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**Direct Transcriptional Regulation of Human Hepatic Cytochrome P450 3A4  
(CYP3A4) by Peroxisome Proliferator-Activated Receptor Alpha (PPAR $\alpha$ )**

Maria Thomas, Oliver Burk, Britta Klumpp, Benjamin A. Kandel, Georg Damm, Thomas S.  
Weiss, Kathrin Klein, Matthias Schwab, Ulrich M. Zanger

Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany (MT,  
OB, BK, BAK, KK, MS, UMZ)

Charité University Medicine Berlin, Berlin, Germany (GD)

Department of Pediatrics and Juvenile Medicine, University of Regensburg Hospital,  
Regensburg, Germany (TSW)

Department of Clinical Pharmacology, University of Tuebingen, Tuebingen, Germany (MS)

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**Running title:** *PPAR $\alpha$  is a direct transcriptional regulator of CYP3A4*

**Address correspondence to:**

Ulrich M. Zanger, Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology,  
Auerbachstr. 112, 70376 Stuttgart, Germany, Tel.: +49-(0)711-81013704; FAX: +49-(0)711-  
859295; E-mail: [uli.zanger@ikp-stuttgart.de](mailto:uli.zanger@ikp-stuttgart.de)

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PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; CYP, cytochrome P450; PBR, PPAR $\alpha$ -binding region; POPC, 16:0/18:1-PC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; PXR, pregnane X receptor; PPRE, peroxisome proliferator response element; PHH, primary human hepatocytes; ADME, absorption, distribution, metabolism and excretion; CHIP, Chromatin immunoprecipitation; RXR, retinoid X receptor; shRNA, small hairpin RNA; DR, direct repeat; ACOX1, acyl-CoA oxidase 1; CAR, constitutive androstane receptor

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## Abstract

The nuclear receptor peroxisome proliferator-activated receptor (PPAR) $\alpha$  is primarily known as a regulator of fatty acid metabolism, energy balance, and inflammation, but there is evidence suggesting a wider role in regulating biotransformation of drugs and other lipophilic chemicals. We investigated whether PPAR $\alpha$  directly regulates the transcription of cytochrome P450 (CYP) 3A4, the major human drug metabolizing enzyme. Using chromatin immunoprecipitation in human primary hepatocytes as well as electrophoretic mobility shift and luciferase reporter-gene assays we identified three functional PPAR $\alpha$ -binding regions (PBR)-I, II, III within ~12kb of *CYP3A4* upstream sequence. Furthermore, a humanized CYP3A4/3A7 mouse model showed in vivo induction of CYP3A4 mRNA and protein by WY14,643 in liver but not in intestine, while hepatic occupancy of PBRs by PPAR $\alpha$  was ligand-independent. Using lentiviral gene knock-down and treatment with WY14,643 in primary human hepatocytes, PPAR $\alpha$  was further shown to affect the expression of a distinct set of CYPs including 1A1, 1A2, 2B6, 2C8, 3A4, and 7A1, but not 2C9, 2C19, 2D6 or 2E1. Interestingly, the common phospholipid 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC, 16:0/18:1-PC), previously proposed to reflect nutritional status and shown to be a specific endogenous ligand of PPAR $\alpha$ , induced *CYP3A4* (up to 4-fold) and other biotransformation genes in hepatocytes with similar selectivity and potency as WY14,643. These data establish PPAR $\alpha$  as a direct transcriptional regulator of hepatic CYP3A4. This finding warrants investigation of known and newly developed PPAR $\alpha$ -targeted drugs for their drug-drug interaction potential. Furthermore, our data suggest that nutritional status can influence drug biotransformation capacity via endogenous phospholipid signalling.

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## Introduction

Genes encoding enzymes and transporters that protect higher animals from toxic effects of lipophilic xenobiotics have evolved not only to optimize their substrate selectivity and catalytic properties but also their regulation in order to allow flexible responses to environmental changes. In particular the lipid- and xenobiotics-sensing nuclear receptors pregnane X-receptor PXR (NR1I2), the constitutive androstane receptor CAR (NR1I3), and the aryl hydrocarbon receptor AhR, together with liver-enriched transcription factors HNF4 $\alpha$  (NR2A1), C/EBP and others form networks of transcription factors that coordinately regulate hepatic expression of the majority of drug metabolizing cytochromes P450 (CYP), phase II enzymes and transporters in response to xenobiotic exposure (Handschin and Meyer, 2005; Pascussi et al., 2008; Pelkonen et al., 2008). Recent research elucidated extensive crosstalk between these receptors and other nuclear receptors and transcription factors, linking xenobiotic metabolism to the homeostasis of lipids, bile acids, glucose and other endogenous processes (Moreau et al., 2008; Gao and Xie, 2012). Such insight into the connections between xenobiotic metabolism and endogenous homeostasis can therefore enhance our mechanistic understanding of drug toxicity as well as that of pathophysiological conditions of the liver such as dyslipidemia, nonalcoholic fatty liver disease and others (Anderson and Borlak, 2008; Buechler and Weiss, 2011).

The most important class of nuclear receptors for lipid homeostasis are the peroxisome proliferator-activated receptors (PPAR)  $\alpha$  (NR1C1),  $\beta/\delta$  (NR1C2), and  $\gamma$  (NR1C3), which act as lipid sensors to control the expression of gene networks involved in lipid and energy homeostasis, adipocyte differentiation, and inflammatory responses (Lalloyer and Staels, 2010; Wahli and Michalik, 2012). PPAR $\alpha$  expression is high in tissues with active fatty acid catabolism, including liver, intestine, heart, muscle and others, where it primarily regulates intracellular trafficking and metabolism of lipids including mitochondrial and peroxisomal  $\beta$ -

