Induction of Xenobiotic Receptors, Transporters and Drug Metabolizing Enzymes by Oxycodone

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Abbreviations: XR, xenobiotic receptor; DME, drug metabolizing enzyme; DDI, drug-drug interaction; MDR, multidrug resistance proteins; Abcb1, P-glycoprotein; Q-PCR, quantitative real time polymerase chain reaction; PK, pharmacokinetics; PD, pharmacodynamics
Abstract

Perturbations of the expression of transporters and drug-metabolizing enzymes (DMEs) by opioids can be the locus of deleterious drug-drug interactions (DDIs). Many transporters and DMEs are regulated by xenobiotic receptors (XR) [e.g., Pregnane X receptor (PXR), Constitutive androstane receptor (CAR) and Aryl hydrocarbon receptor (AhR)]; however, there is a paucity of information regarding the influence of opioids on XRs. The objective of this study was to determine the influence of oxycodone administration (15 mg/kg i.p. b.i.d/8 days) on liver expression of XRs, transporters and DMEs in rats. Microarray, quantitative RT-PCR and immunoblotting analyses were used to identify significantly regulated genes. Three XRs (e.g. PXR, CAR and AhR), twenty-seven transporters (e.g., ABCB1, SLC22A8) and nineteen DMEs (e.g., CYP2B2, CYP3A1) were regulated ($p<0.05$) with fold changes ranging from -46.3 to 17.1. Using MetaCore™ (computational platform), we identified a unique gene-network of transporters and DMEs assembled around PXR, CAR, and AhR. Hence, a series of transactivation/translocation assays were conducted to determine if the observed changes of transporters/DMEs are mediated by direct activation of PXR, CAR or AhR by oxycodone or its major metabolites (noroxycodone and oxymorphone). Neither oxycodone nor its metabolites activated PXR, CAR or AhR. Taken together, these findings identify a signature hepatic gene-network associated with repeated oxycodone administration in rats. And demonstrate that oxycodone alters the expression of many transporters and DMEs (without direct activation of PXR, CAR and AhR) which could lead to undesirable DDIs upon co-administration of substrates of these transporters/DMEs with oxycodone.
Introduction

Inter-individual variability in the expression patterns of genes encoding for transporters and/or drug metabolizing enzymes (DMEs) could lead to subtherapeutic or toxic drug levels. As a result, extensive studies have been directed towards elucidating the mechanisms that govern the expression of these genes. One of the major mechanisms involved in the regulation of these genes is transcriptional regulation by xenobiotic receptors (XRs) [e.g., Pregnane X receptor (PXR), Constitutive androstane receptor (CAR) and Aryl hydrocarbon receptor (AhR)] (Kim, 2002; Tirona and Kim, 2005). For example, ABCB1, CYP3A1 (CYP3A4) and CYB2b2 (CYP2B6) are regulated by PXR. CYP3A1 (CYP3A4) and CYB2B2 (CYP2B6) are regulated by CAR while GSTA and UGR1A are regulated by AhR (Tirona and Kim, 2005). As such, PXR, CAR and AhR are promiscuous XRs that translate chemical activation into coordinated induction of transporters and DMEs. The expression of XRs, DMEs and transporters is prominent in liver where PK-based DDIs occur. Co-administration of XR-activators with transporters/DMEs-substrates can lead to undesirable drug-drug interactions (DDIs). For example, co-administration of rifampin (PXR-activator) with oxycodone (CYP3A4 and ABCB1 substrate) caused a significant reduction in oxycodone plasma concentrations in humans (Nieminen et al., 2009).

Opioids have been extensively used for decades for the management of pain; nonetheless, few studies have examined their ability to regulate the expression of XRs, transporters and/or DMEs. We have previously reported that methadone activates both PXR and CAR in human primary hepatocytes which led to the induction of ABCB1, CYP3A4, CYP2B6, and UGT1A1 (Tolson et al., 2009) while buprenorphine and
diprenorphine activate PXR and CAR in HepG2 cells resulting in CYP3A4 and CYP2B6 induction (Li et al., 2010). Morphine induced the efflux transporter, ABCB1, in rat brain (Aquilante et al., 2000) while oxycodone regulated many transporters (e.g., ABCB1, ABCG2) and DMEs (e.g., rGSTA5) in various rat tissues (Hassan et al., 2007; Hassan et al., 2010; Myers et al., 2010). Changes in the expression of transporters/DMEs by opioids can be the locus of pharmacokinetic (PK)- and pharmacodynamic (PD)- based DDIs. For example, we demonstrated that oxycodone induced Abcb1 in brain, kidney, liver and intestine which hindered paclitaxel (ABCB1 substrate) accumulation in these tissues (Hassan et al., 2007). Additionally, we reported that the efflux transporter, ABCG2, was significantly upregulated in brain tissue of oxycodone-treated rats (Hassan et al., 2010) restricting the uptake of mitoxantrone, an ABCG2 substrate.

 Nonetheless, the characterization of the complete spectrum of XRs, transporters, and DMEs affected upon repeated administration of opioids has not been elucidated in rodents or humans. We hypothesize that repeated opioid administration affects the hepatic expression of XRs, transporters and DMEs which can lead to deleterious DDIs. The significance of identifying changes in the expression of these disposition-controlling genes comes from the fact that polypharmacy is a serious clinical concern today, and opioids are commonly co-administered with therapeutic agents (e.g., efavirenz and paclitaxel) or illicit drugs (e.g., cocaine) that are substrates/inhibitors of these genes. As such, identifying these expression changes is crucial for adjusting therapeutic doses and avoiding DDIs. To test our hypothesis, we investigated the influence of repeated oxycodone (representative opioid agonist) administration on the hepatic expression of XRs, transporters, and DMEs in rats. Oxycodone is the opioid of choice for management
of pain in the United States (Hays, 2004). The severity of oxycodone use/misuse was well documented by the Drug Abuse Warning Network (DAWN) which reported a total of 1014 oxycodone-related deaths in a three year period (Cone et al., 2003). Ninety-percent of these oxycodone-related deaths were attributed to DDIs that can be transporters/DMEs-mediated (Burrows et al., 2003; Cone et al., 2003; Cone et al., 2004; Lee et al., 2006; Nakazawa et al., 2010; Nieminen et al., 2010). We used microarrays to obtain a global profile of genes (including XRs, transporters and DMEs) regulated in liver tissue of oxycodone treated rats. Then we validated the changes in expression of many genes by quantitative real time PCR (Q-PCR) and immunoblotting analysis. All regulated genes were imported into MetaCore™, a web-based computational platform, to build a highly interconnected gene network of XRs, transporters, and DMEs in the context of rat regulatory interactions reported in the literature. Finally, a series of cell-based XR reporter and translocation assays were conducted to determine the magnitude of differential activation of XRs by oxycodone and its two major metabolites, noroxycodone and oxymorphone.
Materials and Methods

Materials:

HepG2 cells were obtained from ATCC (Manassas, VA). Rifampicin, omeprazole, β-naphthoflavone (BNF), dexamethasone, phenobarbital sodium salt (PB) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Lipofectamine 2000 transfection reagent was obtained from GIBCO/Invitrogen (Carlsbad, CA). Luciferase substrate (Steady-Glo) and Dual Luciferase Reporter Assay Systems were purchased from Promega (Madison, WI). Alamar blue (resazurin) was purchased from Trek Diagnostics (Chicago, IL). 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl oxime (CITCO) was purchased from BIMOL research laboratories (Plymouth meeting, PA). Oxycodone hydrochloride was generously donated by Dr. Andrew Coop (School of Pharmacy, UMB) while noroxycodone and oxymorphone were purchased from Cerilliant Co. (Round Rock, Texas). FuGENE 6 transfection reagent was obtained from Roche (Mannheim, Germany). Fetal bovine serum (FBS) was purchased from Hyclone (Lonan, UT), whereas other cell culture reagents were from Invitrogen (Carlsbad, CA).

