DMD Fast Forward. Published on June 11, 2009 as doi:10.1124/dmd.109.027854

Methadone Induces the Expression of Hepatic Drug-Metabolizing Enzymes through the Activation of Pregnane X Receptor and Constitutive Androstane Receptor

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DMD Fast Forward. Published on June 11, 2009 as DOI: 10.1124/dmd.109.027854
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Running Title: Methadone Induces DMEs in Human Hepatocytes

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Number of text pages: 30 Number of tables: 1

Number of figures: 6 References: 32

Words in abstract: 243 Words in introduction: 696

Words in the discussion: 1281

ABBREVIATIONS: Ad/EYFP-hCAR, adenovirus/enhanced yellow fluorescent protein tagged-hCAR; CAR, constitutive androstane receptor; DDIs, drug-drug interactions; DMEs, drug-metabolizing enzymes; MD, methadone; MDR1, multidrug resistance 1 ; NRs, nuclear receptors; PK11195, 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinoline-carboxamide; PXR, pregnane X receptor; CYP, cytochrome P450; UGT, UDP-glucuronosyltranferases; CITCO, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichloro-benzyl)- oxime; RIF, rifampicin.

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Abstract

Methadone (MD) is the most established substance abuse pharmacotherapy of choice for the management of heroin dependence. To date, drug-drug interactions involving MD have been characterized asymmetrically among existing reports, which describe how other drugs affect the metabolic or pharmacokinetic profiles of MD, yet limited information is available regarding the potential for MD to influence similar fates of co-administered drugs. Moreover, little to no mechanistic evidence has been explored. Here, we demonstrate that MD induces hepatic drug-metabolizing enzymes (DMEs) through the activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR). Real-time PCR analysis of human hepatocyte cultures revealed that MD induces the mRNA expression of CYP2B6, CYP3A4, UGT1A1, and MDR1 in a concentration-related manner, with the maximal induction of CYP2B6 challenging that of the induction by rifampicin (RIF). Further, MD-mediated induction of CYP2B6 and CYP3A4 proteins was observed in Western blotting analysis. In cell-based reporter assays, MD significantly increased hPXR-mediated CYP2B6 reporter activities, but exhibited minimal effect on hCAR activation due to the constitutive activity of hCAR in HepG2 cells. Further studies revealed that treatment with MD resulted in significant nuclear accumulation of Ad/EYFP-hCAR in human hepatocytes, which has been regarded as the initial step of CAR activation. Additional analysis of the two enantiomers of MD, R-(-)-MD (active) and S-(+)-MD (inactive) indicates the lack of stereoselectivity pertaining to MD-mediated DME induction. Overall, our results demonstrate that MD induces the hepatic expression of multiple DMEs by activating PXR- and CAR-mediated pathways.

Introduction

Methadone (MD), a synthetic opioid which possesses enduring effects due to its long half-life, is a critical clinical drug therapy that continues to be used for the management of heroin addiction and the treatment of chronic pain. Approximately 20% of an estimated 810,000 addicts in the United States receive long term MD maintenance treatment (American Methadone Treatment Association, 1999), and oftentimes MD maintenance patients consume a myriad of concomitant medication as part of an aggressive polypharmacy approach of therapy. For example, MD users are likely also treated with pain medications such as oxycodone and codeine; or anti-retroviral therapies such as efavirenz and ritonavir (Fornataro, 1999; Ferrari et al., 2004). Thus, the potential for drug-drug interactions (DDIs) involving MD is high.

MD is almost exclusively metabolized in the liver (Nilsson et al., 1982). The primary metabolic route involves hepatic N-demethylation and cyclization to its stable metabolite, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine, which is pharmacologically inactive (Ferrari et al., 2004). Some controversy exists in the literature regarding which CYP isoform(s) play the primary role in hepatic metabolism and clearance of MD. Previously, the predominant mediator of MD metabolism was thought to be CYP3A4; and numerous in vitro and in vivo studies have demonstrated CYP3A4 involvement in the metabolism of MD in human liver or intestines (Wang and DeVane, 2003; Gerber et al., 2004; Kharasch et al., 2004). Recent studies, however, suggest that CYP2B6 is also involved in the metabolism of MD, and may possess a higher affinity for MD metabolism compared to that of CYP3A4 (Gerber et al., 2004; Kharasch et al., 2008; Weschules et

al., 2008). Dramatic variations in the pharmacokinetic profile of MD are speculated to be due largely to the variable expression and activity of CYP2B6 and CYP3A4, given that both of these CYPs are highly inducible isozymes that exhibit significant inter-individual variations (Ingelman-Sundberg et al., 2007).

Notably, though MD has been used extensively for several decades for the management of narcotic dependence, characterization of its interaction with other co-administered or co-abused drugs is largely incomplete and heavily one-sided. To date, mounting literature, which heavily focuses on describing how other drugs affect the metabolic and pharmacokinetic profiles of MD, has been published (Kharasch et al., 2008; Linderbeck, 2008; Weschules et al., 2008). For instance, a number of antiretrovirals, such as efavirenz and nevaripine, have been identified as potent inducers of CYP2B6 and CYP3A4 while ritonavir and indinavir are efficacious inhibitors of CYP3A4. Subsequently, co-treatment of these antiretrovirals with MD has resulted in decrease or increase of the plasma concentrations of MD in HIV-positive heroin addicts (Clarke et al., 2001; Ernest et al., 2005; Faucette et al., 2007). Conversely, only limited data exists regarding the effects of MD on the metabolic fate of co-administered other drugs, and little to no mechanistic evidence has been explored.

Metabolism-based DDIs have received increasing attention over the last two decades, due in large part to the increased incidence of multidrug therapy necessitated by overlapping disease states. The majority of clinically significant DDIs occur via induction or inhibition of DMEs. In the case of enzyme induction, increased expression and activity of DMEs such as CYPs can lead to accelerated drug clearance and premature termination

of drug actions. Furthermore, enzyme induction is often governed by the activation of several nuclear receptors, such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which coordinately control the drug-induced expression of multiple DMEs and drug transporters, including the highly inducible CYP2B6 and CYP3A4 (Sueyoshi et al., 1999; Moore et al., 2000).

The primary objective of the current study was to characterize the effects of MD on the expression of hepatic DMEs and transporters, as well as the activation of the xenobiotic sensors PXR and CAR. MD-mediated induction of CYP2B6, CYP3A4, UGT1A1, and MDR1 was assessed in human primary hepatocytes, which express physiologically relevant liver-enriched transcription factors. Cell-based reporter assays in HepG2 cells were used to determine the differential activation of nuclear receptors. A newly generated adenovirus containing EYFP-tagged hCAR (Ad/EYFP-hCAR) was utilized to assess drug induced hCAR nuclear accumulation in human primary hepatocytes (Li et al., 2009). Overall, our results demonstrate that MD induces the hepatic expression of multiple DMEs through the activation of PXR- and CAR-mediated pathways.

