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Suppression of PXR Expression by Endoplasmic Reticulum Stress and Microrna

Thaveechai Vachirayonstien
University of Rhode Island, aikaik7@yahoo.com

SUPPRESSION OF PXR EXPRESSION BY

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THAVEECHAI VACHIRAYONSTIEN

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THAVEECHAI VACHIRAYONSTIEN

APPROVED:

Dissertation Committee

Major Professor Prof. Dr. Bingfang Yan

Assoc. Prof. Dr. Ruitang Deng

Prof. Dr. Joel M. Chandlee

Prof. Dr. Nasser H. Zawia

Dean, The Graduate School - URI

UNIVERSITY OF RHODE ISLAND

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ABSTRACT

The pregnane X receptor is a nuclear receptor, functioning as a ligand activated transcriptional factor. PXR is recognized as a major regulator of xenobiotic metabolism. Several important Phase I and Phase II enzymes, and drug transporters are regulated by PXR. PXR is also a master regulator of CYP3A4, which is one of the most important enzymes in drug metabolism. Thus, knowledge of regulation of PXR expression is crucial to understand alterations in drug metabolism. Many liver diseases such as steatosis and inflammatory liver diseases alter the expression of drug metabolizing enzymes and transporters. CYP3A4 is down-regulated in these diseases. Endoplasmic reticulum (ER) stress is recognized as one important cause of the diseases and it also accompanies with other diseases such as viral infections. ER stress is also interconnected with inflammation. CYP3A4 and some other P450 enzymes are also down-regulated in inflammation. Since PXR is the master regulator of CYP3A4, this dissertation investigated the effect of ER stress on the PXR expression and its consequence on PXR-mediated CYP3A4 induction. Thapsigargin and brefeldin A were used to induce ER stress in cell cultures. ER stress down-regulated the expression of PXR in primary hepatocytes and HepG2 cells by repressing transcription. A promoter study revealed that the HNF4α protein level decreased in ER stress whereas C/EBPβ LIP increased. HNF4α and C/EBPβ were bound to the promoter of PXR suggested that both HNF4α and C/EBPβ involved in down-regulation of PXR by ER stress. PXR-mediated CYP3A4 induction significantly decreased in ER stress. ER stress also induced the expression of IL-6 in primary hepatocytes. This finding established an interconnection between ER stress and

inflammation. Interestingly, IL-6 also repressed PXR expression at the same regulatory sequences as thapsigargin. Furthermore, it has been shown elsewhere that some other nuclear receptors are regulated by microRNAs (miRNAs). miRNAs regulate gene expression at the post-transcriptional level, adding complexity to the gene regulation. This dissertation identified a miRNA that regulated the expression of PXR. miR-30c-1 down-regulated PXR. miR-30c-1 interacted at the 3'-UTR of PXR and decreased the PXR mRNA level. The potential binding site of miR-30c-1 on the 3'-UTR of PXR was located.

In summary, ER stress, which is the cause and consequence of several diseases, down-regulated the expression of PXR. As a result, CYP3A4 induction by PXR was reduced. Since CYP3A4 and some others drug metabolizing enzymes regulated by PXR are crucial in drug metabolism, ER stress may alter metabolism of drugs, leading to increasing drug toxicity or decreasing drug efficacy. miR-30c-1 regulated the expression of PXR, adding complexity to the regulation on PXR. Although the regulation on the expression of miR-30c-1 is not known, miR-30c-1 might be related to diseases or biological conditions that could eventually affect PXR expression.

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PREFACE

This dissertation is writing in the manuscript format. Chapter 3 “Suppression of the pregnane X receptor during endoplasmic reticulum stress is achieved by down-regulting hepatocytes nuclear factor-4α and up-regulating liver-enriched inhibitory protein” is prepared for submission to the Journal of Biological Chemistry. Chapter 4 is prepared for submission.

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CHAPTER 1 INTRODUCTION

1 Pregnane X receptor

The pregnane X receptor (PXR or SXR) is the member of the nuclear receptor superfamily. PXR is categorized in subfamily 1, group I and member 2 (NR1I2). The human PXR gene is located on chromosome 3q12q13.3. The gene consists of ten exons, including exon 1a, 1b, 2, 3, 4, 5, 6, 7, 8, and 9. PXR is highly expressed in the liver, small intestine, and colon. It is also found in others tissues including, the lung, stomach, peripheral blood monocytes, blood-brain barrier, uterus, placenta, breast, osteoclasts, adrenal gland, ovary, heart, and specific regions of the brain (Bauer et al., 2004 and Lamba et al., 2004a, b).

Several decades ago, exogenous steroids were shown to modulate expression of cytochrome P450 enzymes (P450s) that protect human and animals against toxic substances. The expression of the P450 enzymes is induced by several xenobiotic steroids such as pregnenolone-16α-carbonitrile (PCN) and the enzymes metabolize both natural and synthetic compounds including herbal and synthetic drugs. PXR was discovered due to some evidence related to glucocorticoids, a class of steroid hormones, and the glucocorticoid receptor (GR). PXR was first identified as the nuclear receptor that induces CYP3A4 expression. Before the discovery of PXR, it was known that CYP3A and some other P450 enzymes were induced by the glucocorticoids such as dexamethasone. It was believed that this induction was mediated through GR. However, PCN which is an anti-glucocorticoid is shown to induce rat CYP3A (Schuetz and Guzelian, 1984). In addition, treatment with

dexamethasone and PCN shows a synergistic effect, inducing CYP3A in rat hepatocytes (Burger et al., 1992). This evidence suggested that there was another signaling pathway that induced CYP3A other than the glucocorticoid receptor pathway and led to the discovery of human PXR (Bertilsson et al., 1998, Kliewer et al., 1998, Lehmann et al., 1998).

2 Structure of PXR

PXR shares a common structure to the nuclear receptors (NR), containing a DNA binding domain (DBD), a ligand binding domain (LBD), and an activation function domain 2 (AF-2). The DBD of PXR is highly conserved in mammals, sharing more than 94% of their amino acid sequences (Fig. 1-1) (Kliewer et al., 2002). The DBD contains 2 zinc fingers, approximately 70 amino acids. The DBD of PXR can recognize and

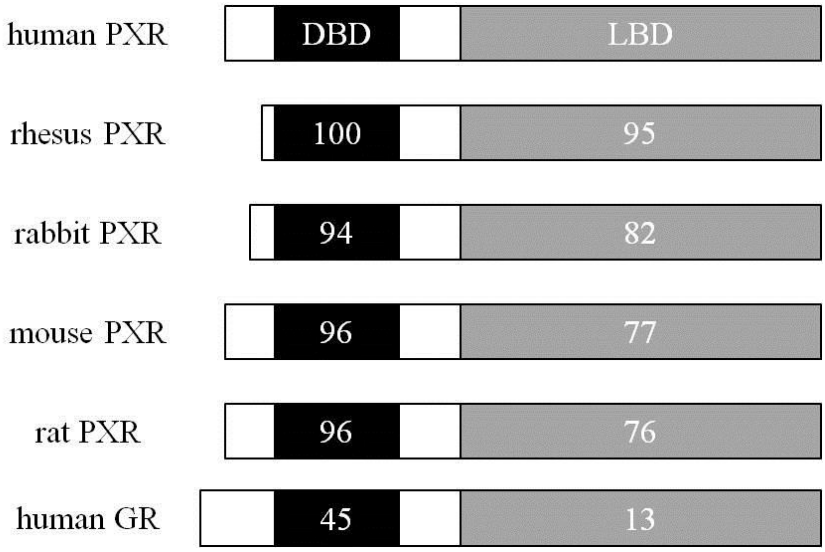

Fig. 1-1 Sequence comparison of PXR among species. The human glucocorticoid receptor is shown for comparison.

bind to a specific DNA sequence so called PXR responsive elements (XREMs). The LBD of PXR contains about 250 amino acids. In contrast to other nuclear receptors, the LBD of PXR is diverse across species. The LBD of human and mouse PXR shares

only 76% amino acid identity. The LBD can fold to form a hydrophobic pocket which can bind to a wide range of ligands. The AF-2 domain, located at the C-terminal, is responsible for the dimerization and transcriptional activation. Several isoforms of PXR have been identified. Some certain isoforms are generated due to alternative splicing and usage of an alternative start codon on exon 1a or 1b. hPXR (hPAR-1, SXR), the most abundant isoform of human PXR (about 93.04% of total PXR in the human livers), contains 434 amino acids. The hPXR transcript contains exon 1a but not 1b and uses the start codon in exon 2. Next, hPXR.2 is the most abundant alternative isoform of PXR (approximately 6.7% of total PXR in the human livers), lacking 111 nucleotides and deleting 37 amino acids from the PXR LBD (Lamba et al., 2004). Thus, hPXR.2 contains 397 amino acids. hPAR-2, which is created by alternative splicing using exon 1b but not 1a, uses initiation codon in exon 1b. hPAR-2 is 473 amino acids with an additional 177 nucleotides compared to hPXR (Bertilsson et al., 1998). In the fourth place, PXR.3 lacks 123 nucleotides, deleting 41 amino acids from the LBD compared to hPXR. As a result, hPXR.3 contains 393 amino acids.

3 Function of PXR

PXR functions as a ligand-activated transcriptional factor. It is found to heterodimerize with the retinoid X receptor-α (RXRα). The PXR/RXR heterodimer can recognize and bind to the PXR responsive element (XREM). XREM contains 2 half sites and spacer sequences on a promoter of a target gene. XREMs include direct repeat 3 (DR-3), direct repeat 4 (DR-4), everted repeat 6 (ER-6), and everted repeat 8 (ER-8) elements. The PXR/RXR heterodimer can also be found with or without promoter binding (Noble et al., 2006). Without a ligand, PXR is associated with transcriptional corepressors such as nuclear corepressor 1 (NCoR1) and NCoR2, resulting in repression of transcription (Ding et al., 2005 and Staudinger et al., 2011). PXR ligands such as rifampicin (RIF) can bind to the LBD of PXR. Following ligand binding, the AF-2 helix undergoes a conformational change leading to dissociation of corepressors and recruitment of coactivators such as steroid receptor coactiavator 1 (SRC-1). SRC-1 has intrinsic histone acetyltransferase activity, resulting in chromatin remodeling and transcriptional activation (Kliewer et al., 1998). The figure 1-2 shows schematic diagram of the mechanistic function of PXR.

PXR is bound and activated by various xenobiotics and endobiotics. 17-β-estradiol, progesterone, and progesterone are examples of endobiotics activating PXR (Blumberg et al., 1998). A number of genes in Phase I and II enzymes, and drug transporters have been identified as PXR target genes. These enzymes and transporters serve as defense mechanisms, transforming and eliminating xenobiotics form bodies. Therefore, PXR is recognized as a key regulator of xenobiotic metabolism. There are studies proving that PXR is a master regulator of CYP3A. PCN, which is known to

induce CYP3A in mice, does not induce CYP3A in mice lacking PXR (Xie et al., 2000). High induction of CYP3A is observed when transgenic mice expressing PXR are treated with rifampicin, proving that PXR regulated CYP3A expression. PXR is a master regulator of CYP3A which is one of the most important Phase I enzyme subfamily. The ensuing discussion will provide more details of the roles of PXR in drug metabolism.

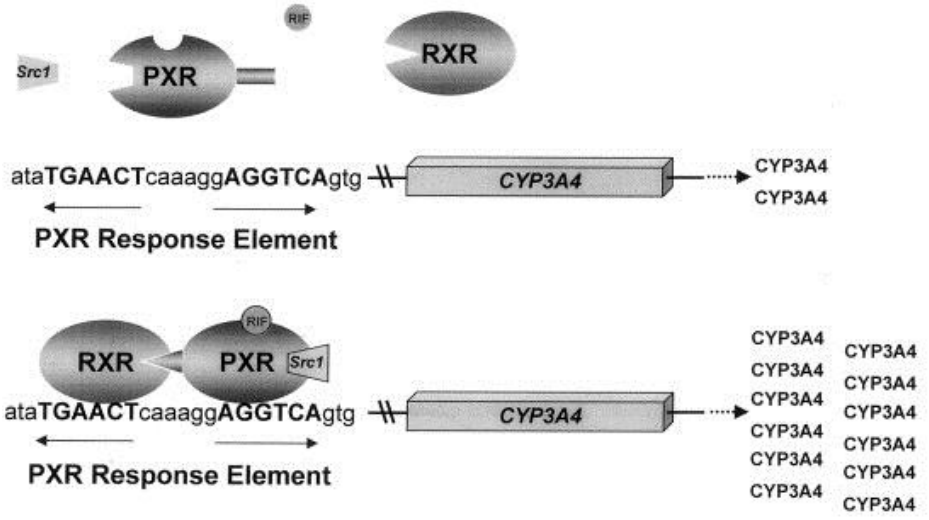

Fig. 1-2 A mechanistic diagram for PXR activity. The figure shows activation of PXR by rifampicin and subsequent transcriptional activation of the CYP3A4 gene (LeCluyse, 2001).

4 Ligand promiscuity and species differences of PXR

A number of compounds, including drugs, herbal medicines, environmental pollutants and endobiotics, have been identified to activate PXR. It has been shown that PXR ligands are structurally diverse (Fig. 1-3 and Table 1-1). Examples of PXR agonists are rifampicin, clotrimazole, phenobarbital, troglitazone, lithocholic acid, hyperforin, and paclitaxel (Kliewer et al., 2002). The ligand promiscuity of PXR is explained by characteristics of PXR binding pocket. The ligand binding pocket of PXR is larger than many other nuclear receptors (Kumar and Thompson, 1999 and Watkins et al., 2001). It has been estimated that the binding pocket volume ranges from 1200 Angstroms in the absence of ligands to about 1600 Angstorms in the presence of rifampicin (di Masi et al., 2009 and Moore et al., 2003). Thus, the binding pocket is large and also flexible. The species-specific activation of PXR by certain compounds has been demonstrated. For example, pregnenolone 16α-carbonitrile (PCN) is a strong PXR agonist of mouse and rat PXR but it does not activate human or rabbit PXR. Dexamethasone strongly activates mouse PXR and also moderately activates human PXR. In contrast, rifampicin and phenobarbital activate human PXR but show very little effect on mouse and rat PXR (Jones et al., 2000). In addition, transgenic mice possessing human PXR responds to rifampicin, resulting in high induction of CYP3A by rifampicin (Xie et al., 2000). This evidence proves that the species-specific induction of CYP3A by rifampicin is due to differences in the amino acid sequences of PXR. An amino acid mutation in the LBD of PXR can alter activation of PXR by rifampicin and PCN both in mice and human (Ostberg et al.,

2002). This study supports that the species-specific induction is due to the differences in the LBD.

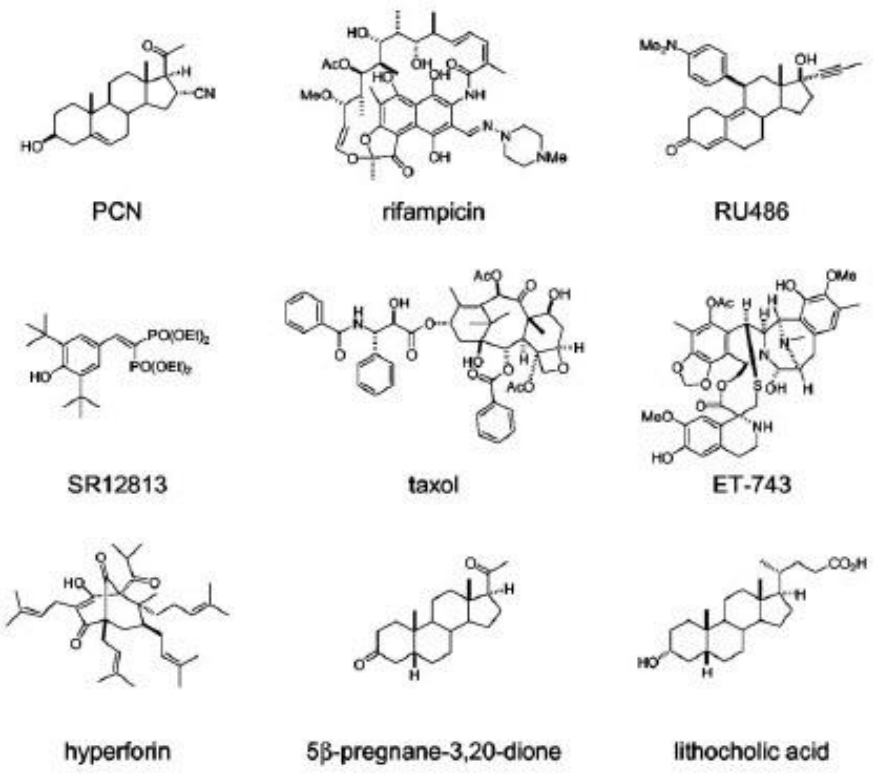

Fig. 1-3 PXR ligands. All compounds except ET-743 are PXR agonists. ET-743 is a PXR antagonist (Kliewer et al., 2002).

5 PXR in drug metabolism and detoxification

Human and animals have been always exposed to xenobiotics, including drugs, environmental pollutants, and naturally-occurring compounds. Our bodies have been evolved to employ various mechanisms to transform and detoxify these foreign compounds. The biotransformation process can be divided into two phases, phase I and II metabolism. Phase I metabolism involves oxidation, reduction, and hydrolysis reactions. The cytochrome P450 (CYP or P450) is the most important enzyme superfamily conducting phase I oxidation reactions. For examples, cytochrome P450 3A subfamily (CYP3A) is responsible for metabolizing approximately 50% of all clinically approved drugs (Guengerich, 2003 and Redinbo, 2004). CYP3A is also the most abundant P450 enzyme in the human liver and intestine (Paine et al., 2006). An important characteristic of CYP enzymes such as CYP2B and CYP3A is that they can be dramatically induced by xenobiotics. This induction is to response to transform and detoxify those foreign compounds. CYP3A are also involved in metabolism of highly toxic and narrow therapeutic window drugs and since many drugs are metabolized by CYP3A, drug interactions among there drugs are life-threatening. PXR is a key regulator of CYP3A expression (Bertilsson et al., 1998, Kliewer et al., 2002, and Lehmann et al., 1998). PXR can be activated by a wide range of structurally diverse compounds and then transactivate CYP3A gene, increasing CYP3A expression. CYP3A can metabolize a wide range of compounds which correlates with the promiscuity of PXR. In human, CYP3A4 is the most abundant CYP3A isoforms. PXR also regulates CYP2B6, CYP2C9, and CYP2C19. Therefore, PXR involves in

metabolism of a number of xenobiotics, including drugs toxic substances, and supplements. Examples of compounds that activate PXR and are shown in table 1.

Table 1-1 Drugs activating human PXR

Drug	**Therpeutic use**
Clotrimazole	Antimycotic
Dexamethasone	Anti-inflammatory
Lovastatin	Antihypercholesterolemic
Nifedipine	Antihypertensive
Paclitaxel	Anticancer
Phenobarbital	Anticonvulsant, sedative
Rifampicin	Antibiotic
Ritonavir	HIV-protease inhibitor
St. John's wort	Antidepressant
Spironolactone	Antihypertensive
Tamoxifen	Anticancer
Troglitazone	Antidiabetic
Warfarin	Anticoagulant

PXR also regulates important genes in phase II metabolism. Phase II enzymes generally increase hydrophilicity of xenobiotics by conducting conjugation reactions of hydrophilic groups to a compound. Substrates of Phase II enzymes can be either metabolites from Phase I enzymes or compounds which are not metabolized by Phase I enzymes. The substrates will be conjugated with charged species such as sulfate, glucuronic acid, and glutathione (GSH). This is to enhance their excretion into urine or bile and the products from Phase II reactions tend to be less active than the substrates. PXR has been shown to regulate some isoforms of sulfotransferase (SULT), UDP-glucoronosyltransferase (UGTA), and glutathione-S-transferase (GST) (Xu et al., 2005).

Xenobiotic biotransformation is also regulated by uptake and efflux transporters. Some certain drug transporters have been shown to be regulated by PXR. PXR is a major regulator of multidrug resistant protein-1 (MDR1, P- glycoprotein, P-gp) (Mills et al., 2004). PXR also control expression of other transporters such as resistance- associated protein 2 (MRP2), MRP3, MRP4, MRP5

Table 1-2 PXR target genes in humans involved in drug metabolism. CYP7A1 involves in bile acids synthesis.

Target gene	Effect on target gene
CYP1A1	Induction
CYP1A2	Induction
CYP1A6	Induction
CYP2B6	Induction
CYP2B10	Induction
CYP2C9	Induction
CYP2C19	Induction
CYP3A4	Induction
CYP3A11	Induction
CYP7A1	Repression
CYP11A1	Induction
CYP11B1	Induction
CYP11B2	Induction
Sult2A1	Induction
UGT1A1	Induction
UGT1A3	Induction
UGT1A4	Induction
MDR1	Induction
MRP2	Induction

and organic anion transporting polypeptide 2 (OATP2) (Jigorel et al., 2006 and Schrenk et al., 2001). This information, altogether, shows that PXR regulates a number of genes involved in drug adsorption, distribution, metabolism, and elimination (Fig. 1-4). These genes are controlled by PXR in response to xenobiotic

challenge so that our bodies can detoxify and eliminate potentially toxic compounds from the bodies. Since PXR regulates a number of genes involved in drug metabolism, what could happen if the PXR expression level is altered? The ensuing discussion will provide more details of certain conditions and factors that could alter PXR expression.

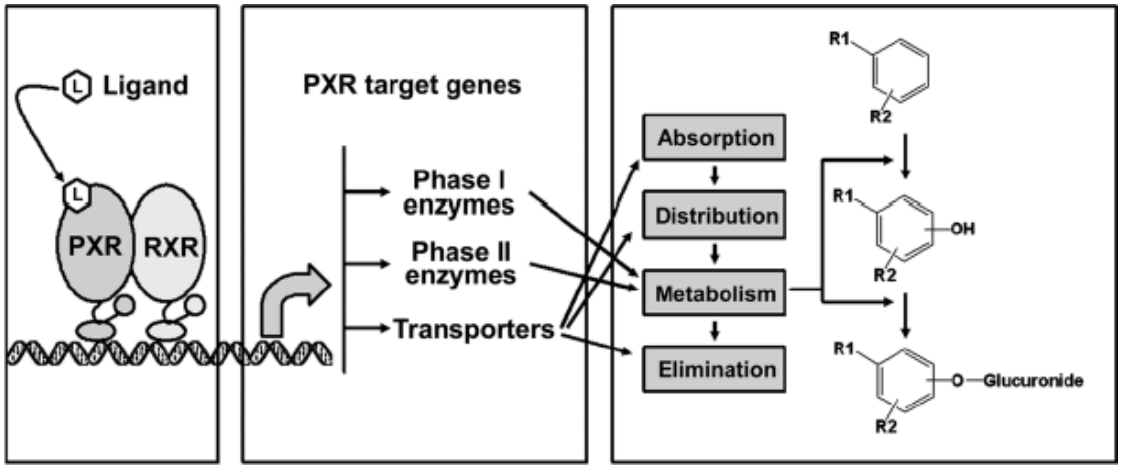

Fig. 1-4 Following ligand binding, PXR binds to XREM of the target genes resulting in transcriptional activation. The PXR target genes involve in drug adsorption, distribution, metabolism, and elimination (Ma et al., 2008).

6 PXR mediated drug-drug interactions

PXR functions as a xenobiotic sensor binding to its ligand and activating transcription of the target genes which result in increasing drug metabolizing enzymes that can metabolize the ligand and also other compounds. Drug interactions frequently occur when multiple drugs are administered such as anti-HIV and anti-tuberculosis. Discovery of PXR reveals molecular mechanism of how one drug increases clearance and decrease therapeutic efficacy of a co-administered drug. Thus, PXR directly involves in drug interactions and causes important clinical implication. There are several examples of PXR mediated drug-drug interactions. Rifampicin is an anti-tuberculosis drug that is usually used for several months in tuberculosis patients and it induces CYP3A4 expression. Midazolam is a short-acting sedative drug used as a premedication for sedation before minor surgical procedures, treatment of acute seizures, and insomnia. It has been shown that midazolam is not effective with patients treated with rifampicin. This is because midazolam is mainly metabolized by CYP3A and rifampicin induces CYP3A expression. Pretreatment with rifamycin, an anti-tuberculosis drug, or rifampicin can dramatically decrease therapeutic efficacy of anti-HIV protease inhibitors (i.e. indinavir and nelfinavir), resulting in loss of HIV suppression (Niemi et al., 2003).

PXR not only involves in drug-drug interaction but also herb-drug interactions. St John's wort is an herbal medicine that is widely used for depression, sleep disorders, and anxiety. St John's wort contains PXR ligands such as hyperforin and therefore induces CYP3A expression (Moore et al., 2000). St John's wort caused serious herb-drug interaction with cyclosporine, an immunosuppressant used in organ

transplant patients to decrease the risk of organ rejection. It has been reported that organ transplant failed in patients using St John's wort with cyclosporine. Since the herb induces CYP3A and cyclosporine is metabolized by CYP3A. As a result, cyclosporine blood levels decreased, so it was not effective (Murakami et al., 2006 and Mai et al., 2004).

