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Expression of the human *UGT1* locus in transgenic mice by WY-14643 and implications on drug metabolism through PPARα activation.

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Running Title: Fibrate induction of the *UGT1* locus in Tg-*UGT1* mice.

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Abbreviations: UGT, UDP-glucuronosyltransferase; Tg-*UGT1*, transgenic *UGT1*; CYP3A4, cytochrome P450 3A4; RXR, retinoid X-receptor; GI, gastrointestinal tract; PPARα, peroxisome proliferator-activated receptor alpha; PPRE, PPAR responsive element; PXR, pregnane X-receptor; CAR, constitutive androstane receptor; LXR, liver X-receptor; Ah receptor, aryl hydrocarbon receptor; DR1, direct repeat response element that is separated by 1 nucleotide; PREM, phenobarbital-responsive enhancer module

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ABSTRACT

The *UDP-glucuronosyltransferase* (*UGT*) *1A* genes in humans have been shown to be differentially regulated in a tissue specific fashion. Transgenic mice carrying the human *UGT1* locus (Tg-*UGT1*) were recently created demonstrating that expression of the 9 *UGT1A* genes closely resembles the patterns of expression observed in human tissues. In the present study, UGT1A1, UGT1A3, UGT1A4 and UGT1A6 have been identified as targets of the peroxisome proliferator-activated receptor alpha (PPARα) in human hepatocytes and Tg-*UGT1* mice. Oral administration of the PPARα agonist WY-14643 to Tg-*UGT1* mice led to induction of these proteins in either the liver, gastrointestinal tract or kidney. The levels of induced *UGT1A3* gene transcripts in liver and UGT1A4 in small intestine correlated with induced lamotrigine glucuronidation activity in these tissues. With UGT1A3 previously identified as the major human enzyme involved in human C24-glucuronidation of lithocholic acid (LCA), the dramatic induction of liver UGT1A3 RNA in Tg-*UGT1* mice was consistent with the formation of LCA-24G in plasma. Furthermore, PPAR responsive elements (PPREs) were identified flanking the *UGT1A1*, *UGT1A3* and *UGT1A6* genes by a combination of site directed mutagenesis, specific binding to PPARα and RXRα, and functional response of the concatenated PPRE elements in HepG2 cells overexpressing PPARα. In conclusion, these results suggest that oral fibrate treatment in humans will induce the UGT1A family of proteins in the gastrointestinal tract and liver, influencing bile acid glucuronidation and first pass metabolism of other drugs that are taken concurrently with hypolipidemic therapy.

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The catalytic reaction that utilizes UDP-glucuronic acid as a cosubstrate for the formation of glucuronides from substrates such as steroids, bile acids, bilirubin, fatty acids, hormones, dietary constituents and thousands of xenobiotics that include drugs, environmental toxicants and carcinogens, has evolved as a highly specialized function in higher organisms (Tukey and Strassburg, 2000). There exists in humans the Superfamily of UGTs which is encoded by the *UGT1* and *UGT2* gene families (Mackenzie *et al.*, 2005). The *UGT1* locus is unique in that it encodes 9 functional UGTs on a 220 kb stretch of DNA on chromosome 2 that has been shown to be regulated in humans in a strict tissue specific pattern (Gregory *et al.*, 2004;Tukey and Strassburg, 2000). The organization of the *UGT1* locus allows for each of the *UGT1A* genes to be regulated uniquely based upon tissue as well as the ability to undergo induction, with no two tissues expressing the same complement of the 9 UGT1A proteins.

Recently, we have cloned the entire *UGT1* locus from a bacterial artificial chromosome and using this DNA created transgenic mice (Tg-*UGT1*) that express each of the 9 *UGT1A* genes (Chen *et al.*, 2005). The *UGT1A* genes in Tg-*UGT1* mice have been shown to be regulated in a tissue specific fashion that is similar to the regulatory patterns observed in humans (Tukey and Strassburg, 2000). Preliminary findings have shown that the treatment of Tg-*UGT1* mice with agents that activate the Ah receptor or the pregnane X receptor (PXR) lead to the induction of one or all of the *UGT1A* gene transcripts, depending on the tissue (Chen *et al.*, 2005). These animals were also used to demonstrate that induction of UGT1A3 by the liver X-receptor (LXR) correlated with increased glucuronidation levels of plasma primary and secondary bile acids (Verreault *et al.*, 2006). Thus, these mice offer an opportunity to examine the regulatory or expression patterns of the human *UGT1A* genes in response to environmental or drug induced exposure.

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Fibrate hypolipidemic drugs are used worldwide in the treatment of dyslipidemia and are synthetic ligands for the peroxisome proliferator-activated receptor alpha (PPARα). Along with other members of the PPAR family of nuclear receptors γ and δ, PPARα activation is linked to regulation of lipid metabolism (Duval *et al.*, 2004). After activation, PPARα heterodimerizes with the 9-*cis*-retinoic acid receptor (RXR) to bind to PPAR response elements in DNA leading to transcriptional activation of target genes. PPARα is widely expressed in liver, muscle, kidney and intestine (Auboeuf *et al.*, 1997) and mediates expression of genes involved in fatty acid β-oxidation (Desvergne and Wahli, 1999). In cell culture studies, activation of PPARα regulates *UGT1A9* (Barbier *et al.*, 2003b) as well as *UGT2B4* expression (Barbier *et al.*, 2003a). Thus, the *UGT* genes appear to be additional targets for PPARα. Interestingly, several of the human UGT1A proteins including UGT1A9 and UGT2B4 were shown to metabolize polyunsaturated fatty acids (Turgeon *et al.*, 2003), providing evidence that the family of UGTs regulated by activated PPARα play an important role in lipid metabolism. Since several of the *UGT* genes that are targeted for regulation by PPARα have been implicated in fatty acid metabolism, confirming the identity of the UGTs that are subject to regulation by PPARα in vivo will advance an understanding of the role of glucuronidation in dyslipidemia.

In the following study, we examined the influence of the fibrate and PPARα agonist WY-14643 on its control of the *UGT1* locus in Tg-*UGT1* mice. Significant regulation of UGT1A proteins was observed in the gastrointestinal tract and liver. The outcome of these results might predict that patients taking fibrates for clinical reasons will result in considerable up-regulation of the UGTs in the GI tract, accelerating first-pass metabolism and impacting on oral bioavailability of other drugs or nutrients that are consumed while on fibrate medication.

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MATERIALS AND METHODS

Reagents-Pirinixic acid (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid), commonly known as WY-14643 and DMSO were purchased from Sigma-Aldrich. An antibody to rabbit anti-human UGT1A (Albert *et al.*, 1999) was kindly provided by Dr. Alain Belanger from Laval University, Quebec, Canada. A rat CYP4A1 antibody originally generated as described (Okita and Okita, 1992) was donated to us by Dr. Eric Johnson from the Scripps Research Institute. The rat CYP4A1 is most homologous to mouse Cyp4a10 (Okita and Okita, 2001) but in experiments outlined here the mouse protein (s) identified with the rat CYP4A1 antibody are referred to as Cyp4a. Human HepG2 cells that express a stably integrated PPARα cDNA were provided by Dr. Eric Johnson.

Animal Studies with the PPARα Ligand WY-14643-Animal studies were performed in compliance with the National Institutes of Health guidelines regarding the use of laboratory animals. Animals were provided with food and water *ad libitum* and maintained under controlled temperature (23^{o}C) and lighting (12-hour light, 12-hour dark cycles). Mice were pooled in groups of three for each treatment. WY-14643 was dissolved in DMSO/corn oil (50/50) at a concentration of 8 mg/ml. Wild-type (WT) and Tg-*UGT1* mice were administered 100 µl of WY-14643 (40 mg/kg) by the oral route once a day for 3 days. Mice were sacrificed the day after the last dose, and the organs were collected and stored at -80^{o}C until RNA and microsomal protein could be prepared. For plasma 24G-LCA analysis, mice were anesthetized by isoflurane inhalation 24 hr after the last dose of WY-14643, and blood collected by cardiac puncture into heparinized tubes. The formation of glucuronide conjugates was quantified by LC/ESI-MS/MS as previously described (Verreault *et al.*, 2006)

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Isolation and Treatment of Primary Hepatocytes-Human hepatocytes were purchased from InVitro Technologies (Baltimore,MD), seeded in 24-well collagen I-coated plates (VWR), and cultured in InVitro Gro CP medium for 48 h. The cells were then treated with either vehicle (DMSO) or Wy-14643 (75 μM) for 48 h. The isolation of primary hepatocytes from Tg-*UGT1* mice has previously been described (Chen *et al.*, 2005). After plating of hepatocytes on 6-well collagen I-coated plates, the cells were allowed to attach for 3 h. The hepatocytes were then treated for 48 h with 30 μM Wy-14643. The cells were replenished with fresh media and Wy-14643 after 24 hours. For analysis of expressed UGT1A1 by Western blot analysis, hepatocytes were collected and lysed in buffer containing 0.05 M Tris-HCL, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40 and a complement of protease and phosphatase inhibitors. The solubilized lysate was centrifuged for 20 min in a refrigerated Eppendorf centrifuge at 16,000 x g, and the supernatant collected for Western blot analysis.