Plasmid constructions

The pCR3-hCAR and pCR3-mCAR expression vectors were obtained from Dr. Masahiko Negishi (NIEHS, NIH, RTP, NC). The CYP2B6-PBREM/XREM reporter construct was generated as reported previously (Wang et al., 2003). The rat PXR and human AhR-DRE transactivation kits were obtained from Puracyp (Carlsbad, CA). The generation of adenovirus containing EYFP-tagged hCAR was described previously (Li et
The pRL-Tk renilla luciferase construct used to normalize the firefly activity was purchased from Promega.

**Experimental animals**

Male Sprague Dawley rats (250-275 g, 8-9 weeks) were purchased from Harlan Laboratories (Indianapolis, IN). Female sex steroid hormones can induce protein expression (Campbell and Febbraio, 2002; Kim and Benet, 2004). As a result, males only were used to avoid potential influences on protein expression caused by fluctuations in ovarian hormone concentrations that are associated with different stages of the estrus cycle in females. They were fed chow and water *ad libitum* and maintained on a 12 hr light/dark cycle. The animals were housed individually and allowed to acclimate for at least one week before the experiments were conducted. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland IACUC.

**Repeated administration of oxycodone**

Sprague Dawley rats were sorted into one of two groups (n=6/group) and administered either a 15 mg/kg i.p. dose of oxycodone hydrochloride (dissolved in saline) or 1 ml/kg saline control twice daily every 12 hr for 8 days. Oxycodone dosage regimen was selected based on reports that oxycodone is 1.5 times more potent than morphine (Kalso et al., 1990; Heiskanen et al., 1998; Nielsen et al., 2000); and a dose of 15 mg/kg oxycodone is equivalent to a dose of 20 mg/kg morphine, which is commonly used for morphine administration (Guitart and Nestler, 1989; Poyhia and Kalso, 1992; Aquilante et al., 2000; Ammon et al., 2003). Since oxycodone has an elimination half-life (t\(_{1/2}\)) of
3.0 ± 0.5 hr and requires 5 half-lives (>15 hr) for ~95% of the drug to be eliminated (Chan et al., 2008), twice daily dosing (Holtman and Wala, 2006; Hassan et al., 2007; Hassan et al., 2010) was employed. Dosing every 12 hr ensures that enough oxycodone is administered before its complete elimination. Finally, an eight day dosing regimen was adopted since rats under similar experimental conditions developed tolerance to the analgesic effect of oxycodone (Hassan et al., 2007). The oxycodone treated group was compared directly to saline treated control, which was handled, treated, and sacrificed in parallel under the same experimental conditions.

**Tissue preparation and RNA extraction**

Rats in both groups were euthanized by CO₂ asphyxiation and decapitated 12 hr after administration of the last dose. Total RNA samples were isolated immediately from fresh liver tissues using TRIzol reagent (Qiagen, Valencia, CA) according to manufacturer’s instructions. The concentration of total RNA was measured by UV spectrophotometry at 260/280 nm. Thirty micrograms of total RNA was then subjected to DNase treatment for 10 min using the RNase-free DNase set (Qiagen) according to the manufacturer’s instructions. Samples were then subjected to RNA clean up using RNeasy® MinElute™ Kit (Qiagen) according to the manufacturer’s instructions. The concentration of purified RNA was measured by UV spectrophotometry and RNA quality was assessed by electrophoresis on a 1% agarose gel. Further assessment of the integrity of the RNA was tested using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Only RNA samples with sharp and defined 28S and 18S ribosomal peaks indicating good RNA integrity were used.
**Microarray hybridization and staining**

A GeneChip® rat genome 230 2.0 array (Affymetrix, Santa Clara, CA) containing 31,000 probe sets that analyze the expression level of over 30,000 transcripts encoding for over 28,000 well-substantiated rat genes was used to evaluate RNA expression. RNA samples (six liver tissues/group) were combined so that two RNA samples were pooled into one sample resulting in a total of three pooled RNA samples/group. Pooled RNA samples from both groups were labeled and run on the arrays at the same time for each experiment (n=3 arrays/group). Briefly, total RNA (5 µg/array) was converted to cDNA, amplified, and labeled according to the Affymetrix protocols. Hybridization and washing were performed using the Affymetrix Fluidics Station 450 and Hybridization Oven 640 under standard conditions. The arrays were then scanned with a GeneChip® Scanner 3000 (Affymetrix).

**Microarray data analysis**

Based on six individual Affymetrix CEL files (three/group), gene expression measures were calculated using the Background Adjusted Robust Multiple Average method (Wu, 2004) and implemented in the Bioconductor R packages Affy and gcrma (Gentleman R., 2005). This process includes background correction, probe-level quantile normalization and expression measures calculated by using median polish. A detection P value by Affymetrix Microarray suite version 5 was used to make a reliable prediction of gene expression (present, marginal or absent). Fold changes in the transcript levels and statistical analysis were calculated using Spotfire® DecisonSite® 8.2.1 algorithm (Spotfire, Inc. Somerville, MA). Significance Analysis of Microarrays (SAM) (Stanford
University, Stanford, CA) was used to investigate differentially expressed genes. Then we applied rigorous, conservative criteria to select candidate genes for further analysis as follows: 1) genes must have present or marginal calls in all arrays; 2) genes must have raw signals > 40 in all arrays; 3) genes must be up/down regulated by ≥ 1.5-fold relative to the control; and 4) genes must have mean differences that are statistically significant (p < 0.05) from the control. Only genes that met the above criteria were considered significantly regulated and were used for further analysis. Under the above conditions, Affymetrix arrays have been shown to achieve a statistical power of > 0.8 to detect 1.5-fold changes and >0.99 to detect 2.0-fold changes (Shippy et al., 2004). For each gene, fold change values were calculated by dividing normalized mean log-transformed probe set intensities of oxycodone treated rats vs. saline treated rats.

**Quantitative Real Time-PCR (Q-PCR)**

Expression levels of 18 genes [14 significantly regulated (p < 0.05) and 4 non-significantly regulated (p > 0.05)] (Supplemental Table 1) were tested by Q-PCR to validate the microarray findings. This approach was adopted to test the reliability of the normalization and statistical methods used for the microarray data analysis. Q-PCR was conducted following the manufacturer’s recommendation using the iCycler instrument (Bio-Rad, Hercules, CA). Briefly, 1 µg of total RNA was used to synthesize cDNA in a final reaction volume of 20 µl using the iScript reverse transcription kit (Bio-Rad). Two microliters of the reverse transcription reaction were used as template for Q-PCR reactions, which were carried out in a total volume of 25 µl using iQ SYBR Supermix (Bio-Rad) with a final primer concentration of 200 nM. Primers for Q-PCR were
designed using the Beacon Designer software (Biosoft International, Palo Alto, CA). Primers were then tested for specificity by blasting the candidate sequence against the whole rat database (Annereau et al., 2004). Designing of primers were performed in an iterative fashion until a specific primer for each gene was obtained. After an initial denaturation step at 95°C for 3 min, PCR cycles (n = 40) consisted of a 30 sec melt at 95°C, followed by annealing and extension at 60°C for 45 sec. All reactions were conducted in triplicate. The threshold cycles (Ct) were calculated using the second derivative of the reaction and were automatically set by the iCycler iQ real time detection system software. The Ct of each gene was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which showed no change upon oxycodone treatment in our microarray study. Fold changes were determined using the Livak method (delta-delta Ct method). The Student’s t-test was used to determine the statistical significance ($p<0.05$) of the normalized Ct using SigmaStat™ statistical package V2.03 (Systat software Inc., San Jose, CA).

