THE CYTOCHROME P450 INHIBITORY EFFECTS OF ANDROGRAPHOLIDE AND 14-DEOXY-11, 12-DIDEHYDROANDROGRAPHOLIDE IN HEPG2 CELLS: DEVELOPMENT OF A MODEL FOR *IN VITRO* STUDIES OF DRUG-HERB INTERACTIONS

OOI JER PING

UNIVERSITI SAINS MALAYSIA

2010

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by

OOI JER PING

Thesis submitted in fulfillment of requirements for the degree of Master of Science

NOV 2010

ACKNOWLEDGEMENT

I would like to sincerely thank my supervisor, Dr. Tan Mei Lan, for her guidance, support, constructive ideas, understanding and most of all her patience. My special thanks to Dr. Tengku Sifzizul Tengku Muhammad and Dr. Shaida Fariza Sulaiman for giving me the support and encouragement when I needed them the most. I would like to express my gratitude to all lab members of 218, IPPT Clinical lab, and IPHARM for their kindness and support. I would like to take this opportunity to say a big thank-you to Kementarian Pengajian Tinggi for supporting this project under the Fundamental Research Grant Scheme (FRGS). Also, I would like to say thank-you to USM for the financial support under the USM Fellowship Scheme. Heartfelt thanks to my parents and wife, who never fail in providing me with their constructive "criticism".

Jer Ping

(2nd Nov 2010)

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LIST OF ABBREVIATIONS

ADR	Adverse drug reaction
AF-1	Activation function 1
AF-2	Activation function 2
AhR	Aryl hydrocarbon receptor
AhRR	AhR repressor
Arnt	Aryl hydrocarbon receptor nuclear translocator
ASC-2	Apoptotic speck protein-2
ATCC	American Type Culture Collection
bHLH	Basic-helix-loop-helix
BSA	Bovine serum albumin
BTE	Basic transcription element
CAR	Constitutive androstane receptor
CBP	CREB binding protein
CCRP	CAR cytoplasmic retention protein
CREB	cAMP response element binding
Ct	Threshold cycle
CYP	Cytochrome P450
Cys	Cysteine
DBD	DNA binding domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EM	Extensive metabolizer
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
Gly	Glycine
GR	Glucocorticoid receptor
GRIP	Glucocorticoid receptor-interacting protein
GTF	General transcription factor
Hsp20	Heat shock protein 20
ICZ	Indolo [2,3-*b*] carbazole
IM	Intermediate metaboliser
LBD	Ligand binding domain
MEM/EBSS	Minimum essential medium with Earle's salts
NES	Nuclear export sequence
NLS	Nuclear localization signal
p/CIP	p300/CBP/cointegrator-associated protein
p23	23-kDa heat shock protein
p300	E1A binding protein p300
PAS	Per-ARNT-Sim
PBP	PPAR-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC-1	PPARγ coactivator-1
PGC-1α	PPARγ coactivator-1 α
Phe	Phenylalanine
PKA	Protein kinase A
PKC	Protein kinase C
PM	Poor metaboliser

PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptors
PXR	Pregnane X receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NCoR	Nuclear receptor corepressor
NES	Nuclear export sequence
NR	Nuclear receptor
RE	Response element
RIP140	Receptor interacting protein 140
RNA	Ribonucleic acid
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RXR	Retinoic acid X receptor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHP	Short heterodimer partner
SMC-1	Structural maintenance of chromosome-1
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SNP	Single nucleotide polymorphism
SRC-1	Steroid receptor co-regulator-1
TBE	Tris-borate-ethylenediaminetetraacetic acid
TCDD	2',3',7',8'-Tetrachlorodibenzo-*p*-dioxin
TEMED	N, N, N', N'-tetramethylethylenediamine
TRIS	Tris(hydroxylmethyl)aminomethane
UM	Ultrarapid metaboliser
VDR	Vitamin D receptor
XAP2	X-associated protein 2

KESAN INHIBITORI TERHADAP SISTEM SITOKROM P450 OLEH ANDROGRAPHOLIDE DAN 14-DEOXY-11,12-DIDEHYDROANDROGRAPHOLIDE DALAM SEL HEPG2, PEMBINAAN *IN VITRO* MODEL UNTUK INTERAKSI DRUG-HERBA

ABSTRAK

Penggunaan herba sebagai rawatan alternatif dan/atau rawatan tambahan semakin meningkat di seluruh dunia. Apabila herba digunakan bersama ubatan moden ia mungkin akan menyebabkan interaksi drug-herba yang serius. Kebanyakan interaksi drug-herba dipengaruhi oleh sistem enzim sitokrom P450 (CYP), terutamanya CYP1A2, CYP2D6, dan CYP3A4. *Andrographis paniculata* Nees merupakan herba tradisional yang digunakan dalam rawatan demam, malaria, dan penyakit kencing manis. Dengan itu, kesan andrographolide dan 14-deoxy-11,12-didehydroandrographolide, sebatian aktif daripada *A. paniculata* ke atas ekspresi mRNA dan protein CYP1A2, CYP2D6, and CYP3A4 dalam sel HepG2 telah dikaji. Selepas 48 jam rawatan sel HepG2, analisis qRT-PCR dan western blot telah menunjukkan bahawa 20µg/mL andrographolide dan 5µg/mL 14-deoxy-11,12-didehydroandrographolide telah mengurangkan ekspresi mRNA and protein CYP1A2, CYP2D6, dan CYP3A4. Rawatan konkomitan diterpenoid dan agen pengaruh CYP (β-naphthoflavone dan dexamethasone) juga mengurangkan ekspresi mRNA dan protein CYP1A2 dan CYP3A4. Kedua-dua diterpenoid telah merencatkan kesan aruhan β-naphthoflavone dan dexamethasone ke atas ekspresi CYP. Aktiviti enzim CYP3A4 juga dikurangkan oleh kedua-kedua diterpenoid tersebut. Kesimpulannya, penyelidikan ini telah menunjukkan bahawa kesan inhibitori andrographolide and 14-deoxy-11,12-didehydroandrographolide ke atas

ekspresi mRNA dan protein CYP1A2, CYP2D6, dan CYP3A4 mungkin akan menghasilkan interaksi drug-herba sekiranya herba ini digunakan bersama dengan ubatan moden yang dimetabolismekan oleh enzim CYP yang sama. Pesakit dan profesional kesihatan perlu dinasihatkan tentang potensi interaksi drug-herba tersebut.