For isolation of RNA from human and Tg-*UGT1* hepatocytes, the supernatant was removed and a 1 ml solution of phenol/quanidinium isothiocyanate solution (TRIzol, Invitrogen) was added directly to the wells. After allowing the solution to set for 30 min, it was removed and a folsh separation was established after the addition of 200 μl of chloroform. The sample was briefly vortexed and after a short pulse in an Eppendorf centrifuge the water phase was removed. The RNA was collected after the addition of 2 vol of isopropyl alcohol and centrifugation at 16,000 x g in an Eppendorf centrifuge.

Isolation of Microsomes from Mouse Tissues-Combining three animals per group, the liver, small and large intestines, kidney, heart, and skeletal muscle were collected from treated and untreated WT and Tg-*UGT1* mice. The intestinal tract tissues were dissected lengthwise and

rinsed in 1.15% KCl before freezing on dry ice. The pooled tissues from each treatment group were pulverized under liquid nitrogen in a porcelain mortar. A sample of the pulverized tissue was homogenized in 5 volumes of chilled 1.15% KCl using a motorized glass Teflon homogenizer. The homogenate was centrifuged at 2,000 x g for 10 min at 4 ^{o}C and the supernatant was further centrifuged at 9,000 x g for 10 min at 4 ^{o}C. The resulting supernatant centrifuged at 100,000 x g for 60 min at 4 ^{o}C in a floor model Beckman SW-40Ti Rotor. The pellet was resuspended in buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride) and protein concentration was determined by the Bradford method prior to storing at -80 ^{o}C. For lamotrigine glucuronidation activity using microsomes, the method as previously described was employed (Chen *et al.*, 2005).

Western Blot Procedures-All Western blots were performed using 4-12% NuPAGE BisTris-polyacrylamide gels as outlined by the supplier (Invitrogen). Twenty µg of microsomal protein was heated at 70 ^{o}C for 10 min in loading buffer and resolved under denaturing conditions (50 mM MOPS, 50 mM Tris base pH 7.7, 0.1% SDS, 1 mM EDTA) prior to transferring the proteins onto nitrocellulose membrane using a semidry transfer system (Novex). The membrane was blocked with 5% nonfat dry milk in washing buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, and 0.05% Tween 20) for 1 h at room temperature, followed by overnight incubation at 4 ^{o}C with primary antibodies (1:5000) prepared in washing buffer containing 0.02% sodium azide and 5% BSA. An anti-UGT1 common carboxyl terminus antibody made from an expressed fusion protein encoding amino acids 312 to 531 was produced in rabbits (Albert *et al.*, 1999). The anti-human UGT1A1, UGT1A4 and UGT1A6 have been described (Chen *et al.*, 2005;Ritter *et al.*, 1999). Membranes were washed and exposed to horseradish

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peroxidase-conjugated secondary antibodies (Cell Signaling). Following additional washes in washing buffer, the conjugated horseradish peroxidase was detected using the ECL plus Western blot detection system (PerkinElmer) and detected after exposure to x-ray film.

Measurements of *UGT1A* Gene Expression by Real-Time PCR. For RNA analysis, tissues from three animals were pooled, frozen in liquid nitrogen and then pulverized to a fine powder. A sample of the mixture was added to 1 ml of acidic phenol/quanidinium isothiocyanate solution (TRIzol, Invitrogen) and total RNA extracted previously described (Chen *et al.*, 2005). For each reverse transcription (RT) reaction, 2 μg of RNA was first denatured by heating and cDNA synthesized in 20 μl using the Omniscript RT kit according to the manufacturer's instructions (Qiagen). Each real-time PCR reaction was performed using a Stratagene Mx4000 Multiplex Quantitative PCR System. For each reaction, the final volume was 20 μl and was comprised of 10 μl of SYBR Green PCR mix, 2 μl of each primer (200 nM final) and 2 μl of each RT reaction. Each reaction was run in triplicate. Quantitative PCR analysis for UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9 mRNA levels were determined with the sense and anti-sense primers listed in Table 1. For UGT1A1 and UGT1A6, each PCR reaction was carried out at 95 °C for 10 minutes, 9 5°C for 30 seconds and 63 °C for 60 seconds, followed by 72 °C for 45 seconds, processed through 40 cycles. Amplification was followed by DNA melt at 95 °C for 1 min and a 41 cycle dissociation curve starting at 55 °C and ramping one degree every 30 seconds. For UGT1A3, each PCR reaction was carried out at 95 °C for 10 minutes, 95 °C for 30 seconds and 58 °C for 60 seconds, followed by 72 °C for 45 seconds, processed through 40 cycles. To determine the quantitation of gene expression, the comparative threshold cycle method (ΔC_T) was used (Livak and Schmittgen, 2001). To control for variation in mRNA

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quantity and quality, β-actin RNA was used as an internal control to calculate a relative C_t for the target molecules. Subtracting the C_t of the housekeeping gene β-actin from the C_t of the *UGT* gene of interest yields the ΔC_t.

Electrophoretic Mobility Shift Assays (EMSAs). These experiments were carried out as previously described (Barbier *et al.*, 2003b), by examining the ability of PPARα and RXRα to associate with putative DR1 binding sequences on the human *UGT1A3* and *UGT1A6* genes. The *UGT1A3* DR1 oligonucleotides -5790 (5'-ggtaAGGTCACAGATCAacag-3'), the *UGT1A6* promoter oligonucleotides -2680 (5'-ttatGGGTCGTAGGTCAtac-3') and the UGT1A9 PPRE (5'-gacaTCACCTCTGACCTcaaggag-3') were end-labeled with [γ-^{32}P]ATP using T4-polynucleotide kinase, followed by incubation with PPARα and/or RXRα synthesized in vitro using the TNT Quick Coupled Transcription/Translation System (Promega). For competition experiments showing specificity, various concentrations of cold UGT1A9 DR1 sequence was included in the binding assays. The protein-DNA complexes were resolved by 4% nondenaturing polyacrylamide gel electrophoresis in Tris-borate-EDTA buffer.

***UGT1A1* Promoter Cloning and Expression**. Enhancer DNA fragments have previously been described (Yueh *et al.*, 2003). All experiments were conducted in triplicate using HepG2 cells and HepG2 cells that carry an integrated PPARα cDNA (PPARα-HepG2) (Hsu *et al.*, 2001). For analysis of functional DR1 elements, oligonucleotides encoding 3-copies of the UGT1A1 (5'-gccaAGGGTAGAGTTCAgtgt-3'), UGT1A3 (5'-ggtaAGGTCACAGATCAacag-3') and UGT1A6 (5'-ttatGGGTCGTAGGTCAtac-3') DR1 sequences were synthesized and cloned into the SV40pGL3 expression vector. HepG2 and PPARα-HepG2 cells were cultured to

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approximately 40% confluence in 12-well plates and transfected with 200 ng of reporter plasmid along with 5 ng phRL-SV40 using Lipofectamine 2000 reagent as described by the manufacturer's protocol (Invitrogen). After 24 h, the cells were treated for 48 h with 100 μM WY-14643 dissolved in DMSO. Dual luciferase assays were conducted following the manufacturer's instructions (Promega). While the cells were still attached, 200 μl of Passive Lysis Buffer (Promega) was added and 20 μl of lysate used for dual luciferase analysis using an LMax^{TM}II^{384} luminometer. Firefly luciferase values were normalized to *Renilla* luciferase and protein concentration and reported as fold increase over vehicle treated cells.

RESULTS

***Induction of UGT1A proteins by the PPARα activator WY-14643 in primary hepatocytes*.**

The treatment of human primary hepatocytes with WY-14643 (75 uM) led to the induction of UGT1A1, UGT1A3, UGT1A4 and UGT1A6 RNA, with the largest response being observed for UGT1A1 and UGT1A3 (Figure 1). In comparison, when primary liver hepatocytes isolated from Tg-*UGT1* mice were cultured in the presence of WY-14643 and the *UGT1A* gene transcripts analyzed by real time RT-PCR, the *UGT1A1* gene transcript was found to be significantly induced (Figure 1). A 2.5 fold induction of UGT1A6 was also observed. However, the *UGT1A3* and *UGT1A4* genes were not responsive to WY-14643 in hepatocytes from Tg-*UGT1* mice. This result might reflect a species difference in control of these genes, or possibly could be attributed to differences in the optimized tissue culture conditions used with the human and transgenic hepatocytes. Regardless, there appears to be consistency in the induction of UGT1A1 and UGT1A6.