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oxidation, microsomal  $\omega$ -hydroxylation, and also cholesterol and bile acid biosynthesis (Pyper et al., 2010). Activating ligands include synthetic fibrates used to treat dyslipidemia and the investigative specific agonist [4-chloro-6-(2,3-xylylidino)-2-pyrimidinylthio]acetic acid (WY14,643), as well as various endogenous lipids (Forman et al., 1997; Kliewer et al., 1997; Chakravarthy et al., 2009). Elucidation of PPAR $\alpha$  target genes by treatment of human and mouse hepatocytes with WY14,643 revealed that regulation of hepatic lipid metabolism is generally well-conserved, while other regulated genes were largely divergent (Rakhshandehroo et al., 2009, 2010). For example, CYP4 family members, which function as microsomal fatty acid omega-hydroxylases, are more inducible in rodents compared to humans (Waxman 1999; Richert et al., 2003; Hsu et al., 2007), while drug metabolizing CYPs of families 1 to 3, including CYP3A4, CYP2B6, CYP2C8, and CYP1A2, appeared to be inducible by fibrates in humans only (Richert et al., 2003; Prueksaritanont et al., 2005; Rakhshandehroo et al., 2009). Although functional PPREs were identified in *CYP4* genes (Johnson et al., 1996) as well as in several phase II enzymes, including members of the UDP-glucuronosyltransferases (Barbier et al., 2003; Senekeo-Effenberger et al., 2007), the basis for PPAR $\alpha$ -dependent hepatic expression of human CYPs of families 1 to 3 remained unclear, in particular whether regulation occurs directly or indirectly, e.g. by involvement of other nuclear receptors such as PXR (Aouabdi et al., 2006). Further evidence suggesting an involvement of PPAR $\alpha$  as a regulator of *CYP3A4*, the most important drug metabolizing P450 of human liver and intestine (Zanger et al., 2008), includes a systems biology approach which analyzed time-dependent expression profiles of primary human hepatocytes (PHH) treated with statins (Schröder et al., 2011) and a pharmacogenomics study, which identified PPAR $\alpha$  polymorphisms as a predictive marker of hepatic CYP3A4 expression and function (Klein et al., 2012).

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Here we used PHH and a CYP3A4/3A7-humanized mouse model to investigate whether PPAR $\alpha$  directly regulates transcription of CYP3A4. Our data suggest a previously unrecognized role of PPAR $\alpha$  as a direct transcriptional regulator of drug metabolizing CYPs of families CYP1-3. We also tested whether a specific phospholipid, that has recently been shown to act as endogenous ligand of PPAR $\alpha$  and that is believed to serve as an indicator of nutritional status (Chakravarthy et al., 2009), is able to induce CYP3A4 and other ADME (absorption, distribution, metabolism and excretion) genes. Our data suggest intricate interactions between lipid homeostasis, nutritional status and drug biotransformation capacity that may be of relevance under diseased conditions such as obesity and hepatic steatosis. Furthermore, our findings suggest a largely uninvestigated potential for PPAR $\alpha$ -dependent drug-drug interactions.

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## Materials and Methods

**Primary human hepatocytes and treatments.** The use of human hepatocytes for research was approved by the local ethics committees of Berlin and Regensburg, and written informed consent was obtained from all patients. Hepatocytes were isolated and cultured essentially as described (Klein et al., 2012) except that 12-well plastic dishes precoated with collagen were used (OMNILAB, Bremen, Germany). Cells were treated for 24h with the indicated substances in 0.1% DMSO (Dimethyl Sulfoxide), Rifampicin (5,6,9,17,19,21-Hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[*N*-(4-methyl-1-piperazinyl)formimidoyl]-2,7-(epoxypentadeca-[1,11,13]trienimino)-naphtho[2,1-*b*]furan-1,11(2*H*)-dion-21-acetat (R3501), CITCO (6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime) (C6240), or WY14,643 ([4-chloro-6-(2,3-xylylidino)-2-pyrimidinylthio]acetic acid) (C7081) from Sigma Aldrich, Steinheim, Germany or in 4% BSA in PBS (for phospholipids) purchased from Avanti Polar Lipids, Hamburg, Germany.

**Chromatin immunoprecipitation (ChIP) assay.** ChIP was performed using MAGnify Chromatin Immunoprecipitation Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's description. DNA was sheared by sonication to an average length of ~300bp using Bioruptor (diagenode, Liege, Belgium) and incubated with 10µg of anti-PPARα antibody (PP-H0723-00; R&D Systems, Wiesbaden, Germany), previously bound to 10µl of magnetic beads at 4°C for 2 hours and DNA was purified using DNA purification beads and eluted in 150µl of elution buffer. Promoter occupation was analyzed with 10µl of immunoprecipitated DNA by Sybr-Green PCR. Primers are listed in the Supplemental Table 1. The results were normalized to HMGCR (3-hydroxy-3-methyl-glutaryl-CoA reductase) promoter region (-1970/-2200bp) used as positive control (van der Meer et al., 2010) and the untranscribed region Untr-5 as negative control (Hariparsad et al., 2009). For ChIP analysis of fresh frozen mouse livers we applied the same protocol using specific manufacturer

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recommendations for tissue handling. Binding to Gys2 (glycogen synthase 2) promoter was assessed in parallel as a positive control for binding of mouse Ppara (Mandard et al., 2007).

**Plasmids.** Previously described plasmids: human PPAR $\alpha$  expression plasmid pcDNA3-hPPAR $\alpha$ , kindly provided by T. Tanaka, University of Tokyo, Japan (Tanaka et al., 2002); pcDhsRXRA, encoding human RXR $\alpha$  (Mathäs et al., 2012); CYP3A4 luciferase reporter gene constructs (Tegude et al., 2007). PPREX3-TK-luc construct was purchased from Addgene (#1015) (Kim et al., 1998). Mutations in the identified PPREs were generated using QuickChange™ II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The wild-type and contextual mutation constructs (12-17) were generated from self-designed gene fragments which were synthesized by GeneArt (Life Technologies, Darmstadt, Germany) and cloned into construct 1.

**Electrophoretic mobility shift assays (EMSA).** Human PPAR $\alpha$  and RXR $\alpha$  proteins were synthesized using expression plasmids and TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI). Complementary single stranded oligonucleotides (Supplemental Table 2) were annealed and radiolabeled as described (Tegude et al., 2007). Binding reactions and gel electrophoresis were performed as described (Geick et al., 2001). Retarded complexes were quantified using BAS1800 II phosphor-storage scanner (Fuji, Kanagawa, Japan) and AIDA software, version 4.19 (Raytest, Straubenhardt, Germany).

**In silico analysis.** For predictions of potential PPAR $\alpha$  binding sites, NUBIScan version 2.0 (cut-off Z-score 7; Podvinec et al., 2002) at [www.nubiscan.unibas.ch](http://www.nubiscan.unibas.ch) and PPRESearch (defaults settings; Venkatachalam et al., 2009) at [www.classicus.com/PPRE](http://www.classicus.com/PPRE) were used.