THE CYTOCHROME P450 INHIBITORY EFFECTS OF ANDROGRAPHOLIDE AND 14-DEOXY-11,12-DIDEHYDROANDROGRAPHOLIDE IN HEPG2 CELLS, DEVELOPMENT OF A MODEL FOR *IN VITRO* STUDIES OF DRUG-HERB INTERACTIONS

ABSTRACT

The use of herbs as alternative and/or complementary therapy is increasing worldwide. When herbs are co-administered with modern medicine it may lead to drug-herb interactions that may be clinically significant. Cytochrome P450 (CYP) enzymes have been implicated in a large number of these preventable drug-herb interactions, especially CYP1A2, CYP2D6, and CYP3A4. *Andrographis paniculata* Nees is a tropical herb traditionally used for fever, malaria, and diabetes mellitus treatments and has an extensive ethnobotanical history in Asia. Hence, the ability of andrographolide and 14-deoxy-11,12-didehydroandrographolide, the most medicinally active compound isolated from *A. paniculata* to modulate the CYP1A2, CYP2D6, and CYP3A4 mRNA and protein expressions were examined in HepG2 cells. After treatment of HepG2 cells for 48hr, the qRT-PCR and western blot analysis showed that 20μg/mL andrographolide and 5μg/mL 14-deoxy-11,12-didehydroandrographolide resulted in significant decreased in CYP1A2, CYP2D6, and CYP3A4 mRNA and protein expressions respectively. Interestingly, concurrent treatment of diterpenoid and CYP inducer (β-naphthoflavone and dexamethasone) also resulted in significant decreased in CYP1A2 and CYP3A4 mRNA and protein expressions implicating that both diterpenoids were able to inhibit the induction ability of CYP1A2 and CYP3A4 inducers. As for CYP3A4, both diterpenoids also caused a decrease in CYP3A4 enzymatic activity. In conclusion, this study clearly

indicated that andrographolide and 14-deoxy-11,12-didehydroandrographolide inhibited the mRNA and protein expressions of CYP1A2, CYP2D6, and CYP3A4. Hence co-administration of this herbal preparation together with modern drugs which are known to be metabolized via the same CYP pathway may cause drug-herb interactions. Patients and healthcare professional should be advised of the possible drug-herb interactions.

INTRODUCTION

1.1 Drug interactions

Drug interactions refer to adverse drug reactions (ADR) produced by the administration of a drug or co-exposure of the drug with another substance which modifies the patient's response to the drug (Shargel, 2004). When a regimen of two or more drugs are prescribed, drug interactions become an important consideration for patients and physicians (Shapiro and Shear, 1999). Non-prescription drugs, herbs or alternative medicine, nutrient, and foods may also be implicated in drug interactions leading to drug-herb interactions, drug-nutrient interactions, and drug-food interactions respectively (Sorensen, 2002). These interactions could lead to therapeutic failure, adverse drug reactions, and even death.

As of January 1, 2002, there were more than 3200 prescription drugs, 300 dietary supplements, and 600 herbal products available in the market in the United States of America (USA) (Prybys, 2004). This equates to trillions of possible drug combinations and potential drug interactions (Prybys, 2004). Widespread use, especially marketing approval of modern drug, sometimes results in the discovery of new drug interactions. For several drugs, the late identification of drug interactions has led to restrictions being placed on the indications, or even withdrawal of drug from the market. For example, Mibefradil (Posicor®), a calcium channel blocker used to treat hypertension, was voluntarily withdrawn from the market early in June 1998 because of severe drug interactions (SoRelle, 1998). Mibefradil was found to potently inhibit cytochrome P450 (CYP), namely CYP2D6 and CYP3A4, a major group of haem containing monooxygenases involved in the metabolism of endogenous substances (SoRelle, 1998). Cardiac shock and death was reported in a number of cases when mibefradil was administered with other cardiotropic drugs

such as calcium channel blockers and β-blockers (SoRelle, 1998). Other examples such as cisapride (Prepulsid®, Janssen-Ortho), astemizole (Hismanal®, Janssen-Pharmaceutica), and terfenadine (Seldane®, Aventis) were also withdrawn from the USA market due to drug interactions (Dresser and Bailey, 2003). Since specific diagnostic codes for drug interactions are lacking, it is difficult to obtain precise rates of incidence and prevalence (Shapiro and Shear, 1999).

1.1.1 Drug-herb interactions

Herbs and botanically derived therapies have been used for variety of ailments for thousands of years, dating to 50,000 B.C (Venkataramanan et al., 2006). As a matter of fact, a lot of modern drugs were actually derived from plants. For example, morphine which is used as a painkiller is derived from opium poppy (*Papaver somniferum* L.); digitalis used in heart medication is from foxglove (*Digitalis purpurea*), and reserpine, an antihypertensive drug, is from rauwolfia (*Rauwolfia serpentine*). According to World Health Organization (WHO), millions of people today use herbal therapies along with prescription and non-prescription medications. 70-80% of the world population especially in developing countries relies on traditional medicine, mostly plant drugs for their primary health care needs (Barrett et al., 1999, Gedif and Hahn, 2002). In 1999, the global market for herbal supplements exceeded US$15 billion, with US$7 billion in Europe, US$2.4 billion in Japan, US$2.7 billion in Asia and US$3 billion in North America (Raskin et al., 2002). In USA, it is reported that from 1990-1997, the total visits to alternative medicine practitioners has increased from 427 million to 629 million (Eisenberg et al., 1998). The number of visits to alternative medicine practitioners was even higher than the total visits to primary care physicians (Eisenberg et al., 1998).

In Malaysia, traditional products form the largest group of medicines that was registered (37.1%), as compared with prescription drugs (32.2%) and non-prescription (over-the-counter) drugs (23.9%) (Ang, 2005). From 2000 to 2005, annual sales for traditional medicines in Malaysia has increased from US$ 385 million to US$ 1.29 billion and the herbal medicines industry was expected to be worth US$ 2.5 billion by 2010 (Aziz and Tey, 2009). This specific industry is growing faster than the general economy, reported at more than 15-20% per year (Aziz and Tey, 2009). Such huge market size, globally and locally, had led to worldwide interest in the scientific validation of the therapeutic efficacy of traditional plant-based medicines.

A high percentage of patients (up to 60%) using alternative therapies are reported to have never informed their physician of their herb use (Halsted, 2003). In most cases, the widespread usage of herbs by the patients are solely based on the basic of claims and anecdote and this is particularly worrying because herbs are often used concurrently with prescribed or over-the-counter medications without the knowledge of the pharmacists and physicians (Huang et al., 2004, Eisenberg et al., 1998, Kaufman et al., 2002). Most patients assume "natural" products are harmless and not worth mentioning, or they fear being ridiculed by doctors sceptical about their use (Kaufman et al., 2002). There is little public understanding or appreciation of the fact that these “natural” herbs are actually a combination of potentially biologically active compounds that exist in unknown quantities. It is reported that an estimated 15 million adults in 1997 took prescription medications concurrently with herbal remedies and/or high-dose vitamins (Eisenberg et al., 1998). As such, patients taking prescription medications metabolized through the same pathways as the herbs

are at an increased risk of drug-herb interactions that could lead to therapeutic failure, adverse drug reactions, and even death.