***The oral administration of WY-14643 induces liver and gastrointestinal tract UGT1A proteins in Tg-UGT1 mice*.**

To examine the potential of activated PPARα to induce human *UGT1A* gene expression in vivo, Tg-*UGT1* mice were treated with WY-14643 by oral gavage (40 mg/kg) every 24 hours for 3 consecutive days. Mice were sacrificed the day after the last dose and the tissues (three per treatment group) were collected and pooled. Following pulverization of the tissue under liquid nitrogen, microsomal preparations were made from liver, small intestine, large intestine and kidney. A sample of protein from each tissue was processed for Western blot analysis and

UGT1A protein detected with an anti-UGT1A antibody that was produced to the common carboxyl terminus region of the human UGT1A proteins (Albert *et al.*, 1999). Results from these experiments demonstrated that oral treatment with WY-14643 induced the human UGT1A family of proteins in the liver, small intestine, large intestine, and kidney (Figure 2). Induction of Cyp4a protein, which is induced following activation of PPARα (Muerhoff *et al.*, 1992), was demonstrated in the liver, small intestine, and kidney, but there was no detectable Cyp4a in large intestine where we observed considerable UGT1A protein induction.

Using antibodies specific for human UGT1A1, UGT1A4 and UGT1A6 (Chen *et al.*, 2005), oral WY-14643 treatment to Tg-*UGT1* mice resulted in prominent induction of UGT1A1 and UGT1A6 in liver microsomes (Figure 3). A very mild but observable induction of UGT1A4 was also evident. In human (Strassburg *et al.*, 1999) and Tg-*UGT1* liver (Chen *et al.*, 2005), basal levels of UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9 RNA were detected. When we carried out real time RT-PCR with specific primers to each of the human transcripts using liver RNA from male WY-14643 treated Tg-*UGT1* mice, very prominent induction of UGT1A1 (500-fold) and UGT1A3 (120-fold) was observed (Figure 4). The induction of UGT1A1 RNA corresponded to induction of protein (Figure 3). A measurable induction of UGT1A4 (3-fold) and UGT1A6 (4 fold) gene transcripts were also observed, and increases in these proteins were demonstrated. Although a specific UGT1A3 antibody is not available to examine protein expression, the 120 fold induction of UGT1A3 RNA by WY-14643 would lead us to speculate that UGT1A3 is highly expressed in liver following fibrate treatment.

When we examined extrahepatic microsomal preparations by Western blot analysis, UGT1A1 and UGT1A4 were induced in small intestine, with no observable induction of UGT1A6 (Figure 3). A small amount of induced UGT1A1, UGT1A4 and UGT1A6 are each

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seen in large intestine. In kidney, a tissue that contains substantial concentrations of PPARα, induction of only UGT1A6 was observed. There was no induction of these UGTs in other tissues such as heart or muscle. The differential response in induction of the *UGT1A* genes by WY-14643 most likely results from coordination of tissue specific regulatory factors with PPARα.

It is interesting to note that the induction pattern of UGT1A6 in the different tissues closely resembles induction of the mouse Cyp4a protein (Figure 3). This result indicates that PPARα is working in concert with additional tissue specific transcriptional mechanisms to coordinately regulate these two genes. Also, while induction of gene expression by activated PPARα is dependent upon tissue abundance of the receptor, the observations that UGT1A1, UGT1A4 and UGT1A6 are differentially induced clearly indicates that receptor abundance is not the sole factor dictating expression of target genes in the respective tissues.

***The role of induced UGT1A3 and UGT1A4 in lamotrigine metabolism*.**

The formation of quaternary ammonium linked glucuronides results from the attachment of glucuronic acid to aliphatic tertiary amino groups. In human liver microsomes, the kinetics of the reaction forming quaternary ammonium glucuronides was shown to be biphasic, displaying a high and low K_m profile (Breyer-Pfaff *et al.*, 1997). The enzyme responsible for the low K_m formation was shown to be UGT1A4, while UGT1A3 exhibited low efficiency and contributed to the high K_m (Green *et al.*, 1998;Green and Tephly, 1998). Interestingly, the formation of N-linked quaternary ammonium glucuronides does not proceed efficiently in rodent models such as rats (Chiu and Huskey, 1998), so Tg-*UGT1* mice may serve as a humanized animal model to examine the contribution of UGT1A expression towards detoxification of xenobiotics containing a tertiary amine moiety.

With the prominent induction of UGT1A4 protein in the gastrointestinal tract following WY-14643 treatment, we sought to examine the contribution of lamotrigine glucuronidation in liver and small intestinal microsomes. Lamotrigine is used therapeutically in humans as an anti-epileptic drug, and forms a quaternary ammonium glucuronide which is excreted in the urine (Remmel and Sinz, 1991). For these studies, Tg-*UGT1* and WT mice (n=3 per group) were treated orally with either vehicle or WY-14643 (40 mg/kg) for three days followed by the preparation of total RNA for real-time PCR quantification as well as microsomal preparation for analysis of lamotrigine glucuronidation.

As shown in Table 2, lamotrigine glucuronidation was detectable and induced in microsomes from WT mice, but the level of induced activity was 16 fold lower than that detected in untreated Tg-*UGT1* mouse liver microsomes. Basal levels of either UGT1A3 or UGT1A4 are responsible for the elevated lamotrigine glucuronidation in Tg-*UGT1* liver (Chen *et al.*, 2005). In liver and small intestinal microsomes from male Tg-*UGT1* mice, the basal levels of lamotrigine glucuronidation were comparable. Following the oral administration of WY-14643, the rates of lamotrigine glucuronidation were dramatically induced in liver (10.4 fold). In light of Western blot findings that induction of UGT1A4 in liver was minimal (Figure 3), the induction of lamotrigine glucuronidation must be dictated by induction of UGT1A3, as determined by real time RT-PCR (Figure 4). In small intestinal microsomes, WY-14643 elicited an 11.6 fold induction of lamotrigine glucuronidation activity. Since UGT1A4 is induced in this tissue as a result of WY-14643 treatment (Figure 3), it can be speculated that the abundance of UGT1A4 underlies the majority of the induced catalytic activity in the gastrointestinal tract.

Induced UGT1A3 and bile acid glucuronidation. During enterohepatic circulation, bile acids (BAs) enter the liver and are targets for glucuronidation (Pauli-Magnus *et al.*, 2005). In humans, UGT1A3 has previously been demonstrated to be the primary enzyme underlying the hepatic C24-glucuronidation of lithocholic acid (LCA) (Gall *et al.*, 1999). In Tg-*UGT1* mice, activation of the liver X-receptor (LXR) leads to induction of UGT1A3, which is followed by the accumulation of LCA-24G in blood (Verreault *et al.*, 2006). In collaboration with our studies, recent findings have demonstrated that WY-14643 treatment of human hepatocytes induces UGT1A3 mRNA (Trottier *et al.*, 2006). To evaluate if the induction of UGT1A3 by PPARα activation in Tg-*UGT1* mice can be correlated with increases in serum LCA-24G levels, the effects of WY-14643 treatment on LCA glucuronidation were evaluated in WT and Tg-*UGT1* mice. Although there were detectable levels of LCA-24G in several WT and DMSO treated Tg-*UGT1* mice (Figure 5), the treatment of Tg-*UGT1* mice with WY-14643 strongly induced the levels of circulating LCA-24G over the other treatment groups. These experiments reinforce previous findings that upregulation of *UGT1A3* will have a significant impact on BA glucuronidation in vivo.

Identification of PPARα responsive regions within the UGT1A1, UGT1A3 and UGT1A6 promoters.