Materials and Methods

Chemicals and Reagents. MD, PK11195 and Rifampicin (RIF) were purchased from Sigma-Aldrich (St. Louis, MO); 6-(4-chlorophenyl)imidazo- [2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichloro-benzyl)-oxime (CITCO) was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Methadone enantiomers, R-(-)-MD and S-(+)-MD, were generously provided by the National Institute on Drug Abuse Drug Supply Program. Oligonucleotide primers and TaqMan fluorescent probes were synthesized by Sigma Genosys (The Woodlands, TX) and Applied Biosystems (Foster, CA), respectively. The Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI). Matrigel, insulin and ITS^+ were obtained from BD Biosciences (Bedford, MA). Horseradish peroxidase-labeled anti-rabbit antibody was purchased from Amersham Biosciences (Pittsburgh, PA). Other cell culture reagents were purchased from Invitrogen (Carlsbad, CA) or Sigma-Aldrich.

Plasmid Constructs and Ad/EYFP-hCAR. The CYP2B6 reporter constructs, containing both PBREM and the distal XREM (CYP2B6-2.2kb) were generated as described previously (Wang et al., 2003). The pCR3-hCAR and the enhanced yellow fluorescent protein tagged hCAR (EYFP-hCAR) expression plasmids were kindly provided by Dr. Masahiko Negishi (National Institute of Environmental and Health Sciences, National Institutes of Health, Research Triangle Park, NC). The pSG5-hPXR, and CMV2-hCAR3 expression plasmids were from Dr. Steve Kliewer (University of Texas, Southwestern Medical Center, Dallas, TX), and Dr. Curtis Omiecinski (The Pennsylvania State University, University Park, PA), respectively. The adenoviral-

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EYFP-hCAR (Ad/EYFP-hCAR) was generated as described previously (Li et al., 2009). The pRL-TK Renilla luciferase plasmid used to normalize firefly luciferase activities was purchased from Promega (Madison, WI).

Induction Studies in Human Primary Hepatocyte Cultures. Human liver tissues were obtained following surgical resection by qualified pathology staff after diagnostic criteria were met and prior approval was obtained from the Institutional Review Board at the University of Maryland at Baltimore. Hepatocytes were isolated by a modification of the two-step collagenase digestion method as described previously (LeCluyse et al., 2005). Hepatocytes were seeded at 1.5×10^6 cells/well in six-well biocoat plates in DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 μg/ml insulin, and 1 μM dexamethasone. After attachment at 37°C in a humidified atmosphere of 5% CO_2, hepatocytes were cultured in complete William's E Medium (WEM), then overlaid with Matrigel (0.25mg/ml). Hepatocytes were maintained for 36 hrs before treatment with different compounds.

Real-Time PCR Analysis. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster, CA) per manufacturers' instructions. Primers and probes for CYP2B6, CYP3A4, UGT1A1, and MDR1 genes (Table 1) were designed using Primer Express Version 2.0; and entered into the NCBI Blast to ensure specificity as described previously (Maglich et al., 2002; Smith et al., 2005; Faucette et al., 2007; Li, 2008). The mRNA expression of CYP2B6, CYP3A4, UGT1A1, and MDR1, was normalized against that of human β-actin, which was detected using a pre-developed primer/probe mixture (Applied

Biosystems, Foster, CA). TaqMan PCR assays were performed in 96-well optical plates on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Fold induction values were calculated according to the equation $2^{\Delta\Delta CT}$, where ΔCT represents the differences in cycle threshold numbers between the target gene and β-actin; and ΔΔCT represents the relative change in these differences between control and treatment groups.

Transient Transfection in HepG2 Cells. HepG2 cells seeded in 24-well plates were transfected with CYP2B6-2.2kb reporter construct in the presence of hPXR, hCAR1 or hCAR3 expression vector using Fugene 6 Transfection Kit according to the manufacturer's protocol. Twenty-four hours post-transfection, cells were treated with solvent (0.1% DMSO) or test compounds at the concentrations of RIF (10 μM), CITCO (1 μM), or MD (10 μM, 25 μM, or 50 μM) for another 24 hrs. Subsequently, cell lysates were assayed for firefly activities normalized against the activities of Renilla luciferase using the Dual-Luciferase Kit (Promega, WI). Data are represented as mean ± S.D. of three individual transfections.

Translocation of Ad/EYFP-hCAR in Human Primary Hepatocyte Cultures. Human hepatocytes were seeded at 3.75×10^5 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 hrs before treatment with vehicle control (0.1% DMSO) or test compounds for another 12 hrs. Confocal laser scanning microscopy was performed with a Nikon C1-LU3 instrument based on an inverted Nikon Eclipse TE2000 microscope. The subcellular localization of Ad/EYFP-hCAR was visualized, and quantitatively characterized as nuclear (N), cytosolic (C), and mixed (N + C) by counting

100 Ad/EYFP-hCAR expressing hepatocytes from each group.

Western Immunoblot Analysis. Homogenate proteins (40 μg each) from treated human hepatocytes were separated on a NuPAGE® 4-12 % Bis-Tris Gel (Invitrogen, Carlsbad, CA), and transferred onto PVDF Transfer Membrane (Pierce, Rockford, IL). Subsequently, membranes were incubated with specific antibodies against CYP2B6 or CYP3A4 (Millipore-Chemicon, CA) diluted 1:4000 and 1:5000, respectively. β-actin (Sigma-Aldrich. St. Louis, MO) was used as internal control. Blots were washed and incubated with horseradish peroxidase goat antirabbit IgG antibody diluted 1:4000. Films were developed using ECL Western blotting detection reagent (GE Healthcare).

Statistical Analysis. All data represent three independent measurements and are expressed as the mean ± S.D. Statistical comparisons were made using the Student's *t*-test, and statistical significance was assessed at 3 levels (***, $p \leq 0.001$; **, $p \leq 0.01$, and *, $p \leq 0.05$).

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Results

Induction of DMEs and Transporter mRNA Expression in Human Primary Hepatocytes. In the current study, the effects of MD on the expression of CYP2B6, CYP3A4, UGT1A1, and MDR1 genes were evaluated in human primary hepatocyte cultures using real-time RT-PCR analysis. In human hepatocytes prepared from two donors (HL#3, HL#6), the mRNA expression of CYP2B6, CYP3A4, UGT1A1, and MDR1 was increased significantly upon treatment by MD at 10, 25 and 50 (μM) (Fig. 1). Notably, the maximal induction of CYP2B6 at higher concentrations of MD treatment equals or exceeds that induced by the positive control RIF (10 μM). Potent induction of CYP3A4 mRNA was also observed in HL#3, where 6-, 25-, and 33-fold of CYP3A4 mRNA increases resulted from 10, 25, and 50 (μM) of MD exposure, respectively. Compared with the highly inducible CYP2B6 and CYP3A4 genes, the induction of UGT1A1 and MDR1 was relatively moderate (Fig. 1C and 1D). As expected, the positive control RIF efficiently induced CYP2B6, CYP3A4, UGT1A1, and MDR1 expression in both hepatocyte preparations, despite obvious inter-individual variations.

