Transcriptional Regulation of CYP3A4 and CYP2D6 by Small Heterodimer Partner

BY

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THESIS

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LIST OF ABBREVIATIONS

ABCB4	ATP-Binding Cassette, Subfamily B, Member 4
ATRA	All-trans Retinoic Acid
CAR	Constitutive Androstane Receptor
cDNA	Complementary DNA
CYP	Cytochrome P450
DME	Drug Metabolizing Enzyme
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EC_{50}	Half Minimum Effective Concentration
FASN	Fatty Acid Synthase
FXF	Farnesoid X Receptor
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GR	Glucocorticoid Receptor
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic Acid
HNF4α	Hepatic Nuclear Factor 4 alpha
HPLC	High Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography–Mass Spectrometry
LXR	Liver X Receptor
MEM	Minimum Essential Media
mi-RNA	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NR	Nuclear Receptor
PCR	Polymerase Chain Reaction

LIST OF ABBREVIATIONS (CONTINUED)

PPARα	Peroxisome Proliferator-activated Receptor alpha
PXR	Pregnane X Receptor
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Element
RXR	Retinoid X Receptor
SHP	Small Heterodimer Partner
SREBF1	Sterol Regulatory Element Binding Transcription Factor 1
TNFα	Tumor Necrosis Factor alpha
UGT	UDP Glucuronosyltransferase

SUMMARY

Small heterodimer partner (SHP) is a transcriptional corepressor of a number of ligand regulated nuclear receptors (NR) and orphan receptors, and represses their target genes expression. Transcriptional regulation tightly controls the mRNA expression of drug-metabolizing enzymes (DMEs), and many crucial DMEs, including cytochrome P450 (CYP) 3A4 and 2D6, showed significant correction between enzyme activity and messenger ribonucleic acid (mRNA) expression. Consequently, studying transcriptional regulation mechanism of important DMEs can help better understand and predict of the elimination of drugs, and effectively achieve personalized medicine.

Farnesoid X receptor (FXR) functions as a regulator of bile acid and lipid homeostasis, and is recognized as a promising therapeutic target for metabolic diseases. The biological function of FXR is in part mediated by SHP ligand-activated FXR enhances SHP expression, and SHP in turn represses activity of multiple transcription factors. Therefore, The effect of FXR activation on major DMEs, including CYP3A4, is of great importance.

Aside from FXR activation, SHP is the target gene of several signaling pathways, and various inducers significantly increase its expression. Previous study in Jeong laboratory showed that elevated CYP2D6 level during pregnancy is associated with reduced all-trans retinoic acid (ATRA) level, and SHP is a key modulator of CYP2D6. Taken together, we hypothesized that ATRA regulates CYP2D6 expression through SHP.

In Chapter 1, we investigated the effect of FXR activation on the expression of the major drug-metabolizing enzyme, CYP3A4. The effects of GW4064, a synthetic agonist of FXR, on expression and activity of CYP3A4 were examined in primary human hepatocytes by using quantitative real-time polymerase chain reaction (qRT-PCR) and S9 phenotyping.

The results in Chapter 1 showed that in human hepatocytes, treatment of GW4064 (1 μM) for 48 hours resulted in 75% decrease in CYP3A4 mRNA expression and 25% decrease in CYP3A4 activity, accompanied by ~3-fold increase in SHP mRNA expression. In HepG2 cells, SHP repressed transactivation of CYP3A4 promoter by pregnane X receptor (PXR), constitutive androstane receptor (CAR), and glucocorticoid receptor (GR). Interestingly, GW4064 did not repress expression of CYP2B6, another target gene of PXR and CAR; GW4064 was shown to enhance CYP2B6 promoter activity. In conclusion, GW4064 represses CYP3A4 expression in human hepatocytes, potentially through upregulation of SHP expression and subsequent repression of CYP3A4 promoter activity. Clinically significant drug-drug interaction involving FXR agonists and CYP3A4 substrates may occur.

In Chapter 2, we characterized the effect of ATRA, another potential inducer of SHP, on CYP2D6 expression *in vitro* and *in vivo*. mRNA expression levels of various genes in primary human hepatocytes and transgenic mice treated with ATRA or control were examined using qRT-PCR. The effect of ATRA on *CYP2D6* and *SHP* promoter transcription activity was further evaluated using luciferase assay.

The results showed in both human hepatocytes and transgenic mices, CYP2D6 mRNA expression level was significantly repressed by ~70% *in vitro*, and by ~50% *in vivo*. The repressed CYP2D6 expression is accompanied by induced SHP expression which was elevated by ~3-fold *in vitro* and ~5-fold *in vivo*, respectively. The correlation between SHP and CYP2D6 was further confirmed using mice with Tg-*CYP2D6* with *Shp* knockout compared with Tg-CYP2D6 wild-type mice. The reduction of CYP2D6 mRNA expression by ATRA is abrogated in mice with *Shp*(-/-) genotype. Overall, the results indicated that SHP is a key regulator in ATRA-

induced CYP2D6 repression, and the molecular mechanism of the effect ATRA on SHP expression needs further study and validation.

1. Introduction

1.1 Transcriptional regulation of CYP3A4 by FXR activation.

1.1.1 Function of FXR as a transcription factor.

FXR is a ligand-activated nuclear receptor and a member of the steroid/thyroid hormone receptors. FXR is highly expressed in liver, intestine, kidney and adrenal gland (Forman, Goode et al. 1995, Parks, Blanchard et al. 1999, Wang, Chen et al. 1999). Endogenous bile acids are ligands for FXR, and binding of bile acids to FXR leads to regulation of genes involved in bile acid homeostasis and glucose metabolism (Wang, Chen et al. 1999, Eloranta and Kullak-Ublick 2008). Based on the biological function of FXR as a regulator of lipid and glucose metabolism, many FXR agonists are currently being investigated as potential therapeutic agents for the treatment of metabolic disorders including hypercholesterolemia (Thomas, Pellicciari et al. 2008, Fiorucci, Zampella et al. 2012).

1.1.2 Transcriptional regulation of FXR target genes by FXR activation.

FXR is known to cause both transactivation and transrepression of its target gene promoters (Hollman, Milona et al. 2012). For example, ligand-activated FXR transrepresses the promoter of a drug-metabolizing enzyme UDP gllucuronosyltransferase (UGT) 2B7 in Caco-2 cells (Lu, Heydel et al. 2005) while FXR transactivates the promoter of SHP by direct binding to –333/-320 of *SHP* (Goodwin, Jones et al. 2000). SHP is a nuclear receptor that lacks a DNA-binding domain, and it represses expression of genes involved in bile acid synthesis by interfering with action of other transcriptional factors (Lee, Dell et al. 2000, Wang, Lee et al. 2002).

1.1.3 Transcriptional regulation of CYP3A4, the major DME.

Cytochrome P450s are the major drug-metabolizing enzymes responsible for the oxidative biotransformation of drugs, and CYP3A4 is the most prevalent among all CYP

enzymes. Expression of P450 enzymes is highly inducible by endo- and xeno-biotics, mainly via actions of ligand-activated transcription factors PXR and CAR (Gao and Xie 2010). PXR and CAR, upon binding to respective ligands, transactivate CYP3A4 and CYP2B6 promoters and increase their expression (Zhou 2008). This subsequently leads to clinically significant drug-drug interactions such that the doses of CYP3A4 substrate drugs need to be adjusted to prevent a lack of drug efficacy. Additionally, corticosteroids (at physiological concentrations) enhance PXR transactivation of CYP3A4 promoter by increasing the expression of PXR and its binding partner retinoid X receptor (RXR) as well as the transcriptional activity of PXR, via GR (Pascussi, Drocourt et al. 2001).

Previously, FXR was shown to transactivate CYP3A4 promoter in HepG2 cells by binding to response elements located in the distal regulatory region of *CYP3A4* (Gnerre, Blattler et al. 2004). Also, in mice, GW4064 (a synthetic agonist of FXR) was shown to enhance expression of Cyp3a11, the murine homolog of human CYP3A4 (Gnerre, Blattler et al. 2004). Together, these results suggest that FXR activators that are currently under development will likely increase CYP3A4-mediated drug metabolism in humans. However, extrapolation of the results to humans appears difficult due to minimal expression of key transcription factors involved in P450 regulation (e.g., PXR or CAR) in HepG2 cells (Hart, Li et al. 2010, Guo, Dial et al. 2011) and well-known interspecies differences in the regulation of hepatic drug-metabolizing enzymes between humans and rodents (Martignoni, Groothuis et al. 2006). Taken together, the effects of FXR activation on CYP3A4 expression and the underlying regulatory mechanisms remain unclear.

1.1.4 Aim of the study.

In this study, we investigated the effect of FXR activation on CYP3A4 expression, using primary human hepatocytes. Our results show that GW4064 represses CYP3A4 expression,

potentially through upregulating SHP expression and subsequent repression of PXR and CAR transactivation of CYP3A4 promoter.

1.2 Transcriptional regulation of CYP2D6 by ATRA.

1.2.1 Importance of CYP2D6 as a major DME.

Most lipophilic drugs and xenobiotics go though metabolism catalyzed by Phase I drug-metabolizing enzymes during biotransformation and elimination, and CYP2D6 is one of the most extensively studied and characterized CYPs (Ingelman-Sundberg 2005).

The reason behind the recognition of the importance of CYP2D6 in drug metabolism is that it metabolizes around 25% of currently prescribed drugs, including beta-blockers, antidepressants and opioids (Bertilsson, Dahl et al. 2002). Additionally CYP2D6 shows significant inter-individual variability in its enzyme activity and expression levels which let to the discovery of multiple polymorphisms within CYP2D6 (Teh and Bertilsson 2012).