7 PXR and inter-individual variability in drug response

CYP3A are major enzymes contributing to a large portion of metabolism of drugs and herbal medicines. CYP3A expression in the human liver and intestine varies as much as 40 folds (Lamba et al., 2002). It is predicted that 90% of the variation in CYP3A4 activity is due to genetic factors (Ozdemir et al., 2000). Genetic variation in the CYP3A4 gene has been extensively studied; however, the genetic variation in the CYP3A4 gene alone fails to explain the large variability of CYP3A4 expression (Lamba et al., 2002). Since PXR is the major transcriptional regulator of CYP3A4 it has been thought that the variability in the PXR expression and the PXR gene could contribute to the variability in the CYP3A4 expression. Some studies have shown that there is significant correlation between the PXR and CYP3A4 expression. In the human livers, the PXR mRNA level is associated with CYP3A4 mRNA (Lamba et al., 2010).

The expression of PXR has been associated to metabolism and elimination of certain drugs in human. Atazanavir, a HIV protease inhibitor, is metabolized by CYP3A4 and MDR1, which are the PXR target genes. Plasma clearance of atazanavir is associated with a PXR SNP, rs2472677 (Siccardi et al., 2008, Schipani et al., 2010). The study shows that patients with homozygous T allele exhibit higher atazanavir clearance than those with C allele. In addition, The SNP rs2472677 has previously been reported that it is also associated with PXR and CYP3A4 expression in the human livers (Lamba et al., 2008). Thus, the association between atazanavir clearance and the PXR SNP is likely due to expression of PXR and its target gene CYP3A4. A human study shows that a haplotype of the PXR gene is associated with doxorubicin

clearance (Sandanaraj et al., 2008). The study is conducted in Asian populations (Chinese, Malay and Indian) and the result shows that the haplotype is correlated with the expression of PXR and its downstream target genes CYP3A4 and MDR1. The haplotype is associated with reduced doxorubicin clearance. These studies suggest that PXR involves in inter-individual variability in drug response of certain drugs. The expression of PXR is related to the drug clearance.

8 Endoplasmic reticulum stress in diseases and drug metabolism

Endoplasmic reticulum (ER) is an organelle in eukaryotic cells that serve several functions, including protein synthesis, folding and trafficking, post-translational modification, calcium storage and regulation, and xenobiotic metabolism. Newly synthesized proteins translocate to the ER where they are folded into the functional structures. The ER also functions as the calcium storage which responds to stimuli that alter cellular energy level or redox status. Some certain conditions such as oxidative stress, viral infection, and inflammation can disrupt the ER function and cause stress in ER (Zhang and Kaufman, 2008). ER stress occurs when there is accumulation of unfolded or misfolded proteins in ER. Accumulation of these unfolded proteins can be caused by an increase of demand for protein folding or disruption of reactions of protein folding. In mammalian cells, the ER responds to stress by triggering unfolded protein response (UPR), which is signaling pathway aiming to relief the stress from the ER and to resume its function. Generally, the UPR will activate signaling pathways that lead to increasing expression of molecular chaperones involved in protein folding. UPR also increase protein degradation to remove unfolded or misfolded proteins. Protein translation is also reduced to decrease influx of new proteins into the ER (Xu et al., 2005). In mammalian cells, the UPR signaling pathways are initiated by ER protein double-stranded RNA-dependent protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring 1α (IRE1α). All three proteins are up-regulated during ER stress and have been used as indicators of stress in ER. Also, IRE1 is a ribonuclease that can splices X box-binding protein 1 (XBP1) mRNA. In ER stress, IRE1 splices XBP1

mRNA, producing spliced XBP1 mRNA that will be translated to XBP1 protein that induces UPR target genes such as chaperones. Thus, the presence of spliced XBP1 mRNA has also been used as an ER stress indicator. When the stress in the ER prolongs and cannot be relived, apoptosis is triggered. The UPR is an adaptive program responding to stimuli. The ER stress-induced apoptosis can be considered as a mechanism to protect tissues and organs by eliminating the stressed cells that produce malfunctioning proteins.

It is recognized that ER stress and UPR implicate in various diseases, including inflammatory diseases, metabolic diseases, neurodegenerative diseases, and cancer (Wang and Kaufman, 2012 and Yoshida, 2007). One reason is because ER stress and UPR can cause apoptosis and may damage functioning tissues. Secondly, the UPR alters expression of many genes. Thus, ER stress and UPR have been extensively studied in pathological aspects of several diseases. The liver is the major organ responsible for drug metabolism. Many diseases can impair liver functions such as decreasing capability of xenobiotic detoxification. ER stress has been associated with these diseases such as diabetes and steatosis (Kolwankar et al., 2007). However, studies of ER stress related to drug metabolism are lacking and there are only few studies about P450 enzymes. In rat hepatocytes, ER stress down-regulates CYP3A (Acharya et al., 2009). Nevertheless, effect of ER stress on many of Phase I and II enzymes and drug transporters has not been investigated. In addition, effect of ER stress on PXR and most of PXR target genes has not been reported.

There is evidence showing that there would be connections between ER stress and drug metabolism. Furthermore, several studies demonstrated that there is cross-

talk between UPR and inflammation (Adolph et al., 2012, Zhang and Kaufman, 2008). The link between UPR and inflammation involves NF-κB signaling pathway and inflammatory cytokines. The inflammatory cytokines such as interleukin-1 (IL-1β), interleukin-6 (IL-6), tumor necrosis factorα (TNFα) are pro-inflammatory cytokines that can lead to inflammation. It has been reported that thapsigargin, a natural compound which is widely used to induce ER stress in cell culture, induced IL-6 expression in rat peritoneal macrophages (Ichinowatari et al., 2002). The study showed the link between ER stress and inflammation and the inflammatory cytokine such as IL-6 in rat. In addition, the inflammatory cytokines are known to down-regulate P450 enzymes. Therefore, ER stress and UPR could affect P450 enzyme expression. Effect of inflammation on drug metabolism is explained in a following topic.

9 Effect of Inflammation on PXR and drug metabolism

Inflammation is a response to harmful stimuli such as infection by pathogens or tissue injuries from physical or chemical damages. Inflammation can occur in short term (acute inflammation) about a few days or long term (chronic inflammation) which can last for months or years. Inflammation at organs involving in drug metabolism such as the liver and small intestine can cause a large impact on drug metabolism. This is because the pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα alter expression of drug metabolizing enzymes. Both in humans and animals, inflammation causes changes in the expression levels of several P450 in the liver and small intestine as well as other organs such as the kidney and brain (Morgan, 1997, Renton, 2004). In most cases, the expression of P450 enzymes is suppressed due to inflammation. These decreases in the P450 enzymes significantly alter drug metabolism and can cause serious clinical consequences especially for drugs with a narrow therapeutic index. Therefore, inflammation can increases drug toxicities.

It is known that interleukin-6 (IL-6), a pro-inflammatory cytokine, down-regulates CYP3A4, 2B6, 2C9, and 2C19 expression in human hepatocytes (Aitken et al., 2007). PXR which regulates these P450 enzymes is also down-regulated by IL-6 in human hepatocytes (Pascussi et al., 2000). The information of inflammation together with ER stress from the previous topic suggests that ER stress may affect PXR expression and function.

10 microRNA

A 3'-untranslated region (3'-UTR) of mRNA is involved in several gene regulatory processes, including mRNA stability, transcript cleavage, translation, and mRNA localization. It is a binding site of many regulatory proteins and microRNA (miRNA) (Barrett et al., 2012). The length of the 3'-UTR in mammalian mRNA varies from 60 – 4000 nucleotides. The average length of the 3'-UTR in human genes is about 740 nucleotides which are longer than that in other mammalian and vertebrate genes (420 – 500 nucleotides) and in plants and fungi (240-274 nucleotides) (Pesole et al., 1997). The longer 3'-UTR in human suggests that human has evolved to possess more complexity of post-transcriptional control which is another important step to regulate gene expression.

miRNA is a short single strand RNA about 21-23 nucleotides. It is transcribed by RNA polymerase II (pol II) in the nucleus as long primary transcripts (pri-miRNA) (Kim, 2005). These transcripts can be several kilobases in length. miRNA is contained in pri-miRNA as a stem-loop hairpin structure about 60-80 nucleotides. This miRNA hairpin in pri-miRNA are recognized and excised by a microprocessor protein complex. The miRNA hairpin (pre-miRNA) is transported to the cytoplasm and further processed. The hairpin portion of pre-miRNA is excised by an RNase III enzyme dicer, producing a 21-23 nucleotide duplex. The miRNA duplex is incorporated into the RNA-induced silencing complex (RISC). One strand of the miRNA duplex is degraded, leaving the other strand in the RISC complex. miRNA in the RISC complex serves as a guide that directs RISC to target mRNAs. Binding of miRNA-RISC complex to mRNA results in blocking translation or cleaving of the

target mRNA (Bartel, 2004). Thus, miRNAs function as post-transcriptional regulators. There is growing evidence showing that many miRNAs can both repress translation and degrade mRNA (Lee et al., 2012). miRNA has been shown to target at the 3'-untranslated region (3'UTR) of mRNA. It has been estimated that human may encode more than 1000 miRNAs (Bartel, 2009). Approximately 70% of miRNAs are located in introns or exons and about 30% are located in intergenic regions (Rodriguez et al., 2004). At present, biological functions of most miRNAs are not known. The role of miRNAs is thought to be for fine-tuning of gene expression (Sevignani et al., 2006). In addition, it is predicted that a gene can be targeted by several miRNAs and binding sites of those miRNAs might overlap (John et al., 2004). miRNAs have been associated with several types of cancers, genetic diseases, and inflammatory diseases. miRNAs have been extensively studied; however, identifying and validating miRNA targets is still challenging. Many in silico algorithms have been developed to predict targets and binding sites of miRNAs but they still predict a large number of false positives (Da Costa Martins and De Windt, 2012). Certain approaches have been used to validate miRNA targets. For instance, a plasmid-based reporter system is used to identify targets and binding sites of miRNAs. Generally, A 3'-UTR of a gene of interest is fused downstream of a luciferase reporter open reading frame. In case that a miRNA can bind to the 3'-UTR of the gene, luciferase expression will be reduced compared to the control.

miRNAs have been shown to involve in regulation of various biological processes such as cell proliferation, tumor development, immune response, metabolism, and disease development. Several groups have demonstrated that

expression of some certain nuclear receptors and liver-enriched proteins are regulated by miRNAs. For example, GR receptor is regulated by miR-18 and miR-124a (Vreugdenhil et al., 2009). Estrogen receptor-α (ERα) is regulated by miR-206 (Adams et al., 2007), miR-221, miR-222 (Zhao et al., 2008), and miR-22 (Xiong et al., 2010). Hepatocyte nuclear factor 4α (HNF4α), an important transcriptional factor regulating several genes in the liver, is regulated by miR-24 and miR-34a (Takagi et al., 2010). PXR has been shown to be regulated by miR-148a (Takagi et al., 2008). miR-148a decreased PXR expression and attenuated PXR-mediated CYP3A4 induction *in vitro*. In fact, a number of miRNAs have been computationally predicted to bind to PXR mRNA (microRNA.org, 2013). Other nuclear receptors such as ERα and HNF4α are also regulated by more than one miRNA. Thus, PXR might be regulated by some other miRNAs other than miR-148a.

REFERENCES

Acharya P, Engel JC, Correia MA (2009) Hepatic CYP3A suppression by high concentrations of proteasomal inhibitors: a consequence of endoplasmic reticulum (ER) stress induction, activation of RNA-dependent protein kinase-like ER-bound eukaryotic initiation factor 2alpha (eIF2alpha)-kinase (PERK) and general control nonderepressible-2 eIF2alpha kinase (GCN2), and global translation shutoff. *Mol Pharmacol.* **76(3):**503-15.

Adams BD, Furneaux H, White BA (2007) The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-α (ERα) and represses ERα messenger RNA and protein expression in breast cancer cell lines. *Mol Endocrinol.* **21(5):**1132-47.

Adolph TE, Niederreiter L, Blumberg RS, Kaser A (2012) Endoplasmic reticulum stress and inflammation. *Dig Dis.* **30(4):**341-6.

Aitken AE, Morgan ET (2007) Gene-specific effects of inflammatory cytokines on cytochrome P450 2C, 2B6, and 3A4 mRNA levels in human hepatocytes. *Drug Metab Dispos.* **35(9):**1687-93.

Aouabdi S, Gibson G, Plant N (2006) Transcriptional regulation of the PXR gene: identification and characterization of a functional peroxisome proliferator-activated receptor alpha binding site within the proximal promoter of PXR. *Drug Metab Dispos.* **34(1):**138-44.

Barrett LW, Fletcher S, Wilton SD (2012) Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. *Cell Mol Life Sci.* **69(21):**3613-34.

Bartel DP. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* **116:**281-97.

Bartel DP. (2009) MicroRNAs: Target recognition and regulatory functions. *Cell.* **136:**215-233.

Bauer B, Hartz AM, Fricker G, Miller DS (2004) Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood brain barrier. *Mol. Pharmacol.* **66:**413-419.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci USA.* **95(21):**12208-13.

Blumberg B, Sabbagh W Jr, Juguilon H, Bolad J Jr, van Meter CM, Ong ES, Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **12(20):**3195-205.

Da Costa Martins PA, De Windt LJ (2012) Targeting microRNA targets. *Circ Res.* **111(5):**506-8.

di Masi A, De Marinis E, Ascenzi P, Marino M (2009) Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Mol Aspects Med.* **30(5):** 297-343.

Ding X, Staudinger JL (2005) Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. J Pharmacol Exp Ther. **312:**849-856.

Guengerich FP (2003) Cytochromes P450, drugs, and diseases. *Mol Interv.* **3(4):**194-204.

Ichinowatari G, Yamada M, Yaginuma H, Tsuyuki K, Tanimoto A, Ohuchi K (2002) Participation of prostaglandin E2 and platelet-activating factor in thapsigargin-induced production of interleukin-6. *Eur J Pharmacol.* **434(3):**187-96.

Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O (2006) Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug Metab Dispos.* **34(10):**1756-63.

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004) Human microRNA targets. *PLoS Biol.* **2(11):**e363.

Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinaol.* **14(1):**27-39.

Kim VN (2005). MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol.* **6:**376-85.

Kliewer SA, Goodwin B, Wilson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev*. **23(5):**687-702.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstorm RH, Perlmann T, Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell.* **92:**73-82.

Kolwankar D, Vuppalanchi R, Ethell B, Jones DR, Wrighton SA, Hall SD, Chalasani N (2007) Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin Gastroenterol Hepatol*. **5:**388-93.

Kumar R, Thompson EB (1999) The structure of the nuclear hormone receptors. *Steroids.* **64:**310-19.

Lamba JK, Lamba V, Yasuda K, Lin YS, Assem M, Thompson E, Strom S, Schuetz E (2004a) Expression of constitutive androstane receptor splice variants in human tissues and their functional consequences. *J Pharmacol Exp Ther.* **311:**811-821.

Lamba JK, Lin YS, Schuetz EG, Thummel KE (2002) Genetic contribution to variable human CYP3A-mediated metabolism. Adv Drug Deliv Rev. **54(10):**1271-94.

Lamba J, Lamba V, Strom S, Venkataramanan R, Schuetz E (2008) Novel single nucleotide polymorphisms in the promoter and intron 1 of human pregnane X

receptor/NR1I2 and their association with CYP3A4 expression. *Drug Metab Dispos.* **36(1):**169-81.

Lamba V, Panetta JC, Strom S, Schuetz EG (2010) Genetic predictors of interindividual variability in hepatic CYP3A4 expression. *J Pharmacol Exp Ther.* **332(3):**1088-99.

Lamba V, Yasuda K, Lamba JK, Assem M, Davila J, Strom S, Schuetz EG. PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators (2004b) *Toxicol Appl Pharmacol*. **199(3):**251-65.

LeCluyse EL (2001) Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chem Biol Interact.* **134(3):**283-9.

Lee D, Shin C (2012) MicroRNA-target interactions: new insights from genome-wide approaches. *Ann N Y Acad Sci.* **1271:**118-28.

Ma X, Idle JR, Gonzalez FJ (2008) The pregnane X receptor: from bench to bedside. *Expert Opin Drug Metab Toxicol.* **4(7):**895-908.

Mai I, Bauer S, Perloff ES, Johne A, Uehleke B, Frank B, Budde K, Roots I (2004) Hyperforin content determines the magnitude of the St John's wort-cyclosporine drug interaction. *Clin Pharmacol Ther.* **76(4):**330-40.

Mills JB, Rose KA, Sadagopan N, Sahi J, de Morais SM (2004) Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. *J Pharmacol Exp Ther.* **309(1):**303-9.

Moore JT, Moore LB, Maglich JM, Kliewer SA (2003) Functional and structural comparison of PXR and CAR. *Biochim Biophys Acta.* **1619:**235-8.

Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, Kliewer SA (2000) St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci USA.* **97(13):**7500-2.

Morgan ET (1997) Regulation of cytochromes P450 during inflammation and infection. *Drug Metab Rev.* **29:**1129-1188.

Murakami Y, Tanaka T, Murakami H, Tsujimoto M, Ohtani H, Sawada Y (2006) Pharmacokinetic modeling of the interaction between St John's wort and ciclosporin A. *Br J Clin Pharmacol.* **61(6):**671-6.

Niemi M, Backman JT, Fromm MF, Neuvonen PJ, Kivisto KT (2003) Pharmacokinetic interactions with rifampicin : clinical relevance. *Clin Pharmacokinet.* **42(9):**819-50.

Noble SM, Carnahan VE, Moore LB, Luntz T, Wang H, Ittoop OR, Stimmel JB, Davis-Searles PR, Watkins RE, Wisely GB, LeCluyse E, Tripathy A, McDonnell DP, Redinbo MR (2006) Human PXR forms a tryptophan zipper-mediated homodimer. Biochemistry. **45(28):**8579-89.

Ostberg T, Bertilsson G, Jendeberg L, Berkenstam A, Uppenberg J (2002) Identification of residues in the PXR ligand binding domain critical for species specific and constitutive activation. *Eur J Biochem.* **269(19):**4896-904.

Ozdemir V, Kalow W, Tang BK, Paterson AD, Walker SE, Endrenyi L, Kashuba AD (2000) Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method. *Pharmacogenetics.* **10(5):**373-88.

Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC (2006) The human intestinal cytochrome P450 pie. *Drug Metab Dispos.* **34(5):**880-6.

Pan YZ, Gao W, Yu AM (2009) MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab Dispos.* **37:**2112-117.

Pascussi JM, Gerbal-Chaloin S, Pichard-Garcia L, Daujat M, Fabre JM, Maurel P, Vilarem MJ (2000) Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. *Biochem Biophys Res Commun.* **274(3):**707-13.

Pesole G, Liuni S, Grillo G, Saccone C (1997) Structural and compositional features of untranslated regions of eukaryotic mRNAs. *Gene.* **205(1-2):**95-102.

Redinbo MR (2004) Promiscuity: what protects us, perplexes us. *Drug Discovery Today.* **9:**431-32.

Renton KW (2004) Cytochrome P450 regulation and drug biotransformation during inflammation and infection. *Curr Drug Metab.* **5(3):**235.

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004) Identification of mammalian microRNA host genes and transcription units. *Genome Res.* **14(10A):**1902-10.

Sandanaraj E, Lal S, Selvarajan V, Ooi LL, Wong ZW, Wong NS, Ang PC, Lee EJ, Chowbay B (2008) PXR pharmacogenetics: association of haplotypes with hepatic CYP3A4 and ABCB1 messenger RNA expression and doxorubicin clearance in Asian breast cancer patients. *Clin Cancer Res.* **14(21):**7116-26.

Schipani A, Siccardi M, D'Avolio A, Baietto L, Simiele M, Bonora S, Rodriguez Novoa S, Cuenca L, Soriano V, Chierakul N, Saguenwong N, Chuchuttaworn C, Hoskins JM, Dvorak AM, McLeod HL, Davies G, Khoo S, Back DJ, Di Perri G, Owen A (2010) Population pharmacokinetic modeling of the association between 63396C->T pregnane X receptor polymorphism and unboosted atazanavir clearance. *Antimicrob Agents Chemother.* **54(12):**5242-50.

Schrenk D, Baus PR, Ermel N, Klein C, Vorderstemann B, Kauffmann HM (2001) Up-regulation of transporters of the MRP family by drugs and toxins. *Toxicol Lett.* **120(1-3):**51-7.

Sevignani C, Calin GA, Siracusa LD, Croce CM (2006) Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm Genome.* **17(3):**189-202.

Siccardi M, D'Avolio A, Baietto L, Gibbons S, Sciandra M, Colucci D, Bonora S, Khoo S, Back DJ, Di Perri G, Owen A. (2008) Association of a single-nucleotide polymorphism in the pregnane X receptor (PXR 66396C→T) with reduced concentrations of unboosted atazanavir. *Clin infect Dis.* **47(9):**1222-5.

Staudinger JL, Xu C, Biswas A, Mani S (2011) Post-translational modification of pregnane X receptor. *Pharmacol Res*. **64(1):**4-10.

Takagi S, Nakajima M, Kida K, Yamamura Y, Fukami T, Yokoi T (2010) MicroRNAs regulate human hepatocytes nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle. *J Biol Chem.* **285(7):**4415-22.

Takagi S, Nakajjima M, Mohri T, Yokoi T (2008) Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem*. **283:**9674-80.

Vreugdenhil E, Verissimo CS, Mariman R, Kamphorst JT, Barbosa JS, Zweers T (2009) MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: implications for glucocorticoid responsiveness in the brain. *Endocrinology.* **150(5):**2220-8.

Wang S, Kaufman RJ (2012) The impact of the unfolded protein response on human disease. *J cell Biol.* **197(7):**857-67.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA, Redinbo MR (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Sciences.* **292(5525):**2329-33.

Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, Evans RM (2000) Humanzied xenobiotic response in mice expressing nuclear receptor SXR. *Nature.* **406(6794):**435-9.

Xiong J, Yu D, Wei N, Fu H, Cai T, Huang Y, Wu C, Zheng X, Du Q, Lin D, Liang Z (2010) An estrogen receptor alpha suppressor, microRNA-22, is downregulated in estrogen receptor alpha-positive human breast cancer cell lines and clinical samples. *FEBS J.* **277(7):**1684-94.

Xu C, Bailly-Maitre B, Reed JC (2005) Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest.* **115(10):**2656-64.

Xu C, Li CY, Kong AN (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res.* **28(3):**249-68.

Yoshida H (2007) ER stress and diseases. *FEBS J.* **274(3):**630-58.

Zhang K, Kaufman RJ (2008) From endoplasmic-reticulum stress to the inflammatory response. *Nature.* **454(7203):**455-62.

Zhao JJ, Lin J, Yang H, Kong W, He L, Ma X, Coppola D, Cheng JQ (2008) MicroRNA-221/222 negatively regulates estrogen receptor alpha and is associated with tamoxifen resistance in breast cancer. *J Biol Chem.* **283(45):**31079-86.

CHAPTER 2 STATEMENT OF PURPOSE

It has been shown in human that certain liver diseases involving inflammation such as viral hepatitis and steatosis decrease the liver's capability to metabolize a number of drugs. CYP3A4 expression is also decreased in these diseases. PXR is recognized as a xenobiotic sensor, binding to a wide range of compounds and functioning as a ligand-activated transcriptional factor. PXR regulates many important genes in drug metabolism, including drug metabolizing enzymes such as CYP3A and drug transporters such as MDR1. CYP3A4 is a major isoform of the CYP3A subfamily responsible for metabolizing about 50% of marketed drugs. CYP3A4 is known to possess high inter-individual variability in expression due to several contributing factors such as genetic and disease related factors. Variation in CYP3A4 expression has been shown to affect therapeutic efficacy and adverse reaction of drugs. CYP3A4 expression and activity in humans is significantly associated with PXR expression. Therefore, alteration in PXR expression has been accepted as a cause of interindividual variability in CYP3A (Sandanaraj et al., 2008 and Urguhart et al., 2007). Nevertheless, factors causing changes in PXR expression have not been clearly understood. This dissertation studies certain factors altering the PXR expression which may lead to variability in CYP3A expression.

The goal of Chapter 3 of this dissertation is to study effect of ER stress on PXR expression and function. ER stress has been linked to chronic liver diseases such as steatosis and type 2 diabetes. These liver diseases have been shown to alter capacity of drug elimination. CYP3A4 is an example of drug metabolizing enzymes that is known to decrease in the chronic liver diseases. However, the effect of ER stress on

PXR, which is the major regulator of CYP3A4, has not been investigated. A mechanism of how ER stress down-regulates CYP3A4 expression has not been elucidated. Thus, Chapter 3 will study the effect of ER stress on expression of PXR and its consequence on induction of CYP3A4. The study will also investigate the mechanism of how ER stress impacts expression of PXR. The effect of ER stress on PXR at the transcriptional level will be studied. Since PXR functions as the ligand-dependent transcriptional factor binding to its ligand and transactivating PXR target genes, the impact of ER stress on PXR function will be determined by monitoring the transcriptional level of CYP3A4.