Induction of CYP2B6 and CYP3A4 Protein in Human Primary Hepatocytes. To assess whether MD could induce CYP2B6 and CYP3A4 expression at the protein level, whole cell homogenate isolated from two preparations of human primary hepatocytes (HL #14, HL #16) treated with different concentrations of MD were analyzed for CYP2B6 and CYP3A4 protein content by Western blot analysis. As demonstrated in Fig. 2, MD robustly induced the protein expression of CYP3A4 in a dose-dependent manner, while the CYP2B6 protein was only induced at higher concentrations of MD (25 and 50

μM) to a relatively minor extent (Fig. 2A and 2B). In both hepatocyte preparations, RIF displayed the greatest extent of CYP2B6 and CYP3A4 protein induction.

Activation of Human PXR and CAR by Racemic MD. Drug-induced expression of CYP2B6 and CYP3A4 is predominantly controlled at the transcriptional level by the two nuclear receptors hCAR and hPXR. Here, we investigated the ability of racemic MD to activate these receptors in cell-based reporter assays conducted in HepG2 cells. As shown in Fig. 3A, MD showed significant increases of hPXR-mediated CYP2B6 reporter activities over the concentration range of 10 to 50 (μM), where significant CYP2B6 and CYP3A4 gene induction was observed in human primary hepatocytes. In contrast to hPXR, in vitro assessment of hCAR activation has been problematic, due to the constitutive activation nature of CAR in all immortalized cell lines. Recently however, several reports indicate that a human CAR splicing variant (hCAR3) displays low basal activity, while still retaining chemical-mediated activation in several cell lines (Auerbach et al., 2005; Faucette et al., 2007). Thus, in the current study, we also evaluated hCAR3 activation in HepG2 cells. As expected, the hCAR-selective agonist CITCO strongly enhanced hCAR3 activity, while MD resulted in moderate but statistically significant activation of hCAR3 (Fig. 3B). Additionally, earlier work conducted in this laboratory demonstrated that the constitutive activity of the wild type hCAR in HepG2 cells could be substantially repressed by PK11195, a typical ligand for peripheral benzodiazepine receptor, and this inhibitory effect was only recovered by co-treatment with direct hCAR activator CITCO, but not by indirect activators such as phenobarbital (PB) (Li et al., 2008). Utilizing this system, our current results showed that MD has no effect on the PK11195-mediated deactivation of hCAR (Fig. 3C).

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Both R-(-)-MD and S-(+)-MD Contribute to the Activation of hPXR in HepG2 Cells. MD is clinically administered as a mixture of two stereoisomers, R-(-)-MD and S-(+)-MD, with opioid activity resides in the R-enantiomer. To assess the contribution of each enantiomer to the observed increases of hPXR-mediated CYP2B6 reporter activities, similar reporter assays in HepG2 cells were conducted as described above using R-(-)-MD (active) and S-(+)-MD (inactive). As demonstrated in Fig. 4, both the active R-enantiomer and the inactive S-enantiomer achieved potent and dose-dependent activation of hPXR with maximal activity occurring at 50 μM. These results suggest that although the metabolism and clearance of racemic MD are highly variable, the potential for each enantiomer to achieve DME induction is most likely non-stereoselective.

Translocation of Ad/EYFP-hCAR by MD in Human Primary Hepatocytes. The high constitutive activation of hCAR in immortalized cell lines can be attributed to the spontaneous nuclear accumulation of hCAR in these cells. In contrast, in primary hepatocytes and in vivo, CAR is sequestered predominantly in the cytoplasm, and translocated to the nucleus only upon exposure to xenobiotics. Recently, our laboratory has generated an adenovirus-driven EYFP-hCAR, which demonstrated exceptional efficiency in transducing human primary hepatocytes (Li et al., 2009). Utilizing this system, we sought to determine if MD can translocate hCAR as the initial step of hCAR activation. Cultured hepatocytes were infected with Ad/EYFP-hCAR and treated with vehicle control, known hCAR indirect activator PB, or 50 μM MD. Fluorescence microscopy analysis demonstrated that both PB and MD treatment result in abundant nuclear accumulation of hCAR (Fig. 5A). Of the hepatocytes expressing hCAR prepared from two liver donors (HL#8, HL#9), 87 to 93% displayed cytoplasmic localization, 7 to

13% exhibited mixed (cytoplasm and nucleus) localization, while without cells in the vehicle control group showed pure nuclear localization (Fig. 5B). The Ad/EYFP-hCAR expression was predominantly accumulated inside the nucleus after the treatment with known indirect hCAR activator PB, where 63 to 80% exhibited nuclear localization, 7 to 38% exhibited a mixed distribution pattern, and only 0 to 13% displayed cytoplasmic localization. Notably, MD (50 μM) treatment also resulted in 62 to 81% nuclear, 11 to 13% mixed, and 8 to 25% cytoplasmic localizations (Fig. 5B). Subsequently, a parallel experiment was conducted to expand the scope of MD mediated hCAR translocation by including both racemic MD and its constituent isomers, at 10 μM, 25 μM and 50 μM concentrations. As demonstrated in Fig. 6, all isomers of MD translocated hCAR efficiently from the inactive cytoplasmic localization to the nucleus at all three concentrations. The maximal translocation extent mimicked that of the positive control PB. Overall, these results indicate that MD and its constituent isomers are capable of accumulating hCAR inside the nucleus of human hepatocytes in a non-stereoselective manner.

Discussion

Methadone is widely prescribed for the management of heroin dependence and different types of chronic pain. Due to frequent co-administration with other therapeutics, DDIs involving MD resulting from a polypharmacy approach to therapy are common. Nevertheless, mounting evidence demonstrates thus far that MD-drug interactions have been characterized asymmetrically among existing literature, where focus has been placed heavily on describing how other drugs affect the metabolic or pharmacokinetic profiles of MD. However, limited data exists regarding the potential for MD to influence similar fates of co-administered drugs, and little to no mechanistic evidence has been provided. Although MD is not a new medication, to our knowledge, the current study is the first to demonstrate that MD can induce the expression of multiple hepatic DMEs through the activation of PXR- and CAR-dependent pathways.