1.2.2 Increased CYP2D6 mRNA and enzyme activity levels during pregnancy.

CYP2D6 had long been recognized as a largely non-inducible DME. The expression of CYP2D6 is tightly controlled under the regulation of HNF4α and its expression and activity levels are primarily associated with genetic polymorphism that allelic variations result in altered protein expression and activity (Heim and Meyer 1990, Wadelius, Darj et al. 1997, Ingelman-Sundberg 2005). However, clinical evidences indicate that pregnancy increases the elimination of CYP2D6 substrate drugs (Hogstedt, Lindberg et al. 1985, Wadelius, Darj et al. 1997). Previous studies in Jeong's group have identified that SHP as a key modulator in the transcription regulation of CYP2D6 (Koh, Pan et al. 2014, Pan, Lee et al. 2015). During pregnancy, decreased ATRA level may act through SHP and eventually alter expression and activity of CYP2D6 (Koh, Pan et al. 2014).

1.2.3 ATRA potentially regulates CYP2D6 via SHP.

ATRA, the biologically active metabolite of vitamin A, is the endogenous activator for nuclear receptors retinoic acid receptor (RARα, β, and γ)/RXR and it exerts various functions, including proliferation, differentiation, development and immune response (Chiang, Misner et al. 1998, Stephensen 2001, Fields, Soprano et al. 2007). ATRA has been widely used as therapeutic agent in the topical treatment for dermatologic disease (Zouboulis, Korge et al. 1991) and acute promyelocytic leukemia (Huang, Ye et al. 1988, Castaigne, Chomienne et al. 1990). When activated by ATRA, RAR and RXR forms heterodimer and bind to specific retinoic acid response element RARE sites which are located in the transcription regulatory regions of target genes, and transcriptionally represses or induces target gene expression (Bastien and Rochette-Egly 2004, Blomhoff and Blomhoff 2006).

Besides activation of RAR/RXR, ATRA has also been shown to activate Farnesoid x receptor (FXR)/RXR and represses CYP7A1 expression, a known target genes which is repressed upon FXR activation (Cai, He et al. 2010). Previous studies have identified the effect and mechanism of FXR activation on transcription regulation of CYP2D6, and the key role of SHP in CYP2D6 regulation in both *in vitro* and *in vivo* models. In brief, FXR activation by its agonist GW4064 induces SHP expression, and SHP transcriptionally represses CYP2D6 expression by inhibiting hepatic nuclear factor 4 alpha (HNF4α) transactivation of CYP2D6, and decreases HNF4α and polymerase II recruitment to CYP2D6 promoter (Koh, Pan et al. 2014, Pan, Lee et al. 2015). Additionally, *in vivo* model showed that observed ATRA level was significantly reduced during pregnancy, indicating an important correlation of CYP2D6 induction and reduced hepatic retinoid levels (Koh, Pan et al. 2014). These data suggest the important role of ATRA level change during pregnancy in the alteration of CYP2D6 expression, and potential transcription regulation acted via ATRA on CYP2D6.

1.2.4 Aim of the study.

Combining these evidences, we hypothesize that ATRA may transcriptionally represses CYP2D6 expression and activity via SHP, therefore significant drug-drug interaction involving ATRA and CYP2D6 substrates may occur. In this study, we investigated the effect of ATRA on CYP2D6 transcription regulation *in vitro* and *in vivo*, and results show that ATRA represses CYP2D6 expression and SHP plays an essential role in this process.

2. Effects of FXR activation on CYP3A4 expression and activity on human hepatocytes

2.1 Materials and methods.

2.1.1 Chemicals and reagents.

GW4064, midazolam, dimethyl sulfoxide (DMSO), 1-hydroxymidazolam, cholic acid, rifampin and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO).

2.1.2 Plasmids.

A luciferase vector harboring distal and proximal xenobiotic response elements of *CYP3A4* (i.e., pGL3-CYP3A4) was obtained from Dr. Hongbing Wang (Faucette, Sueyoshi et al. 2006). The luciferase vector containing 1.8-kb of CYP2B6 upstream regulatory region (i.e., pGL3-CYP2B6) and the luciferase vector containing 2.2-kb of SHP upstream regulatory region (i.e., pGL2-SHP) (Kim, Kim et al. 2003) were kindly provided by Drs. Masahiko Negishi (NIEHS) and Hueng-Sik Choi (Chonnam National University), respectively. Expression vectors for SHP (Koh, Pan et al. 2014), PXR, or CAR (Koh, Jurkovic et al. 2012) were previously described. GR and FXR expression vectors were kindly provided by Drs. Alan McLachlan (University of Illinois at Chicago), and Yoon-Kwang Lee (Northeast Ohio Medical University), respectively.

2.1.3 Cell culture.

HepG2 cells from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in complete DMEM supplemented with 10% fetal bovine serum (Gemini, Sacramento, CA), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% minimum essential media (MEM) nonessential amino acids. HEK293T cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA), 10 mM HEPES, 100 μM non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA).

2.1.4 Primary hepatocyte culture.

Freshly isolated human hepatocytes were obtained from Liver Tissue Cell Distribution System (Pittsburgh, PA). Briefly, human hepatocytes were shipped overnight in cold preservation media. Upon receipt, the media were replaced with serum-free Williams' E media (without phenol red) containing 0.1 μM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin, 15 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 2 mM L-glutamine, 5.5 μg/mL transferrin, and 5 ng/mL sodium selenite. Cells were allowed to recover from shipping for 18 hr at 37 °C in an atmosphere containing 5% CO_2, and used for experiments on the next day.

2.1.5 Quantitative real-time (qRT)-PCR.

Total RNA was isolated from human hepatocytes using Trizol (Life Technologies) and used as template for the synthesis of complementary DNA using High Capacity complementary DNA (cDNA) Archive Kit (Applied Biosystems, Foster City, CA). With the cDNA as template, qRT-PCR was performed using SYBR Green reagents and the primers listed in supplemental Table S1. The fold change in mRNA levels was determined after normalizing the gene expression levels to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ($2^{-\Delta\Delta Ct}$ method).

2.1.6 Luciferase reporter assays.

HepG2 and HEK293T cells were seeded in 24-well plates at a density of $5x10^5$ or $1x10^5$ cells per well, respectively. On the next day, the cells were transfected with 0.3 μg of luciferase construct, 0.1 μg of expression plasmid (or empty vector as a control) for a transcription factor, and 0.002 μg of *Renilla* expression plasmid, using Fugene HD transfection reagent (Roche Applied Sciences) according to the manufacturer's protocol. At 48 hr post-transfection, the

transfected cells were harvested for determination of luciferase activities using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Luciferase activity was normalized to the *Renilla* luciferase activity. At least two independent experiments were performed in triplicate.

2.1.7 Determination of CYP3A4 activity.

S9 fractions were prepared as described previously (Felmlee, Lon et al. 2008). Midazolam (final concentration 3 μM) was mixed with S9 fractions (10 μg) in nicotinamide adenine dinucleotide phosphate (NADPH) -regenerating system (5 mM isocitric acid, 0.2 unit/mL isocitric acid dehydrogenase, and 5 mM magnesium chloride in 100 mM Tris-HCl buffer, pH 7.4; 100 μL total volume). The reactions were started by addition of $NADP^+$ (10 mM) and further incubated for 1 hr. The reactions were quenched by adding cold acetonitrile (120 μL) that contains phenytoin (0.5 μM) as internal standard. The mixture was kept on ice for 30 min, followed by centrifugation at 16,100g for 15 min at 4 ^oC to obtain the supernatant. The concentrations of 1-hydroxymidazolam in the surpernatants were determined by LC-MS/MS (Agilent 1200 HPLC interfaced with Agilent 6410 Triple Quadrupole tandem MS equipped with an electrospray ion source. Chromatographic separation was carried out by using a Waters XTerra MS C18 column (2.1×50 mm, 3.5 μm; Waters Corporation, Milford, MA). Mobile phase was delivered at 250 μL/min, and the gradient was initiated at 90% A and 10% B [A, 0.1% (v/v) formic acid in water; B, acetonitrile]. The proportion of mobile phase B was increased to 90% over 1 min, held constant for 2 min, and then restored to the initial composition. The injection volume was 10 μL. Midazolam was detected by MS/MS (341.9/324.0), and phenytoin was used as the internal standard (253.2/182.2) in positive ion mode. All data were acquired employing Agilent 6410 Quantitative Analysis version analyst data processing software.

2.1.8 Statistical Analysis.

Each experiment with primary human hepatocytes was conducted in triplicate, and data were expressed as mean ± standard deviation (S.D.). Student's t-test was performed for statistically analysis.

2.2 Results

2.2.1 GW4064 represses CYP3A4 expression and activity.

To determine whether FXR activation alters CYP3A4 expression, human hepatocytes from three different donors were treated with GW4064 (a FXR agonist, 1 μM) or DMSO for 48 hr, and then CYP3A4 mRNA expression and enzyme activity levels were examined. GW4064 significantly repressed mRNA expression levels (Fig. 1A) and enzyme activity (Fig. 1B) of CYP3A4.

FXR is capable of causing transactivation or transrepression of its target gene promoters. To determine whether FXR directly transrepresses CYP3A4 promoter, luciferase reporter assays were performed in HepG2 cells. The cells were transfected with a CYP3A4 promoter vector, and treated with GW4064 (or vehicle control), after which luciferase activities were measured. GW4064 did not decrease CYP3A4 promoter activity (Fig. 1C), suggesting that direct FXR transrepression of CYP3A4 promoter is an unlikely mechanism for CYP3A4 repression by GW4064. On the other hand, in human hepatocytes, CYP3A4 repression by GW4064 was accompanied by increased SHP expression (Fig. 1D). Downregulation of CYP3A4 and upregulation of SHP were also observed in human hepatocytes treated with cholic acid, a natural ligand of FXR (Fig. 1E), suggesting that FXR activation leads to CYP3A4 repression. In HEK293T cells co-transfected with a luciferase vector where *luc* expression is driven by 2.2-kb of *SHP* promoter (along with FXR and RXR expression plasmids), GW4064 enhanced SHP promoter activity in a concentration-dependent manner with an estimated EC_{50} of 75 nM.