The aim of Chapter 4 of this dissertation is to identify a miRNA that regulates expression of PXR. miRNA adds complexity of how a gene is regulated by acting on the mRNA post-transcriptionally. miRNA have been recognized to cause interindividual variation in a gene expression (Lu et al., 2012). miRNA is also involved in inflammatory diseases such as inflammatory bowel disease and liver cancer (Ranjha et al., 2013). Other nuclear receptors such as ERα and HNF4α and some genes involving in drug metabolism are regulated by miRNA. Certain P450 enzymes have been showed to be regulated by miRNA (Nakajima et al., 2011). CYP3A4 is regulated by miR-27b and miR-378 regulates CYP2E1 (Pan et al., 2009 and Mohri et al., 2010) miR-148a has been identified to target at the PXR mRNA (Takagi et al., 2008). It is predicted that a gene can be targeted by several miRNAs (John et al., 2005). Identifying a miRNA that regulates PXR will further improve knowledge of how PXR is regulated. A target site of miRNA on PXR will be identified and the effect of miRNA on PXR expression will be studied.

Since ER stress is related to the diseases and could have an impact on drug metabolism, studying the effect of ER stress on PXR, the master regulator of xenobiotic metabolism, could help understand the effect of ER stress on PXR and drug metabolism. The study may reveal the mechanism of regulation of PXR. Identifying miRNA regulating PXR could provide a better understanding of the regulation on PXR. These studies may provide a better understanding in alteration in PXR expression and its effect on drug metabolism.

REFERENCES

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004) Human MicroRNA targets. *PLoS Biol.* **2(11):**e363.

Lu J, Clark AG (2012) Impact of microRNA regulation on variation in human gene expression. *Genome Res.* **22(7):**1243-54.

Mohri T, Nakajima M, Fukami T, Takamiya M, Aoki Y, Yokoi T (2010) Human CYP2E1 is regulated by miR-378. *Biochem Pharmacol.* **79(7):**1045-1052.

Nakajima M, Yokoi T (2011) MicroRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. *Pharmacol Ther.* **131(3):**330-7.

Pan YZ, Gao W, Yu AM (2009) MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab Dispos.* **37:**2112-117.

Ranjha R, Paul J (2013) Micro-RNAs in inflammatory diseases and as a link between inflammation and cancer. *Inflamm Res.* Feb 17 [Epub ahead of print].

Sandanaraj E, Lal S, Selvarajan V, Ooi LL, Wong ZW, Wong NS, Ang PC, Lee EJ, Chowbay B (2008) PXR pharmacogenetics: association of haplotypes with hepatic CYP3A4 and ABCB1 messenger RNA expression and doxorubicin clearance in Asian breast cancer patients. *Clin Cancer Res.* **14(21):**7116-26.

Takagi S, Nakajjima M, Mohri T, Yokoi T (2008) Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem*. **283:**9674-80.

Urguhart BL, Tirona RG, Kim RB (2007) Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *J Clin Pharmacol.* **47(5):**566-78.

CHAPTER 3

MANUSCRIPT 1: formatted for the journal of biological chemistry

SUPPRESSION OF THE PREGNANE X RECEPTOR DURING ENDOPLASMIC RETICULUM STRESS IS ACHIEVED BY DOWN-REGULATING HEPATOCYTE NUCLEAR FACTOR-4α AND UP-REGULATING LIVER-ENRICHED INHIBITORY PROTEIN

Abbreviations: BFA, Brefeldin A; C/EBPβ, cytidine-cytidine-adenosine-adenosine-thymidine enhanced binding protein-β; CYP3A4, cytochrome P450 3A4; DMEM, Dulbecco's modified eagle medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNF-4α, Hepatocyte nuclear factor-4α; LAP, Liver-enriched activator protein; LIP, Liver-enriched inhibitory protein; PXR, pregnane X receptor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Thaps, thapsigargin; XBP1, X-box binding protein 1 gene.

ABSTRACT

ER-stress is recognized as a common theme in the development of metabolic syndrome and other diseases. Chronic liver diseases develop ER-stress and also show decreased capacity of drug metabolism. The pregnane X receptor (PXR) is a master regulator of genes involved in drug elimination. This study was performed to determine whether ER-stress condition decreases the expression of PXR and whether the decrease alters the induction of cytochrome P450 3A4 (CYP3A4). Primary hepatocytes and HepG2 cell line (human hepatocellular carcinoma) were treated with brefeldin A and thapsigargin, two well-established ER-stressors. Without exception, both stressors significantly decreased the expression of PXR. The decrease led to reduced induction of CYP3A4. Reporter dissection, electrophoretic mobility shift assay and chromatin immunoprecipitation located in the PXR promoter region two adjacent elements recognized by hepatocyte nuclear factor-4α (HNF-4α) and cytidine-cytidine-adenosine-adenosine-thymidine enhanced binding proteins (C/EBP proteins), respectively. Over-expression of HNF-4α or liver-enriched activator protein (an activator of C/EBPβ) restored the expression of PXR. Interestingly, the very same sequence also responded to interleukin-6 (IL-6), and primary hepatocytes treated with thapsigargin significantly increased the level of IL-6 mRNA. These findings establish a functional interconnection between ER-stress and signaling of proinflammatory cytokines in the regulated expression of PXR.

1. INTRODUCTION

The endoplasmic reticulum (ER) is an organelle involved in diverse cellular functions including protein synthesis/transportation, membrane generation, calcium concentration regulation and xenobiotic metabolism (Johnson et al., 2012; Li et al., 2012). Therefore, ER homeostasis is critical in maintaining the overall cellular functions. On the other hand, many factors such as oxidative stress disrupt ER homeostasis, leading to ER stress (Cali et al., 2011; Adolph et al., 2012; Xu and Zhu, 2012). While the precise mechanisms of ER-stress remain to be determined, one of the outcomes is the accumulation of unfolded proteins in the ER (Benbrook and Long, 2012; Haeri and Knox, 2012). The unfolded protein response (UPR), occurring at the initial stage of ER-stress, is triggered to slow down protein synthesis, improve protein folding capacity and enhance degradation of unfolded proteins. Nevertheless, persistent ER-stress has been linked to the development of various conditions such as type 2 diabetes and chronic liver diseases (Back and Kaufman, 2012; Flamment et al., 2012; He and Chen, 2012; Pagliassotti, 2012).

The liver is the largest internal organ and plays the primary role in drug metabolism (Santoro et al., 2007; Bock and Bock-Hennig, 2010; Villarroya et al., 2010). The prevalence of hepatic dysfunction is high and it affects more than 10% of Americans (Liver foundation, 2009). Worldwide, liver cancer and chronic liver diseases are the seventh leading cause of death (IPA, 2007). Many liver diseases are accompanied with ER-stress and exhibit decreased capacity of drug metabolism and detoxification. Steatotic livers, for example, were found to have significant decreases in CYP3A

activity (Kolwankar et al., 2007), the most robust catalytic system in the oxidative metabolism. In cultured primary hepatocytes, lipid-loading significantly decreased the expression of CYP3A4 (Donato et al., 2006). Furthermore, livers from diabetic patients showed significantly lower expression of CYP3A4 (Dostalek et al., 2011).

The expression of CYP3A4 is regulated by several major transcription factors. Among these proteins, the pregnane X receptor (PXR) has been established to play the primary role (Ihunnah et al., 2011). This receptor forms a heterodimer with the retinoid X receptor-α and binds to PXR response elements that contain a half-site AG(G/T)TCA or related sequence. We and other investigators have functionally characterized four PXR elements in the CYP3A4 gene and some of the elements operate in a coordinate manner (Goodwin et al., 1999; Song et al., 2004; Liu et al, 2008; Toriyabe et al., 2009). While PXR regulates the expression of CYP3A4 and many other drug-eliminating genes, the expression of PXR varies by drugs and disease mediators. Importantly, the expression of PXR directly affects CYP3A induction. Dexamethasone, a synthetic glucocorticoid, induces PXR and synergistically induces CYP3A (Shi et al., 2010). Likewise, clofibrate, the lipid-lowering agent, causes super-induction of CYP3A23 (Ma et al., 2005). Conversely, interleukin-6 (IL-6), a proinflammatory cytokine, decreases PXR expression and reduce CYP3A4 induction (Yang et al., 2010).

This study was performed to determine whether ER-stress condition decreases the expression of PXR and whether the decrease alters the induction of CYP3A4. Brefeldin A (BFA) and thapsigargin (Thaps), two well-established ER-stressors, significantly decreased the expression of PXR in both primary hepatocytes and HepG2 cell line (human hepatocellular carcinoma). The decrease led to reduced induction of CYP3A4. The decreased expression of PXR was achieved by transcriptional repression via two adjacent elements recognized by hepatocyte nuclear factor-4α (HNF-4α) and cytidine-cytidine-adenosine-adenosine-thymidine enhanced binding proteins (C/EBP proteins), respectively. Over-expression of either protein restored the expression of PXR. Interestingly, the adjacent elements also responded to interleukin-6 (IL-6), suggesting a functional interconnection between ER-stress and signaling of proinflammatory cytokines.

2. MATERIALS AND METHODS

2.1. Chemicals and supplies

IL-6 and Thaps were from R&D Systems (Minneapolis, MN). BFA, Hanks balanced salt solution and the antibody against glyceradehyde-3-phosphate dehydrogenase (GAPDH) were from Sigma (St. Louis, MO). Dulbecco's modified eagle medium (DMEM) and high fidelity Platinum *Taq* DNA polymerase were from Life Technology (Carlsbad, CA). The antibodies against HNF4α or C/EBPβ were from Abcam Inc (Cambridge, MA). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce (Rockford, IL). Plated human primary hepatocytes were obtained from the Liver Tissues Procurement and Distribution System (University of Minnesota) or CellzDirect (Pittsboro, NC). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). Expression constructs were from OriGene Technologies Inc (Rockville, MD). Unless otherwise specified, all other reagents were purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA (1 μg) was subjected to the synthesis of the first strand cDNA as described previously (Xiao et al., 2012). cDNAs were then diluted 8 times and RT-qPCR was conducted with *TaqMan* Gene Expression Assay (Applied Biosystems, Foster City, CA). The *TaqMan* probes were: PXR, Hs00243666_m1; HNF4α, Hs00230853_m1; C/EBPβ, Hs00942496_s1; IL-6, Hs00985639_m1; and GAPDH, 4352934E; and RNA polymerase II, Hs00172187_m1. The normalization of RT-qPCR was performed

primarily based on the signal of GAPDH mRNA and selective samples were analyzed for the level of RNA polymerase II to confirm the normalization. The PCR amplification was conducted in a total volume of 20 μl containing universal PCR master mixture (10 μl), gene-specific *TaqMan* assay mixture (1 μl), and cDNA template (6 μl). The mRNA levels were normalized according to the level of GAPDH and the normalization of selected samples was confirmed based on the signal of RNA polymerase II. Amplification and quantification were done with the Applied Biosystems 7500 Real-Time PCR System.

2.3. Reporter constructs and cotransfection assays

PXR promoter reporters were prepared to contain various lengths of PXR genomic sequence. All promoter reporters were subcloned from the PXR-1286-Luc reporter through Mlu I and BamH I sites. All cloning and subcloning experiments were performed by PCR with high fidelity Platinum *Taq* DNA polymerase. To prepare reporters with a disruption of the element HNF4α, C/EBPβ, or both, oligonucleotides with the wild type or mutant sequences were synthesized and annealed. The resultant double-stranded oligonucleotides were ligated to the pGL3 promoter vector through Nhe I and Xho I sites. The sequences of oligonucleotides for the reporters are shown in Table I. All reporter constructs were subjected to sequence analysis. To determine the reporter activities, cotransfection in HepG2 cells was performed. Transfection mixtures contained 100 ng of a reporter plasmid and 5 ng of null-*Renilla* luciferase plasmid. In some cases, an expression construct was used including HNF4α and C/EBPβ in the transfection mixtures. The corresponding vector was used to equalize

the total amount of plasmid DNA in transfection. Typically, cells were transfected for 24 h and the medium was replaced with fresh medium supplemented with 1% FBS. The treatment lasted for 24 h and the cells were washed once with phosphate buffered saline and collected by scraping. The reporter enzyme activities were assayed with a Dual-Luciferase Reporter Assay System as described previously (Yang et al., 2011).

2.4. Electrophoretic mobility shift assay (EMSA)

The EMSA experiment was performed as described previously (Yang et al., 2012). Nuclear extracts of HepG2 cells treated with Thaps (50 nM) for 24 h were prepared with the nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). The sense and antisense oligonucleotides (Table I) were annealed by heating at 94°C for 5 min followed by gradually cooling to room temperature. The sense strand was synthesized as labeled or non-labeled form (for competition). Nuclear protein (5 μg) was incubated with a double-stranded biotinylated probe (0.1 pmol) at room temperature for 20 min. In competition assays, nuclear extracts were first incubated with an unlabeled probe at a 25x or 100x excess for 20 min before addition of the labeled probe. For antibody-disruption assay, the nuclear extracts were first incubated with an antibody against HNF4α or C/EBPβ on ice for 20 min and then with the labeled probe. The protein-DNA complexes were resolved by non-denaturing polyacrylamide gel electrophoresis (5%) and transferred onto a Biodyne® nylon membrane. The biotinylated probe was detected with streptavidin-conjugated horseradish peroxidase and chemiluminescent substrate (PIERCE, Rockford, IL). The chemiluminescent signal was captured by KODAK Image Station 2000, and the relative intensities were

quantified by KODAK 1D Image Analysis Software (KODAK Molecular Imaging Software, Version 4.0, Rochester, NY).

2.5. Chromatin immunoprecipitation (ChIP)

ChIP experiment was performed, essentially as described previously (Chen et al., 2012). HepG2 Cells were treated with DMSO or Thaps (50 nM) for 24 h, washed and underwent cross-linking for 15 min by 1.0% formaldehyde at room temperature, and the cross-linking was terminated with glycine (final concentration of 125 mM). The soluble chromatin preparations were prepared as described previously (Chen et al., 2012). For the ChIP experiment, chromatin preparations were pre-cleared for 2 h at 4°C with protein G beads pre-treated with herring sperm DNA (0.2 mg/ml) and BSA (0.5 mg/ml). A fraction of the pre-cleared chromatins was stored at -80°C for later use as an input. An antibody against HNF4α or C/EBPβ was added into the pre-cleared chromatins, and an overnight incubation at 4°C was performed. As a negative control, incubation was performed with pre-immune IgG. The antibody-bound chromatin and DNA input were analyzed by PCR for the presence of the genomic fragments containing the HNF4α or C/EBPβ-bound element with primers shown in Table I. The PCR was performed with Platinum *Taq* DNA polymerase for a total of 32 cycles at 94°C for 30 s, 58°C for 30 s and 68°C for 60 s. A 3-min initial denaturation was performed.

2.6. Other analyses

Protein concentrations were determined with BCA assay (Pierce) based on albumin standard. Western blotting was performed as described previously (Shi et al., 2011) and the preparation of antibodies were described elsewhere (Sachdeva et al., 2003). Data are presented as mean ± SD of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Statistical significance between two means was made according to One-way ANOVA followed by a DUNCAN's multiple comparison test ($p < 0.05$).

3. RESULTS

3.1. Down-regulation of PXR by ER-stressors

ER-stress is a phenomenon in various chronic diseases and many PXR-target genes are down-regulated by disease conditions associated with ER-stress (Cali et al., 2011; Johnson et al., 2012). To test whether the expression of PXR itself is decreased during ER-stress, HepG2 cells were treated with Thaps and BFA. Thaps induces ER-stress by depleting calcium in the ER (Salido et al., 2009), whereas BFA by retrograde-transporting proteins from the Golgi apparatus to the ER (Nickel, 2010). As shown in Fig. 1A, treatment with either stressor significantly decreased the level of PXR mRNA. BFA was slightly more potent than Thaps (Fig. 1A). Next, we determined the decrease of PXR mRNA as a function of the amount of Thaps. As shown in Fig. 1B, Thaps at 1 nM caused a 20% decrease of PXR mRNA and at 10 nM caused a 40% decrease. Further increased concentrations of Thaps up to 250 nM caused only a 10% additional decrease (Fig. B). To ascertain the cellular ER-stress level, semi-quantitative PCR was performed to detect the presence of spliced XBP1 mRNA (X-box binding protein 1 gene), a widely used marker for ER-stress (Ri et al., 2012). As shown in Fig. 1B (bottom), little spliced XBP1 mRNA was detected in cells treated with solvent or 1 nM Thaps. Comparable levels of spliced and non-spliced XBP1 mRNA were detected in cells treated at 10 nM (Fig. 1B). In contrast, cells treated at 25 or 250 nM exhibited the presence of spliced XBP1 mRNA only. To gain in vivo relevance, primary hepatocytes were treated with Thaps, and the expression of PXR was determined. As shown in Fig. 1C, Thaps decreased PXR at both mRNA and

protein levels, and the decrease was even greater than that in HepG2 cells (Figs. 1A and 1B).

3.2. Transcriptional repression of PXR by Thaps

The decreases in PXR mRNA pointed to two possibilities: ER-stressors enhanced PXR mRNA degradation and/or reduced PXR transactivation. To shed light on the second possibility, various PXR reporters containing the promoter or along with up-stream regulatory sequences at varying length were tested for the repression in response to Thaps. As shown in Fig. 2A, all PXR reporters, compared with the vector control, were significantly repressed. However, the reporter PXR-56Luc, compared with PXR-106Luc, was repressed to a significantly less extent, suggesting that the DNA segment from -106 to -56 nucleotides is critical for the repression. Based on element predication with computer program, this DNA segment contains an HNF4α and a C/EBP binding site. These two elements are spaced by three nucleotides (Fig. 2A). It should be noted that as many as five transcription start sites (filled triangles in Fig. 2A) are located in the PXR promoter region (Zhang et al., 2001; Kurose et al., 2005; Tompkins et al., 2008).

To test whether these two elements support Thaps-repression, element reporters were prepared to contain this segment or segment with one or both elements disrupted. The resultant reporters were tested for the abolished response to Thaps. As shown in Fig. 2B, disruption of the HNF4α element largely abolished the repressive response to

Thaps. In contrast, disruption of the C/EBP element reversed Thaps-repression by 20%. These results suggested that both HNF4α and C/EBP supported the transactivation of PXR with HNF4α playing a greater role for the repression. To shed light on this possibility, cells were transfected with HNF4α or LAP (liver-enriched activator protein: a form of C/EBPβ), treated with Thaps, and detected for the level of PXR mRNA. As shown in Fig. 2C, transfection of HNF4α or LAP reversed the suppression of PXR mRNA in response to Thaps. Interestingly, LAP showed a greater reversal activity than HNF4α.

3.3. Effect of Thaps on the expression of HNF4α and C/EBP (LAP and LIP)

The transfection study demonstrated that HNF4α and LAP were positive regulators of PXR expression. Next we tested whether Thaps decreased PXR expression by down-regulating HNF4α and C/EBPβ. Cells were treated with Thaps and the expression of HNF4α and C/EBPβ were determined by RT-qPCR and Western blotting. As shown in Fig. 3A, Thaps surprisingly caused a 9-11 fold induction of C/EBPβ mRNA. It has been well established that C/EBPβ mRNA produces several in-frame polypeptides including LAP* (38 kDa), LAP (35 kDa) and LIP (liver-enriched inhibitory protein: 20 kDa) (Tsukada et al., 2011). Importantly, these polypeptides are functionally distinct with LAP* and LAP being activators and LIP being an inhibitor. To determine whether the increased C/EBPβ mRNA by Thaps differentially increases in these polypeptides, Western blotting was performed. As shown in Fig. 3A (Right), little changes were detected on the levels of LAP and LAP*. In contrast, LIP was

markedly increased. These results established that induction of C/EBPβ mRNA by Thaps increased the production of LIP but not activator LAP* and LAP. The level of HNF4α mRNA, in contrast to the level of C/EBPβ mRNA, was significantly decreased by Thaps (Fig. 3B) and the decrease was less with prolonged treatment. The 12 h time-point showed a 60% decrease whereas the 24 h time-point showed a 40% decrease (Fig. 3B). Consistent with the decease in HNF4α mRNA, the level of HNF4α protein was drastically decreased (Fig. 3B).

3.4. Occupancy of the PXR promoter by HNF4α and C/EBPβ

The reporter and expression studies collectively suggested that the PXR promoter is targeted by HNF4α and C/EBPβ. To directly test this possibility, EMSA and ChIP experiments were performed. The EMSA experiment was performed with two probes: one containing the putative HNF4α site and the other the C/EBP site. As shown in Fig. 4A, incubation with the HNF4α probe led to the detection of a major shifted band (Fig. 4A). The intensity of this band was weaker when incubation was performed with nuclear extracts from Thaps-treated cells, consistent with the fact that Thaps down-regulated HNF4α. This band was competed by non-labeled probe and abolished by the antibody against HNF4α. Addition of the antibody also intensified the band on the top of the gel (Fig. 4A). Incubation with the C/EBPβ probe, on the other hand, led to the detection of several bands (Fig. 4B). Incubation with extracts from Thaps-treated cells produced a new shifted band (probably bound by LIP) and decreased the intensities of two shifted bands (arrowed in column 3). All shifted bands except the

top one were competed by non-labeled probe but not by the corresponding non-labeled probe with disrupted C/EBP binding site. Nonetheless, the putative LAP and LIP binding bands were abolished by the antibody against C/EBPβ (Fig. 3).

The EMSA experiment established that the PXR promoter contained HNF4α and C/EBP binding site. Next we tested whether both proteins occupy the PXR promoter. ChIP experiment was performed in cells treated with solvent or Thaps. In addition to HNF4α and C/EBP element-containing segment, a segment of the PXR gene containing either element was subjected to PCR-amplification as a control. As shown in Fig. 4C, PCR detected the amplification of both segments with input DNA. However, PCR detected the HNF4α-C/EBP but not the control segment with ChIPed-DNA. The amplification was observed with ChIPed DNA from control but not Thaps-treated cells (Fig. 3C). It should be noted that pre-immune IgG for ChIP experiment did not yield any amplification.

3.5. Interconnection between Thaps and IL-6 in the suppression of PXR

We have previously showed that PXR was down-regulated by the proinflammatory cytokine IL-6 (Yang et al., 2010). To determine whether Thaps and IL-6 use similar genomic sequence in the down-regulation, HepG2 cells were transfected with various PXR reporters, treated with IL-6 as shown, and detected for luciferase activity. BFA, another commonly used ER stressor, was also included in this study. As predicted,

both IL-6 and BFA produced a similar responding pattern as Thaps among these reporters (Figs, 2A and 5A). Two additional experiments were performed to shed slight on the mechanistic connection between ER stress and IL-6. Firstly, the suppression of PXR by IL-6 and Thaps was determined as a function of the time of treatment. Secondly, the expression of IL-6 was determined in Thaps-treated primary hepatocytes. As shown in Fig. 5B, both IL-6 and Thaps significantly decreased PXR mRNA. However, the decrease by IL-6 occurred sooner than that by Thaps (Fig. 5B). We next tested whether human primary hepatocytes treated with Thaps actually support the induction of IL-6. As shown in Fig. 5C, treatment with Thaps significantly increased IL-6 mRNA (Fig. 5C).

3.5. Effect of ER-stress on CYP3A4 induction

The enhanced production of IL-6 by Thaps suggested that IL-6 is a contributor to Thaps-mediated down-regulation of PXR. It is well established that signal transducer and activator of transcription-3 (STAT3) supports the activity of IL-6 (Bode et al., 2012). We next tested whether Z-guggulsterone, a blocker of STAT3 (Leeman-Neill et al., 2009), antagonizes Thaps in down-regulating PXR. On the other hand, Z-guggulsterone is a known antioxidant (Chen et al., 2012), therefore, emodin was included in this study as a control for antioxidant property (Shia et al., 2010). HepG2 cells were treated with Thaps, Z-guggulsterone, emodin or in combination, and then the level of PXR mRNA was determined. As expected, Thaps significantly decreased PXR mRNA (Fig. 6A). The decrease, however, was almost completely reversed by Z-

guggulsterone but not by emodin. It should be noted that Z-guggulsterone and emodin alone showed no effect on the level of PXR mRNA (Fig. 6A).

