ACTIVATION OF PREGNANE X RECEPTOR BY *GINKGO BILOBA* EXTRACT

by

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ABSTRACT

Pregnane X receptor (PXR) is a ligand-activated transcription factor that plays a role in a broad array of biological processes, including drug metabolism and transport. *Ginkgo biloba* is an herb commonly used to improve cognitive function. In primary cultures of rat hepatocytes, *Ginkgo biloba* induces the mRNA expression of CYP3A23, a target gene for rat PXR. The present study tested the hypothesis that *Ginkgo biloba* activates PXR. Cultured HepG2 human hepatoma cells were transfected with the full-length human PXR (pCR3-hPXR), the full-length mouse PXR (pCR3-mPXR), or an empty vector (pCR3) in addition to a reporter plasmid (XREM-CYP3A4-LUC; firefly luciferase) and an internal control plasmid (phRL-TK; *Renilla* luciferase). At 24 h after transfection, cells were treated for 24 h with *Ginkgo biloba* extract and luciferase activity was measured. The extract at 200 μg/ml increased mouse and human PXR activity by 3.0-fold and 9.5-fold, respectively, indicating that *Ginkgo biloba* more effectively activates human PXR. Dose-response experiments showed that the extract produced a log-linear increase over the range of 200–800 μg/ml. To determine whether *Ginkgo biloba* extract induces human PXR target gene expression, cultured LS180 human colon adenocarcinoma cells were treated for 72 h with the extract. Total cellular RNA was isolated and reverse transcribed. CYP3A4, CYP3A5, and ABCB1 cDNAs were amplified by real-time PCR. *Ginkgo biloba* extract at 200, 400, and 800 μg/ml increased CYP3A4 mRNA expression by 1.7-, 2.4-, and 2.5-fold, respectively. The extract at the same concentrations increased the mRNA expression of CYP3A5 (1.3 to 3.6-fold) and ABCB1 (2.7 to 6.4-fold). To determine whether the increased expression involved PXR activation, cells were treated with a PXR antagonist, L-sulforaphane, and *Ginkgo biloba* extract. L-sulforaphane at 5, 10, and 20 μM decreased CYP3A4 mRNA expression by 54%,

78%, and 93%, respectively, in cells co-treated with the extract. A similar pattern of response was obtained with CYP3A5 and ABCB1. In cells co-treated with the extract, L-sulforaphane (5 and 10 μM) was not cytotoxic and did not decrease PXR mRNA expression. Our data from cell culture experiments indicate that *Ginkgo biloba* activates PXR and increases CYP3A4, CYP3A5, and ABCB1 mRNA expression.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABCB1	P-glycoprotein
ABCC2	multidrug resistance-associated protein 2
AhR	aryl hydrocarbon receptor
$AUC_{0-\infty}$	area under the plasma concentration-time curve from time 0 to infinity
CAR	constitutive androstane receptor
CCRP	cytoplasmic CAR retention protein
Cl	clearance
Cl/F	apparent oral clearance
C_{max}	peak concentration in plasma
C_{ss}	steady-state concentration
CYP	cytochrome P450
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DR	direct repeat
DRE	DNA response element
EC_{50}	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
ER	everted repeat
F	bioavailability
FBS	fetal bovine serum
FXR	farnesoid X receptor
GC/MS	gas chromatography mass spectrometry

GST	glutathione *S*-transferase
hPXR	human pregnane X receptor
HPLC	high-performance liquid chromatography
HSP90	heat shock protein 90
IC_{50}	half maximal inhibitory concentration
LC/MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LXR	liver X receptor
MEM	minimal essential medium
MDR	multi-drug resistance
MRP	multi-drug associated resistance protein
NADPH	nicotinamide adenine dinucleotide phosphate
NCoR	nuclear receptor corepressor protein
NR	nuclear receptor
OATP	organic anion-transporting polypeptide
mPXR	mouse pregnane X receptor
PBS	phosphate-buffered saline
PCN	pregnenolone 16α-carbonitrile
PCR	polymerase chain reaction
PXR	pregnane X receptor
r^2	coefficient of determination
RAC3	receptor-associated coactivator 3
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction

RXRα	retinoid X receptor α subtype
SEM	standard error of the mean
SLC	organic anion-transporting polypeptide
SMRT	silencing mediator of retinoid and thyroid hormone receptors
SRC-1	steroid receptor coactivator 1
SULT	sulfotransferase
SXR	steroid and xenobiotics receptor
$t_{1/2}$	terminal elimination half-life
$t_{1/2a}$	absorption half-life
t_{max}	time to reach C_{max}
UDP	uridine 5'-diphosphate
UGT	UDP-glucuronosyltransferase
V_d	volume of distribution
VDR	vitamin D receptor
XREM	xenobiotic response enhancer module

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1. INTRODUCTION

1.1 The Superfamily of Nuclear Receptors

Members of the superfamily of nuclear receptors are a group of soluble proteins that reside within the interior of cells. Nuclear receptors regulate gene expression through binding to a ligand (Novac and Heinzel, 2004). The receptors play a vital role in normal cellular physiology, such as embryonic development, organ physiology, cell differentiation and homeostasis, and also many pathological processes, such as cancer, diabetes, rheumatoid arthritis, asthma, or hormone resistance syndrome (Novac and Heinzel, 2004). Receptors in this superfamily are categorized into different families according to their phylogeny and sequence homology (Germain et al., 2006; Nuclear Receptors Nomenclature Committee, 1999):

1) NR1: Thyroid hormone receptor-like
2) NR2: Retinoid X receptor-like
3) NR3: Estrogen receptor-like
4) NR4: Nerve growth receptor-like
5) NR5: Steroidogenic factor-like
6) NR6: Germ cell nuclear factor-like
7) NR7: Miscellaneous

Gene families are designated by Arabic numerals (e.g., NR1) and subfamilies are grouped by capital letters (e.g., NR1I). Individual genes have Arabic numerals as their designations (e.g., NR1I2). When functionally and structurally distinct variants derived from the same gene are discovered, a lowercase letter is put at the end of the name (e.g., NR5A1a and NR5A1b) (Nuclear Receptors Nomenclature Committee, 1999).

1.2 Pregnane X Receptor (PXR)

PXR, encoded by the *NR1I2* gene, is a ligand-activated transcription factor that belongs to the superfamily of nuclear receptors (Kliewer et al., 1998). It was given the name "pregnane X receptor" because a steroid metabolite, pregnane, binds to it (Kliewer et al., 1998). This receptor was discovered at the same time by another group of scientists, who named this protein the steroid and xenobiotics receptor (SXR) because a wide range of steroids and xenobiotics bind to it (Blumberg et al., 1998). In the same year, the receptor was discovered using computer modeling and was termed hPAR (Bertilsson et al., 1998). It was believed to be the human homologue of mouse PXR and is equivalent to SXR. Upon the presence of steroids, xenobiotics, and certain endogenous and toxic substances, PXR increases expression of specific proteins that are important in mammalian biology (Willson and Kliewer, 2002).

PXR has three major domains (Fig. 1.1). At the amino acid terminus is an activation function (AF-1) domain that has no functional activity (Timsit and Negishi, 2007). Next to AF-1 is a DNA-binding domain (DBD) containing two zinc fingers, which bind to specific DNA response elements (DRE) on target genes (Urquhart et al., 2007). Separating the DBD and ligand-binding domain (LBD) is a flexible hinge region, which allows receptor dimerization and DNA binding concurrently (Stanley et al., 2006). Located at the carboxy-terminus is the ligand-binding domain that contains the ligand-dependent activation function 2 (AF-2) region, which undergoes conformational changes for recruitment of co-regulatory proteins essential for transcription of target genes (Watkins et al., 2003). The crystal structure of PXR is illustrated in Fig. 1.2.

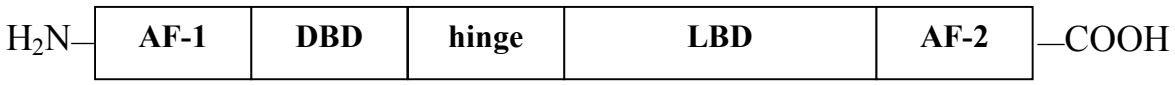


Fig. 1.1. The domain structure of PXR.

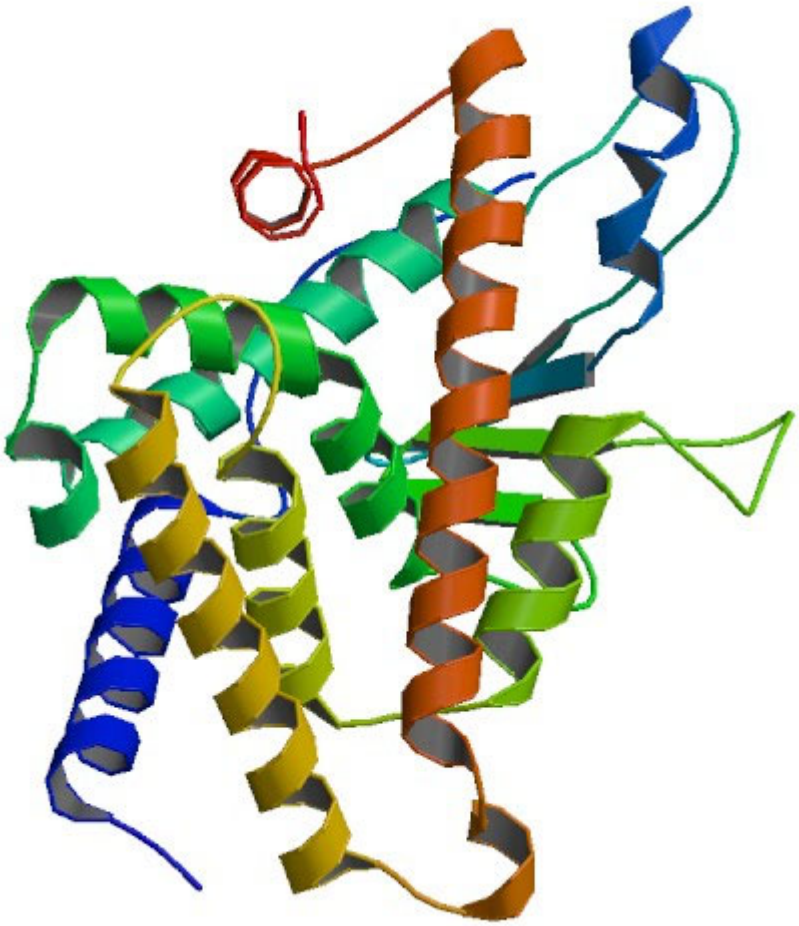


Fig. 1.2. The crystal structure of PXR [Protein Data Bank (ID 1ilg)].

1.2.1 Mechanism of Activation

In the absence of a ligand, PXR resides in the cytoplasm and is in a complex form with cytoplasmic constitutive androstane receptor retention protein (CCRP) and heat shock protein 90 (HSP90) (Squires et al., 2004). When a ligand binds to the PXR, the receptor dissociates from the complex and translocates from the cytoplasm to the nucleus

(Squires et al., 2004). Next, PXR forms a heterodimer with retinoid X receptor-α (RXRα) (Kliewer et al., 2002). The PXR-RXRα heterodimer reorients its DNA binding domain, which then binds to DNA response elements (DRE) in the promoter or enhancer region of a PXR target gene (Lehmann et al., 1998). The DRE region contains an additional high-affinity PXR binding site, which co-ordinately works with the promoter proximal region of the target gene (Goodwin et al., 2002). Conformational changes to this heterodimer allow dissociation of a corepressor, such as silencing mediator for retinoid and thyroid receptors (SMRT), and thereby binding of a coactivator, such as receptor-associated coactivator 3 (RAC3) (Johnson et al., 2006). PXR ligands also recruit steroid receptor coactivator 1 (SRC-1) that helps to stabilize the ligand-heterodimer complex (Mani et al., 2005; Masuyama et al., 2005). Glucocorticoid receptor-interacting protein 1 (GRIP1) is another coactivator involved in PXR activation (Ding et al., 2006; Takeshita et al., 2002), whereas the nuclear receptor corepressor protein (NCoR) is suggested to be a PXR corepressor (Takeshita et al., 2002). Overall, PXR activation results in increased expression of target genes. Fig. 1.3 illustrates the current understanding of how PXR is activated.

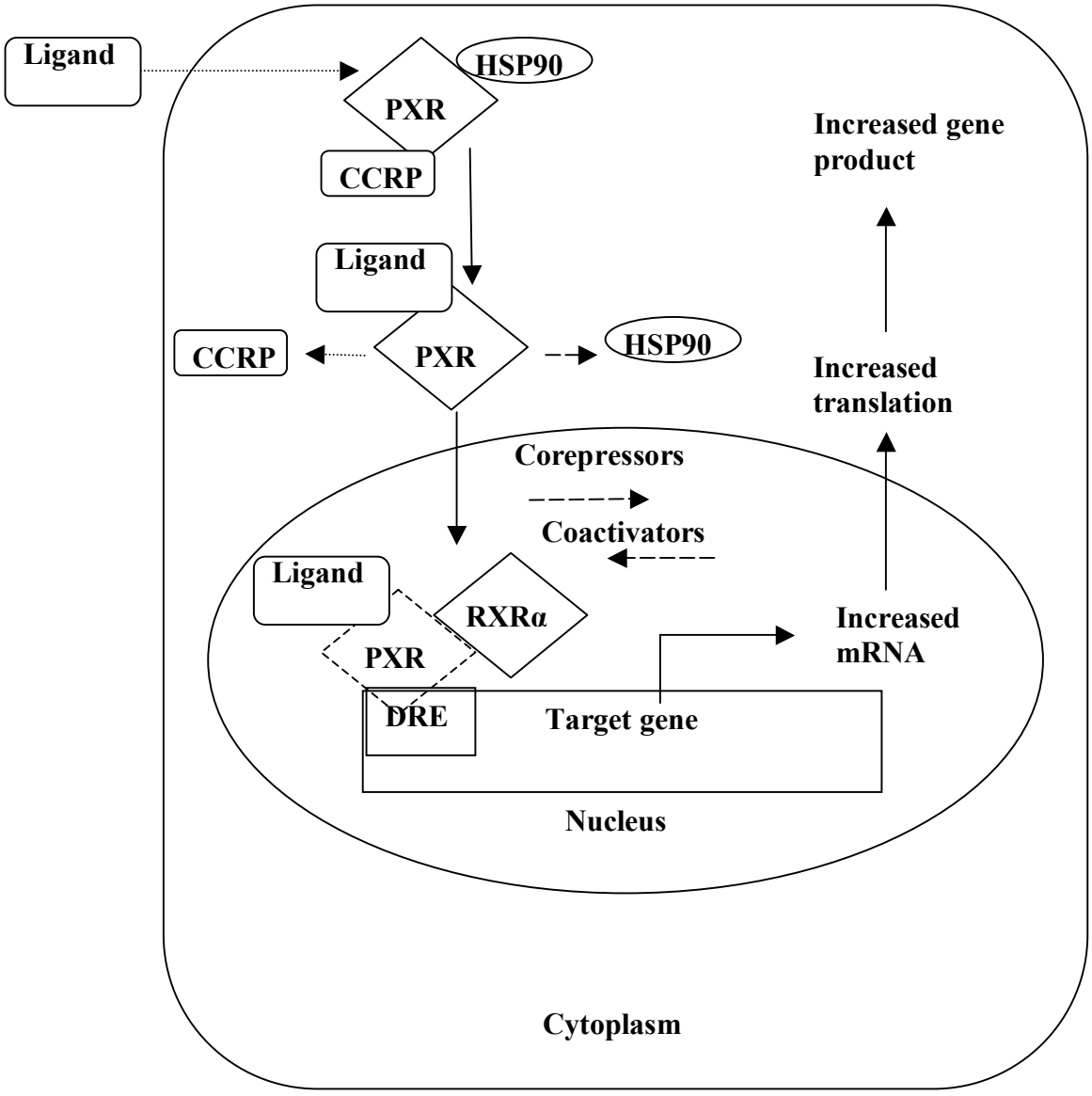


Fig. 1.3. Activation of PXR.

Legends: CCRP, cytoplasmic retention protein; DRE, DNA response elements; HSP90, heat shock protein 90; mRNA, messenger RNA; PXR, pregnane X receptor; RXRα, retinoid X receptor α subtype

1.2.2 Target Genes

PXR target genes encode many clinically important drug-metabolizing enzymes and transporters, including phase I oxidative enzymes, such as the cytochrome P450 (CYP) 3A4 enzyme, which is the most highly expressed CYP enzyme in human liver (Lehmann et al., 1998). PXR also regulates CYP3A5 (Burk et al., 2004), CYP3A7 (Gardner-Stephen et al., 2004), CYP2C9 (Gerbal-Chaloin et al., 2001), CYP2B6 (Goodwin et al., 2001), and various phase II conjugating enzymes, such as UDP-glucuronyltransferases (UGT) 1A1, 1A3, 1A4 (Gardner-Stephen et al., 2004) and 1A6 (Xie et al., 2003), glutathione *S*-transferase A2 (GSTA2) (Falkner et al., 2001), and sulfotransferase (SULT) 1A1 (Duanmu et al., 2002). The expression of various transporters, such as P-glycoprotein (ABCB1, MDR1) (Geick et al., 2001), multidrug resistance-associated protein 2 (ABCC2, MRP2) (Kast et al., 2002) and organic anion-transporting polypeptide 1A2 (OATP1A2, SLC1A2) (Miki et al., 2006), are also regulated by PXR.

Not only does PXR regulate the expression of various drug-metabolizing enzymes and transporters, but it also modulates hepatic energy metabolism (Konno et al., 2008; Moreau et al., 2008). PXR decreases gluconeogenesis through cross-talking with a hormone responsive transcription factor called forkhead transcription factor 1 (FoxO1), which binds to insulin response sequence (IRS) and activates its target genes (Kodama et al., 2004; Kodama et al., 2007). PXR also cross-talks with forkhead transcription factor 2 (FoxO2) to increase lipogenesis and decrease fatty acid oxidation and ketogenesis (Nakamura et al., 2007). Moreover, PXR is suggested to suppress the NF-kappaB signalling pathway (Zhou et al., 2006a), which regulates inflammation and innate and adaptive immune response.

PXR also regulates other homeostasis pathways through cross-talking with other receptors and signalling pathways. For example, ligand-activated PXR competes with HNF-4α signalling for a common co-activator called PGC-1α (Bhalla et al., 2004; Li and Chiang, 2005). This competition leads to an increase of PXR/PGC-1α complex formation and a concomitant decrease of HNF4α/PGC-1α complex formation. The consequence is the down-regulation of CYP7A1 and CYP8B1 protein expression, which control gluconeogenesis, and bile acid and fatty acid homeostasis. In transgenic mice expressing human PXR, PXR activation causes hepatic steatosis, an impairment of the normal synthesis and elimination of triglyceride (Zhou et al., 2006b). PXR activation is associated with up-regulation of a nuclear receptor called peroxisome proliferator-activated receptor γ (PPARγ), which when it is over-expressed, is associated with hepatic steatosis in mice (Yu et al., 2003). Furthermore, PXR and thyroid hormone receptors (TRα and TRβ) were recently found to share DNA response element and target genes (Pascussi et al., 2008). It suggests that PXR may cross-talk with thyroid hormone receptors, which control skeletal muscle development and growth. PXR and vitamin D receptor (VDR) share DNA response element of certain target genes, such as CYP24 (Pascussi et al., 2005), CYP3A4, CYP2B6, and CYP2C9 (Drocourt et al., 2002). Both CYP24 (Kato, 2000) and CYP3A4 (Xu et al., 2006) catalyze hydroxylation of 1α,25-dihydroxyvitamin D_3. Therefore, PXR may regulate the homeostasis of 1α,25-dihydroxyvitamin D_3, and thereby mediate its biological functions, such as bone mineralization.

1.2.3 Modulators

Many endogenous and foreign substances are reported to be modulators of PXR activity. An agonist is a ligand that binds to a receptor and increases the intrinsic activity of a

receptor. Agonists of PXR include natural ligands such as cholesterol (Dussault et al., 2003) and vitamin E (Traber, 2004). Agonists of PXR also consist of synthetic drugs. Based on *in vitro* assays, rifampin (Bertilsson et al., 1998), ritonavir (Dussault et al., 2001), paclitaxel (Synold et al., 2001) and nifedipine (Bertilsson et al., 1998) are examples of human PXR agonists. In addition, clotrimazole (Lehmann et al., 1998), phenobarbital (Moore et al., 2000b) and dexamethasone (Pascussi et al., 2001) are agonists of both human and mouse PXR. Various herbal extracts are found to activate PXR. Based on *in vitro* assays, examples are St. John's wort (Wentworth et al., 2000), gugulipid (Brobst et al., 2004), *Coleus forskohlii* (Ding and Staudinger, 2005), various Tanzanian plant extracts (van den Bout-van den Beukel et al., 2008), and some traditional Chinese medicine such as Tian Xian (Lichti-Kaiser and Staudinger, 2008), Wu Wei Zi, and Gan Cao (Mu et al., 2006). Individual chemicals present in herbal extracts have been identified as PXR activators. Hyperforin in St. John's Wort (Wentworth et al., 2000) (Fig. 1.4), *cis*- and *trans*-guggulsterone in gugulipid (Brobst et al., 2004) (Fig. 1.5) and forskolin in *Coleus forskohlii* (Ding and Staudinger, 2005) (Fig. 1.6) activate both human and mouse PXR activities *in vitro*. In contrast, artemisinin in Qing Hao activates human but not mouse PXR activities (Burk et al., 2005b) (Fig. 1.7).

An antagonist is a ligand that binds to a receptor, but does not alter the intrinsic activity of the receptor. Its binding to receptor prevents the binding between an agonist and the receptor, and thereby blocks the function of an agonist. Human PXR antagonists include L-sulforaphane (Zhou et al., 2007) (Fig. 1.8), ketoconazole (Huang et al., 2007) (Fig. 1.9), and trabectedin, also known as ecteinascidin-743 or ET-743 (Synold et al., 2001) (Fig. 1.10).

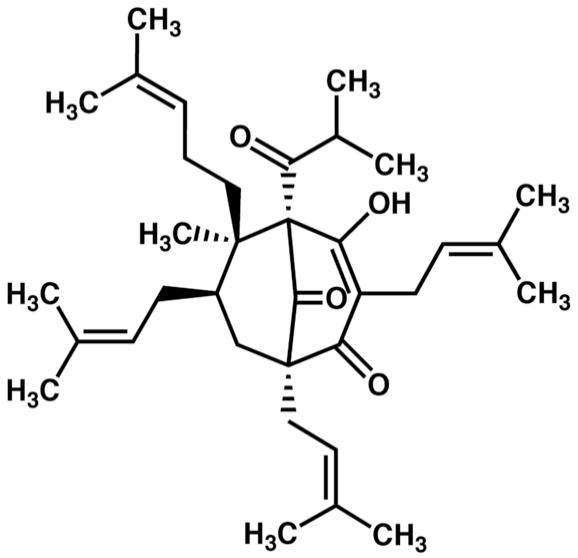
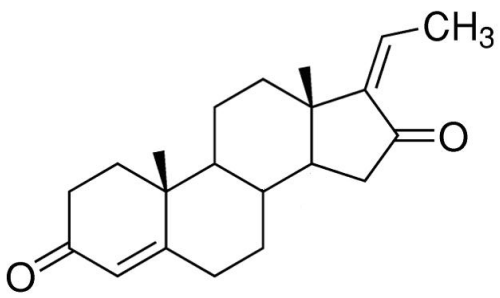
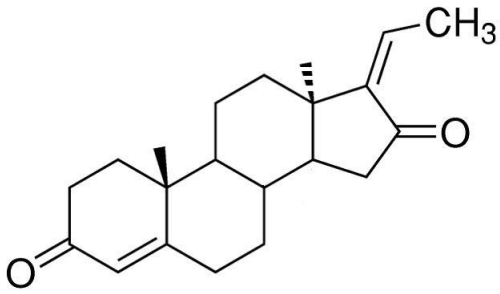


Fig. 1.4. Chemical structure of hyperforin.



***cis*-guggulsterone**



***trans*-guggulsterone**

Fig. 1.5. Chemical structures of *cis*- and *trans*-guggulsterone.

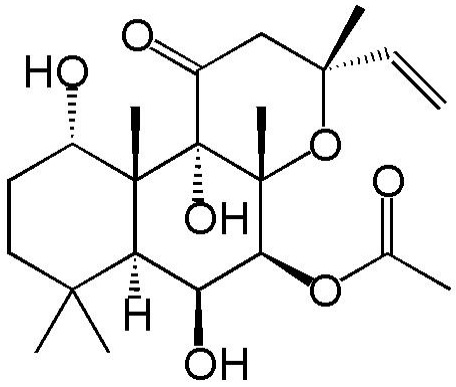


Fig. 1.6. Chemical structure of forskolin.

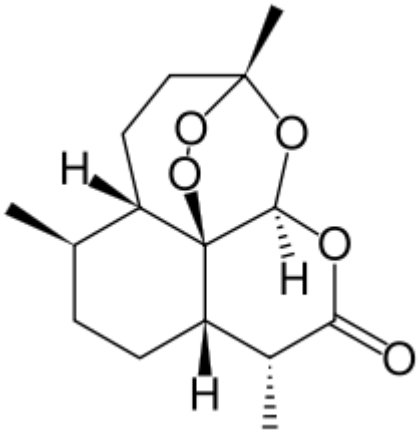


Fig. 1.7. Chemical structure of artemisinin.

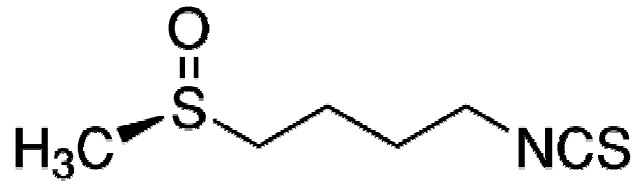


Fig. 1.8. Chemical structure of L-sulforaphane.

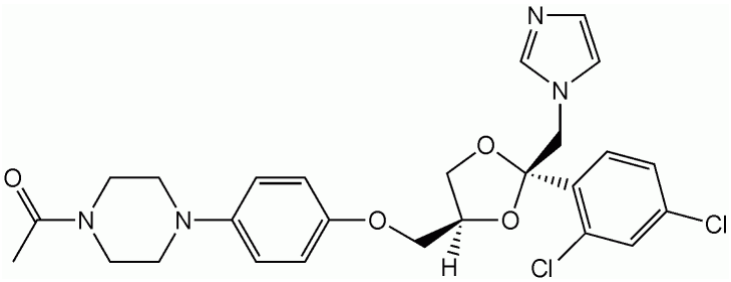


Fig. 1.9. Chemical structure of ketoconazole.

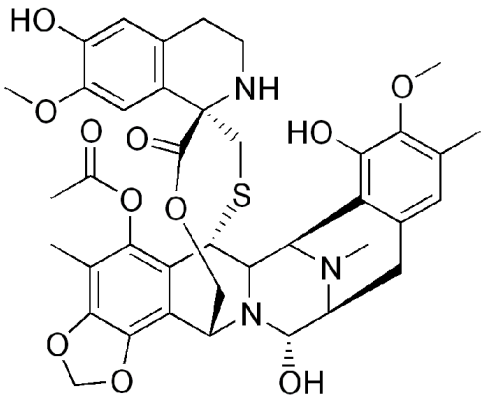


Fig. 1.10. Chemical structure of trabectedin (ET-743).

1.2.3 Species Difference in PXR Activation

It is important to note there are species differences in PXR activation by drugs and other chemicals. The ligand-binding domain of human PXR is only 77% and 76% identical in amino acid sequence with those of mouse and rat, respectively, whereas the ligand-binding domains of mouse and rat PXR are 97% identical (Jones et al., 2000). In reporter gene assays, rifampin (10 μM) activates human PXR, but not mouse PXR or rat PXR (Vignati et al., 2004), because the ligand-binding site of human PXR contains residue Leu 308 (Tirona et al., 2004). Conversely, pregnenolone-16α-carbonitrile (PCN) activates rat and mouse PXR, but not human PXR (Vignati et al., 2004), because the rat and mouse PXR have residues Phe 305 and Asp 318 (Song et al., 2005). Hyperforin, a constituent in St. John's wort, is an example of a compound that strongly activates human PXR (> 20 fold), but not mouse PXR (< 5 fold) *in vitro* (Vignati et al., 2004). On the other hand, exemestane strongly activates mouse PXR (> 20 fold), but not human PXR (< 5 fold) (Vignati et al., 2004).