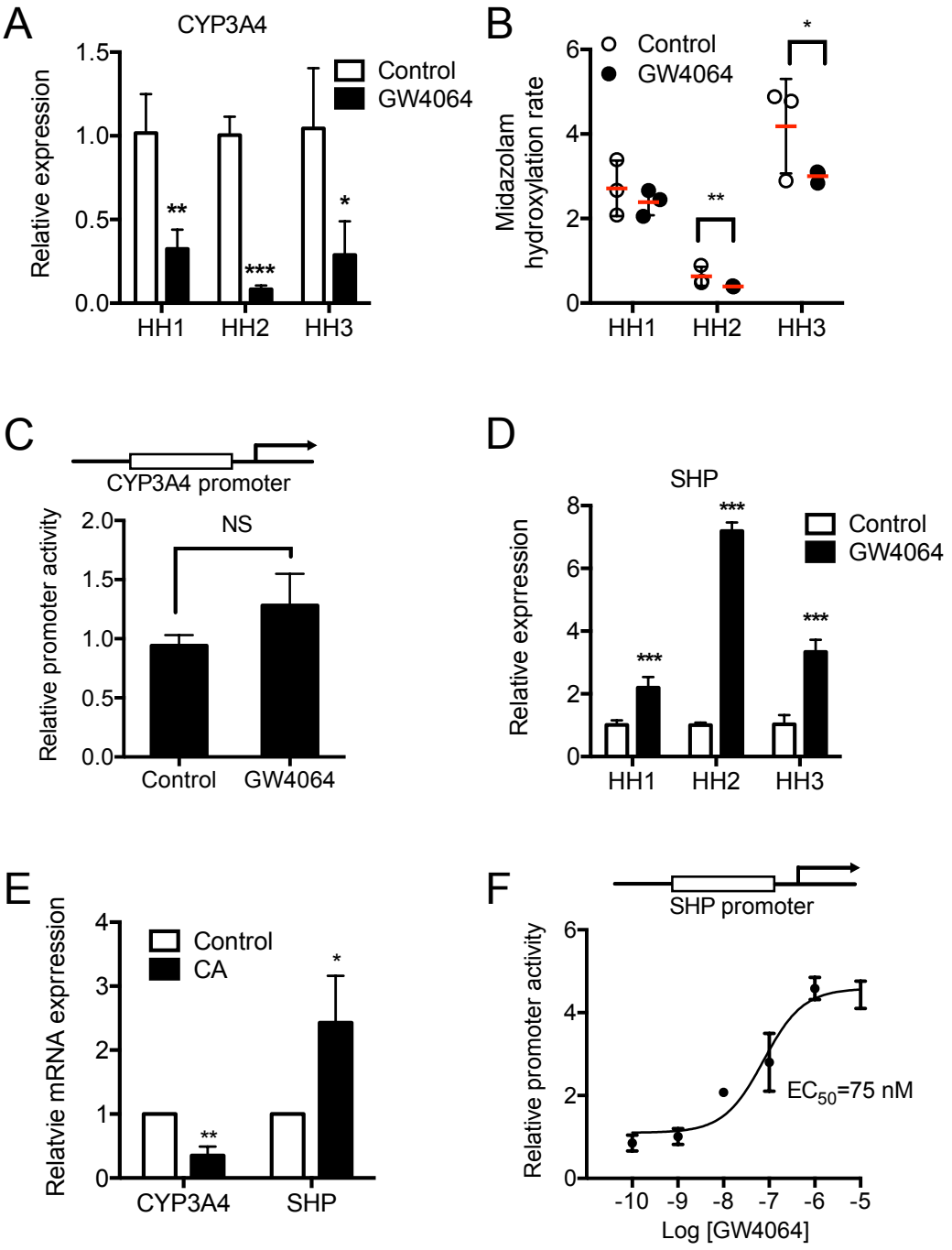

Fig. 1. GW4064 represses CYP3A4 expression. Primary human hepatocytes (HH) from three different donors were treated with vehicle (DMSO) or GW4064 (1 μM) for 48 hr in triplicate. RNAs were collected and mRNA levels of CYP3A4 (A) and SHP (D) were determined by using qRT-PCR. (B) CYP3A4 activity was measured in S9 fractions from the GW4064- or vehicle-treated hepatocytes using midazolam. Data shown are production rates of hydroxymidazolam in pmol/hr/mg protein. (C) HepG2 cells were co-transfected with pGL3-CYP3A4 and *Renilla* luciferase vector. On the next day, the cells were treated with vehicle (DMSO) or GW4064 (1 μM) for 24 hr, and dual luciferase assays were performed. (E) Primary human hepatocytes were

treated with vehicle (DMSO) or cholic acid (50 μM) for 48 hr in triplicate. RNAs were collected to measure CYP3A4 and SHP mRNA expression by qRT-PCR. (F) HEK293T cells were co-transfected with pGL2-SHP and *Renilla* luciferase vectors, along with FXR and RXR expression vectors. On the next day, the cells were treated with vehicle (DMSO) or GW4064 (0.1, 1, 10, 100, 1000, or 10,000 nM) for 24 hr, and dual luciferase assays were performed. Data shown are mean ± standard deviation. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ vs. vehicle-treated group; NS, statistically not significant.

2.2.2 SHP represses PXR and CAR transactivation of CYP3A4 promoter.

SHP is known to repress activity of multiple transcription factors, which potentially include PXR, CAR, and GR that are involved in the regulation of CYP3A4 expression. Considering the absence of prominent PXR or CAR ligands, but the presence of dexamethasone, in the culture media for human hepatocytes, we first examined whether SHP represses regulation of CYP3A4 promoter activity via GR, by using promoter reporter assays. HepG2 cells were co-transfected with a luciferase vector where *luc* expression is driven by xenobiotic response element of *CYP3A4* that binds directly to PXR or CAR (Faucette, Sueyoshi et al. 2006), along with expression vectors of GR and/or SHP. The transfected cells were treated with vehicle control or dexamethasone, and luciferase activity was measured. The results showed that activation of GR (by dexamethasone treatment) led to a significant increase in CYP3A4 promoter activity (Fig. 2A, lane 2 vs. 4), consistent with the previously reported data (Pascussi, Drocourt et al. 2001). The enhanced CYP3A4 promoter activity, however, was abrogated by SHP (Fig. 2A, lane 4 vs. 8).

GR activation leads to increased CYP3A4 expression via enhancing both the expression and activity of PXR and CAR (Pascussi, Drocourt et al. 2001). To determine whether GW4064 alters the GR-mediated regulation of PXR or CAR expression, mRNA expression levels of PXR and CAR were measured in human hepatocytes treated with GW4064 (or vehicle control) by using qRT-PCR. The result showed that PXR or CAR expression did not differ between the

groups (data not shown), suggesting that GW4064 does not repress GR action on PXR or CAR expression. Then, to examine whether SHP decreases PXR or CAR transactivation of CYP3A4, the promoter reporter assay was performed. The results showed that rifampin-activated PXR dramatically enhanced CYP3A4 promoter activity as expected (Fig. 2B, lane 2 and 4), and this was significantly repressed by SHP (Fig. 2B, lane 4 vs. 8). CAR transfection led to increases in CYP3A4 promoter activity (Fig. 2C, lane 1 vs. 2), consistent with the previous report that CAR is constitutively active even in the absence of ligands when transfected into immortalized cells (Baes, Gulick et al. 1994). The enhanced CYP3A4 promoter activity was decreased upon co-expression of SHP (Fig. 2C, lane 4 vs. 8). Together, these results indicate that SHP represses PXR/CAR transactivation of CYP3A4 promoter.