**Determination of the protein expression of representative metabolizing enzymes (CYP3A1 and CYP2B2) and transporter (ABCB1)**

**Preparation of Rat Hepatic Microsomes:**

Isolation of liver microsomes was adopted from standard procedures (van der Hoeven and Coon, 1974; Waxman, 1984). Briefly, frozen liver tissues (n=3/group) from both treatment groups were rapidly thawed at 37°C, and immediately homogenized with 2 volumes of 20 mM Tris-HCl (pH 7.4) buffer containing 1 mM DTT and 1 tablet (Complete Mini, EDTA free protease inhibitor cocktail) (Roche Diagnostics; Mannheim,
Germany). After differential centrifugation, the final microsomal fraction was suspended in buffer containing 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA and 20% glycerol and stored at -80°C until further analysis. Protein concentration was measured using BSA as a standard in the Bio-Rad DC Protein Assay.

**SDS-PAGE and Immunoblotting:**

SDS-PAGE and immunoblotting of representative metabolizing enzymes (CYP3A1 and CYP2B2) and the transporter (ABCB1) were conducted. CYP3A1 immunodetection was performed as previously described using the Laemmli discontinuous buffer system in a 10% polyacrylamide gel with a 5% stacking gel (Laemmli, 1970). Each well (n=3/group) was loaded with 40 µg of microsomal protein isolated from oxycodone or saline treated rats. In addition, 2 wells (controls) were loaded with 10 µg of standard rat liver microsomes (RLM) purchased from BD Biosciences (San Jose, CA). Resolved proteins were transferred electrophoretically to Bio-Rad Immun-Blot™ polyvinylidene difluoride membrane (PVDF) membranes. PVDF membranes were washed several times with TBS-T washing buffer consisting of 1X tris buffered saline (TBS) and 0.05 % Tween 20 (T), and incubated for 1 hr at 25°C in a solution of 5% nonfat dry milk in TBS-T to block protein-binding sites. Membranes were incubated overnight at 4°C with antisera raised in rabbits against CYP3A1 (1:800 dilution) that was purchased from Abcam, Inc. (Cambridge, MA). The PVDF membrane was exposed to goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology; Santa Cruz, CA) at a dilution of 1:10,000. CYP2B2 microsomal protein was detected using 1.0 µg/ml mouse monoclonal anti-CYP2B2 antibody (Abcam), and goat anti-mouse secondary antibody (Santa Cruz). Immuno-reactive protein was detected using the SuperSignal® West Pico
Chemiluminescent Substrate Kit (Pierce; Rockford, IL). The drug efflux transporter protein P-glycoprotein (ABCB1) was detected by a similar immunoblotting method using 1.3 µg/ml of mouse monoclonal Abcb1 antibody clone C219 (ID Labs, Inc.; London, Ontario, Canada), and a goat anti-mouse IgG secondary antibody (Santa Cruz). Band density was quantified by densitometry using Quantity One 4.4.1 software (Bio-Rad). A separate membrane was used for immunoblotting of each protein. For protein normalization, each membrane was probed in an analogous fashion for immuno-reactive β-actin using monoclonal anti-β-actin (1:5,000) produced in mice (Sigma Chemical Co.) and 1:10,000 dilution of goat anti-mouse IgG horseradish peroxidase (KPL; Gaithersburg, MD).

Gene network identification by the computational platform, MetaCore™

MetaCore™ (GeneGo, St Joseph, MI) is a computational resource that uses logic operation algorithms to interpret changes in gene expression for a given condition in terms of biological processes and molecular functions. Genes (XRs, transporters and DMEs) in rat liver with altered expression (change ≥ 1.5, \( p < 0.05 \)) were characterized by Gene Ontology (GO). GO describes gene products in terms of their associated biological processes, cellular components, and molecular functions. The GO entries are hierarchically linked, thus allowing construction of cluster genes of crossed pathways. MetaCore™ also generates statistically significant gene networks. These networks represent gene interactions compiled from a curated database of rat protein interactions, metabolism and bioactive compounds, and thus provides a useful approach to identify
new interactions/relationships using data obtained from numerous reported genomic studies (Ekins et al., 2006).

Many algorithms are integrated into MetaCore™ to enable construction and analysis of gene networks (Ekins et al., 2006). Due to the large number of selected genes (metabolic enzymes, nuclear receptors, and transporters) within the dataset, the Direct Interactions algorithm was used to create a gene network. Each connection between objects in the created network represents a direct, experimentally confirmed, physical interaction between the objects (Ekins et al., 2006). The Direct Interactions algorithm is advantageous when used for initial insight about the dataset because it can determine if uploaded genes in the list cluster together by interacting with each other (Ekins et al., 2006).

Transactivation Assays

Human PXR transactivation assay

For the human PXR transactivation assay, the methodology of Zhu et al. was followed (Zhu et al., 2004). Briefly, HepG2 cells were transfected with pcDNA3-hPXR and pGLcyp2B6-Luc plasmids using Lipofectamine 2000. Oxycodone, noroxycodone and oxymorphone (each dissolved in DMSO), were added to the wells of a 96-well plate at concentrations ranging from 1 - 30 μM in triplicate (final concentration of DMSO was 0.5% v/v). Rifampicin (10 μM), a well-known hPXR agonist, was added to the plate as both a positive control and an internal standard. CITCO (1 μM), a known hCAR activator, was used as a negative control while 0.5% DMSO was used as a blank control. The plate was incubated overnight at 37°C before the addition of luciferase substrate. After 30 min incubation with luciferase, the plate was read on a Packard Topcount plate.
reader (GMI, inc., Ramsey, MN) for luminescence intensity. Cytotoxicity was assessed by Alamar blue as previously described (Zhu et al., 2007).

**Rat PXR and human AhR-DRE transactivation assays**

Culturing of the rat PXR and human AhR-DRE cell lines and transactivation assays were performed according to the manufacturer’s instructions. Briefly, cryopreserved cells were plated in 96-well plates and allowed to attach overnight prior to drug treatment. Oxycodone, noroxycodone and oxymorphone were added at concentrations ranging from 0.1 - 30 μM dissolved in DMSO (0.1% v/v). Dexamethasone (5 μM), a well-known rPXR agonist and BNF, a well-known human AhR agonist were added to the plates as positive controls 0.1% DMSO was used as a blank control. After a 24-48 hr exposure to vehicle, compounds, and positive controls, the luminescent intensity was measured.