In Malaysia, the number of adverse reaction cases attributable to traditional medicine was reported to be 31 cases in 2007 and 26 cases in 2008 according to a report by the Malaysian Adverse Drug Reaction Advisory Committee (MADRAC), National Pharmaceutical Control Bureau, Ministry of Health (http://portal.bpfk.gov.my/bpfk/). However, these numbers of reported cases most probably do not reflect the actual frequency of adverse reactions caused by traditional herbal preparations as most cases go unreported. Moreover, the number of adverse reaction due to drug interactions was not known.

One of the most extensively reported example for drug-herb interactions involved *Hypericum perforatum* (St. John's wort), a popular herbal remedy for depression and mood disorders (Sorensen, 2002). St. John's wort is reported to be involved in a number of drug-herb interactions when consumed with cyclosporine, ethinyl estradiol/desogestrel, theophylline, phenprocoumon, warfarin, amitriptyline, indinavir, digoxin, nefazodon, sertraline, or paroxetinev causing undesired outcomes (Di Carlo et al., 2001). St. John's wort extracts activate CYP3A4 which is the most important of the CYP family of hepatic enzymes involved in the metabolism of many common drugs (Ernst, 1999, Moore et al., 2000a). Concomitant use of St John's wort with drugs metabolized by CYP3A4 will bring about an accelerated clearance of these compounds which results in reduced efficacy.

Given the widespread use of herbs worldwide, documented drug-herb interactions are sparse. In many cases, the drug-herb interactions may increase drug toxicity or even be fatal. Thus, as an integrated part of medical treatment, monitoring of adverse events when herbs are co-administered with drugs could be carried out

and potential drug-herb interactions could be identified. This would enable more accurate product labelling and provision of useful information on potential drug-herb interactions to medical professionals.

1.2 Cytochromes P450 (CYPs)

1.2.1 Biochemistry of cytochromes P450

An analysis revealed that 59% of drugs cited in adverse drug reaction (ADR) studies are metabolized by phase I drug-metabolizing enzymes and that CYP enzymes account for 80% of phase I drug-metabolizing enzymes (Phillips et al., 2001). Thus, understanding of drug interactions involving CYP enzymes and their mechanism can help to evaluate potential ADRs when herbs are prescribed concurrently and this will contribute to a much more effective and safe management of medications.

Cytochrome P450 (CYP) enzymes are a major group of haem containing monooxygenases involved in the metabolism of endogenous substances, such as corticosteroids and fatty acids, and xenobiotics, including carcinogens and medications (Mansuy, 1998). CYP enzymes are a superfamily of oxidative enzymes with 57 different active genes encoding them (Ingelman-Sundberg, 2004). The CYP enzymes were first reported in 1958 from two independent researchers as an unusual cytochrome (Garfinkel, 1958, Klingenberg, 1958), which later became known as P450 due to its characteristic soret absorption maximum at 450nm in the UV spectrum of its carbon monoxide adduct (Omura and Sato, 1962, Omura and Sato, 1964a, Omura and Sato, 1964b). The enzymes exist as multiple forms with different properties in respect of their substrate selectivity and certain physicochemical characteristics (Lewis, 2001). The multiplicity of forms led to the enzymes being

termed “mixed-function” oxidases and “monooxygenases” to describe their ability to insert oxygen into a large variety of substrates and wide range of structural classes of compounds (Kanamura and Watanabe, 2000, Porter and Coon, 1991). The CYP enzymes are haemoproteins that generally function in mixed-function oxidase reactions, using the simplified stoichiometry as shown in Figure 1.1 (Lewis, 2001).

The CYP enzymes mixed-function oxidases catalyze a wide variety of reactions including epoxidations, N-dealkylations, O-dealkylations, S-oxidations, and hydroxylations of aliphatic and aromatic substrates (Lewis, 2001). The diversity of the reactions catalyzed can be understood by considering that the initial reaction in all cases involves insertion of a hydroxyl group into the substrate (RH) to form a hydroxylated intermediate (ROH) which can then, depending on the nature of the substrate and the stability of the intermediate, undergo phase II metabolism (Figure 1.1). The first step involves binding of the substrate to the ferric form of the CYP enzyme. The second step involves transfer of one electron from the NADPH-CYP reductase to the iron of the ferric CYP enzyme to give a ferrous enzyme- substrate complex. The reduced CYP-substrate complex then binds with O_2 to form a ferrous enzyme-O_2-substrate ternary complex. The addition of a second electron to this tertiary complex by the reductase results in the formation of an iron peroxo- species. The next step involves cleavage of the oxygen-oxygen bond. One of the oxygen is released with the uptake of two protons, resulting in the formation of water. The retained oxygen remains associated with the heme iron as activated oxygen. The activated oxygen atom associated with the iron is then inserted into the substrate, resulting in a two-electron oxidation of the substrate to the alcohol. The product is then released, regenerating the native ferric CYP enzyme that is available to begin another catalytic cycle.

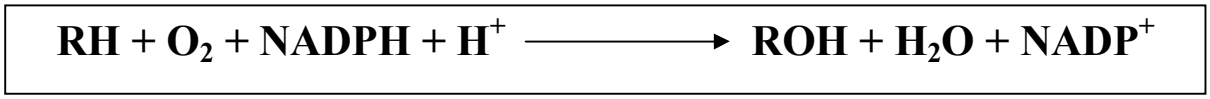

Figure 1.1 Stoichiometry of CYP enzymes mixed-function oxidase reaction

All CYP enzymes, or more correctly called as heme-thiolate monooxygenases (Bachschmid et al., 2005), have a common characteristic of a conserved peptide motif, Phe – $X_{(6-9)}$ – Cys – X – Gly (where X denotes any amino acid), near the C terminus (Nebert and Dalton, 2006). An octahedral heme iron binds to the fifth position of the cysteine and is capable of transferring one atom of atmospheric oxygen into the substrate during phase I metabolism. The oxygenated product is then hydroxylated in phase II metabolism (Lewis, 2001).

1.2.2 Nomenclature system of cytochromes P450

Early naming system for CYP enzymes was developed based on designations of CYP enzyme column fractions obtained by each investigator. For example, CYP2D1, CYP2D2, CYP2D3, CYP2D4, and CYP2D5 of rat were named as db1, db2, db3, db4, and db5 respectively (Lewis, 2001). Such naming system was confusing because the designations of the CYP enzymes were not always unified and result in the presence of multiple designations for a same CYP enzyme, e.g. human CYP3A4 was called in different name as hPCNI, nf-25, and nf-10. A more systematic nomenclature system for human superfamily CYP enzymes was developed and periodically updated by Nebert (Nebert, 1997, Nelson et al., 1993). In this nomenclature system designed by Nebert, human CYP enzymes are now arranged into 18 families and 57 subfamilies based on their amino-acid sequence identity. This "new" nomenclature system makes use of the symbol CYP as an abbreviation of cytochrome P450, which is italicized when referring to the gene. Proteins that have >40% sequence similarity are members of the same gene family, whereas those with >70% similarity are members of the same subfamily. An alphanumerical designation is employed for naming P450 families, subfamilies, and

individual proteins as listed in Table 1.1. This CYP nomenclature system is not only meant to be used on classification of human CYPs but also other species CYPs such as rat, mouse, guinea pig, toadfish, drosophila, mosquito etc ((Lewis, 2001). Nebert's CYP nomenclature system has successfully unified all the CYP enzymes designation and reduce confusion and enable the inter-species comparison of CYP enzymes.