Sandwich cultures of human primary hepatocytes have been used extensively for evaluating the ability of drugs to induce the expression and activity of DMEs and transporters in humans (LeCluyse, 2001). Enzyme induction in human primary hepatocytes possesses the distinct advantage of mimicking physiological in vivo conditions, and exhibiting species-specific induction patterns. Using human primary hepatocytes as a model, our data revealed that the expression of CYP2B6 and CYP3A4 was potently and dose-dependently induced upon treatment by MD, whereas the expression of phase II enzyme UGT1A1 and efflux transporter MDR1 were only moderately induced. Given that the clinical use or abuse of MD is usually chronic, and prescribed dosages could range from approximately 30-180 mg/day up to as much as

1300 mg/day under certain circumstances (Ali and Woods, 2008), exposure levels could vary dramatically. The current in vitro studies have tested MD over a concentration range of 10 to 50 μM. Although it is difficult to quantitatively correlate in vitro data to in vivo conditions, our findings lead to the speculation that MD may also induce CYP2B6 and CYP3A4 expression to a clinically significant level in vivo. Because the occurrence of HIV disease is common among injection drug users and opioid dependants who often require MD maintenance therapy, DDIs occurring between MD and anti-HIV agents have been investigated previously. However, the particular DDI between MD and antiretrovirals has been commonly examined in a unilateral manner, where anti-HIV agents have been reported to affect MD disposition (Clarke et al., 2001; Kharasch et al., 2009), but little has been written about how MD can affect antiretrovirals. A number of anti-HIV agents such as efavirenz and nevirapine are primarily metabolized in the liver by CYP3A4 and CYP2B6 (Desta et al., 2007). Thus, MD induction of CYP2B6 and CYP3A4 may result in altered pharmacokinetics of such agents, and contribute to the frequently observed efavirenz adaptation in clinical settings. Likewise, MD itself is metabolized predominantly by CYP2B6, CYP3A4, and CYP2D6, with CYP2B6 exhibiting the highest affinity and efficacy (Gerber et al., 2004; Kharasch et al., 2004). Therefore, MD-mediated autoinduction of CYPs may enhance the clearance of MD itself. Overall, the current in vitro observations warrant further in vivo and clinical investigations, with a focus on how the chronic administration of MD may affect the efficacy and toxicity of concomitantly administered medications.

Transcriptional regulation of CYP2B6 and CYP3A4, as well as a number of other DMEs and drug transporters, has been attributed to the cross-talk of the two major

xenobiotic receptors, PXR and CAR. In response to structurally diverse xenobiotics, hPXR displays promiscuous ligand binding ability and indiscriminant induction of CYP2B6 and CYP3A4, while hCAR exhibits limited ligand binding capacity with preferential induction of CYP2B6 over CYP3A4 (Faucette et al., 2006; Faucette et al., 2007). In delineating the molecular mechanisms underlying MD-mediated induction of CYP2B6 and CYP3A4, our cell-based reporter assays in HepG2 cells showed that treatment of MD resulted in potent and dose dependent activation of hPXR-mediated luciferase reporter gene expression. Given that activation of PXR coordinately induces the expression of a plethora of DMEs and drug transporters besides CYP2B6 and CYP3A4, MD treatment holds great potential for causing DDIs by interacting with a broader spectrum of DMEs and transporters.

In contrast to PXR, CAR is spontaneously accumulated in the nucleus and constantly activated in immortalized cells prior to xenobiotic activation, which makes the in vitro assessment of hCAR activation extremely difficult. Recently, one of the hCAR splicing variants (hCAR3) has been reported as ligand-responsive hCAR with low constitutive activity in several cell lines (Auerbach et al., 2005; Faucette et al., 2007). Additionally, recent work conducted in this laboratory demonstrated that the constitutive activity of wild-type hCAR in HepG2 cells was sufficiently repressed by hCAR antagonist PK11195, and this repression was reactivated only by the hCAR agonist CITCO, but not by indirect activators such as PB (Li et al., 2008). Utilizing the hCAR3 reporter, and PK11195-based hCAR reactivation assays, our data showed that MD mediated a moderate but dose-dependent activation of hCAR3, while failing to reactivate PK11195-suppressed hCAR activity in HepG2 cells, indicating that MD may not function

as a direct agonist of hCAR. Nevertheless, it is noteworthy that the majority of known hCAR activators are PB-like compounds that activate hCAR through indirect mechanisms without direct ligand binding. As a matter of fact, CITCO and artemisinin are the only two hCAR agonists identified thus far (Maglich et al., 2003; Simonsson et al., 2006). Compared to the cell-based hCAR reporter assays, chemical-mediated hCAR nuclear translocation in hepatocytes of primary culture or in vivo appears to correlate well with hCAR activation and target gene induction, regardless of the distinction between direct or indirect mechanisms (Wang et al., 2004; Faucette et al., 2007). In vitro transfection of human primary hepatocytes, however, has been extremely challenging due mostly to the quiescent nature of hepatocyte in cultures. Most recently, we have generated an adenovirus construct containing enhanced yellow fluorescent-tagged hCAR (Ad/EYFP-hCAR) that transduces human primary hepatocytes with high efficiency, and exhibits a physiologically relevant hCAR distribution pattern (Li et al., 2009). Further evaluation of MD in Ad/EYFP-hCAR-infected human primary hepatocytes revealed that MD treatment resulted in remarkable nuclear accumulation of hCAR at all tested concentrations, which achieves an extent similar to the positive control PB. Combined with the reporter assays, these results indicate that MD actives hCAR most likely through indirect, PB-like mechanisms.

MD is clinically administered as a racemic mixture of two stereoisomers with only the R-enantiomer possessing opioid activity. Several lines of evidence indicate that MD metabolism and clearance are stereoselective, where CYP2B6, but not CYP3A4, contributes significantly to the highly variable plasma R/S-MD ratios (Totah et al., 2007). To gain insight into the potential stereoselectivity between R-(-)-MD and S-(+)-MD in

the induction of DMEs, the current study further evaluated the active R-(-)-MD and the inactive S-(+)-MD isomers for their activation of hPXR and hCAR. Intriguingly, both isomers robustly activated hPXR in cell-based reporter assays in HepG2 cells and translocated Ad/EYFP-hCAR in human primary hepatocytes. These results suggest that the two enantiomers of MD may equally induce hepatic DMEs through similar molecular mechanisms, even though their own disposition is considerably stereoselective.

In conclusion, the current study demonstrates that MD can induce the expression of multiple hepatic DMEs and drug transporters, including CYP2B6, CYP3A4, UGT1A1, and MDR1, through the activation of xenobiotic receptors PXR and CAR. Further, both constituent isomers, R-(-)-MD and S-(+)-MD, contributed significantly and equivalently to the activation of both hPXR and hCAR. These findings indicate that racemic MD may induce multiple hepatic DMEs and drug transporters in a non-stereoselective manner. Given that opioid drug abuse is a rapidly escalating problem, which has the potential to lead to clinically significant DDIs and adverse effects, the in vitro discoveries from the current studies warrant more systematic future in vivo and clinical studies which focus on how MD can mediate changes in the metabolic and pharmacokinetic profiles of other drugs.