**Human CAR and mouse CAR transactivation assays**

HepG2 cells were cultured in 24-well plates in DMEM supplemented with 10% FBS and antibiotics prior to transfection. Cells were co-transfected with 30 ng of nuclear receptor expression vector (mCAR or hCAR1+A), 60 ng of luciferase CYP2B6 reporter plasmids, and 10 ng of control plasmid (pRL-TK) using FuGENE 6 reagent following the manufacturer’s protocol. After 18 hr of transfection, cells were treated for 24 hr in triplicate with oxycodone, noroxycodone and oxymorphone at concentrations ranging from 1 - 30 μM. CITCO (1 μM) or TCPOBOP (250 – 500 nM) were used as positive controls for hCAR and mCAR, respectively, while rifampicin (10 μM), the hPXR agonist, was used as a negative control in the hCAR assay. Cell lysates were assayed for firefly luciferase activities and normalized against the activities of co-transfected Renilla
luciferase. Ratios of the two luciferase activities were expressed as fold activation relative to the vehicle control.

Translocation of Ad/EYFP-hCAR in Human Primary Hepatocyte Cultures

Liver tissues were obtained by qualified medical staff after donor consent and prior approval from the Institutional Review Board at the University of Maryland at Baltimore. Isolation of human hepatocytes was performed following the two-step collagenase method described previously (LeCluyse et al., 2005). Human hepatocytes donated by a 77-year-old female were seeded at $3.75 \times 10^5$ cells/well in 24-well biocoat plates and cultured as described previously (Wang et al., 2003). Twenty four hours later, hepatocyte cultures were infected with Ad/EYFP-hCAR for 12 hr before treatment with vehicle control (0.1% DMSO), phenobarbital (1000 µM) or oxycodone (30 µM) for another 12 hr. Then, the subcellular localization of Ad/EYFP-hCAR was visualized by confocal laser scanning microscopy using a Nikon C1-LU3 instrument based on an inverted Nikon Eclipse TE2000 microscope (Mellville, NY).
Results

Oxycodone-induced gene expression changes in liver tissue of Sprague Dawley rats

For the oxycodone-treated rats, no behavioral or withdrawal effects were noticed during or after oxycodone treatment except for the common opioid-induced Straub tail effects. Using a microarray approach, we examined the effect of repeated oxycodone administration on gene expression in liver tissue of Sprague Dawley rats. Considering all 31,000 probe sets (gene identifiers) corresponding to 28,000 genes represented on each array, oxycodone treatment significantly ($p<0.05$) induced changes in 1,973 probe sets (~6% of probe sets), of which, 670 probe sets had $p$ values <0.01 (data not shown). Among those 670 probe sets, 140 gene identifiers (corresponding to 130 genes) were up/down-regulated by $\geq$ 2-fold [sixty-eight gene identifiers were upregulated by $\geq$ 2-fold (Supplemental Table 2) while seventy-two gene identifiers were downregulated by $\geq$ 2-fold (Supplemental Table 3)]. Three XRs (PXR, CAR and AhR) were significantly ($p<0.05$) upregulated by $2.9 \pm 0.02$ fold, $6.1 \pm 0.1$ fold and $\sim 2.3 \pm 0.2$ fold, respectively (Table 1). Twenty-nine probe sets (Table 2) encoding twenty-seven transporters were significantly ($p<0.05$) regulated with fold changes ranging from (-46.3 to 3.6). Five of these transporters belong to the ATP binding cassette (ABC) superfamily (e.g., ABCB1 and ABCB4) while the remainder belong to the Solute Linked Carrier (SLC) superfamily (e.g., SLC16A1 and SLC22A8) (Table 2). On the other hand, twenty two probe sets encoding nineteen metabolizing enzymes (e.g., CYP2B2, CYP3A1, UGTA1) were significantly ($p<0.05$) altered with fold changes ranging from (-3.3 to 17.1) (Table 3). SAM based statistical analysis of the microarray data revealed a minimal false discovery
rate (FDR) of < 0.01 indicating the presence of < 1% chance of false positives in the microarray data (data shown).

**Validation of microarray data by Quantitative Real Time-PCR (Q-PCR)**

To validate the microarray data, the expression levels of fourteen significantly ($p<0.05$) and four non-significantly ($p>0.05$) regulated genes (as determined by the microarray analysis) were analyzed using Q-PCR analysis. After ascertaining the amplicon specificity by first derivative melting curve analysis, the values obtained for each gene were normalized to the values of the corresponding GAPDH expression. All significantly (n=14) and non-significantly (n=4) regulated genes as indicated by the microarray analysis showed similar patterns (comparable fold changes and $p$ values) upon evaluation of their expression levels using Q-PCR analysis (i.e., Q-PCR data were comparable to the microarray data). Computation of Pearson correlation coefficients (for the fourteen significantly regulated genes) indicated that there was a significant ($r = 0.973, p<0.000001$), correlation between the Q-PCR and the microarray data (data not shown). For the four non-significantly regulated genes there was also a significant correlation between fold changes obtained from microarray analysis and Q-PCR ($r=0.956, p<0.043$) (data not shown). These results confirm the validity of the microarray data and give confidence in the normalization and the statistical methods used for the microarray data analysis.

**Influence of repeated oxycodone administration on the protein expression of representative metabolizing enzymes (CYP3A1 and CYP2B2) and transporter (ABCB1)**
To further validate the microarray data, protein expression of key DMEs and efflux transporter that play major roles in mediating DDIs (Weinstein and Gaylord, 1979; Lin, 2003; Yue et al., 2009; Choi et al., 2010; Giacomini et al., 2010; Calcagno et al., 2012) were determined. Western blot analyses identified CYP3A1 protein (57-59 kDa) in liver samples isolated from saline, oxycodone and standard rat liver microsomes (RLM). The optical density calculations (mean ± SD, n=3) of CYP3A1 bands after normalization to β-actin for saline and oxycodone groups were 6.0 ± 2.9 and 12.2 ± 1.4, respectively (p<0.01) (data not shown). This indicates a significant upregulation (2.0 fold) in CYP3A1 protein levels in oxycodone treated rats comparable with fold changes obtained from the microarray (1.7 fold) and the Q-PCR (2.7 fold) analyses. Also, the normalized optical density calculations (mean ± SD, n=3) of CYP2B2 protein bands (56 kDa) were 0.18 ± 0.05 and 0.84 ± 0.15 for saline and oxycodone samples, respectively (p<0.001) (data not shown). These results demonstrate that CYP2B2 protein expression was significantly induced by ~5.0 fold in oxycodone treated rats consistent with the microarray (6.0 fold) and the Q-PCR (5.8 fold) data. Finally, ABCB1 protein was identified around 168-170 kDa with normalized optical density values (mean ± SD, n=3) of 0.38 ± 0.09 and 1.2 ± 0.3 for saline and oxycodone samples, respectively (p<0.01) (data not shown). This indicates upregulation of ABCB1 (~3.0 fold) in oxycodone treated rats, consistent with the microarray (~3.2 fold), Q-PCR (4.0 fold) and our previously reported study (4.0 fold) (Hassan et al., 2007).