Next we tested whether overexpression of PXR itself reverses the effect of Thaps in terms of the induction of CYP3A4. Both transfected and non-transfected HepG2 cells were used, and the transfection was performed with a PXR expression construct or the corresponding vector. The cells were treated with Thaps, rifampicin or both for 24 h and analyzed for the mRNA level of CYP3A4, a prototypical target of PXR (Klein and Zanger, 2013). The results were expressed as fold of induction. As shown in Fig. 6B, Thaps significantly decreased the induction of CYP3A4 in both vector- and nontransfected cells. However, the decrease was reversed in PXR transfected cells.

4. DISCUSSION

ER-stress is recognized as a common theme in the development of metabolic syndrome and other diseases (Johnson et al., 2012; Lin et al., 2012), and emerging evidence has pointed to decreased capacity of metabolism in liver diseases associated with ER-stress (He and Chen, 2012; Pagliassotti, 2012). PXR is a master regulator of genes in xenobiotic elimination. In this study, we have shown that Thaps and BFA, two well-characterized ER-stressors significantly decreased the expression of PXR. The decrease was mediated through transcriptional repression and led to reduced induction of CYP3A4, a prototypical target gene of PXR (Klein and Zanger, 2013). The decrease of PXR expression by Thaps was reversed by Z-guggulsterone, an active ingredient of the hypolipidemic herb guggul (Yang et al., 2012).

It is likely that the reversal by Z-guggulsterone was achieved by blocking STAT3 activity. Several lines of evidence support this possibility. Firstly, Z-guggulsterone is an antioxidant and many antioxidants reportedly protect against ER-stress (Ding et al., 2012; Li et al., 2012), however, emodin (an antioxidant) showed no reversal activity on the Thaps-mediated downregulation of PXR (Fig. 6A; Harlev et al., 2012), excluding an involvement of the antioxidant property in the reversal of PXR downregulation. Secondly, we have shown that IL-6 and Thaps targeted the same regulatory sequence (Figs. 2A and 5A) and IL-6 is known to activate the STAT3 signaling pathway (Bode et al., 2012). Thirdly, treatment with Thaps induced the expression of IL-6 (Fig. 5C), suggesting that increased expression of IL-6 at least in part plays a role in Thaps-mediated downregulation of PXR. On the other hand, it

remains to be determined whether increased expression of IL-6 by Thaps represents a general phenomenon among ER-stressors and diseases associated with ER-stress. The connection between Thaps and IL-6, nevertheless, provides a mechanistic understanding of how ER-stress conditions may exert differential effect on the expression of PXR depending on the increased secretion of cytokines such as IL-6.

STAT3 is a DNA-sequence specific transcription factor (Bode et al., 2012). However, the PXR promoter regulatory sequence targeted by Thaps and IL-6 does not harbor a consensus STAT3 element. Instead, this sequence contains two adjacent elements that were recognized by HNF4α and C/EBP proteins, respectively. It is therefore assumed that STAT3 decreases the expression of PXR by regulating the expression of HNF4α, C/EBP proteins or both. While it is not clear whether STAT3 down-regulates HNF4α, it was reported that STAT3 up-regulated the expression of C/EBPβ (Anastasov et al., 2010). Furthermore, STAT3 was shown to interact directly with C/EBPβ. Given the fact that co-transfection of LAP increased PXR expression (Fig. 2C), the STAT3-C/EBPβ complex likely exerts repressive activity. Alternatively, such complex no longer acts on the PXR promoter, thus functioning as a dominant negative in comparison with LAP. The C/EBP family has several members and they all bind to same or similar DNA elements (Tsukada et al., 2011). It is conceivable that other C/EBP members likely participate in the regulated expression of PXR during ER-stress.

One of the interesting findings in this study is the unique interplay between C/EBPβ and HNF4α. In the reporter experiment, disruption of the HNF4α element almost

completely eliminated the repressive activity in response to Thaps (Fig. 2B). In contrast, disruption of the C/EBP element diminished the repression to a much lesser extent (Fig. 2B). These observations suggested that HNF4α played an essential or a greater role than a C/EBP protein (probably C/EBPβ) in supporting the expression of PXR. However, transfection of HNF4α surprisingly caused less increases of PXR mRNA than co-transfection of LAP (an active form of C/EBPβ) (Fig. 2C). One explanation is that LAP functioned as a transactivator of HNF4α and/or LAP enhanced the activity of HNF4α. In support of the last possibility, LAP was shown to increase nuclear translocation of HNF4α (Shen et al., 2000).

C/EBPβ mRNA produces several in-frame translated polypeptides including LAP*, LAP and LIP. Under normal conditions, LAP is the most abundant form. While LAP* and LAP are transactivators, LIP acts as a transcriptional repressor (Tsukada et al., 2011). It is generally accepted that the repressive activity of LIP is achieved by forming non-functional dimmer with C/EBP activating members and/or a DNA-binding dominant negative. It is also accepted that the relative abundance of various C/EBPβ forms (e.g., LAP versus LIP) largely depends on the relative efficiency of the initiation codons for translation. Interestingly, Thaps treatment caused an 11-fold increase of C/EBPβ mRNA (Fig. 3A), and yet the increase in proteins was detected on LIP but not LAP or LAP* (Fig. 3A). One explanation is that the initiation codon for LIP was more efficient under ER-stress condition induced by Thaps. It has been reported that LAP can be converted into LIP (LAP is bigger than LIP) through proteolytic digestion through an unknown protease. It is likely that such a protease(s)

is up-regulated and/or activated by Thaps (Welm at el., 1999). Alternatively, LIP was relatively more stable than LAP in the presence of Thaps (Li et al., 2008).

Nevertheless, EMSA experiment detected increases in DNA binding, apparently by LIP (Fig. 4B). In contrast, the intensity of the shifted bands by LAP and LAP* was slightly decreased in nuclear extracts of cells treated with Thaps. Based on ChIP experiment, however, the increased LIP did not lead to increases in the occupancy of the C/EBP element in the PXR promoter, although the same antibody was used in both EMSA and ChIP experiments. One explanation is that chromatin-bound LIP (ChIP) posed a configuration that hided the epitope from being recognized by this antibody. Alternatively, LIP normally does not bind to the C/EBP element in the PXR promoter under the native condition (i.e., cell), although it did so under non-cellular context (i.e., EMSA). Nonetheless, cotransfection of LIP indeed conferred potent repressive activity toward the PXR promoter reporter (data not shown).

In summary, our study presents several important conclusions. Firstly, ER-stressors decreased the expression of PXR and the induction of CYP3A4, pointing to the possibility of reduced capacity of drug metabolism and detoxication during ER-stress condition. Secondly, the decreased expression of PXR was a sequence-specific event through adjacent HNF4α-C/EBP elements. Cotransfection of HNF4α or LAP restored PXR expression, suggesting that factors, altering the activity of these transcription factors, likely affect the expression of PXR and its target genes. Thirdly, ER-stressors and IL-6 targeted the same element in repressing PXR, establishing a novel functional link. This is particularly of significance as such connection suggests that ER-stress

conditions may vary in suppressing PXR expression depending on the enhanced secretion of cytokines such as IL-6.

Footnotes

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[2] To whom correspondence should be addressed.

[3] The authors indicate no potential conflict of interest.

REFERENCES

Adolph TE, Niederreiter L, Blumberg RS, Kaser A (2012) Endoplasmic reticulum stress and inflammation. *Dig Dis*. **30:**341-6.

Anastasov N, Bonzheim I, Rudelius M, Klier M, Dau T, Angermeier D, Duyster J, Pittaluga S, Fend F, Raffeld M, Quintanilla-Martinez L (2010) C/EBPβ expression in ALK-positive anaplastic large cell lymphomas is required for cell proliferation and is induced by the STAT3 signaling pathway. *Haematologica*. **95:**760-7.

Back SH, Kaufman RJ (2012) Endoplasmic reticulum stress and type 2 diabetes. *Annu Rev Biochem*. **81:**767-93.

Benbrook DM, Long A (2012) Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis. *Exp Oncol*. **34:**286-97.

Bock KW, Bock-Hennig BS (2010) UDP-glucuronosyltransferases (UGTs): from purification of Ah-receptor-inducible UGT1A6 to coordinate regulation of subsets of CYPs, UGTs, and ABC transporters by nuclear receptors. *Drug Metab Rev*. **42:**6-13.

Bode JG, Albrecht U, Häussinger D, Heinrich PC, Schaper F (2012) Hepatic acute phase proteins--regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF-κB-dependent signaling. *Eur J Cell Biol*. **91:**496-505.

Calì T, Ottolini D, Brini M (2011) Mitochondria, calcium, and endoplasmic reticulum stress in Parkinson's disease. *Biofactors*. **37:**228-40.

Chen YZ, Shi D, Yang D, Yan B (2012) Antioxidant sulforaphane and sensitizer trinitrobenzene sulfonate induce carboxylesterase-1 through a novel element transactivated by nuclear factor-E2 related factor-2. *Biochem Pharmacol*. **84:**864-71.

Ding Y, Dai X, Jiang Y, Zhang Z, Bao L, Li Y, Zhang F, Ma X, Cai X, Jing L, Gu J, Li Y (2012) Grape seed proanthocyanidin extracts alleviate oxidative stress and ER stress in skeletal muscle of low-dose streptozotocin- and high-carbohydrate/high-fat diet-induced diabetic rats. *Mol Nutr Food Res*. In press.

Donato MT, Jiménez N, Serralta A, Mir J, Castell JV, Gómez-Lechón MJ (2007) Effects of steatosis on drug-metabolizing capability of primary human hepatocytes. *Toxicol In Vitro*. **21:**271-6.

Dostalek M, Court MH, Yan B, Akhaghi F (2011) Significantly reduced cytochrome P4503A4 expression and activity in liver from human with diabetes mellitus. *Brit J Pharmacol*. **163:**937-47.

Flamment M, Hajduch E, Ferré P, Foufelle F (2012) New insights into ER stress-induced insulin resistance. *Trends Endocrinol Metab*. **23:**381-90.

Goodwin B., Hodgson E, Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol. Pharmacol.* **56:**1329-39.

Haeri M, Knox BE (2012) Endoplasmic Reticulum Stress and Unfolded Protein Response Pathways: Potential for Treating Age-related Retinal Degeneration. *J Ophthalmic Vis Res*. **7:**45-59.

Harlev E, Nevo E, Lansky EP, Ofir R, Bishayee A (2012) Anticancer potential of aloes: antioxidant, antiproliferative, and immunostimulatory attributes. *Planta Med*. **78:**843-52.

Ihunnah CA, Jiang M, Xie W (2011) Nuclear receptor PXR, transcriptional circuits and metabolic relevance. *Biochim Biophys Acta*. **1812:**956-63.

IPA, The World Health Organization (2007) Top 20 Causes of Mortality Throughout the World, *The World Health Report*. Retrieved March 3, 2013 from http://www.infoplease.com/ipa/A0779147.html

Johnson N, Powis K, High S (2012) Post-translational translocation into the endoplasmic reticulum. *Biochim Biophys Acta*. In press

Ke PY, Chen SS (2012) Hepatitis C virus and cellular stress response: implications to molecular pathogenesis of liver diseases. *Viruses*. **4:**2251-90.

Kolwankar D, Vuppalanchi R, Ethell B, Jones DR, Wrighton SA, Hall SD, Chalasani N (2007) Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin Gastroenterol Hepatol*. **5:**388-93.

Kurose K, Koyano S, Ikeda S, Tohkin M, Hasegawa R, Sawada J (2005) 5' diversity of human hepatic PXR (NR1I2) transcripts and identification of the major transcription initiation site. *Mol Cell Biochem.* **273:**79-85.

Leeman-Neill RJ, Wheeler SE, Singh SV, Thomas SM, Seethala RR, Neill DB, Panahandeh MC, Hahm ER, Joyce SC, Sen M, Cai Q, Freilino ML, Li C, Johnson DE, Grandis JR (2009) Guggulsterone enhances head and neck cancer therapies via inhibition of signal transducer and activator of transcription-3. *Carcinogenesis*. **30:**1848-56.

Li C, Wang L, Huang K, Zheng L (2012) Endoplasmic reticulum stress in retinal vascular degeneration: protective role of resveratrol. *Invest Ophthalmol Vis Sci*. **53:**3241-9.

Li Y, Bevilacqua E, Chiribau CB, Majumder M, Wang C, Croniger CM, Snider MD, Johnson PF, Hatzoglou M (2008) Differential control of the CCAAT/enhancer-binding protein beta (C/EBPbeta) products liver-enriched transcriptional activating protein (LAP) and liver-enriched transcriptional inhibitory protein (LIP) and the regulation of gene expression during the response to endoplasmic reticulum stress. *J Biol Chem*. **283:**22443-56.

Lin S, Sun S, Hu J (2012) Molecular basis for sculpting the endoplasmic reticulum membrane. *Int J Biochem Cell Biol*. **44:**1436-43.

Liver Foundation (2009) Liver awareness month. Retrieved March 3, 2013 from http://www.liverfoundation.org/chapters/lam2009.

Ma Y, Song X, Sachdeva K, Liu J, Li Y, Yang D, Deng R, Chichester CO, Yan B (2005) Clofibrate and perfluorodecanoate both up-regulate the expression of the pregnane X receptor but only clofibrate enhances its ligand-dependent induction of cytochrome P4503A23. *Biochem Pharmacol*. **69:**1363-71.

Nickel W (2010) Pathways of unconventional protein secretion. *Curr Opin Biotechnol*. **21:**621-6.

Pagliassotti MJ (2012) Endoplasmic reticulum stress in nonalcoholic fatty liver disease. *Annu Rev Nutr*. **32:**17-33.

Ri M, Tashiro E, Oikawa D, Shinjo S, Tokuda M, Yokouchi Y, Narita T, Masaki A, Ito A, Ding J, Kusumoto S, Ishida T, Komatsu H, Shiotsu Y, Ueda R, Iwawaki T, Imoto M, Iida S (2012) Identification of Toyocamycin, an agent cytotoxic for multiple myeloma cells, as a potent inhibitor of ER stress-induced XBP1 mRNA splicing. *Blood Cancer J*. **2:**e79.

Sachdeva K, Yan B, Chichester CO (2003) Lipopolysaccharide and cecal ligation/puncture differentially affect the subcellular distribution of the pregnane X receptor but consistently cause suppression of its target gene CYP3A. *Shock*. **19:**470-75.

Salido GM, Sage SO, Rosado JA (2009) Biochemical and functional properties of the store-operated Ca2+ channels. *Cell Signal*. **21:**457-61.

Santoro A, Mancini E, Ferramosca E, Faenza S (2007) Liver support systems. *Contrib Nephrol*. **156:**396-404.

Shen CN, Slack JM, Tosh D (2000) Molecular basis of transdifferentiation of pancreas to liver. *Nat Cell Biol*. **2:**879-87.

Shi D, Yang D, Yan B (2010) Dexamethasone transcriptionally increases the expression of the pregnane X receptor and synergistically enhances pyrethroid deltamethrin in the induction of cytochrome P450 3A23. *Biochem Pharmacol*. **80:**1274-83.

Shi D, Yang D, Prinssen EP, Brian E. Davies BE, Yan B (2011) Surge in expression of carboxylesterase-1 during the post-natal stage enables a rapid gain of the capacity to activate the anti-influenza prodrug oseltamivir. *J Infect Dis*. **203:**937-42.

Shia CS, Hou YC, Tsai SY, Huieh PH, Leu YL, Chao PD (2010) Differences in pharmacokinetics and ex vivo antioxidant activity following intravenous and oral administrations of emodin to rats. *J Pharm Sci*. **99:**2185-95.

Song X., Xie M, Zhang H, Li Y, Sachdeva K, Yan B (2004) The pregnane X receptor binds to response elements in a genomic context-dependent manner and its activator rifampicin selectively alters the bindings among target genes. *Drug Metab Dispos*. **32:**35-42.

Tompkins LM, Sit TL, Wallace AD (2008) Unique transcription start sites and distinct promoter regions differentiate the pregnane X receptor (PXR) isoforms PXR 1 and PXR 2. *Drug Metab Dispos*. **36:**923-9.

Toriyabe T, Nagata K, Takada T, Aratsu Y, Matsubara T, Yoshinari K, Yamazoe Y (2009) Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics. *Mol Pharmacol*. **75:**677-84.

Tsukada J, Yoshida Y, Kominato Y, Auron PE (2011) The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine*. **54:**6-19.

Villarroya F, Domingo P, Giralt M (2010) Drug-induced lipotoxicity: lipodystrophy associated with HIV-1 infection and antiretroviral treatment. *Biochim Biophys Acta*. **801:**392-9.

Welm AL, Timchenko NA, Darlington GJ (1999) C/EBPalpha regulates generation of C/EBPbeta isoforms through activation of specific proteolytic cleavage. *Mol Cell Biol*. **19:**1695-704.

Xiao D, Yang D, Charpentier M, Yan B (2012) Regulation of carboxylesterase-2 expression by p53 family proteins and enhanced anticancer activities among 5-fluorouracil, irinotecan and doxazolidine prodrug. *Brit J Pharmacol*. In press

Xu K, Zhu XP (2012) Endoplasmic reticulum stress and prion diseases. *Rev Neurosci*. **23:**79-84.

Yang D, Shi D, Yang J, Deng R, Yan B (2011) Scoparone potentiates transactivation of the bile salt export pump gene and this effect is enhanced by cytochrome P450 metabolism but abolished by a PKC inhibitor. *Brit J Pharmacol*. **164:**1547-57.

Yang D, Yang J, Shi D, Black C, Deng R, Yan B (2012) The hypolipidemic agent Z-guggulsterone: metabolism interplays with induction of cholesteryl ester hydrolase CES1 and bile salt export pump. *J Lipid Res*. **53:**529-39.

Yang J, Hao C, Yang D, Shi D, Song X, Luan X, Hu G, Yan B (2010) Pregnane X receptor is required for interleukin-6-mediated down-regulation of cytochrome P450 3A4 in human hepatocytes. *Toxicol Lett*. **197:**219-26.

Zhang J, Kuehl P, Green ED, Touchman JW, Watkins PB, Daly A, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Wrighton SA, Hancock M, Kim RB, Strom S, Thummel K, Russell CG, Hudson JR Jr, Schuetz EG, Boguski MS (2001) The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics*. **11:**555-72.

Table I Sequences of Oligonucleotides

Oligonucleotide	Sequence
Native promoter reporters [numbered according to Kurose et al., *Mol Cell Biochem*. (2005) 273:79-85]	
PXR-1286-MluI	5'-tcctagccctagcagaatcccatgtggata-3’
PXR-506-MluI	5'-gttctgagatcaaagtggtggggtcacatt-3’
PXR-206-MluI	5'-atttgccactctcttcccct-3’
PXR-106-MluI	5'-atttgctagttcaagtgctg-3’
PXR-56-MluI	5'-gcttagtgcctacatctgac-3’
PXR+14-BamHI	5'-gacaagattgtctcatatccggggaaat-3’
Element reporters	
PXR-wild type	5’-gctagttcaagtgctggacttgggacttaggaggggcaatgg-3’
PXR-HNF4α mutant	5’-gctagttcaagtgctggaaccgggacttaggaggggcaatgg-3’
PXR-C/EBP mutant	5’-gctagttcaagtgctggacttgggacttaggattttaccggg-3’
PXR-double mutant	5’-gctagttcaagtgctggaaccgggacttaggattttaccggg-3’
EMSA	
PXR-HNF4α	5’-tagttcaagtgctggacttgggacttagga-3’
PXR-HNF4α(mutant)	5’-tagttcaagtgctggaaccgggacttagga-3’
PXR-C/EBP	5’-cttgggacttaggaggggcaatggagccgcttag-3’
PXR-C/EBP(mutant)	5’-cttgggacttaggattttaccgggagccgcttag-3’

ChIP

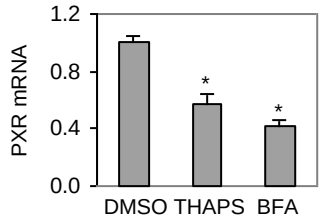
Element sense	5’-gcggatatttgccactctctt-3’
Element reverse	5’-cggatatgagacaatcttgtc-3’
Non-element sense	5’-gagtcttttcattgctacctc-3’
Non-element reverse	5’-tggatgcagagacacagaatg-3’

Semi-quantitative PCR

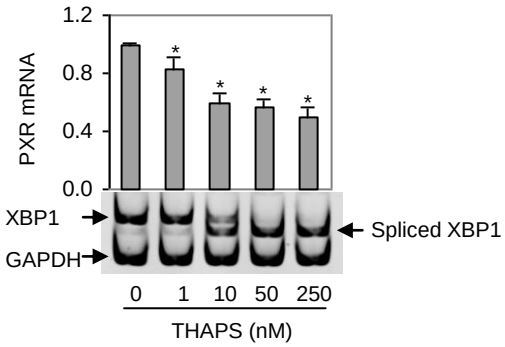
XBP1-sense	5’-ttacgagagaaaactcatggcc-3’
XBP1-reverse	5’-gggtccaagttgtccagaatgc-3’
GAPDH-sense	5′-agggctgcttttaactctggt-3′
GAPDH-reverse	5′-ccccacttgattttggaggga-3′

Fig. 1. Effect of Thaps or BFA on the expression of PXR (A) Suppression of PXR mRNA by BFA and Thaps in HepG2 cell line Cells were treated with Thaps and BFA at 1 μM or DMSO for 24 h. The level of PXR mRNA was determined by RT-qPCR. The level of PXR mRNA was normalized according to the level of GAPDH mRNA and expressed as fold of induction. Asterisk signs indicate statistical significance ($P < 0.05$). (B) Suppression of PXR mRNA as a function of Thaps HepG2 cells were treated with Thaps at 0, 1, 10, 50 or 250 nM and the level of PXR mRNA was determined. Asterisk signs indicate statistical significance ($P < 0.05$). To ascertain the magnitude of ER-stress, the presence of spliced XBP1 mRNA was determined by semi-quantitative RT-PCR with the level of GAPDH mRNA as a control. The PCR amplification was performed with two pairs of primers designed to target XBP1 and GAPDH. A preliminary study established that these primers did not interfere with each other. (C) Suppression of PXR expression in primary hepatocytes Human primary hepatocytes ($n = 4$) were treated with Thaps at 0.1 or 2 μM for 24 h. Total RNA was analyzed for the level of PXR mRNA by RT-qPCR. Cell lysates (25 μg) from pooled samples were analyzed by Western blotting for the level of PXR protein. Asterisk signs indicate statistical significance from the vector ($P < 0.05$).

A



B



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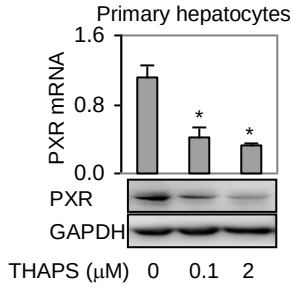
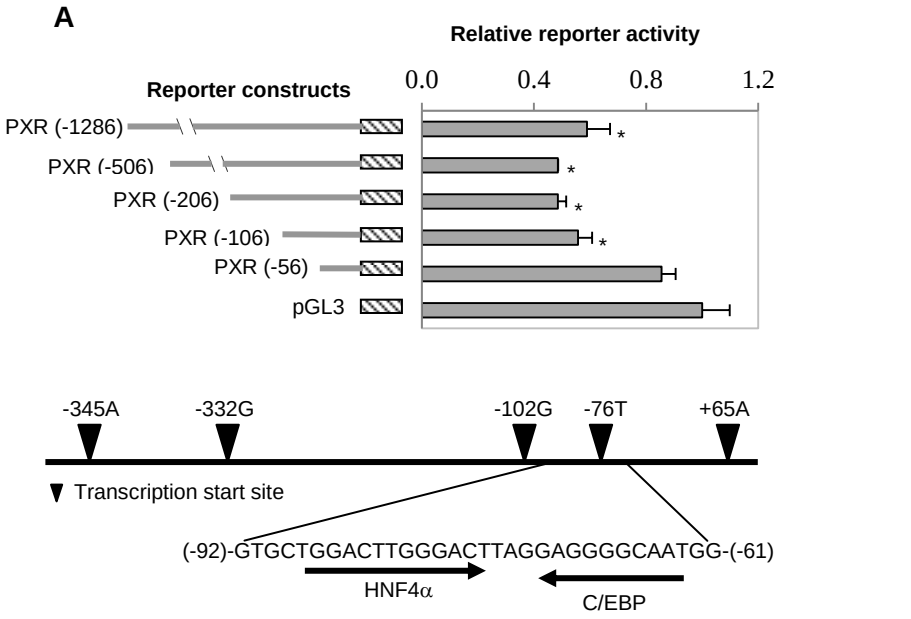
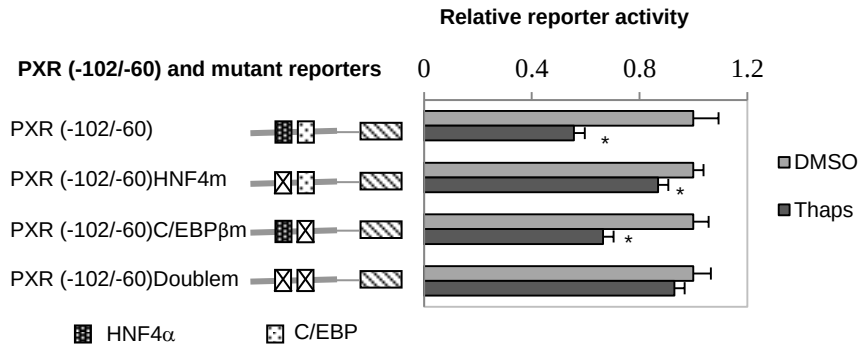


Fig. 1, Vachirayonstien (2013)

Fig. 2. Repression of PXR reporters and reversal of Thaps-suppression of PXR expression (A) Repression of PXR promoter reporters HepG2 cells were transiently transfected by FuGene HD with a mixture containing 50 ng of a reporter, or the vector along with 5 ng of the null-Renilla luciferase plasmid. The transfected cells were then treated with DMSO or Thaps at 50 nM for 24 h. Luciferase activities were determined with a Dual-Luciferase Reporter Assay System and the signals were expressed as percentages of the normalized luciferase activity of the vector reporter. Below is the diagram with reported transcription start sites numbered according to Kurose et al (2005). Asterisk signs indicate statistical significance ($P < 0.05$). (B) Repression of PXR element reporters HepG2 cells were transfected with an element reporter (wild type of a mutant) as described above. Once gain, the luciferase activity was expressed after normalization. Asterisk signs indicate statistical significance from the wild type reporter ($P < 0.05$). (C) Reversal of Thaps-suppression of PXR by LAP or HNF4α. HepG2 cells were transfected with an expression construct (LAP or HNF4α) or the corresponding vector. After an overnight incubation, the transfected cells were treated with Thaps at 50 nM for 24 h. Cells were collected and total RNA was isolated. The level of PXR mRNA was determined by RT-qPCR. The level of PXR in vector-transfected and DMSO-treated cells was expressed as 100%. Asterisk signs indicate statistical significance from the vector ($P < 0.05$).