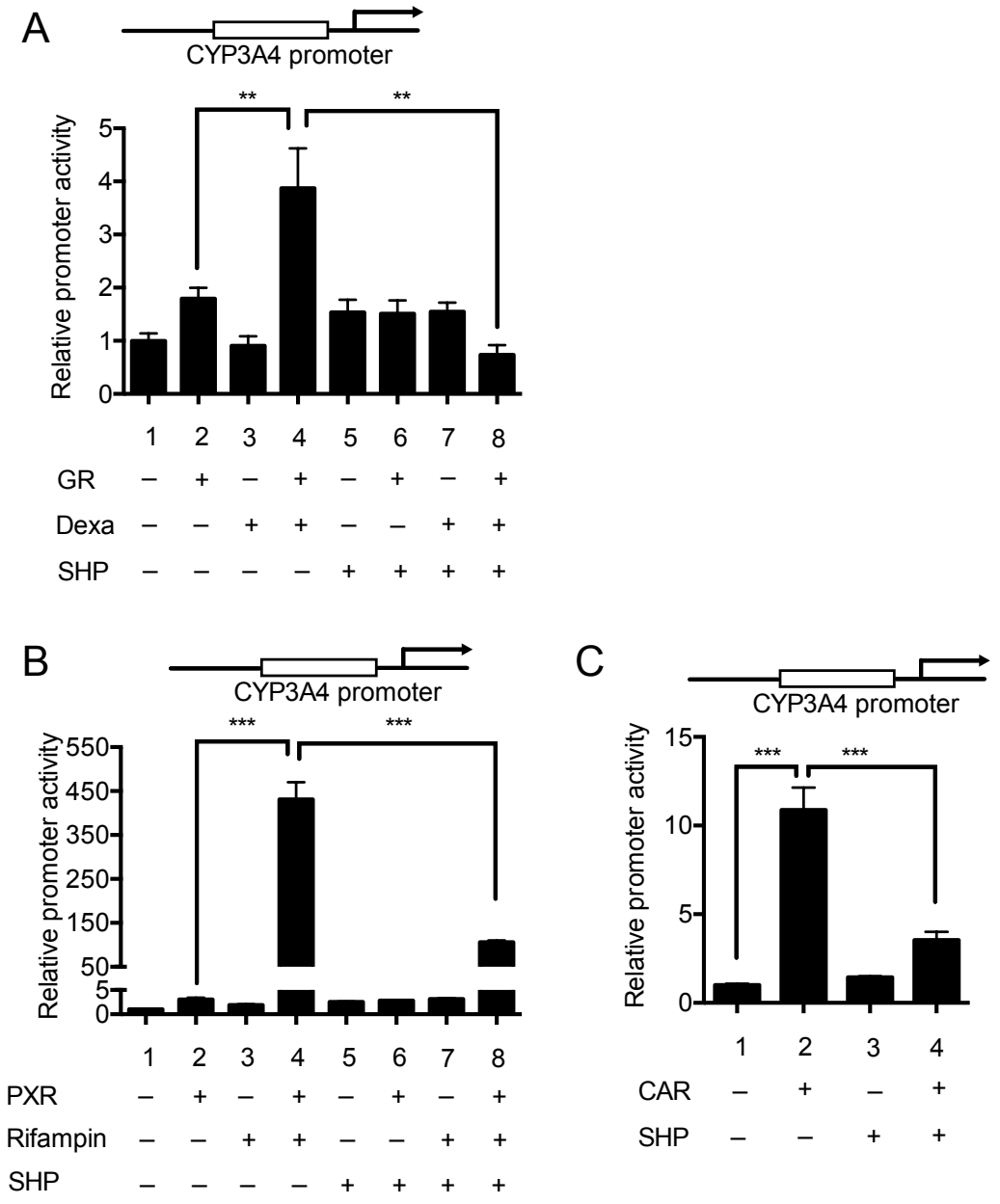

Fig. 2. SHP represses PXR and CAR transactivation of CYP3A4 promoter. (A) HepG2 cells were co-transfected with pGL3-CYP3A4 and *Renilla* luciferase vector, along with expression vectors for GR or SHP. On the next day, the cells were treated with vehicle (DMSO) or dexamethasone (1 μM) for 24 hr, and dual luciferase assays were performed. (B) HepG2 cells were co-transfected with pGL3-CYP3A4 and *Renilla* luciferase vector, along with expression vectors for PXR or SHP. On the next day, the cells were treated with vehicle (DMSO) or rifampin (5 μM) for 24 hr, and dual luciferase assays were performed. (C) HepG2 cells were co-transfected with pGL3-CYP3A4 and *Renilla* luciferase vector, along with expression vectors for CAR or SHP. The cell lysates were collected after 24 hr, and dual luciferase assays were performed. **, $p<0.01$; ***, $p<0.001$.

2.2.3 GW4064 enhances CYP2B6 promoter activity.

PXR and CAR enhance expression of many hepatic drug-metabolizing enzymes including CYP2B6 (Faucette, Sueyoshi et al. 2006). To determine whether the SHP-mediated repression of PXR and CAR action alters expression of other target genes, we examined expression of CYP2B6 in human hepatocytes treated with GW4064. Interestingly, CYP2B6 mRNA levels were significantly increased (rather than decreased) in two of three different batches of hepatocytes (Fig. 3A). To examine whether GW4064 can directly activate CYP2B6 promoter, promoter reporter assays were performed. HepG2 cells were transfected with a luciferase vector where *luc* expression is driven by 1.8-kb upstream regulatory region of *CYP2B6*, treated with vehicle control or GW4064, and luciferase activity was measured. The results showed that GW4064 treatment led to ~3-fold increase in CYP2B6 promoter activity (Fig. 3B).

To determine whether the enhanced CYP2B6 promoter activity is through FXR-transactivation of CYP2B6 promoter, promoter reporter assays were performed in HEK293T cells co-transfected with a CYP2B6-promoter driven luciferase vector and expression vector for FXR and RXR. The cells were treated with GW4064 (or vehicle control), and luciferase activity was measured. A luciferase vector harboring 2.2-kb *SHP* promoter was included as a control. Results showed that GW4064 enhanced CYP2B6 promoter activity ~2.5-fold (Fig. 3C, lane 1 and 4). This induction was of a similar magnitude as the increase in SHP promoter activity by GW4064 (Fig 3C, lane 5 and 6). Collectively, these data indicate that the enhanced CYP2B6 expression could be due to increased CYP2B6 promoter activity by FXR activation.

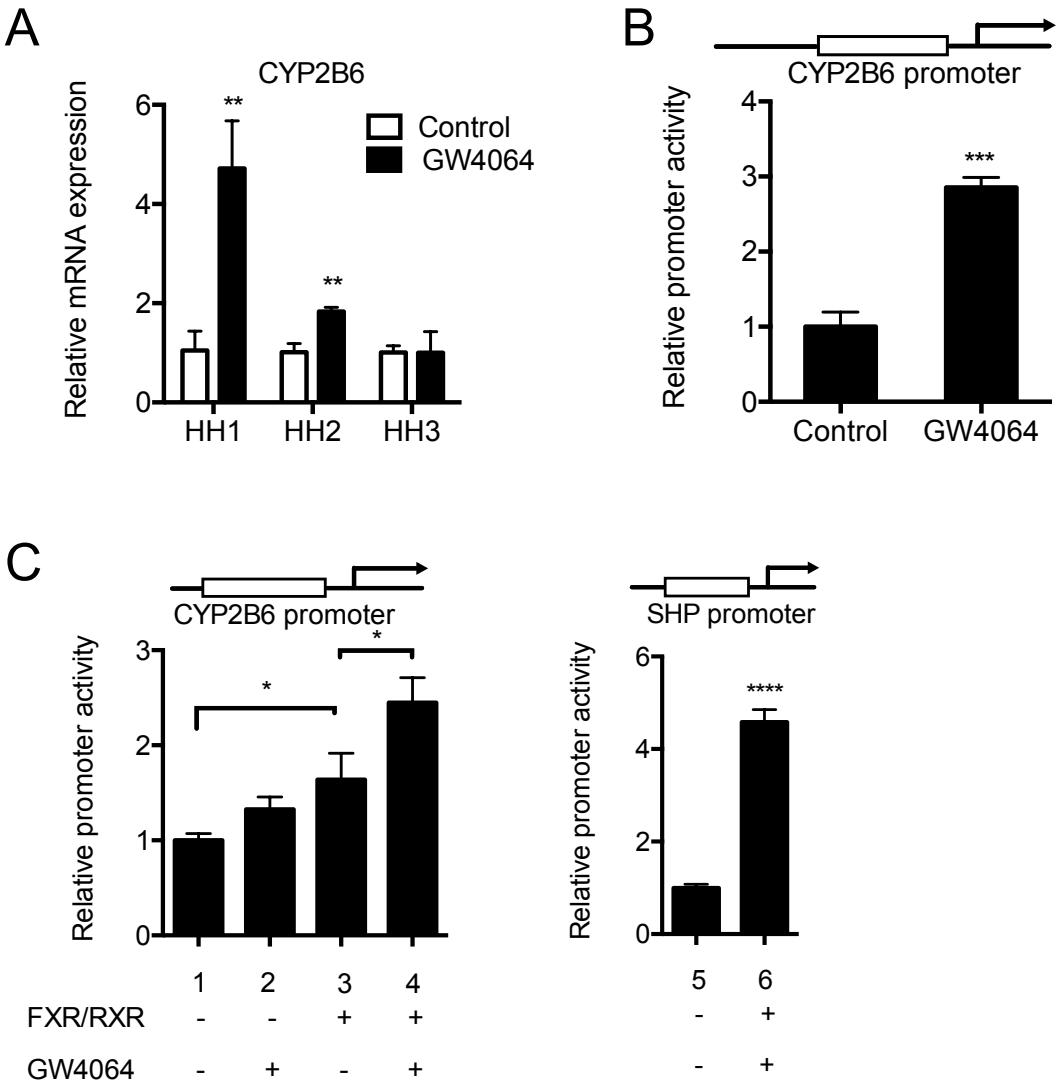

Fig. 3. GW4064 upregulates CYP2B6 expression via FXR. (A) Primary human hepatocytes from three different donors were treated with vehicle (DMSO) or GW4064 (1 μM) for 48 hr in triplicate. RNA was collected and CYP2B6 mRNA expression level was measured by using qRT-PCR. (B) HepG2 cells were co-transfected with pGL3-CYP2B6 and *Renilla* vector, treated with vehicle or GW4064 (1 μM), and dual luciferase assay was performed. (C) HEK293T cells were co-transfected with pGL3-CYP2B6 (or pGL2-SHP) and *Renilla* vector, along with FXR and RXR expression vectors. On the next day, the cells were treated with vehicle (DMSO) or GW4064 (1 μM). The cell lysates were collected after 24 hr, and dual luciferase assays were performed. **, $p<0.01$; ***, $p<0.001$.