The significant induction of UGT1A1, UGT1A3, and UGT1A6 by WY-14643 in Tg-*UGT1* liver would suggest these genes are targets of activated PPARα, and that the PPARα/RXRα transcriptional complex facilitates this induction by binding to PPAR response elements (PPREs) on these genes. The PPREs that bind PPARα contain imperfect direct repeats separated by a single base (DR1). In scanning these genes for consensus DR1 like elements,

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EMSAs were performed using these response elements as probes to determine if PPARα and RXRα were capable of binding to the PPREs. This approach resulted in the identification of functional DR1 elements located at -5790 on the *UGT1A3* gene and -2692 on the *UGT1A6* gene (Figure 6). Both of these sequences were unable to bind expressed PPARα, but when incubated with PPARα and RXRα, their binding elicited an induced gel shift. In addition, binding of PPARα/RXR to the UGT1A3 and UGT1A6 PPREs was inhibited when the binding reaction included wild type UGT1A9 DR1 (Barbier *et al.*, 2003b), but not when the incubations included oligonucleotide towards the mutated UGT1A9 DR1 sequence. Functional expression of the DR1 sequences were confirmed when they were concatenated and cloned into expression plasmids and shown to elicit WY-14643 induced promoter activity in HepG2 cells that overexpress PPARα.

To identify the potential DR1 sequence on the *UGT1A1* gene, a *UGT1A1* promoter construct (-3712 to -7) was cloned into the expression plasmid pGL3-basic and transfected into either HepG2 cells or PPARα-HepG2 cells. When these cells were treated with WY-14643, HepG2 cells showed no induction of luciferase activity (Figure 7). In contrast, PPARα-HepG2 cells displayed a 2 fold induction of luciferase activity when treated with WY-14643 in comparison to vehicle (DMSO)-treated cells.

To localize the responsive element, a series of enhancer sequences were generated and transfected into the PPARα-HepG2 cells followed by treatment with WY-14643. WY-14643 treatment led to induced luciferase activity with those enhancer sequences (clones E1 and E3) that included the PXR, NR1, XRE and putative DR1 binding regions (Figure 8). This region has been identified as the phenobarbital responsive enhancer module (PREM). When the PREM region was deleted (clone E3), no induction of luciferase activity was noted. DNA sequence

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comparisons to other DR1 elements demonstrated that the consensus recognition DR1 sequence identified on the *UGT1A1* gene (Figure 8) was identical to the *CYP4A1* DR1 motif (Palmer *et al.*, 1995). To further regionalize the binding region, a construct was made (clone E4, bases -3430 to -3272, Figures 6 and 7) that contained the PXR, NR1 and XRE sequences, but with the identified DR1 element mutated by removing the 3' region of the consensus binding region. Following WY-14643 treatment of transfected HepG2-PPARα cells with E4 (Figure 7), there was no increase in luciferase activity over DMSO treated cells. Similar results were observed when the DR1 sequence was mutated in clone E3 ($E3_{mt}$). Further evidence that the DR1 sequence provides functional activity in the presence of PPARα was confirmed by cloning the DR1 sequence into an expression plasmid and demonstrating that WY-14643 was capable of inducing reporter gene activity in PPARα-HepG2 cells. Combined, the putative UGT1A1 DR1 motif was shown to be essential for induction of *UGT1A1* transcription by PPARα agonists.

DISCUSSION

One of the more complicated model systems to study is presented with the human *UGT1* locus, since 9 functional *UGT1A* genes are individually regulated in a tissue specific and inducible fashion through a process of exon sharing (Tukey and Strassburg, 2000). Through tightly controlled tissue specific regulation, each of the 9 exon 1 elements are individually spliced to common exons 2-5, producing UGTs that are encoded by an amino terminal region that is highly variable and a carboxy region that is identical. In the present study, the oral administration of the PPARα activator and fibrate WY-14643 to Tg-*UGT1* mice induced UGT1A1, UGT1A3, UGT1A4 and UGT1A6 in liver. It is unclear why UGT1A9 was not induced in liver, since WY-14643 had been shown previously to induce this protein in both human primary hepatocytes and HepG2 cells (Barbier *et al.*, 2003b). Surprisingly, UGT1A9 was not induced in human hepatocytes in this study, indicating that separate batches of human hepatocytes may respond differently to PPARα activators. However, the significant induction of UGT1A3 and UGT1A6 RNA in Tg-*UGT1* liver was attributed to functional PPREs identified in the flanking region of these genes. Mapping of the responsive element on the *UGT1A1* gene demonstrated that induction was regulated by the same 290-bp distal PREM region that controls induction through activated Ah receptor (Yueh *et al.*, 2003), PXR and CAR (Xie *et al.*, 2003). This region is specific for activated PPARα since previous studies had indicated that the DR1 region played little role in either CAR or PXR activation of the *UGT1A1* gene (Sugatani *et al.*, 2005).

WY-14643 treatment also facilitated the induction of UGT1A1, UGT1A4 and UGT1A6 in either the liver, gastrointestinal tract or kidney. In kidney, only UGT1A6 was induced by oral administration of WY-14643. While UGT1A1, UGT1A4 and UGT1A6 are induced in liver,

there is virtually no induction of UGT1A6 in response to WY-14643 treatment in small intestine where UGT1A1 and UGT1A4 are induced. Clearly, WY-14643 activation of PPARα and induction of the *UGT1A* gene products is occurring in concert with other tissue specific regulatory factors. Although PPARα is abundant in tissues such as liver, small intestine, kidney, heart and muscle (Desvergne and Wahli, 1999), activation by oral treatment with WY-14643 leads to a unique complement of induced glucuronidation activity in each tissue.

In humans, the small intestine is rich in glucuronidation activity as evident from the detection of multiple *UGT1A* gene transcripts (Strassburg *et al.*, 1999;Strassburg *et al.*, 2000). A similar pattern of expression is observed in Tg-*UGT1* mice. The predominant induction of UGT1A1 and UGT1A4 in the small intestine by WY-14643 may be indicative of the expression patterns in humans following oral exposure to drugs that are PPARα agonists, such as those encountered with therapeutic doses of hypolipidemic drugs. Overexpression of the UGTs in tissues such as the small intestine and liver would be expected to accelerate metabolism of dietary constituents and therapeutic drugs that are ingested simultaneously with PPARα agonists. But this possibility can only be suggestive, since the role of PPARα in human liver is unclear. It has been suggested that functional capabilities of PPARα in humans may not resemble those observed in mice since the hepatic concentrations of PPARα in mice are higher than those detected in human liver (Desvergne and Wahli, 1999). However, it can be noted from these studies that WY-14643 treatment does not facilitate a dramatic induction of mouse UGT1A protein expression in small intestine or liver, leading us to conclude that the human *UGT1* locus is a more responsive gene target toward activated PPARα. This difference in sensitivity may result from PPREs being conserved on the human *UGT1* locus, and not conserved on the mouse *Ugt1* locus.

The 3-5 fold increase in human hepatocyte UGT1A3 mRNA levels by WY-14643 noted previously (Trottier *et al.*, 2006) as well as in these studies are reflected by greater than a 100-fold induction in liver UGT1A3 mRNA following oral treatment to Tg-*UGT1* mice. This is significant since UGT1A3 has been identified as the primary enzyme linked to the human glucuronidation of the BAs chenodeoxycholic acid (CDCA) and LCA (Trottier *et al.*, 2006;Verreault *et al.*, 2006). During cholestasis, plasma concentrations of conjugated BAs are elevated and excreted in the urine, since normal bile flow is interrupted. Clinically, cholestasis are sometimes treated by the administration of drugs that activate the family of xenobiotic receptors, such as PXR and PPARα (Dohmen *et al.*, 2004;Oo and Neuberger, 2004). Interestingly, we have shown previously that the treatment of Tg-*UGT1* mice with the PXR activator pregnenolone-16α-carbonitrile dramatically induces UGT1A3 in both the liver and gastrointestinal tract (Chen *et al.*, 2005). Induction of liver UGT1A3 in Tg-*UGT1* mice by the oral administration of LXR activators also resulted in significant accumulation of plasma CDCA-24G and LCA-24G (Verreault *et al.*, 2006). In the present studies, induction of UGT1A3 by WY-14643 and activation of PPARα is followed by increases in plasma LCA-24G levels. Since functional interruption in normal bile acid elimination can lead to cholestatic liver disease, increases in primary and secondary bile acid glucuronidation by induced UGT1A3 may be considered an additional detoxification step in reducing the accumulation of toxic levels of hepatic BAs.