Stereoselectivity could be a factor in the species-dependent activation of PXR. One example is S20, a *C*-cyclopropylalkylamide. In transient transfection assays, (−)−S20 enantiomer preferentially activates mouse PXR, whereas (+)−S20 preferentially activates human PXR (Mu et al., 2005). In a dose-response experiment, (−)−S20 and PCN had similar EC_{50} (half maximal effective concentration), but (−)−S20 showed greater E_{max} (maximal response) in mouse PXR activity. On the other hand, (+)−S20 had a smaller EC_{50} and greater E_{max} than rifampin in human PXR activity. Mutagenesis analysis shows that the ligand binding domain residue Phe305 is a critical component for the preferential binding of the rodent PXR to (−)−S20.

Moreover, mutagenesis analysis shows that the ligand binding domain residue Gly285 is critical for the preferential binding of human PXR ligands (Ostberg et al., 2002). When Gly285 in human PXR was mutated to Ile, which is the equivalent residue in mouse PXR, it has increased responsiveness to PCN and decreased responsiveness to rifampicin. In mouse PXR, the Ile285 to Gly mutation makes the mouse PXR less responsive to PCN than the wild type, and has no response to rifampicin or SR12823, both of which are prototypical PXR agonists. The Gly285 to Ile mutation changes the ligand binding domain residue from hydrophilic to hydrophobic.

1.2.4 Tissue Distribution

PXR mRNA expression is detectable at high levels in liver, colon, small intestine, stomach and fetal liver, and weakly in heart and bone marrow, as determined in pooled tissues from multiple individuals of different ethnicities (Lamba et al., 2005). PXR mRNA is also expressed in distinct regions of the human brain, such as thalamus, spinal cord, and to a lesser extent, pons and medulla. As assessed by polymerase chain reaction (PCR) analysis, PXR mRNA expression is detectable in adult liver, kidney, and small and large intestines, but undetectable in lung (Miki et al., 2005). In fetal tissue, PXR mRNA is also detectable in liver, kidney, and small and large intestines, but not in lung. Similarly, as determined by northern blot analysis, PXR mRNA is most highly expressed in adult liver tissue, and also in colon and small intestine tissue, but to a lesser extent (Bertilsson et al., 1998; Lehmann et al., 1998).

Miki *et al.* (2005) used immunoblotting to measure PXR protein expression. PXR protein was detectable in human liver, kidney, lung, small intestine, and large intestine, but undetectable in spleen and stomach. Immunoreactivity was greatest in human liver and small

intestines, and, to a lesser extent, in kidney, but below the limit of detection in spleen and stomach.

1.2.5 Ontogeny

In human liver, PXR mRNA expression is low in fetal and neonatal groups (i.e., before birth and 0 years old, respectively) (Miki et al., 2005). It reaches its highest level in the young group (i.e., 15-38 years old), starts to decrease in middle age (i.e., 45-65 years old), and decreases to the fetal level in the elderly group (i.e., 67-85 years old). In human kidney tissues, PXR mRNA expression is relatively high in fetus, but decreases after birth. In human lung tissues, PXR mRNA expression is variable throughout different groups, but is relatively low in general. In human small intestine tissues, PXR mRNA expression is highest in young-middle age, but is relatively low in fetal, neonatal, and elderly. In contrast, in human large intestines, PXR mRNA expression is highest in fetal group, but is relatively low in neonatal, young-middle age, and elderly groups.

PXR protein is weakly expressed in fetal and neonatal liver but highly expressed in adult liver (Miki et al., 2005). Western blot analysis shows that the PXR protein expression on embryonic day 20 is at about 40% of adult expression, and on postnatal day 28, is at 80% of adult protein expression (Balasubramaniyan et al., 2005). In addition, PXR protein expression in the elderly (i.e., 67-85 years old) is less than 50% of adult protein expression (Miki et al., 2005).

Levels of hepatic PXR mRNA expression show no difference in 8, 14, 20, and 24 month-old mice, as determined by RT-PCR (Echchgadda et al., 2004). Mouse hepatic PXR mRNA expression does not change during senescence. In contrast to mouse PXR, rat hepatic PXR mRNA expression is subject to developmental regulation. From embryonic days 17 to

21, the PXR mRNA expression in rat liver tissue is between 2.4 and 6.2% of the adult mRNA expression, detected using RT-PCR analysis (Balasubramaniyan et al., 2005). PXR mRNA expression rises to 20% of adult mRNA expression at one day after birth, and reaches 83.4% of adult mRNA expression by day 28 of age.

1.2.6 Allelic Variants

Human PXR transcript variants result from alternative splicing, a process in which multiple protein isoforms are generated from a single gene. Bertilsson *et al.* (1998) first discovered the variants T1 (or PXR wild type) and T2 (or hPAR-2) by isolating their cDNAs. The 5′ end of the T2 has an open reading frame that is 39 amino acids longer than that of the T1. Later, a third human PXR variant mRNA (T3) was discovered using RT-PCR and found to have an in-frame deletion of 111 nucleotides (base pairs 823-933 relative to T1) (Fukuen et al., 2002). Similar to T1, T3 is highly expressed in neoplastic breast tissue. The mRNA expression of all three isoforms is detectable by RT-PCR in cultured HepG2 human hepatoma cells and normal human liver and intestine tissues; however, only T1 mRNA is present in cultured Caco-2 colorectal adenocarcinoma cells (Gardner-Stephen et al., 2004).

There are two variants of mouse PXR transcripts, PXR.1 (wild type) and PXR.2, which are differentially activated by steroids (Kliewer et al., 1998). The cDNA sequence of PXR.2 has a deletion of 123 nucleotides (base pairs 661 to 783 relative to PXR.1). It encodes a 390 amino acid protein, which does not have a 41 amino acid region normally present in the ligand-binding domain of PXR.1.

Changes in the amino acid residues within or adjacent to the PXR ligand-binding domain, such as Val-140-Met, Asp-163-Gly and Ala-370-Thr, are shown to increase the transcriptional activity of CYP3A4, a PXR target gene, in reporter gene assays (Hustert et al.,

2001). RT-PCR demonstrated that, T3, along with mouse PXR.2, has an in-frame 123-nucleotide deletion in the ligand-binding domain (Dotzlaw et al., 1999). Mouse PXR.2 has a reduced response to steroids that normally activate PXR.2 in reporter gene assay (Kliewer et al., 1998). Examples of those steroids are dexamethasone, mifepristone (RU486), and PCN.

1.3 Cytochrome P450 (CYP)

CYP is a superfamily of heme-containing trans-membrane proteins found in virtually all organisms (Danielson, 2002). It was first reported to be a carbon monoxide-binding pigment in rat liver microsomes (Klingenberg, 1958). It was later named "cytochrome P450" because it is a colored ("chrome") cellular ("cyto") protein, with a characteristic peak formed by absorbance of light at 450 nm when the heme iron, in the reduced state, bound to carbon monoxide (Omura and Sato, 1962; Omura and Sato, 1964). CYP enzymes are transmembrane protein mostly located in smooth endoplasmic reticulum, although a few of them are found in the mitochondria (Fig 1.11). CYP enzymes are present most abundantly in the liver, and at lower levels of expression in many other organs, such as intestine, lung, adrenal, and kidney (Danielson, 2002).

NADPH-cytochrome P450 reductase functions as an electron carrier that transfers electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to CYP, which is the substrate and oxygen-binding site of the system (Danielson, 2002; Guengerich, 2001). Phospholipids in the endoplasmic reticulum facilitate the interaction between NADPH-cytochrome P450 reductase and CYP. The most common CYP reaction involves interaction between drug substrate (RH) and an oxygen molecule (O_2); an oxygen atom is inserted into RH, whereas the other oxygen atom is reduced to water (H_2O). Examples of oxidation reactions include aliphatic hydroxylation, aromatic hydroxylation, alkene

hydroxylation, sulphur/nitrogen oxidation, *N*-dealkylation, *O*-dealkylation, and *S*-dealkylation.

The generally accepted major events in the CYP catalytic cycle are those shown in Fig. 1.12 (Guengerich, 2001). The cycle starts with iron in the ferric state (Fe^{3+}). In step 1, the substrate (RH) binds to the enzyme near the distal region of the heme. In step 2, electrons are transferred from NADPH via the NADPH-cytochrome P450 reductase. The NADPH-cytochrome P450 reductase becomes oxidized, whereas the Fe^{3+} ion becomes reduced. In step 3, the O_2 molecule binds to the Fe^{3+} ion forming the Fe^{2+}-O_2 complex. In step 4, a second electron, coming from NADPH-cytochrome P450 reductase or cytochrome b_5, enters the cycle. In step 5, a proton enters the cycle. In step 6, the proton cleaves the O-O bond and generates H_2O and FeO^{3+}. In step 7, the electron-deficient FeO^{3+} complex draws either a hydrogen atom or an electron from the substrate to form a complex with the substrate. In step 8, the intermediate collapses and subsequently generate the product (ROH). In step 9, the product is released from the active site of the enzyme, which returns to its initial state.

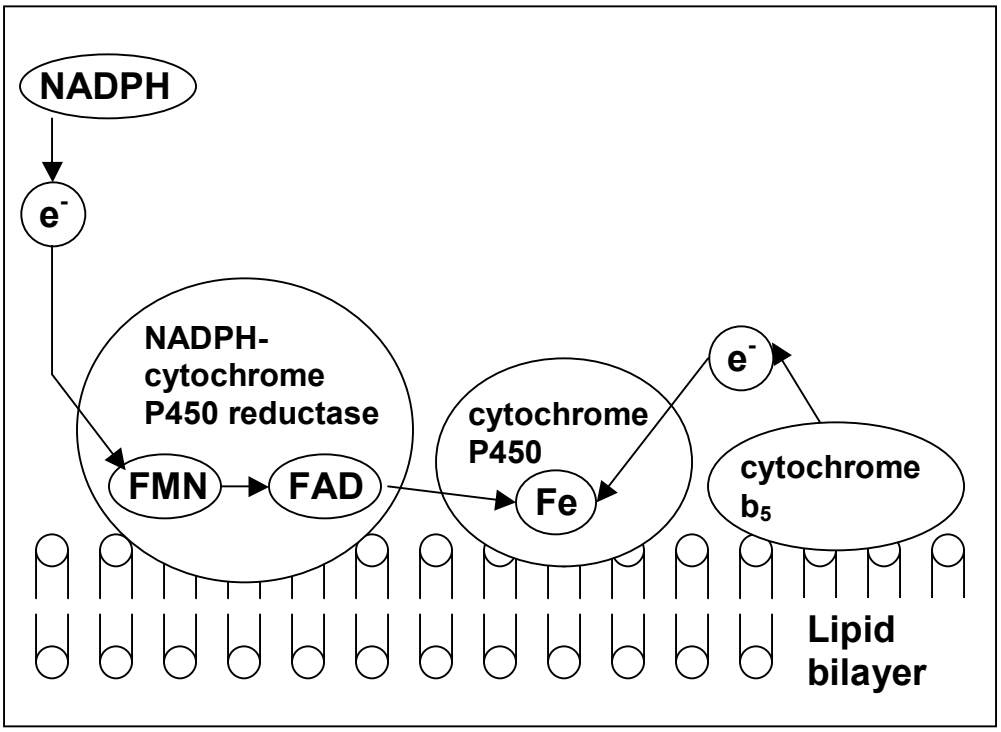


Fig. 1.11. Localization of CYP in cell membrane.

Legend: NADPH = nicotinamide adenine dinucleotide; e^- = electrons; FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; Fe = iron atom (in the reduced state) in heme

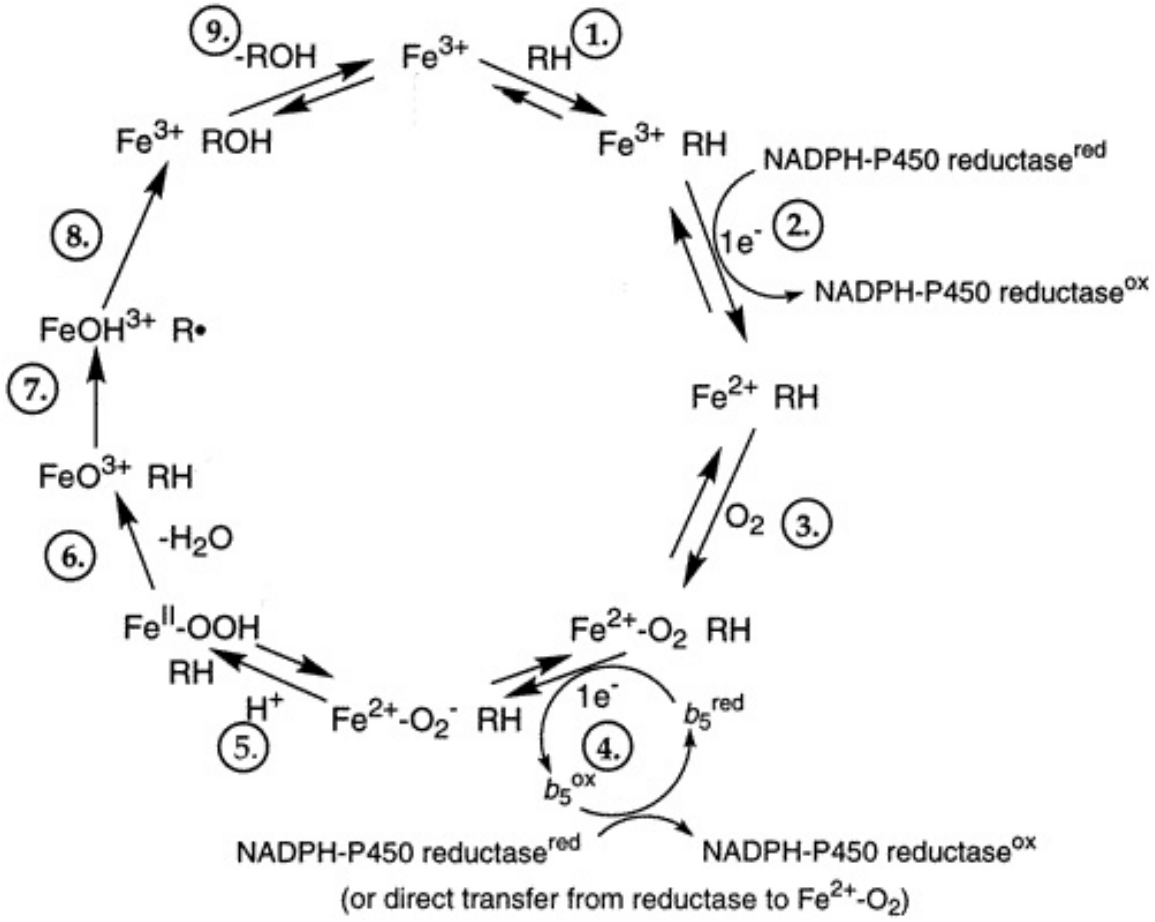


Fig. 1.12. The generally accepted catalytic mechanism for the CYP catalytic cycle.

Legend: Fe = iron atom in heme; RH = substrate; e^- = electrons; ROH = product; ox and red = oxidized and reduced states of the reductase involved in the electron transfers, respectively

[Reproduced from Guengerich (2001) with permission]

1.3.1 Nomenclature

The P450 Nomenclature Committee (http://www.cypalleles.ki.se/) has a standardized system to name and assign individual CYP member into families and subfamilies (Nelson, 2006). All CYP enzymes have the root designation "CYP," followed by an Arabic numeral for the gene family, a capital letter for the subfamily, and another numeral

for the individual gene. The numerals within a subfamily are numbered chronologically as they are reported to the nomenclature committee. CYP1A1 was the first officially named CYP. For the genes encoding the CYP enzymes, the designations are italicized (e.g. *CYP1A1*) (Nelson, 2006). As a general convention, members of CYP families share greater than 40% amino acid homology, whereas members of subfamilies display greater than 55% amino acid identity (Nelson, 2006).

1.3.2 Major CYP Enzymes in Drug Metabolism

The Human Genome Project has identified 57 full genes and 5 pseudogenes, which are defective genes that do not produce functional proteins (Brown et al., 2008). Enzymes in the CYP1, CYP2, and CYP3 families are involved mainly in drug metabolism. CYP3A enzymes are abundantly expressed in liver (Lamba et al., 2002; Shimada et al., 1994), which is the most important site for drug metabolism. CYP3A4 and CYP3A5 together make up the major portion (40%) of total CYP content in the human liver (Shimada et al., 1994) and intestine (80%) (Paine et al., 2006). CYP3A (mainly CYP3A4 and CYP3A5) is involved in metabolism of approximately 50% of drugs on the market (Rendic, 2002).

1.3.3 CYP3A4

CYP3A4 is a phase I enzyme that transforms lipophilic substrates into more hydrophilic metabolites by adding or exposing polar functional groups. Among all CYP enzymes, CYP3A4 is responsible for the metabolism of the largest number of xenobiotics and endogenous compounds.

1.3.3.1 Tissue Distribution

Immunochemistry demonstrates that CYP3A protein accounts for the majority of CYP proteins (40%) in human liver samples from 30 subjects (Shimada et al., 1994). CYP3A4 is the major CYP3A mRNA (Bork et al., 1989) and protein (Lamba et al., 2002) expressed in adult human liver. Other than being in the liver, CYP3A4 protein is abundantly expressed in jejunum, colon, and pancreas (Zhang et al., 2004). Moreover, CYP3A4 mRNA is detected in adult human liver, lung, and intestine using Northern blot analysis (Kolars et al., 1992). CYP3A4 mRNA is also detected in human liver, kidney, lung, and small intestine, as determined by RT-PCR (Miki et al., 2005). In addition, CYP3A microsomal protein is found in human kidney, as determined by western blotting (Haehner et al., 1996).

1.3.3.2 Ontogeny

As determined by RT-PCR, human hepatic CYP3A4 mRNA expression reaches its highest level in the young group (i.e., 15-38 years old), starts to decrease in middle age (i.e., 45-65 years old), and decreases to its lowest level in the elderly group (i.e., 67-85 years old) (Miki et al., 2005). In human kidney tissues, CYP3A4 mRNA expression is relatively low in general, and is variable within and across age groups. In human lung tissues, CYP3A4 mRNA expression is variable throughout different age groups and is relatively low in general. Hines (2007) retrospectively analyzed the western blot analysis data from three independent studies (Overby et al., 1997; Shimada et al., 1994; Wrighton et al., 1990), and determined that the average CYP3A4 enzyme expression was 128.9 pmol/mg of protein in those panels of human adult (>18 years) liver samples. The adult level was not reached until at least 2-3 years of age.

1.3.3.3 Substrates

Some examples of CYP3A4 substrates include nifedipine, clarithromycin, cyclosporine, alprazolam, citalopram, and simvastatin. Substrates that have shown to be CYP3A-selective include alfentanil (Klees et al., 2005b), cortisol (Huang et al., 2004a), testosterone (Kamdem et al., 2004), midazolam, and triazolam (Patki et al., 2003). Quinidine (3-hydroxylation) (Mirghani et al., 2003) is a CYP3A4-selective substrate, but is not a substrate of CYP3A5.

1.3.3.4 Enzyme Inhibitors

Drugs and other chemicals that inhibit the catalytic activity of CYP3A4 include 1-aminobenzotriazole (Emoto et al., 2003), erythromycin (McGinnity et al., 2006), clarithromycin (Zhao et al., 2006), fluconazole (Gibbs et al., 1999), itraconazole (Huang et al., 2004b), ketoconazole, SKF-525A (Emoto et al., 2003), troleandomycin (Klees et al., 2005a), and verapamil (Yeo and Yeo, 2001). In addition to inhibiting CYP3A4 catalytic activity, fluconazole (Gibbs et al., 1999), ketoconazole (Klees et al., 2005a), and troleandomycin (Chang et al., 1994) also inhibit CYP3A5 activity. CYP3A4-selective inhibitors are chemicals that are shown to inhibit only CYP3A4-mediated metabolism, after examination on various CYP isoform-specific probe substrates. For example, itraconazole is a selective inhibitor of CYP3A4 but not CYP3A5 activity (Huang et al., 2004b). Dimethyl-4,4'-dimethoxy-5,6,5',6'-dimethylenedioxybiphenyl-2,2'-dicarboxylate (DDB) is also a selective inhibitor of CYP3A activity (Kim et al., 2001).

1.3.3.5 Enzyme Inducers

Enzyme inducers increase protein synthesis and thereby its catalytic activity. Based on *in vitro* assays, CYP3A4 inducers include dexamethasone (Krusekopf et al., 2003), sulfinpyrazone (Luo et al., 2002a), carbamazepine, phenobarbital, phenytoin, and rifampin (Usui et al., 2003). Dexamethasone (Krusekopf et al., 2003), carbamazepine, phenobarbital, phenytoin and rifampin increase both CYP3A4 and CYP3A5 activity, but the extent of CYP3A4 induction is greater than that of CYP3A5 (Usui et al., 2003).

1.3.3.6 Mechanisms of CYP3A4 Induction

CYP3A4 induction occurs by the activation of pregnane X receptor (PXR) (Kliewer et al., 1998). Upon activation by a ligand, PXR translocates from the cytoplasm to the nucleus and forms a heterodimer with a retinoid X receptor alpha (RXRα) (Tompkins and Wallace, 2007). The PXR-RXRα heterodimer binds to the everted repeat 6 motif (ER6; two copies of the same nucleotide sequence oriented in opposite directions and separated by 6 bp) in the proximal promoter region of the CYP3A4 gene (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). However, maximal induction of the CYP3A4 promoter is achieved when a distal xenobiotic responsive enhancer module (XREM), at ~8 kb upstream of the transcription initiation site, coordinately works with the promoter proximal region of CYP3A4 (Goodwin et al., 1999). Increased synthesis of CYP3A4 mRNA then occurs and leads to induction of CYP3A4 protein and thereby increasing enzyme activity.

Other nuclear receptors also regulate CYP3A4 induction. Transient transfection of HepG2 cells with human constitutive androstane receptor (CAR) causes increased expression of CYP3A4 mRNA (Goodwin et al., 2002). CYP3A4 induction by CAR is associated with two high-affinity binding motifs located within the 5'-flanking region of the

CYP3A4 gene, which is about 7720 and 150 bases upstream of the transcription initiation site. In addition, CAR trans-activates the steroid/rifampicin-response element of the transfected CYP3A4 gene in HepG2 cells (Sueyoshi et al., 1999). Since the human CAR response elements regulate trans-activation of CYP3A4 by the human PXR, it is suggested that cross-talk between PXR and CAR is important in determining CYP3A4 gene expression (Goodwin et al., 2002).

Farnesoid X receptor (FXR), also known as the bile acid receptor, is another signaling molecule suggested to activate CYP3A4 transcription (Gnerre et al., 2004). In trans-activation assays, ligand-activated FXR causes eight-fold increase in the activity of the XREM reporter gene, which was previously shown to mediate drug-induced mRNA expression of CYP3A4 (Goodwin et al., 1999). In gel-mobility shift assays, two functional FXR recognition sites are identified in a 345-bp element within the 5'-flanking region of CYP3A4. Mutation of these sites abolishes binding of human FXR to the 345-bp element.

The vitamin D receptor (VDR) is also believed to regulate CYP3A4 mRNA expression. Treatment of untransfected LS180 human colorectal adenocarcinoma cells with 1α,25-dihydroxyvitamin D_3 (1 to 10 nM) increases CYP3A4 protein and CYP3A4 mRNA expression in a dose-dependent fashion (Thummel et al., 2001). Increase in CYP3A4-promoter-luciferase reporter activity is also observed in LS180 cells transiently transfected with VDR. The activation of CYP3A4 reporter is inhibited by mutation of the nuclear hormone receptor-binding motif (ER6) in the CYP3A4 promoter. ER6 is an everted repeat with a spacer of 6 bp located in the proximal region of the promoter. Vitamin D receptor (VDR)-retinoid X receptor (RXR) heterodimer binds specifically to ER6. Although the CYP3A4 ER6 promoter element also binds to PXR, the 1α,25-dihydroxyvitamin D_3 induction of CYP3A4 is independent of PXR, because 1α,25-dihydroxyvitamin D_3 does not

activate the ligand-binding domain of human PXR (Thummel et al., 2001). In addition to ER6, a DR3-type element in the distal region of the promoter is identified as another functional vitamin D responsive element in CYP3A4 gene (Thompson et al., 2002).

1.3.4 CYP3A5

Similar to CYP3A4, CYP3A5 is a phase I enzyme that belongs to the CYP3A subfamily and transforms lipophilic substrates into more hydrophilic metabolites by adding or exposing polar functional groups. It metabolizes drugs such as such as midazolam, triazolam, nifedipine, and testosterone (Patki et al., 2003). The amino acid sequence of CYP3A5 and CYP3A4 are 84% similar, and thus, they have substantial overlapping substrate specificities between them (Emoto and Iwasaki, 2006).

1.3.4.1 Tissue Distribution

Hepatic CYP3A5 mRNA expression was detected in 19 Caucasian donors, as determined by RT-PCR (Jounaidi et al., 1996). However, western blot analysis demonstrated that only 60% of African Americans and 33% of Caucasians had hepatic CYP3A5 protein (Kuehl et al., 2001). The discrepancy could be due to the lack of specificity of the CYP3A5 probe in western blot analysis and truncated CYP3A5 proteins present in individuals. CYP3A5 protein is more commonly expressed in the small intestine, stomach, and kidneys (Zhang and Benet, 2001). It is the second most abundantly expressed CYP protein in small intestine, as determined by western blot analysis of human intestine microsomes pooled from 31 Caucasian, African American, Asian, and Hispanic donors (Paine et al., 2006). CYP3A5 protein is found in human kidney microsomes, as determined by western blotting (Haehner et al., 1996).

1.3.4.2 Ontogeny

Immunoblot analyses detected CYP3A5 proteins in about 50% of all infant livers and 29% of adult livers studied (Wrighton et al., 1990). Wrighton *et al*. (1989) also found CYP3A5 proteins in the livers of a 2-month-old and a 14-year-old patient. In another study, only two of the nine first trimester fetal liver specimens (11.9 weeks) had detectable CYP3A5 protein expression, as determined by western blot analysis (Hines, 2007). CYP3A5 protein expression remains unchanged in pre-puberty (4.9 years), adolescent (15.2 years), and adult (>18 years) livers (Hines, 2007).

1.3.4.3 Substrates

Some examples CYP3A5 substrates include alfentanil (Klees et al., 2005b), cortisol (Huang et al., 2004a), testosterone (Kamdem et al., 2004), midazolam and triazolam (Patki et al., 2003). Drug reactions that are found to be CYP3A5-selective include alprazolam α-hydroxylation (at position-2 of the alpha ring) (Hirota et al., 2001; Williams et al., 2002), tacrolimus demethylation (Dai et al., 2006; Kamdem et al., 2005), and vincristine oxidation (Dennison et al., 2006). All three of them are probe markers for CYP3A5 enzyme activity (Liu et al., 2007).

1.3.4.4 Enzyme Inhibitors

CYP3A5 inhibitors include fluconazole (Gibbs et al., 1999), ketoconazole (Emoto et al., 2003), and troleandomycin (Chang et al., 1994). There has yet to be a small molecule inhibitor determined that specifically inhibits CYP3A5 but not CYP3A4 enzyme activity (Liu et al., 2007).

1.3.4.5 Enzyme Inducers

Based on *in vitro* assays, CYP3A5 inducers include dexamethasone (Krusekopf et al., 2003), carbamazepine, phenobarbital, phenytoin, and rifampin (Usui et al., 2003). They induce both CYP3A4 and CYP3A5 activity, but the extent of CYP3A4 induction is greater in than that of CYP3A5.

1.3.4.6 Mechanisms of CYP3A5 Induction

Very little is known about mechanism of CYP3A5 induction. Similar to CYP3A4, CYP3A5 induction is believed to be mainly due to ligand binding of PXR. PXR activates the CYP3A5 reporter gene in transfected LS174T human intestinal cell line; the reporter activity increases 2-fold when treated with rifampin, a PXR ligand (Burk et al., 2004). The same study also showed that CAR activates the CYP3A5 reporter. Both PXR and CAR bind to the ER6 motif (two copies of the same nucleotide sequence oriented in opposite directions and separated by 6 bp) as heterodimers with RXRα. CYP3A5 transactivation is dependent on the ER6 motif, which is located -100 bp from the transcription start site. The location and sequence of the ER6 motif are identical to the proximal ER6 motif of CYP3A4, where binding of PXR and CAR have been demonstrated. Therefore, it is believed that CYP3A4 and CYP3A5 induction involve a similar mechanism.