Ac**knowledgments**

The authors would like to acknowledge Drs. Masahiko Negishi (National Institute of Environmental Health Sciences, National Institute of Health, RTP, NC), Steve Kliewer (University of Texas, Southwestern Medical Center, Dallas, TX), and Curtis Omiecinski (The Pennsylvania State University, University Park, PA) for generously providing pCR3-hCAR, pSG5-hPXR, and CMV2-hCAR3 expression vectors, respectively. We thank the Drug Supply Program at the National Institute on Drug Abuse for providing the methadone enantiomers, and Dr. Andrew Coop (Chair, Department of Pharmaceutical Sciences, University of Maryland at Baltimore) for assistance with obtaining methadone. Human liver tissues were procured with the aid of John Cottrell from the University of Maryland at Baltimore Medical Center (Baltimore, MD).

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Footnotes

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Figure Legends

Figure 1. **Induction of DMEs and Drug Transporter in Human Primary Hepatocytes.** Human hepatocytes (HL#3 and HL#6) cultured in WEM were treated for 24 h with vehicle, RIF (10 μM), CITCO (1 μM), or MD (10 μM, 25 μM and 50 μM). Total RNA was isolated, reverse transcribed, and subjected to TaqMan real-time PCR. The expression levels of mRNA for (A) CYP2B6, (B) CYP3A4, (C) UGT1A1, and (D) MDR1, were determined and normalized against β-actin. Induction of these enzymes relative to vehicle control was calculated as described under "MATERIALS AND METHODS". All data are expressed as mean ± SD. (n = 3) ***, ###: p≤0.001; **, ##: p≤0.01; *, #: p≤0.05

Figure 2. **Effects of MD on the Expression of CYP3A4 and CYP2B6 Immunoreactive Proteins.** Human hepatocytes, (A) HL#14 and (B) HL#16, were treated for 72 hrs with vehicle control, RIF (10 μM), or MD (10, 25, and 50 μM). Whole cell homogenates were subjected to Western blot and densitometric analyses as described under "MATERIALS AND METHODS."

Figure 3. **Effects of MD on hPXR-, hCAR3-, and hCAR-mediated CYP2B6 Reporter Gene Activation.** HepG2 cells were transfected with (A) hPXR, (B) hCAR3, or (C) hCAR, expression vectors in the presence of CYP2B6-2.2 kb reporter construct. Transfected cells were then treated with vehicle, or MD (10 μM, 25 μM, or 50 μM) for 24

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h. RIF (10 μM) and CITCO (1 μM) were used as positive controls for hPXR and hCAR, respectively. Luciferase activities were determined and expressed relative to vehicle control. Data represent the mean ± SD. (n=3) ***: p≤0.001; **: p≤0.01

Figure 4. **MD Enantiomers Increase the Activities of hPXR in HepG2 Cells.** HepG2 cells were transfected with hPXR expression vector in the presence of CYP2B6-2.2 kb reporter construct, then treated with vehicle control, (A) R-(-)-MD, or (B) S-(+)-MD (10, 25 or 50 μM), for 24 h. RIF (10 μM) was used as positive control. Luciferase activities were determined and expressed relative to vehicle control. Data represent the mean ± SD. (n=3) ***: p≤0.001; **: p≤0.01

Figure 5. **Methadone Ttranslocates Ad/EYFP-hCAR in Human Primary Hepatocytes.** Human hepatocytes (HL#8 and HL#9) were infected with Ad/EYFP-hCAR as described in "MATERIALS AND METHODS" and treated with vehicle, PB (1 mM) or MD (50 μM). After 24 hrs of treatment, hepatocytes were subjected to confocal microscopy. (**A**) Representative Ad/EYFP-hCAR localizations from vehicle control, PB (1 mM), and MD (50 μM) treated hepatocytes. (**B)** For each treatment, over 60 hCAR-expressing cells were counted and classified based on cytosolic, nuclear, or mixed (cytosolic + nuclear) hCAR localizations.

Figure 6. **MD and Constituent Isomers Promote Nuclear Translocation of Ad/EYPF-hCAR in Human Hepatocytes**. Hepatocytes were infected with Ad/EYFP-hCAR and

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treated with vehicle (0.1% DMSO), or racemic MD and its constituent isomers at 10 μM, 25 μM, and 50 μM for 24 hrs. For each treatment group, 100 hCAR-expressing cells were counted and classified based on cytosolic, nuclear, or mixed (cytosolic + nuclear) hCAR distributions.

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Table 1 Primer and probe sequences for real-time PCR assays

Gene		Sequence	Reference
CYP2B6	Forward primer	5-AAGCGGATTTGTCTTGGTGAA-3	(Faucette et al. 2007)
	Reverse primer	5-TGGAGGATGGTGGTGAAGAAG-3	
	Probe	6-FAM-CATCGCCCGTGCGGAATTGTTC-TAMRA	
CYP3A4	Forward primer	5-TCAGCCTGGTGCTCCTCTATCTAT-3	(Faucette et al. 2007)
	Reverse primer	5-AAGCCCTTATGGTAGGACAAAATATTT-3	
	Probe	6-FAM-TCCAGGGCCCACACCTCTGCCT-TAMRA	
UGT1A1	Forward primer	5-GGCCCATCATGCCCAATAT-3	(Smith et al. 2005)
	Reverse primer	5-TTCAAATTCCTGGGATAGTGGATT-3	
	Probe	6-FAM-TTTTTGTTGGTGGAATCAACTGCCTTCAC-TAMRA	
MDR1	Forward primer	5-GTCCCAGGAGCCCATCCT-3	(Maglich et al. 2002)
	Reverse primer	5-CCCGGCTGTTGTCTCCAT-3	
	Probe	6-FAM-TGACTGCAGCATTGCTGAGAACATTGC-TAMRA	