The activation of PPARα in different tissues is thought to affect insulin sensitivity, lipoprotein synthesis and metabolism, inflammation and cholesterol flux (Li and Palinski, 2006). Thus, the use of therapeutic agents such as fibrates that modulate these events has been keenly investigated. For example, type 2 diabetes predisposes individuals to coronary artery disease

(CHD), a predictor that has called for the treatment of diabetes with fibrates. Recent clinical findings have demonstrated that fenofibrate treatment led to a significantly reduced rate of the progression of coronary atherosclerosis in patients with type 2 diabetes (Rubins *et al.*, 1996). In another study, gemfibrozil therapy resulted in the reduction of major cardiovascular events in patients with coronary artery disease (Rubins *et al.*, 1996). In this same study, those individuals with the highest tertile of body mass and triglyceride level had the most dramatic risk reduction in CHD. This clinical study also indicated that fibrate therapy would be useful in those who display one or more of the major features associated with the metabolic syndrome: obesity, high triglyceride level, insulin resistance, or diabetes. From our studies, it might be anticipated that the gastrointestinal tract and hepatic levels of the UGT1A proteins will be significantly induced when individuals are on therapeutic levels of fibrate therapy. Since glucuronidation in these tissues plays an important role in facilitating the metabolism and elimination of hundreds of known therapeutic agents (Tukey and Strassburg, 2000), individuals on fibrate therapy may be predisposed to accelerated metabolism of those drugs that are taken concurrently with fibrate therapy.

In conclusion, transgenic mice that express the *UGT1* locus serve as a sensitive model to examine the contribution of PPARα activators on UGT1A expression. These data implicate the *UGT1* locus as a target in the GI tract and liver for activated PPARα, allowing us to speculate that the Tg-*UGT1* mice may serve as a biological model to study the impact of human UGT induction on drug metabolism and other physiological events.

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LEGENDS FOR FIGURES

Figure 1. Expression of human UGT1A1 in primary hepatocytes. A) Human and B) Tg-*UGT1* primary hepatocytes from Tg-*UGT1* mice were cultured in media containing 75 μM WY-14643 for 48 h. As a control, media contained only the vehicle, DMSO. After 48 h, whole cell extracts and total RNA was prepared. Total RNA was used following reverse transcription for real time PCR analysis of UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9. Values are expressed as the means ± S.E.M. (n=3). Significance is represented by the (*) with a $p<0.05$ (student t-test).

Figure 2. Western immunoblot analyses of UGT1A expression in Tg-*UGT1* mice. Wild-type (WT) and Tg-*UGT1* mice were treated every 24 h with either vehicle or WY-14643 (40 mg/kg) orally for 3 days. Liver, small intestine, large intestine and kidney from each treatment group (n=3) were combined and microsomes were isolated. Samples of microsomal protein (20 μg) were separated through 4-12% SDS-PAGE. UGT1A protein was identified with an anti-UGT1A antibody that was generated to the conserved region of the human UGT1A proteins. Human liver microsomes (20 μg) were included in the blot as a positive control. To monitor the positive actions of PPARα activation, mouse Cyp4a protein was detected using a rat CYP4A1 antibody.

Figure 3. Western immunoblot analysis for human UGT1A1, UGT1A4, and UGT1A6 in Tg-*UGT1* mice treated with WY-14643. Using the same tissues as outlined in Figure 2, human

UGT1A1, UGT1A4 and UGT1A6 were identified. As above, each lane contained 20 μg of microsomal protein.

Figure 4. Analysis of *UGT1A* gene transcripts from liver of WY-14643 treated Tg-*UGT1* mice. Tg-*UGT1* mice were treated by oral gavage with Wy-14634 (40 mg/kg) and UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9 gene transcripts identified by real time PCR. For these experiments, livers from untreated (n=3) and treated (n=3) were pooled prior to RNA isolation. Values are expressed ± S.E.M. (n=6). Statistical significance was determined by the student t-test ($p<0.05$).

Figure 5. Analysis of LCA-24G metabolites in plasma from WY-14643 treated mice. Wild-type (WT) and Tg-*UGT1* mice were given vehicle (DMSO) and WY-14643 (40 mg/kg) orally for three days. Twenty four hr after the last dose, mice were anesthetized and blood samples collected by cardiac puncture. Plasma was collected and LCA-24G concentrations determined by LC/ESI-MS/MS.

Figure 6. PPARα is functional in the presence of UGT1A3 and UGT1A6 DR1 sequences. A) EMSAs were conducted with end-labeled PPRE probes in the presence of unprogrammed reticulocyte lysate, PPARα, or both PPARα and RXRα. Following binding, the labeled proteins were resolved on a 4% nondenaturing polyacrylamide gel. Competition EMSA with the radiolabeled DR1 sequences were performed by adding 1-, 10 or 50-fold molar excess of the indicated cold consensus DR1 sequences. The arrow indicates the retarded band with PPARα and RXRα are added to the binding reactions. B) Oligonucleotides encoding three copies of the

UGT1A3 and UGT1A6 DR1 elements were cloned into expression plasmids. Following transfection into either HepG2 cells or PPARα-HepG2 cells, the cells were treated with WY-14643 for 48 h and luciferase activity measured. The induced levels are expressed as fold induction over DMSO treated cells.

Figure 7. Activation of the *UGT1A1* promoter in response to WY-14643. A portion of the *UGT1A1* gene containing the promoter and -3712 bases (-3712/-7 PR) of the regulatory region previously shown to be responsive to activated PXR, CAR and the Ah receptor (Xie *et al.*, 2003;Yueh *et al.*, 2003) was transiently transfected into either HepG2 cells or PPARα-HepG2 cells (Hsu *et al.*, 2001). Additional enhancer constructs containing the PREM region (E1 and E3) as well as constructs that were deficient the DR1 element (E2 and E4) were subcloned into the pGL3-promoter vector. Each of these plasmids was transfected into PPARα-HepG2 cells. Following transfections, the cells were treated with WY-14643 (30 μM) for 48 h prior to analysis of luciferase activity. Fold induction was calculated by dividing the activity generated from Wy-1643 treatment with the activity from the same set of transfected cells treated with DMSO. Each value is represented by the S.E.M. Statistical significance was calculated by the student t-test, and indicated by an asterisk ($p<0.05$).

Figure 8. DNA sequence of the Phenobarbital Responsive Enhancer Module (PREM) flanking the *UGT1A1* gene. A). DNA sequence of the *UGT1A1* gene from bases -3430 to -3230 is shown. Included in this sequence is the DNA binding region for the PXR, CAR (NR1), Ah receptor (XRE) and PPARα (DR1), as indicated by the sequence in bold. Enhancer element E4 spans DNA sequence from -3420 to -3275. B). The UGT1A1 DR1 sequence containing three

copies of the DR1 (agggtagagttca) was cloned into the SV40-pGL3 reporter plasmid and transfected into HepG2 and PPARα-HepG2 cells. The cells were treated with vehicle (DMSO) or WY-14643 (100 μM) for 48 h followed by analysis of luciferase activity.

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TABLES AND FIGURES

TABLE 1. Oligonucleotides used in this study for Real Time PCR analysis

Target	*GenBank™ accession number*	*Orientation*	*Sequence(5'-3')*	*Nucleotide Location*
UGT1A1	NM_000463	Forward	AACAAGGAGCTCATGGCCTCC	412-432
		Reverse	GTTCGCAAGATTCGATGGTCG	1056-1036
UGT1A3	NM_019093	Forward	CAGTGGTGGATATTCTCAGTC	731-751
		Reverse	CCATGTTCTCCAGAAGCATTA	905-885
UGT1A4	AY435139.1	Forward	ACGCTGGGCTACACTCAAGG	277-296
		Reverse	TCATTATGCAGTAGCTCCACACAA	404-381
UGT1A6	AY435141.1	Forward	CTTTTCACAGACCCAGCCTTAC	439-460
		Reverse	TATCCACATCTCTCTTGAGGACAG	727-704
UGT1A9	AY435144.1	Forward	GAGGAACATTTATTATGCCACCG	643-665
		Reverse	GCAACAACCAAATTGATGTGTG	760-739

TABLE 2. Glucuronidation of lamotrigine in liver and small intestine microsomes from wild type (WT) and Tg-*UGT1* mice from untreated and WY-14643 treated mice.

Strain	*Treatment*	*Liver*	*Fold*	*Small Intestine*	*Fold*
		nmoles/min/mg		***nmoles/min/mg***	
WT	DMSO	0.010 ± 0.004	--	0.0095 ± 0.003	--
	WY-14643	0.026 ± 0.002	**2.6**	0.027 ± 0.001	**2.8**
Tg-*UGT1*	DMSO	0.42 ± 0.025	**--**	0.38 ± 0.03	--
	WY-14643	4.40 ± 0.34	**10.4**	4.42 ± 0.54	**11.6**

Liver and small intestine was collected from three animals per group. Each value represents the mean ± S.D. from three separate determinations.

