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**Synergistically enhanced CYP2B6 Inducibility between a Polymorphic Mutation in
CYP2B6 Promoter and PXR Activation**

Haishan Li, Stephen S. Ferguson, and Hongbing Wang

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, 20
Penn Street, Baltimore, MD, USA 21201 (H. L., H. W.) and Life Technologies Corporation,
4301 Emperor Blvd, Durham, NC, USA 27703 (S. S. F.)

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Address corresponding to:

Hongbing Wang Ph.D.,

Department of Pharmaceutical Sciences

University of Maryland School of Pharmacy

20 Penn Street, Baltimore MD 21201.

Fax: 410-706-5017; email: hwang@rx.umaryland.edu

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Abbreviations: CAR, constitutive androstane receptor; CYP, cytochrome P450; C/EBP, CCAAT/enhancer-binding protein; CHIP, chromatin immunoprecipitation assay; CoIP, co-immunoprecipitation assay; 3C, chromosome conformation capture assay; DMSO, dimethylsulfoxide; NR, nuclear receptor; PB, phenobarbital; PBREM, phenobarbital-responsive enhancer module; PXR, pregnane X receptor; RIF, rifampicin; XREM, xenobiotics-responsive enhancer module.

ABSTRACT

CYP2B6 is a highly inducible and polymorphic enzyme, involved in the metabolism of an increasing number of clinically important drugs. Significant interindividual variability in CYP2B6 expression has been attributed to either genetic polymorphisms or chemical-mediated induction through the activation of constitutive androstane receptor and/or pregnane X receptor (PXR). It was reported that the -82T>C substitution within the CYP2B6*22 allele creates a functional CCAAT/enhancer-binding protein (C/EBP) binding site and enhances the basal expression of the *CYP2B6* gene. Here, we explored whether this polymorphic mutation could affect drug-mediated induction of *CYP2B6*. Cell-based promoter reporter assays demonstrated that CYP2B6 luciferase activity was synergistically enhanced in the presence of both -82T>C mutation and rifampicin (RIF) activated PXR. Conversely, this synergism was attenuated by disrupting the C/EBP binding site or knocking down C/EBP α expression. Mechanistic studies revealed that C/EBP α plays an important role in such synergism by directly interacting with PXR; enhancing RIF-mediated recruitment of PXR to the -82T>C harboring the CYP2B6 promoter; and looping the PXR-bound distal phenobarbital-responsive enhancer module towards the proximal C/EBP binding site. Furthermore, the genotype-phenotype association was evaluated in cultured human primary hepatocytes from 44 donors. Interestingly, RIF-mediated induction of CYP2B6 in four -82T/C carriers was higher compared to that in the reference -82T/T homozygotes. Collectively, our results demonstrate, for the first time, a synergistic interplay between a CYP2B6 polymorphism and PXR-mediated induction, which may contribute to the large individual variations and inducibility of CYP2B6 in humans.

Cytochrome P450 2B6 (CYP2B6) participates in the metabolism of a growing number of clinically important drugs, such as cyclophosphamide, ifosfamide, efavirenz, selegiline, bupropion, tamoxifen, and methadone, as well as a wide variety of environmental chemicals (Hidestrand et al., 2001; Hodgson and Rose, 2007; Lang et al., 2001; Wang and Tompkins, 2008). Notably, significant interindividual variations in CYP2B6 expression have been observed previously. In these studies, CYP2B6 exhibits 20- to 280-fold interindividual variability in its gene expression and 25- to 80-fold variations in its enzymatic activity (Code et al., 1997; Ekins et al., 1998; Faucette et al., 2000; Hanna et al., 2000; Hesse et al., 2004; Hesse et al., 2000; Lamba et al., 2003; Stresser and Kupfer, 1999). These findings suggest that the remarkable variability in CYP2B6 expression and activity may be a major determinant of the efficacy and toxicity of these widely used drugs that are characterized by narrow therapeutic indices.

During the past several years, numerous investigations have focused on elucidating the predominant mechanisms underlying interindividual variability in CYP2B6 expression. Among the factors studied such as age, gender, ethnics, genetics, and xenobiotics, it appears that receptor-mediated gene regulation and genetic polymorphisms are the major contributors. Currently, it has been well established that CYP2B6 is highly inducible through the activation of nuclear receptors (NR) such as constitutive androstane receptor (CAR, NR1I3) and pregnane X receptor (PXR, NR1I2) (Goodwin et al., 2001; Sueyoshi et al., 1999). By cross-talking, CAR and PXR coordinately control the induction of CYP2B6 expression via recognizing and binding to two putative xenobiotic response clusters located at – 1.7 kb [phenobarbital-responsive enhancer module (PBREM)] and – 8.5 kb [xenobiotics-responsive enhancer module (XREM)] of CYP2B6 promoter (Sueyoshi et al., 1999; Wang et al., 2003). PXR and CAR are considered promiscuous xenobiotic sensors that recognize a broad array of xenobiotics, and translate chemical activation into enhanced expression of target genes.

In addition to chemical induction, CYP2B6 is also characterized as a highly polymorphic gene. Presently, 29 alleles of CYP2B6 have been identified (<http://www.cypalleles.ki.se/>) from

over 50 mutations in different combinations. Among these alleles, extensive studies have centered on the non-synonymous single nucleotide polymorphisms (SNPs), which often result in decreased expression or malfunction of CYP2B6 protein (Lang et al., 2004; Rotger et al., 2007). Comparatively, little is known regarding the function of polymorphisms in the promoter of *CYP2B6* gene. Initial genotyping of 83 and 108 human samples led to the identification of 10 and 14 mutations in the promoter of *CYP2B6*, respectively (Hesse et al., 2004; Lamba et al., 2003). Sequence analysis of these mutations purported that a number of them may influence *CYP2B6* expression by altering consensus transcription factor binding sites. Subsequently, Zukunft et al. reported that the -82T>C, a mutation within the *CYP2B6**22 allele, enhances the basal expression of *CYP2B6* by introducing a functional CCAAT/enhancer-binding protein (C/EBP) binding site and shifting the transcription start site downstream (Zukunft et al., 2005).

C/EBPs belong to the basic region leucine zipper (bZIP) protein family, and play pivotal roles in cellular differentiation, liver regeneration, apoptosis, and liver-specific gene expression (Schrem et al., 2004). An earlier study showed that introduction of C/EBP α to HepG2 cells augments the basal expression of *CYP2B6*, *CYP2C9*, and *CYP2D6* mRNA (Jover et al., 1998). Disruption of the C/EBP α binding sites in the promoter of *CYP3A4* repressed the basal expression of *CYP3A4* reporter construct (Rodriguez-Antona et al., 2003). Nevertheless, whether and how introduction/disruption of C/EBP binding site would affect NR-mediated induction of CYP enzymes was largely unexplored.

Given that *CYP2B6* expression exhibits dramatic intra- and inter-individual variability, we hypothesized that the polymorphic (-82T>C) substitution may affect drug induced expression of *CYP2B6* by facilitating communication between C/EBP and PXR. Here we provide experimental evidence to show the synergistic effect between this polymorphic mutation and PXR-mediated induction of *CYP2B6* gene expression. By employing co-immunoprecipitation (CoIP), chromatin immunoprecipitation (ChIP), chromosome conformation capture (3C) assays, and transient luciferase promoter reporter experiments, our studies revealed, for the first time, that C/EBP α

directly interplays with xenobiotic receptor PXR and CAR to coregulate the synergistic induction of the *CYP2B6* gene. Additionally, a pilot genotype-phenotype study showed a possible correlation between this polymorphism and the potent induction of CYP2B6 through PXR activation. Together, these findings reveal a novel role of C/EBP-PXR in the maximal induction of CYP2B6, and may have pharmacological significance in the efficacy and toxicity of drugs as CYP2B6 substrates.

MATERIALS AND METHODS

Materials. phenobarbital (PB), dimethylsulfoxide (DMSO), RIF, and collagenase type IV were purchased from Sigma-Aldrich (St. Louis, MO). Oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, IA). The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). FuGENE[®] 6 and FuGENE[®] HD transfection reagents were from Roche (Basel, Switzerland). Lipofectamin 2000 transfection reagent was from Invitrogen (Calsbad, CA). Matrigel, insulin and ITS⁺ were obtained from BD Biosciences (Bedford, MA). Other cell culture reagents were purchased from Invitrogen or Sigma-Aldrich.