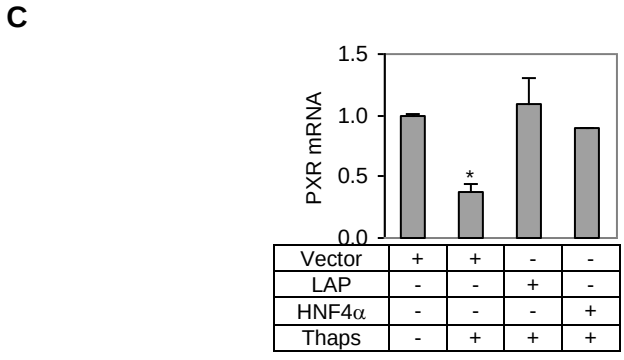
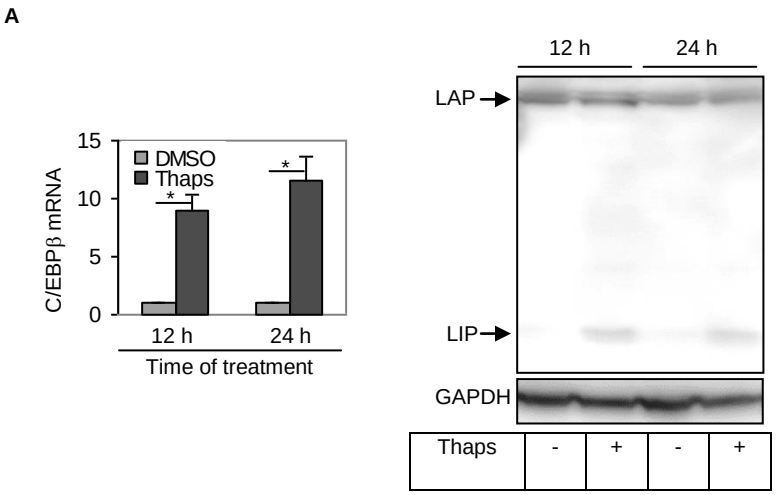


Fig. 2, Vachirayonstien (2013)

Fig. 3. Effect of Thaps on the expression of HNF4α and C/EBPβ (LAP*, LAP and LIP) (A) Effect of Thaps on the expression of C/EBPβ (LAP*, LAP and LIP) HepG2 cells were treated with Thaps at 50 nM for 12 or 24 h. Cells were collected, total RNA was isolated and lysates were prepared. The level of C/EBPβ mRNA was determined by RT-qPCR (Left). Lysates (20 μg) were resolved by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The blots were incubated with a carboxylesterase antibody and developed with chemiluminescent substrate and re-probed by GAPDH antibody. The signal was captured by Carestream 2200 PRO Imager. Asterisk signs indicate statistical significance ($P < 0.05$). (B) Effect of Thaps on the expression of HNF4α Cells were treated and samples were processed as described above. RT-qPCR was performed to determine the level of HNF4α mRNA whereas Western blotting was performed to determine the level of HNF4α protein. Asterisk signs indicate statistical significance ($P < 0.05$).



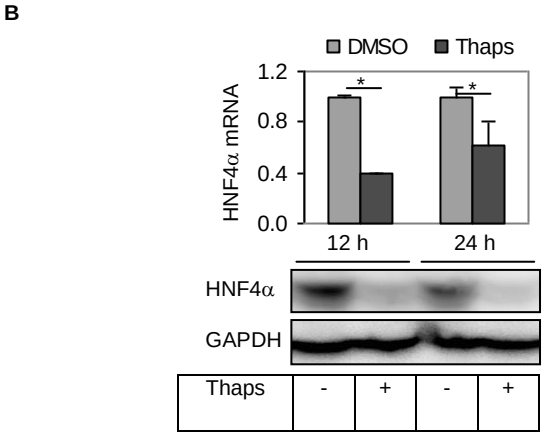


Fig. 3, Vachirayonstien (2013)

Fig. 4. Characterization of HNF4α and C/EBP elements by EMSA and ChIP (A) EMSA analysis of HNF4α element Nuclear extracts (5 μg) from HepG2 cells treated with DMSO or Thaps (50 nM) for 24 h were incubated with a biotinylated HNF4α probe for 20 min. In the competition assay, nuclear extracts were pre-incubated with the unlabeled element (100x), for 20 min, and then incubated with the biotinylated probe. In disruption assay, nuclear extracts were incubated first with an antibody against HNF4α on ice for 20 min and then with the biotinylated probe. The protein-DNA complexes were electrophoretically resolved, transferred to a Biodyne® nylon membrane and located with streptavidin-conjugated horseradish peroxidase and chemiluminescent substrate. (B) EMSA analysis of C/EBPβ element Incubations were performed as characterization of the HNF4α element. However, the C/EBP probe and an antibody against C/EBPβ were used. (C) ChIP analysis HepG2 cells were treated with DMSO or Thaps at 50 nM for 24 h, washed and underwent cross-linking for 15 min by 1% formaldehyde, and the cross-linking was terminated with 125 mM glycine. The soluble chromatins were prepared, pre-cleared with protein G beads and incubated with an antibody against HNF4α or C/EBPβ. As a control, the antibody was replaced with pre-immune IgG. The antibody-bound chromatins and DNA input (1/20 of the antibody-bound chromatins) were analyzed by PCR for the presence of the genomic fragment containing the HNF4α and C/EBPβ adjacent elements. The location of the primers is shown in the diagram and the sequences of primers are shown in Table I. All experiments in this figure were performed three times.

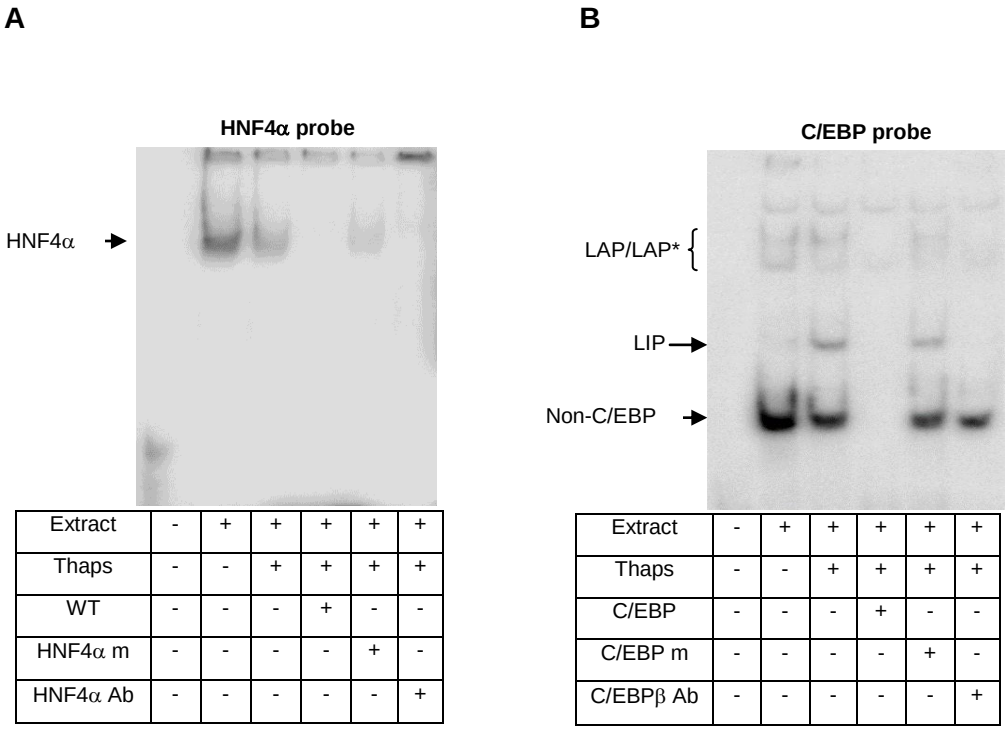


Fig. 4, Vachirayonstien (2013)

Fig. 5. Regulated expression of PXR by IL-6 (A) Repression of PXR promoter reporters by IL-6 and BFA HepG2 cells were transiently transfected as described in the legend of Figure 2. The transfected cells were then treated with IL-6 (10 ng/ml), BFA (20 nM) or the corresponding solvent for 24 h. Luciferase activities were determined with a Dual-Luciferase Reporter Assay System and the signals were expressed as percentages of the normalized luciferase activity of the vector reporter. (B) Suppression of PXR mRNA as a function of time of treatment by Thaps and IL-6 HepG2 cells were treated with Thaps at 50 nM, IL-6 at10 ng/ml or the corresponding solvent for 0-24 h. The level of PXR mRNA was determined by RT-qPCR. Asterisk signs indicate statistical significance from the corresponding zero-time points ($P < 0.05$). (C) Effect of Thaps at the level of IL-6 mRNA Primary hepatocytes ($n = 4$) were treated with Thaps at 0.1 or 2 μM for 24 h. The level of IL-6 mRNA was determined. Asterisk signs indicate statistical significance ($P < 0.05$).

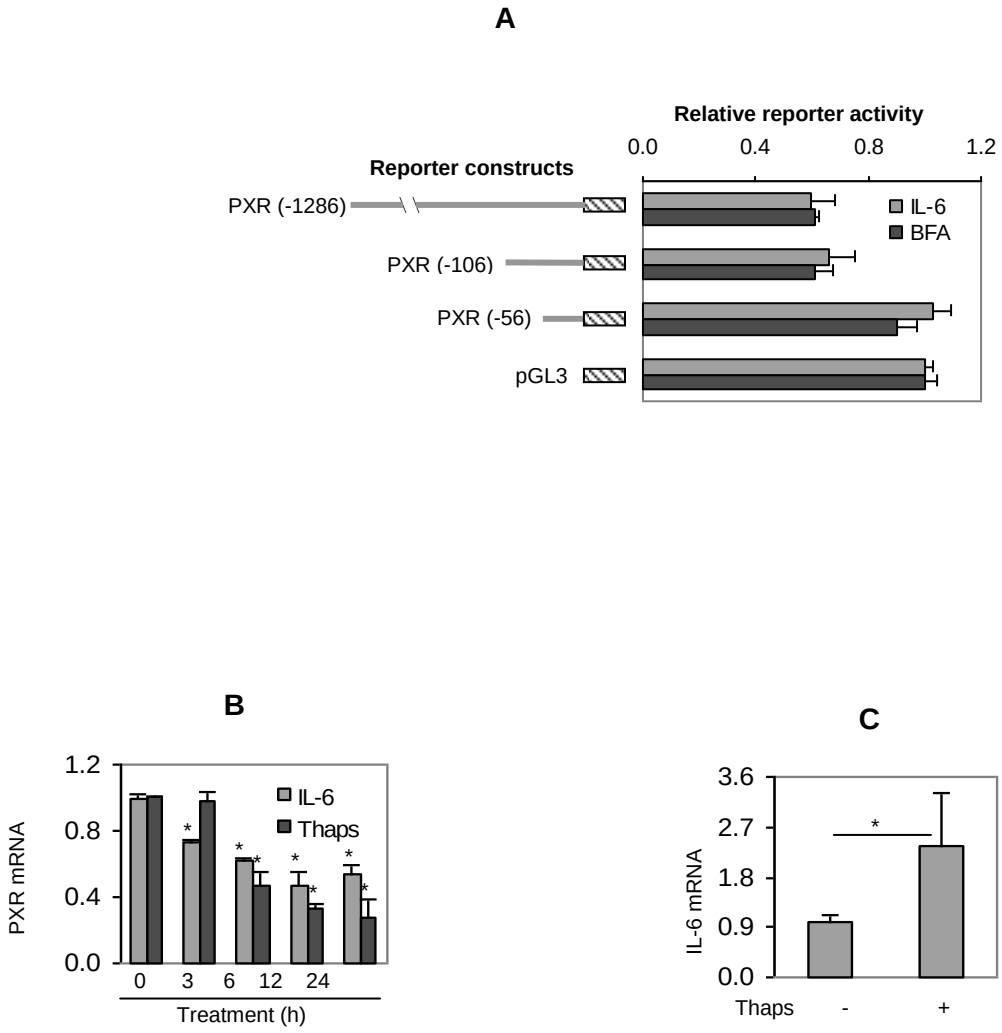
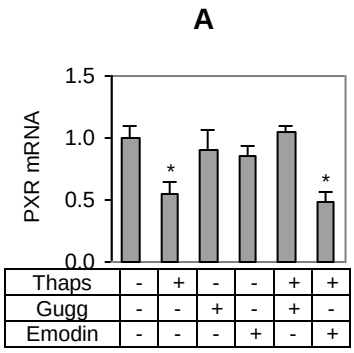


Fig. 5, Vachirayonstien (2013)

Fig. 6. Interplay of Thaps with antioxidants and rifampicin (A) Reversal of Thaps-mediated suppression of PXR by Z-guggulsterone HepG2 cells were treated Thaps at 50 nM, Z-guggulsterone (10 μM), emodin (10 μM), or in various combinations. The treatment lasted for 24 h and the expression of PXR mRNA was determined by RT-qPCR. The results were from three individual experiments in triplicate. Asterisk signs indicate statistical significance from the solvent control ($P < 0.05$). (B) Effect of PXR transfection on the reduced induction of CYP3A4 by Thaps HepG2 cells were treated with rifampicin (10 μM), Thaps (50 nM). Both transfected and non-transfected cells were used. The transfection was performed with a PXR expression construct or the corresponding vector. The treatment lasted for 24 h, cells were collected and total RNA was isolated. The level of CYP3A4 mRNA was determined. The results were expressed as fold of induction. Asterisk signs indicate statistical significance from nontransfected and RIF-treated cells ($P < 0.05$).



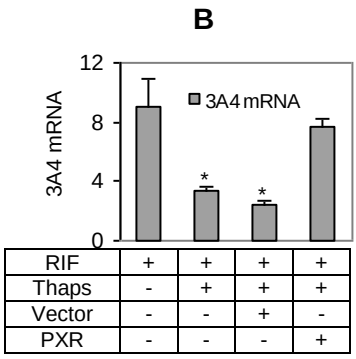


Fig. 6, Vachirayonstien (2013)

CHAPTER 4

MANUSCRIPT 2: formatted for the journal of biological chemistry

MicroRNA-30c-1 IS A SILENCER OF THE PREGNANE X RECEPTOR BY TARGETING THE 3'-UNTRANSLATED REGION AND ALTERS THE EXPRESSION OF ITS TARGET GENE CYTOCHROME P450 3A4

Abbreviations: CYP3A4, cytochrome P450 3A4; DMEM, Dulbecco's modified eagle medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR, microRNA; PXR, pregnane X receptor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; UTR, untranslated region

ABSTRACT

The pregnane X receptor (PXR) is a master regulator of genes involved in drug elimination. Activation of PXR is linked to the development of a spectrum of tumor behaviors, particularly chemoresistance. MicroRNAs (miRs) have emerged as important molecular species involved in tumor progression or suppression. This study was undertaken to test a large number of oncogenically implicated miRs for their ability to regulate PXR expression. A total 58 miRs (tumor enhancers or suppressors) were tested and miR-30c-1 was identified to suppress PXR expression. The suppression was achieved by targeting the 3'-untranslated region, 438 nucleotides from the stop codon. The suppression was detected in multiple cell lines from different organ origins. In addition, miR-30c-1 altered the expression of cytochrome P450 3A4 (CYP3A4), a prototypical target gene of PXR. The alteration varied depending on the time and amounts of miR-30c-1. CYP3A4 is responsible for the metabolism of more than half of drugs in the market. Interestingly, miR-30a, sharing the seed sequence with miR-30c-1 in the guide strand, failed to suppress PXR, suggesting that the suppression miR-30c-1 is mediated with the passenger strand. It has been reported that the expression of miR-30c is low in chemoresistant cell lines. The interconnection between miR-30c-1 and PXR likely plays an important role in regulating tumor behaviors, particularly related to chemoresistance.

1. INTRODUCTION

All organisms are exposed constantly to toxic chemicals from both foreign and endogenous sources. Organisms such as humans have evolved several defensive systems against chemical insults (Parkinson, 2006). In mammals, these systems are generally referred to as phase I (Lewis, 2003), phase II (Deenen et al., 2011) and phase III (van Waterschoot and Schinkel, 2011). Phase I and II consist of drug-metabolizing enzymes, whereas phase III is drug transporters. The expression of these genes undergoes constant changes in response to chemical stimuli. The pregnane X receptor (PXR, NR1I2) is established as a master transcription factor intimately involved in the regulated expression of these genes (Krasowski et al., 2005; Ihunnah et al., 2011). Structurally, PXR belongs to the nuclear hormone receptor superfamily (Timsit and Negishi, 2007; Ihunnah et al., 2011). Like other nuclear receptors, PXR consists of a variable N-terminal domain, a highly conserved DNA-binding domain, a hinge region and a multifunctional C-terminal ligand-binding domain (Timsit and Negishi, 2007). The DNA-binding domain recognizes the canonical sequence AGG/TTCA (Modica et al., 2009). The major portion of the ligand-binding domain is helical in structure, and the C-terminal helix (helix 12) is directly involved in switching from repressing to activating status of a target gene (Carnahan and Redinbo, 2005). Binding to an agonist induces conformational changes of this helix, leading to a platform favoring association with coactivators, namely transactivation.

The expression of PXR itself, like its target genes, is drastically altered by certain xenobiotics and disease conditions (Sachdeva et al., 2003; Song et al., 2004; Ma et al.,

2005; Shi et al., 2010). For example, the hypolipidemic agent clofibrate and synthetic glucocorticoid dexamethasone have been shown to induce PXR (Ma et al., 2005; Shi et al., 2010). The induction synergistically increased the expression of cytochrome P450 3A genes (CYP3A) (Shi et al., 2010), the prototypical targets of PXR (Timsit and Negishi, 2007). Dexamethasone induced PXR in both rodents and humans (Cooper et al., 2008; Shi et al., 2010). Proinflammatory stimuli, on the other hand, have been shown to suppress the expression of PXR (Sachdeva et al., 2003; Yang et al., 2010). The level of PXR mRNA was rapidly decreased in rodents treated with lipopolysaccharide, a potent immunostimulant (Sachdeva et al., 2003). In human hepatocytes, pro-inflammatory cytokine interleukin-6 markedly reduced the levels of PXR mRNA (Yang et al., 2010). The suppression was accompanied by reduced induction on the expression of PXR-regulated genes such as CYP3A23 (Beigneux et al., 2002; Sachdeva et al., 2003).

While transactivation and repression are recognized the major mechanisms in the regulated expression of PXR (Ma et al., 2005; Shi et al., 2010; Yang et al., 2010), post-transcriptional mechanisms have been increasingly implicated in the regulated expression of this nuclear receptor. MicroRNA (miR)-148a reportedly down-regulated PXR post-transcriptionally, manifested by a significant induction of PXR mRNA (Takagi et al., 2008). On the other hand, miRs are important regulators in a wide spectrum of diseases including malignancies and metabolic syndrome (Rottiers and Näär, 2012; Kim and Reitmair, 2013; Yu et al., 2013). These diseased conditions are inherently interconnected with inflammatory process, although the magnitude of

inflammation may vary depending on the stage of diseases (Ranjha and Paul, 2013). Interestingly, the majority of studies with human malignant tissues indicated increases in PXR expression, and activation of PXR has been associated with the development of a wide spectrum of tumor behaviors, particularly chemoresistance (Pondugula and Mani, 2013).

In this study, we took a comprehensive approach and tested a large number of miRs for their ability to regulate PXR expression. These miRs are implicated in the development of cancer, inflammation or both. A total 58 miRs were tested and miR-30c-1 was identified to suppress PXR. The suppression was achieved by targeting the 3'-untranslated region (UTR) and detected in multiple cell lines from different organ origin. Importantly, miR-30c-1 was shown to alter the expression of CYP3A4, a prototypical target gene of PXR. CYP3A4 is responsible for the metabolism of more than half medicines and many chemotherapeutic agents are PXR activators. Therefore, the PXR and miR-30c-1 connection is likely a major determinant in regulating tumor behaviors, particularly related to chemosensitivity.

2. MATERIALS AND METHODS

2.1. Plasmid constructs

All miR precursor clones were purchased from System Biosciences Inc (Mountain View, CA). The CYP3A4-DP-Luc reporter was described in our previous publication (Song et al., 2005). The PXR cDNA reporters harboring a 3'-UTR segment were prepared with the pGL3 promoter vector (Promega, Madison, WI) through Xba I and Fse I restriction endonuclease sites. The 3'-UTR segments were amplified by PCR with high fidelity Platinum *Taq* DNA polymerase (Life Technology Co., Carlsbad, CA). A cDNA clone encoding human PXR, used as the PCR template, was described elsewhere (Song et al., 2004). The PXR 3142/3691Luc and 3690/4408Luc reporters were prepared initially and the PXR 3142/3691Luc reporter was used as the template for preparing deletion mutants at 5' end. The primers for PCR amplification are listed in Table I. All reporter constructs were subjected to sequence analysis.

2.2. Cell transfection and luciferase assay

Three cell lines were used in this study including 293T (human embryonic kidney), HepG2 (human hepatocellular carcinoma) and LS180 (human colon adenocarcinoma). HepG2 and LS180 lines were purchased from American Type Culture Collection (Manassas, VA), but the 293T line was from GenHunter Corporation (Nashville, TN). All cell lines were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, penicillin and streptomycin, 1x non-essential amino acids. Unless otherwise indicated, cells were plated in 48 well-plates and

transiently transfected by GenJet version II from SignaGen Laboratories (Rockville, MD). For reporter assays, the transfection mixture typically contained 50 ng of a reporter, 50 ng of a miR construct and 5 ng of the null-*Renilla* luciferase plasmid. After incubation at 37°C for 24 h, cells were extensively washed, collected and assayed for luciferase activities with the Dual-Luciferase Reporter Assay System as described previously (Song et al., 2004; Yang et al., 2011). The reporter luciferase activity was normalized with *Renilla* luciferase activity, and the vector-transfected cells served as the basal reporter activity for miRs-transfected cells. It should be noted that the same amount of total plasmids were used in all reporter assays.

2.3. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The LS180 cell line was primarily used for the RT-qPCR and because this cell line expresses high-levels of PXR and has been shown to robustly support PXR-directed transactivation (Zheng et al., 2012). Cells were typically plated in 24-well plates for overnight and then transfected with the miR-30c-1 construct or the corresponding vector. Cells were harvested 72 or 96 h after the transfection. In some cases, cells were treated with DMSO or rifampicin (10 μM) after transfection. Harvested cells were used for the preparation of total RNA. For the determination of PXR or CYP3A4 mRNA, total RNA (1 μg) was subjected to the synthesis of the first strand cDNA as described previously (Xiao et al., 2012). cDNAs were then diluted 8 times and RT-qPCR was conducted with *TaqMan* Gene Expression Assay (Applied Biosystems, Foster City, CA). The *TaqMan* probes were: PXR, Hs00243666_m1; CYP3A4, Hs00604506_m1; glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

4352934E; and RNA polymerase II, Hs00172187_m1. The PCR amplification was conducted in a total volume of 20 μl containing universal PCR master mixture (10 μl), gene-specific *TaqMan* assay mixture (1 μl), and cDNA template (6 μl). The mRNA levels were normalized according to the level of GAPDH and the normalization of selected samples was confirmed based on the signal of RNA polymerase II. Amplification and quantification were done with the Applied Biosystems 7500 Real-Time PCR System.