Identification of cause-effect relationship among XRs, transporters and DMEs by MetaCore™
Genes that were significantly \( p<0.05 \) up/down regulated by at least 1.5-fold (Tables 1-3) were uploaded into MetaCore\textsuperscript{TM}. Using Direct Interactions algorithm, we identified a unique cluster of interacting gene encoded proteins representing XRs, transporters, and DMEs. Only genes that exhibit one or more interaction are shown. Non-interacting genes were omitted from the figure since they do not reveal possible cause-effect relationships (Figure 1). The corresponding sub-network was assembled around PXR, CAR, and AhR showing direct interactions among genes and revealing potential cause-effect relationships. In the network, positive interactions are indicated by green hexagons (e.g., interaction between PXR and MDR1 (ABCB1)), negative interactions are indicated by red hexagons (e.g., interaction between CAR and CYP2E1), and unspecified interactions are indicated by black hexagons (e.g., interaction between AhR and MDR1) (Figure 1). Each of these interactions/relationships represent validated, experimentally confirmed interactions (Ekins et al., 2006). Differentially expressed genes can be visualized simultaneously and identified by expression signs at the top right of each node where red small circles indicate up-regulation (depicted in Figure 1) and blue small circles indicate downregulation (not shown in the figure since no interactions with other genes were detected). In addition to illustrating the interactions between each XR and the regulated transporters and DMEs, the MetaCore\textsuperscript{TM}-generated network also illustrates the intra-relationships among XRs (Figure 1). It indicates that XRs can interact and perhaps regulate each other (e.g., positive interaction between PXR and AhR) consistent with previous reports (Tirona and Kim, 2005). Interestingly, the MetaCore\textsuperscript{TM} analysis also indicated that the transcription factor FKHR (upregulated by 2.9-fold, \( p<0.001 \),
Supplemental Table 2) may also be involved in modulating many XRs, transporters and DMEs (e.g., PXR, CAR, MDR1 and CYP2B6) (Figure 1).

**Transactivation/translocation assays**

In order to identify whether oxycodone or its two main metabolites (noroxycodone or oxymorphone) can activate XRs we conducted a series of cell-based transactivation assays. For the rPXR, hPXR, hAhR and mCAR all positive control compounds responded appropriately in each transactivation assay (Figure 2). Neither oxycodone nor its metabolites demonstrated significant ($p>0.05$) transactivation in any assay up to a concentration of 30 µM. In contrast to other receptors, hCAR is constitutively activated in all immortalized cell lines and is insensitive to chemical stimulation even by the hCAR agonist, CITCO (Figure 2E). As a result, hCAR1+A transfected cells were employed. Oxycodone and its metabolites did not activate hCAR1+A (Figure 2F).

Recently, chemical-mediated nuclear translocation of hCAR in human primary hepatocyte cultures has been established as a novel tool for predicting in vitro CAR activation (Li et al., 2009). So we conducted additional experiments to evaluate the ability of oxycodone to mediate translocation of hCAR in this model. As depicted in Figure 2G, Ad/EYFP-hCAR is predominantly expressed in the cytoplasm of human primary hepatocytes prior to activation, and accumulated within the nucleus in the presence of phenobarbital, a known hCAR activator. In contrast, oxycodone treatment (30 µM) did not translocate hCAR from the cytoplasm to the nucleus which confirmed the inability of oxycodone to activate hCAR.
Discussion

The objective of this study was to evaluate the influence of repeated oxycodone administration on the expression of XRs, transporters, and DMEs. The results reported herein demonstrate that oxycodone indeed modulates the expression of XRs (e.g., PXR, CAR, AhR), transporters (e.g., ABCB1, SLC16A1), and DMEs (e.g., CYP3A1, CYP2B2) (Tables 1–3) as well as many other genes (Tables S2, S3). These findings are supported and validated by: First, Q-PCR analyses of 18 genes demonstrated a strong correlation between Q-PCR and microarray data. Second, Western blot analysis of representative transporter (ABCB1) and DMEs (CYP3A1 and CYP2B2) confirmed the mRNA data. Third, we identified herein several oxycodone-regulated genes (Tables 2, S2) that were previously reported in responses to oxycodone and/or morphine (μ-opioid agonist similar to oxycodone) (e.g., ABCB1, SLC16A1, SLC22A8, SLC1A2, SLC37A4, FKBP5, ADAMTS1) (Aquilante et al., 2000; Homayoun et al., 2003; McClung et al., 2005; Hassan et al., 2010). Finally, our previous report (Hassan et al., 2007) supports the present data and demonstrates that ABCB1 was upregulated in liver tissue of oxycodone treated rats. Collectively, these factors confirm the validity of the gene sets described in this study.

Twenty seven transporters were significantly regulated by oxycodone (Table 2). The number of transporters altered by oxycodone underscores the importance of understanding the impact of concomitant administration of analgesics with other drugs. Five of these transporters belong to the ATP-binding cassette (ABC) superfamily (e.g., ABCB1 and ABCB4). The efflux transporter ABCB1 was significantly upregulated (Table 2). This is consistent with our previous report (Hassan et al., 2007) where a lower
dose of oxycodone (5 mg/kg) upregulated ABCB1 and impeded the accumulation of paclitaxel (ABCB1 marker) in liver tissue of oxycodone treated rats. Paclitaxel is a chemotherapeutic agent often used for the management of hepatic cancer (Tono et al., 2004). Thus, our results raise the possibility that the co-administration of paclitaxel with oxycodone may diminish paclitaxel’s chemotherapeutic effect. It should also be noted that oxycodone is an ABCB1 substrate (Hassan et al., 2007). Thus, the upregulation of ABCB1 could be a defensive mechanism to enhance oxycodone elimination, which in turn, can contribute to tolerance development to its analgesic activity.

The other altered transporters belong to the Solute-Linked Carrier (SLC) superfamily (e.g., SLC22A8 and SLC16A1). A major finding of this study is dramatic downregulation of the organic anion transporter, SLC22A8 (OAT3) by >46-fold. SLC22A8 is a transporter of special interest since it transports a wide spectrum of substrates including, anticancer agents, antiretroviral agents, organic anions, and organic cations (Giacomini et al., 2010). SLC22A8 is reported to be involved in clinical DDI (Giacomini et al., 2010). If any of the SLC22A8-substrates (e.g., methotrexate, adefovir, cidofovir, penicillin G) (Vanwert et al., 2007; VanWert and Sweet, 2008; Giacomini et al., 2010) are co-administered with oxycodone, their excretion could be impeded, which may lead to toxic levels in the plasma. As such, our data would suggest that co-administered drugs with oxycodone should be tested for SLC22A8 substrate specificity prior to their prescription.

Another notable SLC transporter altered by oxycodone treatment was SLC16A1 (Table 2). SLC16A1 is a proton-linked monocarboxylate transporter (MCT) which transports lactates, pyruvates, mevalonates and branched-chain oxo acids derived from
leucine, isoleucine and valine across plasma membranes (Fang et al., 2006). It regulates intracellular pH via symporting protons along with monocarboxylates. SLC16A1 was downregulated in both hepatic (Table 2) and brain tissues (Hassan et al., 2010). Downregulation of SLC16A1 results in acidic intracellular environment and affects many vital physiological processes (e.g., protein folding, ligand binding, enzyme activity and metabolism) that could lead to cell death (Fang et al., 2006). Therefore, our findings suggest that the apoptosis commonly observed in opioid-treated rodents could be, in part, due to SLC16a1 downregulation (Emeterio et al., 2006).