Cell lines. HepG2, HepG2-stable expression of hPXR (PXR-HepG2), HepG2-stable expression of hCAR (Yh18), Huh7, and COS1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The Yh18 cell line was obtained from Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, National Institutes of Health, RTP, NC) (Swales et al., 2005). PXR-HepG2 was a cell line generated by stable transfection of hPXR (pCR3-hPXR) expression vector, and selected for neomycin resistance. A single cell clone was selected and functionally evaluated (Supplemental Fig. 1).

Human primary hepatocytes. Human liver tissues (15 donors) were obtained following surgical resection by qualified pathology staff after diagnostic criteria were met and prior approval from the Institutional Review Board at the University of Maryland School of Medicine. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described previously (LeCluyse et al., 2005). Another 29 human primary hepatocyte preparations were obtained from Life Technologies Corporation (Durham, NC). Hepatocytes were seeded at 1.5×10^6 cells/well in 6-well Biocoat (BD Biosciences) plates in DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml insulin, and 1 µM dexamethasone, and then cultured in serum-free Williams' E medium as described previously (Wang et al., 2003). All human primary hepatocytes

were treated with RIF (10 μ M) or vehicle control (0.1% DMSO) for 24 or 72 hrs before harvested for mRNA or CYP2B6 activity analysis, respectively.

Plasmids. The pSG5-hPXR expression vector was obtained from Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). The CYP2B6-1.8 kb luciferase reporter vector (Swales et al., 2005) as well as the pMEX-C/EBP α and pcDNA3-C/EBP β expression vectors were kindly provided by Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC). A 2kb fragment spanning -1 to -1993 bp of the native CYP2B6 promoter region was PCR amplified using specific primers listed in Table 1. This product was subcloned into the NheI-HindIII site of pGL3-basic vector, resulting in the construct termed CYP2B6-2kb. Polymorphic variant -82T>C and C/EBP disruption -82M were constructed using the QuickChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA) (Fig. 1A). The CYP2B6-2kb construct and all the mutants were sequencing confirmed. The pRL-TK *Renilla* luciferase plasmids used to normalize firefly luciferase activities were from Promega (Madison, MI).

Transient Transfection and Luciferase assay. HepG2 or Huh7 cells in 24-well plates were transfected with hPXR expression vector, and CYP2B6-2kb reporter construct using Fugene[®] 6 transfection reagent following the manufacturer's instruction. Twenty four hours after transfection, cells were treated with solvent (0.1% DMSO) or 10 μ M RIF for another 24 hrs. Subsequently, cell lysates were assayed for firefly activities normalized against the activities of cotransfected *Renilla* luciferase using Dual-Luciferase Kit (Promega, WI). Data were represented as mean \pm S.D. of three individual transfections.

Small Interfering RNA. The predesigned siRNA specific for C/EBP α (mixture of Hs_CEBPA_2 and Hs_CEBPA_4) and a non-targeting siRNA were obtained from Qiagen (Valencia, CA). To detect knockdown of endogenous C/EBP α , HepG2 cells plated in 12-well plates were transfected with siRNA-CEBP α (40 pmol) or non-targeting siRNA (40 pmol), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent. Forty-eight hours after

transfection, cells were harvested and total RNA was isolated and reverse-transcribed into cDNA. C/EBP gene expression was measured using SYBR real-time polymerase chain reaction (PCR) as described in the section of *Quantitative RT-PCR*. In cell-based reporter assays, following 24 hrs of siRNA transfection, PXR expression vector and CYP2B6-2kb reporter vector were also transfected in HepG2 cells using FuGENE[®] HD transfection reagent. The double transfected cells were treated with RIF (10 μ M) or vehicle control (0.1% DMSO) for 24 hrs, then subjected to dual-luciferase assays as described above.

Co-immunoprecipitation assays. COS1 cells were transfected with C/EBP α and PXR expression vectors, and treated with RIF (10 μ M) for 24 hrs. Subsequently, cells were washed with phosphate-buffered saline buffer and scraped into the lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulphonyl fluoride, and 1 \times complete protease inhibitor cocktail). After incubation on ice for 15 min, the lysate was centrifuged at 13,000 \times g for 15 min at 4°C. The supernatant fractions were collected and incubated with antibodies (anti-PXR rabbit polyclonal antibody, sc-25381x; anti-C/EBP α mouse monoclonal antibody, sc-81558x. Santa Cruz, CA) and protein A-Sepharose beads overnight at 4°C. Corresponding isotype IgG was used as a negative control. The beads were washed three times, and the precipitated protein complexes were analyzed by Western blotting with PXR and C/EBP α antibodies, respectively.

Chromatin immunoprecipitation assays. Experiments were performed using a ChIP assay kit according to the manufacturer's protocol (Millipore, Bedford, MA). Briefly, 2 \times 10⁶ PXR-HepG2 cells were seeded into 10 cm dish, grown to 80% confluence, and transfected with constructs containing -82T, -82C, or -82M. Transfected cells were treated with RIF (10 μ M) or DMSO (0.1% v/v) for 3 hrs. Subsequently, cells were crosslinked with 1% formaldehyde for 10 min at 37 °C, washed with ice-cold phosphate-buffered saline containing a protease inhibitor cocktail, lysed, and sonicated. Immunoprecipitation was performed overnight at 4°C using 2.5 μ g rabbit anti-human PXR polyclonal antibody (sc-25381x) or isotype control IgG followed by

precipitation using protein A coupled to agarose beads. After de-crosslink and protease digestion, DNA fragments were recovered by QIAquick PCR purification kit (Qiagen, Valencia CA). Quantitative PCR was performed using specific sets of primers (Table 1) (Song et al., 2004). PCR products were also resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Chromosome Conformation Capture (3C). 3C assays were performed as described previously with minor modification (Babu et al., 2008; Inoue and Negishi, 2009). Briefly, 100 μ g of nuclear extract prepared from PXR-HepG2 cells were shaken with 50 ng of the CYP2B6-1.8k construct containing -82T, -82C, or -82M, respectively, at room temperature for 45 min and were cross-linked with formaldehyde then terminated by the addition of glycine. Subsequently, these protein-DNA complexes were precipitated by ethanol at -20°C. Precipitates were dissolved in 80 μ l of Tris-EDTA buffer, digested by *Xho*I at 37°C for 4 hrs and then incubated for 20 min at 70°C to inactivate the enzyme activity of *Xho*I. Digests were ligated by T4 DNA ligase (Fermentas, Hanover, MD) for 5 min at room temperature. After ligation, protein-DNA complexes were decross-linked by adding sodium dodecyl sulfate and NaCl up to 1% and 0.3M, respectively, and treated overnight at 55°C and then treated with proteinase K for additional 1 h at 65°C. DNA was purified by PCR purification kit (Qiagen, Valencia CA). Purified DNA was subjected to amplification using two primer sets (Table 1).

Quantitative RT-PCR. Total RNA was isolated from treated hepatocytes using the RNeasy Mini kit (Qiagen, Valencia CA) and reverse transcribed using High Capacity cDNA Archive kit (Applied Biosystems, Foster, CA) following the manufacturers' instructions. CYP2B6 mRNA expression was normalized against the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems). Primers for CYP2B6 and GAPDH, as well as C/EBP mRNA detection were shown in Table 1 (Spandidos et al., 2009). Fold induction values were calculated according to the

equation $2^{\Delta\Delta Ct}$, where ΔCt represents the differences in cycle threshold numbers between the target gene and GAPDH, and $\Delta\Delta Ct$ represents the relative change in these differences between control and treatment groups.

CYP2B6 activity assay. CYP2B6 basal and induced enzymatic activities were determined in situ from cultures of human primary hepatocytes using the probe substrate bupropion. Incubations were performed with 0.5 mL of HBSS containing 250 μ M bupropion for 15 minutes for each preparation of hepatocytes following 72 hours treatment with 0.1% DMSO or 10 μ M RIF. After incubations were complete, supernatants were transferred to deep well blocks and frozen at -80°C for subsequent LC-MS/MS analysis of hydroxybupropion formation using standard analytical methods as described previously (Lau and Chang, 2009).