1.4 ATP-Binding Cassette (ABC) Transporters

ABC transporters are categorized according to the sequence and organization of their ATP-binding domains (Dean et al., 2001). They are the largest family of transmembrane proteins. ABC transporters use the energy of ATP hydrolysis to transport various substrates

across cellular membranes, including lipids, steroids, and drugs. Some ABC transporters play an important role in drug disposition, tumour resistance and multidrug resistance. Some are linked to development of cystic fibrosis and a range of genetic diseases, such as immune deficiency.

1.4.1 Nomenclature

The ABC Nomenclature Committee (www.genenames.org/genefamily/abc.php) and Human Genome Organization (HUGO) have identified 50 known ABC transporters present in humans (Dean et al., 2001). ABC transporters have the root designation "ABC." They are grouped into seven families (i.e., ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG). An Arabic numeral is assigned to individual members (e.g., ABCB1).

1.4.2 P-Glycoprotein (*ABCB1*)

P-Glycoprotein is a membrane efflux transporter encoded by the *ABCB1* gene, which is also known as the multi-drug resistance 1 (MDR1) gene. ABCB1 functions as an energy-dependent transporter that effluxes substrates from the intracellular to extracellular compartment (Ambudkar et al., 1999). ABCB1 is a major determinant of drug bioavailability, renal secretion, biliary secretion, and central nervous system entry of a variety of hydrophobic substrates (Faber et al., 2003; Ho and Kim, 2005). ABCB1 is expressed in healthy, normal cells, and it is over-expressed in various tumour cells. Over-expression results in decreased intracellular concentration of drugs, including a wide range of chemotherapeutic drugs (Zhang and Benet, 2001). ABCB1 is highly expressed in the villus tip of enterocytes in the gastrointestinal tract (Zhang and Benet, 2001). Therefore, ABCB1 is a major determinant of oral bioavailability of drugs.

1.4.2.1 Tissue Distribution

ABCB1 mRNA is detected in human liver, kidney, lung, and small and large intestine, as determined by RT-PCR (Miki et al., 2005). Immunohistochemistry shows that ABCB1 is present in liver, pancreas, kidney, colon, jejunum, and adrenal gland (Thiebaut et al., 1987) (Cordon-Cardo et al., 1990). The protein expression of ABCB1 is highest in small intestine, followed by liver, kidney, colon, lung, spleen, brain and skeletal muscle (Silverman and Schrenk, 1997).

1.4.2.2 Ontogeny

In human liver, ABCB1 mRNA expression is low in fetal and neonatal tissue (i.e., before birth and right after birth, respectively), as determined by RT-PCR (Miki et al., 2005). It slightly increases in the young group (i.e., 15-38 years old) and plateaus in the middle age (i.e., 45-65 years old) and elderly groups (i.e., 67-85 years old). In human kidney, ABCB1 mRNA expression is low in fetal and neonatal groups, increases to its maximal level in the young group, and plateaus in the middle age and elderly groups. In human lung, ABCB1 mRNA expression is low in fetal tissue, but increases after birth, reaches its maximal level in the young group, and decreases thereafter. In human small intestine, ABCB1 mRNA expression is at its peak in young-middle age, but remains relatively low in all other groups. In human large intestines, ABCB1 mRNA expression is also relatively low in all groups.

1.4.2.3 Substrates

ABCB1 transports a board range of structurally diverse substrates. The substrates include several chemotherapeutic agents (e.g., doxorubicin), some antiretroviral therapy

agents (e.g., indinavir), cardiac glycosides (e.g., digoxin), immunosuppressive agents (e.g., cyclosporin), and corticosteroids (e.g., dexamethasone, hydrocortisone) (Marchetti et al., 2007). They are generally hydrophobic and amphipathic in nature and uncharged or basic. However, they can also be zwitterionic (e.g. [$_{D}$-penicillamine^{2,5}]enkephalin) (Hoffmaster et al., 2005), and negatively charged (e.g. methotrexate) (Norris et al., 1996).

1.4.2.4 ABCB1 Inhibitors

Many clinically used drugs have been identified as ABCB1 inhibitors. They include various calcium antagonist (e.g., diltiazem, nicardipine, and verapamil), hydrocortisone, immunosuppresants (cyclosporine and tacrolimus), and HIV protease inhibitors (e.g., indinavir, nelfinavir, ritonavir, and saquinavir) (Zhang and Benet, 2001). Erythromycin and mifepristone are not ABCB1 substrates, but are able to inhibit ABCB1 function. Moreover, naturally occurring compounds have been identified as ABCB1 inhibitors (Marchetti et al., 2007). For example, capsaicin (Nabekura et al., 2005), ginsenoside Rg(3) (Kim et al., 2003), piperine from black pepper (Bhardwaj et al., 2002), resveratrol (Nabekura et al., 2005), and silymarin from milk thistle (Zhang and Morris, 2003) inhibit ABCB1 activity *in vitro*. Curcumin (Anuchapreeda et al., 2002), curcuminoids (Limtrakul et al., 2004), and several catechins from green tea (Jodoin et al., 2002; Mei et al., 2004) not only reduce ABCB1 function, but also reduce ABCB1 mRNA expression *in vitro*.

1.4.2.5 ABCB1 Inducers

Some clinically used drugs and naturally occurring compounds have been identified as ABCB1 inducers (Urquhart et al., 2007). For example, rifampin, phenobarbital, and triacetyloleandomycin increase ABCB1 protein expression in the LS180 human colon

carcinoma cell line (Schuetz et al., 1996). By the use of real-time PCR, artemisinin was shown to increase ABCB1 mRNA expression in primary human hepatocytes and in the human intestinal cell line LS174T (Burk et al., 2005b). Similarly, treatment of human LS180 colorectal adenocarcinoma cells with flucloxacillin causes a dose-dependent induction of ABCB1 mRNA in primary human hepatocytes (Huwyler et al., 2006). In addition, ABCB1 protein expression and transport activity are significantly increased when isolated brain capillaries of PXR-transgenic mice were treated *in vitro* with hyperforin, a constituent in St. John's wort (Bauer et al., 2006).

1.4.2.6 Mechanisms of ABCB1 Induction

PXR has been shown to regulate ABCB1 induction (Geick et al., 2001). Rifampin, a prototypical PXR agonist, induces *ABCB1* gene expression in the LS174T human colon carcinoma cell line through activation of ABCB1 promoter. The activation requires a DR4 motif, where PXR/RXRα heterodimer specifically binds. Likewise, CAR has been shown to regulate ABCB1 induction (Burk et al., 2005a). Using gel shift and transfection experiments, Burk *et al.* (2005a) demonstrated that CAR binds to distinct nuclear receptor response elements in the enhancer region of ABCB1, and also transactivates *ABCB1* gene expression.

1.5 Herbal Medicines

Herbal medicines are taken to prevent and treat diseases. They are made of plant or plant parts and formulated as tablets, capsules, powder, teas, and extracts. Herbal medicines are part of complementary and alternative medicine (CAM), which is generally defined as medical products and practices that are not part of conventional medicine. In a

survey conducted in Lenox Hill Hospital, New York, over a 10-week period, 57% of the 2,186 surgical patients admitted they had used herbal medicines at some point in their life (Adusumilli et al., 2004). Examples of some commonly used herbs and their uses include St. John's wort for treating depression (Kasper et al., 2008), *Ginkgo biloba* for improving cognitive function (Birks and Grimley Evans, 2007; DeFeudis, 2003), valerian for treating insomnia (Bent et al., 2006), ginseng for boosting immune system (Block and Mead, 2003), echinacea for treating and preventing colds and flu (Roxas and Jurenka, 2007), and garlic for reducing cholesterol and blood pressure (Borek, 2006; Rahman and Lowe, 2006). Other examples of herbs and their uses include trabectedin for soft tissue sarcoma (Schoffski et al., 2008), gugulipid for cholesterol (Ulbricht et al., 2005), Tian Xian for cancer (Lichti-Kaiser and Staudinger, 2008), Qing Hao for malaria (van Agtmael et al., 1999), Gan Cao for inflammation (Staudinger et al., 2006), Wu Wei Zi for infection, coughing, and thirst (Staudinger et al., 2006), and *Coleus forskohlii* for eczema, convulsion, hypothyroidism, hypertension, and congestive heart failure (Ammon and Muller, 1985).

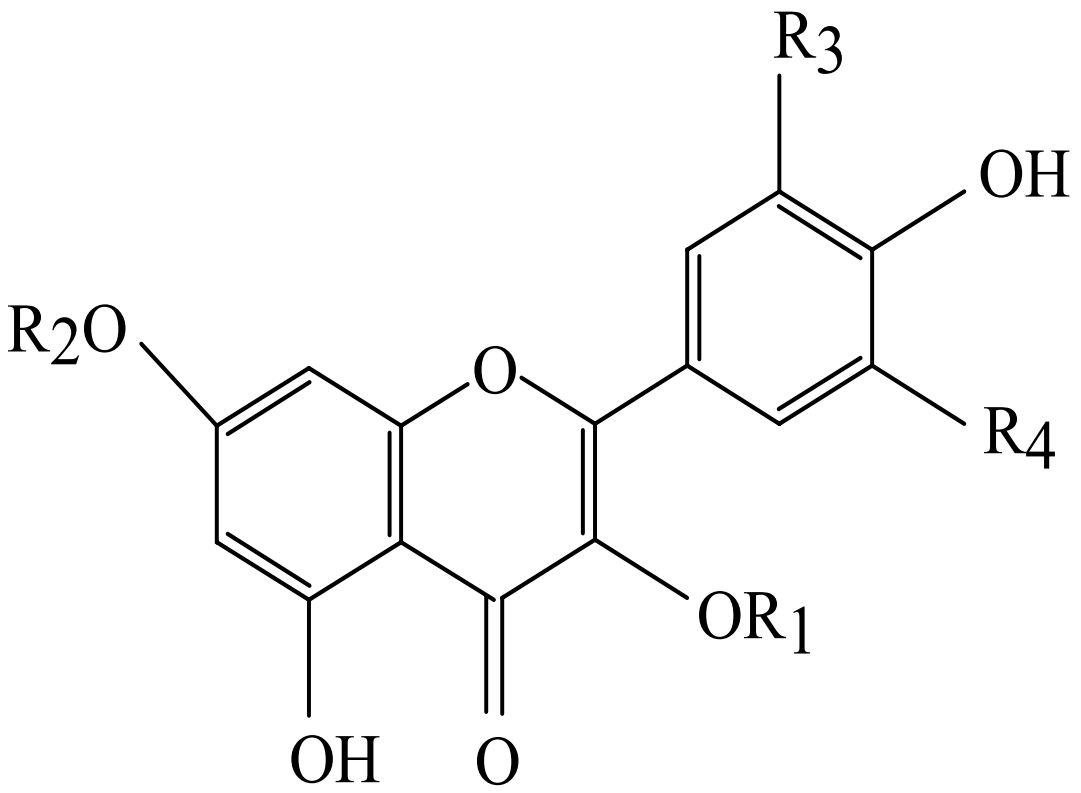
Many herbal products are available on the market, advertised to prevent and treat many different symptoms, but most of them lack scientific evidence to support their advertised claims. Some of them can interact with drugs and other herbs and cause serious health problems. Therefore, before taking herbal products, consumers should always pay attention to all possible risks, and consider if the benefits for taking them outweigh the risks. Unlike prescription and over-the-counter medicines, herbal products usually lack extensive research on their safety and advertised claims. Consequently, it is important to conduct research on the safety and efficacy of herbal medicines.

1.6 *Ginkgo biloba*

Ginkgo biloba is a tree that is originally from China. It is the only surviving species of the order *Ginkgoales* (class *Gymnospermae*), which is 200 million years old and existed in the Jurassic period (Smith and Luo, 2004). The leaf extract was introduced into medical practice in 1965 (DeFeudis, 2003). It is commonly used to improve age-related memory impairment, improve cognitive functions, increase attention and enhance mental concentration (Birks and Grimley Evans, 2007; DeFeudis, 2003). *Ginkgo biloba* was reported to be the third most commonly used herb in a survey conducted in the United States in 2002 (Barnes et al., 2004).

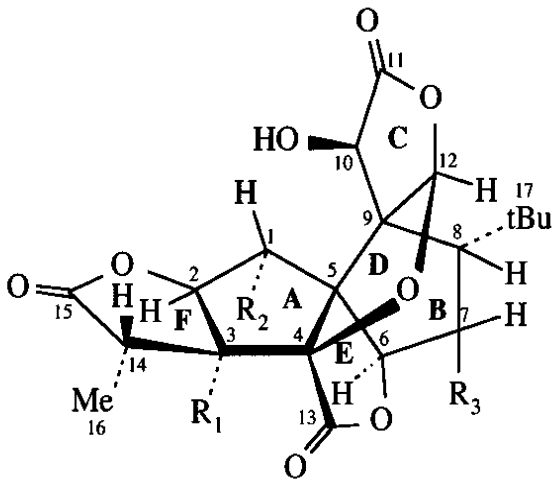
1.6.1 Chemical Constituents

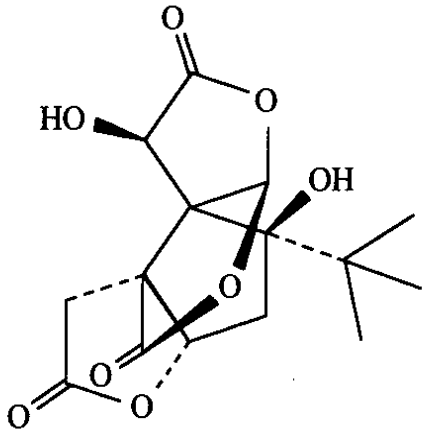
Most of the commercially available *Ginkgo biloba* extracts contain approximately 6% terpene trilactones (e.g., bilobalide and ginkgolides A, B, C, and J) and approximately 24% flavonol glycosides (e.g., those of kaempferol, quercetin, and isorhamnetin) (van Beek, 2002). They also contain proanthocyanidins, carboxylic acids, catechins, non-flavonol glycosides, high molecular mass compounds, inorganic constituents, alkylphenols, and unknown substances. Shown in Fig. 1.13 and Fig 1.14 are the chemical structures of some flavonols and terpene trilactones, respectively. It is important to note that quercetin is a metabolite of kaempferol, whereas isorhamnetin is a metabolite of quercetin.



	R_1 (C-3)	R_2 (C-7)	R_3 (C-3')	R_4 (C-5')
Kaempferol	H	H	H	H
Quercetin	H	H	OH	H
Isorhamnetin	H	H	OCH_3	H

Fig. 1.13. Chemical structures of kaempferol, quercetin, and isorhamnetin.





Ginkgolides **Bilobalide**

	R1	R2	R3
Ginkgolide A	**H**	**OH**	**H**
Ginkgolide B	**H**	**OH**	**OH**
Ginkgolide C	**OH**	**OH**	**OH**
Ginkgolide J	**OH**	**OH**	**H**

Fig. 1.14. Chemical structures of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide.

1.6.2 Pharmacokinetics of Terpene Trilactones and Flavonols in Animals Administered *Ginkgo biloba* Extract

In rats, no intact flavonol glycosides were present in whole blood samples, which were withdrawn every 30 min for 6 h after oral administration of 4 g/kg dosage of *Ginkgo biloba* extract (Pietta et al., 1995). Similarly, no intact flavonol glycosides were present in urine or feces samples collected for 5 days after administration. The following metabolites were present: 3,4-dihydroxy-phenylacetic acid, hippuric acid, 3-hydroxyphenylacetic acid, homovanillic acid, and benzoic acid (Fig 1.15). In another study, 12, 7, and 50 ng/ml of quercetin, kaempferol, and isorhamnetin, respectively, were quantified in plasma of rats provided with food pellets containing *Ginkgo biloba* extract (300 mg/kg of pellet) (Watanabe et al., 2001). On average, each rat consumed 3.8 g of feed per day, which is equivalent to a dosage of 36 mg/kg of *Ginkgo biloba* extract. In addition, quercetin metabolism has been investigated in animal models. Rats consumed ~13 g/d diet, which provided ~58.5 mg/d of quercetin, for 6 weeks (Graf et al., 2006). Quercetin was present as sulphated (e.g. quercetin-3'sulfate), glucuronidated (e.g. isorhamnetin-3-glucuronide), and methylated (e.g. isorhamnetin and tamarixetin) metabolites in the rat plasma and gastrointestinal (stomach, small intestine, cecum, and colon), liver, and kidney tissues. The chemical structures of quercetin metabolites are illustrated in Fig. 1.16.

The pharmacokinetic parameters of ginkgolide A, ginkgolide B, and bilobalide were characterized in rats after oral administration of 30, 55, and 100 mg/kg of *Ginkgo biloba* extract (Biber and Koch, 1999). The peak concentrations in plasma (C_{max}) were highest for bilobalide (159, 275, and 398 ng/ml, respectively), second highest for ginkgolide B (40, 69, and 103 ng/ml, respectively), and lowest for ginkgolide A (68, 147, and 301 ng/ml,

respectively). The terminal half-lives of elimination ($t_{1/2}$) of ginkgolide A, ginkgolide B, and bilobalide were 1.8, 2.2, and 2.3 h, respectively.

The pharmacokinetic parameters of ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide were determined in rats receiving intravenous injection of *Ginkgo biloba* extract via a tail vein at a dosage of 8 mg/kg (Xie et al., 2008). The areas under the plasma concentration-time curve from time 0 to infinity ($AUC_{0-\infty}$) for ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide were 258.6, 157.6, 15.3, and 39.06 μg h/l, respectively. The terminal half-lives of elimination ($t_{1/2}$) for ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide were 0.97, 1.02, 0.67, and 1.13 h, respectively. The clearances (Cl) for ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide were 36.83, 112.1, 136.8, and 301.1 L/kg, respectively. The apparent volumes of distribution (V_d) for ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide were 49.33, 163.9, 130.4, and 478.0 L/kg, respectively. In addition, the chemical structures of ginkgolide B metabolites were identified in the urine of rats, which received a single dose of ginkgolide B emulsion (4 mg/kg) injected via the caudal vein (Wang et al., 2008) (Fig 1.17). However, there is currently no study on the metabolism of other terpene trilactones in animals.

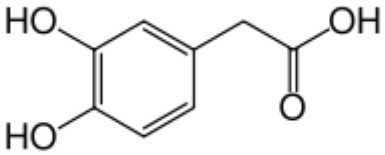
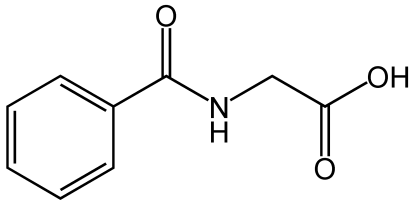
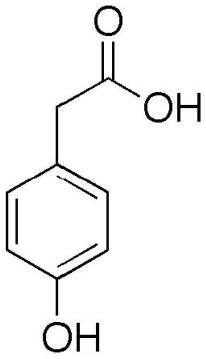
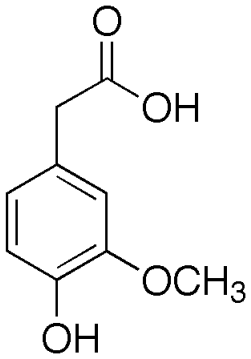
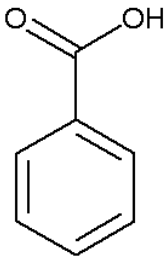
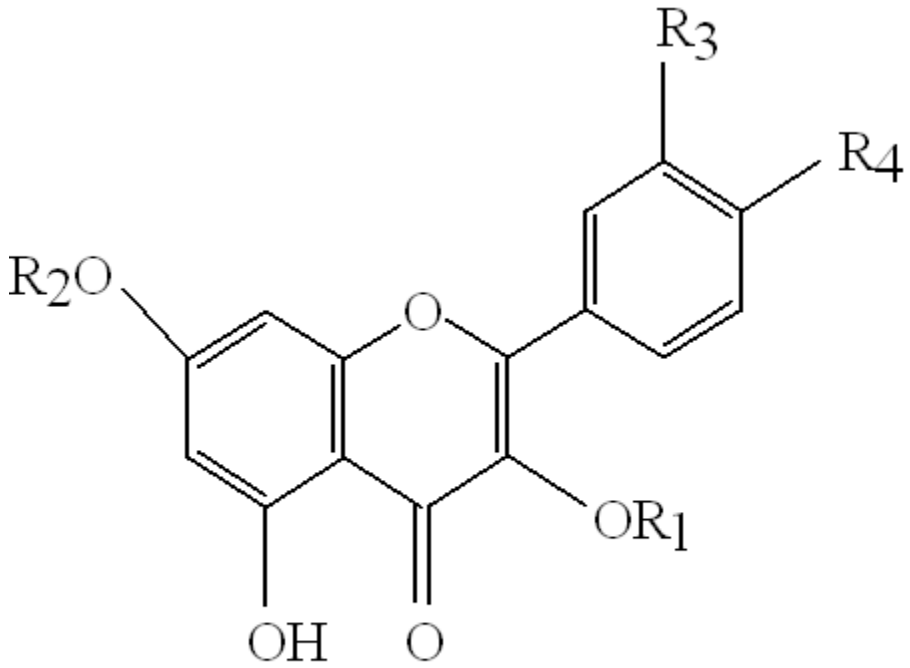
3,4-dihydroxy-phenylacetic acid 	hippuric acid 
3-hydroxyphenylacetic acid 	homovanillic acid 
benzoic acid 	

Fig. 1.15. Chemical structures of 3,4-dihydroxy-phenylacetic acid, hippuric acid, 3-hydroxyphenylacetic acid, homovanillic acid, and benzoic acid.



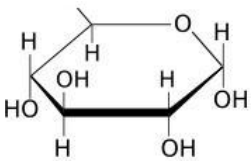
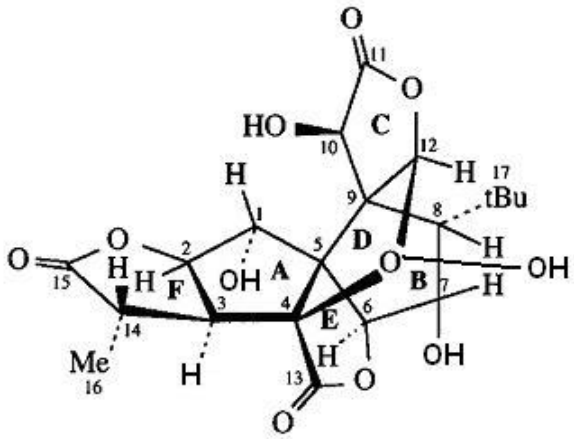
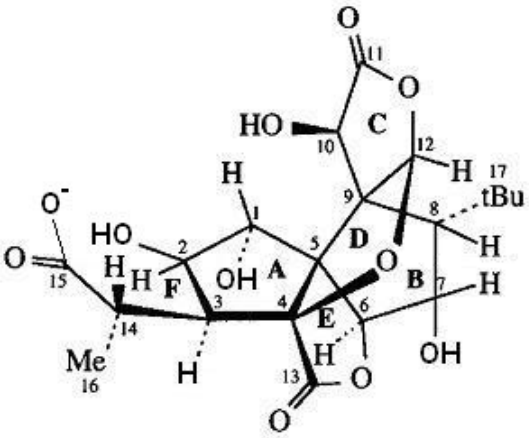
	R_1	R_2	R_3	R_4
Quercetin	H	H	OH	OH
Isorhamnetin	H	H	OCH_3	OH
Tamarixetin	H	H	OH	OCH_3
Quercetin-3'sulfate	H	H	SO_3	OH
Isorhamnetin-3-glucuronide		H	OCH_3	OH

Fig. 1.16. Chemical structures of the sulfated, glucuronidated, and methylated metabolites of quercetin.

Metabolite M_1



Metabolite M_2



Metabolite M_3

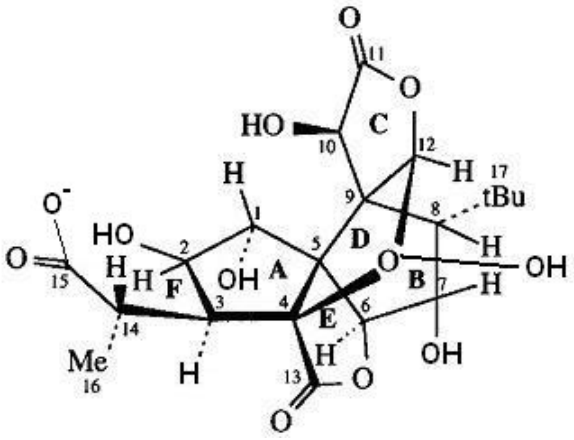


Fig. 1.17. Chemical structures of ginkgolide B metabolites in rat urine (Wang et al., 2008).

1.6.3 Pharmacokinetics of Terpene Trilactones and Flavonols in Humans Administered *Ginkgo biloba* Extract

The aglycones of quercetin, kaempferol, and isorhamnetin were found in the urine of 3 volunteers who received oral administration of 400 mg standardized *Ginkgo biloba* extract while on a flavones-free diet (Biber, 2003). Studies showed flavonols (measured as the sum of quercetin, kaempferol and isorhamnetin) had elimination half-lives of 2 to 4 h in human subjects receiving 50, 100, and 300 mg of *Ginkgo biloba* extract (Biber, 2003). In ten volunteers (mean age 28 years) receiving a single oral dose of six tablets of *Ginkgo biloba* extract (1.134 mg of quercetin and 1.233 mg kaempferol per tablet), quercetin had slightly greater elimination rate constant (k) and absorption rate constant (ka), but smaller absorption half-life ($t_{1/2a}$), elimination half-life ($t_{1/2}$) and t_{max} than kaempferol (Wang et al., 2003b). However, the differences were not statistically significant. The study also found that quercetin and kaempferol were excreted in the human urine mainly as glucuronides. Table 1.1 summarizes the pharmacokinetic parameters of quercetin and kaempferol in the study by Wang *et al.* (2003b). In another study, the mean urine concentrations of quercetin and kaempferol were 34.38 and 44.45 ng/ml, respectively, in seven volunteers 4 h after receiving a single oral dose of six tablets of *Ginkgo biloba* extract (1.134 mg of quercetin and 1.233 mg kaempferol per tablet) (Wang et al., 2003a). However, 11 h after the dose, the cumulative excretion amounts of quercetin (4 μg) was less than that of kaempferol (8 μg). In addition, the following metabolites were found in human subjects 7 days after a single oral administration of 4 g *Ginkgo biloba* extract while on a flavones-free diet: hippuric acid, 4-hydroxyhippuric acid, 3-methoxy-4-hydroxyhippuric acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, and vanillic acid (Pietta et al., 1997) (Fig. 1.18).

Table 1.2, Table 1.3 and Table 1.4 summarize the pharmacokinetic parameters of ginkgolide A, ginkgolide B, and bilobalide, respectively, in human volunteers administered *Ginkgo biloba* extract. In general, ginkgolide B has the lowest peak concentrations in plasma (C_{max}). Ginkgolide A and bilobalide have the highest C_{max}. The time to reach C_{max} (t_{max}) for all three constituents is approximately 1 h. Ginkgolide B has the smallest area under the plasma time-concentration curve from time 0 to infinity ($AUC_{0-\infty}$), whereas ginkgolide A and bilobalide have the greatest AUC. Ginkgolide B also has the longest terminal elimination half-life ($t_{1/2}$), whereas ginkgolide A and bilobalide have the shortest $t_{1/2}$. Ginkgolide A has the greatest urinary excretion ratio (U) and bilobalide has the smallest ratio. The bioavailability (F) is approximately 82% for all three constituents. Bilobalide has the highest clearance (Cl), whereas ginkgolide A and ginkgolide B have virtually the same clearance. The elderly subjects have lower clearance than the young subjects probably because clearance decreases with age (Biber, 2003). The pharmacokinetic parameters are not different between single and multiple dosing administrations in elderly subjects (Biber, 2003).