1.2.3 Cytochromes P450 in liver

CYP enzymes play a huge role in metabolism process of many xenobiotics and endogenous substrate in the liver (Table 1.1). The CYP enzymes are highly conserved in all five biological kingdoms from Archebacteria to humans (Nebert et al., 1989), with the apparent exception of enterobacteria (Nelson et al., 1993, Nelson et al., 1996). It is thought that the CYP enzymes responsible for foreign compound metabolism evolved about 400-500 million years ago to enable animals to detoxify chemicals in plants (Gonzalez and Gelboin, 1994, Gonzalez and Nebert, 1990, Nebert, 1997). In mammalian species, CYP enzymes are present predominantly in the smooth endoplasmic reticulum (ER) membrane of hepatocytes in liver (Stier, 1976). They are also expressed in most other organs and tissues such as the kidney, breast, prostate, skin, nasal epithelium, gonads, placenta, brain, lung, spleen, pancreas, and gastro-intestinal tract (Lewis, 2001).

However, liver remains as the major site for CYPs expression and different human CYP isoforms vary in their abundance within liver, as shown in Table 1.2. CYP3As are the most abundant CYP enzymes in liver (28%), follow by CYP2Cs (18%), and CYP1A2 (13%) (Lewis, 2001). These three CYPs sum up for about 60% of all CYPs while other CYPs are only expressed in a relatively low percentage.

Table 1.1 Human CYP enzymes (Nebert and Dalton, 2006)

Family	Number of subfamilies	Number of genes	Substrates/function
CYP1	2	3	Metabolism of xenobiotics and eiscosanoids
CYP2	13	16	Metabolism of xenobiotics and eiscosanoids
CYP3	1	4	Metabolism of xenobiotics and eiscosanoids
CYP4	6	12	Metabolism of xenobiotics and eiscosanoids
CYP5	1	1	Thromboxane A_2 synthase
CYP7	2	2	Cholesterol, bile acid synthesis
CYP8	2	2	Prostacyclin synthase, bile acid synthesis
CYP11	2	3	Steroidogenesis
CYP17	1	1	Steroid 17-hydroxylase, 17/20-lyase
CYP19	1	1	Oestrogen aromatization
CYP20	1	1	Expressed in gastula, neural patterning and somitogenesis, organogenesis, fetus and nasopharynx.
CYP21	1	1	Steroid 21-hydroxylase
CYP24	1	1	Vitamin D_3 24-hydroxylase
CYP26	3	3	Retinoic acid hydroxylation
CYP27	3	3	Bile acid biosynthesis, vitamin D_3 hydroxylations
CYP39	1	1	24-hydroxycholesterol 7-hydroxylase
CYP46	1	1	Cholesterol 24-hydroxylase in the central nervous system
CYP51	1	1	Lanosterol 14-demethylase

Interestingly, the highly abundance number of certain CYP enzymes may not exactly explain their "real" capacity in drug metabolism in human liver. For example, even though CYP2D6 is only a relatively minor form, 2.5%, in human liver, it metabolizes up to 18.8% of all prescribed drugs as compared to CYP1A2 enzymes (Table 1.2). The relative data given in Table 1.2 may differ among individuals as the expression of CYPs are generally affected by various intrinsic and extrinsic factors such as genetic variation, drug consumption, nutrient and diet status which may affect the expression of CYPs (Lewis, 2001).

1.2.4 Regulation of cytochromes P450

Four of CYP gene families (families CYPs 1–4) code for liver-expressed enzymes that metabolize foreign compounds (drugs, environmental chemicals, and other xenobiotics) and endogenous lipophilic substrates. These CYP genes are regulated in a variety of ways and at multiple levels: they exhibit tissue-specific expression, are regulated by endogenous hormones and cytokines, and respond to structurally diverse foreign chemicals, which often increase CYP protein levels by stimulating P450 gene transcription initiation and are regulated by nuclear receptors (NR) (Honkakoski and Negishi, 2000). The induction of CYP1 family member expression is regulated by a heterodimer composed of the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator (Arnt) (Fujii-Kuriyama and Mimura, 2005, Kawajiri and Fujii-Kuriyama, 2007) while the expression of CYP2, 3, and 4 family members are regulated by the nuclear receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR), and peroxisome proliferator-activated receptors (PPAR), respectively (Waxman, 1999, Karpen, 2002, di Masi et al., 2009). CAR, PXR, and PPAR belong to nuclear receptor (NR)

Table 1.2 **Relative abundance of CYP isoenzymes in human liver and relative contribution of CYP enzymes to drug metabolism (Lewis, 2001)**

CYP	Relative abundance in human liver	Relative contribution to drug metabolism
1A1	< 1%	2.5%
1A2	~ 13%	8.2%
1B1	< 1%	Unknown
2A6	~ 4%	2.5%
2B6	< 1%	3.4%
2C8, 2C9	~ 18%	15.8%
2C18, 2C19	~ 1%	8.3%
2D6	≤ 2.5%	18.8%
2E1	≤ 7%	4.1%
2F1	< 1%	1.3%
3A4, 3A5	≤ 28%	34.1%

superfamily while AhR belong to basic helix loop helix (bHLH) family of transcription factors.

1.2.4.1 Overview of nuclear receptors (NR)

In humans, 49 members of the nuclear receptor (NR) family have been definitively cloned and identified (Robinson-Rechavi et al., 2001, Kininis and Kraus, 2008). Members of the family share a common structure that includes a variable amino-terminal domain, a highly conserved central DNA binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Mangelsdorf et al., 1995). Typically, there are two transcriptional activation domains in a NR: the activation function 1 (AF-1), which resides in the N-terminal domain and the activation function 2 (AF-2), which is present in the C-terminal portion of the LBD as shown in Figure 1.2 (Mangelsdorf et al., 1995, Mangelsdorf and Evans, 1995).