2.3 Discussion

In this study, we examined the effects of FXR activation on expression of CYP3A4 by using freshly isolated primary human hepatocytes. Our results showed that GW4064 significantly decreases the expression of the major drug-metabolizing enzyme CYP3A4 in human hepatocytes, and that this is in part attributable to increased SHP expression upon FXR activation. We found that a different inducer of SHP, all-trans retinoic acid (Koh, Pan et al. 2014), also repressed CYP3A4 expression in human hepatocytes (data not shown; a manuscript in preparation), further supporting the important role of SHP in CYP3A4 regulation. CYP3A4 expression is regulated by nuclear receptors including PXR and CAR. While PXR and CAR are activated to the fullest extent upon binding of respective ligands to the receptors, both transcription factors appear activated at basal levels in human hepatocytes (Zamule, Strom et al. 2008, Hariparsad, Chu et al. 2009). This could be in part due to the presence of corticosteroids in culture media that are known to enhance the expression as well as transcriptional activity of PXR and CAR, via GR (Pascussi, Gerbal-Chaloin et al. 2000, Pascussi, Drocourt et al. 2001). Our results from luciferase reporter assays showed that SHP represses actions of GR as well as those of PXR and CAR on CYP3A4 promoter, suggesting that repression of these transcriptional activators on CYP3A4 promoter by SHP may potentially trigger CYP3A4 repression by GW4064. Interestingly, such repressive action of SHP on PXR/CAR target gene promoter was not shown for CYP2B6, in part because GW4064 activates CYP2B6 promoter. The model for differential regulation of CYP3A4 and CYP2B6 by GW4064 is shown in Fig. 4.

In addition to PXR and CAR, multiple other transcription factors including peroxisome proliferator-activated receptor peroxisome proliferator-activated recpter alpha (PPARα) and liver X receptor (LXR) upregulate CYP3A4 expression (Drocourt, Ourlin et al. 2002, Duniec-Dmuchowski, Ellis et al. 2007, Thomas, Burk et al. 2013). However, potential involvement of

PPARα and LXR in CYP3A4 repression by GW4064 appears unlikely. Our results from human hepatocytes indicate that GW4064 does not alter the mRNA expression of the representative target genes of these nuclear receptors, including ATP-binding cassette, subfamily B, member 4 (ABCB4) (Ghonem, Ananthanarayanan et al. 2014), fatty acid synthase (FASN), and sterol regulatory element binding transcription factor 1 (SREBF1) (Wagner, Valledor et al. 2003) (data not shown). Inflammation is also known to alter CYP3A4 expression; CYP3A4 expression is down-regulated in the state of inflammation (Aitken, Richardson et al. 2006). In GW4064-treated human hepatocytes, there were no increases in the expression levels of inflammatory marker tumor necrosis factor alpha (TNFα) (data not shown) and no significant changes in the gross morphology of hepatocytes (that are indicative of drug toxicity or inflammation). Together, these findings suggest that PPARα, LXR, or inflammation plays minimal roles, if any, in CYP3A4 repression by GW4064.

Previous studies using HepG2 cells have shown that GW4064 *increases* (not decreases as shown in our study) CYP3A4 expression (Gnerre, Blattler et al. 2004); GW4064 (at 1 μM) increased CYP3A4 mRNA levels by 1.8-fold in HepG2 cells, potentially by FXR binding to ~7.8-kb upstream region of *CYP3A4* and transactivating the promoter (Gnerre, Blattler et al. 2004). Also, GW4064 enhanced expression of Cyp3a11 (a CYP3A4 homolog gene) in mice by ~2-fold (Gnerre, Blattler et al. 2004). While the reason for discrepancy between the previous and our study is unclear, it appears possible that in HepG2 cells, the expression levels of key hepatic transcription factors including PXR and CAR may have been low for SHP to exhibit any repressive action. Also, significant interspecies differences in the regulation of drug-metabolizing enzymes expression hamper the extrapolation of animal data to humans (Lu and Li 2001, Vignati, Bogni et al. 2004). Considering that primary human hepatocytes have served as a gold standard tool to study regulation of hepatic drug-metabolizing enzymes, it appears

likely that GW4064 decreases CYP3A4-mediated drug metabolism in humans through SHP-mediated repression of CYP3A4 expression.

FXR agonists including GW4064 are currently under development as potential therapeutic agents for metabolic diseases such as obesity, type 2 diabetes, hypertriglyceridaemia, atherosclerosis, and non-alcoholic steatohepatitis (reviewed in (Thomas, Pellicciari et al. 2008)). As CYP3A4 repression by GW4064 is potentially triggered by increased expression of SHP and that SHP induction is a class effect of FXR agonists, other FXR agonists are also expected to repress CYP3A4 expression, potentially causing drug-drug interactions with CYP3A4 substrates. It remains difficult, however, to quantitatively predict the clinical outcome of these interactions based only on the results from human hepatocytes. This is in part due to the long degradation half-lives of CYP3A4 protein (i.e., 36-50 hr (Fromm, Busse et al. 1996)) such that CYP3A4 protein levels in human hepatocytes do not reach the steady state after the typical time of drug treatment (e.g., 48 hr) passes. In accordance, CYP3A4 mRNA expression decreased >50% in GW4064-treated hepatocytes in this study while the decrease in catalytic activity of CYP3A4 (as determined in S9 fraction of hepatocytes) was only 10-30%. The clinical consequences of FXR agonists repressing CYP3A4 expression, thus, remain to be examined.

In conclusion, we showed that GW4064 represses CYP3A4 expression, potentially through upregulating SHP expression and subsequent repression of PXR and CAR transactivation of CYP3A4 promoter. This suggests that drug-drug interactions may occur clinically between CYP3A4 substrates and FXR agonists that are currently under development for the treatment of metabolic diseases.

Fig. 4

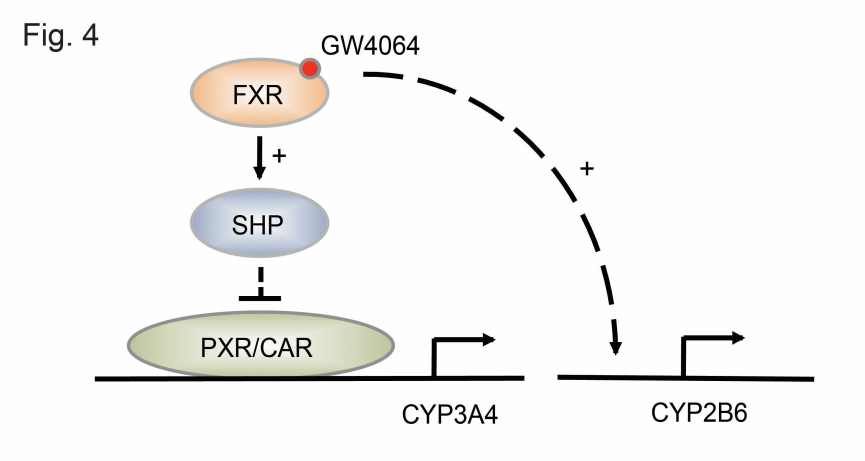

Fig. 4. Differential regulation of CYP2B6 and CYP3A4 by GW4064. FXR activation by GW4046 leads to decreased CYP3A4 expression, potentially through SHP upregulation and subsequent repression of PXR and CAR transactivation of CYP3A4 promoter. On the other hand, GW4064 enhances CYP2B6 promoter activity.

3. Transcription regulation of CYP2D6 by all-trans retinoic acid (ATRA)

3.1 Materials and Methods

3.1.1 Animals.

CYP2D6-humanized transgenic (Tg-*CYP2D6*) and *Shp*-null mice were previously described (Corchero, Granvil et al. 2001, Park, Kim et al. 2011). Tg-*CYP2D6* mice harbor human CYP2D6 gene with ~2.5 kilobase of promoter region which replaces mouse *Cyp2d.* Both Tg-*CYP2D6* and *Shp*-null mice were on C57BL/6 background. *Shp*-null mice and Tg-*CYP2D6* mice were crossed to produce offspring of two genotypes (*CYP2D6*, $Shp^{-/-}$; *CYP2D6*, $Shp^{+/+}$). Offspring were genotyped using PCR (described below), and adult male mice of each genotype (8 – 12 weeks of age; ~25 g body weight) were used for the experiments. ATRA (5 mg/kg) or vehicle (olive oil) was injected intraperitoneally daily for 5 days (n = 4 per group). Mice were sacrificed on the sixth day, and liver tissues were collected. All procedures were approved by the Institutional Animal Care and Committee at the University of Illinois at Chicago.

3.1.2 Genotyping.

Tg-*CYP2D6* mice genomic DNA samples were isolated from tail snip, and unknown *Shp* copy number were genotyped by PCR using customized primers (wild-type forward: 5'-CTCTGCAGGTCGTCCGACTATTCTG-3'; wild-type reverse: 5'-CCTCGAAGGTCACAGCATCCTG-3'; *Shp*-null forward: 5'-CTAGCTAGAGGATCCCCGGGTACC-3'; *Shp*-null reverse: 5'-AATTCGCGTCTGGCCTTCCTGTAG-3'). PCR products were analyzed using southern blot, and PCR bands of product from *Shp*-null and wild-type mice were identified at 500 base pair and 300 base pair, respectively.

3.1.3 Primary hepatocyte culture.

Freshly isolated human hepatocytes, derived from three different donors, were obtained from Liver Tissue Cell Distribution System (Pittsburgh, PA). Briefly, human hepatocytes were shipped overnight in cold preservation media. Upon receipt, the media were replaced with serum-free Williams' E media (without phenol red) containing 0.1 μM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin, 15 mM HEPES, 2 mM L-glutamine, 5.5 μg/mL transferrin, and 5 ng/mL sodium selenite. Cells were allowed to recover from shipping for 18 hr at 37 °C in an atmosphere containing 5% CO_2, and used for experiments on the next day. After recover, the hepatocytes were treated with vehicle control (DMSO) or ATRA (1 μM) for 48 hours. Cell lysates were collected for the preparation of RNA and protein samples.

3.1.4 Reagents.

ATRA, GW4064, cycloheximide and DMSO were purchased from Sigma-Aldrich (St. Louis, MO).