Genotyping. Genomic DNA samples from human primary hepatocytes of 44 liver donors (Supplemental Table 1) were isolated and purified by QIAamp DNA Mini kit (Qiagen, Valencia CA). Genotyping of CYP2B6 - 82T>C was undertaken using ABI Prism 7000 Sequence Detection System, and probes and primers were also designed by Applied Biosystems (TaqMan Drug Metabolism SNP Genotyping assay, C_27830964_10). In brief, 50 ng of whole genomic DNA was used as a template for amplification of the CYP2B6 target sequence. The PCR reactions were conducted in a reaction volume of 10 μ l (DNA template, 2 \times genotyping master mix, and 20 \times mixture of primers and probes) under the following conditions: initial denaturation step of 10 min at 95°C followed by 50 cycles of denaturation 92°C for 15 s, annealing and extending for 90 s at 60°C . The allelic discrimination was performed by post read.

Statistics. All reporter assay data represent at least three independent experiments and are expressed as the mean \pm S.D. Statistical comparisons were made using Student's *t*-test. Because CYP2B6 activity data were not normally distributed, nonparametric method (rank-sum test) was used to compare phenotypic data. Statistical significance was set at $p < 0.05$.

RESULTS

Synergistic enhancement of CYP2B6 reporter expression by CYP2B6 -82T>C and PXR activation. Initial investigation of the potential interplay between CYP2B6 -82T>C (CYP2B6-82C) polymorphisms and PXR activation was carried out using cell-based reporter assays in HepG2 and Huh7 cells, two human hepatoma cell lines. As expected, in HepG2 cells, CYP2B6-82C mutation alone enhanced the basal reporter expression by 4.4-fold over the reference CYP2B6-82T, and RIF-mediated activation of PXR resulted in 14-fold increase in CYP2B6-82T expression (Fig. 1B). Surprisingly, combination of CYP2B6-82C mutation and activation of PXR led to a 64-fold augmentation of reporter expression over the reference CYP2B6-82T, which is substantially higher than the additive effects of the polymorphism and PXR activation individually (4.4- plus 14-fold). In Huh7 cells, a similar trend but more robust synergistic effect has been observed, where CYP2B6-82C and PXR activation resulted in 10- and 3-fold increases, respectively, whereas in combination they enhance the reporter activity by 80-fold over the reference CYP2B6-82T control (Fig. 1C). Notably, without ligand activation, over-expression of PXR had no effects on either of these two CYP2B6 reporter constructs (Fig. 1B and 1C), suggesting that ligand-based conformational changes in PXR are required for these observed synergistic enhancement of CYP2B6 reporter expression.

C/EBP α plays an important role in the observed synergism. Because the polymorphic -82C was reported to form a new C/EBP binding site, we further evaluated the role of C/EBP in this synergistic effect in HepG2 and Huh7 cells. As depicted in Fig. 1A, a construct containing mutations disrupting the C/EBP binding site without affecting the polymorphic -82C was generated and termed CYP2B6-82M. Reporter assays revealed that PXR-mediated activation of CYP2B6-82M expression was significantly reduced compared with CYP2B6-82C in transfected HepG2 and Huh7 cells (Fig. 2A and 2B). To further test the effects of C/EBP α expression on PXR-mediated activation of CYP2B6-82C expression, a siRNA specific for C/EBP α was used to knock down the C/EBP α expression in HepG2 cells. Forty-eight hours after transfection, the

gene expression of C/EBP α was down-regulated by 65% in comparison with the control group transfected with nontargeting siRNA (Fig. 2C). This knockdown of C/EBP α significantly decreased the synergistic effects between CYP2B6-82C and PXR activation (Fig. 2D). Conversely, over-expression of transfected C/EBP α expression vector led to an enhancement of PXR-mediated activation of CYP2B6-82C (Fig. 2E). Collectively, these results support the pivotal role of C/EBP in the observed synergism of CYP2B6-82C and PXR activation. Additionally, because the major isoform of C/EBP expressed in HepG2 cells is C/EBP α (Fig. 2C), this isoform is the focus of the current studies.

Direct interaction between PXR and C/EBP α . To determine whether the two proteins interact in a cellular context, we performed Co-IP assays using COS-1 cells transiently transfected with PXR and C/EBP α expression vectors. As demonstrated in Fig. 3A, immunoprecipitation (IP) of PXR protein using PXR antibody resulted in coimmunoprecipitation of C/EBP α protein, a result that was not observed when normal rabbit IgG was used for IP instead. Alternatively, coimmunoprecipitation of PXR was also observed when C/EBP α antibody was used for IP. These findings suggest that the two proteins are components of an intracellular complex.

-82T>C mutation increased recruitment of PXR to the promoter of CYP2B6. To demonstrate whether the presence of the polymorphic -82C mutation would enhance the binding of PXR to the PBREM in the native CYP2B6 promoter, a modified ChIP assay was conducted in PXR-HepG2 cells. CYP2B6 promoter construct CYP2B6-82T, CYP2B6-82C, or CYP2B6-82M was transfected into the PXR-HepG2 cells, respectively, and treated with RIF (10 μ M) for 3 hrs as described in *Experimental Procedures*. To eliminate the interference of the genomic wild-type CYP2B6 inherited in HepG2 cells, a pair of primers specific to the plasmid-expressed PBREM region was designed (Table 1). In particular, the forward primer complements with the backbone sequence of pGL3-basic plasmid but not the CYP2B6 promoter. Using this unique system, we observed that recruitment of PXR to the PBREM in CYP2B6-82C

construct was clearly stronger than that to the CYP2B6-82T and CYP2B6-82M after RIF stimulation (Fig. 3B, 3C).

Looping the distal PBREM towards the proximal C/EBP binding site. It appears that both the distal PBREM and the -82C formed proximal C/EBP α binding site are required for the synergistic effects of PXR-mediated transcription of CYP2B6. An *in vitro* chromosome conformation capture (3C) assay was employed to explore whether a looping mechanism exists by which the PBREM-bound PXR would have physical access to the C/EBP α sitting on -82C generated proximal binding site (Hagege et al., 2007; Inoue and Negishi, 2009). As depicted in Fig. 4A, the -1.8kb CYP2B6 promoter constructs containing either -82T, -82C, or -82M were cross-linked with nuclear proteins and digested to open the circled plasmids. After ligation, PCR reaction should produce a 80bp fragment if looping occurs. When the -1.8k CYP2B6 promoter was incubated with nuclear proteins prepared from PXR-HepG2 cells, a dramatically increased amplification of the 80bp fragments was observed in the construct containing -82C, whereas no PCR product can be detected in either the reference -82T or the -82M construct (Fig. 4B).

Since CAR induces CYP2B6 through binding to the PBREM, we also examined whether -82T>C mutation could enhance CAR-mediated induction of CYP2B6 in the same manner as with PXR. In cell-based reporter assays, CYP2B6-82C and PB-stimulated CAR activation resulted in 2.5- and 4-fold increases over the reference CYP2B6-82T control, respectively, while combined they enhanced the reporter activity by 10-fold (Fig. 5A). Similar to the results from PXR, a parallel 3C experiment using nuclear extract from the Yh18 cells revealed that CAR-bound PBREM was looped towards the C/EBP-occupied -82C region, which has led the amplification of an 80 bp PCR product in CYP2B6-82C only (Fig. 5B).