Figure 1

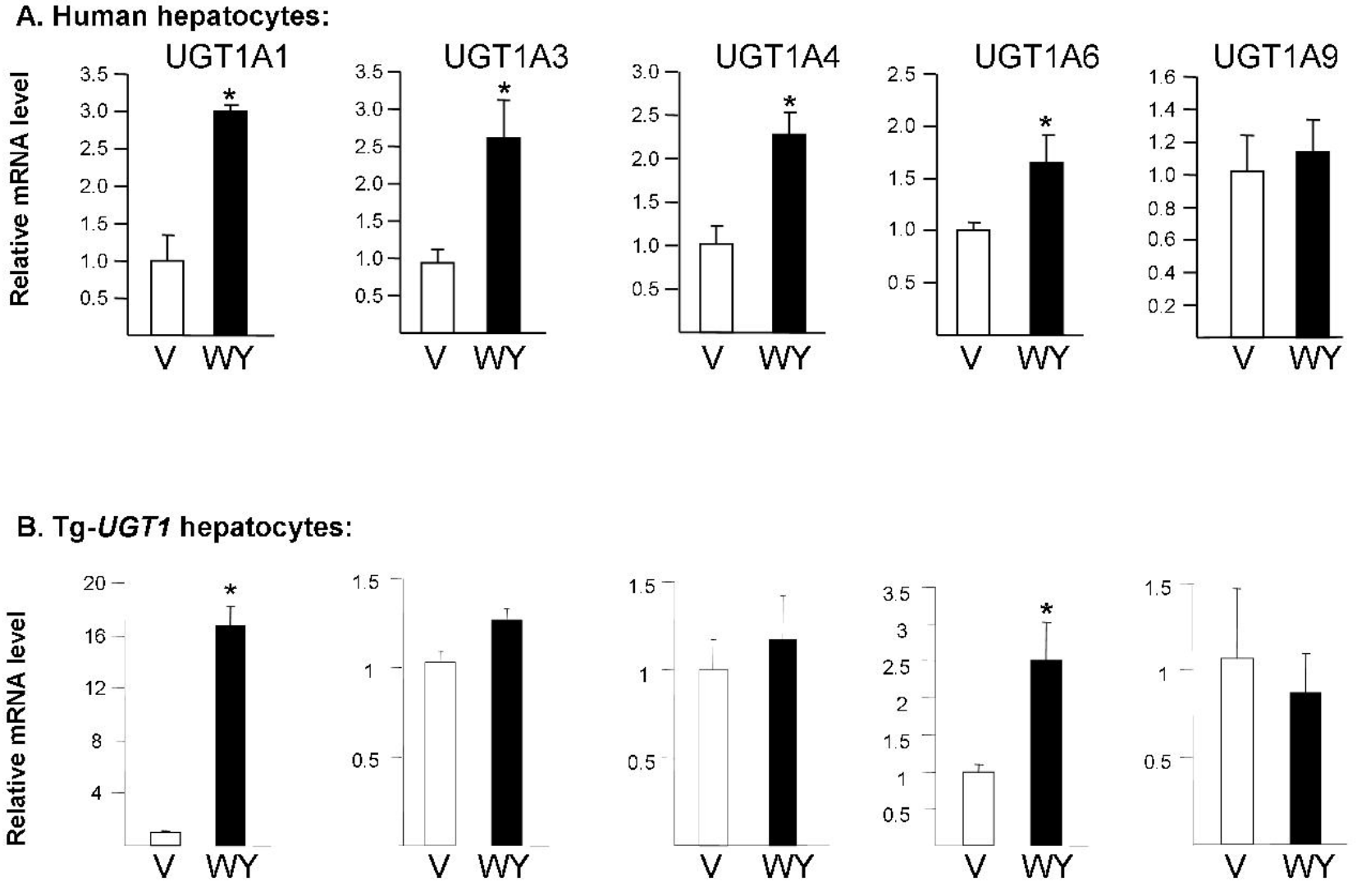

A. Human hepatocytes:
UGT1A1
UGT1A3
UGT1A4
UGT1A6
UGT1A9
Relative mRNA level
V
WY
B. Tg-UGT1 hepatocytes:
Relative mRNA level
V
WY

Figure 2

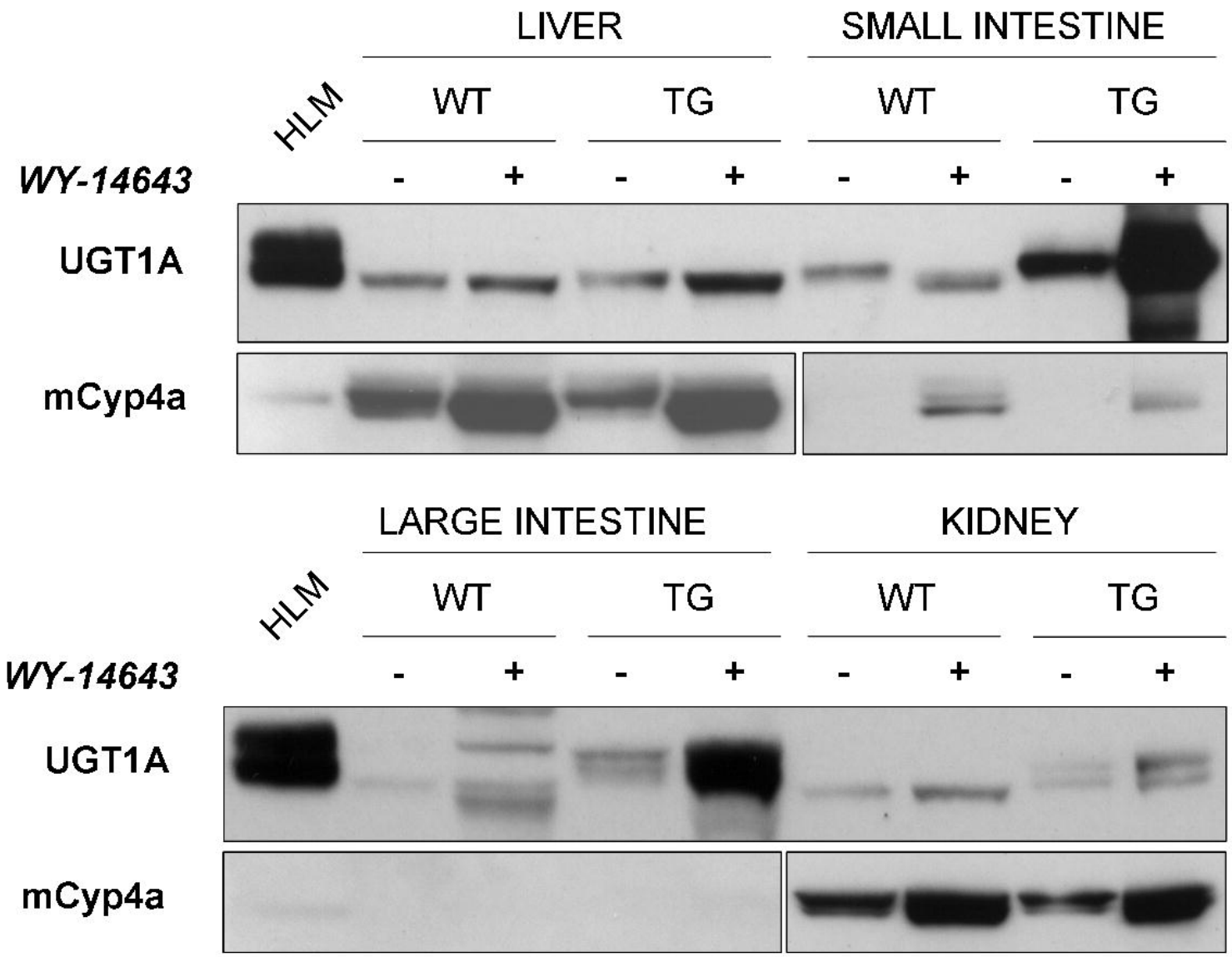

LIVER
SMALL INTESTINE
HLM
WT
TG
WT
TG
WY-14643
- + - + - + - +
UGT1A
mCyp4a
LARGE INTESTINE
KIDNEY
HLM
WT
TG
WT
TG
WY-14643
- + - + - + - +
UGT1A
mCyp4a