3. RESULTS

3.1. Identification of miR-30c-1 as a suppressive miR of PXR

PXR is a master regulator of gene expression and has been known to modulate the tumor behaviors, particularly related chemoresistance (Pondugula and Mani, 2013). Like its target genes, we and other investigators have shown that the expression of PXR is regulated by factors such as age and therapeutic agents (Pascussi et al., 2000; Ma et al., 2005; Vyhlidal et al., 2006; Shi et al., 2010). While transactivation and repression are involved in regulated expression of PXR, emerging evidence suggests an involvement of post-transcriptional mechanisms, particularly through miR species (Takagi et al., 2008). miRs constitute a superfamily of small RNA species, and many of them are implicated in the development of various types of tumor behaviors (Rottiers and Näär, 2012; Kim and Reitmair, 2013; Yu et al., 2013). To shed light on the missing link between PXR expression and miRs, we tested a large number of miRs (cancer enhancers or suppressors) for their ability to regulate the expression of PXR. Some of the miRs are known to regulate inflammatory reaction. We took the advantage of miRs as predominantly post-transcriptional regulators (Kim and Reitmair, 2013; Yu et al., 2013), mRNA-based PXR reporters (cDNA reporters) were used for the initial study.

While there are exceptions, miRs usually target the 3'-UTR sequences. Therefore, we constructed two luciferase reporters that together harbor the entire 3'-UTR sequence of the dominant human PXR transcripts (Fig. 1A) (NM_003889). These two reporters

were designated PXR3142/3691Luc and PXR3690/4408Luc, respectively. Cotransfection was performed in 293T cells to determine the effect of a miR on the reporter activity. In addition, a *renilla* luciferase construct was included in the transfection. As shown in Fig. 1B, all miRs, with an exception of miR-30c-1, affected the activity of both reporters to a similar extent. Transfection of miR-30c-1, compared with the vector, resulted in decreased activity of PXR3142/3691Luc by 50%, but only 10% decrease on the activity of PXR3690/4408Luc. Some miRs such as miR-26A-2, compared with the vector, caused significantly increases in the activity of both reporters. Some others such as miR-433 significantly decreased the activity of both reporters (Fig. 1B). These miRs generally affected the *renilla* luciferase activity, which was used for normalizing transfection efficiency.

3.2. Sequence-specific targeting by miR-30c-1 independently of cell types

The screening study clearly demonstrated that human PXR mRNA is a sequence-specific target of miR-30c-1. To locate the sequence that supports the action of this miR, a serial of deletions from the 5' end were made on the PXR3142/3691Luc reporter and the resultant reporters were tested for the lost activity toward miR-30c-1. As shown in Fig. 2A, all deletion mutants, except PXR3592Luc and PXR3642Luc, were repressed by miR-30c-1. These results established that the 50 base pairs, namely from 3542 to 3592 support the repression of miR-30c-1 on PXR expression. We are in the process of specifying the precise sequence and nucleotides that support the action of miR-30c-1 by site-directed mutagenesis.

It is well known that some miRs are processed in a cell-specific manner (Finnegan and Pasquinelli, 2013). We next tested whether the repression by miR-30c-1 occurs in HepG2 and LS180 cell line. HepG2 was derived from hepatocellular carcinoma whereas LS180 from colon adenocarcinoma. Importantly, both organs abundantly express PXR (Lehmann et al., 1998; Zhang et al., 1999). In addition, the cotransfection was performed with various amounts of miR-30c-1 to establish the concentration-response relationship. As expected, the PXR3542Luc but not PXR3592Luc reporter was repressed in both cell lines (Fig. 2B). HepG2 cells supported greater repression than LS180 cells. Overall, the magnitude of the repression occurred in a concentration-dependent manner (Fig. 2B).

3.3. Effect of miR-30c-1 on the expression of CYP3A4

The study with the reporters established that the action of miR-30c-1 on PXR suppression is sequence-specific but not cell-specific (Kim and Reitmair, 2013; Yu et al., 2013). Next we tested whether the repressive activity of the reporter can be recaptured on the expression of the endogenous gene of PXR. Initially, cells were transfected with miR-30c-1 or the corresponding vector, cultured for various lengths of time, and the levels of PXR mRNA were determined. Fig. 3A shows representative results of this study. The level of PXR mRNA was decreased in miR-30c-1 transfected cells, and the 96 h time-point showed less decrease than the 72 h time-point (Left of Fig. 3A). The decrease in cells transfected with 20 ng plasmid of miR-30c-1 was less profound than that in cells transfected with 200 and 800 ng.

Nonetheless, 200 and 800 ng caused a comparable decrease in both time-points. Next we tested whether miR-30c-1 alters the mRNA level of CYP3A4, a prototypical target of PXR (Klein and Zanger, 2013). While miR-30c-1 caused changes in the level of CYP3A4 mRNA, the changes varied depending on the amount of miR-30c-1 plasmid used for the transfection as well as the time after the transfection (Fig. 3A, Right). At the 72 h time-point, cells transfected with the miR-30c-1 plasmid at 20 and 200 ng exhibited increases in CYP3A4 mRNA, whereas a slight decrease was detected in cells transfected with 800 ng. At the 96 h time-points, significant decreases were detected in cells transfected with 200 and 800 ng plasmid (Fig. 3A, Right).

We next tested whether miR-30c-1 also alters the induction of CYP3A4. Cells were transfected with miR-30c-1 or the vector, and then treated with DMSO or rifampicin, a prototypical activator of human PXR. As shown in Fig. 3B (Left), the overall expression of CYP3A4 mRNA was decreased at both basal and induced level with an exception of the basal level in cells transfected with 20 ng plasmid of miR-30c-1. As described above, marked decreases in basal expression were detected in cells transfected with miR-30c-1 at 200 and 800 ng. As a result, these cells showed no changes in terms of fold of induction (Fig. 3B, Right). To further establish the role in reduced induction of CYP3A4, a CYP3A4 reporter was tested for the reduced activation in response to miR-30c-1 (Song et al., 2005). Consistent with the result on the level of CYP3A4 mRNA, cotransfection of miR-30c-1 decreased the activation of this reporter by as much as 60% (Fig. 3C).

4. DISCUSSION

PXR has been established to play a major role in the expression of genes involved in drug elimination (Krasowski et al., 2005). However, emerging evidence has suggested that this nuclear receptor, in addition to drug elimination, is integrally connected with endobiotic signaling and homeostasis of energy balance (Ihunnah et al., 2011). While PXR is recognized as a master regulator of gene expression, we and other investigators have reported that the expression of PXR is regulated as well (Pascussi et al., 2000; Ma et al., 2005; Vyhlidal et al., 2006; Shi et al., 2010). Multiple mechanisms are reportedly involved in the regulation of PXR expression including transactivation, repression and miR-148a silencing (Takagi et al., 2008). This study identified and characterized miR-30c-1 as a silensor of PXR. A set of experiments have shown that miR-30c-1 targeted the PXR 3'-UTR and decreased the level of PXR mRNA, suggesting that miR-30c-1 suppresses PXR by decreasing the mRNA stability of this nuclear receptor. Importantly, this miR altered the expression of CYP3A4, a prototypical target of PXR (Klein and Zanger, 2013), pointing to the functional consequences of miR-30c-1 drug elimination.

miR-30c-1 belongs to the miR-30 family, and the human genome has six miR-30 genes (Lewis et al., 2003; Bridge et al., 2012). However, these genes produce only four distinct mature guide strands. Importantly, these guide strands have the identical seed sequence, allowing them to regulate the expression of the same target genes, at least through the guide strand. This study, however, has demonstrated that miR-30a, a member of this family, failed to suppress the PXR reporters (Fig. 1B), suggesting that

miR-30c-1 uses the passenger strand to target the PXR sequence. In addition to miR-30c-1, the miR-30c class has another member, namely miR-30c-2 (Karbiener et al., 2011). Both miRs are derived from intronic sequences of other genes. The miR-30c-1 gene is located at chromosome 1 and the miR-30c-2 gene is located at chromosome 6. Importantly, miR-30c-1 and miR-30c-2 differ in the sequence of their passenger strand (Fig. 4A). Interestingly, the passenger strand of miR-30c-2 matches better than that of miR-30c-1 with the PXR 3-UTR region (Fig. 4A). Therefore, it is likely that miR-30c-2 is more potent than miR-30c-1 in silencing PXR, although the relative expression of these two miRs likely determines their contribution to PXR silencing. In addition to miR-30c-2, miR-148a, another miR, reportedly silenced PXR (Takagi, 2008). Based on the recognition sequences (Fig. 4B), both miR-30c and miR-148a target the same RNA species of PXR, although their recognition elements are 218 nucleotides apart (Fig. 4B). Nevertheless, it remains to be determined whether and how miR-148a networks with miR-30c in terms of regulating PXR expression.

The suppression of PXR by miR-30c-1 may have important clinical consequences. In this study, we have shown that miR-30c-1 decreased the level of PXR mRNA accompanied by altered expression of CYP3A4, a prototypical gene of PXR (Klein and Zanger, 2013). The altered expression of CYP3A4, however, varied depending on time and the amounts of miR-30c-1. The overall expression of CYP3A4 mRNA was decreased under both basal and induction conditions (Fig. 3B, Left), but the decrease was not evident until later time-point (Fig. 3A, Right). Many investigators reported delayed response in miR-mediated suppression (Haenishch et al., 2011). The altered

expression of CYP3A4 by miR-30c-1 was in particular as it represented a mechanism secondarily to the suppression of PXR. Such an indirect mechanism required longer time to achieve the anticipated effect. On the other hand, the basal level of CYP3A4 mRNA at the early time-point was actually increased when small amounts of miR-30c-1 were used (Fig. 3A, Right). The precise mechanism on the increase remains to be determined. Nuclear receptors including PXR are known to interact with co-repressors and coactivators (Carnahan and Redinbo, 2005). It is the presence of a ligand that induces conformational changes from repressing to activating status of a target gene.

The functionality of PXR has been linked to a wide range of behavior changes of malignancy and some of them are opposing to each other (Pondugula SR, Mani, 2013). In some cases, activation of PXR up-regulates the expression of proapoptotic genes thus shows tumor suppressive activity. In other cases, PXR is linked to upregulation of antiapoptotic genes and favors tumor progression. Nevertheless, up-regulated expression of genes involved in drug eliminations is generally considered to be a major contributing factor to the development of chemoresistance. Interestingly, miR-30c has been shown to promote cell apoptosis, inhibit cell proliferation, reduce tumor clonogenicity and suppress metastatic potentials (Li et al., 2012; Bockhorn et al., 2013). In addition, the expression of miR-30c was significantly decreased in many chemoresistant cell lines (Sorrentino et al., 2008). It remains to be determined whether miR-30c members can overcome PXR-directed chemoresistance.

Interestingly, miR-148a, another silencer of PXR, has also been down-regulated in advanced cancer (Takahashi et al., 2012).

In summary, our study presents several important conclusions. Firstly, identification of miR-30c-1 as a negative regulator of PXR, along with the previous reporter on miR-148a, points the existence of a miR-network in regulating the functionality of PXR. Secondly, miR-30c-1 but not miR-30a suppressed PXR, underscoring the importance of the passenger strand in gene silencing. Thirdly, activation of PXR has been closely linked to the development of a spectrum of tumor behaviors, particularly in chemo resistance. miR-30c and miR-148a, on the other hand, have been associated with less aggressive behaviors of malignancy. These findings suggest that miR-30c/miR-148a-PXR represents favorable outcomes of chemotherapy, particularly those with a potent activating activity of PXR.

Footnotes

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[2] To whom correspondence should be addressed.

[3] The authors indicate no potential conflict of interest.

REFERENCES

Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR (2002) Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. *Biochem Biophys Res Commun*. **293:**145-49.

Bockhorn J, Yee K, Chang YF, Prat A, Huo D, Nwachukwu C, Dalton R, Huang S, Swanson KE, Perou CM, Olopade OI, Clarke MF, Greene GL, Liu H (2013) MicroRNA-30c targets cytoskeleton genes involved in breast cancer cell invasion. *Breast Cancer Res Treat*. **137:**373-82.

Bridge G, Monteiro R, Henderson S, Emuss V, Lagos D, Georgopoulou D, Patient R, Boshoff C (2012) The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis. *Blood*. **120:**5063-72.

Carnahan VE, Redinbo MR (2005) Structure and function of the human nuclear xenobiotic receptor PXR. *Curr Drug Metab*. **6:**357-67.

Cooper BW, Cho TM, Thompson PM, Wallace AD (2008) Phthalate induction of CYP3A4 is dependent on glucocorticoid regulation of PXR expression. *Toxicol Sci*. **103:**268-77.

Deenen MJ, Cats A, Beijnen JH, Schellens JH (2011) Part 3: Pharmacogenetic variability in phase II anticancer drug metabolism. *Oncologist*. **16:**992-1005.

Finnegan EF, Pasquinelli AE (2013) MicroRNA biogenesis: regulating the regulators. *Crit Rev Biochem Mol Biol*. **48:**51-68.

Haenisch S, Laechelt S, Bruckmueller H, Werk A, Noack A, Bruhn O, Remmler C, Cascorbi I (2011) Down-regulation of ATP-binding cassette C2 protein expression in HepG2 cells after rifampicin treatment is mediated by microRNA-379. *Mol Pharmacol*. **80:**314-20.

Ihunnah CA, Jiang M, Xie W (2011) Nuclear receptor PXR, transcriptional circuits and metabolic relevance. *Biochim Biophys Acta*. **1812:**956-63.

Karbiener M, Neuhold C, Opriessnig P, Prokesch A, Bogner-Strauss JG, Scheideler M (2011) MicroRNA-30c promotes human adipocyte differentiation and co-represses PAI-1 and ALK2. *RNA Biol.* **8:**850-60.

Kim T, Reitmair A (2013) Non-Coding RNAs: Functional Aspects and Diagnostic Utility in Oncology. *Int J Mol Sci*. **14:**4934-68.

Klein K, Zanger UM (2013) Pharmacogenomics of Cytochrome P450 3A4: Recent Progress Toward the "Missing Heritability" Problem. *Front Genet*. **4:**12.

Krasowski MD, Yasuda K, Hagey LR, Schuetz EG. (2005) Evolution of the pregnane x receptor: adaptation to cross-species differences in biliary bile salts. *Mol Endocrinol*. **19:**1720-39.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest*. **102:**1016-23.

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell.* **115:**787-98.

Lewis DF (2003) Human cytochromes P450 associated with the phase 1 metabolism of drugs and other xenobiotics: a compilation of substrates and inhibitors of the CYP1, CYP2 and CYP3 families. *Curr Med Chem*. **10:**1955-72.

Li XH, Ha CT, Fu D, Xiao M (2012) Micro-RNA30c negatively regulates REDD1 expression in human hematopoietic and osteoblast cells after gamma-irradiation. *PLoS One*. **7:**e48700.

Ma Y, Song X, Sachdeva K, Liu J, Li Y, Yang D, Deng R, Chichester CO, Yan B (2005) Clofibrate and perfluorodecanoate both up-regulate the expression of the pregnane X receptor but only clofibrate enhances its ligand-dependent induction of cytochrome P4503A23. *Biochem Pharmacol.* **69:**1363-71.

Modica S, Bellafante E, Moschetta A (2009) Master regulation of bile acid and xenobiotic metabolism via the FXR, PXR and CAR trio. *Front Biosci*. **14:**4719-45.

Parkinson A (2006) Biotransformation of xenobiotics, in Klaassen, C.D. the Casarett & Doull's Toxicology, the Basic Science of Poisons McGraw-Hill, New York, pp 139-62.

Pascussi JM, Drocourt L, Fabre JM, Maurel P, Vilarem MJ (2000) Dexamethasone induces pregnane X receptor and retinoid X receptor-alpha expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. *Mol Pharmacol*. **58:** 361-72.

Pondugula SR, Mani S (2013) Pregnane xenobiotic receptor in cancer pathogenesis and therapeutic response. *Cancer Lett*. **328:**1-9.

Ranjha R, Paul J (2013) Micro-RNAs in inflammatory diseases and as a link between inflammation and cancer. *Inflamm Res*. 2013 Apr;62(4):343-355.

Rottiers V, Näär AM (2012) MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol*. **13:**239-50.

Sachdeva K, Yan B, Chichester CO (2003) Lipopolysaccharide and cecal ligation/puncture differentially affect the subcellular distribution of the pregnane X receptor but consistently cause suppression of its target gene CYP3A. *Shock.* **19,** 469-74.

Song X, Li Y, Liu J, Mukundan M, Yan B (2005) Simultaneous substitution of phenylalaine-305 and aspartate-318 of rat PXR by the corresponding human residues abolishes the ability to transactivate the cytochrome P450 3A23 promoter. *J Pharmacol Exp Ther*. **312:**571-82.

Song X., Xie M, Zhang H, Li Y, Sachdeva K, Yan B (2004) The pregnane X receptor binds to response elements in a genomic context-dependent manner, and PXR activator rifampicin selectively alters the bindings among target genes. *Drug Metab Dispos.* **32:**35-42.

Sorrentino A, Liu CG, Addario A, Peschle C, Scambia G, Ferlini C (2008) Role of microRNAs in drug-resistant ovarian cancer cells. *Gynecol Oncol*. **111:**478-86.

Takagi S, Nakajima M, Mohri T, Yokoi T (2008) Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem*. **283:**9674-980.

Takahashi M, Cuatrecasas M, Balaguer F, Hur K, Toiyama Y, Castells A, Boland CR, Goel A (2012) The clinical significance of MiR-148a as a predictive biomarker in patients with advanced colorectal cancer. *PLoS One.* **7:**e46684.

Timsit YE, Negishi M (2007) CAR and PXR: the xenobiotic-sensing receptors. *Steroids*. **72:**231-46.

Vyhlidal CA, Gaedigk R, Leeder JS (2006) Nuclear receptor expression in fetal and pediatric liver: correlation with CYP3A expression. *Drug Metab Dispos*. **34:**131-7.

Yang J, Hao C, Yang D, Shi D, Song X, Luan X, Hu G, Yan B (2010) Pregnane X receptor is required for interleukin-6-mediated down-regulation of cytochrome P450 3A4 in human hepatocytes. *Toxicol Lett*. **197:**219-26.

Yang D, Shi D, Yang J, Deng R, Yan B (2011) Scoparone potentiates transactivation of the bile salt export pump gene and this effect is enhanced by cytochrome P450 metabolism but abolished by a PKC inhibitor. *Brit J Pharmacol.* **164,** 1547-1557.

Yu HW, Sze DM, Cho WC (2013) MicroRNAs Involved in Anti-Tumour Immunity. *Int J Mol Sci*. 2013 Mar 11;14(3):5587-607.

Xiao D, Chen YZ, Yang D, Yan B (2012) Age-related inducibility of carboxylesterases by the antiepileptic agent phenobarbital and implications in drug metabolism and lipid accumulation. *Biochem Pharmacol.* **84:**232-9.

Zheng XE, Wang Z, Liao MZ, Lin YS, Shuhart MC, Schuetz EG, Thummel KE (2012) Human PXR-mediated induction of intestinal CYP3A4 attenuates 1α,25-dihydroxyvitamin D_3 function in human colon adenocarcinoma LS180 cells. *Biochem Pharmacol*. **84:**391-401.

Table I Sequences of Oligonucleotides

Oligonucleotide	Sequence
PXR3142/3691Luc (XbaI)	5’-tgagcggctgcccttggg-3’
PXR3142/3691Luc (FseI)	5’-agaggactcccacagata-3’
PXR3690/4408Luc (XbaI)	5’-ggagtcctctagagagatgagaagccagga-3’
PXR3690/4408Luc (FseI)	5’-gtacattatttaattcct-3’
PXR3192/3691Luc (XbaI)	5’-gccctctgagccgccact-3’
PXR3242/3691Luc (XbaI)	5’-gacaatgccctgctggcc-3’
PXR3292/3691Luc (XbaI)	5’-ggctagcattcctcagga-3’
PXR3342/3691Luc (XbaI)	5’-ctgtagggagtgaagcca-3’
PXR3392/3691Luc (XbaI)	5’-aggtcaggaccatcagag-3’
PXR3442/3691Luc (XbaI)	5’-tgtggtctggggagaaat-3’
PXR3492/3691Luc (XbaI)	5’-aagggaccaagcgaccaa-3’
PXR3542/3691Luc (XbaI)	5’-ccacgtttgttcgcttcc-3’
PXR3592/3691Luc (XbaI)	5’-gtctcccacttcccactc-3’
PXR3642/3691Luc (XbaI)	5’-tccaggcctgtactcatc-3’

Numbered according to NM_003889.

Fig. 1. Suppression of PXR reporters derived from the 3'-untranslated region (UTR) (A) Diagrammatic presentation of PXR reporters The PXR3142/3691Luc reporter contains the cDNA segment from nucleotide 3142 to 3691, whereas the PXR3690/4408Luc reporter contains from nucleotide 3690 to 4408. The hatched box represents the luciferase coding sequence. (B) Identification of PXR suppressive miR(s) Cells (293T) were transiently transfected by GenJet version II with a mixture containing 50 ng of a miR precursor construct, 50 ng of a reporter, or the vector along with 5 ng of the *Renilla* luciferase plasmid. The transfected cells were cultured for 24 h, harvested and analyzed for luciferase activities with a Dual-Luciferase Reporter Assay System. The results were from one of two experiments in triplicate.

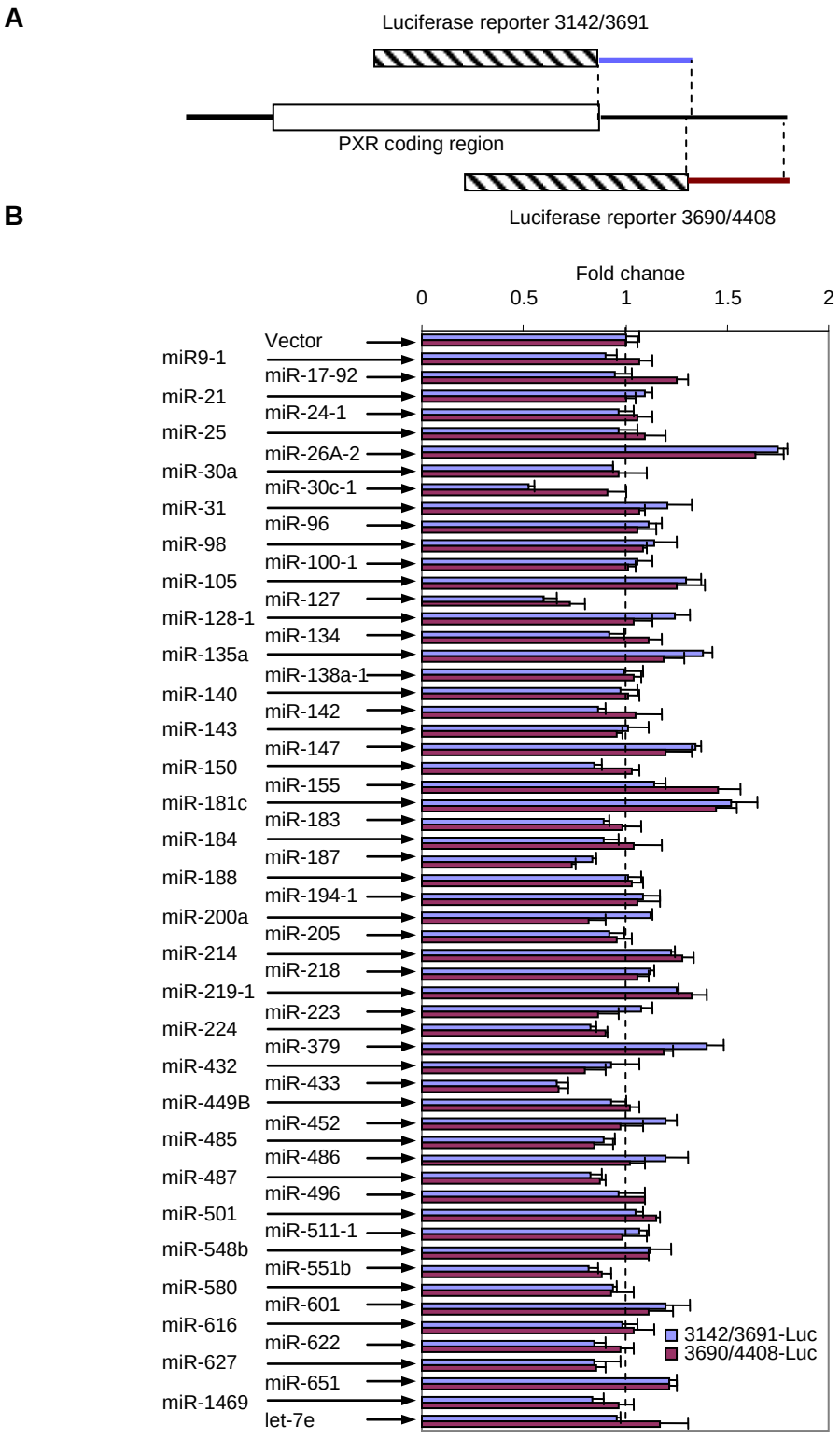

Figure 1 (Vachirayonstien, 2013)

Fig. 2. Characterization of miR-30c-1 on the suppression of PXR (A) Dissection of The PXR3142/2691Luc reporter for the identification of miR-30c-1 response Cells (293T) were transiently transfected by GenJet version II with a mixture containing 50 ng of the miR-30c-1 construct, 50 ng of a reporter, or the vector along with 5 ng of the *Renilla* luciferase plasmid. The transfected cells were cultured for 24 h, harvested and analyzed for luciferase activities with a Dual-Luciferase Reporter Assay System. (B) Suppression of PXR3542/3691Luc by miR-30c-1 in HepG2 and LS180 cell lines Cells were transfected by GenJet version II with a mixture containing 5-150 ng of the miR-30c-1 construct, 50 ng of a reporter (PXR3542/3691 or PXR3592/3691Luc, or the vector along with 5 ng of the *Renilla* luciferase plasmid. The vector plasmid was used to equalize the total amount of constructs. The transfected cells were cultured for 24 h, harvested and analyzed for luciferase activities as described above. Asterisk signs indicate statistical significance ($P < 0.05$).

