Temecula, US
12.	TOPO® cloning kit	Invitrogen, California, US

All other reagents and supplies were obtained from standard sources. CYP2D6 cDNA of wild type sequence was a gift from Professor Frank J Gonzalez from the National Institute of Health, USA.

Table 2.2 Instrumentation for cloning, expression and characterisation of CYP CYP2D6*1 and NADPH-Cytochrome P450 reductase

No.	Instruments	Supplier
1.	Analytical balance	Sartorius, Germany
2.	Autoclave machine	Hirayama Manufacturing Cooperation, Japan.
3.	Double beam recording spectrophotometer, model Varian CARY 50 UV/VIS spectrophotometer	Varian, Inc., Palo Alto, CA, U.S.A.
4.	Dry evaporating unit, model 18780	Pierce, Rockford, Illinois.
5.	Electrophoresis power supply, EC 250-90	ThermoQuest, E-C Apparatus Division, Holbrook, New York.
6.	Electrophoresis tank, model Mini and Maxicell®	Primo Submarine Gel system, ThermoQuest, E-C Apparatus Division, Holbrook, New York.
7.	Gel documentation system	Vilber Lourmat, Cedex, France
8.	Gel drying kit	Promega, Promega Corporation, Madison U.S.A.
9.	HPLC pump, Perkin Elmer series 200	PerkinElmer Life and Analytical Services, U.S.A.