Figure 3

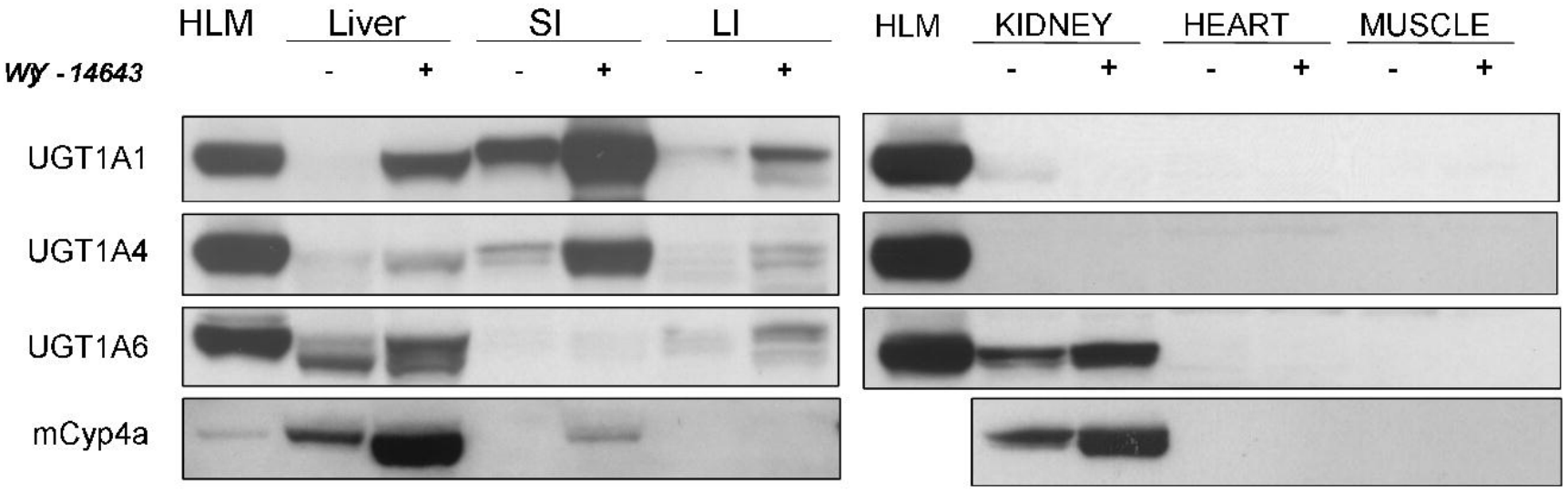

HLM
Liver
SI
LI
HLM
KIDNEY
HEART
MUSCLE
WY - 14643
- + - + - +
- + - + - +
UGT1A1
UGT1A4
UGT1A6
mCyp4a

Figure 4

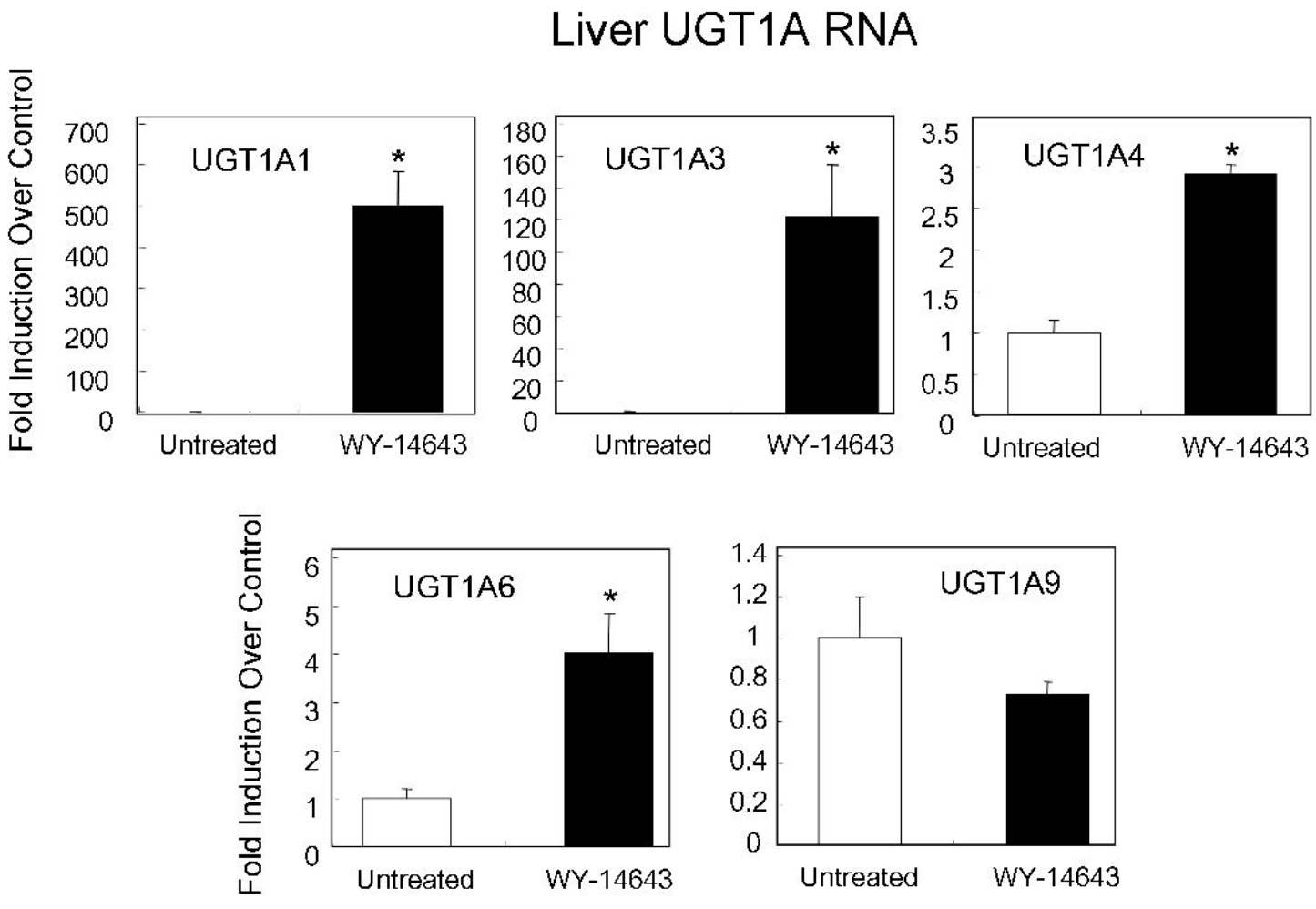

Liver UGT1A RNA
Fold Induction Over Control
UGT1A1
*
700
600
500
400
300
200
100
0
Untreated
WY-14643
UGT1A3
*
180
160
140
120
100
80
60
40
20
0
Untreated
WY-14643
UGT1A4
*
3.5
3
2.5
2
1.5
1
0.5
0
Untreated
WY-14643
Fold Induction Over Control
UGT1A6
*
6
5
4
3
2
1
0
Untreated
WY-14643
UGT1A9
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
WY-14643