Genotype-phenotype association of CYP2B6-82T/C polymorphism and RIF-mediated induction of CYP2B6 in human primary hepatocytes. To further characterize the role of the -82T>C mutation in drug-mediated CYP2B6 induction in humans, a collection of fresh human primary hepatocytes from 44 liver donors was treated with RIF for 24 hrs, and the RIF-

mediated induction of CYP2B6 mRNA (15 donors) or bupropion hydroxylation activity (29 donors) was analyzed (Supplemental Table 1). Overall, four donor preparations were identified with the CYP2B6-82T/C genotype, while the remaining 40 donors were reference carriers of CYP2B6-T/T (Fig. 6C). Real-time PCR analysis of 15 human hepatocyte preparations from University of Maryland Medical Center showed that RIF induction of CYP2B6 mRNA was most robustly in HL#2 (30-fold), which happens to be the only CYP2B6-82T/C carrier in these 15 donors (Fig. 6A). Additionally, CYP2B6 activity (bupropion hydroxylation) was analyzed in 29 human primary hepatocytes from Life Technologies Corporation. As demonstrated in Fig. 6B, the average induction of RIF in three CYP2B6-82T/C heterozygotes (11-fold) was significantly higher than that observed in the 26 donors with reference CYP2B6-82T/T (6.5-fold).

DISCUSSION

Remarkable inter- and intra-individual variations in CYP2B6 expression and catalysis have been documented through an array of *in vivo* and *in vitro* studies (Code et al., 1997; Ekins et al., 1998; Hanna et al., 2000; Hesse et al., 2000). These variations are relevant to clinical outcomes in context of interindividual drug safety and efficacy with CYP2B6-metabolized drugs such as bupropion, efavirenz, and cyclophosphamide (Gatanaga et al., 2007; Rotger et al., 2007). To date, mounting evidence indicates that both nuclear receptor-mediated induction and genetic variation are important factors in variable interindividual CYP2B6 expression (Lamba et al., 2003; Lang et al., 2001; Sueyoshi et al., 1999; Wang and Tompkins, 2008). Nevertheless, these two pivotal factors have predominantly been considered separately with a clear lack of studies investigating the interplay between genetic polymorphisms and NR-mediated drug induction for CYP2B6. In this report, we have demonstrated that the SNP -82T>C that creates a functional C/EBP binding site in the proximal promoter of CYP2B6, exerts synergistic effects with PXR on RIF-mediated induction of CYP2B6 expression. Moreover, a novel mechanism has been revealed by which ligand activated PXR could mediate the synergistic response through interaction with C/EBP α protein harboring the -82T>C introduced C/EBP binding site.

Several previous studies have indicated that polymorphic changes in the promoter of drug-metabolizing genes may affect the basal expression and/or activity of these isozymes. For instance, UGT1A1*28, which introduces an extra TA to the TATA box of this gene, has resulted in a lower UGT1A1 expression and activity (Biason et al., 2008; Lampe et al., 1999); SNPs of -750T>C and -82T>C in the promoter of CYP2B6 have led to decreased and increased basal expression and activity of CYP2B6, respectively (Hesse et al., 2004; Lamba et al., 2003; Zukunft et al., 2005). Interestingly, further analysis of the CYP2B6 promoter by Zukunft et al (Zukunft et al., 2005) revealed that the -82T>C substitution introduces a functional C/EBP binding site and shifts the transcription start site downstream. Knowing that C/EBP proteins play pleiotropic roles in hepatic gene expression, we initially explored whether the C/EBP site

forming SNP (-82T>C) would affect the PXR-mediated CYP2B6 expression. To our surprise, a potent synergism between -82T>C mutation and ligand-activated PXR has been observed in our cell-based reporter experiments using both HepG2 and Huh7 cells. This is further evidenced by the synergistic response attenuated by both site-directed disruption of the C/EBP binding site and by genetic knockdown of the C/EBP α expression by siRNA. Conversely, overexpression of C/EBP α in Huh7 cells was found to augment this synergistic response. Together these results and the elevated induction responses observed in primary hepatocyte cultures from -82T/C individuals suggest the existence of a positive interdependence between PXR and C/EBP α leading to synergism.

It has been well established that induction of *CYP2B6* gene expression is predominantly regulated at the transcriptional level. To date most findings suggest that induction of *CYP2B6* gene by xenobiotics is mediated by activation of the nuclear receptors CAR and/or PXR through interactions with the PBREM/XREM located in the upstream of *CYP2B6* transcriptional start site (Goodwin et al., 2001; Sueyoshi et al., 1999; Wang et al., 2003). Nevertheless, several significant phenomena regarding *CYP2B6* induction could not be fully explained by this simplified model. For instance, in contrast to the potent induction of *CYP2B6* gene, relatively moderate induction has been observed for the majority of other PXR/CAR target genes such as *CYP2Cs* and *UGT1A1* (Chen and Goldstein, 2009; Ferguson et al., 2005; Sugatani et al., 2004). Moreover, dramatic variations of PXR/CAR-mediated induction of *CYP2B6* occurred among individuals even with the same PBREM/XREM sequences. This suggests that other regulatory factors, in addition to PXR/CAR proteins and the PBREM/XREM sites, may be also involved in the maximal induction of *CYP2B6* gene. In addition to the synergy between the -82T>C mutation and PXR activation in cell-based reporter assays, our coimmunoprecipitation data clearly showed that C/EBP α and PXR cross-coupled together in an intracellular environment. Of importance, recruitment of PXR to the PBREM of *CYP2B6* was remarkably enhanced only when the -82T>C was presented in the modified ChIP assays, suggesting both C/EBP protein and the

novel binding site are crucial for the beneficial interaction of PXR with the CYP2B6 promoter. C/EBPs are liver-enriched transcription factors with pleiotropic influences on hepatic gene expression (Schrem et al., 2004). Previous studies indicate that cross-talk with other transcriptional factors exists for C/EBPs in modulating their target gene expression. For example, the formation of a complex between the glucocorticoid receptor and the C/EBP α involves the regulation of glucocorticoids on lymphocytic and mesenchymal cell proliferation (Rudiger et al., 2002); while coupling of C/EBPs and nuclear factor NF- κ B affects the expression of genes with putative Kappa-B enhancer motifs via protein-protein interactions (Stein and Baldwin, 1993; Stein et al., 1993). Recently, Song and colleagues (Song et al., 2006) illustrated the essential role of C/EBP α in VDR-mediated induction of SULT2A1 by directly interacting with each other through adjacent VDRE and C/EBP sites. In contrast, the known PXR/CAR interacting PBREM and the novel C/EBP binding site in the promoter of CYP2B6 are distally spaced by around 1.7kb.

Insight into the genomic organization of mammals reveals that regulatory elements, enhancers in particular, in the promoter of genes are often dispersed remotely encompassing a long-scale of kilobase-pairs (Hagege et al., 2007). Proper folding of the sequence at this scale is extremely important for gene regulation. In light of the recent development of an *in vitro* 3C assay which allows the detection of physical interactions of protein-bound DNA segments and DNA looping (Hagege et al., 2007; Inoue and Negishi, 2009), we applied this assay to test the hypothesis that PXR-bound PBREM is physically folded towards the proximal C/EBP binding site through a looping mechanism. Our 3C experiments revealed that both PXR- and CAR-bound PBREMs were efficiently looped to the C/EBP harboring -82C site but not to the -82T region (Fig. 4B, 5B). Given that DNA looping is a widely accepted mechanism by which distantly separated cis-acting elements were physically coupled, it is reasonable to speculate that constitutive factors such as C/EBPs and HNFs may interact with xenobiotic factors such as PXR, CAR, AhR, and VDR through the common looping mechanisms to facilitate coregulation of their

target genes.

To further investigate the association of -82T>C with RIF-mediated induction of CYP2B6, a pilot genotype-phenotype association study has been conducted in human primary hepatocytes collected from 44 donors. Notably, four CYP2B6-82T/C heterozygous and no CYP2B6-82C/C homozygous carriers were identified from these donors. It is in agreement with an earlier report in that -82T/C heterozygotes were the dominant form of this SNP with no -82C/C being identified, yet the basal expression of CYP2B6 was significantly increased in the -82T/C carriers (Zukunft et al., 2005). In our functional experiments, RIF induction of CYP2B6 mRNA or activity was clearly elevated in hepatocyte cultures from the four -82T/C heterozygous than from the reference donors (Fig. 6). Although we cannot reach a conclusive correlation between -82T>C SNP and RIF-mediated induction of CYP2B6 in humans at this moment due to the limited sample size and the difficult nature of obtaining human primary hepatocytes, these pilot results indicate that the -82T/C carrier might be more sensitive to chemical induction of *CYP2B6* gene.