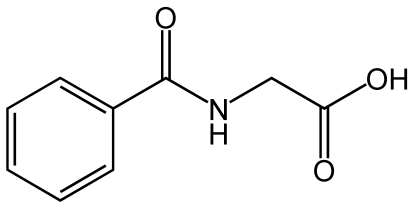
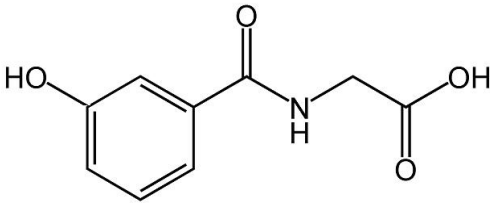
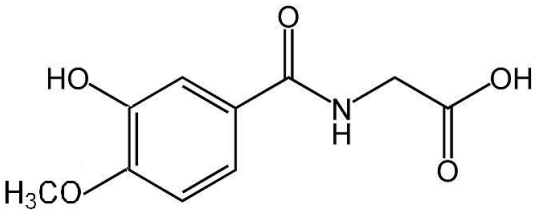
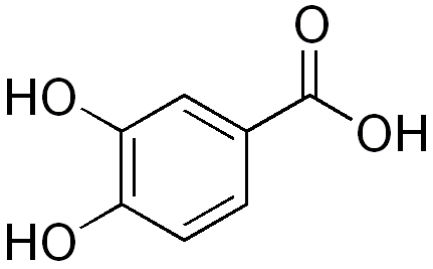
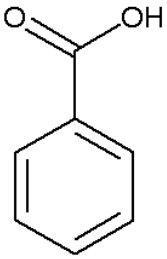
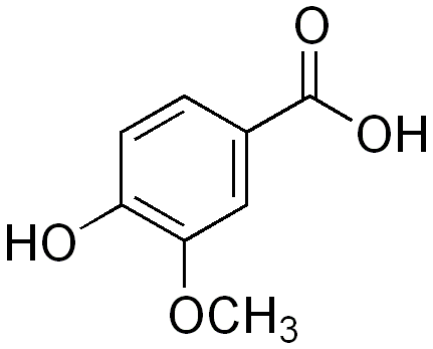
hippuric acid 	4-hydroxyhippuric acid 
3-methoxy-4-hydroxyhippuric acid 	3,4-dihydroxybenzoic acid 
4-hydroxybenzoic acid 	vanillic acid 

Fig. 1.18. Chemical structures of hippuric acid, 4-hydroxyhippuric acid, 3-methoxy-4-hydroxyhippuric acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, and vanillic acid.

Table 1.1. Pharmacokinetic parameters of quercetin and kaempferol in human volunteers administered *Ginkgo biloba* extract

Study: Ten healthy volunteers (mean age 28 years) received a single oral dose of six tablets of *Ginkgo biloba* extract (1.134 mg of quercetin and 1.233 mg kaempferol per tablet) (Wang et al., 2003b)

	ka (h^{-1})	$t_{1/2a}$ (h)	k (h)	t_{max} (h)	$t_{1/2}$ (h)
Quercetin	0.61	1.51	0.37	2.30	2.17
Kaempferol	0.55	1.56	0.31	2.68	2.76

Abbreviations: absorption rate constant (ka), $t_{1/2a}$, absorption half-life; k, elimination rate constant; t_{max}, time to reach C_{max}; t1/2, terminal elimination half-lives

Table 1.2. Pharmacokinetic parameters of ginkgolide A in human volunteers administered *Ginkgo biloba* extract

Ginkgolide A									
	C_{max} (ng/ml)	t_{max} (h)	AUC (ng h ml^{-1})	$t_{1/2}$ (h)	U (%)	F (%)	Cl (l/h)	V_d (l)	C_{ss} (ng/ml)
Study 1	22.22	1.17	121.35	3.93	NA	NA	NA	NA	NA
Study 2	33.29	1.06	146.04	4.5	72.3	80	10.07	NA	NA
Study 3	73.04	0.24	146.75	3.75	72.6	NA	8.02	36.9	NA
Study 4A	15.2	0.61	69.9	4.5	NA	NA	NA	NA	NA
Study 4B	25.3	0.60	103.2	4.5	NA	NA	NA	NA	NA
Study 4C	42.9	0.75	211.1	5.1	NA	NA	NA	NA	NA
Study 5A	20.77	0.71	113.3	6.09	50	NA	7.1	NA	NA
Study 5B	21.58	0.75	113.1	9.06	NA	NA	NA	NA	9.43

Abbreviations: C_{max}, the peak concentrations in plasma; tmax, time to reach C_{max}; $AUC_{0-\infty}$, areas under the plasma time concentration curve from time 0 to infinity; $t_{1/2}$, terminal elimination half-lives; U, urine excretion ratio; F, bioavailability; Cl, clearance; V_d, volume of distribution; C_{ss}, steady-state concentration; NA, not available

A description of the design for each study is given in page 48.

Table 1.3. Pharmacokinetic parameters of ginkgolide B in human volunteers administered *Ginkgo biloba* extract

Ginkgolide B									
	C_{max} (ng/ml)	t_{max} (h)	AUC (ng h ml^{-1})	$t_{1/2}$ (h)	U (%)	F (%)	Cl (l/h)	V_d (l)	C_{ss} (ng/ml)
Study 1	8.27	1.54	59.88	6.04	NA	NA	NA	NA	NA
Study 2	16.48	1.17	109.9	10.6	41.4	88	9.67	NA	NA
Study 3	42.42	0.26	111.84	5.23	45.25	NA	8.27	53.6	NA
Study 4A	6.53	1.29	43.75	6.5	NA	NA	NA	NA	NA
Study 4B	9.12	0.92	70.03	8.5	NA	NA	NA	NA	NA
Study 4C	18.11	1.21	140.69	9.9	NA	NA	NA	NA	NA
Study 5A	8.54	1.04	70.81	9.78	8.4	NA	28	NA	NA
Study 5B	10.73	1.29	73.74	12.53	NA	NA	NA	NA	6.15

Abbreviations: C_{max}, the peak concentrations in plasma; tmax, time to reach C_{max}; $AUC_{0-\infty}$, areas under the plasma time concentration curve from time 0 to infinity; $t_{1/2}$, terminal elimination half-lives; U, urine excretion ratio; F, bioavailability; Cl, clearance; V_d, volume of distribution; C_{ss}, steady-state concentration; NA, not available

A description of the design for each study is given in page 48.

Table 1.4. Pharmacokinetic parameters of bilobalide in human volunteers administered *Ginkgo biloba* extract

Bilobalide									
	C_{max} (ng/ml)	t_{max} (h)	AUC (ng h ml^{-1})	$t_{1/2}$ (h)	U (%)	F (%)	Cl (l/h)	V_d (l)	C_{ss} (ng/ml)
Study 1	54.42	1.21	217.24	3.19	NA	NA	NA	NA	NA
Study 2	18.81	1.17	78.97	3.2	3.12	79	52.18	NA	NA
Study 3	57.08	0.23	97.46	3.19	33.21	NA	36.26	150.0	NA
Study 4A	30.2	0.86	114.7	5.5	NA	NA	NA	NA	NA
Study 4B	35.2	0.67	128.1	4.0	NA	NA	NA	NA	NA
Study 4C	58.6	0.72	247.1	4.9	NA	NA	NA	NA	NA
Study 5A	21.85	0.71	91.15	4.00	25	NA	20.1	NA	NA
Study 5B	25.85	0.92	105.0	4.12	NA	NA	NA	NA	8.74

Abbreviations: C_{max}, the peak concentrations in plasma; tmax, time to reach C_{max}; $AUC_{0-\infty}$, areas under the plasma time concentration curve from time 0 to infinity; $t_{1/2}$, terminal elimination half-lives; U, urine excretion ratio; F, bioavailability; Cl, clearance; V_d, volume of distribution; C_{ss}, steady-state concentration; NA, not available

A description of the design for each study is given in page 48.

Study Designs:

Study 1. Twelve healthy male volunteers (mean age 33.3 years, mean weight 78.6 kg) received 120 mg standardized *Ginkgo biloba* extract tablets (contained 1.78% ginkgolide A, 0.83% ginkgolide B, and 3.35% bilobalide) (Kressmann et al., 2002).

Study 2. Twelve healthy volunteers (6 males and 6 females, mean age 25.1 years, mean weight 58.6 kg) received oral administration of 120 mg standardized *Ginkgo biloba* extract (contained 1.16% ginkgolide A, 0.90% ginkgolide B, and 3.00% bilobalide) (Fourtillan et al., 1995).

Study 3. Twelve healthy volunteers (6 males and 6 females, mean age 25.1 years, mean weight 58.6 kg) received intravenous administration of 100 mg standardized *Ginkgo biloba* extract (contained 1.16% ginkgolide A, 0.90% ginkgolide B, and 3.00% bilobalide) (15 minutes perfusion) (Fourtillan et al., 1995).

Study 4. Twelve healthy male volunteers (mean age 22 years, mean weight 71.9 kg) received oral administration of 80 (A), 120 (B), and 240 (C) mg standardized *Ginkgo biloba* extract (contained 1.32% ginkgolide A, 0.70% ginkgolide B, and 2.85% bilobalide) in single doses (Biber, 2003).

Study 5. Twelve healthy elderly volunteers of both sexes (mean age 72.7 years, mean weight 64.7 kg), who (A) orally received a single dose of 60 mg standardized *Ginkgo biloba* extract (2 batches; contained 0.765 mg ginkgolide A, 0.600 mg ginkgolide B, and 1.605 mg bilobalide for 8 subjects, and 0.750 mg ginkgolide A, 0.480 mg ginkgolide B, and 1.600 mg

bilobalide for 4 subjects) after an overnight fast, and (B) 60 mg of the same extract twice daily from day 3 to day 8 (Biber, 2003).

1.6.4 Biological Activities and Mechanisms of Action

Various biochemical and pharmacological activities of *Ginkgo biloba* have been identified. Since the 1970's, *Ginkgo biloba* has been found to have beneficial effects on cerebral ischemia in animals (Larsen et al., 1978). Rats treated with *Ginkgo biloba* extract for three weeks showed reduced neurological deficits and death that follow cerebral embolism. *Ginkgo biloba* has also been found to regulate contraction and relaxation in isolated blood vessels (Auguet et al., 1982). *Ginkgo biloba* (100 μg/ml) potentiated the contractile effect of norepinephrine in rabbit aorta; the extract reduced the EC_{50} (concentration required to elicit 50% of the maximal effect) of norepinephrine from 75 to 36 nM. *Ginkgo biloba* is also able to contract isolated rabbit vena cava, suggesting it may play a role in maintaining venous tone (Hellegouarch et al., 1985). *Ginkgo biloba* (200 or 300 μg/ml) relaxes isolated rabbit aorta that has been pre-contracted by phenylepherine. The relaxant effect is believed to be due to stimulation of endothelium-derived relaxing factor (EDRF) by *Ginkgo biloba*. The results indicate that *Ginkgo biloba* is able to dilate blood vessels, thereby increasing blood supply to the brain. This would provide more oxygen and nutrients supply that are beneficial for blood cerebral insufficiency.

Ginkgo biloba acts as an antioxidant that scavenges toxic oxygen free radicals, thereby decreasing levels of reactive oxygen species in tissue and prevents membrane lipid peroxidation (DeFeudis and Drieu, 2000). Free radicals can lead to oxidative damage, which is associated with disorders such as age-associated neurological diseases, cardiovascular disorders, cancer, and peripheral arterial occlusive disease (DeFeudis, 2003). *In vitro*, *Ginkgo biloba* has been shown to possess a superoxide anion (O^{2-}) scavenging effect (Pincemail et al., 1989), which is an important defence in nearly all cells exposed to oxygen. The scavenging effect of *Ginkgo biloba* has also been confirmed by reduction of lipid peroxidation in mouse

brain tissue with experimental cerebral ischemia (Pierre et al., 1999). In addition, reactive oxygen species production is significantly increased in a cellular genetically engineered Alzheimer's disease model, and in an *Caenorhabditis elegans* Alzheimer's disease model; pre-treatment with *Ginkgo biloba* extract significantly reduced oxygen species production in both models (Smith and Luo, 2003). The results indicate that the antioxidant effect of *Ginkgo biloba* may be beneficial in treating cerebral ischemia and neurological disorders; however, further clinical tests are required to validate this finding.

The antioxidant property of *Ginkgo biloba* might also be beneficial for cardiovascular disorders. Post-ischemic levels of reactive oxygen species in coronary effluents were significantly decreased after *in vivo* oral treatments or after *in vitro* perfusion with standardized *Ginkgo biloba extract* in rats (Pietri et al., 1997a). The same study also showed anti-ischemic effects after repeated (15-day) oral treatments with *Ginkgo biloba* (60 mg/kg/day). The scavenging effect of *Ginkgo biloba* has also been confirmed by reduction of lipid peroxidation in humans undergoing coronary bypass surgery (Pietri et al., 1997b). Those results indicate that *Ginkgo biloba* can improve myocardial functional recovery.

Ginkgo biloba has been found to possess neuroprotective effects through various mechanisms. As mentioned before, *Ginkgo biloba* decreases levels of reactive oxygen species in tissue and prevents membrane lipid peroxidation. *Ginkgo biloba* has also been shown to possess anti-apoptotic properties in neuronal culture of PC 12 cells, a well-established model for studying neuroprotection (Smith et al., 2002). *Ginkgo biloba* treatments of PC12 cells protects the integrity of the mitochondrial membrane; decreases the release of cytochrome *c* from the mitochondria, which is crucial for initiation of the apoptotic caspase cascade; blocks the activation of caspase-3, a crucial protease that executes apoptosis; increases the transcription of anti-apoptotic Bcl-2-like protein; decreases the transcription of

pro-apoptotic caspase-12, an endoplasmic reticulum protein that mediates stress; and blocks nuclear fragmentation, which is the hallmark of apoptosis (Smith et al., 2002). Another neuroprotective effect of *Ginkgo biloba* is its ability to inhibit aggregation of amyloid β, which appears to be a cause of Alzheimer's disease (Gandy et al., 1994). *Ginkgo biloba* inhibits formation of amyloid β aggregates in a neroblastoma cell line that stably expressed an Alzheimer's disease-related mutation (Luo et al., 2002b). In addition, using primary culture hippocampal neurons, *Ginkgo biloba* extract has been shown to protect against cell death induced by amyloid β (Bastianetto et al., 2000). Those experiments demonstrate a possible neuroprotective effectiveness of *Ginkgo biloba* towards neurodegenerative diseases.

Ginkgo biloba has also been shown to have anti-stress effects. Young (4-month-old) and old (20-month-old) rats are trained to discriminate to obtain a small amount of drinking water as a reward (Rapin et al., 1994). When stress (in form of auditory disturbance) is introduced during their learning phase, both young and old animals have detrimental change in learning and increased plasma concentrations of epinephrine, norepinephrine, and corticosterone. Oral treatment with *Ginkgo biloba* extract (50 or 100 mg/kg/day for 20 days) was able to suppress those effects in both young and old rats. The suppressive effect became statistically significant by the third day of learning phase. Furthermore, oral administration of *Ginkgo biloba* extract (100 mg/kg/day for 10 days) to rats reduced the ligand binding capacity, protein, and mRNA expression of the adrenocortical mitochondrial peripheral-type benzodiazepine receptor (PBR) (Amri et al., 1996). This receptor is a key factor for glucocorticoid synthesis, which is increased by mental and physical stress. Therefore, the anti-stress effect of *Ginkgo biloba* is possibly caused by its influence on the levels of neurotransmitters and hormones circulating the body.

1.6.5 Clinical Uses

The German Commission E, which is equivalent to the United States Food and Drug Administration (FDA) for botanicals, has approved some clinical uses of *Ginkgo biloba* that include symptom relief of impairments in concentration, memory, depression from organic brain syndrome (physical disorders that decreased mental function), and cerebral insufficiency (Diamond et al., 2000). Some of the uses have been tested in clinical trials.

In a meta-analysis of 35 randomized, double-blind trials, including subjects with dementia and cognitive impairment, *Ginkgo biloba* was better than placebo in subjects' clinical global improvement (CGI) at 24 weeks when dosages were greater than 200 mg/day (Birks and Grimley Evans, 2007). *Ginkgo biloba* improves cognition at dosages less than 200 mg/day for 12 weeks. *Ginkgo biloba* was also shown to improve Activities of Daily Living scale (ADL) at dosages less than 200 mg/day for 12 weeks. Despite methodological deficiencies, such as limited sample size, in many of the trials, there is evidence suggesting that intake of *Ginkgo biloba* modestly improves dementia and cognition.

Intermittent claudication (Fontaine's Stage II peripheral arterial occlusive disease) is a complex of symptoms characterized by leg pain and weakness brought on by walking (DeFeudis, 2003). This disorder can be due to restriction of blood supply to the leg muscles. In a meta-analysis of 8 randomized, placebo-controlled, double-blind trials (n = 415), *Ginkgo biloba* extract (120–160 mg per day for 12–24 weeks) increased pain-free walking distance in intermittent claudication by 53.4 m, whereas an increase of 17.4 m was observed in the placebo-treated group (Pittler and Ernst, 2000). The same study showed that *Ginkgo biloba* increased maximal walking distance by 83.3 m, whereas placebo improved the distance by 6 m. The results indicate that *Ginkgo biloba* can be used for symptom relief of intermittent claudication. However, the overall impact on the treatment is modest.

Cerebral insufficiency refers to brain disorders characterized by symptoms such as memory loss, impairment of cognitive functions, dizziness, and dementia (DeFeudis, 2003). In a systematic review of 40 trials of *Ginkgo biloba* for symptoms and signs of cerebral insufficiency, 8 met methodological criteria and the rest were excluded because of deficiencies in methods, such as limited sample size, and vague description of randomization procedures, patient characteristics, effect measurement and data presentation (Kleijnen and Knipschild, 1992). In most trials, subjects ingested a dosage of 120 mg *Ginkgo biloba* extract a day for at least 4-6 weeks. The results showed *Ginkgo biloba* extract improved the mild-to-moderate clinical symptoms of cerebral insufficiency.

A randomized, controlled, double-blind trial was conducted in 55 subjects with computerized tomographic (CT) evidence of acute ischemic infarction (Garg et al., 1995). Twenty-one subjects received *Ginkgo biloba* 40 mg four times daily for four weeks, whereas the other subjects received placebo. Both groups showed improvement in scores on the validated Matthew's neurologic assessment scale at two and four-week intervals. However, *Ginkgo biloba* was not superior to placebo in treatment of acute ischemic stroke.

A randomized, double-blind, placebo controlled, parallel-group, multicenter trial was conducted in elderly subjects with age-associated memory impairment (van Dongen et al., 2003). The group mean changes of primary endpoints were not different between *Ginkgo biloba* and placebo. The primary endpoints include the Syndrome Kurz Test (SKT; psychometric functioning), the Clinical Global Impression of change (CGI-2; psychopathology, assessed by nursing staff), and the Nuremberg Gerontopsychological Rating Scale for Activities of Daily Living (NAI-NAA; behavioral functioning). No dose-effect relationship and no effect of prolonged *Ginkgo biloba* treatment were found. Therefore, *Ginkgo biloba* does not appear to benefit patients with age-associated memory impairment.

According to Natural Standard (www.naturalstandard.com) and Natural Medicines Comprehensive Database (www.naturaldatabase.com), *Ginkgo biloba* has been tested for other clinical uses, such as altitude sickness, chemotherapeutic adjunct, chronic cochleovestibular disorders, impotence, depression, gastric cancer, glaucoma, macular degeneration, memory enhancement, multiple sclerosis, peripheral artery disease, premenstrual syndrome, Raynaud's disease, retinopathy, tinnitus, and vertigo. However, the scientific evidence is unclear or conflicting for these uses.

1.6.6 Adverse Effects

Based on the Cochrane Database of Systematic Reviews on 35 studies, the rates of adverse effects in general are similar between use of *Ginkgo biloba* and placebo (Birks and Grimley Evans, 2007). A few cases of allergic skin reactions, such as contact dermatitis, have been reported (Becker and Skipworth, 1975; Tomb et al., 1988).

Ginkgo biloba has been reported to decrease systolic and diastolic blood pressure in healthy volunteers (Kudolo, 2000). Twenty individuals (14 females and 6 males, ages 21-57 years) ingested 120 mg standardized *Ginkgo biloba* extract (24% flavonol glycosides and 6% terpene trilactones) daily for 3 months. Ingestion of *Ginkgo biloba* extract decreased systolic blood pressure from 125 to 118 mm Hg ($p < 0.05$) and diastolic blood pressure from 86 to 68 ($p < 0.01$). There was a randomized, double-blind crossover design study using oral treatment with *Ginkgo biloba* extract (containing 9.6 mg flavonol glycoside and 2.4 mg terpene trilactones per tablet; administered three times daily) in 16 healthy subjects (nine females, seven males) with a median age of 32 years (range: 21-47) (Mehlsen et al., 2002). The group mean arterial blood pressure was unchanged; however, the forearm blood flow

was significantly greater during treatment, indicating *Ginkgo biloba* may possess vasodilatory effects.

Mild gastrointestinal upset has been documented in clinical trials. In a randomized, placebo-controlled, double-blind, parallel-group, multicenter trial, 513 outpatients with uncomplicated dementia of the Alzheimer's type received placebo or *Ginkgo biloba* extract (120 or 240 mg daily) for 26 weeks (Schneider et al., 2005). Of the 174 subjects taking placebo, eight subjects experienced nausea or vomiting. In comparison, five of the 169 subjects taking 120 mg *Ginkgo biloba* extract experienced nausea or vomiting; 11 out of the 170 subjects taking 240 mg experienced the symptoms. Gastrointestinal side effects appear to be dose-related.

Ginkgo biloba has been shown to inhibit platelet activating factor (PAF)-induced aggregation of human thrombocytes *in vitro* (Koch, 2005). The concentrations required producing half-maximal inhibition by ginkgolide B, ginkgolide A, ginkgolide C, and ginkgolide J are at concentrations of 2.5, 15.8, 29.8 and 43.5 µg/ml, respectively. In general, these concentrations are more than 100 times greater than the peak plasma concentrations measured after ingestion of standardized *Ginkgo biloba* extract at recommended doses (120 to 240 mg). In a prospective, double-blind, randomized, placebo-controlled study in 32 young male healthy volunteers, *Ginkgo biloba* did not alter platelet function or coagulation parameters (Bal Dit Sollier et al., 2003). There was no evidence of inhibition of blood coagulation and platelet aggregation through *Ginkgo biloba* among 29 coagulation and bleeding parameters assessed (Kohler et al., 2004). Despite that, there are several case reports that have documented bleeding associated with *Ginkgo biloba* use (Benjamin et al., 2001; Gilbert, 1997; Matthews, 1998; Meisel et al., 2003; Rosenblatt and Mindel, 1997; Rowin and

Lewis, 1996; Skogh, 1998; Vale, 1998). Therefore, caution is warranted when a patient predisposed to bleeding is taking *Ginkgo biloba*.

Headache, dizziness, and restlessness are infrequent neurological side effects of *Ginkgo biloba* reported in clinical trials (Birks and Grimley Evans, 2007; Pittler and Ernst, 2000). In a randomized, placebo-controlled, double-blind, parallel-group, multicenter trial, 513 outpatients with uncomplicated dementia of the Alzheimer's type received placebo or *Ginkgo biloba* extract (120 or 240 mg daily) for 26 weeks (Schneider et al., 2005). Of the 169 patients taking 120 mg *Ginkgo biloba* extract, 2.4% experienced headache, 10.1% experienced dizziness; 3.0% experienced tinnitus, and 5.9% experienced agitation. Of the 170 subjects taking 240 mg extract, 4.1% experienced headache, 6.5% experienced dizziness; 4.1% experienced tinnitus, and 2.9% experienced agitation. The percentages of either group were not different from the percentages in the placebo group (n = 174), in which 5.2% experienced headache, 6.9% experienced dizziness; 6.9% experienced tinnitus, and 4.6% experienced agitation.

1.6.7 *Ginkgo biloba* Extract as an Inhibitor of the Major Drug-Metabolizing Enzymes Regulated by PXR

Ginkgo biloba extract inhibits the catalytic activity of recombinant CYP3A4 enzymes *in vitro* with a K_i of 155 µg/ml, as determined by the debenzylation of 7-benzyloxy-4-trifluoromethylcoumarin (BFC) to the fluorescent product 7-hydroxy-4-trifluoromethylcoumarin (HFC) (Gaudineau et al., 2004). The flavonol glycosides of the extract (i.e., glycosidic derivatives of quercetin, kaempferol, isorhamnetin, and myricetin, and traces of the aglycones) inhibit CYP3A4 activity with a K_i of 43 µg/ml, whereas the

terpene trilactones of the extract (i.e., ginkgolides A, B, C, J, and M, and bilobalide) do not show inhibition.

Ginkgo biloba extract has also been shown to inhibit CYP3A-catalzyed testosterone 6β-hydroxylation activity *in vitro*. In a concentration-dependent manner, *Ginkgo biloba* (0.01–1.5 mg/ml) decreased testosterone 6β-hydroxylation activity of cDNA-expressed human CYP3A4 enzymes (20 nM), which were prepared from a baculovirus-infected insect cell system (Hellum and Nilsen, 2008). *Ginkgo biloba* showed *in vitro* inhibition with a calculated IC_{50} value of 668 μg/ml. In another study, human hepatocytes are incubated with medium containing testosterone (100 μM) and *Ginkgo biloba* (2.19, 21.9 or 219 μg/ml) for 1 hr at 37°C (Hellum et al., 2007). *Ginkgo biloba* at 2.19, 21.9 and 219 μg/ml showed 73.8%, 65.0%, and 40.6%, respectively, of the activity of the vehicle control.

1.6.8 *Ginkgo biloba* Extract as an Inducer of the Major Drug-Metabolizing Enzymes Regulated by PXR

When rats were fed a diet containing *Ginkgo biloba* extract (0.5% w/w) for 4 weeks, their hepatic CYP3A1 and CYP3A2 mRNA expression increased (Shinozuka et al., 2002). Similarly, oral administration of *Ginkgo biloba* extract (100 mg/kg for 10 days) increased hepatic CYP3A1 mRNA and protein expression in rats (Deng et al., 2008a; Zhao et al., 2006). Oral administration of *Ginkgo biloba* extract (100 mg/kg and 1000 mg/kg for 5 days) also increased hepatic microsomal CYP3A-catalyzed testosterone 6β–hydroxylation activities in rats (Umegaki et al., 2002). In another study, *Ginkgo biloba* extract increased hepatic CYP3A2, CYP3A18, and CYP3A23 mRNA expression and CYP3A-catalyzed testosterone 6β–hydroxylation activity in primary culture of rat hepatocytes (Chang et al., 2006). The same study showed ginkgolide A, one of the constituents in the extract, increases

CYP3A2, CYP3A18, and CYP3A23 mRNA expression. Ginkgolide A was shown to be partially responsible for the induction of CYP3A by the extract. In human hepatocytes, *Ginkgo biloba* extract (100 to 2500 ng/ml) increased the activity, protein and mRNA expression of CYP3A in a concentration-dependent fashion (Deng et al., 2008b). In the same study, bilobalide (2, 10, and 50 ng/ml), a constituent in the extract, also increased CYP3A protein expression in a concentration-dependent fashion.

1.6.9 *Ginkgo biloba* Extract as Both an Inhibitor and Inducer of ABCB1

Not a lot is known regarding the effect of *Ginkgo biloba* on *ABCB1*, which encodes P-glycoprotein. *Ginkgo biloba* extract has been shown to inhibit P-glycoprotein activity *in vitro*. Digoxin is used as a probe substrate for P-glycoprotein activity. *Ginkgo biloba* shows *in vitro* inhibition of ^{3}H-digoxin transport with a calculated IC_{50} value of 23.6 μg/ml (Hellum and Nilsen, 2008). Net flux of digoxin decreases in the presence of *Ginkgo biloba* (0–290 μg/ml) in a concentration-dependent manner. There is currently no experimental data on the effect of *Ginkgo biloba* on ABCB1 mRNA or protein expression.

1.7 Rationale of the Study

In cultured rat hepatocytes, *Ginkgo biloba* extract induces *CYP3A23* gene expression (Chang et al., 2006). The mechanism of CYP3A23 induction by *Ginkgo biloba* extract is unknown. However, CYP3A23 is a target gene for PXR (Zhang et al., 1999). Therefore, the present study was conducted to 1) investigate whether *Ginkgo biloba* extract activates mouse and human PXR; and 2) assess the effect of *Ginkgo biloba* extract on the mRNA expression of some of the PXR target genes.

1.8 Research and Experimental Hypotheses

The research hypothesis is that *Ginkgo biloba* extract activates PXR, and thereby induces the mRNA expression of its target genes. The experimental hypotheses were 1) treatment of PXR-transfected HepG2 human hepatoma cells with *Ginkgo biloba* extract will increase PXR activity; and 2) *Ginkgo biloba* extract will increase the mRNA expression of PXR target genes, such as CYP3A4, CYP3A5, and ABCB1, in LS180 human colorectal adenocarcinoma cells.

1.9 Specific Aims

The first aim was to characterize the effect of *Ginkgo biloba* extract on PXR activity in cultured HepG2 cells. The individual experiments were: 1) to verify the effect of positive and negative controls on mouse PXR activity; 2) to verify the effect of positive and negative controls on human PXR activity; 3) to determine the effect of *Ginkgo biloba* extract on mouse and human PXR activity; 4) to determine the effect of different lots of *Ginkgo biloba* extract on human PXR activity; and 5) to characterize the dose-response relationship in the activation of human PXR by *Ginkgo biloba* extract.