Poorly-conserved AF-1 is allocated at the N-terminus and is responsible for the ligand-independent transcriptional activation and involved in the coordinated interaction of co-activators and co-repressors (Honkakoski and Negishi, 2000). Immediately adjacent to AF-1 is a highly-conserved DBD which contains two zinc finger motifs and responsible for high-affinity recognition and binding to the canonical DNA hexamer sequences comprising the specific response elements (REs) (Mangelsdorf and Evans, 1995, Mangelsdorf et al., 1995). A small hinge region facilitates the three-dimensional functional organization of the multiple domains. The C-terminus region, AF-2, provides ligand-dependent transactivation, via a complex three-dimensional conformational switch to coordinate interactions with co-regulators. The LBD serves the greatest differentiating and identifying function among NR family members, determining the affinity of receptors for various

Figure 1.2 **Nuclear receptor domains and DNA binding elements.** AF-1: Activation function 1; DBD: DNA binding domain; LBD: Ligand binding domain; AF-2: Activation function 2 (Mangelsdorf et al., 1995, Mangelsdorf and Evans, 1995).

potential ligands, and is responsible for the species variability of ligand responsiveness (Edwards, 2000).

The ligands for the NRs are all small and lipophilic in nature, which permits them to diffuse into cells (Handschin and Meyer, 2003). The binding of a ligand to the LBD results in a conformational change in the AF-2 that disrupts interactions with transcriptional co-repressor proteins such as nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) and permits interactions with transcriptional coactivator proteins such as the steroid receptor co-regulator-1 (SRC-1) family members (Edwards, 2000). The activated NR stimulates the expression of target genes by binding to short DNA sequence motifs, termed response elements (RE), located in the regulatory regions of target genes.

1.2.4.2 Regulation of cytochrome P450 2 (CYP2) by constitutive androstane receptor (CAR)

CAR is a member of nuclear receptor superfamily. CAR is sequestered in the cytosol in a multi-protein complex which includes a recently identified protein termed CAR cytoplasmic retention protein (CCRP) and the Hsp90 (Figure 1.3) (Kobayashi et al., 2005, Timsit and Negishi, 2007). CAR is cytoplasmic in the dormant state and translocates to the nucleus upon activation by ligand. CAR can also be activated in a ligand-independent manner which does not necessarily require the direct binding of ligands (Honkakoski and Negishi, 2000). Phenobarbital and bilirubin have been shown to be CAR activators, but they do not appear to interact directly with the CAR-LBD (Moore et al., 2000b, Swales and Negishi, 2004). Activators can promote the nuclear translocation of CAR by a ligand-independent

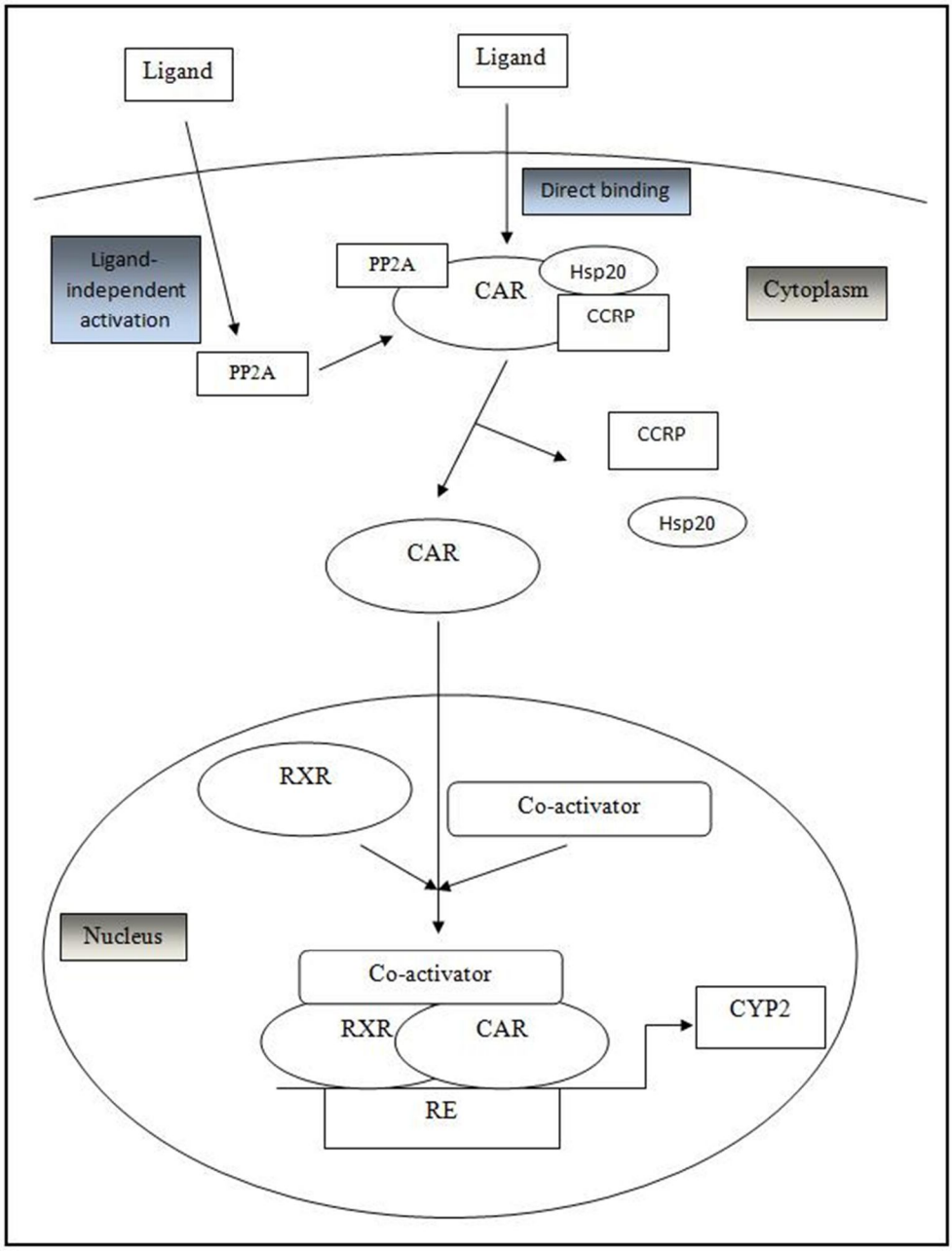

Figure 1.3 **CAR signalling pathway.** CAR: Constitutive androstane receptor; PP2A: protein phosphatase 2a; Hsp20: heat shock protein 20; CCRP: CAR cytoplasmic retention protein; RXR: retinoic acid X receptor; RE: response element. (di Masi et al., 2009)

process, regardless of their receptor-binding ability (Waxman, 1999, di Masi et al., 2009). Over expression of phosphatase 2A (PP2A) and mouse CAR enhances mouse CAR nuclear translocation in hepatoma cells. However, okadaic acid, a protein phosphatase inhibitor, blocks the phenobarbital-induced target gene expression in rodents (Sidhu and Omiecinski, 1997) and prevents nuclear accumulation of CAR (Honkakoski and Negishi, 1998).