3.1.5 Plasmids.

pGL3-CYP2D6 plasmid harboring 2.5 kilobase of CYP2D6 upstream regulatory region and pGL2-SHP plasmid harboring 2.2 kilobase of SHP upstream regulatory region were previously describe ((Koh, Pan et al. 2014, Zhang, Pan et al. 2015).

3.1.6 Cell culture.

HepG2 cells from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in complete DMEM supplemented with 10% fetal bovine serum (Gemini, Sacramento, CA), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% MEM nonessential amino acids. HEK293T cells were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA), 10 mM HEPES, 100 μM non-

essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA).

3.1.7 Quantitative real-time (qRT)-PCR.

Total RNA was isolated from human hepatocytes using Trizol (Life Technologies) and used as template for the synthesis of complementary DNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). With the cDNA as template, qRT-PCR was performed using SYBR Green reagents and the primers listed in supplemental Table S1. The fold change in mRNA levels was determined after normalizing the gene expression levels to those of GAPDH ($2^{-\Delta\Delta Ct}$ method).

3.1.8 Luciferase reporter assay.

HepG2 and HEK293T cells were seeded in 24-well plates at a density of $5x10^5$ or $1x10^5$ cells per well, respectively. On the next day, the cells were transfected with 0.3 μg of luciferase construct, 0.1 μg of expression plasmid (or empty vector as a control) for a transcription factor, and 0.002 μg of *Renilla* expression plasmid, using Fugene HD transfection reagent (Roche Applied Sciences) according to the manufacturer's protocol. At 48 hr post-transfection, the transfected cells were harvested for determination of luciferase activities using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Luciferase activity was normalized to the *Renilla* luciferase activity. At least two independent experiments were performed in triplicate.

3.1.9 Statistical analysis.

Each experiment with primary human hepatocytes was conducted in triplicate, and data were expressed as mean ± standard deviation (S.D.). Student's t-test was performed for statistically analysis.

3.2 Results

3.2.1 ATRA represses CYP2D6 mRNA expression in primary human hepatocytes.

To determine the whether CYP2D6 mRNA expression is represses by ATRA *in vitro*, human hepatocytes from three donors were treated with vehicle control or ATRA (1 μM) for 48 hr, and then mRNA was collected and CYP2D6 expression level were examined using qRT-PCR. Results showed that relative expression of CYP2D6 was repressed by ~4-fold upon ATRA treatment (Fig 5A).

Previous studies have demonstrated the capability of RAR/RXR activation by ATRA to directly cause transactivation or transrepression of target genes, including CYPs (Allegretto, McClurg et al. 1993, Benkoussa, Brand et al. 2002). To determine whether the repression of CYP2D6 mRNA expression is directly regulated by ATRA, dual-luciferase reporter assay was performed in HepG2 cell. Cells were transfected with a CYP2D6 promoter-driven luciferase vector which harbors 2.5 kb of CYP2D6 upstream regulatory region, and were treated with vehicle control or different ATRA doses, after which luciferase activities were measured. Results showed that ATRA significantly inhibited CYP2D6 promoter activity in all three treatments with various ATRA concentrations (Fig 5B). However interestingly, the magnitude of the repression effect decreased with increased ATRA concentration treatment, suggesting that RAR/RXR activation by ATRA may not be the solely nuclear receptors acting in this repression, and additional protein with negative feedback regulatory effect may be involved.

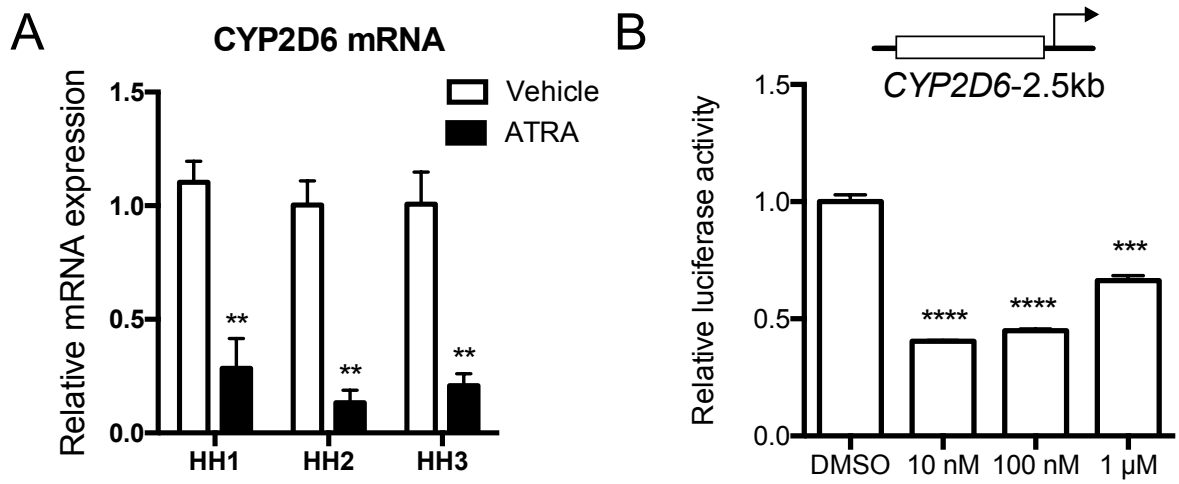

Fig. 5. ATRA represses CYP2D6 mRNA expression and promoter activity. (A) Primary human hepatocytes (HH) from three different donors were treated with vehicle (DMSO) or ATRA (1 μM) for 48 hr in triplicate. RNAs were collected and mRNA levels were determined by using qRT-PCR. (B) HepG2 cells were co-transfected with pcDNA3-CYP2D6 and *Renilla* luciferase vector, and were treated with vehicle (DMSO) or ATRA (1 μM) for 24 hr, and dual luciferase assays were performed. **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

3.2.2 Proximal regulatory region of CYP2D6 promoter mediates the effect of ATRA on CYP2D6 repression.

HNF4α is recognized to play a crucial role in the transcriptional regulation of various genes involved in liver organogenesis and drug metabolism, and it is the only known transcription factor which controls constitutive CYP2D6 expression via binding at its promoter region -53/-41 bp (Cairns, Smith et al. 1996). Because of the essential role of HNFα in the basal induction of CYP2D6, we hypothesized that ATRA act through the disruption of HNF4α to CYP2D6 promoter to repress CYP2D6 expression. To examine which *cis*-element is responsible for the ATRA disruption of HNF4α-mediated CYP2D6 transactivation, luciferase

assays were performed using seven CYP2D6 promoter 5'-deletion constructs harboring 2.5 kb, 2.0 kb, 1.5 kb, 1.0 kb, 0.5 kb, 0.2 kb, and 0.1 kb of CYP2D6 promoter region, respectively. HepG2 cells transfected with basal vector or CYP2D6 promoter construct were treated with vehicle control or ATRA, and luciferase activities were measured. Luciferase assay data indicated that there is significant difference between ATRA-treated and vehicle control-treated all cells, and such difference is abrogated from cells containing 0.1 kb promoter to basic promoter, and from 0.2 kb to 0.1 kb promoter (Fig 6). The results showed the existence of important transactivation site ~-100 bp disrupted by ATRA, which is consistent with previous reported HNF4α binding site at -53/-41. In addition, ATRA may interact with more transcription factor other than HNF4α at ~100-200 bp which control CYP2D6 promoter activity as the results showed. In conclusion, ATRA represses CYP2D6 promoter activity via disruption of HNF4α transactivation of CYP2D6.