In conclusion, our data suggest that polymorphic -82T >C mutation exerts synergistic effects with PXR in xenobiotic-mediated induction of *CYP2B6* gene. This synergy is mediated through a novel mechanism by which the constitutive C/EBP α interplays with xenobiotic sensitive PXR and led to the enhanced recruitment of PXR to the PBREM and folding of the distal PBREM towards the proximal region of CYP2B6 where transcription happens. Moreover, the pilot genotype-phenotype association study seems to support the observation that PXR-mediated induction of CYP2B6 is enhanced in donors with such a gain-of-function allele. Overall, these findings bridge the polymorphism of CYP2B6 with the NR-mediated induction of this gene, and shed light on a potential explanation of the remarkable inter- and intra-individual variations in CYP2B6 expression. Given the increasing importance of CYP2B6 in drug metabolism and detoxification, these findings may also have clinical implications to drug-drug interactions arising from induction of CYP2B6-mediated substrates.

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FOOTNOTES

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FIGURE LEGENDS:

Figure 1. Synergistic activation of CYP2B6 reporter in human hepatoma cells. *A*, three firefly luciferase reporter constructs carrying wild type (-82T), mutant (-82C), or C/EBP binding site disruptive mutation (-82M) of -2kb CYP2B6 promoter. The location of phenobarbital-responsive enhancer module (PBREM) was also indicated. *B and C*, representative reporter assays in HepG2 cells and Huh7 cells. Cells were cotransfected with the reporter constructs carrying -82T or -82C, in the presence of PXR expression vector or empty pGS5 vector. Twenty-four hours after transfection, cells were treated with RIF (10 μ M) or vehicle (0.1%DMSO) for 24 hrs. Luciferase activities were measured using the dual-luciferase kit (Promega). Triplicate samples were performed for each treatment.

Figure 2. Both the -82T>C mutation and C/EBP α protein are required in the synergistic response. Different reporter and/or expression constructs, as well as siRNAs were transfected into HepG2 or Huh7 cells as described under *Materials and Methods*. *A and B*, HepG2 and Huh7 cells were cotransfected with the reporter constructs carrying -82T, -82C or -82M, and PXR expression vector or empty vector. Transfected cells were then treated with vehicle or rifampicin (RIF) for 24 hrs. Luciferase activities were measured using the dual-luciferase kit (Promega). *C*, HepG2 cells were transfected with siRNA-control or siRNA-CEBP α . Expression levels of C/EBP α and C/EBP β were detected using real-time RT-PCR. *D*, In a parallel experiment, 24 hrs after C/EBP α knockdown, HepG2 cells were subsequently cotransfected with CYP-82T, or -82C, and PXR expression vector or empty vector. Luciferase activities were measured as described above after RIF treatment. *E*, Similar reporter assays were conducted in Huh7 cells following the initial transfection of C/EBP α expression vector. All experiments were performed in

triplicate samples. *: $P < 0.05$.

Figure 3. C/EBP α interacts with PXR, and the -82T>C mutation enhances the recruitment of PXR to the PBREM of CYP2B6. *A*, direct protein-protein interaction between PXR and C/EBP α was detected by CoIP assay in COS1 cells transiently transfected with PXR and C/EBP α expression vectors. *B*, PXR recruitment to the PBREM region of CYP2B6 was detected using a modified ChIP assay as described under *Materials and Methods*. T: -82T wild type; C: -82C mutant; M: the C/EBP binding site disruption mutant. *C*, quantity of the de-crosslinked DNA was measured by real-time PCR.

Figure 4. Physical interaction between PXR-bound PBREM and C/EBP α harboring -82C. *A*, schematic presentation of the chromosome conformation capture (3C) experimental procedure. *B*, The -1.8kb CYP2B6 constructs containing -82T (T), -82C (C) or -82M (M) were incubated with nuclear extracts prepared from PXR-HepG2 cell line treated with RIF (10 μ M) or vehicle control, and subjected to 3C experiments as outlined under *Materials and Methods*. The degrees of ligation were determined by PCR using primers listed in table 1. The PCR amplicons were loaded to a 1.5% agarose gel and stained with ethidium bromide.

Figure 5. Interplay between CAR and C/EBP α . *A*, Human CAR-HepG2 stable cell line (Yh18) was transfected with CYP2B6 reporter constructs carrying -82T or -82C, in the presence of pRL-TK vector as internal control. Transfected cells were then treated with vehicle or phenobarbital (PB, 3 mM) for 24 hrs. Luciferase activities were measured using the dual-luciferase kit (Promega). Triplicate samples were performed for each treatment. *B*, similar 3C assays as described above were conducted using nuclear

extracts prepared from Yh18 cells.

Figure 6. Association of CYP2B6 - 82T/C genotyping with the induction of CYP2B6 by rifampicin treatment. *A*, Fifteen human primary hepatocyte preparations were treated with RIF (10 μ M) or vehicle control (0.1% DMSO) for 24 hrs. Realtime RT-PCR was conducted to measure fold induction of CYP2B6 mRNA (HL#2 is -82T/C and all the rest are -82T/T carriers). *B*, Twenty-nine human hepatocyte preparations were treated with RIF (10 μ M) or vehicle control (0.1% DMSO) for 72 hrs. Bupropion hydroxylation was used to detect fold induction of CYP2B6 activity as described in Materials and Methods. *C*, depicts the genotyping distribution of CYP2B6-82T>C SNP in the 44 liver donors. *: $P < 0.05$

Table 1. Primer sequences for PCR assays

Assay	Gene	Sequence (5'— 3")	Reference
RT-PCR	GAPDH	CCCATCACCATCTTCCAGGAG	[Spandidos et al., 2009]
		GTTGTCATGGATGACCTTGGC	
	C/EBP α	TCGGTGGACAAGAACAGCAA	
		TTTCAGGAGGCACCGGAATCT	
C/EBP β	CTTCAGCCCGTACCTGGAG		
	GGAGAGGAAGTCGTGGTGC		
	CYP2B6	AGACGCCTTCAATCCTGACC	
		CCTTCACCAAGACAAATCCGC	
ChIP	RVprimer3	CTAGCAAATAGGCTGTCCC	[Song et al., 2004]
	CYP2B6(PBREM)	GATGCTGATTCAGGGAATGGA	
3C	CYP2B6(Loop)	AGGATAAAAGGCCAGTTGGA	[Inoue et al., 2009]
		CTAAGATTGGGTGCTCATTGCA	
	CYP2B6(Control)	GGCCAGGATGGTCTCGAA	
		CCAGCACGTTGGGAAGCT	
CYP2B6-2kb	CYP2B6-2kNheI	CTAGCTAGCGGACAATGTAGCCCCAACCC	
	CYP2B6-2kHindIII	CCCAAGCTTGGTCCTGGTCTGACTGCCCTG	