**Luciferase reporter gene assays.** Human hepatocarcinoma HepG2 cells were transfected with 250 ng of reporter plasmid and 50 ng of pcDNA3-hPPAR $\alpha$  expression vector using Turbofect (Fermentas, St. Leon-Rot, Germany). pcDNA3 plasmid DNA was added to adjust the total amount of transfected DNA to 300 ng. To control for transfection efficiency, 25 ng



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of pGL3-TK-Renilla (Promega, Mannheim, Germany) was used. Cells were harvested in reporter “passive” lysis buffer (Promega) after 24h of incubation, and cell extracts were analyzed for firefly and renilla luciferase activities using Enspire® Multimode Plate-Reader (Perkin Elmer, Rodgau, Germany) and Luciferase Assay System from P.J.K. GmbH (Kleinblittersdorf, Germany).

**Animal experiment.** Experiments with humanized huCYP3A4/3A7 mice (Hasegawa et al., 2011) were performed at TaconicArtemis GmbH (Cologne, Germany). Animals were maintained in a temperature-controlled room (23±1°C) with a 12h light/dark cycle. All experiments were approved by the Local Ethical Review Committee and conducted in compliance with the United Kingdom “Animals (Scientific Procedures) Act” (1986). Fifty mg of WY14,643 in corn oil per kilogram of body weight was orally administered (with syringe) to five male huCYP3A4/3A7 mice at approximately 3 months of age daily for four days. Control animals received corn oil vehicle only. Mice were then anesthetized, exsanguinated, and tissues removed and flash-frozen in liquid nitrogen.

**Quantitative RT-PCR Analysis.** Isolation of total RNA, quantification, and complementary DNA synthesis were performed as described (Klein et al., 2012). The assays for the detection of PPAR $\alpha$  mRNA (Klein et al., 2012), CYP1A1 (400nM of fw:5'-TGG TCA AGG AGC ACT ACA AAA CC-3', and rev:5'-TCA TCT GAC AGC TGG ACA TTG G-3' primers, and 200nM of probe MGB: 6-FAM-GAT GTG GCC CTT CTC-MGB), and CYP3A4 (Wolbold et al., 2003) were self-designed and oligonucleotides were from Applied Biosystems (Darmstadt, Germany). For all other genes pre-designed Taqman assays were purchased from Applied Biosystems. For comprehensive qPCR profiling we used Fluidigm's Biomark high-throughput qPCR chip platform (Fluidigm Corporation, San Francisco, CA) with 96 gene expression assays (Applied Biosystems; list available on request) assays following

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manufacturer's instructions (Spurgeon et al., 2008). For final evaluation of fold changes, ribosomal gene RPLP0 (human) and beta-actin (mouse) were used for normalization.

**Western blot analysis.** CYP3A4 protein was detected in mouse liver homogenates using 50  $\mu$ g of protein per lane as described earlier (Wolbold et al., 2003). Beta-actin was detected using mouse monoclonal antibody from Sigma-Aldrich (Steinheim, Germany) #A5441 in 1:5000 dilutions.

**Lentivirus-mediated RNAi in human primary hepatocytes.** Treatment of PHH with lentivirus encoding PPAR $\alpha$ -specific small hairpin RNAs (shRNA) was performed as described (Klein et al., 2012). Further details can be found at <http://seek.virtuelle-leber.de/sops/13>.

**Measurement of CYP enzyme activities.** CYP enzyme activities were determined in culture supernatants using an LC/MS/MS-based substrate cocktail assay as reported previously (Feidt et al., 2010).

**Statistical Methods.** For pairwise analysis of statistical significance, the Student's paired t-test (2-tailed) was applied and statistical significance was defined as  $P < 0.05$ . To identify statistically significant differences, one-way ANOVA with Student-Newman-Keuls posttest was performed with the mean values of at least three independent experiments done in triplicates. For correlations, the Spearman rank correlation coefficient ( $r_s$ ) was calculated. All calculations were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

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## Results

**ChIP reveals occupation of *CYP3A4* promoter by PPAR $\alpha$  in PHH.** In silico analysis of the *CYP3A4* promoter and upstream region (20kb) identified a vast number of putative DR1 and DR2 motifs with different degree of homology to the consensus AGGTCA nuclear receptor half site however did not identify any 100% consensus motif. Thus, we systematically screened ~12kb of upstream region by ChIP of isolated chromatin from PHH using a total of 40 primer pairs. As evident from figure 1, three regions, designated as PPAR $\alpha$ -binding regions (PBR-I, -2600/-3100bp; PBR-II, -7200/-7800bp; PBR-III, -8500/-8900bp) showed significant enrichment of promoter binding by PPAR $\alpha$  as compared to *HMGCR* and unoccupied intermediary gene regions. Pre-treatment of hepatocytes with WY14,643 did not change enrichment profiles (data not shown). Region PBR-II overlapped partially with the well-described XREM (xenobiotic-responsive enhancer module), which includes binding sites for several transcriptional regulators of *CYP3A4* including PXR, HNF (hepatocyte nuclear factor) 4 $\alpha$ , and FXR (farnesoid X receptor).

**PPAR $\alpha$  directly binds to distinct motifs within the identified PPAR $\alpha$  binding regions.**

We next investigated whether PPAR $\alpha$  can directly bind to PPREs in the identified PBRs. PPAR $\alpha$  is known to bind as PPAR $\alpha$ /RXR $\alpha$  heterodimer to DR1 and DR2 motifs and in silico analysis of PBRs I to III identified several high-score putative binding motifs (Table 1). PPAR $\alpha$ /RXR $\alpha$  specifically bound to three DR1-type motifs and to one DR1/DR2 motif within the identified PBRs (Fig. 2A). Competition EMSA experiments determined relative in vitro binding affinities of PPAR $\alpha$ /RXR $\alpha$  in the order DR1-B>DR1-D $\geq$  DR1/DR2-C>DR1-A (Fig. 2B).

**Functional redundancy and cooperativity of *CYP3A4* PPREs in transcriptional regulation by PPAR $\alpha$ .** Results of HepG2 cell cotransfection experiments performed in the absence and presence of WY14,643 are shown in figure 3. Deletion analysis of the *CYP3A4*

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5' upstream promoter region showed that only constructs comprising the region between -2.0 kb and -8.8 kb were transcriptionally activated by PPAR $\alpha$  (Fig. 3A). The -8.8kb construct showed 1.9-fold induction of PPAR $\alpha$  activation by WY14,643. Presence of the PBR-I region comprising motifs DR1-A and DR1-B was sufficient for PPAR $\alpha$ -dependent transactivation (construct #4), which was further enhanced if regions PBR-II and -III with motifs DR1/DR2-C and DR1-D were also present (construct #5). All three distal upstream PBRs acted as PPAR $\alpha$ -dependent enhancers as they distance-independently conferred activation by the receptor to the proximal *CYP3A4* promoter (Fig. 3A, constructs #6-11). To dissect the contribution of individual PPRES, contextual mutational analysis was performed. Because construct #5 was too large for manipulation by mutagenesis, an artificial construct #12 was created, which comprises the three PBRs and 360 bp of proximal promoter sequence (Fig. 3B). While mutation of DR1-A and DR1-D did not show any significant effect, mutation of motifs DR1-B and DR1/DR2-C completely abolished constitutive and inducible PPAR $\alpha$ -dependent transactivation.