Translocation of CAR to the nucleus is followed by CAR association with another NR, retinoic acid X receptor (RXR) through heterodimerization forming CAR/RXR complex (di Masi et al., 2009, Waxman, 1999). CAR-DBD and RXR-DBD heterodimerize in the ‘‘head-to-tail” orientation. The interaction between CAR-DBD and RXR-DBD involves Tyr21, Asn24, and Asp79 of CAR-DBD and Arg172 and Arg186 of RXR-DBD. CAR-DBD binds to the RE and recruitment of co-activators such as apoptotic speck protein-2 (ASC-2), GRIP1/TIF2, PPAR gamma co-activator-1 (PGC-1), structural maintenance of chromosome-1 (SMC-1), and SRC-1 occurs leading to the expression of CYP2 (Kim et al., 1998, Muangmoonchai et al., 2001, Min et al., 2002a, Min et al., 2002b, Shiraki et al., 2003, Choi et al., 2005, Inoue et al., 2006).

1.2.4.3 Regulation of cytochrome P450 3 (CYP3) by pregnane-X receptor (PXR)

PXR is also a member of nuclear receptor superfamily and appears to be located in the cytosol in the resting state, and the AF-2 region appears to be involved in pregnenolone 16α-carbonitrile-induced nuclear translocation (Matias et al., 2000). Like CAR, PXR forms a protein complex with CCRP and Hsp90 which, in turn, increase the cytosolic retention of PXR (Figure 1.4) (Squires et al., 2004). Upon

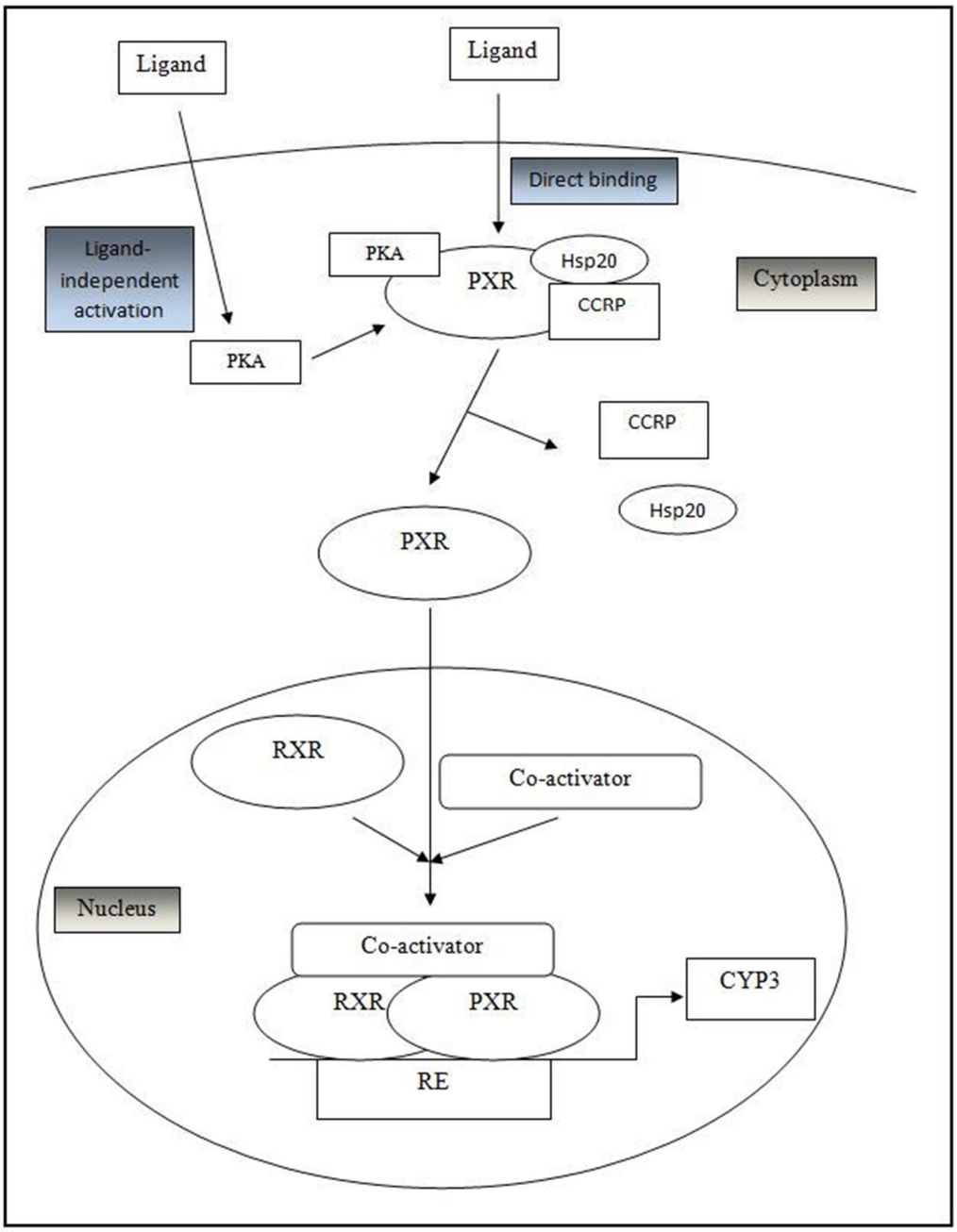

Figure 1.4 **PXR signalling pathway.** PXR: pregnane X receptor; PKA: protein kinase A; Hsp20: heat shock protein 20; CCRP: CAR cytoplasmic retention protein; RXR: retinoic acid X receptor; RE: response element. (Honkakoski and Negishi, 2000)

ligand binding, the PXR dissociates from the multi-protein complex and translocates to the nucleus to activate gene transcription as a heterodimer with RXR (Goodwin et al., 1999, Frank et al., 2005). Activation of PXR by ligands could result in the dissociation of co-repressors, such as the SMRT and NCoR, allowing the binding of the co-activators glucocorticoid receptor-interacting protein (GRIP) and SRC-1. While SMRT and NcoR stabilize chromatin and consequently repress transcription, SRC-1 and GRIP destabilize chromatin allowing the transcription machinery recruitment on DNA (Harmsen et al., 2007).

Increased transcription is mediated by recruitment of the p160 family co-activators, SRC-1, GRIP, PPAR-binding protein (PBP), and proliferator-activated receptors γ (PPARγ) coactivator-1 α (PGC-1α) (Ding and Staudinger, 2005a, Orans et al., 2005). On the other hand, the PXR transcriptional activity is inhibited by interactions with co-repressors including NCoR, RIP140, short heterodimer partner (SHP), and SMRT (Orans et al., 2005). PXR can be phosphorylated by protein kinase A (PKA), resulting in strengthened interaction with co-activators, such as SRC-1 and PBP (Ding and Staudinger, 2005a). In contrast, the activity of PXR can be repressed by the activation of protein kinase C α (PKCα) which alters the phosphorylation status of PXR and/or PXR-interacting proteins (i.e.,co-activators) (Ding and Staudinger, 2005a). In addition, okadaic acid, a protein phosphatase inhibitor, strongly represses PXR transactivation (Ding and Staudinger, 2005b).