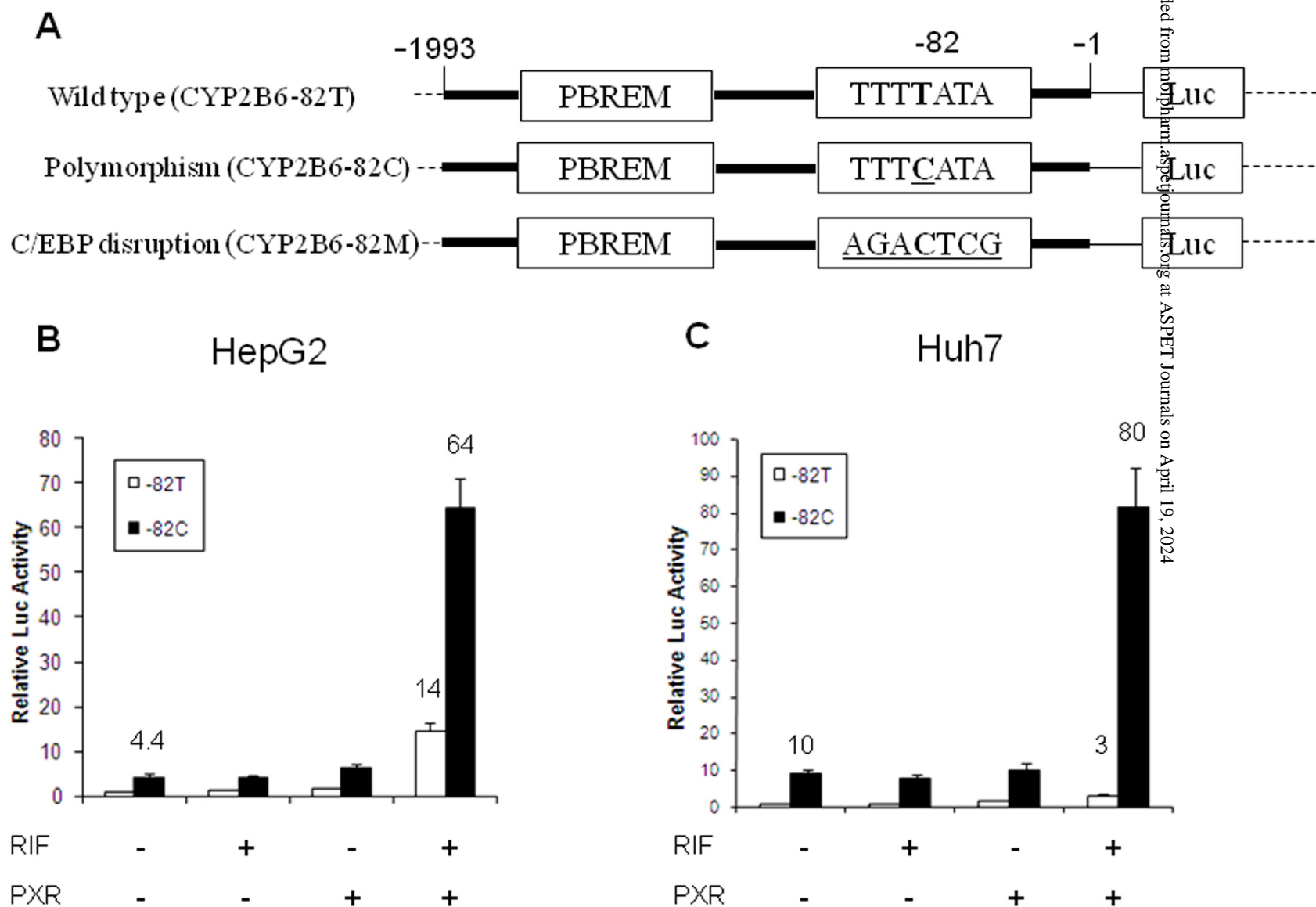
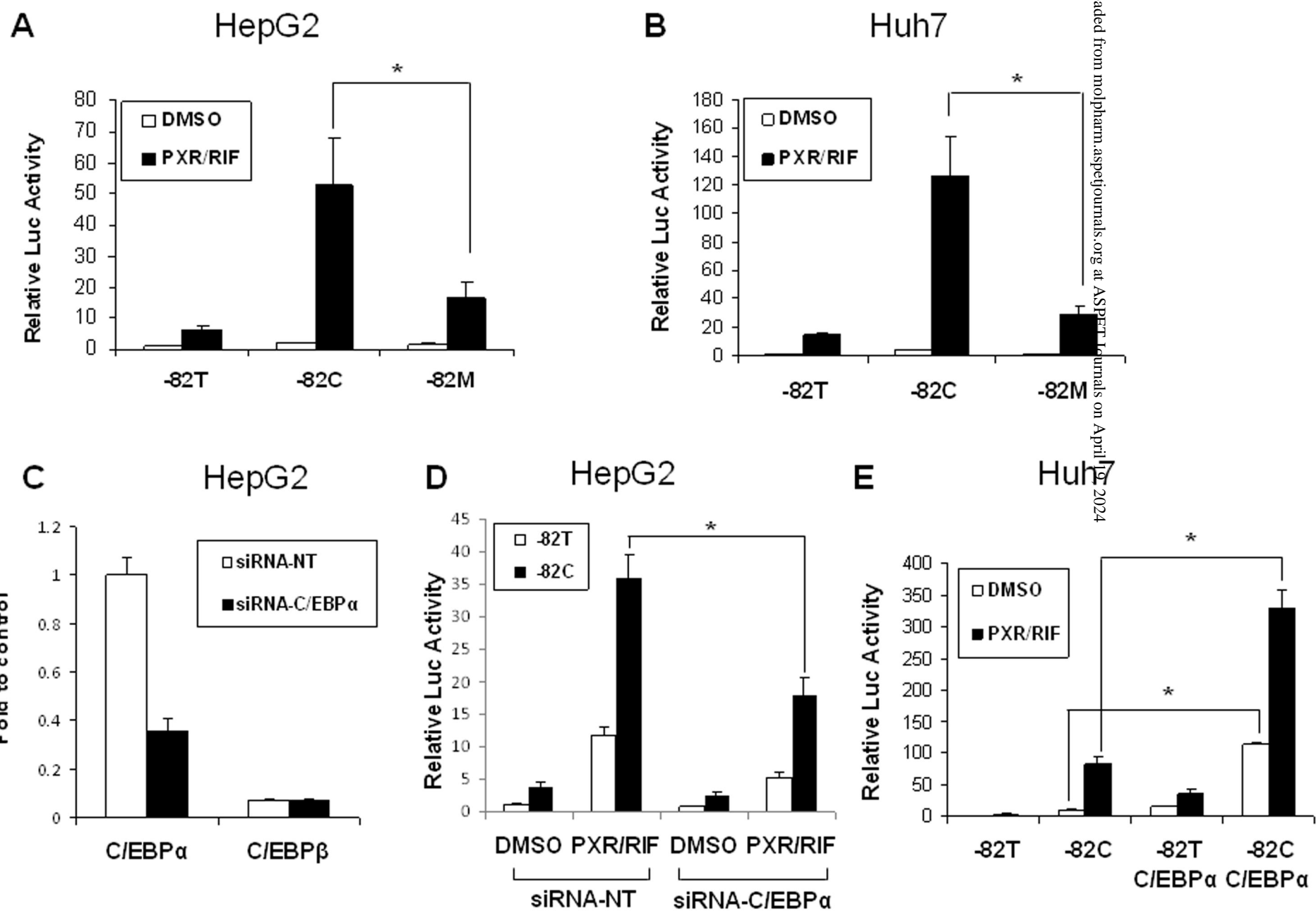