#### **PPAR $\alpha$ regulates CYP3A4 in vivo in a humanized CYP3A4/CYP3A7 mouse model.**

Since it is was shown that mouse *cyp3a* genes were not responsive to the WY14,643 treatment (Rakhshandehroo et al., 2009), we used a CYP3A4-humanized mouse model to investigate whether CYP3A4 is regulated by mouse Ppar $\alpha$  in vivo. Indeed, as shown in figure 4, oral administration of WY14,643 for 4 days resulted in the significant induction of the mouse *Acox1* (4-fold) and *Cyp4a10* (27-fold) genes, as well as approximately 5-fold induction of the human CYP3A4 in the liver (Fig. 4A). Western blot analysis confirmed about 2-fold induction of CYP3A4 protein (Fig. 4C). However, no induction was seen in the intestine, while the mouse Ppar $\alpha$  target genes were still induced, although at lower fold changes (Fig. 4B).

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Because induction of CYP3A4 by WY14,643 treatment of the humanized mice could also be due to indirect mechanisms or to mouse regulatory sequences further upstream of the human *CYP3A4* promoter, we analyzed promoter occupancy by ChIP assay in the resected liver tissues of the humanized mice. As shown in figure 4D, screening of the 5'-flanking sequence confirmed human *CYP3A4* promoter occupancy by mouse Ppar $\alpha$  at binding regions PBR-III and PBR-II, while PBR-I did not demonstrate binding. As already observed with human hepatocytes, the enrichment profile was essentially identical between untreated and WY14,643-treated animals.

**PPAR $\alpha$  regulates a selective set of CYP genes in human primary hepatocytes.** To investigate whether regulation of CYP3A4 by PPAR $\alpha$  was characteristic of a broader scheme, we applied lentiviral vectors for shRNA-mediated downregulation of PPAR $\alpha$  to PHH. As shown in figure 5A, knock-down of PPAR $\alpha$  with two different PPAR $\alpha$ -directed shRNA vectors strongly decreased mRNA expression not only of ACOX and CYP3A4 but also of CYPs 1A1, 1A2, 2B6, 2C8, and 7A1, whereas CYPs 2A6, 2C9, 2C19, 2E1, and 3A5 were only marginally influenced or remained unchanged. Measurement of corresponding enzyme activities following PPAR $\alpha$  gene silencing or treatment with WY14,643 resulted in consistent changes of enzyme activities (Fig. 5B).

**Inducibility of ADME genes by endogenous PPAR $\alpha$  phospholipid-ligands.** To investigate whether the lipid sensor function of PPAR $\alpha$  is of relevance for the regulation of ADME genes, we compared several phospholipids with WY14,643 for inducing potency (Fig. 6A). Indeed, treatment of PHH from three donors with 16:0/18:1PC but not several other phospholipids tested resulted in significant induction of *ACOX1* (1.2 to 1.7-fold), *CYP3A4* (1.8 to 3.1-fold), *CYP2C8* (3.1 to 4.2-fold), *CYP1A2* (0.8 to 2.15-fold) and *CYP2B6* (0.95 to 2.2-fold) genes, whereas the PPAR $\alpha$  target genes *IL1- $\beta$*  and *CYP7A1* were strongly downregulated by both, synthetic and natural ligands. The PPAR $\alpha$  activating properties of

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16:0/18:1PC thus closely resembled those of WY14,643 in terms of target gene selectivity and potency of induction. This was also shown with a broader selection of target genes (Fig. 6B, Supplement Table 2). The most highly induced genes were as expected metabolic genes (*CPT1A*, *HMGCS2*, *PK4*, up to 5-fold), while several CYPs and other ADME genes (*SULT*, *PXR*) were induced by 86% (*CYP3A5*) up to 280% (*CYP2C8*). The high similarity in WY14,643 or 16:0/18:1PC-induced gene expression changes was reflected in a strong correlation ( $r_s=0.83$ ,  $p<0.0001$ ,  $n=91$ ). WY14,643 treatment of PHH further closely mirrored the gene silencing effects as reflected in a negative correlation ( $r_s=-0.56$ ,  $p<0.0001$ ,  $n=91$ ).

#### **Comparative transcriptional profiling of PPAR $\alpha$ and the xenosensors PXR and CAR.**

The broad effects of PPAR $\alpha$  activation on ADME gene expression, which resembled those of the classical xenosensors pregnane X receptor (PXR) and constitutive androstane receptor (CAR), prompted us to further compare these three nuclear receptors. We treated PHH from three different donors with the most specific available agonists (CITCO for CAR, rifampicin for PXR, and WY14,643 for PPAR $\alpha$ ), to analyze their gene regulation patterns in a wider selection of target genes. Figure 7 presents a gene expression heatmap of the three donors. Induction of PXR and CAR resulted in the expected distinct upregulation of ADME genes and only slight changes in genes of energy metabolism. Activation of PPAR $\alpha$  revealed a similar pattern of ADME gene upregulation and stronger influence on metabolic-relevant gene expression.

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## Discussion

Direct regulation of *CYP3A4* expression by PPAR $\alpha$  is supported by three lines of evidence: first, by ChIP of PHH we elucidated three PBRs of 400~600bp length; second, four PPREs were identified by EMSA; and third, reporter gene analysis demonstrated that two of the PPREs (DR1-B and DR1/DR2-C) are essential for transcriptional activation by PPAR $\alpha$ . The DR1-A and DR1-B sites are located closely together in a previously unremarkable region at approximately -3kb. The DR1-B site displayed the strongest binding affinity of all four sites, in agreement with its strong functional role as demonstrated by mutational analysis. It is of interest that the PBR-I region was less occupied in huCYP3A4/3A7 mouse liver as well as in PHHs from two male donors diagnosed with adiposity and diabetes in comparison to one nondiabetic female donor (data not shown). As we can exclude DNA sequence variants at the identified PPAR $\alpha$  binding sites (data not shown), the basis for this difference remains unclear. The DR1/DR2-C site located within the well-documented XREM partially overlaps with an HNF4 $\alpha$ -binding site, which cooperatively interacts with adjacent PXR sites to promote inducibility by PXR ligands (Tirona et al., 2003). While the DR1/DR2-C site appeared to be transcriptionally highly active and inducible, its affinity by EMSA was lower compared to the DR1-B site. The presence of several nearby and overlapping transcription factor binding sites may lead to protein-protein interactions that could explain the apparent discrepancy. The DR1-D site is located in a previously undescribed region of the *CYP3A4* 5'upstream region and apparently functions independently, although in the context of all PPREs it was not essential. Taken together, our experiments suggest redundancy as well as cooperativity of at least three functional PPAR $\alpha$ -dependent enhancers in *CYP3A4*, which mediate both constitutive and inducible transactivation. Interestingly, pre-treatment of hepatocytes with WY14,643 did not change enrichment profiles of PPAR $\alpha$  binding within the upstream regions of *CYP3A4*. Based on our data, we hypothesize that upstream binding regions of *CYP3A4* are