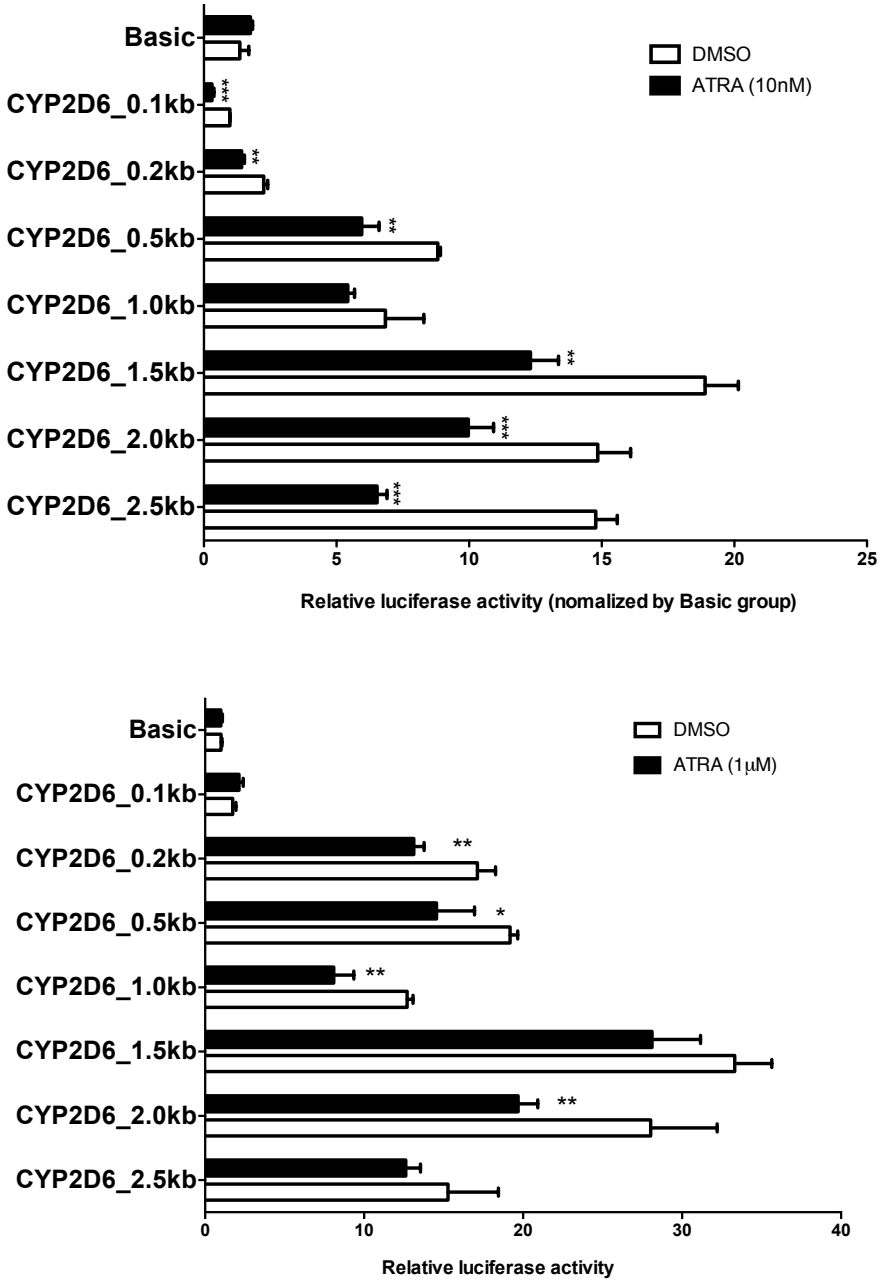

Fig. 6. Repressive effect of ATRA on CYP2D6 promoter activity located between 200 bp to 100 bp, and within 100 bp. Luciferase activity of HepG2 cells co-transfected with different deletion constructs of CYP2D6 promoter treated with ATRA (10 nM or 1μM) or vehicle control. The cell lysates were collected after 24 hr, and dual luciferase assays were performed. *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$.

3.2.3 ATRA induces SHP mRNA and pre-mRNA expression.

Based on the evidences that SHP is a previously reported important co-repressor of CYP2D6, and is also a downstream target gene of FXR/RXR, which is reported to be activated by ATRA (Cai, He et al. 2010, Koh, Pan et al. 2014), we hypothesized that ATRA represses CYP2D6 expression via the induction of SHP. Same primary human hepatocytes samples were used for analysis and SHP mRNA was measured using qRT-PCR. The results showed that SHP mRNA expression was significantly increased by ~3-fold (Fig 7A).

SHP is known to be induced by FXR/RXR activation (Goodwin, Jones et al. 2000). To determine whether ATRA directly increases SHP promoter activity through FXR, luciferase assays were performed in HepG2 and HEK293T cells co-transfected with SHP-promoter driven luciferease vector harboring 2.2 kb SHP upstream regulatory region, and FXR/RXR (only in HEK293T cells). Cells were treated with vehicle control or 1 μM ATRA and luciferase activities were measured. Interestingly, ATRA did not show induction in SHP promoter activity in both cell lines; no significant difference in SHP promoter activity was observed between vehicle control and ATRA group cells (Fig 7B). This observation can be explained by two possible scenarios: 1) the induction of SHP is not via FXR/RXR activation, as explained by the results shown in HEK293T cell line; 2) the auto-regulatory feedback loop of SHP itself may take effect in the cell systems. To further examine whether ATRA directly increases SHP transcriptional expression, pre-mRNA of SHP was measured using the same human hepatocyte samples. Human hepatocyte mRNA samples were pre-treated with DNase I before reverse transcription, and mRNA expression level were quantified using qPCR primers designed to target intron-extron junction of SHP. In all three batches, SHP pre-mRNA was significantly induced by ATRA by ~3-fold, which is consistence with the mature mRNA results presented previously (Fig 7A), indicating significant increase in SHP transcription activity by ATRA. Overall, results showed that ATRA induces SHP expression in human hepatocytes.

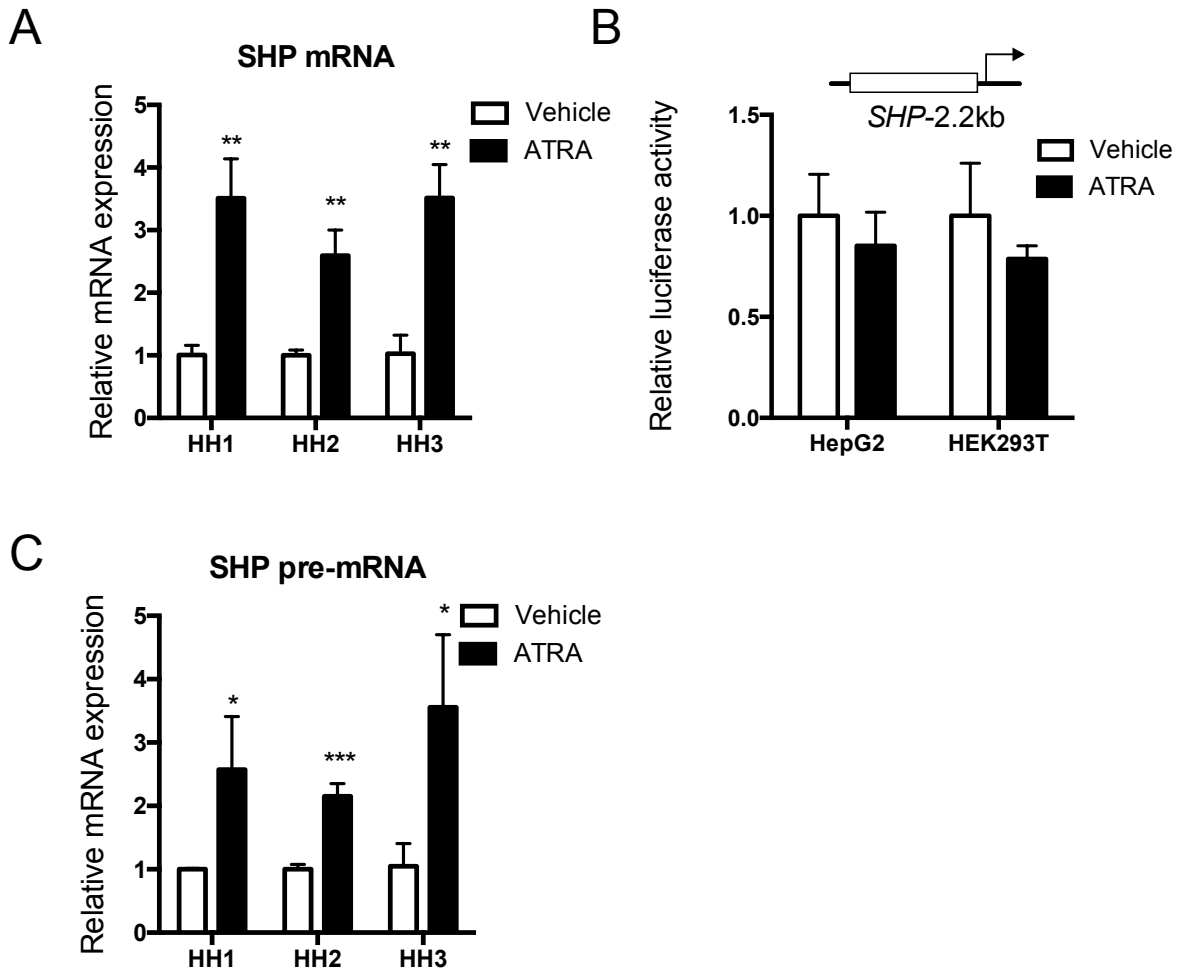

Fig. 7. ATRA induces SHP mRNA and pre-mRNA expression. (A) Primary human hepatocytes from three different donors were treated with vehicle (DMSO) or ATRA (1 μM) for 48 hr in triplicate. RNA was collected and SHP mRNA expression level was measured by using qRT-PCR. (B) HepG2 and HEK293 cells were co-transfected with pGL2-SHP and *Renilla* vector, along with FXR and RXR expression vectors in HEK293T, treated with vehicle or ATRA (1 μM), and dual luciferase assay was performed. (C) Primary human hepatocytes mRNA samples were pretreated with DNase I, and pre-mRNA of SHP was measured using qPCR. **, $p<0.01$;***,$p<0.001$.

3.2.4 CYP2D6 repression by ATRA is abrogated in *Shp* (-/-);CYP2D6 mice.

To investigate the effect of ATRA on CYP2D6 expression *in vivo* and SHP's involvement, we used Tg-*CYP2D6* and *Shp*-null mice in this study. Tg-*CYP2D6* mice which harbor human CYP2D6 and 2.5kb of upstream regulatory region were crossed with *Shp*-null mice to generate mice with *Shp*(+/+);*CYP2D6* or *Shp*(-/-);*CYP2D6* genotypes. Genotyping was performed using PCR and southern blot, and results of each genotype are shown as in Fig 8A.

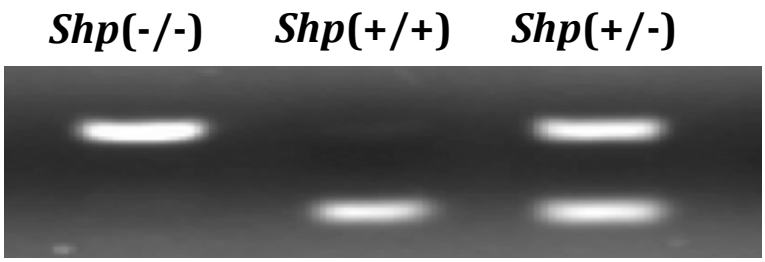

Fig. 8. Genotyping *Tg-CYP2D6* cross *Shp*-null mice.

Mice of *Shp*(+/+);*CYP2D6* and *Shp*(-/-);*CYP2D6* genotypes were intraperitoneally administered vehicle control (olive oil) or ATRA (5m/kg) for 5 days, and hepatic mRNA and protein were collected, and CYP2D6, Shp, Hnf4α levels were measured using qPCR and western blot. Previous study using Tg-CYP2D6 mice indicated that ATRA represses CYP2D6 expression and activity *in vivo* (Koh, Pan et al. 2014).