Figure 1

**Figure 2**

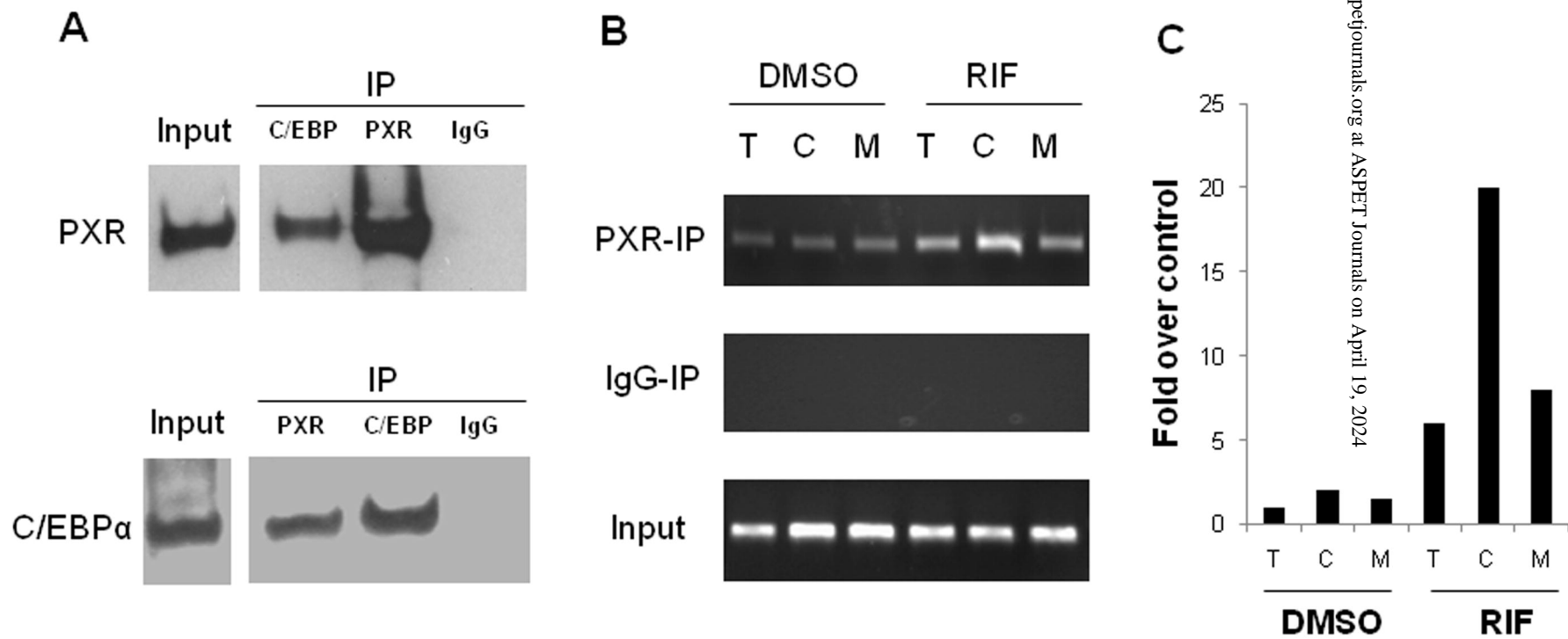


Figure 3

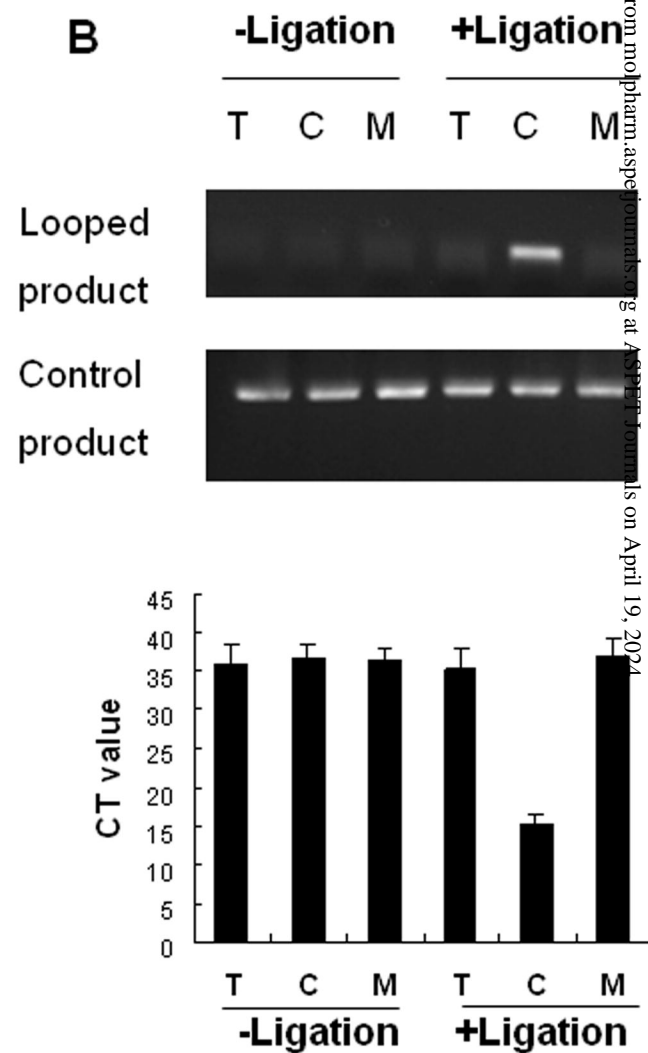
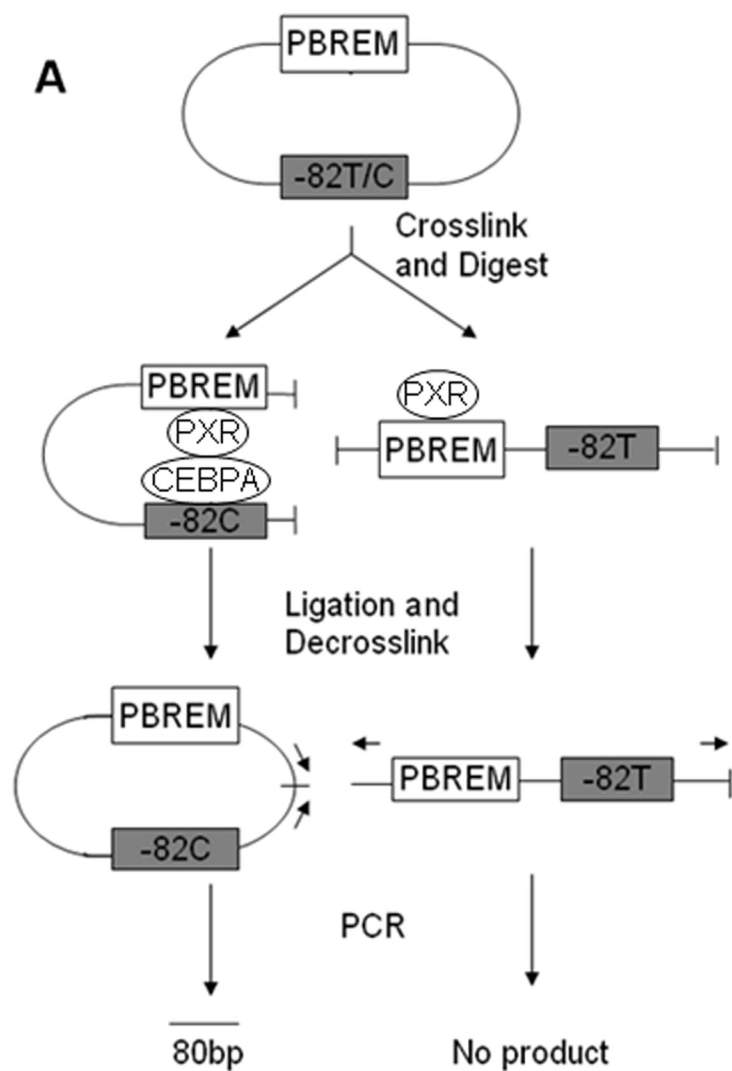