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continuously occupied by PPAR $\alpha$  and the co-factor environment determines repression or activation of CYP3A4 transcription following binding of a ligand. Therefore, addition of a chemical ligand does not lead to the increased binding but rather to the reinforced release of co-repressors and thereby increase in transcription.

We investigated a possible involvement of PPAR $\alpha$  in the regulation of other drug biotransformation genes by gene silencing experiments. Our data show a pattern of selectively regulated CYPs including 1A1, 1A2, 2B6, 2C8, 3A4, and 7A1 but not CYPs 2C9, 2C19, 2D6 or 2E1 that is well in agreement with previous studies using PPAR $\alpha$  agonists such as WY14,643 (Rakhshandehroo et al., 2009) or fibrates (Prueksaritanont et al., 2005). Beyond that, our data imply an involvement of PPAR $\alpha$  in the expression of these genes not only by induction but also at the constitutive level, although we have not formally addressed direct regulation of these genes. Of note, two functional PPREs were found to be responsible for the regulation of *CYP1A1* in intestinal CaCo2 cells (Villard et al., 2011). It is possible that these PPREs are involved in the regulation of both, CYP1A1 and CYP1A2, because they are located within the bidirectional promoter between the two CYPs. Preliminary bioinformatic analyses suggest the presence of potential high-score DR1/DR2-type binding sites in the genes regulated by PPAR $\alpha$  compared to unregulated genes.

Direct transcriptional activation by PPAR $\alpha$  of other drug biotransformation genes has already been reported for several UDP-glucuronosyltransferases (Barbier et al., 2003; Senekeo-Effenberger et al., 2007), some drug transporters (Cheng et al., 2005; Moffit et al., 2006), as well as the rate-limiting enzyme of heme biosynthesis, aminolevulinic acid synthase (34). The regulation of hepatic CYPs by PPAR $\alpha$  can thus be regarded as a “missing link” within the drug biotransformation network. Our direct comparison of ligand-induced gene expression changes mediated by PPAR $\alpha$  and the two prototypic xenosensors PXR and CAR further



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emphasize the similar target profiles of these three nuclear receptors with respect to the regulation of drug biotransformation genes in the liver.

With respect to the pronounced species difference concerning the regulation of human *CYP1-3* and orthologous mouse genes by PPAR $\alpha$  (Rakhshandehroo et al., 2009), our experiment with huCYP3A4/3A7 mice suggests that this difference must be due to the lack of functional PPRE sites within the mouse Cyp genes, because mouse Ppara was able to bind to the CYP3A4 PBRs II and III and activate hepatic transcription. Therefore, the huCYP3A4/3A7 mouse appears to be a useful model to study CYP3A4 regulation by PPAR $\alpha$  and other factors (Hasegawa et al., 2011). The lack of Ppara-dependent *CYP3A4* induction in mouse intestine is probably related to the similar observation in regard to the XREM HNF4 $\alpha$ -binding site that regulates CYP3A4 in human liver (Tirona et al., 2003) but not in intestinal cells, where a specific additional factor was proposed to be missing (Tegude et al., 2007). Lower inducibility of CYP3A4 in the duodenum compared to liver has been described in this and another humanized mouse model before (Hasegawa et al., 2011). Interestingly, rat *CYP3A* and *2C* genes were found to be inducible by the fibrate gemfibrozil (Liu et al., 2011).

The current data on direct CYP induction through PPAR $\alpha$  suggest a link between endogenous lipids and the regulation of drug biotransformation in human liver. We tested this hypothesis by treating PHH with phospholipids, because a recent study identified POPC as an authentic endogenous ligand bound to PPAR $\alpha$  (Chakravarthy et al., 2009). Indeed, we could show induction of CYP3A4 and numerous other CYPs and drug biotransformation enzymes specifically by POPC but not by related phospholipid species. To our knowledge, this is the first observation that a phospholipid acts as a selective inducer of drug biotransformation genes in human hepatocytes. As described by Chakravarthy and colleagues (Chakravarthy et al., 2009), intracellular availability of POPC as a PPAR $\alpha$  ligand is limited by fatty acid synthase (FAS) and choline-ethanolamine phosphotransferase-1 (CEPT1), suggesting that

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metabolic perturbations in this system, e.g. in obesity and hepatic steatosis, may not only affect hepatic lipid metabolism but also drug biotransformation. The fact that externally administered POPC leads to enzyme induction in PHH suggests that dietary phospholipid components may also influence drug biotransformation gene expression. As a variety of dietary and endogenous lipids including saturated and unsaturated fatty acids, phospholipids, eicosanoids, and many derivatives and metabolites have been implicated in PPAR $\alpha$  activation, our findings suggest an intricate interplay between intermediary metabolism, nutritional status and biotransformation.

In conclusion, we have elucidated the mechanistic basis for constitutive and inducible transcriptional regulation of *CYP3A4* by PPAR $\alpha$  and we provided evidence for a broader range of similarly regulated drug metabolizing CYPs. Since fibrate hypolipidemic drugs are used worldwide in the treatment of dyslipidemia, there is clearly a need to readdress the potential for drug-drug interactions, which may depend on nutritional status. This is of particular importance for newly developed PPAR $\alpha$  ligands to target obesity, insulin resistance and diabetes (Lalloyer and Staels, 2010). Our results furthermore demonstrate the direct influence of phospholipids on CYP regulation, indicating a physiological link between lipid homeostasis and drug biotransformation in the liver, which is likely of importance in diseased states such as obesity and hepatic steatosis.