The results showed ATRA represses CYP2D6 expression in *Shp*(+/+);*CYP2D6* mice, along with significant increase in Shp expression (Fig 9A, 9B), and such repression is abrogated in *Shp*(-/-);*CYP2D6* mice (Fig 9A). Along with the repression of CYP2D6 observedin *Shp*(+/+);*CYP2D6* mice, Shp mRNA expression level was notably induced by ~5-fold while no significant difference in Hnf4α expression level (Fig 9C). To verify the effect of ATRA *in vivo,* Cyp26a1 was selected as the positive as it is known to be upregulated by RA signaling pathway

(Kam, Deng et al. 2012, Topletz, Tripathy et al. 2015). The results showed that Cyp26a1 level was increased in mice of both genotypes (Fig 9D). These results suggested that the repressive effect of ATRA on CYP2D6 requires the involvement of Shp *in vivo*, and ATRA does note affect the basal mRNA expression level of CYP2D6. In conclusion, ATRA transcriptionally represses CYP2D6 *in vivo* via SHP.

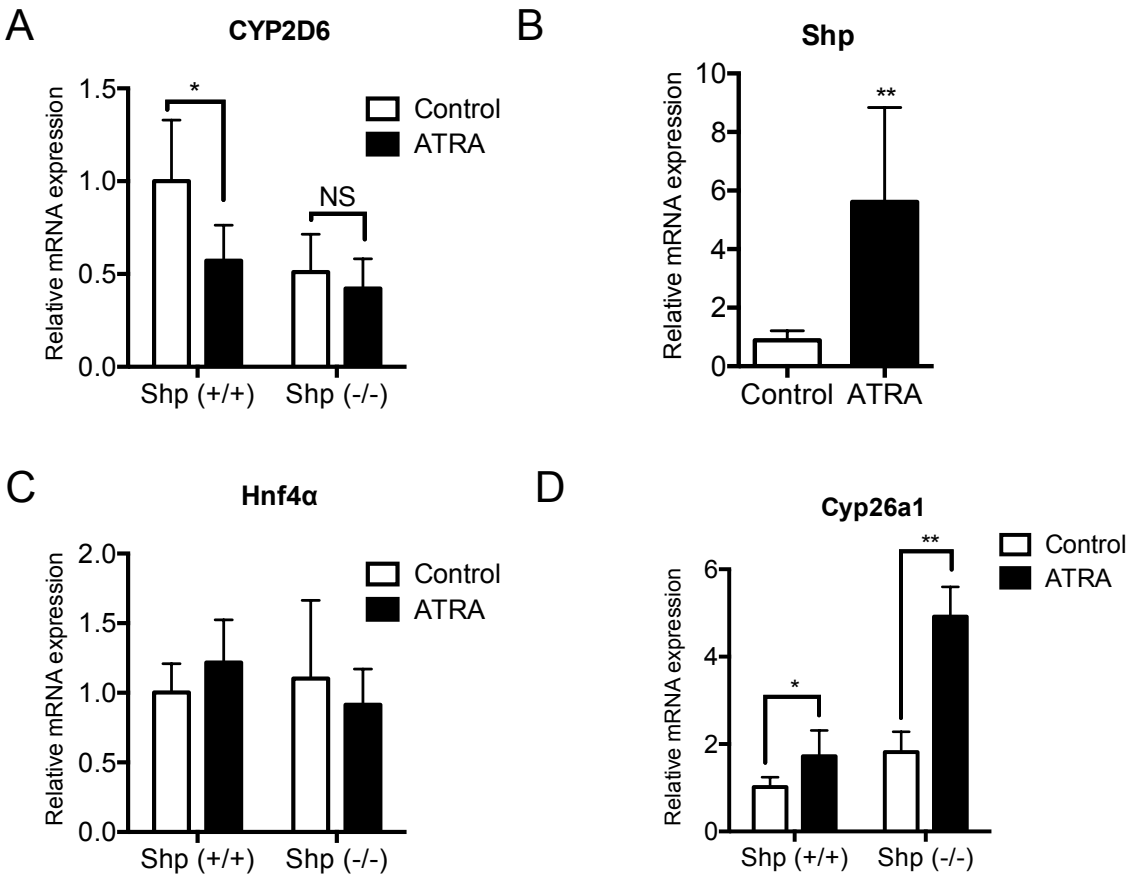

Fig. 9. CYP2D6 repression by ATRA is abrogated in *Shp* (-/-);CYP2D6 mice. *Shp*(+/+);*CYP2D6* or *Shp*(−/−);*CYP2D6* mice were injected with ATRA (10 mg/kg) or vehicle (olive oil) i.p. daily for 5 days (*n* = 4 per group). Hepatic mRNA levels of (B) CYP2D6, (C) Shp, (D) Hnf4α and (E) Cyp26a1 were measured by qPCR and normalized by vehicle control-treated *Shp*(+/+);*CYP2D6* mice. **, *p*<0.01; ***, *p*<0.001; ****, p<0.0001.

3.3 Discussion

Previous studies in Jeong's laboratory have identified the role of SHP as an important regulator in CYP2D6 transcription. SHP represses HNF4α-directed of CYP2D6 transcription (Koh, Pan et al. 2014), and SHP inducers, FXR, represses CYP2D6 expression *in vitro* and *in vivo* (Pan, Lee et al. 2015). ATRA is known to activate RAR/RXR and FXR/RXR heterodimers, and transcriptionally regulates target genes. Aiming to investigate the effect of ATRA as a potential SHP inducer on the transcription regulation of CYP2D6, we utilized both human hepatocytes and Tg-*CYP2D6* models and showed ATRA transcriptionally represses CYP2D6, and this process involves SHP. These findings provide more evidences for the evaluation of co-administration of ATRA, a commonly prescribed drug and supplement, and CYP2D6 substrate drugs. Additionally, investigating the effect of ATRA on CYP2D6 provides better understanding of the altered CYP2D6 expression level during pregnancy, which can be partly attributed to altered hepatic retinoid levels.

In this study, we showed that ATRA represses CYP2D6 by ~4-fold in human hepatocytes and ~2-fold in Tg-*CYP2D6* mice. The decrease in CYP2D6 mRNA was shown to be accompanied by increased SHP expression both *in vitro* and *in vivo.* As an important negative regulator in the transcription of CYP2D6, the role of SHP was further confirmed by utilizing the transgenic mouse model crossing Tg-*CYP2D6* and *Shp*-null mice. The repression of CYP2D6 by ATRA was abrogated in *Shp*(-/-);*CYP2D6* mice, confirming that SHP is involved in the regulation of CYP2D6 by ATRA. This finding is in accordance with previous report on the effect of FXR activation on CYP2D6 *in vivo* (Pan, Lee et al. 2015), that GW4064, a selective FXR agonist, represses CYP2D6 via SHP.

However, it sill remains to be studied the mechanism of ATRA in the induction of SHP. ATRA is known to act through RAR/RXR to transactivate or transrepress target gene expression, and there has not been any RARE reported located on SHP regulatory region yet. Considering that SHP has widely been reported to be induced upon FXR activation (Goodwin, Jones et al. 2000), and ATRA specifically activated FXR/RXR heterodimer (Cai, He et al. 2010), it is reasonable to deduce that ATRA-activated FXR induces SHP expression, and consequently represses CYP2D6 expression. Our results, however, showed that a more complex mechanism of ATRA induction of SHP may exist and FXR is not the only transcription factor responsible for SHP induction. Our luciferase assay data indicate that ATRA does not directly increases SHP promoter activity in HepG2 and HEK293T cells, and the repressive effect of ATRA on CYP2D6 is not dependent. This can be possibly due to the limitation in cell systems that the expression levels of some key transcription factors, other than FXR/RXR, were inadequate. Due to the fact that that the final mRNA level measure is the quantification of mature mRNA product, we considered the possibilities of other regulatory mechanisms involvement. SHP expression level has shown to be regulated by multiple regulatory mechanisms, including transcriptional regulation (Goodwin, Jones et al. 2000), post-transcription regulation via mi-RNAs (Song and Wang 2008), and post-translational modification (Miao, Xiao et al. 2009). We further confirmed our finding using pre-mRNA samples that the induction of SHP by ATRA is via transcriptional activation. Overall, these results indicate that ATRA transcriptionally induces SHP expression, and the detailed mechanism of ATRA in SHP induction is yet to be determined.

Taken together, we showed that ATRA, a commonly prescribed drug represses CYP2D6 expression via SHP induction *in vitro* and *in vivo.* This finding suggests that clinically significant drug-drug interaction may occur between ATRA and CYP2D6 substrates; and altered ATRA level may partially explain the altered CYP2D6 level during pregnancy. These results suggest

the important role of ATRA regulatory effect on important DMEs, and as-yet-known mechanism of ATRA on key hepatic nuclear receptors.

Table 1. Sequences of oligonucleotides used in qRT-PCR

Gene	**Forward primer**	**Reverse primer**
SHP (Nr0b2)	TCAAGTCCATTCCGACCAGC	AAGAAGGCCAGCGATGTCAA
PXR	GATGATCATGTCCGACGAG	GACTTCAGAGTAATGGCGA
MDR3	AATTTATCCTGCCAATCGGA	GCATCAGCAGCAAACAAAAA
FASN	ACAGGGACAACCTGGAGTTC	TGTGGTCCCACTTGATGAGT
SREBF1	TTCTCACCTCCCAGCTCTGT	GGTCCTGGAAGTCAGTCCAT
TNFα	GCCAGAGGGCTGATTAGAGA	TCAGCCTCTTCTCCTTCCTG

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