In fact, the regulation of CYP expressions are found to be more complicated as cross-talk among NRs occurred (Honkakoski and Negishi, 2000). CYP-regulatory NRs belong to the same NR gene family (family NR1), share a common heterodimerization partner, retinoid X-receptor (RXR), and are subject to cross-talk interactions with other nuclear receptors and with a broad range of other intracellular

signalling pathways, including those activated by certain cytokines and growth factors (Honkakoski and Negishi, 2000, Waxman, 1999). These NRs include CAR, PXR, and PPAR. In man, PXR mediates xenobiotic-mediated induction of CYP3A4 (Bertilsson et al., 1998, Blumberg et al., 1998, Lehmann et al., 1998), CYP3A7 (Bertilsson et al., 2001, Pascussi et al., 1999), and CYP2B6 (Goodwin et al., 2001). Some reports suggest that CYP2C8 and CYP2C9 are also regulated by PXR (Gerbal-Chaloin et al., 2002, Gerbal-Chaloin et al., 2001, Synold et al., 2001). Thus, PXR coordinately regulates genes involved in the metabolism and elimination of potentially harmful xenobiotics. PXR interacts with its cognate response elements in the 5'-flanking region of target genes by forming a heterodimer with the 9-cis-retinoid acid receptor (RXR). In rat CYP3A23 and human CYP3A4, these elements consist of two copies of the AG(G/T)TCA hexanucleotide organized as a direct repeat with a three-nucleotide spacer and an everted repeat separated by 6 bp respectively. It is reported that these cognate response elements are also recognized by CAR (Sueyoshi et al., 1999) and vitamin D receptor (VDR) (Drocourt et al., 2002, Thummel et al., 2001) after heterodimerization with RXR. These observations suggest that these receptors are capable of regulating a same series of genes through the same cis-acting elements.

1.2.4.4 Regulation of cytochrome P450 1 (CYP1) by aryl hydrocarbon receptors (AhR)

Unlike CAR and PXR, aryl hydrocarbon receptor (AhR), a member of the basic-helix-loop-helix (bHLH)–Per-ARNT-Sim (PAS) gene superfamily, is a ligand-activated transcription factor that functions as an intracellular mediator in the xenobiotic signalling pathway (Hahn, 2002). Normally, AhR exists in a dormant

state within the cytoplasm in association with a complex of heat shock protein 90 (Hsp90), X-associated protein (XAP2), and 23-kDa heat shock protein (p23) (Figure 1.5). Upon ligand binding, AhR in the complex is activated by a conformation change that exposes a nuclear localization signal (NLS). 2',3',7',8'-Tetrachlorodibenzo-*p*-dioxin (TCDD) and indolo [2,3-*b*] carbazole (ICZ) are the most potent inducers of CYP1 expression (Fujii-Kuriyama and Mimura, 2005, Kawajiri and Fujii-Kuriyama, 2007). The ligand-activated AhR in the complex translocates into the nucleus and forms a heterodimer with the closely related Arnt protein already present in the nucleus by dissociating from the complex (Mimura and Fujii-Kuriyama, 2003, Hankinson, 1995).

However, it is also reported that AhR is activated in the absence of obvious ligands in Hepa 1clc7 cells (Sadek and Allen-Hoffmann, 1994b), human keratinocytes (Sadek and Allen-Hoffmann, 1994a), 10T1/2 fibroblasts (Cho et al., 2004), and HaCaT cells (Ikuta et al., 2004) grown under specific culture conditions such as loss of cell-cell contact, low cell densities, and in Ca^{2+}-deficient medium. Under such specific culture condition, the expression of AhR-responsive reporter is activated and further induces the nuclear accumulation of AhR (Cho et al., 2004). The nuclear accumulation of AhR is regulated by the phosphorylation of Ser68 within the nuclear export sequence (NES) of AhR (Ikuta et al., 2004). Tyr320 is another putative phosphorylation site on AhR activated by omeprazole in a ligand-independent manner via a signal transduction pathway that involves protein tyrosine kinases (Backlund and Ingelman-Sundberg, 2005). This pathway is independent from that induced by high-affinity ligands, such as TCDD. Although the protein kinases involved remain unclear, AhR can be activated in a ligand-independent manner.