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### **Authorship Contributions**

*Participated in research design:* Thomas, Burk, Kandel, Damm, Weiss, Klein, Zanger

*Conducted experiments:* Thomas, Burk, Kandel, Klumpp

*Performed data analysis:* Thomas, Burk, Kandel, Klumpp

*Wrote or contributed to the writing of the manuscript:* Thomas, Burk, Schwab, Zanger

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## Footnotes

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**Reprint requests:** Ulrich M. Zanger, Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, Auerbachstr. 112, 70376 Stuttgart, Germany, Tel.: +49-(0)711-81013704; FAX: +49-(0)711-859295; E-mail: [uli.zanger@ikp-stuttgart.de](mailto:uli.zanger@ikp-stuttgart.de)

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## Figure Legends

**Fig. 1.** Binding of PPAR $\alpha$  to the CYP3A4 promoter *in vivo*. Human primary hepatocytes were fixed with formaldehyde in suspension and DNA was sheared by sonication. Sheared chromatin was incubated with PPAR $\alpha$  antibody coupled to magnetic beads. Protein-DNA complexes were precipitated and bound protein was digested by proteinase K. Precipitated DNA was purified and was used, together with input DNA, as template for Sybr-Green PCR using a total of 40 primer pairs spanning approximately 10 kb of the CYP3A4 promoter region. Raw Ct values were normalized to input DNA to calculate the percentage of DNA immunoprecipitated. Primers encompassing the PPRE of the human HMGCR gene were used as positive control. Means relative to intergenic binding (n.c.)  $\pm$  standard deviations (SD) of three independent donors (two male, one female) are shown. Significant enrichment was assessed using ANOVA analysis and is indicated by hashes. IR, intermediary region; PBR, PPAR binding region; n.c., negative control.

**Fig. 2.** PPAR $\alpha$  specifically binds to distinct DR1/DR2 motifs with differential affinities. A. Electrophoretic mobility shift assays using *in vitro* translated proteins bound to radiolabeled doublestranded oligonucleotide probes corresponding to the indicated wild type or mutated (mut) motifs of CYP3A4. As a positive control for binding, the known PPARA binding site of the rat ACOX1 gene (36), here indicated as PPRE, was used. Binding reactions contained (+) or lacked (-) the indicated proteins. Complexes of PPAR $\alpha$  /RXR $\alpha$  heterodimers with the oligonucleotides are marked by an arrow. B. Competition electrophoretic mobility shift assays with radiolabeled wild type motif DR1-B as probe and n-fold molar excess of indicated unlabeled motifs as competitors were performed. Retarded complexes were quantified and expressed as percentage of the complex obtained in the absence of competitors. Data represent means of two independent experiments for each competitor  $\pm$  S.D.

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**Fig. 3.** The PPAR $\alpha$  binding regions in the CYP3A4 promoter show differential and redundant functions. Luciferase reporter gene constructs (A, 1-11; B, 12-17) containing the indicated sequences of the CYP3A4 promoter (in kilobase pairs, kb) were cotransfected with a PPAR $\alpha$  expression plasmid and renilla-luciferase expression vector into HepG2 cells. Cells were treated with either vehicle (DMSO, grey bars) or 50 $\mu$ M WY14,643 (black bars) for 24h prior to measurement of firefly/renilla luciferase activities. Firefly luciferase activities were normalized to renilla luciferase activities. Data are means $\pm$ SD of three independent experiments, each performed in triplicates; •, mutated sites; #, statistically significant ( $p < 0.05$ ) compared to construct 1; \*, statistically significant ( $p < 0.05$ ) compared to untreated. The TK gene promoter fused to 3 copies of the ACOX1-PPRE was used as positive control.

**Fig. 4.** Induction of CYP3A4 by WY 14,643 in humanized huCYP3A4/3A7 mice. Humanized CYP3A4/3A7 mice were fed with WY14,643, or vehicle (corn oil) for 4 days and mRNA expression of target genes was analyzed by real-time RT-PCR in liver (A) and intestine (B) tissue lysates normalized to beta-actin. The bars indicate means  $\pm$  SD relative to vehicle control set at 1, with 5 animals in each group. # indicate significant changes in the expression of target genes. C. Western blot analysis of liver lysates of the huCYP3A4/3A7 mice treated with vehicle (Oil, n=5) or WY14,643 (WY, n=5). An exemplary Western blot is shown. CYP3A4 protein was quantitated and normalized against  $\beta$ -actin. D. ChIP analysis of Ppara binding to the CYP3A4 promoter in huCYP3A4/3A7 mice. ChIP assay was performed with liver tissues (n=3 in each group) as described in Fig. 1. The promoter of glycogen synthase 2 (Gys2) was analyzed as a positive control for binding of mouse Ppara. Statistical significance ( $p < 0.05$ , ANOVA) was assessed for expression changes of WY-treated group vs oil (A-C) or for Ppara binding enrichment of PBRs vs negative control (n.c.).

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**Fig. 5.** PPAR $\alpha$  gene silencing and activation regulates expression and activity of a selective set of CYPs. A. Two shRNA-encoding lentiviral vectors targeted to PPAR $\alpha$  were used to silence PPAR $\alpha$  gene expression in primary human hepatocytes obtained from three donors (light bars, shRNA1; dark bars, shRNA2). mRNA levels were measured by qPCR five days after infection and compared to mRNA levels measured in cells treated with non-targeting shRNA control vector set at 1.0. Data of three independent donors (all male) are shown. B. Enzyme activities measured by LC-MS/MS cocktail assay in the culture supernatants of human hepatocytes following PPAR $\alpha$  gene silencing (means of both shRNAs) (dark bars) or treatment with WY14,643 (light bars); CYP activities measured: CYP1A2, phenacetin O-deethylation; CYP2B6, bupropion 4-hydroxylation; CYP2C8, amodiaquine N-desethylation; CYP2C9, tolbutamide 4'-hydroxylation; CYP2C19, S-mephenytoin 4'-hydroxylation; CYP2D6, propafenone 5-hydroxylation; CYP3A4, atorvastatin o-hydroxylation. Data are means of 3 independent donors (A) and of two donors (B). Statistical significance for RNAi experiments in comparison to control shRNA is indicated by # ( $p < 0.05$ , paired t-test). Two independent experiments were made for CYP activity measurements; error bars indicate the two measurements.