The second aim is to determine whether *Ginkgo biloba* extract induces PXR target gene expression in cultured LS180 cells. The main experiment was to quantify the effect of *Ginkgo biloba* extract on *CYP3A4*, *CYP3A5*, and *ABCB1* gene expression, as assessed by real-time PCR analysis. The secondary experiments were to determine whether the CYP3A4, CYP3A5, and ABCB1-inducing concentrations of *Ginkgo biloba* extract are associated with cytotoxicity and able to increase PXR mRNA expression.

The third aim is to investigate the role of PXR in the increase in *CYP3A4*, *CYP3A5*, and *ABCB1* gene expression by *Ginkgo biloba* in cultured LS180 cells. The main experiment was to determine the effect of L-sulforaphane, which is an antagonist of human PXR, on *CYP3A4*, *CYP3A5*, and *ABCB1* gene expression in LS180 cells treated with *Ginkgo biloba* extract. The secondary experiments were to determine whether the concentrations of L-sulforaphane used in the above experiment are associated with cytotoxicity and able to decrease PXR mRNA expression.

2. EXPERIMENTAL

2.1 Chemicals and Reagents

Ginkgo biloba extract (lot 1306A, 302831, 38121) was obtained from Indena S.A. (Milan, Italy). Shown in Appendix I (Chapter 8) is the amount of bilobalide, ginkgolides, and flavonols in each extract. FuGENE 6 transfection reagent and Cytotoxicity Detection Kit were purchased from Roche (Laval, QB, Canada). Dual-Luciferase Reporter Assay System was bought from Promega (Madison, WI, U.S.A.). L-glutamine, minimum essential medium (MEM), opti-MEM, penicillin G, streptomycin, phosphate buffered saline (PBS, pH 7.4), trypsin-EDTA, heat-inactivated fetal bovine serum (FBS), agarose, TriZol, Platinum *Taq* DNA Polymerase, RiboGreen RNA Quantitation Kit, PicoGreen dsDNA Quantitation Kit, Superscript II reverse transcriptase, and QIAquick Gel Extraction Kit were purchased from Invitrogen Canada (Burlington, ON, Canada). Dimethylsulfoxide (DMSO), pregnenolone-16α-carbonitrile (PCN), rifampin, L-sulforaphane, Triton X-100, dextran, ethidum bromide, ethanol, chloroform, isoamyl alcohol, diethylpyrocarbonate (DEPC), magnesium chloride, bovine serum albumin, and SYBR Green I were ordered from Sigma-Aldrich (Oakville, ON, Canada). Forward and reverse primers for CYP3A4, CYP3A5, ABCB1, and PXR were synthesized at Integrated DNA Technologies (Coralville, IA, U.S.A.).

2.2 Cell Culture

HepG2 human hepatoma cells and LS180 human colorectal adenocarcinoma cells were cultured in T-75 flasks at 37^{o}C in a humidified, 5% CO_2 incubator. The cells were grown in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal

bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Media were changed every 3-4 days, and cells were sub-cultured once a week.

2.3 Transient Transfection

HepG2 cells were cultured in 24-well plates at a density of 100,000 cells per well and then transfected with various plasmids (provided by Dr. M. Negishi at the National Institute of Environmental Health Sciences, Research Triangle Park, NC, U.S.A.) using FuGENE 6 (0.6 μl diluted in 20 μl Opti-MEM per well). hPXR-pCR3 or mPXR-pCR3 (100 ng per well) was used as the PXR expression plasmid. The XREM-LUC (50 ng per well) was used as the reporter plasmid, which contained a firefly luciferase cDNA whose expression is controlled by PXR activation. The *Renilla* luciferase (phRL-TK; 50 ng per well) was used as an internal control to normalize transfection efficiency within each sample. pCR3 (empty vector; 100 ng per well) was used as the expression plasmid to correct background luminescence. After the cells were transfected for 24 hours, culture medium was aspirated and replaced with new culture medium (vehicle control), dimethylsulfoxide (vehicle control; 0.1% DMSO v/v), *Ginkgo biloba* extract (30, 100, 200, 400, or 800 μg/ml in culture medium), rifampin (control; 10 μM in 0.1% DMSO v/v) or PCN (control; 10 μM in 0.1% DMSO v/v), as indicated in each figure legend.

2.4 Luciferase Reporter Assay

The assay was performed using the protocol provided by the manufacturer (Promega Dual-Luciferase Reporter Assay), but with minor modifications. After the transfected HepG2 cells were treated with an extract or a chemical for 24 hours, the culture medium in the 24-well plates was aspirated and 1× passive lysis buffer was added. The plates

were placed on an orbital shaker with gentle shaking for 30 minutes. Cell lysate (5 µl) was transferred to a Lumitrac White 96-well plate (Greiner Bio-one, Frickenhausen, Germany). Firefly and *Renilla* luciferase levels in each sample were measured using the Glomax 96 Microplate Luminometer with Dual Injectors (Promega, Madison, WI, U.S.A.). The luminometer was programmed to inject 25 µl of Luciferase Assay Reagent II and then 25 µl of Stop & Glo Substrate. After a 2-second pre-measurement delay, the luminometer measured each sample for 10 seconds. To calculate the normalized reporter activity, the firefly luciferase level was divided by the *Renilla* luciferase level (internal control).

2.5 Isolation of Total RNA

The isolation of total RNA was conducted using a method described in a previous publication (Cheung et al., 2004). LS180 cells were cultured for 72 hours in T-25 flasks at a density of 1,000,000 cells per flasks. Every 24 hours, the complete culture medium was decanted and replaced with various concentrations (200, 400, or 800 µg/ml) of *Ginkgo biloba* extract (GBE; lot 1306A, Appendix 1), culture medium (vehicle control for *Ginkgo biloba* extract), rifampin (10 µM; positive control), PCN (10 µM; negative control), or DMSO (0.1% v/v; vehicle control for rifampin and PCN). After 72 hours of treatment, the medium was decanted and LS180 cells were lysed with 1 ml Trizol, passed through a 26-gauge needle three times, and extracted with 200 µl choloroform/isoamyl alcohol (4:1 v/v). Then, the mixture was centrifuged (10,500 × *g* for 15 min at 4^{o}C) and the aqueous phase was mixed with 500 µl of ice-cold isopropanol for precipitation of the RNA pellet. The RNA pellet was chilled on ice (5 minutes), centrifuged (10,500 × *g* for 15 min at 4^{o}C), washed (in 1 ml ice-cold 75% ethanol), centrifuged again (6,600 × *g* for 5 min at 4^{o}C), and air-dried (10–15

minutes at room temperature). Subsequently, it was suspended in 50 µl sterile, autoclaved water treated with DEPC and stored at –70°C until use.

2.6 Quantification of Total RNA Concentration

The concentrations of total RNA were quantified using the RiboGreen dsDNA Quantitation Kit (Invitrogen Canada, Burlington, Ontario, Canada), following a method described a publication (Jones et al., 1998). Each RNA sample (2 µl) was mixed with distilled water (6 µl), DNAse I (5 U/µl), and 10× digestion buffer (1 µl), which contained 200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, and 20 mM $CaCl_2$. Each sample mixture was then incubated at 37ºC for 90 min before addition of 1× TE buffer (90 µl; 10 mM Tris–HCl, pH 7.5, 1 mM EDTA). After that, RiboGreen dye (100 µl) and 1× TE buffer (98 µl) was added to an aliquot of the sample mixture (2 µl). The mixture (200 µl) was incubated for 2 min at room temperature, protected from light, in microplate wells. The ribosomal RNA standards (16s and 23s rRNA from *Escherichia coli*) supplied in the kit were used to construct the standard curves (0, 0.25, 0.5, 1, 2.5 µg/ml). One volume of the standard solution (100 µl) was added to one volume of Ribogreen dye (100 µl). The CytoFluor Series 4000 fluorescence microplate reader (Millipore, Bedford, MA, U.S.A.) measured the fluorescence of each sample at excitation wavelength of 485 nm (20 nm band width), emission wavelength of 530 nm (20 nm band width), and a gain of 50.

2.7 Reverse Transcription

Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen Canada, Burlington, Ontario, Canada), following a method described in a previous publication (Cheung et al., 2004). Oligo$(dT)_{12–18}$ primer (1 µl) and tRNA (2 µg)

were incubated in DEPC-treated water (6 µl) at 65°C for 10 min and then quickly chilled on ice. They were mixed with 2 µl of 10× PCR buffer (200 mM Tris–HCl, pH 8.4, and 500 mM KCl), 1 µl of dNTP mix (10 mM), 4 µl of $MgCl_2$ (25 mM), 1 µl of dithiothreitol (0.1 M), and 2 µl of DNase I (1 U/µl). To denature DNase I, the mixture was incubated at 37°C for 30 min, followed by 75°C for 5 min. After that, the mixture was incubated with SuperScript II reverse transcriptase (1 µl) at 42°C for 20 minutes. To stop the reaction, the mixture was heated at 95°C for 5 minutes and then stored at −20°C until PCR analysis.

2.8 Quantification of Total cDNA Concentration

The concentrations of total cDNA were quantified using the PicoGreen dsDNA Quantitation Kit (Invitrogen Canada, Burlington, Ontario, Canada), following a method described in a publication (Singer et al., 1997). Each DNA sample (2.5 µl) was added to 1× TE buffer (247.5 µl; 10 mM Tris–HCl, pH 7.5, 1 mM EDTA). Then, 100 µl of the mixture was diluted with 100 µl PicoGreen dye. The mixture (200 µl) was incubated for 2 min at room temperature, protected from light, in microplate wells. The lambda DNA standards (0.2–40 ng) provided in the kit were used to construct the standard curves (0, 10, 20, 100, 200, 400 µg/ml). One volume of the standard solution (100 µl) was added to one volume of PicoGreen dye (100 µl). The CytoFluor Series 4000 fluorescence microplate reader (Millipore, Bedford, MA, U.S.A.) measured the fluorescence of each sample at excitation wavelength of 485 nm (20 nm band width), emission wavelength of 530 nm (20 nm band width), and a gain of 60.

2.9 PCR Primers

The sequences of the forward and reverse primers to amplify human CYP3A4, CYP3A5, ABCB1, and PXR cDNA are listed in Table 2.1. The BLAST program (http://www.ncbi.nlm.nih.gov) was used to confirm primer specificity.

2.10 Real-time Polymerase Chain Reaction (PCR)

The PCR analyses were conducted in a real-time DNA thermal cycler (LightCycler, Roche Diagnostics, Laval, Quebec, Canada). Each PCR reaction (20 µl) had 1 U Platinum *Taq* DNA polymerase in 1× PCR II buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 3 mM $MgCl_2$ (except for CYP3A4, which used 4 mM), 0.25 mg/ml bovine serum albumin, 0.2 mM dNTP, 0.2 µM forward and reverse primers specific for CYP3A4, CYP3A5, and ABCB1 cDNA, 1:30,000 SYBR Green I, and 10 ng total cDNA. Cycling conditions for the amplication of human CYP3A4, CYP3A5, ABCB1, and PXR cDNA are listed in Table 2.2.

Table 2.1. Sequence of PCR primers

Gene	Primer	Reference
CYP3A4	Forward: 5'-CCT-TAC-ACA-TAC-ACA-CCC-TTT-GGA-AGT-3' Reverse: 5'-AGC-TCA-ATG-CAT-GTA-CAG-AAT-CCC-CGG-TTA-3'	(Schuetz et al., 1996)
CYP3A5	Forward: 5'-CCT-TAC-ATA-TAC-ACA-CCC-TTT-GGA-AC-3' Reverse: 5'-GTT-GAA-GAA-GTC-CTT-GCG-TGT-C-3'	(Yamaori et al., 2005)
ABCB1	Forward: 5'-GGC-CTA-ATG-CCG-AAC-ACA-TT-3' Reverse: 5'-CAG-CGT-CTG-GCC-CTT-C-3'	(Liu et al., 2002)
PXR	Forward: 5'-CAA-GCG-GAA-GAA-AAG-TGA-ACG-3' Reverse: 5'-CAC-AGA-TCT-TTC-CGG-ACC-TG-3'	(Chang et al., 2003)

Table 2.2. Cycling conditions for the amplification of human CYP3A4, CYP3A5, and ABCB1 cDNA by a real time DNA thermal cycler (LightCycler)

Gene	Step	Temperature (^{o}C)	Time (s)
CYP3A4	Denaturation	94	5
	Annealing	60	10
	Elongation	72	15
CYP3A5	Denaturation	95	1
	Annealing	65	6
	Elongation	72	10
ABCB1	Denaturation	95	1
	Annealing	61	6
	Elongation	72	10
PXR	Denaturation	95	5
	Annealing	65	10
	Elongation	72	15

2.11 Purification and Sequencing of CYP3A4, CYP3A5, and ABCB1 Amplicons

The identity of the CYP3A4, CYP3A5, ABCB1, and PXR amplicons was confirmed previously by sequencing analysis (Applied Biosystems 37 DNA Sequencer, Applied Biosystems, Foster City, CA) at the University of British Columbia Nucleic Acid and Protein Service Unit.

2.12 Lactate Dehydrogenase (LDH) Assay

Leakage of LDH from cell culture is an indicator of the level of cytotoxicity (Jauregui et al., 1981). The assay was conducted using a method in a previous publication (Rajaraman et al., 2006), but with minor modifications. LS180 cells were cultured for 72 hours in 24-well microplates at a density of 100,000 cells per well. After that, the complete culture medium was decanted and replaced with treatment every 24 hours. After 72 hours of treatment, the culture plates were centrifuged at 250×g for 10 minutes. Then, 5 µl of the supernatant from each well containing cultured cells was transferred to a well in a 96-well microplate containing 95 µl of PBS (pH 7.4) and 100 µl of the reaction mixture (provided in the Cytotoxicity Detection Kit, Roche Diagnostics; Laval, QC, Canada). To obtain cell lysates, the cells were lysed with 500 µl of lysis buffer (20 mM EDTA and 2% Triton X-100 in PBS, and then placed in an orbital shaker for 2 hours. Then, 5 µl of the supernatant from each well containing cultured cells was transferred to a well in a 96-well microplate containing 95 µl of PBS and 100 µl of the Cytotoxicity Detection Kit reaction mixture. After 30 min of incubation at room temperature, the absorbance of each sample was measured at 492 nm in a Multiskan Ascent multiplate reader (Thermo Electron Corp, Waltham, MA, U.S.A.). The amount of LDH released to the culture medium (i.e., LDH leakage) is expressed

as a percentage of the total cellular LDH content (i.e., sum of the LDH in the culture medium and in the cell lysate).

2.13 Data Analysis

Data are presented as mean ± standard error of the mean (SEM) for the number of independent experiments indicated on each figure legend. One independent experiment means one trial performed on one passage of cells, which were sub-cultured once per week. Student's paired *t*-test was used when comparing data from two treatment groups. When comparing data from more than two treatment groups, statistical comparisons were made using Kruskal-Wallis one-way analysis of variance test followed by Student Newman-Keuls test. The criterion of statistical significance was set at $p < 0.05$. Statistical tests were performed using SigmaStat (SPSS Science, Chicago, IL, U.S.A.).

3. RESULTS

3.1 Control Experiment on the Effect of Rifampin and PCN on Mouse PXR Activity

As a control experiment, I determined the effect of positive and negative controls on mouse PXR activity. PCN activates rat and mouse PXR but not human PXR (Song et al., 2005), whereas rifampin activates human PXR but not mouse PXR (Tirona et al., 2004). Therefore, PCN was the positive control and rifampin was the negative control in this experiment. Cultured HepG2 cells were transfected for 24 hours with pCR3-mPXR (mouse PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with DMSO (0.1% v/v; vehicle control), PCN (10 μM; positive control), or rifampin (10 μM; negative control). PCN increased mouse PXR activity by 13.8 ± 3.9-fold, whereas rifampin did not increase PXR activity (Fig. 3.1). The species-dependent effect of PCN and rifampin on PXR activity was consistent with published data (Vignati et al., 2004).

3.2 Control Experiment on the Effect of Rifampin and PCN on Human PXR Activity

As another control experiment, I determined the effect of positive (rifampin) and negative (PCN) controls on human PXR activity. Cultured HepG2 cells were transfected for 24 hours with pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with DMSO (0.1% v/v; vehicle control), rifampin (10 μM; positive control), or PCN (10 μM; negative control). Rifampin increased human PXR activity by 30.5 ± 10.0-fold, whereas PCN did not increase PXR

activity (Fig. 3.2). The species-dependent effect of rifampin and PCN on PXR activity was consistent with published data (Vignati et al., 2004).

3.3 Effect of *Gingko biloba* Extract on Mouse and Human PXR Activity

This experiment was performed to test whether *Ginkgo biloba* extract activates mouse PXR and human PXR. Cultured HepG2 cells were transfected for 24 hours with pCR3-mPXR (mouse PXR) or pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with complete culture medium (vehicle control) or *Ginkgo biloba* extract (200 μg/ml; lot 302831, Appendix 1). In cultured rat hepatocytes, 48-h treatment of *Ginkgo biloba* extract (100 μg/ml) induces mRNA expression of CYP3A23 (Chang et al., 2006), a target gene of rodent PXR (Zhang et al., 1999). Therefore, it would be logical to use *Ginkgo biloba* extract at a concentration of 200 μg/ml in the initial experiment. *Ginkgo biloba* extract (200 μg/ml) increased mouse PXR activity (Fig. 3.3a) and increased human PXR activity (Fig. 3.3b) by 3.2 ± 0.3-fold and 8.3 ± 1.3-fold, respectively.

3.4 Effect of Different Lots of *Ginkgo biloba* Extract on Human PXR Activity

This experiment was to determine whether different lots of *Ginkgo biloba* extract have a similar activation effect on human PXR. Cultured HepG2 cells were transfected for 24 hours with pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with complete culture medium (vehicle control) or one of the three lots of *Ginkgo biloba* extract (200 μg/ml; lot 1306A, 302831, and 38121; Appendix 1). All three lots of *Ginkgo biloba* extract increased PXR

activity. The extent of the increase by the three lots was similar (10.0 ± 2.6-, 9.5 ± 0.8-, and 10.5 ± 3.6-fold for lot numbers 1306A, 302831, and 38121, respectively, Fig. 3.4).

3.5 Dose-Response Experiment on the Effect of *Ginkgo biloba* Extract on Human PXR Activity

This experiment was to characterize the effect of various concentrations of *Ginkgo biloba* extract on human PXR activity. Cultured HepG2 cells were transfected for 24 hours with pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with complete culture medium (vehicle control) or various concentrations of *Ginkgo biloba* extract (30, 100, 200, 400 or 800 μg/ml; lot 302831, Appendix 1). As shown in Fig. 3.5, *Ginkgo biloba* extract at 30 μg/ml did not increase human PXR activity. At 100, 200, 400, and 800 μg/ml, it increased human PXR activity 2.9 ± 0.3-, 6.4 ± 1.4-, 14.5 ± 2.4-, and 32.2 ± 10.1-fold, respectively. The dose-response curve shows a log-linear increase in PXR activity at concentrations of 200 to 800 μg/ml of *Ginkgo biloba* extract.

3.6 Effect of *Ginkgo biloba* Extract on CYP3A4 mRNA Expression in Cultured LS180 Cells

As shown in Fig. 3.3b, 4, and 5, *Ginkgo biloba* extract was shown to increase human PXR activity. Therefore, it should also increase the mRNA expression of its target genes, such as CYP3A4 (Lehmann et al., 1998). LS180 cells were used because they show endogenous human PXR mRNA expression (Pfrunder et al., 2003) and are an appropriate model to study PXR-mediated CYP3A4 induction (Gupta et al., 2008). Cultured LS180 cells were treated for 72 hours with various concentrations of *Ginkgo biloba* extract (200, 400, or

800 μg/ml; lot 1306A, Appendix 1), culture medium (vehicle control for *Ginkgo biloba* extract), rifampin (10 μM; positive control), PCN (10 μM; negative control), or DMSO (0.1% v/v; vehicle control for rifampin and PCN). As shown in Fig. 3.6a, *Ginkgo biloba* extract at concentrations of 200, 400, and 800 μg/ml increased CYP3A4 mRNA expression by 1.7 ± 0.3-, 2.4 ± 0.2-, and 2.5 ± 0.1-fold, respectively. The positive control, rifampin (10 μM), increased CYP3A4 mRNA expression by 49.4 ± 12.1-fold, whereas the negative control, PCN (10 μM), had no effect.

3.7 Effect of *Ginkgo biloba* Extract on CYP3A5 mRNA Expression in Cultured LS180 Cells

CYP3A5 is a target gene of human PXR (Burk et al., 2004). Therefore, the effect of *Ginkgo biloba extract* on CYP3A5 mRNA expression was determined. Cultured LS180 cells were treated for 72 hours with various concentrations of *Ginkgo biloba* extract (200, 400, or 800 μg/ml; lot 1306A, Appendix 1), culture medium (vehicle control for *Ginkgo biloba* extract), rifampin (10 μM; positive control), PCN (10 μM; negative control), or DMSO (0.1% v/v; vehicle control for rifampin and PCN). *Ginkgo biloba* extract at concentrations of 200, 400, and 800 μg/ml increased CYP3A5 mRNA expression by 1.3 ± 0.1-, 1.6 ± 0.1-, and 3.6 ± 0.2-fold, respectively (Fig. 3.6b). The positive control, rifampin (10 μM), increased CYP3A5 mRNA expression by 2.4 ± 0.3-fold, whereas the negative control, PCN (10 μM), had no effect.

3.8 Effect of *Ginkgo biloba* Extract on ABCB1 mRNA Expression in Cultured LS180 Cells

ABCB1 is also a target gene of human PXR (Geick et al., 2001). Therefore, the effect of *Ginkgo biloba* extract on ABCB1 was determined. Cultured LS180 cells were treated for 72 hours with various concentrations of *Ginkgo biloba* extract (200, 400, or 800 μg/ml; lot 1306A, Appendix 1), culture medium (vehicle control for *Ginkgo biloba* extract), rifampin (10 μM; positive control), PCN (10 μM; negative control), or DMSO (0.1% v/v; vehicle control for rifampin and PCN). *Ginkgo biloba* extract (200, 400, and 800 μg/ml) increased ABCB1 mRNA expression by 2.7 ± 0.3-, 3.4 ± 0.5-, and 6.4 ± 1.2-fold, respectively (Fig. 3.6c). The positive control, rifampin (10 μM), increased ABCB1 mRNA expression by 5.0 ± 0.9-fold, whereas the negative control, PCN (10 μM), had no effect.

3.9 Effect of *Ginkgo biloba* Extract on LDH Release in Cultured LS180 Cells

This experiment was performed to test whether the increase in CYP3A4, CYP3A5, and ABCB1 mRNA expression occurred at cytotoxic concentrations of *Ginkgo biloba* extract. Cultured LS180 cells were treated for 72 hours with culture medium (vehicle control), various concentrations of *Ginkgo biloba* extract (200, 400, or 800 μg/ml; lot 1306A, Appendix 1), Triton X-100 (1% v/v; positive control; a lysis buffer), or dextran (1% w/v; negative control; an osmotically neutral fluid). As a baseline control, cells treated with culture medium had 3.0 ± 0.8% of LDH released into the culture medium (Fig. 3.7). Cells treated with the negative control, dextran, did not increase LDH release (3.6 ± 2.0%) compared to the culture medium control. By comparison, cells treated with the positive control, Triton X-100, had 99.6 ± 0.4% of LDH released. Cells treated with *Ginkgo biloba* extract at concentrations of 200, 400, and 800 μg/ml did not increase LDH release into the

culture medium (1.7 ± 0.4%, 1.4 ± 0.7%, and 2.2 ± 0.9%, respectively), indicating that the PXR-activating concentrations of *Ginkgo biloba* extract were not cytotoxic in cultured LS180 cells.

3.10 Effect of *Ginkgo biloba* Extract on Human PXR mRNA Expression in Cultured LS180 Cells

An increase in PXR mRNA expression may account for the increases in CYP3A4, CYP3A5, and ABCB1 mRNA expression by *Ginkgo biloba* extract. To determine whether *Ginkgo biloba* extract alters human *PXR* gene expression, cultured LS180 cells were treated for 72 hours with culture medium (vehicle control) or various concentrations of *Ginkgo biloba* extract (200, 400, or 800 μg/ml; lot 1306A, Appendix 1). *Ginkgo biloba* extract at the concentrations tested did not affect human PXR mRNA expression (Fig. 3.8). This result indicates that the increases in CYP3A4, CYP3A5, and ABCB1 mRNA expression by *Ginkgo biloba* extract were not due to up-regulation of *PXR* gene expression.

3.11 Effect of a PXR Antagonist, L-Sulforaphane, on Induction of CYP3A4, CYP3A5, and ABCB1 mRNA Expression by *Ginkgo biloba* Extract in Cultured LS180 Cells

This experiment was performed to determine whether the induction of CYP3A4, CYP3A5, and ABCB1 mRNA expression was due to activation of PXR. L-sulforaphane is an antagonist of human PXR (Zhou et al., 2007). Therefore, cultured LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (400 μg/ml; control; lot 1306A, Appendix 1) and DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (5, 10, or 20 μM) dissolved in DMSO (0.1% v/v final concentration). L-sulforaphane at 5, 10, and 20

μM decreased CYP3A4 mRNA expression by 54%, 78%, and 93%, respectively, in cells co-treated with *Ginkgo biloba* extract (Fig. 3.9a). By comparison, L-sulforaphane at 5, 10, and 20 μM decreased CYP3A5 mRNA expression by 33.6%, 36.0%, 66.3%, respectively, in cells co-treated with *Ginkgo biloba* extract (Fig. 3.10a). ABCB1 mRNA expression was also decreased by 32.7%, 44.3%, and 67.9% in cells co-treated with *Ginkgo biloba* extract and L-sulforaphane at 5, 10, and 20 μM, respectively (Fig. 3.11a).

As a positive control, LS180 cells were treated for 72 hours with rifampin (10 μM; control) and DMSO (0.2% v/v final concentration) or L-sulforaphane (20 μM) dissolved in DMSO (0.2% v/v final concentration). L-sulforaphane decreased CYP3A4 and CYP3A5 mRNA expression by 71.1% and 29.1%, respectively, in LS180 cells treated with rifampin (Fig. 3.9b and 3.10b). Overall, these results suggest that the increases in CYP3A4 (Fig. 3.6a) and CYP3A5 (Fig. 3.6b) mRNA expression by *Ginkgo biloba* extract were likely due to activation of PXR by the extract. The effect of L-sulforaphane on CYP3A4 mRNA expression in rifampin-treated cells was consistent with published data (Zhou et al., 2007). However, L-sulforaphane did not decrease ABCB1 mRNA expression by rifampin (Fig. 3.11b).

3.12 Effect of L-Sulforaphane on LDH Release in Cultured LS180 Cells Treated with *Ginkgo biloba* Extract in Cultured LS180 Cells

This experiment was performed to rule out the possibility that the decreases in CYP3A4 (Fig. 3.9), CYP3A5 (Fig. 3.10), and ABCB1 (Fig. 3.11) mRNA expression by L-sulforaphane were a consequence of cellular toxicity. Cultured LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (400 μg/ml; lot 1306A, Appendix 1) and DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (5, 10, or 20 μM)

dissolved in DMSO (0.1% v/v final concentration). L-sulforaphane at these concentrations did not increase LDH release (Fig. 3.12).

3.13 Effect of L-Sulforaphane on Human PXR mRNA Expression in Cultured LS180 Cells Treated with *Ginkgo biloba* Extract

This experiment was performed to rule out the possibility that the decreases in CYP3A4 (Fig. 3.9), CYP3A5 (Fig. 3.10), and ABCB1 (Fig. 3.11) mRNA expression by L-sulforaphane were a consequence of decreased *PXR* gene expression. Cultured LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (400 µg/ml; lot 1306A, Appendix 1) and DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (5, 10, or 20 µM) dissolved in DMSO (0.1% v/v final concentration). L-sulforaphane at 5 and 10 µM did not decrease human PXR mRNA expression, whereas L-sulforaphane at 20 µM decreased human PXR mRNA expression by 55% (Fig. 3.13).