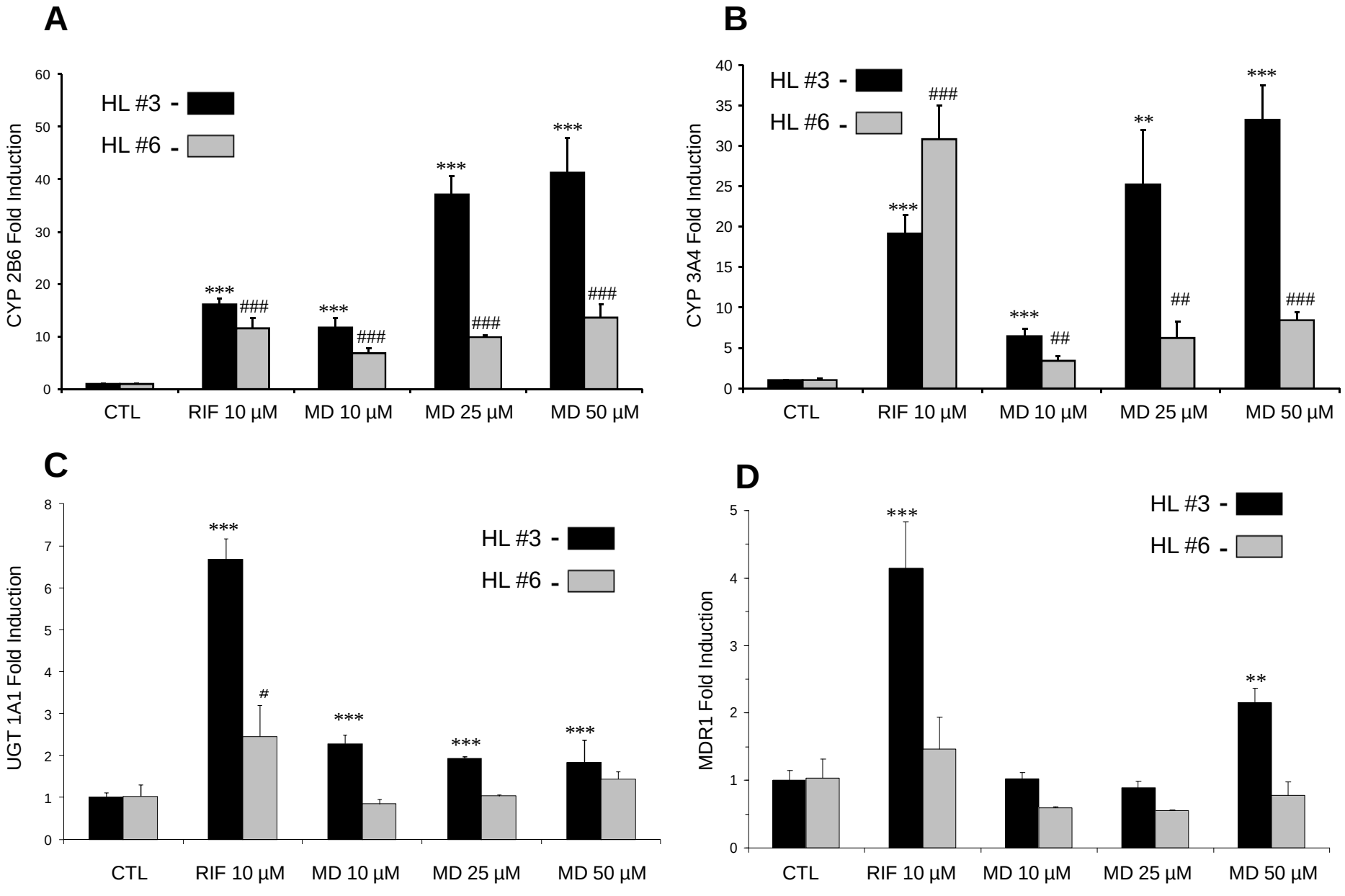


Figure 1

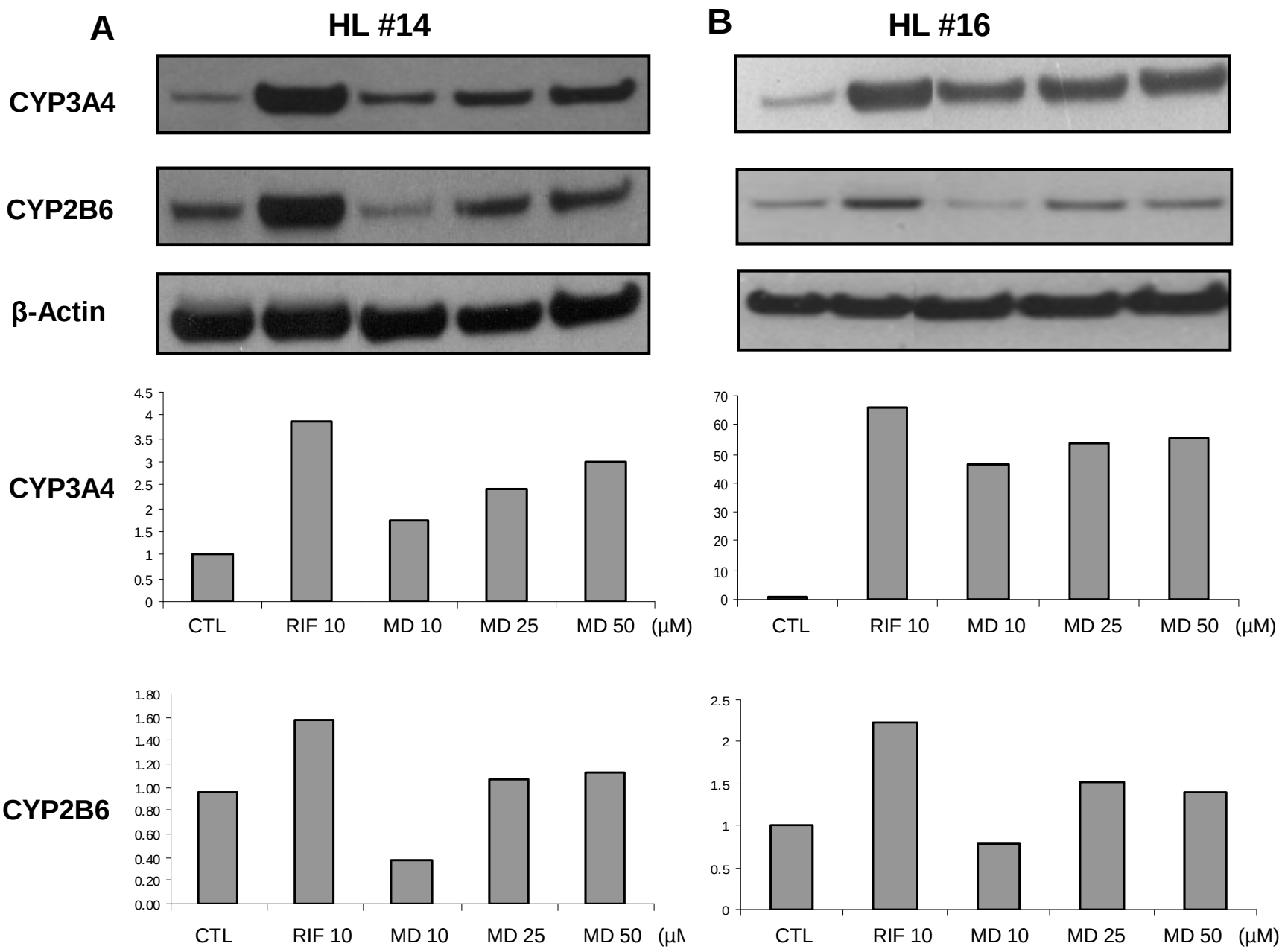


Figure 2