**Fig. 6.** Endogenous phospholipid ligands of PPAR $\alpha$  induce ADME gene expression. Primary human hepatocytes of two independent donors (male) were treated for 24 h with WY14,643 (50  $\mu$ M) or with different phospholipids (50  $\mu$ M): 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0/18:1 GPC); 1-hexadecanoyl-sn-glycero-3-phosphocholine (16:0 GPC); 1-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (18:1 GPC); 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (18:0/18:1 GPC). A. Expression of selected genes determined by qRT-PCR and expressed relative to vehicle (DMSO) control, which was set as 1. Data

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represent means $\pm$ S.D. of two independent experiments measured in triplicates. Statistical significance ( $p < 0.05$ , paired t-test) is indicated by #. B. Heatmap showing mRNA expression changes (fold) in human hepatocytes from three independent donors following PPAR $\alpha$  gene silencing or following treatment with WY14,643 (50  $\mu$ M) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0/18:1 GPC; 50  $\mu$ M). Fold-changes were calculated in relation to control shRNA (RNAi, means of both shRNAs shown) or vehicle treatment (WY14,643, 16:0/18:1PC). The colour scale from blue (downregulation) to red (upregulation) spans from -2.5 to 2.5 (log<sub>2</sub>-scale). Right panel, corresponding Spearman rank correlation analysis of expression profiles of 91 genes analyzed (see Supplemental Table 3).

**Fig. 7.** Comparison of CAR, PXR, PPAR $\alpha$  transcriptional regulation. Heatmap showing color-coded mRNA expression differences in human hepatocytes from three donors (2 male, 1 female) following induction of CAR (CITCO, 1 $\mu$ M), PXR (Rifampicin, 10 $\mu$ M) and PPAR $\alpha$  (WY14,643, 50 $\mu$ M). The individual donor gene expression patterns are shown on the left (1,2,3), the mean values are represented on the right (1-3). The upper panel presents selected ADME genes, the lower panel selected genes of energy metabolism. The colour scale from blue (downregulation) to red (upregulation) spans from -2.5 to 2.5 (log<sub>2</sub>-scale).

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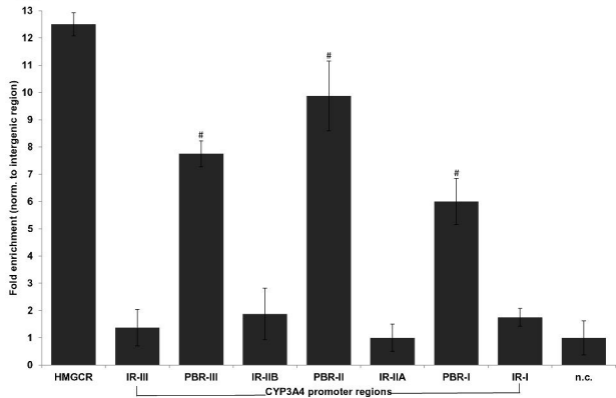
## Tables

**Table 1 PPREs identified in the CYP3A4 upstream region**

Designation	PBR	Position <sup>a</sup>	Sequence (strand)
DR1-A	I	-2915/-2903	GGTACA A AGTTGA (+)
DR1-B	I	-3062/-3050	GGATCA A AGTTCA (-)
DR1/DR2-C	II	-7784/-7764	<i>CAATTA</i> A AGGTCA TA AAGCCC (+)
DR1-D	III	-8816/-8804	AGGGCA A AGGACA (+)

<sup>a</sup>with respect to transcriptional start site; PBR, PPAR $\alpha$  binding region; PPRE, PPAR $\alpha$  response element; DR1, DR2, direct repeat with 1 or 2 bases distance; deviations in base changes from the consensus shown in italicized type.

Figure 1



**A**

probe	PPRE		DR1 A		mut DR1 A		DR1 B		mut DR1 B		DR1/2 C		mut DR1/2 C		DR1 D		mut DR1 D	
PPAR $\alpha$	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
RXR $\alpha$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

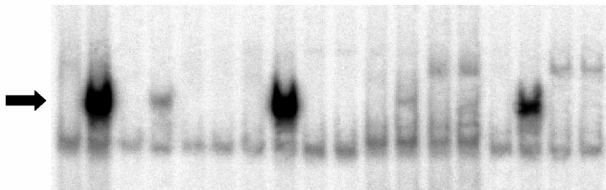
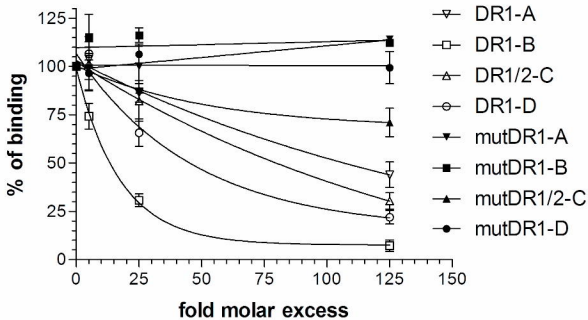
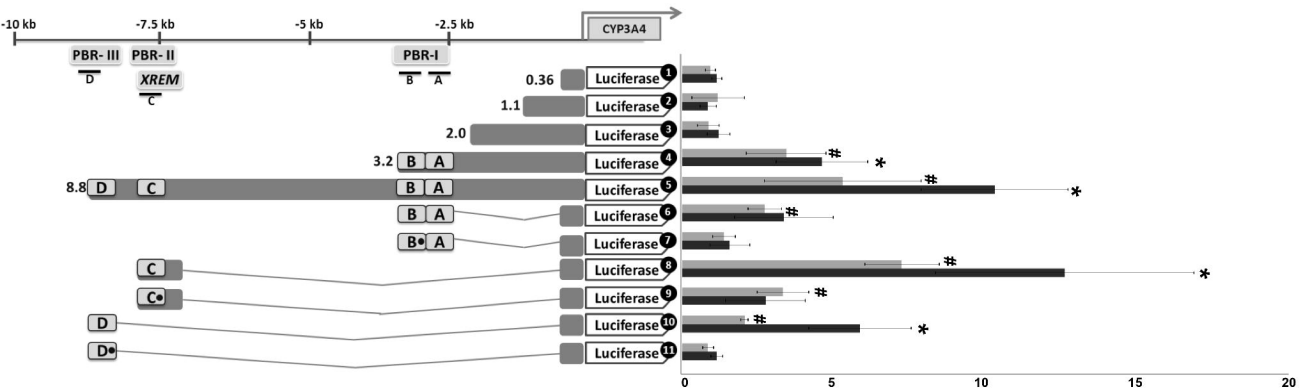
**B**