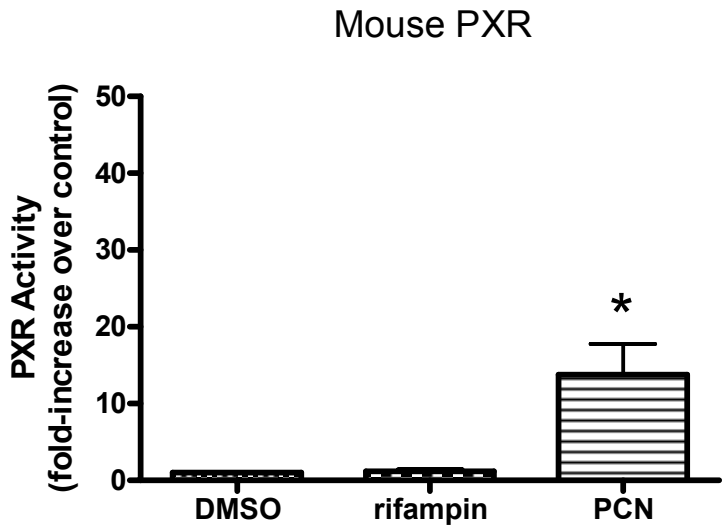


Fig. 3.1. Effect of rifampin and pregnenolone-16α-carbonitrile (PCN) on mouse PXR activity.

HepG2 cells were transfected for 24 hours with pCR3-mPXR (mouse PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with DMSO (0.1% v/v; vehicle control), rifampin (10 μM), or PCN (10 μM). Luminescence generated from cells transfected with XREM-LUC, phRL-TK, and pCR3 (empty vector) was used to correct for the background luminescence. The firefly and *Renilla* luciferase levels were measured and the normalized mouse PXR activity was calculated. Data are presented as mean ± SEM (n = 3). * $p < 0.05$ when compared to the vehicle-treated control. One-way ANOVA showed that the means were significantly different.

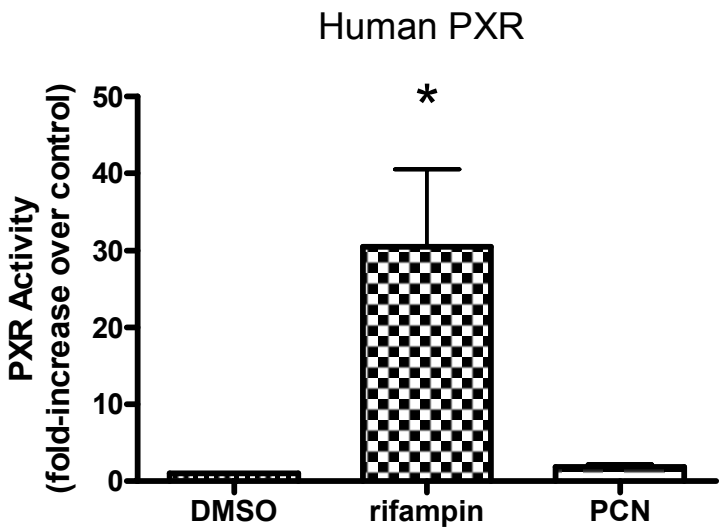


Fig. 3.2. Effect of rifampin and pregnenolone-16α-carbonitrile (PCN) on human PXR activity.

HepG2 cells were transfected for 24 hours with pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with DMSO (0.1% v/v; vehicle control), rifampin (10 μM), or PCN (10 μM). Luminescence generated from cells transfected with XREM-LUC, phRL-TK, and pCR3 (empty vector) was used to correct for the background luminescence. The firefly and *Renilla* luciferase levels were measured and the normalized human PXR activity was calculated. Data are presented as mean ± SEM (n = 3). * $p < 0.05$ when compared to the vehicle-treated control. One-way ANOVA showed that the means were significantly different.

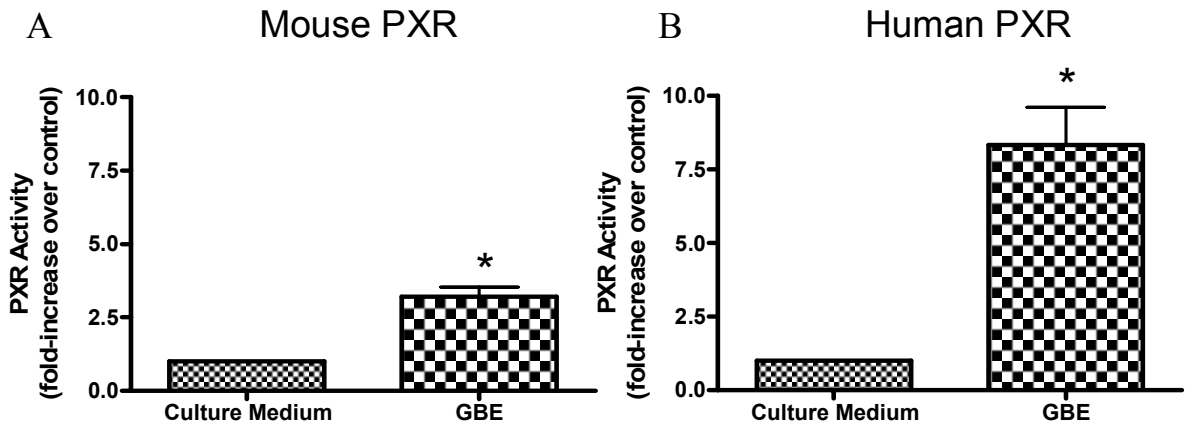


Fig. 3.3. Effect of *Ginkgo biloba* extract (GBE) on mouse and human PXR activity.

HepG2 cells were transfected for 24 hours with pCR3-mPXR (mouse PXR) or pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with DMSO (0.1% v/v; vehicle control) or *Ginkgo biloba* extract (GBE; 200 µg/ml; lot 302831, Appendix 1). Luminescence generated from cells transfected with XREM-LUC, phRL-TK, and pCR3 (empty vector) was used to correct for the background luminescence. The firefly and *Renilla* luciferase levels were measured and the normalized mouse and human PXR activities were calculated. Data are presented as mean ± SEM (n = 4). * $p < 0.05$ when compared to the vehicle-treated control. Student's paired *t*-test showed that the means were significantly different.

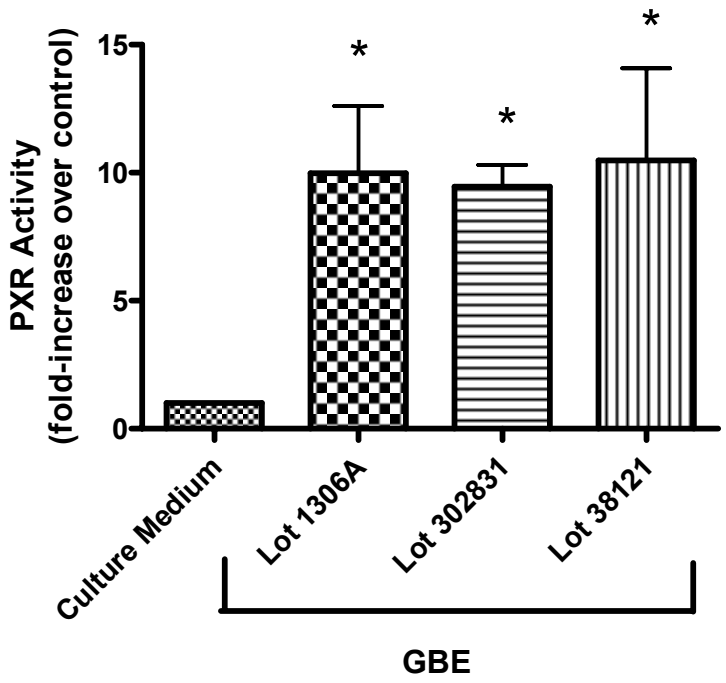


Fig. 3.4. Effect of different lots of *Ginkgo biloba* extract (GBE) on human PXR activity.

HepG2 cells were transfected for 24 hours with pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with complete culture medium (vehicle control) or one of the three lots of *Ginkgo biloba* extract (GBE; lot 1306A, 302831, and 38121, Appendix 1) at a concentration of 200 μg/ml. Luminescence generated from cells transfected with XREM-LUC, phRL-TK, and pCR3 (empty vector) was used to correct for the background luminescence. The firefly and *Renilla* luciferase levels were measured and the normalized PXR activity was calculated. Data are presented as mean ± SEM (n = 4). * $p < 0.05$ when compared to the vehicle-treated control. One-way ANOVA showed that the means were significantly different.

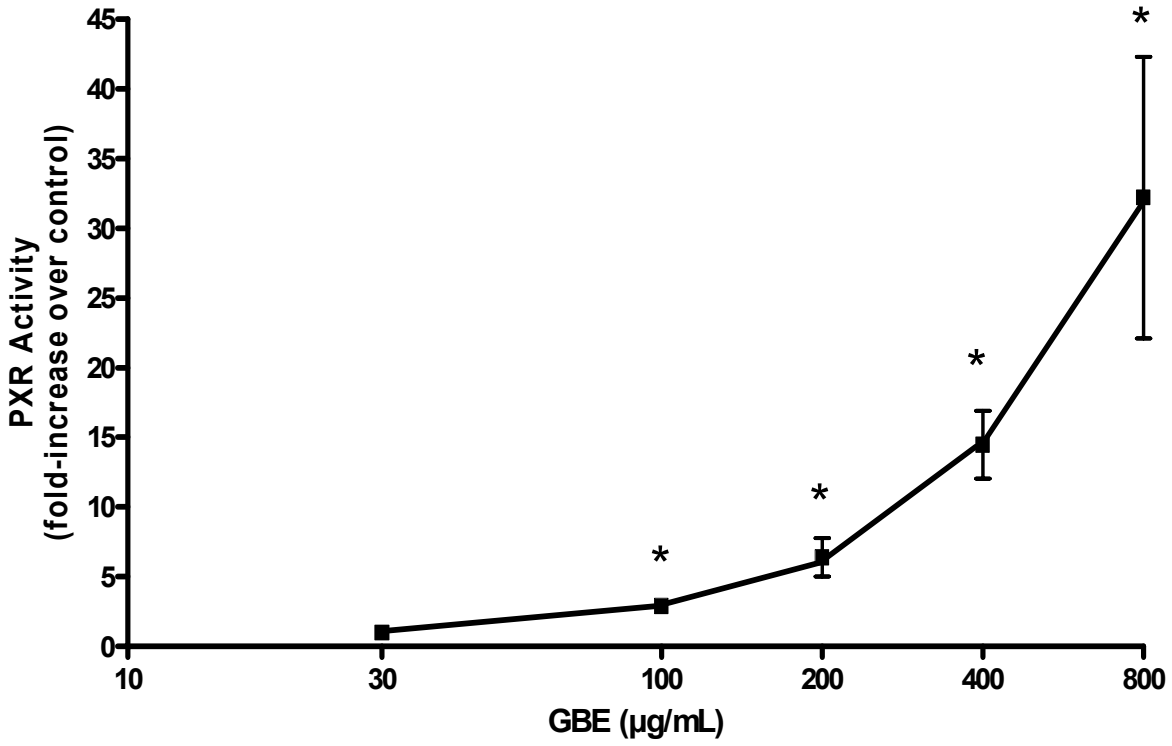


Fig. 3.5. Effect of different concentrations of *Ginkgo biloba* extract (GBE) on human PXR activity.

HepG2 cells were transfected for 24 hours with pCR3-hPXR (human PXR), XREM-LUC (firefly luciferase), and phRL-TK (*Renilla* luciferase), and then treated for another 24 hours with complete culture medium (vehicle control) or *Ginkgo biloba* extract (GBE; lot 302831, Appendix 1) at a concentration of 30, 100, 200, 400 or 800 µg/ml. Luminescence generated from cells transfected with XREM-LUC, phRL-TK, and pCR3 (empty vector) was used to correct for the background luminescence. The firefly and *Renilla* luciferase levels were measured and the normalized PXR activity was calculated. Data are presented as mean ± SEM (n = 4). * $p < 0.05$ when compared to the vehicle-treated control. One-way ANOVA showed that the means were significantly different.

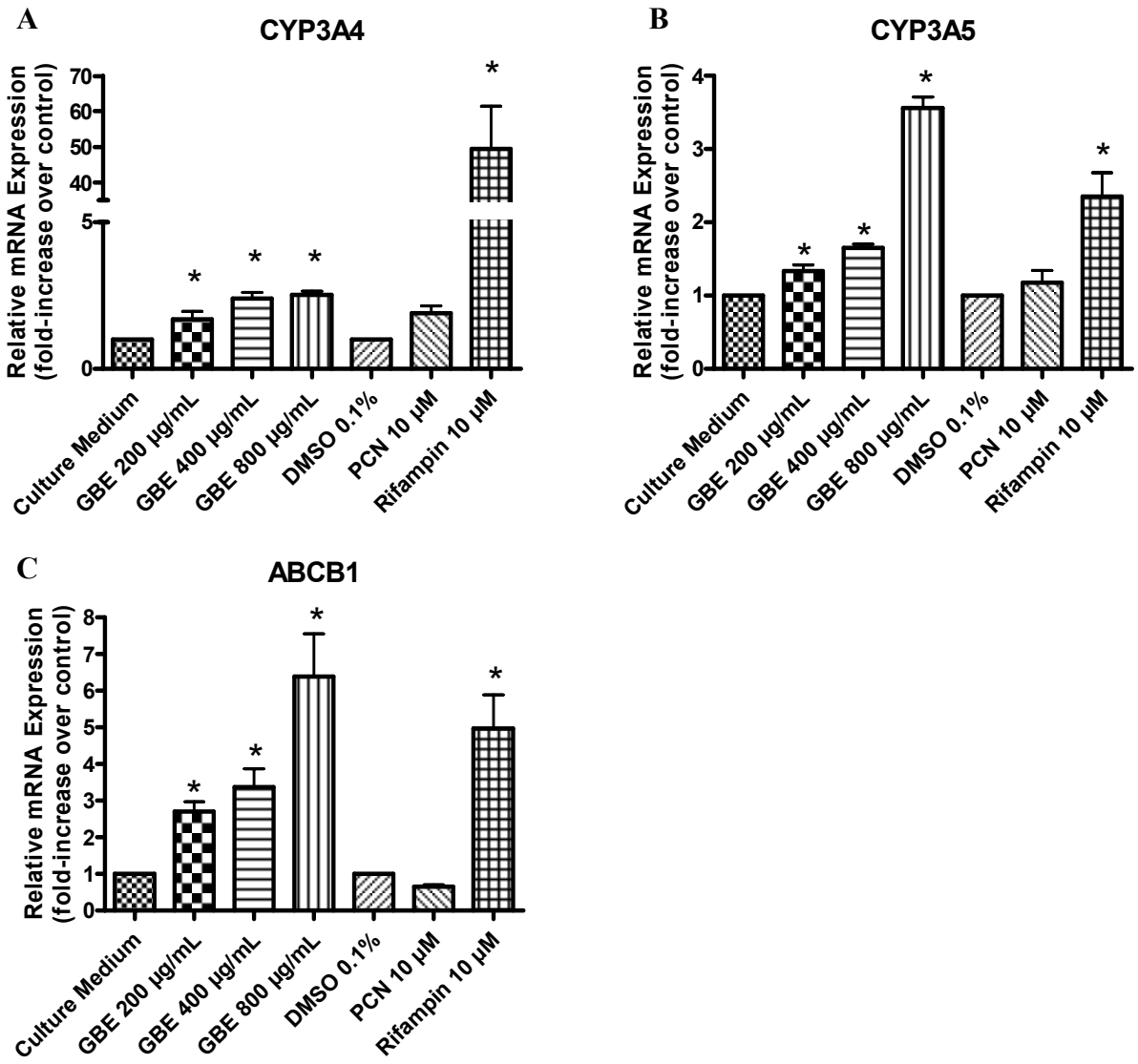


Fig. 3.6. Concentration-dependent effect of *Ginkgo biloba* extract (GBE) on CYP3A4, CYP3A5, and ABCB1 mRNA expression.

LS180 cells were treated for 72 hours with various concentrations (200, 400, or 800 µg/ml) of *Ginkgo biloba* extract (GBE; lot 1306A, Appendix 1), culture medium (vehicle control for *Ginkgo biloba* extract), rifampin (10 µM; positive control), PCN (10 µM; negative control), or DMSO (0.1% v/v; vehicle control for rifampin and PCN). CYP3A4 (A), CYP3A5 (B), and ABCB1 (C) mRNA levels were determined by reverse transcription and real-time PCR. Data

are expressed as mean ± SEM (n = 3-6). * $p < 0.05$ when compared to the vehicle-treated control. One-way ANOVA showed that the means were significantly different.

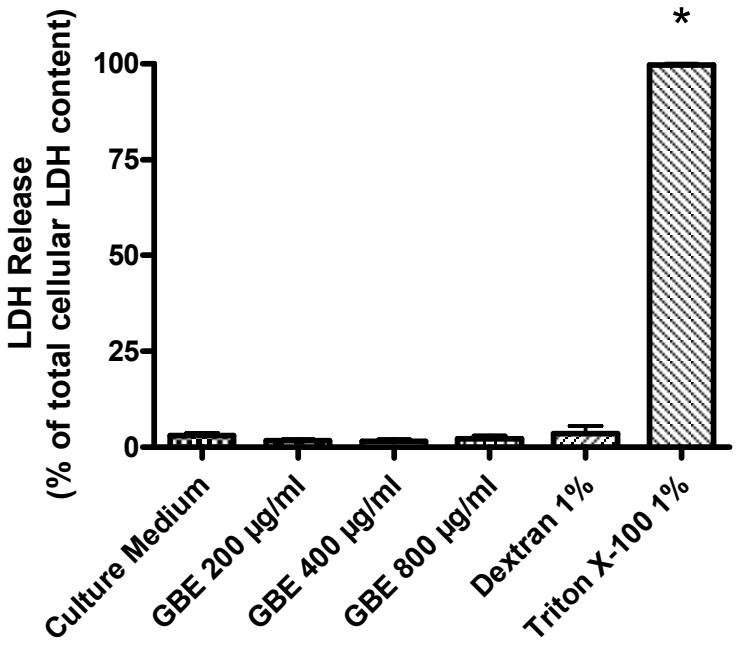


Fig. 3.7. Effect of *Ginkgo biloba* extract (GBE) on LDH release in cultured LS180 cells.

LS180 cells were treated for 72 hours with various concentrations (200, 400, or 800 µg/ml) of *Ginkgo biloba* extract (GBE; lot 1306A, Appendix 1), dextran (1% w/v; negative control), Triton-X 100 (1% v/v; positive control), or culture medium (vehicle control). The amount of LDH released to the culture medium (i.e., LDH leakage) is expressed as a percentage of the total cellular LDH content (i.e., sum of the LDH in the culture medium and in the cell lysate). Data are expressed as mean ± SEM (n = 3). * $p < 0.05$ when compared to the vehicle-treated control. One-way ANOVA showed that the means were significantly different. One-way ANOVA showed that the means were significantly different.

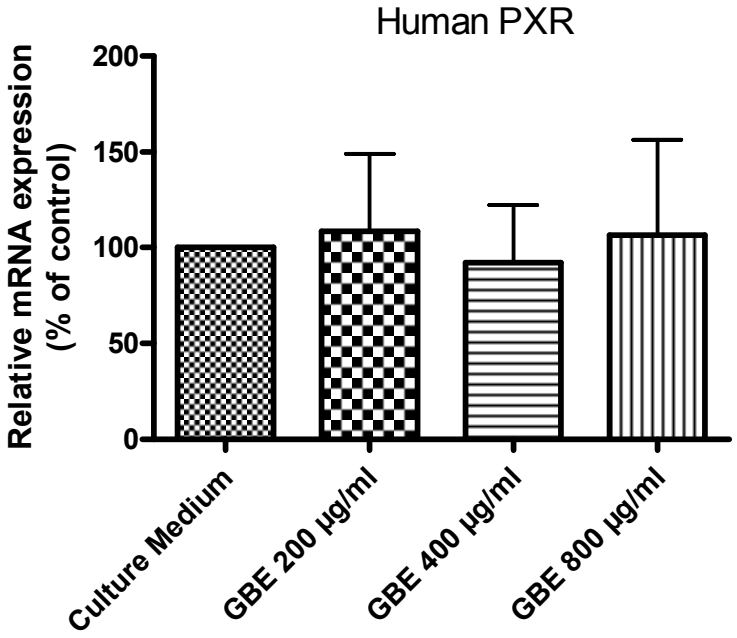


Fig. 3.8. Effect of *Ginkgo biloba* extract (GBE) on human PXR mRNA expression.

LS180 cells were treated for 72 hours with various concentrations (200, 400, or 800 µg/ml) of *Ginkgo biloba* extract (GBE; lot 1306A, Appendix 1) or culture medium (vehicle control). Human PXR mRNA level was determined by reverse transcription and real-time PCR. Data are expressed as mean ± SEM (n = 4). One-way ANOVA showed that the means were not significantly different.

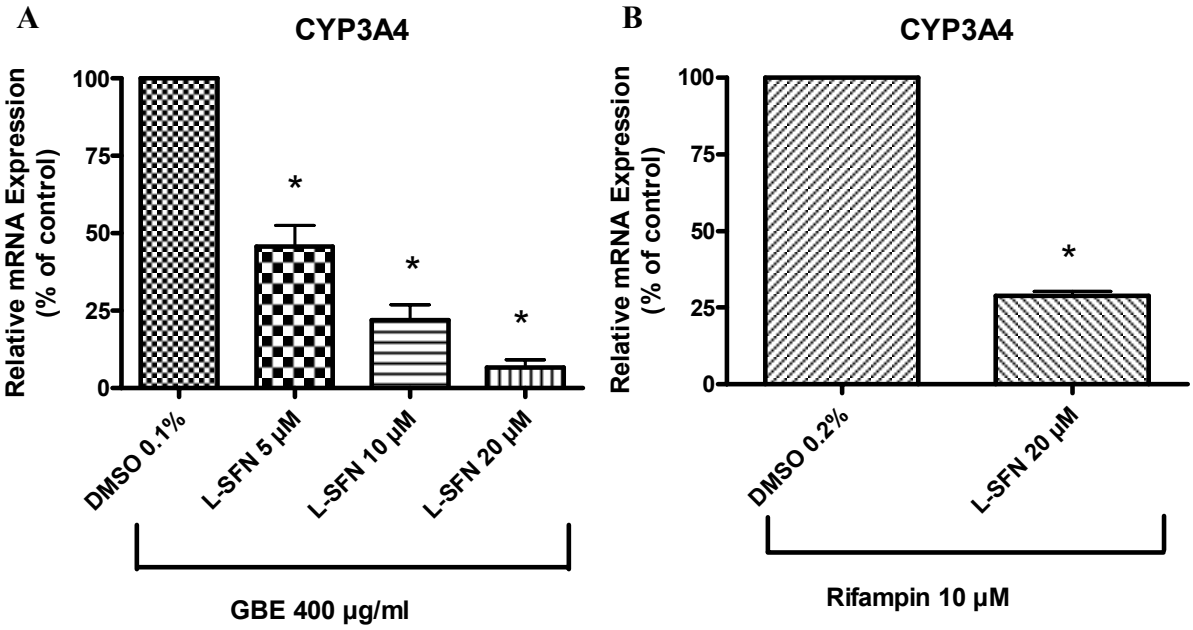


Fig. 3.9. Effect of L-sulforaphane (L-SFN) on induction of CYP3A4 mRNA expression by *Ginkgo biloba* extract (GBE) in cultured LS180 cells.

LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (GBE 400 µg/ml; control; lot 1306A, Appendix 1) and DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (L-SFN; 5, 10, or 20 µM) dissolved in DMSO (0.1% v/v final concentration). As a positive control, LS180 cells were treated for 72 hours with rifampin (10 µM; control) and DMSO (0.2% v/v final concentration) or L-sulforaphane (20 µM) dissolved in DMSO (0.2% v/v final concentration). CYP3A4 mRNA levels were determined by reverse transcription and real-time PCR. Data are expressed as mean ± SEM (n = 4). * $p <$ 0.05 when compared to the control. One-way ANOVA and student's *t*-test showed that the means were significantly different.

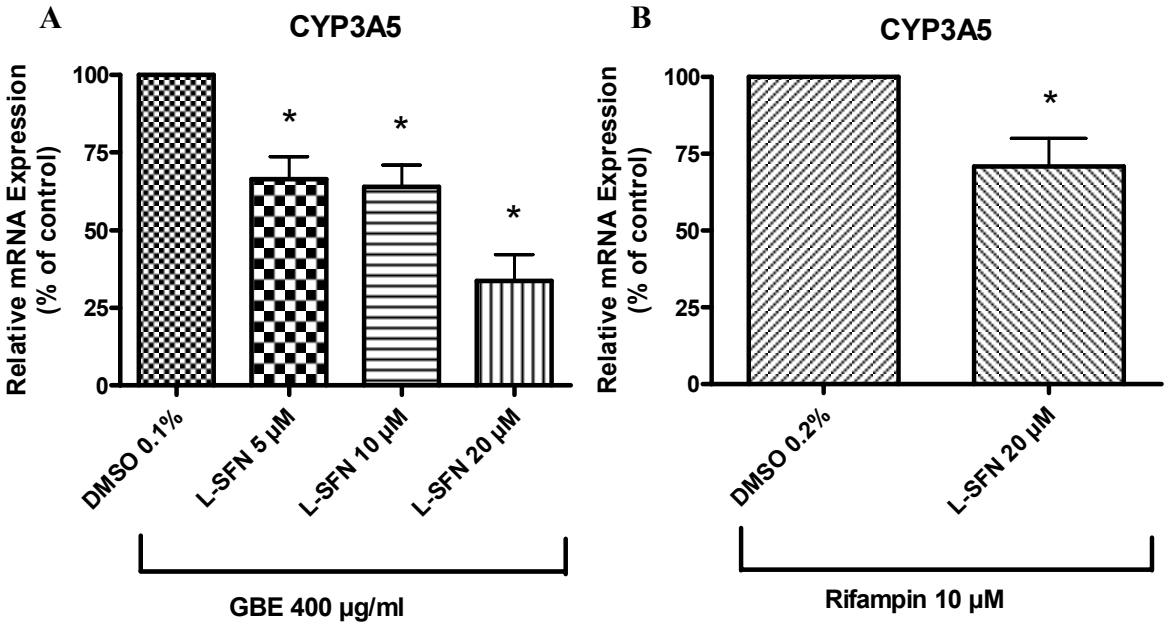


Fig. 3.10. Effect of L-sulforaphane (L-SFN) on induction of CYP3A5 mRNA expression by *Ginkgo biloba* extract (GBE) in cultured LS180 cells.

LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (GBE 400 µg/ml; control; lot 1306A, Appendix 1) with DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (L-SFN; 5, 10, or 20 µM) dissolved in DMSO (0.1% v/v final concentration). As a positive control, LS180 cells were treated for 72 hours with rifampin (10 µM; control) and DMSO (0.2% v/v final concentration) or L-sulforaphane (20 µM) dissolved in DMSO (0.2% v/v final concentration). CYP3A5 mRNA levels were determined by reverse transcription and real-time PCR. Data are expressed as mean ± SEM (n = 4). * $p < 0.05$ when compared to the control. One-way ANOVA and student's *t*-test showed that the means were significantly different.

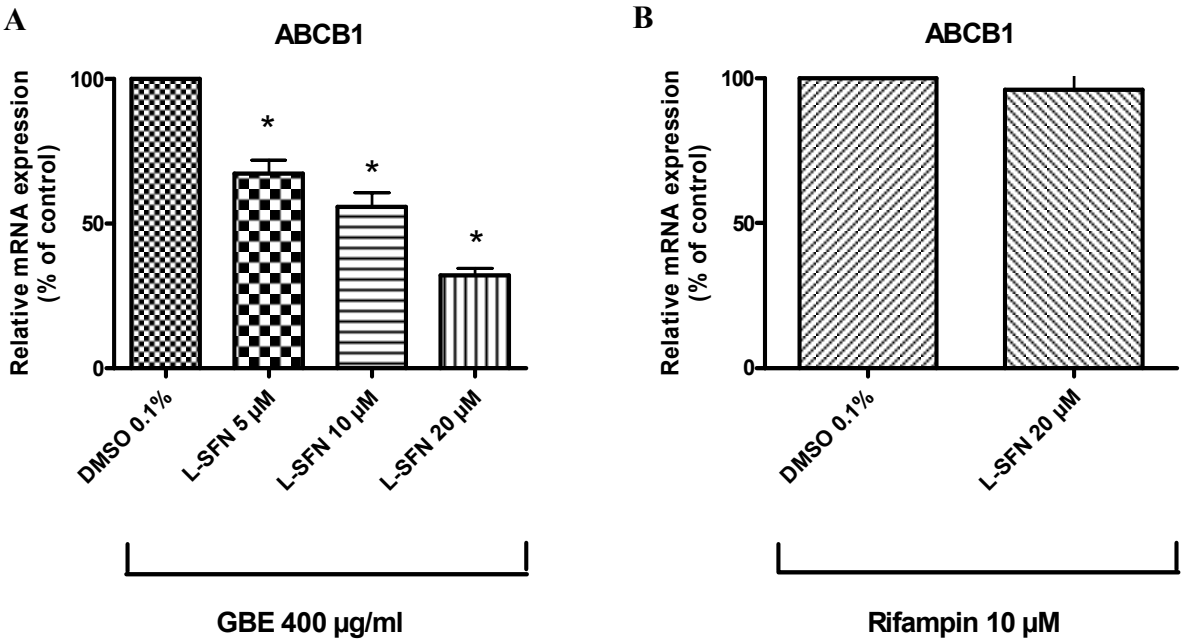


Fig. 3.11. Effect of L-sulforaphane (L-SFN) on induction of ABCB1 mRNA expression by *Ginkgo biloba* extract (GBE) in cultured LS180 cells.

LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (GBE 400 µg/ml; control; lot 1306A, Appendix 1) with DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (L-SFN; 5, 10, or 20 µM) dissolved in DMSO (0.1% v/v final concentration). As a positive control, LS180 cells were treated for 72 hours with rifampin (10 µM; control) and DMSO (0.2% v/v final concentration) or L-sulforaphane (20 µM) dissolved in DMSO (0.2% v/v final concentration). ABCB1 mRNA levels were determined by reverse transcription and real-time PCR. Data are expressed as mean ± SEM (n = 4). * $p < 0.05$ when compared to the control. One-way ANOVA and student's *t*-test showed that the means were significantly different.

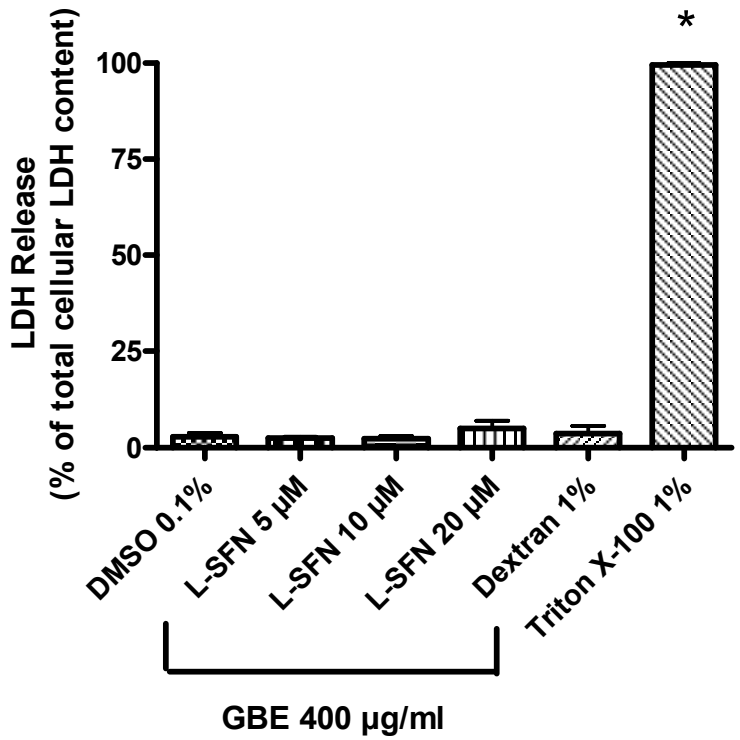


Fig. 3.12. Effect of L-sulforaphane (L-SFN) on LDH release in cultured LS180 cells treated with *Ginkgo biloba* extract (GBE) in cultured LS180 cells.

LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (400 µg/ml; control; lot 1306A, Appendix 1) and DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (L-SFN; 5, 10, or 20 µM) dissolved in DMSO (0.1% v/v final concentration), dextran (1% w/v; negative control) or Triton-X 100 (1% v/v positive control). The amount of LDH released to the culture medium (i.e., LDH leakage) is expressed as a percentage of the total cellular LDH content (i.e., sum of the LDH in the culture medium and in the cell lysate). Data are expressed as mean ± SEM (n = 3). One-way ANOVA showed that the means were significantly different.

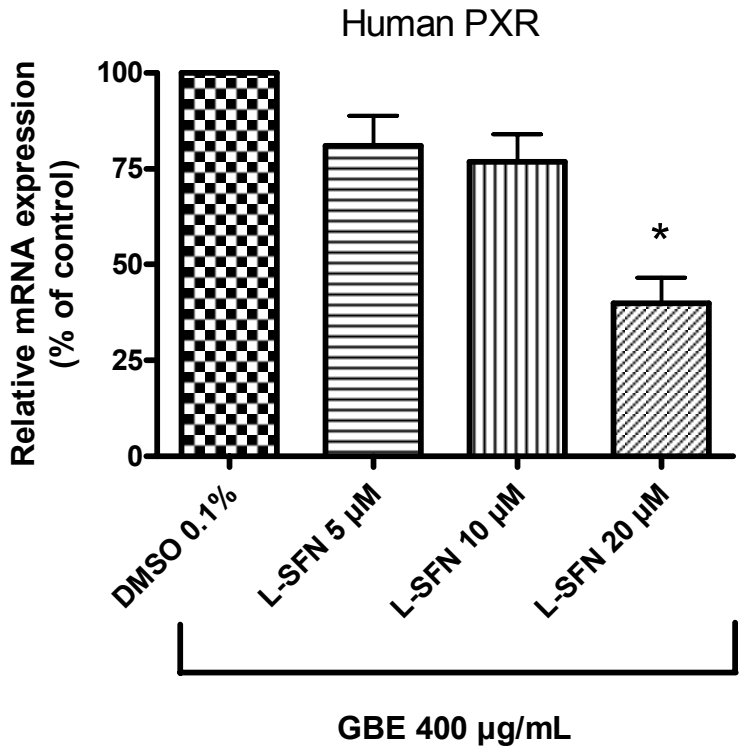


Fig. 3.13. Effect of L-sulforaphane (L-SFN) on human PXR mRNA expression in cultured LS180 cells treated with *Ginkgo biloba* extract (GBE).

LS180 cells were treated for 72 hours with *Ginkgo biloba* extract (400 µg/ml; control; lot 1306A, Appendix 1) and DMSO (0.1% v/v final concentration) or various concentrations of L-sulforaphane (L-SFN; 5, 10, or 20 µM) dissolved in DMSO (0.1% v/v final concentration). Human PXR mRNA levels were determined by reverse transcription and real-time PCR. Data are expressed as mean ± SEM (n = 4). * $p < 0.05$ when compared to the vehicle-treated control. One-way ANOVA showed that the means were significantly different.

4. DISCUSSION

4.1 Evidence that the Reporter Activity Assay Identifies PXR Activators

As a control experiment, PCN was used as a positive control for the mouse PXR activity because it is an agonist for mouse, but not human, PXR (Vignati et al., 2004). In contrast, rifampin was used as a negative control because it is an agonist for human, but not mouse, PXR (Vignati et al., 2004). Treatment of mouse PXR-transfected HepG2 cells with PCN (10 μM) increased PXR activity by 13.8-fold, whereas rifampin (10 μM) showed no increase (Fig. 3.1). The differential effect in PXR-reporter activity was consistent with the published data (Vignati et al., 2004), and thus, indicating that the reporter gene assay was able to identify mouse PXR activators.

Because of their differential PXR activation, PCN and rifampin were also used as negative and positive controls, respectively, for the human PXR activity. Rifampin activates human, but not mouse, PXR, whereas PCN activates mouse, but not human, PXR (Vignati et al., 2004). Treatment of human PXR-transfected HepG2 cells with rifampin (10 μM) increased PXR activity by 30.5-fold, whereas PCN (10 μM) showed no increase (Fig. 3.2). The differential effect in PXR-reporter activity was also consistent with the published data (Vignati et al., 2004), indicating that the assay was able to identify human PXR activators.

4.2 Evidence that *Ginkgo biloba* Activates PXR

An *in vitro* cell-based reporter assay was performed to test whether *Ginkgo biloba* extract activates mouse PXR. At a concentration of 200 μg/ml, *Ginkgo biloba* extract increased mouse PXR activity by 3.2-fold (Fig. 3.3a). This indicates that *Ginkgo biloba* extract activates mouse PXR. Oral administration of *Gingko biloba* has been shown to induce CYP3A-catalyzed activity in mouse hepatic microsomes (Umegaki et al., 2007), but most of

the human pharmacokinetic studies show no increase in clearance of drugs that are metabolized predominantly by CYP3A (Gurley et al., 2002; Markowitz et al., 2003; Uchida et al., 2006; Yin et al., 2004). One possible explanation for the discrepancy could be that *Ginkgo biloba* activates only mouse PXR but not human PXR. In the present study, treatment of human PXR-transfected HepG2 cells with *Ginkgo biloba* extract (200 μg/ml) increased human PXR activity by 8.3-fold (Fig. 3.3b). This indicates *Ginkgo biloba* extract more effectively activated human PXR than mouse PXR.

It was not surprising that *Ginkgo biloba* extract had variable PXR activities in mouse and human PXR reporter gene assays. Hyperforin, a constituent in St. John's wort, is another example that strongly activates human PXR (> 20 fold), but not mouse PXR (< 5 fold) (Vignati et al., 2004). Conversely, exemestane strongly activates mouse PXR (> 20 fold), but not human PXR (< 5 fold) (Vignati et al., 2004). In addition, corticosterone, cortisone, cyclophosphamide, hydrocortisone, lovastatin, mifepristone (RU486), nifedipine, and spironolactone have moderate activities (5–15 fold) in both human and mouse PXR reporter gene assays (Vignati et al., 2004).

To corroborate the results obtained from the *in vitro* cell-based reporter gene assays (Fig 3.3b, Fig 3.4, and Fig 3.5), real-time PCR analyses were performed to determine the effect of *Ginkgo biloba* extract on the mRNA expression of PXR target genes in a cell line that expresses endogenous human PXR. *Ginkgo biloba* extract increased the mRNA expression of CYP3A4, CYP3A5, and ABCB1, all of which are PXR target genes, in LS180 human colon adenocarcinoma cells. LS180 cells have endogenous mRNA expression of PXR and basal and inducible mRNA expression of CYP3A4, CYP3A5, and ABCB1 (Gupta et al., 2008; Schuetz et al., 1996). In addition, the positive control, rifampin (10 μM), increased CYP3A4, CYP3A5, and ABCB1 mRNA expression, whereas the negative control,

PCN (10 μM), had no effect. It indicated that LS180 cells were able to characterize the target gene expression of human PXR, but not mouse PXR.

4.3 Evidence that PXR is Responsible for the Induction of CYP3A4, CYP3A5, and ABCB1 in LS180 cells by *Ginkgo biloba*

L-sulforaphane, a human PXR antagonist (Zhou et al., 2007), was used to determine whether the increase in CYP3A4, CYP3A5, and ABCB1 mRNA expression was due to activation of PXR. In a dose-dependent manner, L-sulforaphane (5, 10, and 20 μM) decreased CYP3A4, CYP3A5, and ABCB1 mRNA expression in cells treated with *Ginkgo biloba* extract (Fig. 3.9a, 3.10a, and 3.11a, respectively). The result suggested the increase in CYP3A4, CYP3A5, and ABCB1 mRNA expression (Fig 3.6a, 3.6b, and 3.6c) was likely due to activation of PXR by *Ginkgo biloba* extract. L-sulforaphane has been shown to reduce the ligand-activation of human PXR (by approximately 50%), but not mouse PXR, rat PXR, human constitutive androstane receptor (CAR), human vitamin D receptor (VDR), human peroxisome proliferator-activated receptor (PPAR) α subtype, human PPAR γ subtype, and human retinoid X receptor (RXR) (Zhou et al., 2007). Therefore, the decrease in CYP3A4, CYP3A5, and ABCB1 mRNA expression was unlikely due to antagonism of VDR, CAR, PPAR, and RXR by L-sulforaphane. It is believed that L-sulforaphane reduces human PXR activity by inhibiting recruitment of coactivators, such as steroid receptor coactivator-1 (SRC-1) and proliferator-activated receptor-binding protein (PBP) (Zhou et al., 2007).

The increase in CYP3A4, CYP3A5, and ABCB1 mRNA expression in LS180 cells treated with *Ginkgo biloba* extract might not have been solely due to PXR activation by *Ginkgo biloba* extract. CYP3A4, CYP3A5, and ABCB1 are also target genes of other receptors. For example, CAR regulates the mRNA expression of some PXR target genes,

such as CYP3A4 (Goodwin et al., 2002; Kliewer et al., 1998), CYP3A5 (Burk et al., 2004), and ABCB1 (Burk et al., 2005a; Geick et al., 2001). However, LS180 cells lack CAR mRNA expression, so the increase in the mRNA expression of CYP3A4, CYP3A5, and ABCB1 in LS180 cells could not be due to CAR activation (Gupta et al., 2008). Vitamin D receptor (VDR) is also believed to regulate *CYP3A4* gene expression (Thummel et al., 2001). Calcitriol (1 and 100 nM), a VDR agonist also known as 1α,25-dihydroxyvitamin D_3 (Schmiedlin-Ren et al., 2001), increases CYP3A4 mRNA and protein expression in LS180 cells (Fukumori et al., 2007; Harmsen et al., 2008). Currently, there is no study to determine whether VDR is expressed in LS180 cells. The present study cannot rule out whether VDR contributes to the increase in CYP3A4 mRNA expression by *Ginkgo biloba* extract. However, only bile acids and vitamin D derivatives have been found to activate VDR, suggesting VDR has a very narrow ligand specificity (Harmsen et al., 2008). In addition, farnesoid X receptor (FXR), also known as the bile acid receptor, is another signaling molecule suggested to activate CYP3A4 transcription (Gnerre et al., 2004). Similar to VDR, there is no study to determine whether FXR is expressed in LS180 cells. The present study cannot rule out whether FXR contributes to the increase in CYP3A4 mRNA expression by *Ginkgo biloba* extract.

L-sulforaphane decreased CYP3A4 and CYP3A5 mRNA expression in cells co-treated with rifampin, a prototypical inducer for the two genes (Usui et al., 2003). However, L-sulforaphane did not decrease ABCB1 mRNA expression by rifampin (Fig. 3.11b). The reason for the lack for effect is not known. Both PXR and CAR regulate ABCB1 mRNA expression (Burk et al., 2005a; Geick et al., 2001), but the increase in ABCB1 mRNA expression by the extract was unlikely due to CAR activation, because CAR is not expressed

in LS180 cells (Gupta et al., 2008). I speculate that rifampin could have induced ABCB1 mRNA expression via another pathway in addition to PXR activation.

The LDH assay was performed to rule out the possibility that the decrease in CYP3A4 (Fig. 3.9), CYP3A5 (Fig. 3.10), and ABCB1 (Fig. 3.11) mRNA expression by L-sulforaphane was a consequence of cellular toxicity. An increase in LDH release would indicate that the co-treatment of L-sulforaphane and *Ginkgo biloba* extract cause tissue breakdown, thereby decreasing the number of cells available for PXR activation and target gene transcription. In the present study, L-sulforaphane (5, 10, or 20 μM) did not increase LDH release in cultured LS180 cells treated with *Ginkgo biloba* extract (400 μg/ml) (Fig. 3.12). The results indicate that the observed decrease in CYP3A4 (Fig. 3.9), CYP3A5 (Fig. 3.10), and ABCB1 (Fig. 3.11) mRNA expression was not due to tissue breakdown.

Human PXR mRNA expression was measured to rule out the possibility that the decrease in CYP3A4 (Fig. 3.9), CYP3A5 (Fig. 3.10), and ABCB1 (Fig. 3.11) was due to L-sulforaphane decreasing the gene expression of *PXR*. As shown in Figure 3.8, L-sulforaphane (5 or 10 μM) did not decrease human PXR mRNA expression in cultured LS180 cells treated with *Ginkgo biloba* extract. This indicated there was no down-regulation of PXR that could be a reason for the decrease in CYP3A4, CYP3A5, and ABCB1 mRNA expression by L-sulforaphane. However, at a concentration of 20 μM, L-sulforaphane decreased human PXR mRNA expression by 55% in cells treated with the extract. This result suggests that the decreases in CYP3A4 (Fig. 3.9), CYP3A5 (Fig. 3.10), and ABCB1 (Fig. 3.11) mRNA levels by 20 μM of L-sulforaphane could have been due to a combination of receptor antagonism and down-regulation of PXR.

Overall, the experiments with L-sulforaphane (5 and 10 μM) suggest that the increase in CYP3A4, CYP3A5, and ABCB1 mRNA expression was due to PXR activation

by *Ginkgo biloba* extract. Moreover, L-sulforaphane at 5 and 10 μM were not cytotoxic and did not decrease PXR mRNA expression, and thereby ruling out two possibilities that could have caused the change in CYP3A4, CYP3A5, and ABCB1 mRNA expression by *Ginkgo biloba* extract.

4.4 Lot-to-Lot Comparison

Various factors may contribute to inter-lot differences in the extent of PXR activity by herbal medicines. As an example, different lots of St. John's wort dry extract (previously stored at 50^{o}C for 0 to 24 days before the experiment) showed decreasing PXR activity in PXR-transfected LS174T human colon adenocarcinoma cells as the number of storage days of the extract increases (Godtel-Armbrust et al., 2007). The decrease in PXR activity is correlated with decreases in amount of hypericin, hyperforin, and flavonols, which are chemical constituents in St. John's wort. Other factors that may cause variable PXR activation between lots include differences in manufacturing processes, variation in the bioactive constituents of the extract, growth condition of the herbal plants, and time of a year when the plants are harvested (Foster et al., 2005).

In studies with herbal products, results may not be reproducible even when different lots of herbal products from the same manufacturer are used. Therefore, a reporter gene assay was used to determine whether different lots of *Ginkgo biloba* extract have a similar activation effect on human PXR. Each of the three lots of *Ginkgo biloba* extract (200 μg/ml; Indena S. A.; lot numbers 1306A, 302831, and 38121) increased human PXR activity (9.5 to 10.5-fold; Fig. 3.4). The activation effects from the three lots were not statistically different from one another.

4.5 Evidence that the PXR-Activating Concentrations of *Ginkgo biloba* were Non-Cytotoxic

LDH release into the culture medium was measured to determine whether the PXR-activating concentrations of *Ginkgo biloba* extract were cytotoxic. An increase in LDH release would indicate that the treatment of *Ginkgo biloba* extract cause tissue breakdown. Cytotoxicity also decreases the number of viable cells and thereby decreases PXR activity and its target gene expression. As shown in Fig. 3.7, LS180 cells treated with *Ginkgo biloba* extract at a concentration of 200, 400, or 800 μg/ml for 72 hours did not increase LDH release into the culture medium, indicating that the PXR-activating concentrations of *Ginkgo biloba* extract were not cytotoxic.

4.6 Reasons for Using HepG2 Cells for Transient Transfection Assays

The HepG2 human hepatoma cell line is commonly used for transient transfection assays involving PXR (Stanley et al., 2006). HepG2 cells have low or negligible endogenous PXR mRNA expression, when compared to LS180 cells (Harmsen et al., 2008). This reduces the possibility of endogenous PXR affecting the reporter activity in PXR transfection assays; therefore, HepG2 cells were chosen over LS180 human colorectal adenocarcinoma cells for the transient transfection assays in my study.

4.7 Reasons for Using LS180 Cells for Gene Expression Assays

LS180 human colorectal adenocarcinoma cell line was chosen over HepG2 cell line for gene expression assays because the LS180 cell line is an appropriate model to study endogenous PXR-mediated CYP3A4 and ABCB1 induction (Gupta et al., 2008; Harmsen et al., 2008). LS180 cells show endogenous PXR mRNA expression (Thummel et al., 2001),

and basal and inducible CYP3A4, CYP3A5, and ABCB1 mRNA expression (Gupta et al., 2008; Schuetz et al., 1996). In contrast, HepG2 cells have low or negligible endogenous PXR mRNA expression and PXR target gene expression compared to LS180 cells (Harmsen et al., 2008). Thus, LS180 cells were chosen over HepG2 cells. Moreover, CAR mRNA is not expressed in LS180 cells (Gupta et al., 2008). It is an advantage because CAR also regulates expression of some PXR target genes, such CYP3A4 (Goodwin et al., 2002; Kliewer et al., 1998), CYP3A5 (Burk et al., 2004), and ABCB1 (Burk et al., 2005a; Geick et al., 2001). Therefore, LS180 cells were chosen over HepG2 cells in the gene expression assays.

4.8 Effect of *Ginkgo biloba* on Human Pharmacokinetics of Drugs Metabolized or Transported by a PXR Target Gene Product

Human PXR has been shown to regulate induction of CYP3A4 (Kliewer et al., 1998) and ABCB1 (Geick et al., 2001). The present study showed that *Ginkgo biloba* activated PXR (Fig. 3.5) and increased mRNA expression of CYP3A4 and ABCB1 (Fig. 3.6). *In vitro* data in human hepatocytes have shown that *Ginkgo biloba* extract (100 to 2500 ng/ml) increases the activity, protein, and mRNA expression of CYP3A4 in a concentration-dependent fashion (Deng et al., 2008b). However, there are conflicting data in human pharmacokinetic studies with respect to the *in vivo* effect of *Ginkgo biloba* on CYP3A-mediated drug clearance. The following sections describe each of the human pharmacokinetic studies that investigated the effect of *Ginkgo biloba* on CYP3A-mediated drug clearance or P-glycoprotein-mediated drug transport. It appears that none of the studies determined whether *Ginkgo biloba* increases CYP3A-mediated drug clearance and P-glycoprotein-mediated drug transport.

4.8.1 CYP3A4

In an open-label study, 12 volunteers received oral administration of *Ginkgo biloba* (60 mg, four times daily) for 28 days (Gurley et al., 2002). They received midazolam, a CYP3A4 substrate, at 1 day prior to the start of *Ginkgo biloba* treatment and 1 day before the end of *Ginkgo biloba* treatment (i.e., Day -1 and 27). Blood samples (10 mL) were collected at 1 hour after administration of midazolam. The subjects' pharmacokinetic parameters before the *Ginkgo biloba* treatment served as the baseline control. The authors used the serum ratio of 1'-hydroxymidazolam/midazolam to estimate CYP3A4 activity, because CYP3A4 enzymes metabolize midazolam to 1'-hydroxymidazolam (Ghosal et al., 1996). At the end of the treatment period, the group mean serum ratio of 1'-hydroxymidazolam/midazolam remained unchanged from baseline. The authors concluded that *Ginkgo biloba* produced no significant change in CYP3A4 phenotype.

The study by Gurley *et al.* (2002) used single blood sampling of midazolam, which failed to predict oral midazolam AUC at 1 h prior to and 1 h after the administration of a CYP3A4-inducing agent, as determined by precision, bias, and coefficient of determination (r^2) for correlations between midazolam AUC and midazolam serum concentrations (Penzak et al., 2008). It happened possibly because midazolam was only partially absorbed at 1 h post-dose. In a crossover study on 18 healthy volunteers who were orally administered with midazolam (15 mg) one hour before, with, and one hour after a standard meal as well as under fasting conditions (control), the time to reach the maximum plasma concentration (t_{max}) was 0.8, 1.7, 1.9, and 1.0, respectively (Bornemann et al., 1986). Therefore, midazolam is not in the elimination phase at 1 h post-dose. The study by Gurley *et al.* (2002) did not indicate whether they restricted the subjects from food consumption before blood sampling.

Consequently, the data from the single blood sampling of midazolam at 1 h post-dose might not be reliable to determine the effect of *Ginkgo biloba* on CYP3A4-mediated drug clearance.

Although the group mean serum ratio of 1'-hydroxymidazolam/midazolam remained unchanged from baseline in the study by Gurley *et al.* (2002), two of the twelve subjects did have increased serum ratios, suggesting increased CYP3A4 metabolism. Ten of the twelve subjects did not have increased serum ratios of 1'-hydroxymidazolam/midazolam. This was possibly due to inhibition of CYP3A4-catalyzed formation of 1'-hydroxymidazolam by *Ginkgo biloba*, which has been shown to inhibit CYP3A4 catalytic activity *in vitro* (Gaudineau et al., 2004; Hellum et al., 2007; Hellum and Nilsen, 2008; Yale and Glurich, 2005). In the study by Gurley *et al.* (2002), the subjects received midazolam, a CYP3A4-selective substrate, at 1 day before the end of *Ginkgo biloba* treatment (i.e., Day 27) (Gurley et al., 2002). The exact time interval between the administration of midazolam and *Ginkgo biloba* was not given. In studies conducted in human volunteers, the elimination $t_{1/2}$ of ginkgolide A, ginkgolide B, bilobalide, quercetin aglycone, and kaempferol aglycone are 3.9–5.1, 6.0–10.6, 3.2–5.5, 2.2, and 2.8 h, respectively (Biber, 2003; Fourtillan et al., 1995; Kressmann et al., 2002; Wang et al., 2003b). It takes seven elimination $t_{1/2}$ to remove 99% of a substance out of the body. When the subjects CYP3A4 phenotypes were assessed at Day 27, some constituents of *Ginkgo biloba* might still have been present in the body and might have masked the inductive effect of *Ginkgo biloba*. This is assuming that ginkgolide A, ginkgolide B, bilobalide, quercetin aglycone, and kaempferol aglycone are responsible for the CYP3A4 inhibition by *Ginkgo biloba* extract. Quercetin aglycone and kaempferol aglycone have shown *in vitro* inhibition on CYP3A4-catalzyed hydroxylation of triazolam in human liver microsomes (von Moltke et al., 2004). However, no comparison was made between the inhibition by those constituents and by *Ginkgo biloba* extract. The same study claims that

ginkgolide A, ginkgolide B, bilobalide, quercetin glycosides, and kaempferol glycosides all have weak or negligible CYP3A4 inhibitory capacity, but did not show the data or illustrate concentrations of constituents used. Because of limitations on single blood sampling of midazolam, interindividual differences in serum ratio of 1'-hydroxymidazolam/midazolam, and the unknown time interval between the administration of midazolam and *Ginkgo biloba* in the study by Gurley *et al.* (2002), it is impossible to conclude that *Ginkgo biloba* does not affect CYP3A4-mediated drug clearance.

In another study, twelve subjects orally received two probe substrates, dextromethorphan 30 mg (CYP2D6-selective substrate) and alprazolam 10 mg (CYP3A4-selective substrate) at baseline (Markowitz et al., 2003). Blood samples (10 ml) were collected immediately prior to the administration of alprazolam (i.e., 0 h) and again at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, and 60 h after the administration of alprazolam. The subjects waited 7 days, and then took *Ginkgo biloba* (120 mg twice daily; 8am and 8pm) for 14 days. The following morning subjects orally received both dextromethorphan 30 mg and alprazolam 10 mg along with *Ginkgo biloba* (120 mg), which was continued for one additional day thereafter. Blood samples (10 ml) were collected immediately prior to the administration of alprazolam (i.e., 0 h) and again at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, and 60 h after the administration of alprazolam. The subjects' pharmacokinetic parameters before the *Ginkgo biloba* treatment served as the baseline control. The AUC of alprazolam decreased after *Ginkgo biloba* treatment, whereas the elimination $t_{1/2}$ of alprazolam remained unchanged. The apparent oral clearance of all subjects slightly increased, but the difference was not statistically significant. The authors concluded that *Ginkgo biloba* is unlikely to significantly alter the disposition of co-administered medications primarily dependent on the CYP3A4 pathways for elimination.

Both alprazolam and dextromethorphan were administered to each subject in the study by Markowitz *et al.* (2003). It is not known whether the substrates affect the pharmacokinetic parameters of each other, as there has been no study of an interaction between alprazolam and dextromethorphan. Dextromethorphan is also metabolized by CYP3A4 to produce an *N*-demethylated metabolite, but it was not measured in the study. It is possible that dextromethorphan competes with alprazolam for the binding sites in CYP3A4 enzymes, and thus inhibits the metabolism of alprazolam. Therefore, the inhibition of CYP3A4-catalyzed metabolism of alprazolam by dextromethorphan could have masked any induction by *Ginkgo biloba*. The unchanged elimination $t_{1/2}$ could be due to no net effect on the intrinsic clearance or metabolism of alprazolam by *Ginkgo biloba*; the decrease in AUC could be due to a decrease in bioavailability of the alprazolam by *Ginkgo biloba*. However, similar to the study by Gurley *et al.* (2002), the CYP3A4 probe and *Ginkgo biloba* were co-administered. Therefore, the inhibitory effect of *Ginkgo biloba* might have masked its inductive effect. It is inappropriate to conclude that *Ginkgo biloba* has no effect on medications primarily dependent on the CYP3A4 pathways for elimination.