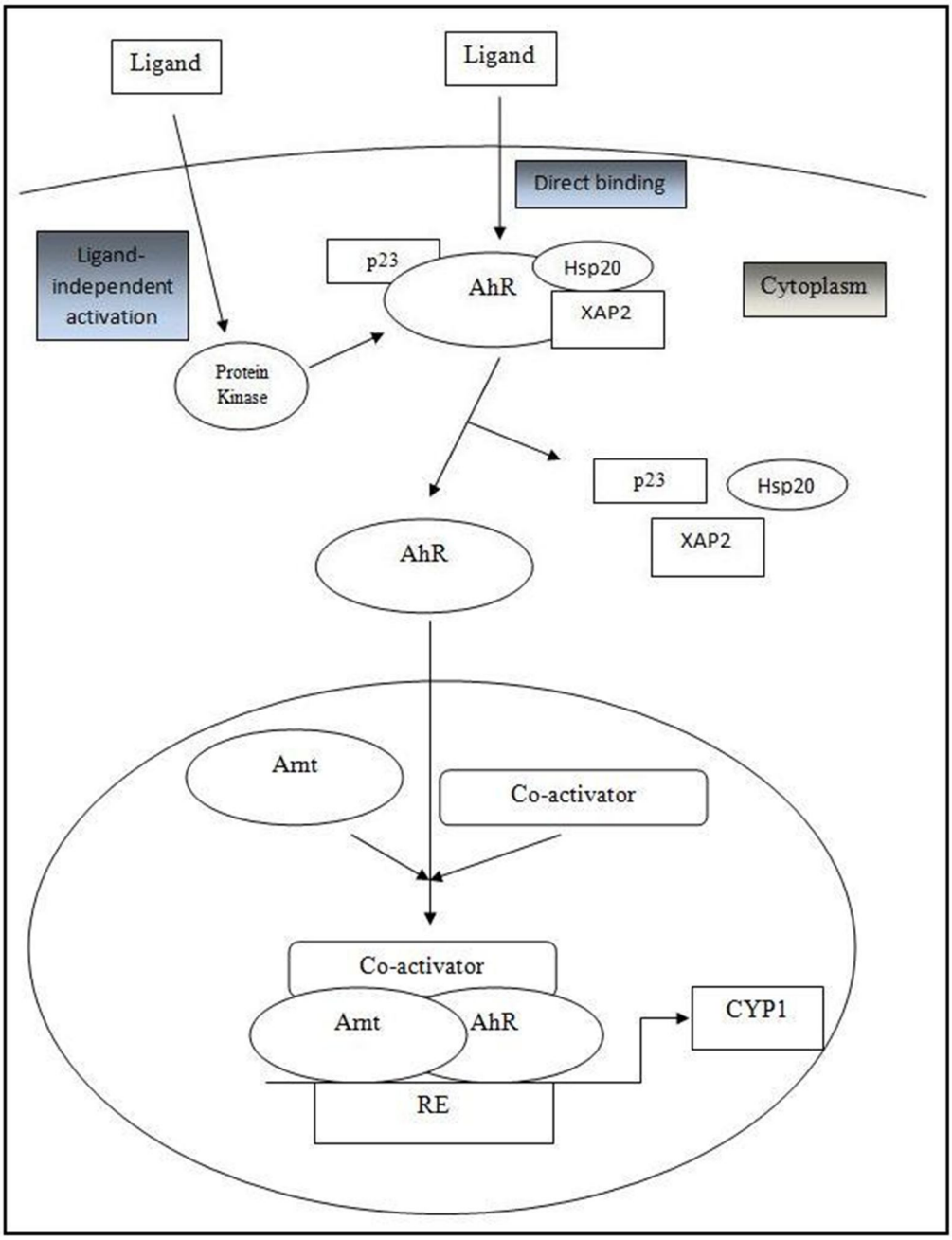

Figure 1.5 **AhR signalling pathway.** AhR: aryl hydrocarbon receptor; p23: 23-kDa heat shock protein; Hsp20: heat shock protein 90; XAP2: X-associated protein; Arnt: aryl hydrocarbon receptor nuclear translocator; RE: response element (Fujii-Kuriyama and Mimura, 2005).

The TCDD-induced expression of CYP1A1 is mediated through the RE (Fujisawa-Sehara et al., 1987). The core consensus sequence of RE is 5'-TNGCGTG-3', and this site is recognized by the AhR/Arnt heterodimer. Approximately 1 kb upstream of the CYP1A1 gene, a cluster of REs functions as an enhancer element, and a basic transcription element (BTE), a GC box sequence localized to the proximal promoter of CYP1A1, is also required for the induction of CYP1A1 (Kobayashi et al., 1996). Chromatin re-modelling is initiated by liganded AhR/Arnt heterodimer binding to the REs in the enhancer region, and this leads to increased DNase sensitivity and the appearance of a DNase hypersensitive site within 300bp upstream of the transcription initiation site. The AhR/Arnt heterodimer transactivates in conjunction with general transcription factors (GTFs) through interactions with coactivator proteins including CREB binding protein/ E1A binding protein p300 (CBP/p300), SRC-1, NCoA-2 and p300/CBP/cointegrator-associated protein (p/CIP), and the coactivator/corepressor protein, receptor interacting protein 140 (RIP140) (Beischlag et al., 2002).

However, less is known about the factors regulating the induction of CYP1A2 expression, although the AhR/Arnt heterodimer is clearly required for induction to occur. One study suggests that the AhR/Arnt heterodimer may function as a coactivator without directly binding the RE. Instead, it may interact with other DNA-binding factors of a novel xenobiotic responsive element termed XREII to induce transcription activation (Sogawa et al., 2004).

AhR is rapidly degraded both *in vivo* and *in vitro* following ligand binding and several studies have examined the regulation of AhR degradation (Fujii-Kuriyama and Mimura, 2005, Kawajiri and Fujii-Kuriyama, 2007). When AhR is fused to the heterologous NLS of nucleoplasmin, it constitutively accumulates in the

nucleus and is degraded in a 26S proteasome-dependent manner (Roberts and Whitelaw, 1999). Conversely, when nuclear export of AhR is blocked by leptomycin B, AhR accumulates in the nucleus following ligand binding and is not efficiently degraded. In this system, AhR degradation requires both an NES and redistribution from the nucleus to the cytoplasm (Davarinos and Pollenz, 1999).

The AhR repressor (AhRR) is identified as a negative regulator of AhR activity. AhRR contains both NLS and NES that are homologous to AhR, but AhRR is localized constitutively to the nucleus (Fujii-Kuriyama and Mimura, 2005, Kawajiri and Fujii-Kuriyama, 2007). Here, AhRR forms a heterodimer with Arnt, but RE binding by the AhRR/Arnt heterodimer leads to transcriptional repression. Finally, AhRR expression is induced in an AhR dependent manner, indicating that AhR and AhRR form a regulatory feedback loop (Mimura et al., 1999).

1.2.5 The role of cytochromes P450 in drug interactions

CYP enzymes are found to be involved in metabolism of 90% drugs available in the market nowadays (Lynch and Price, 2007). During this process, drugs are metabolized and transformed into new chemical entities that may have either toxic or therapeutic effects. The pharmacological effects of a drug are basically based on the result of its effective interaction with the target which is concentration-dependent. Pharmacokinetics (absorption, distribution, metabolism, and excretion) of the compound also play a key role in its efficacy as it governs the drug concentration at the site of action (Donato et al., 2008). Inappropriate pharmacokinetics can result in an inadequate or variable concentration of the drug at the site of action and contribute to great variations in clinical response.

Drug metabolism is a major determinant of drug clearance and inter-individual pharmacokinetic differences, and it is an indirect determinant of clinical efficacy and toxicity of drugs (Donato et al., 2008). Thus, many drug interactions are the result of an alteration of CYP metabolism. Basically, drug interactions involving the CYP enzymes are divided into two types: CYP enzyme induction or CYP enzyme inhibition. Common substrates, inhibitors and inducers of CYP enzymes are listed in Table 1.3.

1.2.5.1 Induction of cytochromes P450

When CYP metabolism is enhanced by inducer, the effect of induction will increase the amount of CYP enzyme present and accelerate the rate of oxidation and clearance of another drug (Bibi, 2008). CYP induction is a slow regulatory process that reduces drug concentrations in plasma and compromise the efficacy of the drug in a time-dependent manner (Pelkonen et al., 2008). In most cases, induction of CYP enzymes is mediated to a major extent by group of nuclear receptor (NR) as discussed in the previous section (Pelkonen et al., 2008). For example, St John's wort is a potent inducer of CYP3A4 and is involved in the most severe drug-herb interactions (Ernst, 1999, Moore et al., 2000a). Thus, the protease inhibitors, cyclosporine, warfarin, digoxin, oral contraceptives, and many other medications can be rendered ineffective with concomitant use of St John's wort. CYP induction causes in a reduction of pharmacological effects caused by an increased in drug metabolism.