DMEs also play a critical role in the disposition of drugs and are involved in DDIs. Numerous DMEs were dramatically altered by oxycodone treatment (Table 3). For example, P450 oxidoreductase (POR) and CYP17A1 (17α-hydroxylase/17,20-lyase) were strikingly upregulated by 8 and 17-fold, respectively. Por plays a pivotal role in facilitating electron transfer from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to microsomal cytochrome P450 (CYP) enzymes, including the steroidogenic enzymes CYP17A1 which plays a crucial role in androgens biosynthesis. Excessive androgen production is involved in the pathogenesis of serious diseases including prostate cancer and benign prostatic hyperplasia (BPH) (Bostwick et al., 1992). The induction of POR and CYP17A1 in oxycodone treated male rats may trigger excessive androgen production.

Our data also indicates a potential risk for drug abusers. For instance, CYP2B2 and FMO were significantly upregulated (Table 3). CYP2B2 plays a key role in the metabolism of cocaine to norcocaine (Boelsterli et al., 1992; Jover et al., 1993), while FMO facilitates the conversion of norcocaine to the hepatotoxic norcocaine nitroxide.
Our results signify a potential risk of serious hepatic injury if drug abusers co-administer cocaine with oxycodone, where upregulation of CYP2B2 and FMO by oxycodone may accelerate the formation of the hepatotoxic norcocaine nitrooxide metabolite (Cone et al., 2003).

CYP3A1 is involved in the metabolism of many therapeutic agents (e.g., paclitaxel, morphine and efavirenz) and is also responsible for \(N\)-demethylation of oxycodone. In fact, more than 80% of oxycodone administered dose undergoes CYP3A1-mediated \(N\)-demethylation to the inactive metabolite noroxycodone in rats. (Weinstein and Gaylord, 1979). Likewise, CYP3A4- (human CYP3A1 isoform) mediated \(N\)-demethylation in humans is the major oxidative pathway for oxycodone metabolism (Lalovic et al., 2006). Our results indicated that CYP3A1 was significantly upregulated (Table 3) by oxycodone which could enhance noroxycodone formation. We hypothesize that CYP3A1 upregulation coupled with ABCB1 upregulation (Table 2) could be an adaptive mechanism that enhances the hepatic elimination of oxycodone and contributes to tolerance development to its analgesic effect (Hassan et al., 2007).

Another important aspect of this study was the induction of three key XRs (PXR, CAR and AhR) by oxycodone (Table 1). MetaCore™ analysis (Figure 1) generated a signature network of hepatic genes assembled around XRs in response to oxycodone administration and demonstrated that XRs in addition to FKHR, a transcription factor, have direct regulatory effects on the transcription of many transporters and DMEs. To the best of our knowledge this is the first report to present a signature gene network in response to opioid treatment which provides insight into hepatic regulatory mechanisms associated with oxycodone treatment. Of particular interest are the interactions among
MDR1 (ABCB1) and the XRs (PXR, CAR, AhR and FKHR), suggesting that oxycodone-induced upregulation of ABCB1 may result from the activation of PXR, CAR, AhR, and/or FKHR. Neither oxycodone nor its metabolites were capable of activating any of the tested XRs (Figure 2). Morphine (structurally-similar to oxycodone) demonstrated oxycodone-like characteristics, where it induced the expression (data not shown) but not the activity of hPXR and hCAR (Li et al., 2010). Likewise, the insulin-like growth factor-1 receptor inhibitor, BMS-665351, induced CYP3A4 and hCAR but did not activate hCAR or hPXR (Li et al., 2012). In summary, these results indicate that the observed induction of transporters/DMEs is not due to the direct activation of PXR, CAR or AhR via oxycodone or its two major metabolites but alternatively could be due to activation of the induced PXR, CAR and AhR via a, as of yet, unrecognized endogenous activators and/or untested oxycodone metabolite.

In conclusion, this is the first report to elucidate the effect of repeated administration of an opioid on global gene expression in rat liver. Our findings demonstrate that oxycodone alters the expression of many drug disposition controlling genes including three XRs, twenty seven transporters and nineteen DMEs. The significance of these findings is four-fold; firstly, it helps in explaining the escalating problem of DDIs when oxycodone is co-administered with substrates/inhibitors of these transporters/DMEs. Many of these DDIs have resulted in fatalities (Burrows et al., 2003; Cone et al., 2003; Cone et al., 2004; Nieminen et al., 2009). Secondly, oxycodone is often prescribed in medical conditions that involve moderate-to-severe pain. Our results underscore the importance of adjusting the therapeutic doses of substrates of the regulated transporters/DMEs that are co-prescribed with oxycodone for management of these
conditions. Thirdly, our findings suggest that oxycodone may indirectly expedite its own elimination via enhancing both its hepatic efflux (through ABCB1 upregulation) and its hepatic metabolism to the inactive metabolite, noroxycodone (through upregulation of CYP3A1). These changes could be underlying mechanisms by which tolerance occurs to its analgesic effect. Lastly, although regulation of transporters/DMEs is not due to direct activation of XRs via oxycodone or its two major metabolites, co-administration of XRs-activators (e.g., methadone) with oxycodone (XRs-inducer) by drug addicts/abusers could lead to synergistic effects on XRs-mediated induction of transporters/DMEs that could lead to deleterious DDIs. We acknowledge that there are species differences between rats and humans, and as a result, these findings should be interpreted with caution when translated to humans.
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Authorship Contributions

*Participated in research design:* Hassan, Lee, H. Wang, Sinz and Eddington

*Conducted experiments:* Hassan, Myers, Lee, D. Wang and Sinz

*Performed data analysis:* Hassan and Mason

*Wrote or contributed to writing of the manuscript:* Hassan, Lee, H. Wang, Sinz and Eddington
References


SNPs on nevirapine plasma concentrations in Burundese HIV-positive patients using dried sample spot devices. *Br J Clin Pharmacol* **74:**134-140.


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Legends for Figures

**Figure 1.** Gene network illustrates interactions among differently expressed genes encoding XRs, transporters, and DMEs due to oxycodone treatment vs. saline treatment in liver tissues of Sprague Dawley rats. Gene network was obtained using MetaCore™ (web-based computational platform) after applying the Direct Interactions algorithm. Only genes that exhibit one or more interactions with others are shown. Genes without direct interactions were omitted from the figure since they do not reveal cause-effect relationships. Colored highlighted symbols (nodes) represent genes. Red small solid circles (top right of each node) correspond to genes with significant \( p < 0.05 \) up-regulation of ≥1.5-fold. The small colored hexagons on vectors between nodes describe functional interactions. For example, positive interactions are indicated by green hexagons (e.g., interaction between PXR and MDR1), negative interactions are indicated by red hexagons (e.g., interaction between CAR and CYP2E1), and unspecified interactions are indicated by black hexagons (e.g., interaction between AhR and MDR1). Each connection (vector) represents a direct, experimentally confirmed, physical interaction between genes (Ekins et al., 2006). Red cone-like shapes represent XRs, purple X-like shapes represent transporters while yellow arrows represent DMEs.