In eighteen subjects receiving 12-day oral treatment of *Ginkgo biloba* (140 mg twice daily), the urinary ratio of 6β-hydroxycortisol to cortisol (CYP3A4 activity marker) was determined before and after the treatment period (Yin et al., 2004). The authors did not mention administration of a CYP3A4 substrate. The subjects' pharmacokinetic parameters before the *Ginkgo biloba* treatment served as the baseline control. The group mean urinary ratio showed no change between baseline and post-treatment. The authors concluded that *Ginkgo biloba* has no effect on CYP3A4 activity.

The study by Yin *et al.* (2004) used urinary data, which has its own limitation when being extrapolated to drug metabolism. The amount of 6β-hydroxycortisol and cortisol

collected are dependent on the urinary excretion of 6β-hydroxycortisol and cortisol by the kidney. If *Ginkgo biloba* compromises a subject's renal function, it may offset the increase in CYP3A4-catalyzed metabolism of cortisol by *Ginkgo biloba*. Moreover, it appeared that the subjects were not administered a CYP3A4 substrate in the study by Yin *et al.* (2004). The timeline for the administration of *Ginkgo biloba* and urine sampling was not clearly described. When the urinary ratio was measured, some constituents of *Ginkgo biloba* extract might still be present in the body that might possess an inhibitory effect on CYP3A4 catalytic activity and masked the inductive effect by *Ginkgo biloba* extract. Because of the limitation of urine data, it is not clear whether *Ginkgo biloba* affects urinary levels of 6β-hydroxycortisol.

There is a study that suggested inhibition of CYP3A4-mediated drug clearance by *Ginkgo biloba* extract (Uchida et al., 2006). Following overnight fasting, each of the ten healthy male subjects received oral administration of tolbutamide (125 mg; CYP2C9 substrate) at 9 a.m.. After 1 hour, they received oral administration of midazolam (8 mg; CYP3A4 substrate), and glucose (75 g) to determine the hypoglycemic effect of tolbutamide. Blood samples were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 24 h after the tolbutamide dose, while urine samples were collected up to 24 h after the dose. Starting the second day, *Ginkgo biloba* extract (120 mg) was taken three times a day (8 a.m., 12 a.m., and 8 p.m.) for 28 days. After the treatment with *Ginkgo biloba* extract, subjects received tolbutamide, midazolam, and glucose, but the time interval between the last dose of *Ginkgo biloba* and the first dose of midazolam was not given. Blood and urine samples were collected the same way as before. The subjects' pharmacokinetic parameters before the *Ginkgo biloba* treatment served as the baseline control. *Ginkgo biloba* decreased the apparent oral clearance (Cl/F) by 26% and increased AUC by 25% of midazolam. The authors suggested that oral

administration of *Ginkgo biloba* might inhibit CYP3A4 catalyzed activity, thereby resulting in the increased plasma concentrations of midazolam. However, both midazolam and tolbutamide were administered to each subject. It is not known whether the substrates affect the pharmacokinetic parameters of each other, as there has been no study of an interaction between midazolam and tolbutamide.

Only one human pharmacokinetic study showed increased clearance of drugs that are metabolized by CYP3A4. In an open-label study, single oral doses of midazolam (120 mg; CYP3A4 substrate) and fexofenadine (8 mg; P-glycoprotein substrate) were administered together to 14 healthy subjects, 21 to 42 days prior to and at the end of *Ginkgo biloba* extract treatment (120 mg orally, twice daily for 28 days) (Robertson et al., 2008). The exact timeline for the dosing of *Ginkgo biloba* extract and the two substrates was not given. In addition, lopinavir (400 mg) and ritonavir (100 mg) were orally administered twice daily with meals for 29.5 days, starting 14 days prior to the first administration of *Ginkgo biloba*. In other words, the subjects first received lopinavir and ritonavir for 15 days (Day 1-15), then received lopinavir, ritonavir, and the extract together for 14 days (Day 16-29), then continued to receive the extract alone for 14 more days (Day 30-43), and finally had blood sampled at the end of the extract treatment (Day 43-44). Blood samples were collected for at 0 (pre-dose), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8 and 24 h after the fexofenadine and midazolam doses. The subjects' pharmacokinetic parameters before the *Ginkgo biloba* treatment served as the baseline control. After *Ginkgo biloba* treatment, the AUC and C_{max} of midazolam, a CYP3A4 substrate, decreased by 33% and 31%, respectively, whereas the elimination $t_{1/2}$ and t_{max} remained unchanged. The oral clearance (Cl/F) increased from 76 to 116 L/h, but the increase was not significant ($p > 0.06$). The author concluded that *Ginkgo biloba* induced CYP3A4 metabolism, as assessed by a decrease in midazolam concentrations.

In the study by Robertson *et al.* (2008), fexofenadine was co-administered with midazolam. It is not known whether fexofenadine affects the pharmacokinetic of midazolam, as there has been no study of an interaction between the two substrates. The increase in Cl/F was probably due to an increase in clearance (Cl) and a decrease in oral bioavailability (F) of midazolam. The decrease in F could be due to an increase in CYP3A4 metabolism in liver and intestine that consequently led to reduction in midazolam AUC and C_{max}. The reduction in midazolam AUC suggested that *Ginkgo biloba* increased CYP3A4-mediated drug clearance of midazolam. However, there was an overlap of 14 days in which subjects took lopinavir, ritonavir, and *Ginkgo biloba* extract together. Ritonavir is an agonist of PXR (Dussault et al., 2001), which regulates *CYP3A* gene expression (Kliewer et al., 1998). It was possible that ritonavir induced CYP3A4 and contributed to the increased CYP3A4-mediated drug clearance of midazolam in the study by Robertson *et al.* (2008).

In vitro data in the present study have shown that *Ginkgo biloba* increases the mRNA expression of CYP3A4 enzymes. For example, in cultured LS180 cells, *Ginkgo biloba* extract (200, 400, and 800 μg/ml) increased CYP3A4 and CYP3A5 mRNA expression (Fig. 3.6a and 3.6b, respectively). In a study with human hepatocytes, *Ginkgo biloba* extract (100 to 2500 ng/ml) increased the activity, protein, and mRNA expression of CYP3A4 in a concentration-dependent fashion (Deng et al., 2008b). The results indicate that *Ginkgo biloba* extract have inductive effect on CYP3A4 *in vitro*. However, there are conflicting data in the literature with respect to the *in vivo* effect of *Ginkgo biloba* on the clearance of drugs metabolized by CYP3A4. *In vitro* studies show that *Ginkgo biloba* inhibits CYP3A4 catalytic activity (Gaudineau et al., 2004; Hellum et al., 2007; Hellum and Nilsen, 2008). Therefore, insufficient time between the last dose of *Ginkgo biloba* extract and the first does of the CYP3A4 probe substrate could explain the lack of increase in CYP3A4-

mediated drug clearance observed in many human pharmacokinetic studies (Gurley et al., 2002; Markowitz et al., 2003; Robertson et al., 2008; Yin et al., 2004). It is because some constituents of *Ginkgo biloba* extract might still be present in the body when the CYP3A4 probe substrates were given to the subjects in these human studies; those constituents might possess inhibitory effect on CYP3A4 catalytic activity that masked the inductive effect by *Ginkgo biloba* extract. In addition, reporting only the group mean values of various pharmacokinetic parameters could have masked interindividual differences in CYP3A4-mediated drug clearance by *Ginkgo biloba* extract. Two of the twelve subjects had increased serum ratios of 1'-hydroxymidazolam/midazolam (Gurley et al., 2002), suggesting increased CYP3A4 metabolism. However, the group mean serum ratios of 1'-hydroxymidazolam/midazolam remained unchanged. It is possible that *Ginkgo biloba* extract *in vivo* does not alter the pharmacokinetic of drugs that are metabolized by CYP3A4. After administration of *Ginkgo biloba* extract *in vivo*, the constituents may not reach the level present in the *in vitro* PXR-activating concentration of the extract (Fig. 3.5). Active transport of *Ginkgo biloba* extract out of hepatocytes could be a factor for the lack of effect on CYP3A4-mediated drug clearance. It is also possible that *Ginkgo biloba* extract undergoes extensive metabolism that only a low concentration of CYP3A4-inducing constituents are available in the systemic circulation. As a result, human pharmacokinetic models fail to predict the *in vivo* effect of *Ginkgo biloba* extract on CYP3A4 metabolism.

4.8.2 ABCB1

In an open-label, crossover trial, eight healthy human volunteers received oral administration of *Ginkgo biloba* (80 mg three times daily) for two weeks (Mauro et al., 2003). After an overnight fast of 10 h, four of the eight volunteers were randomized to

receive 0.5 mg of digoxin, a substrate for P-glycoprotein (Schinkel et al., 1995). The control group, which consisted of the remaining four volunteers, received digoxin alone without having received any *Ginkgo biloba*. After that, the volunteers were crossed over to receive the alternative treatment regimen for 2 weeks. The exact timeline for the dosing of *Ginkgo biloba* extract and digoxin was not given. Blood samples were collected immediately prior to and 1, 2, 4, 6, 8, 12, 24, and 36 h after the ingestion of digoxin. *Ginkgo biloba* was shown to have no effect on the pharmacokinetic parameters, such as C_{max}, t_{max}, or oral clearance, of digoxin. The authors did not comment on the effect of *Ginkgo biloba* on P-glycoprotein-mediated drug transport.

Ginkgo biloba extract has been shown to inhibit P-glycoprotein activity *in vitro* (Hellum and Nilsen, 2008). In the study by Mauro *et al.* (2003), some constituents of the *Ginkgo biloba* extract might still be present in the body during blood sampling, and thereby masked the inductive effect of P-glycoprotein by *Ginkgo biloba*. Furthermore, transport of digoxin may also involve intestinal organic anion-transporting polypeptide (OATP) (Bressler, 2006). *In vitro* studies show that *Ginkgo biloba* extract inhibits OATP-mediated uptake of estrone-3-sulfate in a concentration dependent fashion with an IC_{50} value of 11.2 μg/ml of the extract (Fuchikami et al., 2006). In the study by Mauro *et al.* (2003), *Ginkgo biloba* might have inhibited OATP-mediated uptake of digoxin into enterocytes, and thereby limit the amount of digoxin for P-glycoprotein to efflux, causing no change in the pharmacokinetics of digoxin.

In vitro data in the present study have shown that *Ginkgo biloba* increases the expression of ABCB1 mRNA, which encodes P-glycoprotein. In cultured LS180 cells, *Ginkgo biloba* extract (200, 400, and 800 μg/ml) increased the mRNA expression of ABCB1 (Fig. 3.6c). Therefore, *Ginkgo biloba* extract has an inductive effect on P-glycoprotein *in*

vitro. However, *Ginkgo biloba* extract has also been shown to inhibit P-glycoprotein activity *in vitro* (Hellum and Nilsen, 2008). The inhibitory effect of *Ginkgo biloba* might have masked its induction effect *in vivo*, and thereby caused no net change in the pharmacokinetics of P-glycoprotein-mediated substrates.

4.9 Candidate Chemical Constituents Responsible for PXR Activation by *Ginkgo biloba* Extract

Some terpene trilactones could be constituents responsible for PXR activation by *Ginkgo biloba* extract because they increase CYP3A4 protein expression, which is regulated by PXR (Kliewer et al., 1998). Ginkgolide A (30 μM or 12.3 μg/ml) induces CYP3A protein expression by 2.1-fold and CYP3A4-mediated testosterone 6β-hydroxylation by 2.5-fold in primary cultures of human hepatocytes (He et al., 2007). It gave a clue that ginkgolide A might be a PXR activator, as other receptors also regulate CYP3A4 protein expression. Reporter gene assays are still required to determine whether ginkgolide A activates PXR. Fig. 3.5 shows that *Ginkgo biloba* extract (lot 302832; Appendix 1) increased human PXR activity over a concentration range of 100, 200, 400, and 800 μg/ml, which contained 0.9, 1.8, 3.6, and 7.2 μg/ml of ginkgolide A, respectively. Ginkgolide A (10, 50, and 100 μM or 4.1, 20.4, and 40.8 μg/ml) has recently been shown to increase human PXR activity in a dose-dependent fashion (approximately 1.3-, 2.0-, and 2.5-fold, respectively) after 24-hour of *in vitro* treatment in LS180 cells (Satsu et al., 2008). Ginkgolide A at a concentration of 4.1 μg/ml (corresponding to 456 μg/ml of the extract) increases human PXR activity by approximately 1.3-fold (Satsu et al., 2008), whereas the extract at a concentration of 400 μg/ml (the closest concentration to 456 μg/ml) increased human PXR activity by 14.5-fold

(Fig. 3.5). Ginkgolide A may only be partially responsible for the human PXR-activating effect of *Ginkgo biloba* extract.

Ginkgolide B may also be a constituent responsible for human PXR activation by *Ginkgo biloba* extract, because it increases CYP3A4 protein expression. Ginkgolide B (30 μM or 12.7 μg/ml) induces CYP3A protein expression 2.0-fold and CYP3A4-mediated testosterone 6β-hydroxylation 2.5-fold *in vitro* in primary cultures of human hepatocytes (He et al., 2007). Fig. 3.5 shows that *Ginkgo biloba* extract (lot 302832; Appendix 1) increased human PXR activity over a concentration range of 100, 200, 400, and 800 μg/ml, which contained 0.3, 0.6, 1.2, and 2.4 μg/ml of ginkgolide B, respectively. Ginkgolide B (10, 50, and 100 μM or 4.2, 21.2, and 42.4 μg/ml) has recently been shown to increase human PXR activity in a dose-dependent fashion (approximately 1.8-, 3.0-, and 4.5-fold, respectively) after 24-hour of *in vitro* treatment in LS180 cells (Satsu et al., 2008). Because the effect of ginkgolide B at a concentration of 0.3, 0.6, 1.2, or 2.4 μg/ml was not determined, it was not clear whether ginkgolide B contributes to the human PXR-activating effect of the extract in the present study.

Bilobalide may be responsible for mouse PXR activation by *Ginkgo biloba* extract. In mice, oral ingestion of bilobalide (42 mg/kg) increases CYP3A4-catalyzed testosterone 6β–hydroxylation activities (Umegaki et al., 2007). In Fig. 3.3a, *Ginkgo biloba* extract (lot 302832; Appendix 1) increased mouse PXR activity at a concentration of 200 μg/ml, which contained bilobalide at a concentration of 5.8 μg/ml. However, the effect of bilobalide on mouse PXR activity was not determined in the present study.

Quercetin aglycone (5 μM or 1.5 μg/ml) has been shown to increase CYP3A4 mRNA expression in human hepatocytes (Raucy, 2003). However, in human PXR-transfected HepG2 cells, quercetin aglycone (10 μM or 3 μg/ml) does not increase PXR

activity (Raucy, 2003). Likewise, in LS180 and stable DPX-2 cells transfected with human PXR, quercetin aglycone (10 and 100 μM, respectively) does not increase PXR activity (Yueh et al., 2005). In transient reporter gene assay in CV-1 Normal African green monkey kidney fibroblast cells transfected with human PXR, quercetin aglycone (10 μM or 3 μg/ml) appeared to slightly increase PXR activity (Moore et al., 2000a), but statistical analysis was not performed to determine whether this increase was significant. In the extract used in my study, quercetin was present as various glycosides (Appendix 1). Although the glycosides are absorbed, they are quickly metabolized (Graefe et al., 1999; Hollman and Katan, 1998). It is not known whether quercetin glycosides activate PXR activation. Cultured LS180 cells may or may not be able to convert quercetin glycosides to quercetin aglycone. Experiments are needed to determine the concentration of quercetin aglycone present in cultured LS180 cells. Moreover, reporter gene assays are needed to determine whether quercetin aglycone and quercetin glycosides are able to activate human PXR.

Kaempferol aglycone (100 μM or 28.6 μg/ml) does not increase PXR activity in LS180 cells transfected with human PXR, indicating no PXR involvement (Satsu et al., 2008). In transient reporter gene assay in CV-1 Normal African green monkey kidney fibroblast cells transfected with human PXR, kaempferol aglycone (10 μM or 2.86 μg/ml) increased PXR activity by approximately 1.5-fold (Moore et al., 2000a), but statistical analysis was not performed to determine whether this increase was significant. In the *Ginkgo biloba* extract used in my study, kaempferol was present as various glycosides (Appendix 1). Although the glycosides are absorbed, they are quickly metabolized (Graefe et al., 1999; Hollman and Katan, 1998). It is not known whether kaempferol glycosides activate PXR activation. Cultured LS180 cells may or may not be able to convert kaempferol glycosides to kaempferol aglycone. Experiments are needed to determine the concentration of kaempferol

aglycone present in the cultured LS180 cells. Furthermore, reporter gene assays are needed to determine whether kaempferol aglycone and kaempferol glycosides are able to activate human PXR.

4.10 Limitations of the Study

The present study used an *in vitro* cell-based reporter gene assay to determine whether *Ginkgo biloba* extract activates PXR. However, the assay cannot determine whether a substance binds to PXR. Receptor binding assays are needed to identify PXR "agonists," which are ligands that binds to a receptor. It is possible that PXR agonists and the constituents of *Ginkgo biloba* extract activate PXR by different mechanisms.

Although the present study shows activation of mouse and human PXR activity by *Ginkgo biloba* extract, the results may not be repeatable with *Ginkgo biloba* extract produced by other manufacturers. The present study has not determined the constituents responsible for the activation of PXR by *Ginkgo biloba* extract. *Ginkgo biloba* extract from other manufacturers may not contain sufficient levels of constituents to activate PXR. In addition, LDH release is an indication of tissue breakdown that cannot account for damages within the cell cytoplasm and nucleus.

It is not known whether the PXR-activating concentrations of *Ginkgo biloba* extract in the present study are physiologically relevant concentrations. Fig. 3.5 shows that *Ginkgo biloba* extract (lot 302832; Appendix 1) increased human PXR activity over a concentration range of 100, 200, 400, and 800 μg/ml, which contained ginkgolide A, ginkgolide B, and bilobalide. The corresponding concentrations of ginkgolide A (0.9, 1.8, 3.6, and 7.2 μg/ml, respectively), ginkgolide B (0.3, 0.6, 1.2, and 2.4 μg/ml, respectively), and bilobalide (2.8, 5.6, 11.2, 22.4 μg/ml) are much higher than the C_{max} reported in human

pharmacokinetic studies (Table 1.2, 1.3, and 1.4, respectively). However, it is not known whether ginkgolide A, ginkgolide B, and bilobalide are responsible for the increase in PXR activity by *Ginkgo biloba* extract. Overall, the present study is based on only *in vitro* experiments; additional experiments are needed to determine whether *Ginkgo biloba* extract activates PXR *in vivo*.

5. FUTURE RESEARCH

5.1 Mechanism of PXR Activation by *Ginkgo biloba* Extract

The present study used an *in vitro* cell-based reporter gene assay to determine whether *Ginkgo biloba* extract activates PXR. However, the assay cannot determine whether a substance binds to PXR. A receptor-binding assay is needed to identify PXR "agonists," which are ligands that bind to a receptor. The scintillation proximity assay has been developed as a binding assay that tests whether a compound binds to a receptor (Moore and Kliewer, 2000). For example, [^{3}H]SR12813, a high affinity radiolabelled ligand, interacts specifically with human PXR with a dissociation constant (K_d) of 40 nM (Jones et al., 2000). Rifampin (10 μM) dissociates more than 60% of [^{3}H]SR12813 from human PXR, whereas PCN (10 μM) dissociates less than 30%. The differential effects of rifampin and PCN as shown in the scintillation proximity assay are in good agreement with the results determined from *in vitro* cell-based reporter gene assays (Vignati et al., 2004). The present study has demonstrated that *Ginkgo biloba* activates PXR in cell-based reporter gene assays. The next step would be to use a receptor-binding assay to determine whether the constituents of *Ginkgo biloba* extract bind to PXR, and thereby identify whether the constituents are PXR agonists. If the constituents fail to bind to PXR, it would indicate that *Ginkgo biloba* extract indirectly activates PXR. One possibility is that the constituents bind to another nuclear receptor, and then form a complex that activates PXR. Another possibility is that the metabolites of *Ginkgo biloba* extract, but not the parent compound, bind to PXR.

5.2 Identification of Individual Chemical Constituents Responsible for PXR Activation by *Ginkgo biloba* Extract

Although *Ginkgo biloba* extract was shown to activate mouse and human PXR activity, I still have not determined which constituents are responsible for the activation of PXR by *Ginkgo biloba* extract. It is important to investigate which constituents are essential for PXR activation. Comparisons can be made between the PXR activities of the extract and each of the constituents, at the concentrations present in the extract (Appendix 1). If the PXR activity of a constituent is similar to that of the extract, it suggests that this constituent is responsible for PXR activation by the extract. It is possible that multiple chemical constituents have additive or synergistic effect on PXR activity. Comparisons should also be made between the PXR activities of the extract and various combinations of chemical constituents.

5.3 Determination of the Effects of *Ginkgo biloba* Extract in Humanized-PXR Mice

The present study is based on only *in vitro* experiments; additional experiments are needed to determine whether *Ginkgo biloba* extract activates PXR *in vivo*. A humanized mouse line has been developed by insertion of human PXR cDNA to make a transgenic mouse (Ma et al., 2007; Xie et al., 2000). This mouse line can be used to determine the *in vivo* effects of *Ginkgo biloba* extract on human PXR target gene expression. Oral treatment of *Ginkgo biloba* extract could be given to humanized-PXR mice and human PXR-null mice. At the end of the treatment period, mice would be sacrificed and their liver samples subjected to PCR and western blot analysis. If there were increases in mRNA and protein expression of PXR target genes (e.g. CYP3A4, CYP3A5, and ABCB1) in humanized-PXR mice, but not in

PXR-null mice, it would suggest that *Ginkgo biloba* extract was able to activate human PXR *in vivo*. In addition, a PXR antagonist, such as L-sulforaphane, could be given orally to attenuate the increases in mRNA and protein expression of PXR target genes in humanized-PXR mice. This is to determine whether the increases are due to PXR activation.

5.4 Determination on whether VDR and FXR are Responsible for the Induction of CYP3A4 in LS180 cells by *Ginkgo biloba*

For the gene expression study, PXR might not be the sole receptor responsible for increase in CYP3A4 mRNA expression because other receptors may also be present in LS180 cells. Vitamin D receptor (VDR) is also believed to regulate CYP3A4 mRNA expression (Thummel et al., 2001). Therefore, PCR analysis should be performed to determine whether VDR is expressed in LS180 cells. If VDR mRNA expression is found, the gene expression study could be repeated with the addition of a VDR antagonist, such as 1α,25-dihydroxyvitamin D_3-26,23-lactam analogues (Ishizuka et al., 2008). If the VDR antagonist does not attenuate the increase in CYP3A4 mRNA expression by *Ginkgo biloba* extract, it would rule out VDR activation by the extract in LS180 cells. In contrast, if the VDR antagonist attenuates the increase in CYP3A4 mRNA expression by *Ginkgo biloba* extract, it would suggest that the extract activates VDR in LS180 cells. Subsequent reporter gene assays with VDR-transfected cells should be performed to determine whether *Ginkgo biloba* extract activates VDR. The findings would allow researchers to predict more biological activities of this herbal extract, such as vitamin D homeostasis. Currently, it is suggested that PXR may also regulate the homeostasis of 1α,25-dihydroxyvitamin D_3 (Pascussi et al., 2008) because two PXR target genes, CYP24 (Kato, 2000) and CYP3A4 (Xu et al., 2006), catalyze hydroxylation of 1α,25-dihydroxyvitamin D_3. Determining whether

Ginkgo biloba extract is an activator of both PXR and VDR should lead to more research on the effect of this extract on vitamin D homeostasis.

Farnesoid X receptor (FXR), also known as the bile acid receptor, is another signalling molecule suggested to activate CYP3A4 transcription (Gnerre et al., 2004). Therefore, PCR analysis should be performed to determine whether FXR is expressed in LS180 cells. If FXR mRNA expression is expressed, the present gene expression study could be repeated with the addition of a FXR antagonist, such as stigmasterol, a soy lipid-derived phytosterol (Carter et al., 2007). If the FXR antagonist does not attenuate the increase in CYP3A4 mRNA expression by *Ginkgo biloba* extract, it would rule out FXR activation by the extract in LS180 cells. In contrast, if the FXR antagonist attenuates the increase in CYP3A4 mRNA expression by *Ginkgo biloba* extract, it would suggest that the extract activates FXR in LS180 cells. Subsequent reporter gene assays with FXR-transfected cells should be performed to determine whether *Ginkgo biloba* extract activates FXR. Similar to the present study in PXR, findings on the effect of *Ginkgo biloba* on FXR activity would allow researchers to predict more biological activities of this herbal extract, such as bile acid homeostasis.

Studies have shown relation between PXR and bile acid homeostasis (Pascussi et al., 2008). In wild-type but not in FXR-knockout mice, oral administration of cholic acid or the synthetic FXR agonist GW4064 causes marked increase in total RNA expression of PXR and three rat PXR target genes, CYP3A11, CYP2B10, and ABCB1 (Jung et al., 2006). The study also identified that two of the four FXR binding-sites (IR1) in the mouse PXR gene exhibited binding to and transcriptional activation by FXR protein. The results suggested that FXR is a regulator of PXR expression. Interestingly, PXR and FXR share ligands, such as ursodeoxycholic acid and lithocholic acid and its 3-keto derivative, which activate rodent and

human PXR at physiologically relevant concentrations (Staudinger et al., 2001; Xie et al., 2001). Lithocholic acid increases mouse hepatic total RNA expression of CYP3A (Xie et al., 2001) and OATP2 (Staudinger et al., 2001), which are PXR target genes that regulate 6α- and 6β-hydroxylation and transport of bile acids, respectively (Araya and Wikvall, 1999; Reichel et al., 1999). In addition, lithocholic acid decreases mouse hepatic total RNA expression of CYP7A1 (Staudinger et al., 2001), which is the rate-limiting enzyme for conversion of cholesterol to bile acids. Therefore, determining whether *Ginkgo biloba* extract is an activator of both PXR and FXR should lead to more research on the effect of this extract on bile acid homeostasis.

6. SUMMARY AND CONCLUSIONS

In summary, the present study showed *Ginkgo biloba* extract activated mouse and human PXR in PXR-transfected HepG2 cells. The effective human PXR-activating concentrations of *Ginkgo biloba* extract were between 100 and 800 μg/ml. The finding was corroborated by increased mRNA expression of CYP3A4, CYP3A5, and ABCB1, all of which are PXR target genes. The effects were likely due to PXR activation because L-sulforaphane, a PXR antagonist, was able to attenuate the increased mRNA expression of CYP3A4, CYP3A5, and ABCB1 by *Ginkgo biloba* extract. L-sulforaphane at 5 and 10 μM were not cytotoxic and did not decrease PXR mRNA expression, thereby ruling out two factors that could have caused the decreases in CYP3A4, CYP3A5, and ABCB1 mRNA expression by *Ginkgo biloba* extract.

Since PXR target genes encode many clinically important drug-metabolizing enzymes and transporters, results from the present study may help to identify novel pharmacological and toxicological actions of *Ginkgo biloba*. Moreover, PXR has recently been shown to regulate the action of other receptors that control many important biological actions, such as gluconeogenesis, ketogenesis, inflammation, immune response, bile acid and fatty acid homeostasis, and bone and muscle growth and development (Konno et al., 2008; Moreau et al., 2008; Pascussi et al., 2008). Identification of *Ginkgo biloba* as a PXR activator would lead to more research on the physiological and possible therapeutic actions of this herb.

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8. APPENDIX

Appendix I: Amount of Ginkgolides, Bilobalide, and Flavonol Glycosides in *Ginkgo biloba* Extracts Used in the Present Study

	Ginkgo biloba extract		
	Lot 1306A	Lot 302831	Lot 38121
	Amount (% w/w)		
Ginkgolide A	1.1	0.9	1.3
Ginkgolide B	0.3	0.3	0.6
Ginkgolide C	1.4	1.5	1.4
Ginkgolide J	0.6	0.6	0.5
Bilobalide	2.8	2.9	3.0
Sum of Terpene Trilactones	6.2	6.2	6.8
Quercetin	10.6*	10.9*	N/A
Kaempferol	6.3*	11.2*	N/A
Isorhamnetin	4.1*	2.3*	N/A
Sum of Flavonols and their Glycosides	21.0	24.4	24.4

The levels of terpene trilactones (ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J and bilobalide) in *Ginkgo biloba* extract were quantified by gas chromatography and those of flavonols and their glycosides (quercetin, kaempferol, and isorhamnetin) were quantified by liquid chromatography-mass spectrometry (ChromaDex, Inc., Santa Ana, CA).

*Represents the amount of flavonol glycosides. The aglycones of quercetin, kaempferol, and isorhamnetin were not present.