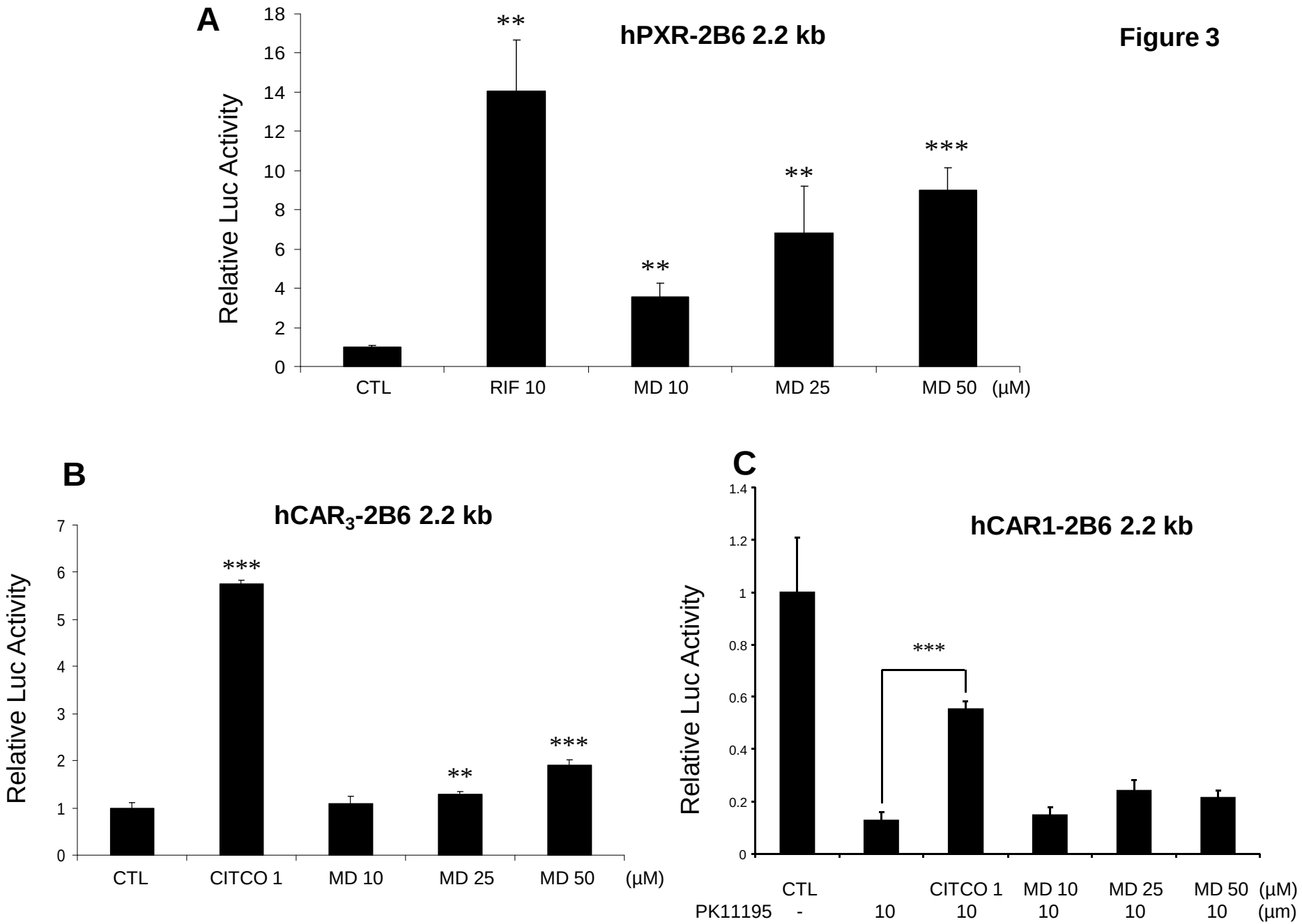


Figure 3

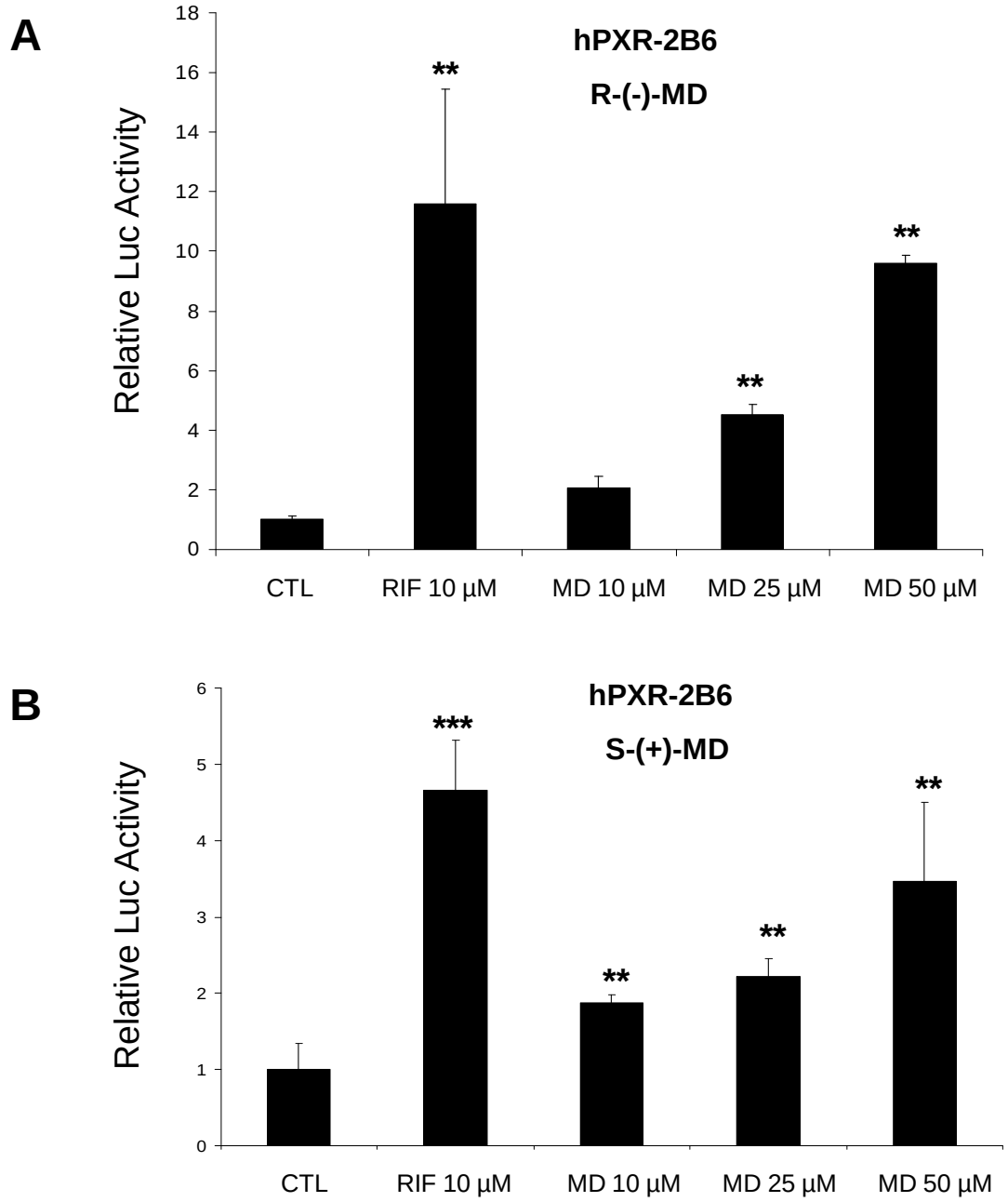
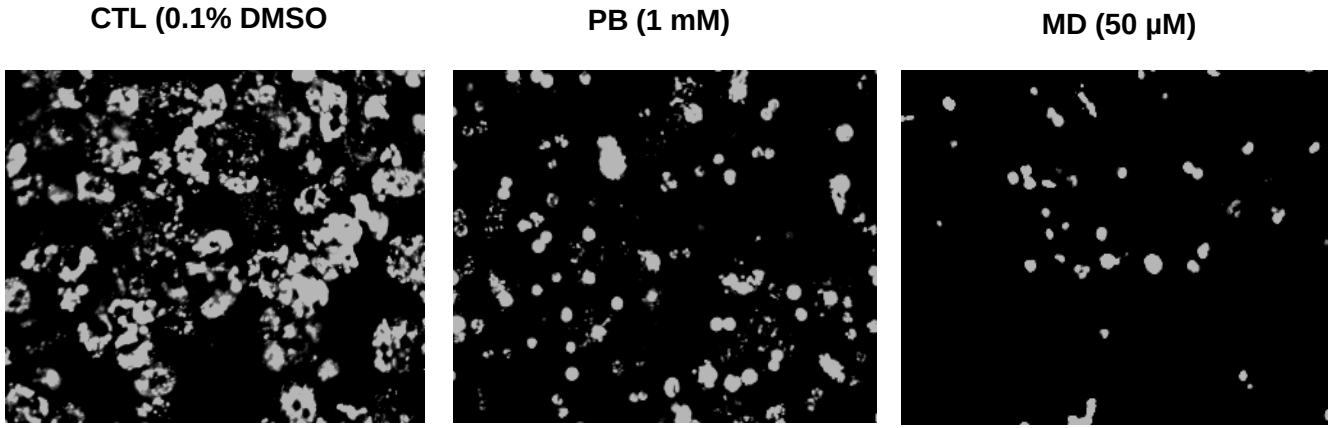