**Figure 2.** Oxycodone (oxy), noroxycodone (noroxy) and oxymorphone (oxymor) are not activators of PXR, CAR or AhR in cell-based experiments. HepG2 cells were co-transfected with CYP2B6 or CYP3A4 luciferase reporter plasmid along with one of the XR expression vectors: rPXR (A), hPXR (B), hAhR (C), mCAR (D), hCAR (E) or
hCAR1+A (F). Following the transfections, cells were treated with oxycodone (oxy), noroxycodone (noroxy), oxymorphone (oxymor) or the corresponding positive or negative controls as indicated in the figure. After treatment, luciferase activities were determined and expressed relevant to vehicle control. Data represent mean ± SD of three independent transfections. For the hCAR translocation assay (G), human primary hepatocytes were infected with Ad/EYFP-hCAR as described in the Material and methods section and treated with a vehicle control (0.1% DMSO), oxycodone (30 µM) or phenobarbital (1 mM). Then the hepatocytes were subjected to analysis by confocal laser scanning microscopy which indicated that translocation of hCAR to the nucleus occurred in response to phenobarbital but not oxycodone treatment.
Table 1. Significantly regulated xenobiotic receptors in liver tissues of oxycodone treated rats

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Fold change</th>
<th>p value</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR1I3 (CAR)</td>
<td>nuclear receptor subfamily 1, group I, member 3</td>
<td>6.1 ± 0.1</td>
<td>0.002</td>
<td>NM_022941</td>
</tr>
<tr>
<td>NR1I2 (PXR)</td>
<td>nuclear receptor subfamily 1, group I, member 2</td>
<td>2.9 ± 0.02</td>
<td>0.001</td>
<td>NM_052980</td>
</tr>
<tr>
<td>AhR*</td>
<td>aryl hydrocarbon receptor</td>
<td>2.3 ± 0.1</td>
<td>0.002</td>
<td>AA858521</td>
</tr>
</tbody>
</table>

* indicates a gene represented twice in the microarray chips

Fold change values are expressed as mean ± SD, n=3

Metacore assigned gene symbols/names are indicated in parentheses (column 1) if different from Affymetrix gene symbols/names
Table 2. Differentially expressed transporters in liver tissues of oxycodone treated rats

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Fold change</th>
<th>p value</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcg5</td>
<td>ATP-binding cassette, sub-family G (WHITE), member 5</td>
<td>3.6 ± 0.1</td>
<td>0.004</td>
<td>NM_053754</td>
</tr>
<tr>
<td>Abcb1a*</td>
<td>ATP-binding cassette, sub-family B (MDR/TAP), member 1A</td>
<td>3.6 ± 0.1</td>
<td>0.005</td>
<td>AY582535</td>
</tr>
<tr>
<td>Slc16a10</td>
<td>solute carrier family 16 (monocarboxylic acid transporters), member 10</td>
<td>3.0 ± 0.03</td>
<td>0.008</td>
<td>AB047324</td>
</tr>
<tr>
<td>Slc22a5</td>
<td>solute carrier family 22 (organic cation transporter), member 5</td>
<td>2.8 ± 0.1</td>
<td>0.013</td>
<td>NM_019269</td>
</tr>
<tr>
<td>Abcb1a</td>
<td>ATP-binding cassette, sub-family B (MDR/TAP), member 1A</td>
<td>2.8 ± 0.1</td>
<td>0.002</td>
<td>AY582535</td>
</tr>
<tr>
<td>Slc1a2</td>
<td>solute carrier family 1 (glial high affinity glutamate transporter), member 2</td>
<td>2.4 ± 0.2</td>
<td>0.015</td>
<td>NM_017215</td>
</tr>
<tr>
<td>Abca8b_predicted</td>
<td>ATP-binding cassette, sub-family A (ABC1), member 8b (predicted)</td>
<td>2.3 ± 0.02</td>
<td>0.001</td>
<td>BF386852</td>
</tr>
<tr>
<td>Slc25a15*_predicted</td>
<td>solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15</td>
<td>2.2 ± 0.01</td>
<td>0.009</td>
<td>BF554040</td>
</tr>
<tr>
<td>Slc25a15_predicted</td>
<td>solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15</td>
<td>2.1 ± 0.01</td>
<td>0.005</td>
<td>BG377383</td>
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<tr>
<td>Slc37a4 (G6PT1)</td>
<td>solute carrier family 37 (glycerol-6-phosphate transporter), member 4</td>
<td>2.0 ± 0.01</td>
<td>0.019</td>
<td>NM_031589</td>
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<tr>
<td>Slc38a4</td>
<td>amino acid transport system A3</td>
<td>1.8 ± 0.01</td>
<td>0.007</td>
<td>NM_130748</td>
</tr>
<tr>
<td>Slc27a5</td>
<td>bile acid CoA ligase</td>
<td>1.6 ± 0.02</td>
<td>0.005</td>
<td>NM_024143</td>
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<tr>
<td>Slc39a4_predicted</td>
<td>solute carrier family 39 (zinc transporter), member 4 (predicted)</td>
<td>1.6 ± 0.01</td>
<td>0.012</td>
<td>AI556941</td>
</tr>
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<td>Gene</td>
<td>Description</td>
<td>Fold Change ± Standard Error</td>
<td>p-Value</td>
<td>Accession Number</td>
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<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------</td>
<td>-----------</td>
<td>------------------</td>
</tr>
<tr>
<td>Slc17a5_predicted</td>
<td>solute carrier family 17 (anion/sugar transporter), member 5 (predicted)</td>
<td>1.5 ± 0.01</td>
<td>0.005</td>
<td>AA900983</td>
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<tr>
<td>Abcb4 (MDR3)</td>
<td>ATP-binding cassette, sub-family B (MDR/TAP), member 4</td>
<td>1.5 ± 0.03</td>
<td>0.049</td>
<td>NM_012690</td>
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<tr>
<td>Slc21a10</td>
<td>solute carrier family 21, member 10</td>
<td>1.5 ± 0.02</td>
<td>0.029</td>
<td>AF147740</td>
</tr>
<tr>
<td>Slc2a2</td>
<td>solute carrier family 2 (facilitated glucose transporter), member 2</td>
<td>1.5 ± 0.01</td>
<td>0.003</td>
<td>NM_012879</td>
</tr>
<tr>
<td>Slc25a29_predicted</td>
<td>solute carrier family 25 (mitochondrial carrier, palmitoylcarnitine transporter), member 29 (predicted)</td>
<td>1.5 ± 0.04</td>
<td>0.034</td>
<td>BF555120</td>
</tr>
<tr>
<td>Slc6a9</td>
<td>solute carrier family 6 (neurotransmitter transporter, glycine), member 9</td>
<td>-1.6 ± 0.02</td>
<td>0.038</td>
<td>AA943735</td>
</tr>
<tr>
<td>Abcg1</td>
<td>ATP-binding cassette, sub-family G (WHITE), member 1</td>
<td>-1.7 ± 0.04</td>
<td>0.020</td>
<td>NM_053502</td>
</tr>
<tr>
<td>Slc25a30</td>
<td>solute carrier family 25, member 30</td>
<td>-1.8 ± 0.01</td>
<td>0.038</td>
<td>H35736</td>
</tr>
<tr>
<td>Slc16a1</td>
<td>solute carrier family 16 (monocarboxylic acid transporters), member 1</td>
<td>-1.9 ± 0.04</td>
<td>0.033</td>
<td>NM_012716</td>
</tr>
<tr>
<td>Slc35b2</td>
<td>solute carrier family 35, member b2</td>
<td>-2.2 ± 0.1</td>
<td>0.006</td>
<td>BI293600</td>
</tr>
<tr>
<td>Slc41a2_predicted</td>
<td>solute carrier family 41, member 2 (predicted)</td>
<td>-2.5 ± 0.1</td>
<td>0.030</td>
<td>BF410740</td>
</tr>
<tr>
<td>Slc15a3</td>
<td>peptide/histidine transporter PHT2</td>
<td>-2.6 ± 0.1</td>
<td>0.006</td>
<td>AB026665</td>
</tr>
<tr>
<td>Slc7a7</td>
<td>solute carrier family 7 (cationic amino acid transporter, y+ system), member 7</td>
<td>-2.8 ± 0.2</td>
<td>0.031</td>
<td>AF200684</td>
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<tr>
<td>Slc10a2</td>
<td>solute carrier family 10, member 2</td>
<td>-6.3 ± 0.8</td>
<td>0.001</td>
<td>NM_017222</td>
</tr>
<tr>
<td>Slc34a2</td>
<td>solute carrier family 34 (sodium phosphate), member 2</td>
<td>-6.6 ± 0.4</td>
<td>0.023</td>
<td>NM_053380</td>
</tr>
<tr>
<td>Slc22a8</td>
<td>solute carrier family 22 (organic anion transporter), member 8</td>
<td>-46.3 ± 2.2</td>
<td>0.001</td>
<td>NM_031332</td>
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</table>
* indicates genes represented more than once