Figure 4

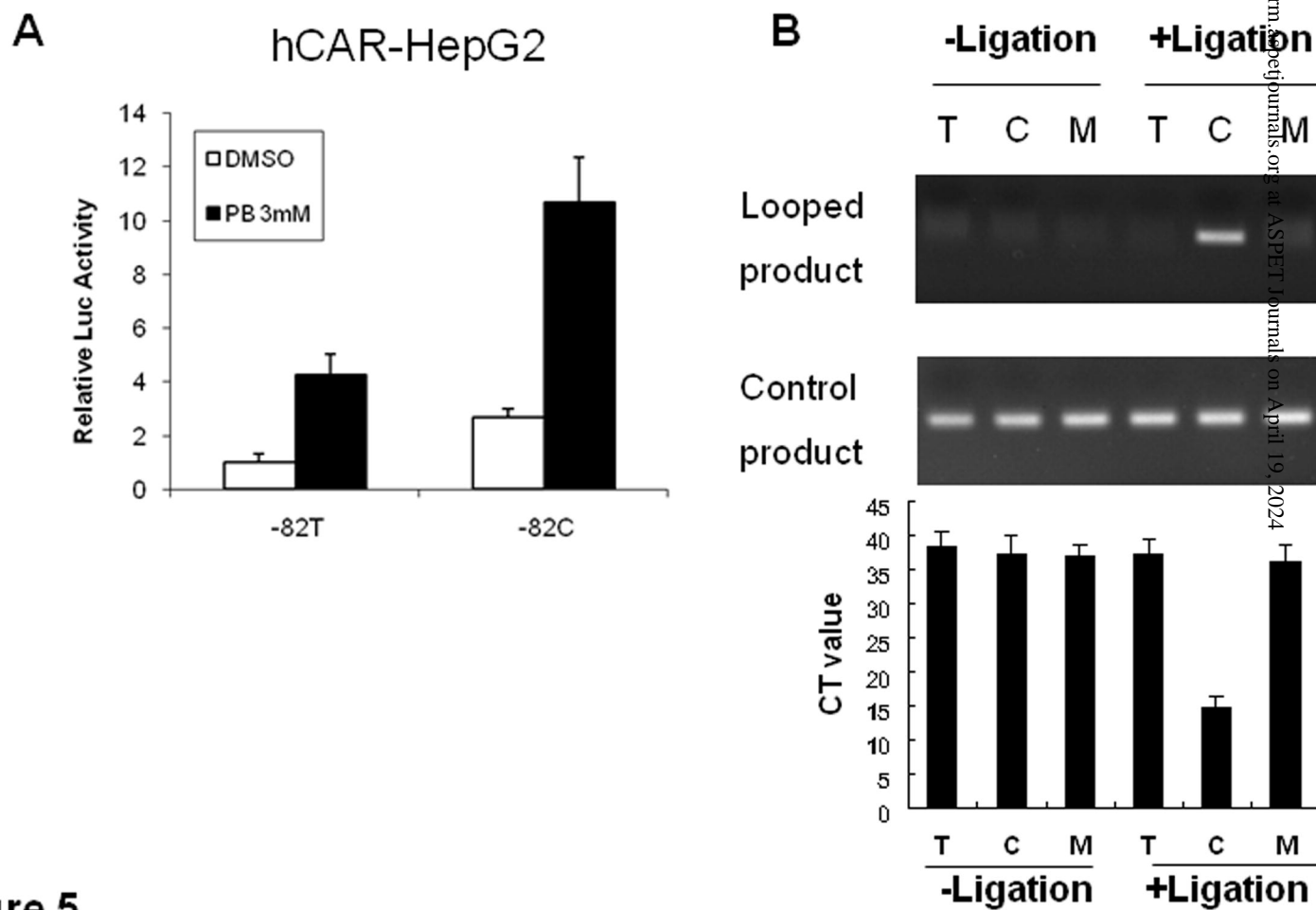


Figure 5

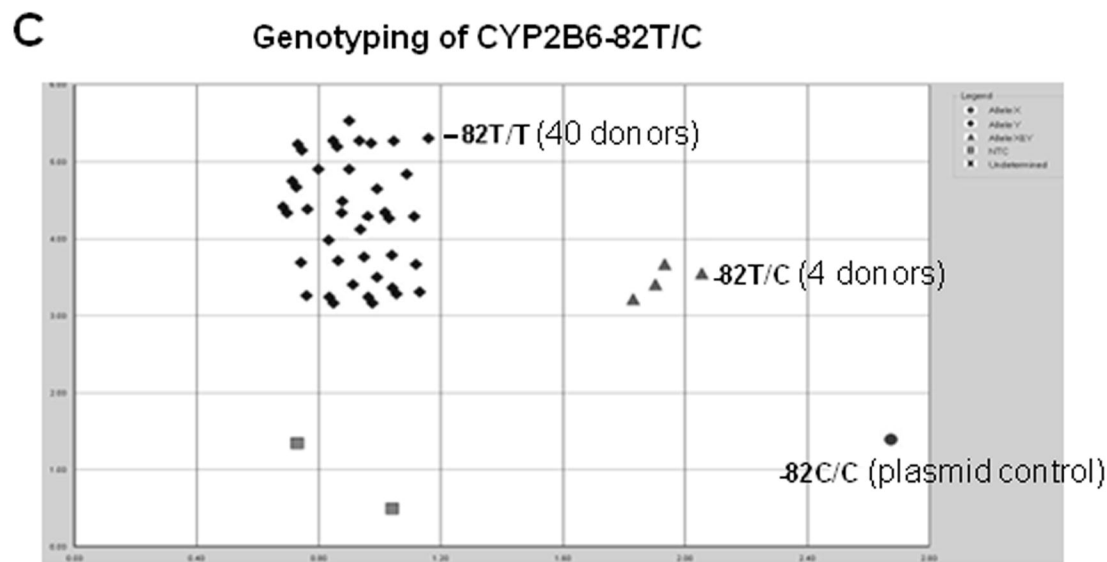
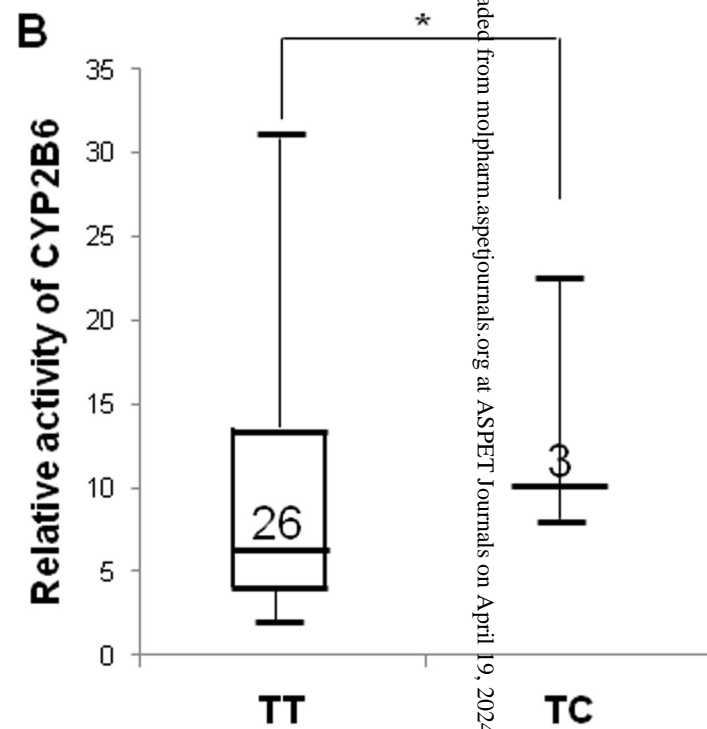
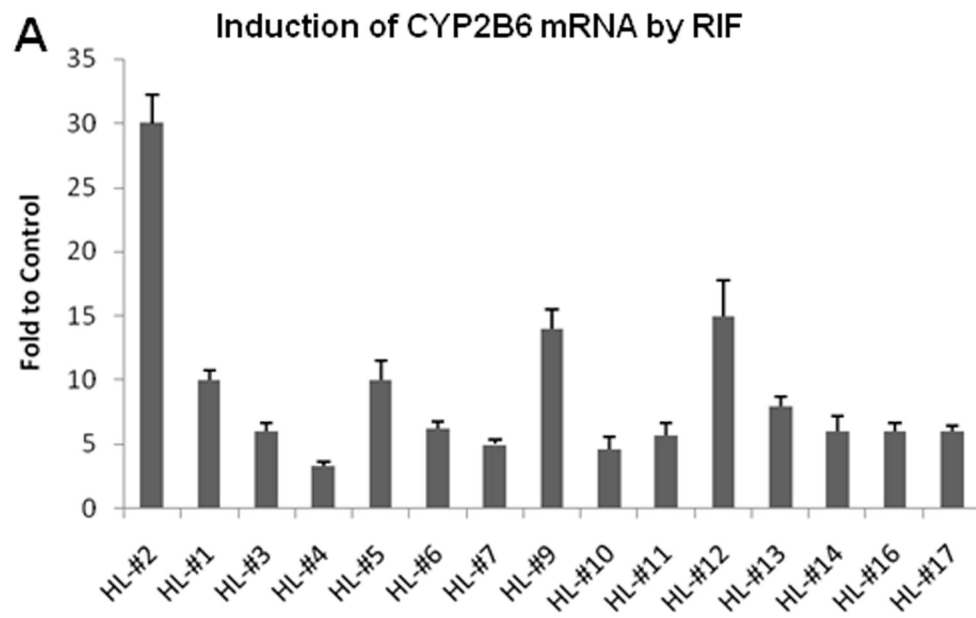


Figure 6