Figura 3



**B**

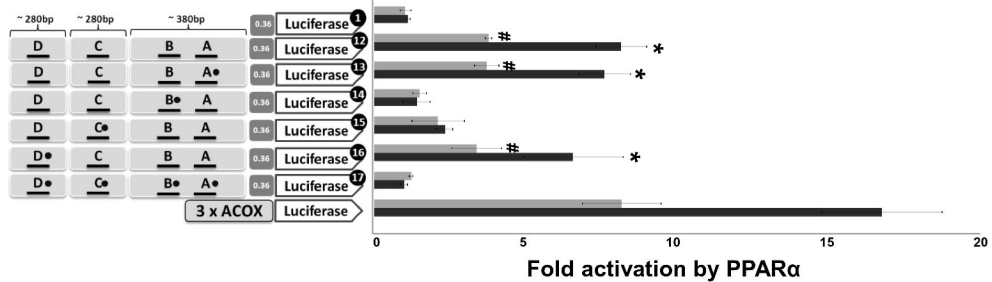
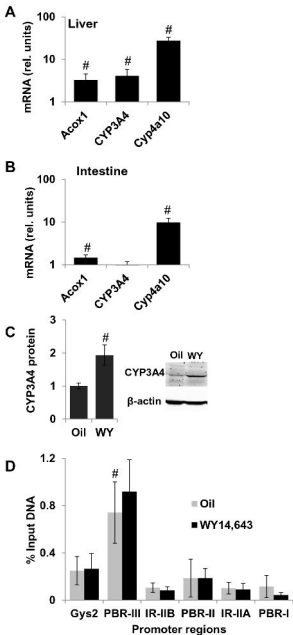
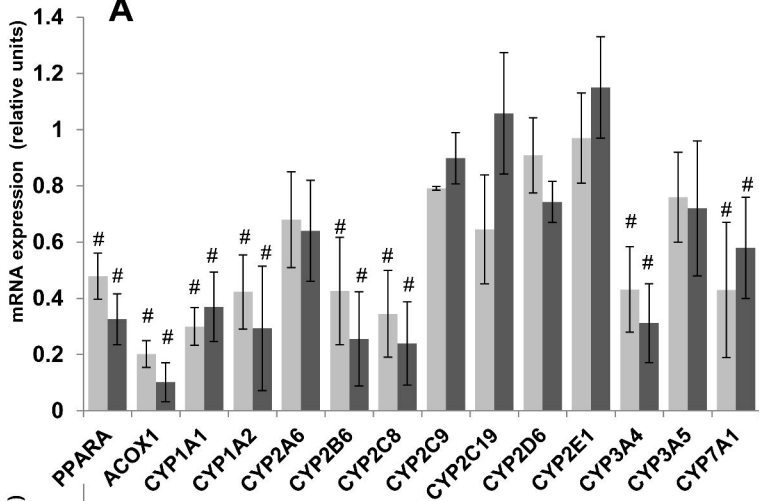
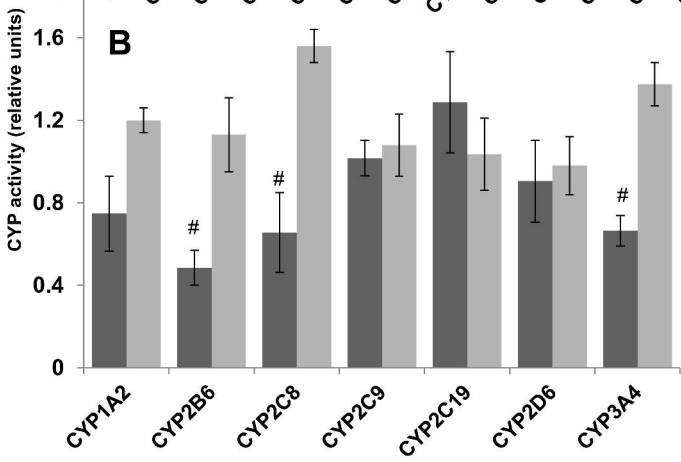
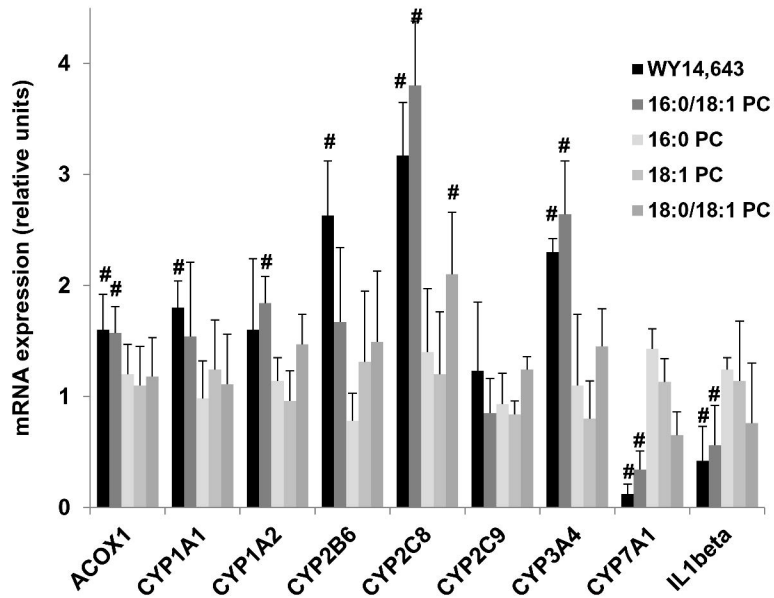


Figure 4



**A****B**

A



B

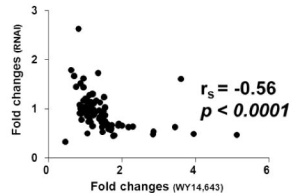
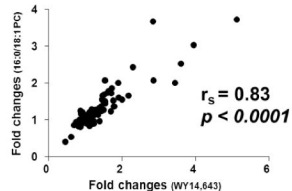
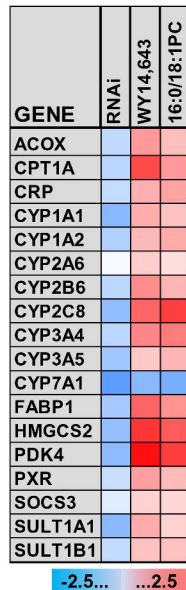
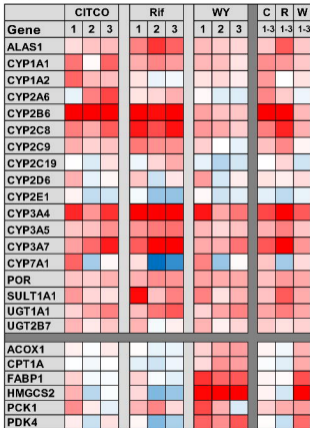


Figure 7



-2.5...

...2.5