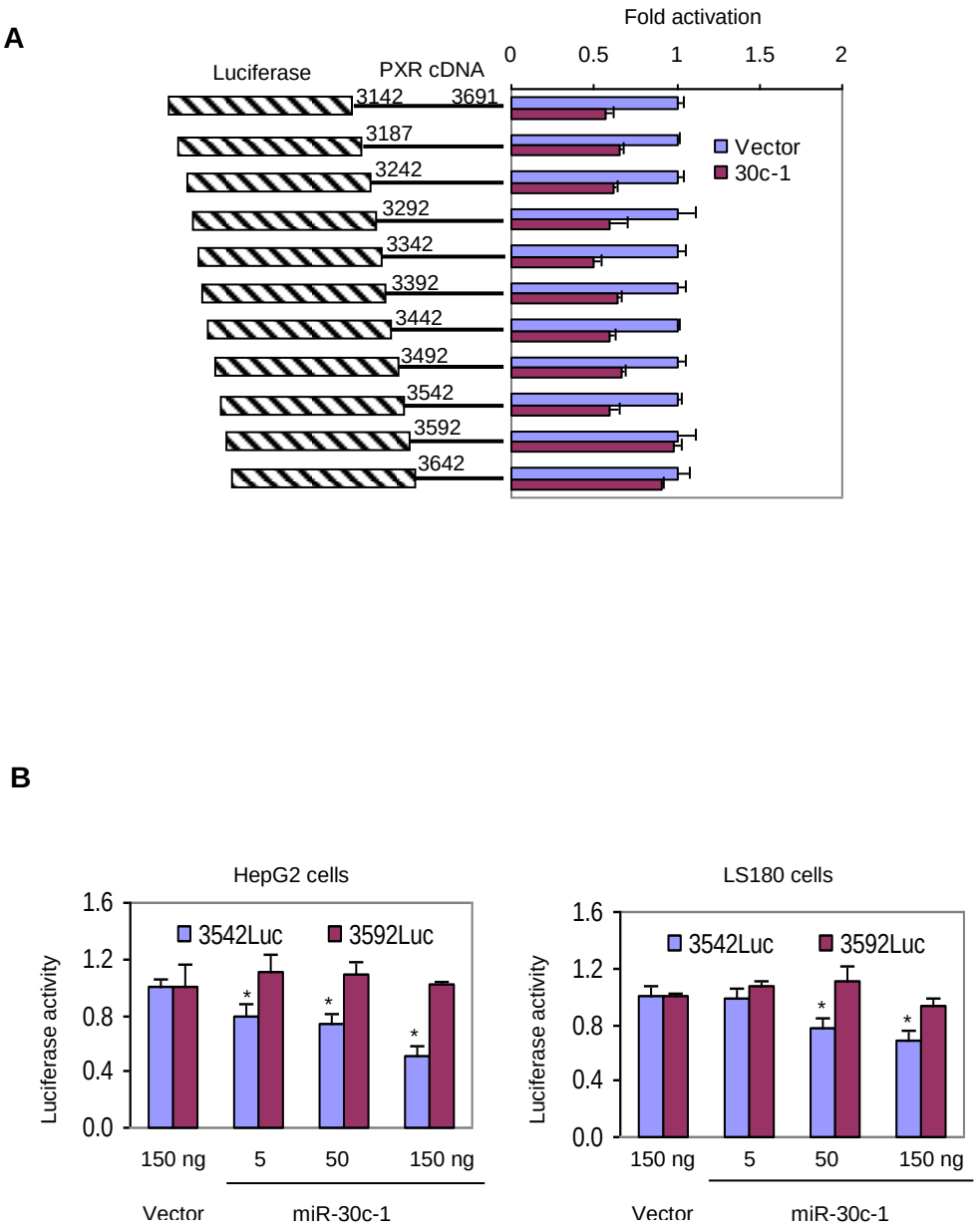

Figure 2 (Vachirayonstien, 2013)

Fig. 3. Effect of miR-30c-1 on the expression of CYP3A4 (A) Effect of miR-30c-1 on the expression of PXR and CYP3A4 Cells (LS180) were transfected by GenJet version II with the miR-30c-1 construct at 20, 200 and 800 ng. The vector plasmid was used to equalize the total amount of constructs. Cells were harvested 72 or 96 h post-transfection. Total RNA was isolated and the mRNA levels of PXR (Left) and CYP3A4 (Right) were determined by RT-qPCR. All experiments were performed three times in triplicate. Asterisk signs indicate statistical significance from vector-transfected cells ($P < 0.05$). (B) Effect of miR-30c-1 on the induction of CYP3A4 Cells (LS180) were transfected as described above and treated with DMSO or 10 μM rifampicin (RIF) 72 h post-transfection. The treated cells were collected 24 h thereafter and the level of CYP3A4 mRNA was determined by RT-qPCR. All experiments were performed three times in triplicate. Asterisk signs indicate statistical significance from vector-transfected cells or cells treated with DMSO ($P < 0.05$). (C) Effect of miR-30c-1 on the activation of CYP3A4-DP-Luc reporter Cells (LS180) were plated in 48 well-plates and transfected with a mixture containing 100 ng of the CYP3A4-DP-Luc reporter, 10 or 100 ng of miR-30c-1 or the vector along with the PXR expression plasmid and 5 ng of the *Renilla* luciferase plasmid. The vector was used to equalize the total amount of constructs. After incubation at 37°C for 24 h, the transfected cells were treated with 10 μM rifampicin (RIF) or the same volume of DMSO for 48 h. Luciferase activities were determined with a Dual-Luciferase Reporter Assay System and the reporter activity was normalized based on the *Renilla* luminescence signal. All experiments were performed three times in

triplicate. Asterisk signs indicate statistical significance from vector-transfected and RIF-treated cells ($P < 0.05$).

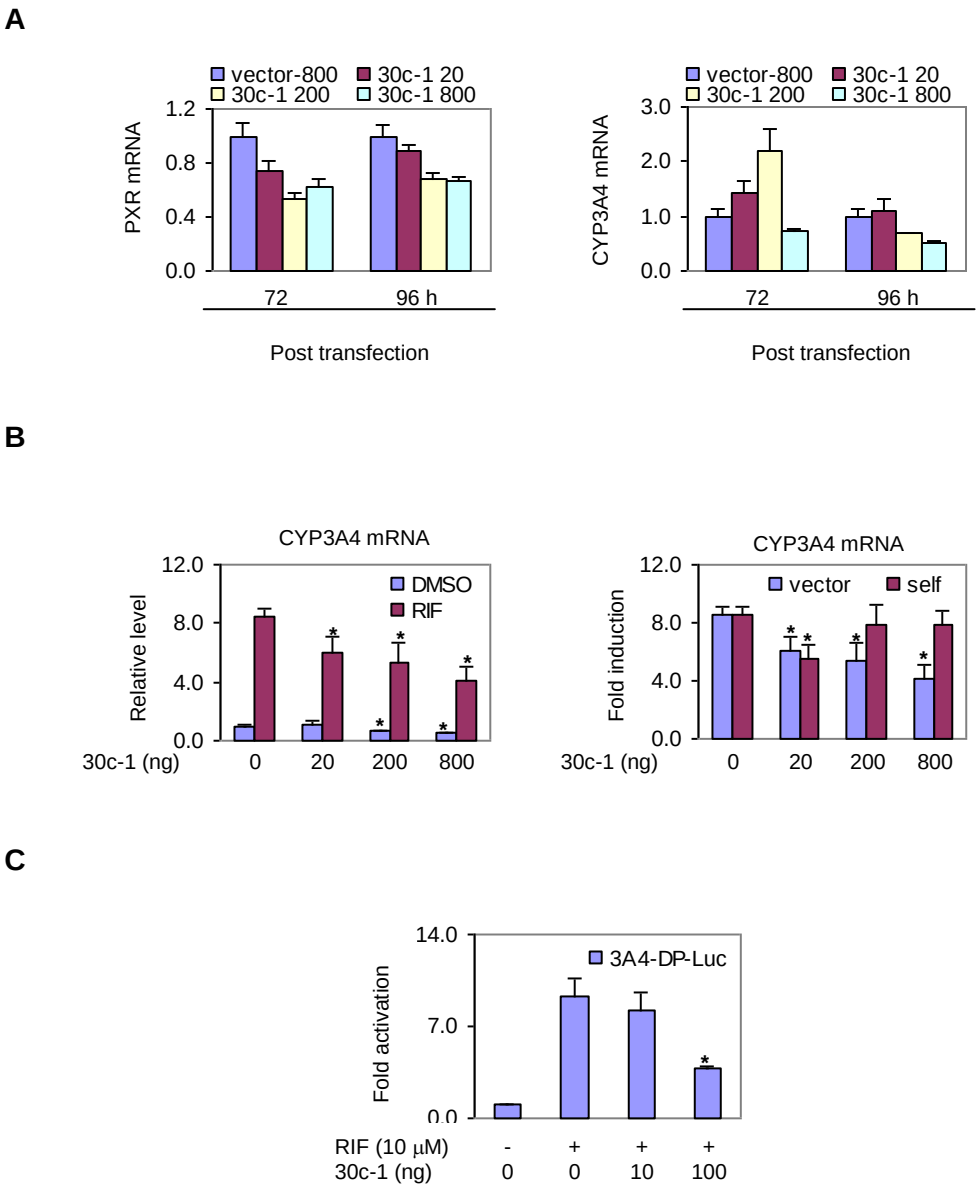

Figure 3 (Vachirayonstien, 2013)

Fig. 4. Sequence targeted by miR-30c-1 and its location in the 3'-UTR of PXR transcript (A) Sequence targeted by miR-30c-1 The sequence potentially targeted by miR-30c is identified by xx. Both miR-30c-1 and miR-30c-2 are shown as the passenger strand. (B) Location of the sequence targeted by miR-30c In addition to the location of the sequence targeted by miR-30c, the location of the sequence targeted by miR-148a is also shown. Specifically, miR-30c (both 30c-1 and 30c-2) likely targets the sequence from nucleotide 3583 to nucleotide 3660, whereas miR148a from 3359 to 3386, respectively. The sequence is numbered according to NM_003889.

A

```
PXR:          5′-ACCUCUAAUAGUCCUGUCUCCCAC-3′
                         || |  ||  |||||||
miR-30c-1:    3′-CCUCAUUUGUUGGG--AGAGGGUC-5′
```

```
PXR:          5′-ACCUCUAAUAGUCCUGUCUCCCAC-3′
                         || ||  ||| |||||||
miR-30c-2:    3′-UCUCAUUUGUC-GGA-AGAGGGUC-5′
```

B

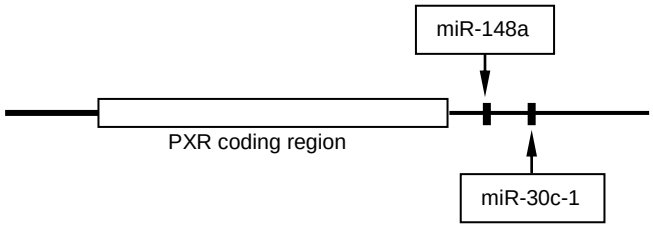

Figure 4 (Vachirayonstien, 2013)

CHAPTER 5 CONCLUSION

PXR is the master regulator of xenobiotic clearance. PXR regulates the expression of important genes in xenobiotic metabolism. CYP3A4, which metabolizes approximately 50% of marketed drugs, is mainly regulated by PXR (Guengerich, 2003 and Redinbo, 2004). Various diseases such as steatosis, diabetes, liver cancer, and viral infections are known to affect the expression of CYP3A4 and other PXR target genes. As a result, drug metabolism and elimination are altered in these conditions. ER stress and unfolded protein response is profoundly involved in the diseases. Thus, the effect of ER stress on PXR was studied to understand the consequences of ER stress on the expression of PXR and CYP3A4.

Effect of ER stress on PXR expression

ER stressors thapsigargin and brefeldin A caused ER stress in human hepatocytes and HepG2 cells, leading to down-regulation of PXR and reduced induction of CYP3A4. The expression of PXR was repressed at the transcriptional level. The promoter study revealed that HNF4α and C/EBPβ bound to the promoter region of PXR. Thapsigargin decreased HNF4α protein level whereas increased C/EBPβ LIP level. It is known that HNF4α generally functions as a positive transcriptional regulator and C/EBPβ LIP represses transcription of target genes (Cereghini 1996, Schrem et al., 2002, and Tsukada et al., 2011). Therefore, down-regulation of HNF4α and up-regulation of C/EBPβ LIP contributed to reduced transcription of PXR. The reporter assay showed that mutation at the HNF4α binding

site abolished the repressive effect of thapsigargin. Overexpression of HNF4α or C/EBPβ LAP alone in the HepG2 cells treated with thapsigargin returned PXR mRNA to the normal level. The EMSA experiment showed that HNF4α bound to the promoter region of PXR and the binding decreased once HepG2 cells were treated with thapsigargin. The experiment proved that HNF4α bound to the binding site and thapsigargin-induced ER stress decreased HNF4α protein level and thus decreased bounded HNF4α. The ChIP assay also showed that HNF4α bound to the binding site in living cells and the binding of HNF4α decreased in thapsigargin-treated cells. Both the EMSA and ChIP assay showed that C/EBPβ bound to the binding site next to the HNF4α binding site. Thus, C/EBPβ would also contribute to the expression of PXR.

Thapsigargin-induced ER stress up-regulated IL-6 expression in human hepatocytes. IL-6 repressed the expression of the reporters at the same sequences as thapsigargin. This finding suggested that IL-6 involved in repression of PXR by thapsigargin. IL-6 is a pro-inflammatory cytokines, inducing acute or chronic inflammation which involves in several diseases such as viral infections and liver cancer (Neurath et al., 2011 and Sun et al., 2012). Many P450 enzymes are known to down-regulated during inflammation. Thus, the evidence that thapsigargin induced IL-6 showed that there were connection between ER stress and inflammation which led to down-regulation of PXR and CYP3A4.

The study showed that ER stress induced by ER-stressors decreased PXR expression and PXR-mediated induction of CYP3A4. The reduction of CYP3A4 induction in ER stress could diminish capability to metabolize and eliminate drugs, which could affect drug toxicity and efficacy. ER stress was connected to

inflammation through the pro-inflammatory cytokine IL-6. Thus, diseases such as steatohepatitis, viral infections, and cancer in the state that involves ER stress and inflammation would decrease PXR expression.

miRNA regulating PXR

miRNAs are recognized as important regulators of gene expression at the post-transcriptional level. In human, more than 1000 miRNAs have been identified. However, target genes of many of these miRNAs are not known and one miRNA tends to interact with more than one mRNA. In addition, a gene can be regulated by several miRNAs. Thus, miRNAs play important roles in regulating protein coding gene. Identifying miRNA that targets a gene of interest is challenging because factors that dictate binding between miRNA and the gene are not well understood. In this study, miR-30c-1 was identified to interact with the 3'-UTR of PXR. The potential binding site of miR-30c-1 was located based on the computational analysis and the reporter assay. Overexpression system showed that miR-30c-1 decreased PXR mRNA level, suggesting that miR-30c-1 decreased stability of PXR mRNA. In the reporter system, miR-30c-1 reduced CYP3A4 induction by PXR. The miRNA also altered the basal expression of CYP3A4.

The present study showed that PXR was regulated by miR-30c-1. The miRNA decreased the expression of PXR and the CYP3A4 induction. Thus, miR-30c-1 may contribute to alteration in drug metabolism. Since the miRNA regulated the expression of PXR, the expression of miR-30c-1 is likely to affect the PXR expression. At

present, the regulation on the expression of miR-30c-1 is not known. miR-30c-1 might be related to diseases or biological conditions that could eventually affect PXR expression.

In summary, the studies showed that ER stress down-regulated the expression of PXR and reduced PXR-mediated CYP3A4 induction. The transcription of PXR was repressed. Decreasing of HNF4α and increasing of C/EBPβ LIP were shown to be the mechanism of repression. Since HNF4α and C/EBPβ bound to the promoter region of PXR, alteration in the levels of HNF4α and C/EBPβ in other conditions would also affect the expression of PXR. The transcriptional activation of CYP3A4 was decreased in ER stress due to reduction of PXR. Inflammation is known to down-regulated CYP3A4 and some other P450s enzymes and impair drug metabolism. Many of them are the PXR target genes. The pro-inflammatory cytokine IL-6 was also induced during ER stress, proving the link between ER stress and inflammation. Thus, ER stress could affect drug toxicity and efficacy. PXR was also regulated by miR-30c-1. The miRNA down-regulated the expression of PXR at the specific binding site on the 3'-UTR. Both the basal expression and induction of CYP3A4 were altered by miR-30c-1. Thus, miR-30c-1 affected both the expression and function of PXR and may contribute to alteration in drug metabolism.

REFERENCES

Cereghini S (1996) Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J.* **10(2):**267-82.

Guengerich FP (2003) Cytochromes P450, drugs, and diseases. *Mol Interv.* **3(4):**194-204.

Neurath MF, Finotto S (2011) IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev.* **22(2):**83-9.

Redinbo MR (2006) Human PXR forms a tryptophan zipper-mediated homodimer. Biochemistry. **45(28):**8579-89.

Schrem H, Klempnauer J, Borlak J (2002) Liver-enriched transcription factors in liver function and development. Part I: the hepatocytes nuclear factor network and liver-specific gene expression. *Pharmacol Rev.* **54(1):**129-58.

Sun B, Karin M (2012) Obesity, inflammation, and liver cancer. *J Hepatol.* **56(3):**704-13.

Tsukada J, Yoshida Y, Kominato Y, Auron PE (2011) The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine.* **54:**6-19.

BIBILOGRAPHY

Acharya P, Engel JC, Correia MA (2009) Hepatic CYP3A suppression by high concentrations of proteasomal inhibitors: a consequence of endoplasmic reticulum (ER) stress induction, activation of RNA-dependent protein kinase-like ER-bound eukaryotic initiation factor 2alpha (eIF2alpha)-kinase (PERK) and general control nonderepressible-2 eIF2alpha kinase (GCN2), and global translation shutoff. *Mol Pharmacol.* **76(3):**503-15.

Adams BD, Furneaux H, White BA (2007) The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-α (ERα) and represses ERα messenger RNA and protein expression in breast cancer cell lines. *Mol Endocrinol.* **21(5):**1132-47.

Adolph TE, Niederreiter L, Blumberg RS, Kaser A (2012) Endoplasmic reticulum stress and inflammation. *Dig Dis.* **30(4):**341-6.

Aitken AE, Morgan ET (2007) Gene-specific effects of inflammatory cytokines on cytochrome P450 2C, 2B6, and 3A4 mRNA levels in human hepatocytes. *Drug Metab Dispos.* **35(9):**1687-93.

Anastasov N, Bonzheim I, Rudelius M, Klier M, Dau T, Angermeier D, Duyster J, Pittaluga S, Fend F, Raffeld M, Quintanilla-Martinez L (2010) C/EBPβ expression in ALK-positive anaplastic large cell lymphomas is required for cell proliferation and is induced by the STAT3 signaling pathway. *Haematologica*. **95:**760-7.

Aouabdi S, Gibson G, Plant N (2006) Transcriptional regulation of the PXR gene: identification and characterization of a functional peroxisome proliferator-activated receptor alpha binding site within the proximal promoter of PXR. *Drug Metab Dispos.* **34(1):**138-44.

Back SH, Kaufman RJ (2012) Endoplasmic reticulum stress and type 2 diabetes. *Annu Rev Biochem*. **81:**767-93.

Barrett LW, Fletcher S, Wilton SD (2012) Regulation of eukaryotic gene expression by the untranslated gene regions and other non-coding elements. *Cell Mol Life Sci.* **69(21):**3613-34.

Bartel DP. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* **116:**281-97.

Bartel DP. (2009) MicroRNAs: Target recognition and regulatory functions. *Cell.* **136:**215-233.

Bauer B, Hartz AM, Fricker G, Miller DS (2004) Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood brain barrier. *Mol. Pharmacol.* **66:**413-419.

Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR (2002) Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. *Biochem Biophys Res Commun*. **293:**145-49.

Benbrook DM, Long A (2012) Integration of autophagy, proteasomal degradation, unfolded protein response and apoptosis. *Exp Oncol*. **34:**286-97.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci USA*. **95(21):**12208-13.

Blumberg B, Sabbagh W Jr, Juguilon H, Bolad J Jr, van Meter CM, Ong ES, Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **12(20):**3195-205.

Bock KW, Bock-Hennig BS (2010) UDP-glucuronosyltransferases (UGTs): from purification of Ah-receptor-inducible UGT1A6 to coordinate regulation of subsets of CYPs, UGTs, and ABC transporters by nuclear receptors. *Drug Metab Rev*. **42:**6-13.

Da Costa Martins PA, De Windt LJ (2012) Targeting microRNA targets. *Circ Res.* **111(5):**506-8.

Bockhorn J, Yee K, Chang YF, Prat A, Huo D, Nwachukwu C, Dalton R, Huang S, Swanson KE, Perou CM, Olopade OI, Clarke MF, Greene GL, Liu H (2013) MicroRNA-30c targets cytoskeleton genes involved in breast cancer cell invasion. *Breast Cancer Res Treat*. **137:**373-82.

Bode JG, Albrecht U, Häussinger D, Heinrich PC, Schaper F (2012) Hepatic acute phase proteins--regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF-κB-dependent signaling. *Eur J Cell Biol*. **91:**496-505.

Bridge G, Monteiro R, Henderson S, Emuss V, Lagos D, Georgopoulou D, Patient R, Boshoff C (2012) The microRNA-30 family targets DLL4 to modulate endothelial cell behavior during angiogenesis. *Blood*. **120:**5063-72.

Calì T, Ottolini D, Brini M (2011) Mitochondria, calcium, and endoplasmic reticulum stress in Parkinson's disease. *Biofactors*. **37:**228-40.

Carnahan VE, Redinbo MR (2005) Structure and function of the human nuclear xenobiotic receptor PXR. *Curr Drug Metab*. **6:**357-67.

Chen YZ, Shi D, Yang D, Yan B (2012) Antioxidant sulforaphane and sensitizer trinitrobenzene sulfonate induce carboxylesterase-1 through a novel element transactivated by nuclear factor-E2 related factor-2. *Biochem Pharmacol*. **84:**864-71.

di Masi A, De Marinis E, Ascenzi P, Marino M (2009) Nuclear receptors CAR and PXR: Molecular, functional, and biomedical aspects. *Mol Aspects Med.* **30(5):** 297-343.

Cooper BW, Cho TM, Thompson PM, Wallace AD (2008) Phthalate induction of CYP3A4 is dependent on glucocorticoid regulation of PXR expression. *Toxicol Sci.* **103:**268-77.

Deenen MJ, Cats A, Beijnen JH, Schellens JH (2011) Part 3: Pharmacogenetic variability in phase II anticancer drug metabolism. *Oncologist*. **16:**992-1005.

Ding X, Staudinger JL (2005) Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. J Pharmacol Exp Ther. **312:**849-856.

Ding Y, Dai X, Jiang Y, Zhang Z, Bao L, Li Y, Zhang F, Ma X, Cai X, Jing L, Gu J, Li Y (2012) Grape seed proanthocyanidin extracts alleviate oxidative stress and ER stress in skeletal muscle of low-dose streptozotocin- and high-carbohydrate/high-fat diet-induced diabetic rats. *Mol Nutr Food Res*. In press.