Fold change values are expressed as mean ± SD, n=3

Metacore assigned gene symbols/names are indicated in parentheses (column 1) if different from Affymetrix gene symbols/names.
Table 3. Differentially expressed metabolizing enzymes in liver tissues of oxycodone treated rats

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Title</th>
<th>Fold change</th>
<th>p value</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP17a1</td>
<td>cytochrome P450, family 17, subfamily a, polypeptide 1</td>
<td>17.1 ± 1.3</td>
<td>0.004</td>
<td>NM_012753</td>
</tr>
<tr>
<td>Por</td>
<td>cytochrome P450 oxidoreductase</td>
<td>8.4 ± 0.03</td>
<td>0.001</td>
<td>NM_031576</td>
</tr>
<tr>
<td>Por</td>
<td>cytochrome P450 oxidoreductase</td>
<td>7.0 ± 0.1</td>
<td>0.002</td>
<td>NM_031576</td>
</tr>
<tr>
<td>Yc2 // Gsta5</td>
<td>glutathione S-transferase Yc2 subunit</td>
<td>7.0 ± 0.6</td>
<td>0.001</td>
<td>AA945082</td>
</tr>
<tr>
<td>CYP2b2</td>
<td>cytochrome P450, family 2, subfamily b, polypeptide 2</td>
<td>6.0 ± 0.02</td>
<td>0.008</td>
<td>AI454613</td>
</tr>
<tr>
<td>FMO1</td>
<td>flavin containing monooxygenase 1</td>
<td>3.7 ± 0.1</td>
<td>0.010</td>
<td>NM_012792</td>
</tr>
<tr>
<td>Ces2</td>
<td>carboxylesterase 2 (intestine, liver)</td>
<td>2.8 ± 0.02</td>
<td>0.001</td>
<td>NM_133586</td>
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<tr>
<td>CYP2j4</td>
<td>cytochrome P450, family 2, subfamily J, polypeptide 4</td>
<td>2.4 ± 0.01</td>
<td>0.001</td>
<td>NM_023025</td>
</tr>
<tr>
<td>Ugt1a1*</td>
<td>UDP glycosyltransferase 1 family, polypeptide A1</td>
<td>2.0 ± 0.02</td>
<td>0.026</td>
<td>AF461738</td>
</tr>
<tr>
<td>CYP4a10</td>
<td>cytochrome P450, family 4, subfamily a, polypeptide 10</td>
<td>2.0 ± 0.04</td>
<td>0.027</td>
<td>NM_016999</td>
</tr>
<tr>
<td>CYP27a1</td>
<td>cytochrome P450, family 27, subfamily a, polypeptide 1</td>
<td>1.9 ± 0.03</td>
<td>0.003</td>
<td>M73231</td>
</tr>
<tr>
<td>CYP4b1</td>
<td>cytochrome P450, family 4, subfamily b, polypeptide 1</td>
<td>1.9 ± 0.03</td>
<td>0.004</td>
<td>M29853</td>
</tr>
<tr>
<td>CYP2a1</td>
<td>cytochrome P450, family 2, subfamily a, polypeptide 1</td>
<td>1.7 ± 0.04</td>
<td>0.002</td>
<td>NM_012692</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Description</td>
<td>Fold Change</td>
<td>SD</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------</td>
<td>-----</td>
<td>-------------</td>
</tr>
<tr>
<td>CYP3a1(CYP3A4)*</td>
<td>cytochrome P450, family 3, subfamily a, polypeptide 1</td>
<td>1.7 ± 0.01</td>
<td>0.026</td>
<td>NM_173144</td>
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<tr>
<td>Ugt1a1 (UGT)</td>
<td>UDP glycosyltransferase 1 family, polypeptide A1</td>
<td>1.6 ± 0.02</td>
<td>0.025</td>
<td>AF461738</td>
</tr>
<tr>
<td>CYP2e1</td>
<td>cytochrome P450, family 2, subfamily e, polypeptide 1</td>
<td>1.5 ± 0.01</td>
<td>0.009</td>
<td>NM_031543</td>
</tr>
<tr>
<td>CYPbb*</td>
<td>cytochrome b-245, beta polypeptide</td>
<td>-2.0 ± 0.02</td>
<td>0.017</td>
<td>BE098739</td>
</tr>
<tr>
<td>CYPba</td>
<td>cytochrome b-245, alpha polypeptide</td>
<td>-2.0 ± 0.02</td>
<td>0.018</td>
<td>AI232788</td>
</tr>
<tr>
<td>CYPbb</td>
<td>cytochrome b-245, beta polypeptide</td>
<td>-2.1 ± 0.05</td>
<td>0.018</td>
<td>BE098739</td>
</tr>
<tr>
<td>CYP2c</td>
<td>cytochrome P450, family 2, subfamily c</td>
<td>-2.1 ± 0.04</td>
<td>0.003</td>
<td>NM_019184</td>
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<tr>
<td>CYP2c13*</td>
<td>cytochrome P450, family 2, subfamily c, polypeptide 13</td>
<td>-2.2 ± 0.04</td>
<td>0.002</td>
<td>J02861</td>
</tr>
<tr>
<td>Sult1a2</td>
<td>sulfotransferase family 1A, member 2</td>
<td>-3.3 ± 0.03</td>
<td>0.001</td>
<td>NM_031732</td>
</tr>
</tbody>
</table>

* indicates genes represented more than once

Fold change values are expressed as mean ± SD, n=3

Metacore assigned gene symbols/names are indicated in parentheses (column 1) if different from Affymetrix gene symbols/names

# indicates previously reported genes (Myers et al., 2010)
Figure 1

cytochrome P-450 reductase

CYP3A4

CYP2E1

CAR

MDR1

MDR3

CYP2B6

UGT

PXR

AHR

FKHR

G6PT1
Figure 2

A. rPXR

B. hPXR

C. hAhR

D. mCAR

E. hCAR

F. hCAR1+A

G. hCAR localization in human hepatocytes

Ctrl  OXY (30 μM)  PB (1 mM)