Figure 4

A



B

Treatment	Cytoplasmic		Nuclear		Mixed		Total	
	HL#8	**HL#9**	**HL#8**	**HL#9**	**HL#8**	**HL#9**	**HL#8**	**HL#9**
CTL	65(93%)	144(87%)	0 (0%)	0 (0%)	5(7%)	21(13%)	70	165
PB (1 mM)	14(13%)	0 (0%)	83(80%)	109(63%)	7(7%)	65(38%)	104	173
MD (50μM)	15(25%)	8 (8%)	38(62%)	87(81%)	8(13%)	12 (11%)	61	107

Figure 5

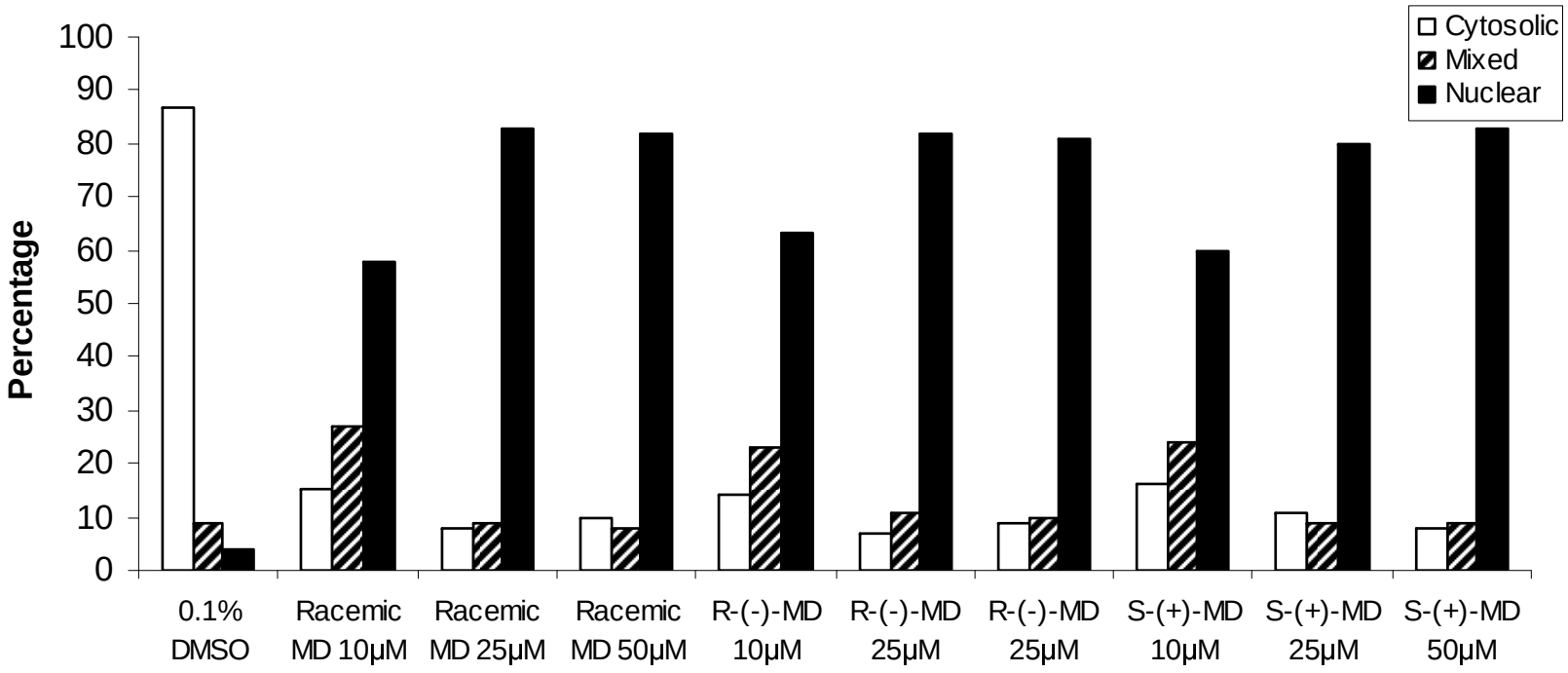


Figure 6