Donato MT, Jiménez N, Serralta A, Mir J, Castell JV, Gómez-Lechón MJ (2007) Effects of steatosis on drug-metabolizing capability of primary human hepatocytes. *Toxicol In Vitro*. **21:**271-6.

Dostalek M, Court MH, Yan B, Akhaghi F (2011) Significantly reduced cytochrome P4503A4 expression and activity in liver from human with diabetes mellitus. *Brit J Pharmacol*. **163:**937-47.

Finnegan EF, Pasquinelli AE (2013) MicroRNA biogenesis: regulating the regulators. *Crit Rev Biochem Mol Biol*. **48:**51-68.

Flamment M, Hajduch E, Ferré P, Foufelle F (2012) New insights into ER stress-induced insulin resistance. *Trends Endocrinol Metab*. **23:**381-90.

Goodwin B., Hodgson E, Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol. Pharmacol*. **56:**1329-39.

Guengerich FP (2003) Cytochromes P450, drugs, and diseases. *Mol Interv*. **3(4):**194-204.

Haenisch S, Laechelt S, Bruckmueller H, Werk A, Noack A, Bruhn O, Remmler C, Cascorbi I (2011) Down-regulation of ATP-binding cassette C2 protein expression in HepG2 cells after rifampicin treatment is mediated by microRNA-379. *Mol Pharmacol*. **80:**314-20.

Haeri M, Knox BE (2012) Endoplasmic Reticulum Stress and Unfolded Protein Response Pathways: Potential for Treating Age-related Retinal Degeneration. *J Ophthalmic Vis Res*. **7:**45-59.

Harlev E, Nevo E, Lansky EP, Ofir R, Bishayee A (2012) Anticancer potential of aloes: antioxidant, antiproliferative, and immunostimulatory attributes. *Planta Med*. **78:**843-52.

Ichinowatari G, Yamada M, Yaginuma H, Tsuyuki K, Tanimoto A, Ohuchi K (2002) Participation of prostaglandin E2 and platelet-activating factor in thapsigargin-induced production of interleukin-6. *Eur J Pharmacol*. **434(3):**187-96.

Ihunnah CA, Jiang M, Xie W (2011) Nuclear receptor PXR, transcriptional circuits and metabolic relevance. *Biochim Biophys Acta*. **1812:**956-63.

IPA, The World Health Organization (2007) Top 20 Causes of Mortality Throughout the World, *The World Health Report*. Retrieved March 3, 2013 from http://www.infoplease.com/ipa/A0779147.html

Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, Fardel O (2006) Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug Metab Dispos.* **34(10):**1756-63.

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS (2004) Human microRNA targets. *PLoS Biol.* **2(11):**e363.

Johnson N, Powis K, High S (2012) Post-translational translocation into the endoplasmic reticulum. *Biochim Biophys Acta*. In press

Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, Moore JT (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinaol.* **14(1):**27-39.

Karbiener M, Neuhold C, Opriessnig P, Prokesch A, Bogner-Strauss JG, Scheideler M (2011) MicroRNA-30c promotes human adipocyte differentiation and co-represses PAI-1 and ALK2. *RNA Biol.* **8:**850-60.

Ke PY, Chen SS (2012) Hepatitis C virus and cellular stress response: implications to molecular pathogenesis of liver diseases. *Viruses*. **4:**2251-90.

Kim T, Reitmair A (2013) Non-Coding RNAs: Functional Aspects and Diagnostic Utility in Oncology. *Int J Mol Sci*. **14:**4934-68.

Kim VN (2005). MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol.* **6:**376-85.

Klein K, Zanger UM (2013) Pharmacogenomics of Cytochrome P450 3A4: Recent Progress Toward the "Missing Heritability" Problem. *Front Genet*. **4:**12.

Kliewer SA, Goodwin B, Wilson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev*. **23(5):**687-702.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstorm RH, Perlmann T, Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell.* **92:**73-82.

Kolwankar D, Vuppalanchi R, Ethell B, Jones DR, Wrighton SA, Hall SD, Chalasani N (2007) Association between nonalcoholic hepatic steatosis and hepatic cytochrome P-450 3A activity. *Clin Gastroenterol Hepatol*. **5:**388-93.

Krasowski MD, Yasuda K, Hagey LR, Schuetz EG. (2005) Evolution of the pregnane x receptor: adaptation to cross-species differences in biliary bile salts. *Mol Endocrinol*. **19:**1720-39.

Kumar R, Thompson EB (1999) The structure of the nuclear hormone receptors. *Steroids.* **64:**310-19.

Kurose K, Koyano S, Ikeda S, Tohkin M, Hasegawa R, Sawada J (2005) 5' diversity of human hepatic PXR (NR1I2) transcripts and identification of the major transcription initiation site. *Mol Cell Biochem.* **273:**79-85.

Lamba JK, Lamba V, Yasuda K, Lin YS, Assem M, Thompson E, Strom S, Schuetz E (2004a) Expression of constitutive androstane receptor splice variants in human tissues and their functional consequences. *J Pharmacol Exp Ther.* **311:**811-821.

Lamba JK, Lin YS, Schuetz EG, Thummel KE (2002) Genetic contribution to variable human CYP3A-mediated metabolism. Adv Drug Deliv Rev. **54(10):**1271-94.

Lamba J, Lamba V, Strom S, Venkataramanan R, Schuetz E (2008) Novel single nucleotide polymorphisms in the promoter and intron 1 of human pregnane X receptor/NR1I2 and their association with CYP3A4 expression. *Drug Metab Dispos.* **36(1):**169-81.

Lamba V, Panetta JC, Strom S, Schuetz EG (2010) Genetic predictors of interindividual variability in hepatic CYP3A4 expression. *J Pharmacol Exp Ther.* **332(3):**1088-99.

Lamba V, Yasuda K, Lamba JK, Assem M, Davila J, Strom S, Schuetz EG. PXR (NR1I2): splice variants in human tissues, including brain, and identification of neurosteroids and nicotine as PXR activators (2004b) *Toxicol Appl Pharmacol*. **199(3):**251-65.

LeCluyse EL (2001) Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chem Biol Interact.* **134(3):**283-9.

Lee D, Shin C (2012) MicroRNA-target interactions: new insights from genome-wide approaches. *Ann N Y Acad Sci.* **1271:**118-28.

Leeman-Neill RJ, Wheeler SE, Singh SV, Thomas SM, Seethala RR, Neill DB, Panahandeh MC, Hahm ER, Joyce SC, Sen M, Cai Q, Freilino ML, Li C, Johnson DE, Grandis JR (2009) Guggulsterone enhances head and neck cancer therapies via inhibition of signal transducer and activator of transcription-3. *Carcinogenesis*. **30:**1848-56.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest*. **102:**1016-23.

Li C, Wang L, Huang K, Zheng L (2012) Endoplasmic reticulum stress in retinal vascular degeneration: protective role of resveratrol. *Invest Ophthalmol Vis Sci*. **53:**3241-9.

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. *Cell.* **115:**787-98.

Lewis DF (2003) Human cytochromes P450 associated with the phase 1 metabolism of drugs and other xenobiotics: a compilation of substrates and inhibitors of the CYP1, CYP2 and CYP3 families. *Curr Med Chem*. **10:**1955-72.

Li XH, Ha CT, Fu D, Xiao M (2012) Micro-RNA30c negatively regulates REDD1 expression in human hematopoietic and osteoblast cells after gamma-irradiation. *PLoS One*. **7:**e48700.

Li Y, Bevilacqua E, Chiribau CB, Majumder M, Wang C, Croniger CM, Snider MD, Johnson PF, Hatzoglou M (2008) Differential control of the CCAAT/enhancer-binding protein beta (C/EBPbeta) products liver-enriched transcriptional activating protein (LAP) and liver-enriched transcriptional inhibitory protein (LIP) and the regulation of gene expression during the response to endoplasmic reticulum stress. *J Biol Chem*. **283:**22443-56.

Lin S, Sun S, Hu J (2012) Molecular basis for sculpting the endoplasmic reticulum membrane. *Int J Biochem Cell Biol*. **44:**1436-43.

Liver Foundation (2009) Liver awareness month. Retrieved March 3, 2013 from http://www.liverfoundation.org/chapters/lam2009.

Lu J, Clark AG (2012) Impact of microRNA regulation on variation in human gene expression. *Genome Res.* **22(7):**1243-54.

Ma X, Idle JR, Gonzalez FJ (2008) The pregnane X receptor: from bench to bedside. *Expert Opin Drug Metab Toxicol.* **4(7):**895-908.

Ma Y, Song X, Sachdeva K, Liu J, Li Y, Yang D, Deng R, Chichester CO, Yan B (2005) Clofibrate and perfluorodecanoate both up-regulate the expression of the pregnane X receptor but only clofibrate enhances its ligand-dependent induction of cytochrome P4503A23. *Biochem Pharmacol*. **69:**1363-71.

Mai I, Bauer S, Perloff ES, Johne A, Uehleke B, Frank B, Budde K, Roots I (2004) Hyperforin content determines the magnitude of the St John's wort-cyclosporine drug interaction. *Clin Pharmacol Ther.* **76(4):**330-40.

Mills JB, Rose KA, Sadagopan N, Sahi J, de Morais SM (2004) Induction of drug metabolism enzymes and MDR1 using a novel human hepatocyte cell line. J *Pharmacol Exp Ther.* **309(1):**303-9.

Mohri T, Nakajima M, Fukami T, Takamiya M, Aoki Y, Yokoi T (2010) Human CYP2E1 is regulated by miR-378. *Biochem Pharmacol.* **79(7):**1045-1052.

Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, Kliewer SA (2000) St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci USA.* **97(13):**7500-2.

Moore JT, Moore LB, Maglich JM, Kliewer SA (2003) Functional and structural comparison of PXR and CAR. *Biochim Biophys Acta.* **1619:**235-8.

Morgan ET (1997) Regulation of cytochromes P450 during inflammation and infection. *Drug Metab Rev.* **29:**1129-1188.

Murakami Y, Tanaka T, Murakami H, Tsujimoto M, Ohtani H, Sawada Y (2006) Pharmacokinetic modeling of the interaction between St John's wort and ciclosporin A. *Br J Clin Pharmacol.* **61(6):**671-6.

Nakajima M, Yokoi T (2011) MicroRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. *Pharmacol Ther.* **131(3):**330-7.

Neurath MF, Finotto S (2011) IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine Growth Factor Rev.* **22(2):**83-9.

Nickel W (2010) Pathways of unconventional protein secretion. *Curr Opin Biotechnol*. **21:**621-6.

Niemi M, Backman JT, Fromm MF, Neuvonen PJ, Kivisto KT (2003) Pharmacokinetic interactions with rifampicin : clinical relevance. *Clin Pharmacokinet.* **42(9):**819-50.

Noble SM, Carnahan VE, Moore LB, Luntz T, Wang H, Ittoop OR, Stimmel JB, Davis-Searles PR, Watkins RE, Wisely GB, LeCluyse E, Tripathy A, McDonnell DP, Redinbo MR (2006) Human PXR forms a tryptophan zipper-mediated homodimer. Biochemistry. **45(28):**8579-89.

Ostberg T, Bertilsson G, Jendeberg L, Berkenstam A, Uppenberg J (2002) Identification of residues in the PXR ligand binding domain critical for species specific and constitutive activation. *Eur J Biochem.* **269(19):**4896-904.

Ozdemir V, Kalow W, Tang BK, Paterson AD, Walker SE, Endrenyi L, Kashuba AD (2000) Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method. *Pharmacogenetics.* **10(5):**373-88.

Pagliassotti MJ (2012) Endoplasmic reticulum stress in nonalcoholic fatty liver disease. *Annu Rev Nutr*. **32:**17-33.

Paine MF, Hart HL, Ludington SS, Haining RL, Rettie AE, Zeldin DC (2006) The human intestinal cytochrome P450 pie. *Drug Metab Dispos.* **34(5):**880-6.

Pan YZ, Gao W, Yu AM (2009) MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab Dispos.* **37:**2112-117.

Parkinson A (2006) Biotransformation of xenobiotics, in Klaassen, C.D. the Casarett & Doull's Toxicology, the Basic Science of Poisons McGraw-Hill, New York, pp 139-62.

Pascussi JM, Drocourt L, Fabre JM, Maurel P, Vilarem MJ (2000) Dexamethasone induces pregnane X receptor and retinoid X receptor-alpha expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. *Mol Pharmacol*. **58:** 361-72.

Pascussi JM, Gerbal-Chaloin S, Pichard-Garcia L, Daujat M, Fabre JM, Maurel P, Vilarem MJ (2000) Interleukin-6 negatively regulates the expression of pregnane X receptor and constitutively activated receptor in primary human hepatocytes. *Biochem Biophys Res Commun.* **274(3):**707-13.

Pesole G, Liuni S, Grillo G, Saccone C (1997) Structural and compositional features of untranslated regions of eukaryotic mRNAs. *Gene.* **205(1-2):**95-102.

Pondugula SR, Mani S (2013) Pregnane xenobiotic receptor in cancer pathogenesis and therapeutic response. *Cancer Lett*. **328:**1-9.

Ranjha R, Paul J (2013) Micro-RNAs in inflammatory diseases and as a link between inflammation and cancer. *Inflamm Res.* Feb 17 [Epub ahead of print].

Redinbo MR (2004) Promiscuity: what protects us, perplexes us. *Drug Discovery Today*. **9:**431-32.

Renton KW (2004) Cytochrome P450 regulation and drug biotransformation during inflammation and infection. *Curr Drug Metab.* **5(3):**235.

Ri M, Tashiro E, Oikawa D, Shinjo S, Tokuda M, Yokouchi Y, Narita T, Masaki A, Ito A, Ding J, Kusumoto S, Ishida T, Komatsu H, Shiotsu Y, Ueda R, Iwawaki T, Imoto M, Iida S (2012) Identification of Toyocamycin, an agent cytotoxic for multiple myeloma cells, as a potent inhibitor of ER stress-induced XBP1 mRNA splicing. *Blood Cancer J*. **2:**e79.

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A (2004) Identification of mammalian microRNA host genes and transcription units. *Genome Res.* **14(10A):**1902-10.

Rottiers V, Näär AM (2012) MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol*. **13:**239-50.

Sachdeva K, Yan B, Chichester CO (2003) Lipopolysaccharide and cecal ligation/puncture differentially affect the subcellular distribution of the pregnane X receptor but consistently cause suppression of its target gene CYP3A. *Shock*. **19:**470-75.

Salido GM, Sage SO, Rosado JA (2009) Biochemical and functional properties of the store-operated Ca2+ channels. *Cell Signal*. **21:**457-61.

Sandanaraj E, Lal S, Selvarajan V, Ooi LL, Wong ZW, Wong NS, Ang PC, Lee EJ, Chowbay B (2008) PXR pharmacogenetics: association of haplotypes with hepatic CYP3A4 and ABCB1 messenger RNA expression and doxorubicin clearance in Asian breast cancer patients. *Clin Cancer Res.* **14(21):**7116-26.

Santoro A, Mancini E, Ferramosca E, Faenza S (2007) Liver support systems. *Contrib Nephrol*. **156:**396-404.

Schipani A, Siccardi M, D'Avolio A, Baietto L, Simiele M, Bonora S, Rodriguez Novoa S, Cuenca L, Soriano V, Chierakul N, Saguenwong N, Chuchuttaworn C, Hoskins JM, Dvorak AM, McLeod HL, Davies G, Khoo S, Back DJ, Di Perri G, Owen A (2010) Population pharmacokinetic modeling of the association between 63396C->T pregnane X receptor polymorphism and unboosted atazanavir clearance. *Antimicrob Agents Chemother.* **54(12):**5242-50.

Schrenk D, Baus PR, Ermel N, Klein C, Vorderstemann B, Kauffmann HM (2001) Up-regulation of transporters of the MRP family by drugs and toxins. *Toxicol Lett.* **120(1-3):**51-7.

Sevignani C, Calin GA, Siracusa LD, Croce CM (2006) Mammalian microRNAs: a small world for fine-tuning gene expression. *Mamm Genome.* **17(3):**189-202.

Shen CN, Slack JM, Tosh D (2000) Molecular basis of transdifferentiation of pancreas to liver. *Nat Cell Biol*. **2:**879-87.

Shi D, Yang D, Prinssen EP, Brian E. Davies BE, Yan B (2011) Surge in expression of carboxylesterase-1 during the post-natal stage enables a rapid gain of the capacity to activate the anti-influenza prodrug oseltamivir. *J Infect Dis.* **203:**937-42.

Shi D, Yang D, Yan B (2010) Dexamethasone transcriptionally increases the expression of the pregnane X receptor and synergistically enhances pyrethroid deltamethrin in the induction of cytochrome P450 3A23. *Biochem Pharmacol*. **80:**1274-83.

Shia CS, Hou YC, Tsai SY, Huieh PH, Leu YL, Chao PD (2010) Differences in pharmacokinetics and ex vivo antioxidant activity following intravenous and oral administrations of emodin to rats. *J Pharm Sci*. **99:**2185-95.

Siccardi M, D'Avolio A, Baietto L, Gibbons S, Sciandra M, Colucci D, Bonora S, Khoo S, Back DJ, Di Perri G, Owen A. (2008) Association of a single-nucleotide polymorphism in the pregnane X receptor (PXR 66396C→T) with reduced concentrations of unboosted atazanavir. *Clin infect Dis.* **47(9):**1222-5.

Song X, Li Y, Liu J, Mukundan M, Yan B (2005) Simultaneous substitution of phenylalaine-305 and aspartate-318 of rat PXR by the corresponding human residues abolishes the ability to transactivate the cytochrome P450 3A23 promoter. *J Pharmacol Exp Ther*. **312:**571-82.

Song X., Xie M, Zhang H, Li Y, Sachdeva K, Yan B (2004) The pregnane X receptor binds to response elements in a genomic context-dependent manner and its activator rifampicin selectively alters the bindings among target genes. *Drug Metab Dispos*. **32:**35-42.

Sorrentino A, Liu CG, Addario A, Peschle C, Scambia G, Ferlini C (2008) Role of microRNAs in drug-resistant ovarian cancer cells. *Gynecol Oncol*. **111:**478-86.

Staudinger JL, Xu C, Biswas A, Mani S (2011) Post-translational modification of pregnane X receptor. *Pharmacol Res*. **64(1):**4-10.

Sun B, Karin M (2012) Obesity, inflammation, and liver cancer. *J Hepatol.* **56(3):**704-13.

Takagi S, Nakajima M, Kida K, Yamamura Y, Fukami T, Yokoi T (2010) MicroRNAs regulate human hepatocytes nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle. *J Biol Chem.* **285(7):**4415-22.

Takagi S, Nakajjima M, Mohri T, Yokoi T (2008) Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem*. **283:**9674-80.

Takahashi M, Cuatrecasas M, Balaguer F, Hur K, Toiyama Y, Castells A, Boland CR, Goel A (2012) The clinical significance of MiR-148a as a predictive biomarker in patients with advanced colorectal cancer. *PLoS One.* **7:**e46684.

Timsit YE, Negishi M (2007) CAR and PXR: the xenobiotic-sensing receptors. *Steroids*. **72:**231-46.

Tompkins LM, Sit TL, Wallace AD (2008) Unique transcription start sites and distinct promoter regions differentiate the pregnane X receptor (PXR) isoforms PXR 1 and PXR 2. *Drug Metab Dispos*. **36:**923-9.

Toriyabe T, Nagata K, Takada T, Aratsu Y, Matsubara T, Yoshinari K, Yamazoe Y (2009) Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics. *Mol Pharmacol*. **75:**677-84.

Tsukada J, Yoshida Y, Kominato Y, Auron PE (2011) The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine*. **54:**6-19.

Urguhart BL, Tirona RG, Kim RB (2007) Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *J Clin Pharmacol.* **47(5):**566-78.

Villarroya F, Domingo P, Giralt M (2010) Drug-induced lipotoxicity: lipodystrophy associated with HIV-1 infection and antiretroviral treatment. *Biochim Biophys Acta*. **801:**392-9.

Vreugdenhil E, Verissimo CS, Mariman R, Kamphorst JT, Barbosa JS, Zweers T (2009) MicroRNA 18 and 124a down-regulate the glucocorticoid receptor: implications for glucocorticoid responsiveness in the brain. *Endocrinology.* **150(5):**2220-8.

Vyhlidal CA, Gaedigk R, Leeder JS (2006) Nuclear receptor expression in fetal and pediatric liver: correlation with CYP3A expression. *Drug Metab Dispos*. **34:**131-7.

Wang S, Kaufman RJ (2012) The impact of the unfolded protein response on human disease. *J cell Biol.* **197(7):**857-67.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA, Redinbo MR (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Sciences.* **292(5525):**2329-33.

Welm AL, Timchenko NA, Darlington GJ (1999) C/EBPalpha regulates generation of C/EBPbeta isoforms through activation of specific proteolytic cleavage. *Mol Cell Biol*. **19:**1695-704.

microRNA.org (2013) microRNA.org – Targets and Expression. Retrieved January 2, 2013 from http://www.microRNA.org.

Xiao D, Chen YZ, Yang D, Yan B (2012) Age-related inducibility of carboxylesterases by the antiepileptic agent phenobarbital and implications in drug metabolism and lipid accumulation. *Biochem Pharmacol.* **84:**232-9.

Xiao D, Yang D, Charpentier M, Yan B (2012) Regulation of carboxylesterase-2 expression by p53 family proteins and enhanced anticancer activities among 5-fluorouracil, irinotecan and doxazolidine prodrug. *Brit J Pharmacol*. In press

Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, Evans RM (2000) Humanzied xenobiotic response in mice expressing nuclear receptor SXR. *Nature.* **406(6794):**435-9.

Xiong J, Yu D, Wei N, Fu H, Cai T, Huang Y, Wu C, Zheng X, Du Q, Lin D, Liang Z (2010) An estrogen receptor alpha suppressor, microRNA-22, is downregulated in estrogen receptor alpha-positive human breast cancer cell lines and clinical samples. *FEBS J.* **277(7):**1684-94.

Xu C, Bailly-Maitre B, Reed JC (2005) Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest.* **115(10):**2656-64.

Xu C, Li CY, Kong AN (2005) Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res.* **28(3):**249-68.

Xu K, Zhu XP (2012) Endoplasmic reticulum stress and prion diseases. *Rev Neurosci*. **23:**79-84.

Yang D, Shi D, Yang J, Deng R, Yan B (2011) Scoparone potentiates transactivation of the bile salt export pump gene and this effect is enhanced by cytochrome P450 metabolism but abolished by a PKC inhibitor. *Brit J Pharmacol*. **164:**1547-57.

Yang D, Yang J, Shi D, Black C, Deng R, Yan B (2012) The hypolipidemic agent Z-guggulsterone: metabolism interplays with induction of cholesteryl ester hydrolase CES1 and bile salt export pump. *J Lipid Res*. **53:**529-39.

Yang D, Shi D, Yang J, Deng R, Yan B (2011) Scoparone potentiates transactivation of the bile salt export pump gene and this effect is enhanced by cytochrome P450 metabolism but abolished by a PKC inhibitor. *Brit J Pharmacol.* **164,** 1547-1557.

Yang J, Hao C, Yang D, Shi D, Song X, Luan X, Hu G, Yan B (2010) Pregnane X receptor is required for interleukin-6-mediated down-regulation of cytochrome P450 3A4 in human hepatocytes. *Toxicol Lett*. **197:**219-26.

Yoshida H (2007) ER stress and diseases. *FEBS J.* **274(3):**630-58.

Yu HW, Sze DM, Cho WC (2013) MicroRNAs Involved in Anti-Tumour Immunity. *Int J Mol Sci*. 2013 Mar 11;14(3):5587-607.

Zhang J, Kuehl P, Green ED, Touchman JW, Watkins PB, Daly A, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Wrighton SA, Hancock M, Kim RB, Strom S, Thummel K, Russell CG, Hudson JR Jr, Schuetz EG, Boguski MS (2001) The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics*. **11:**555-72.

Zhang K, Kaufman RJ (2008) From endoplasmic-reticulum stress to the inflammatory response. *Nature.* **454(7203):**455-62.

Zhao JJ, Lin J, Yang H, Kong W, He L, Ma X, Coppola D, Cheng JQ (2008) MicroRNA-221/222 negatively regulates estrogen receptor alpha and is associated with tamoxifen resistance in breast cancer. *J Biol Chem.* **283(45):**31079-86.

Zheng XE, Wang Z, Liao MZ, Lin YS, Shuhart MC, Schuetz EG, Thummel KE (2012) Human PXR-mediated induction of intestinal CYP3A4 attenuates 1α,25-dihydroxyvitamin D_3 function in human colon adenocarcinoma LS180 cells. *Biochem Pharmacol*. **84:**391-401.