Figure 5

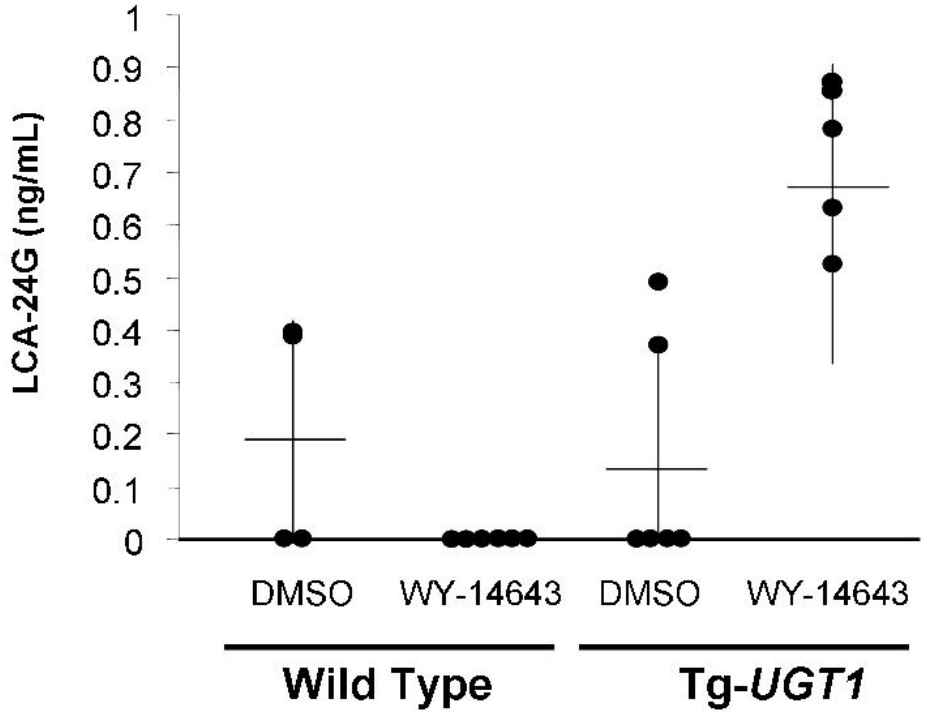
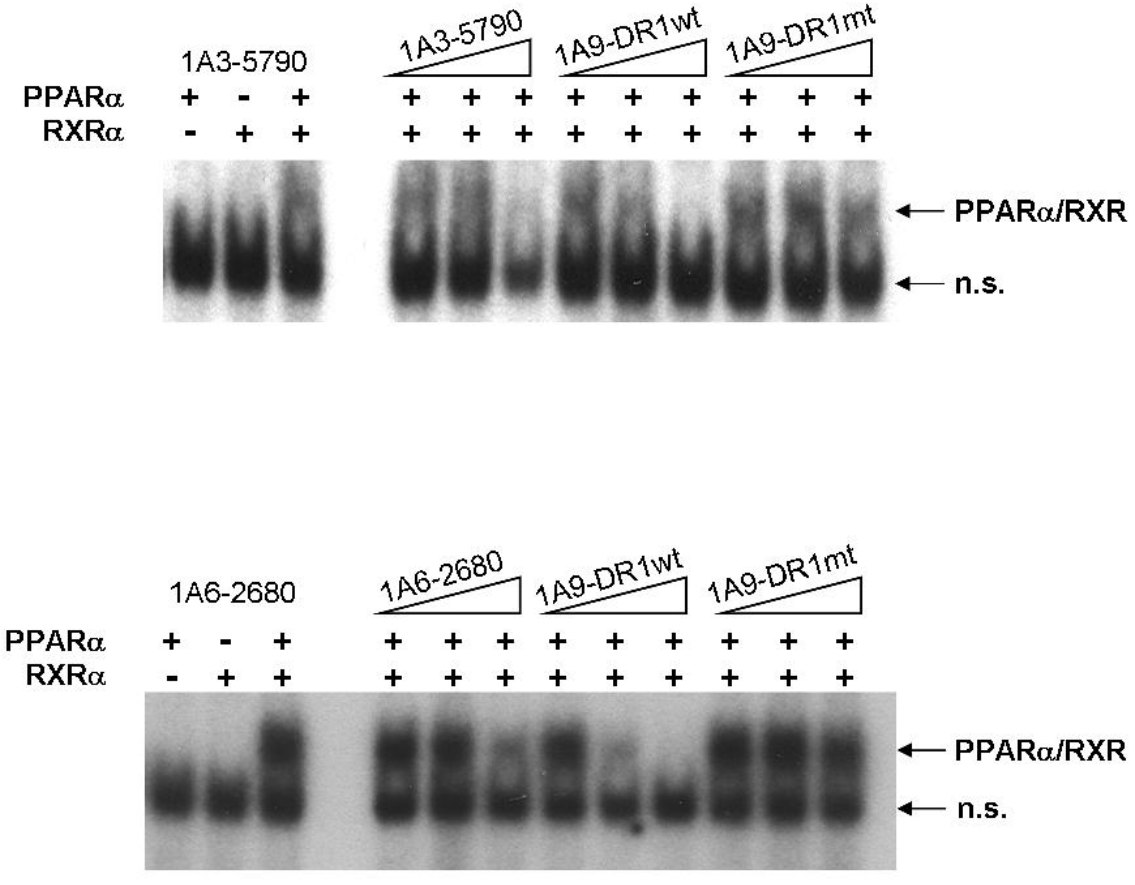

LCA-24G (ng/mL)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DMSO
WY-14643
DMSO
WY-14643
Wild Type
Tg-UGT1

Figure 6


1A3-5790
1A3-5790
1A9-DR1wt
1A9-DR1mt
PPARα + - + + + + + + + + + +
RXRα - + + + + + + + + + + +
PPARα/RXR
n.s.
1A6-2680
1A6-2680
1A9-DR1wt
1A9-DR1mt
PPARα + - + + + + + + + + + +
RXRα - + + + + + + + + + + +
PPARα/RXR
n.s.

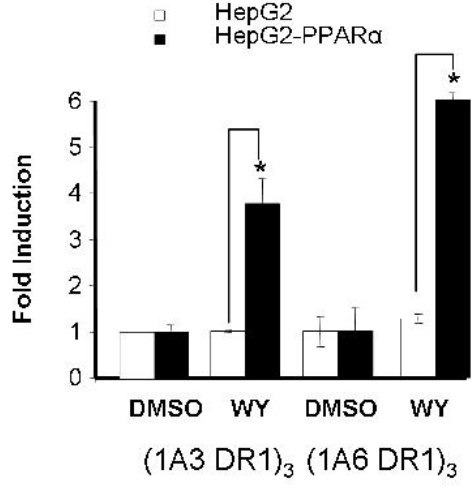

HepG2
HepG2-PPARα
Fold Induction
0
1
2
3
4
5
6
*
*
DMSO
WY
DMSO
WY
(1A3 DR1)3
(1A6 DR1)3

Figure 7

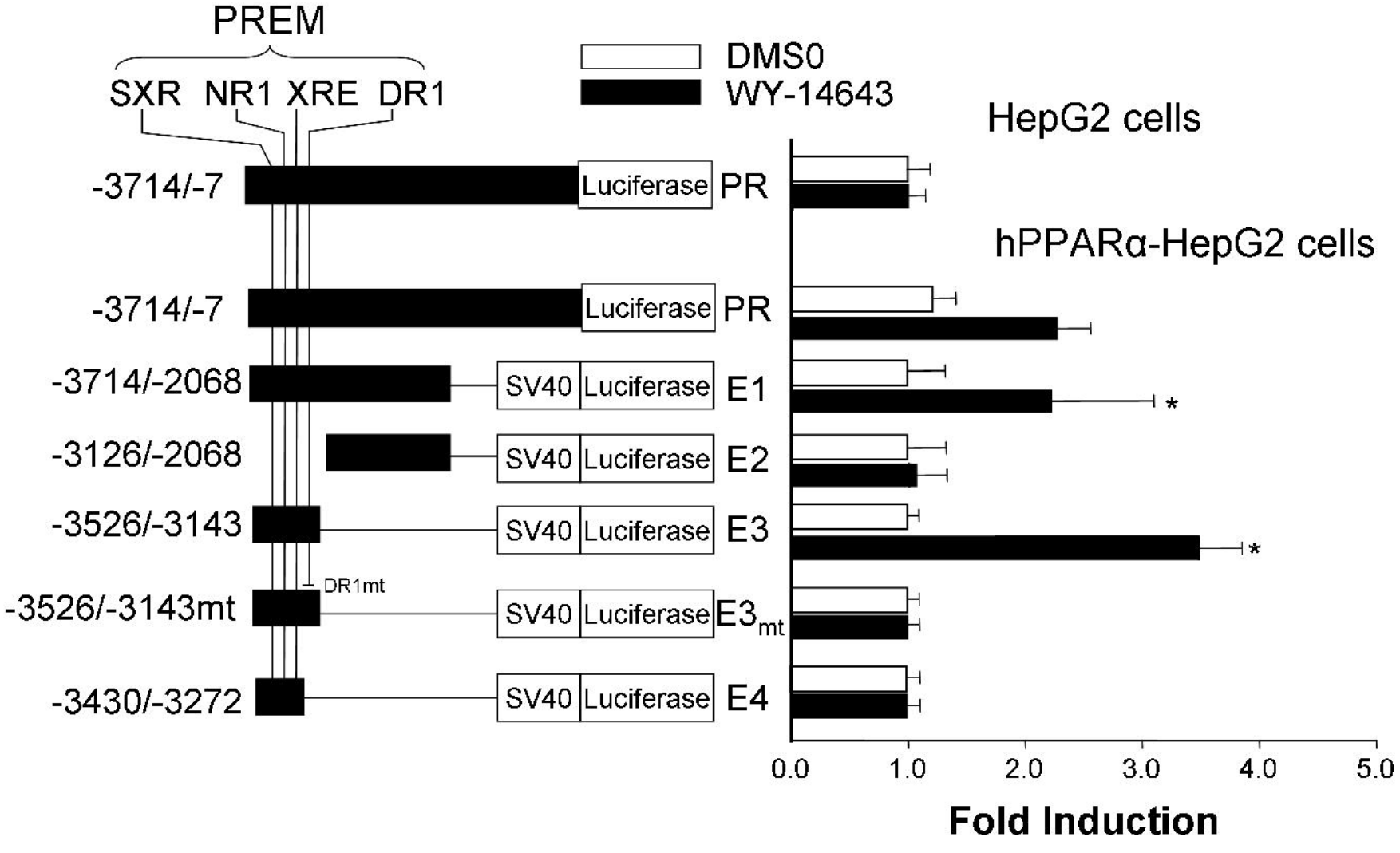
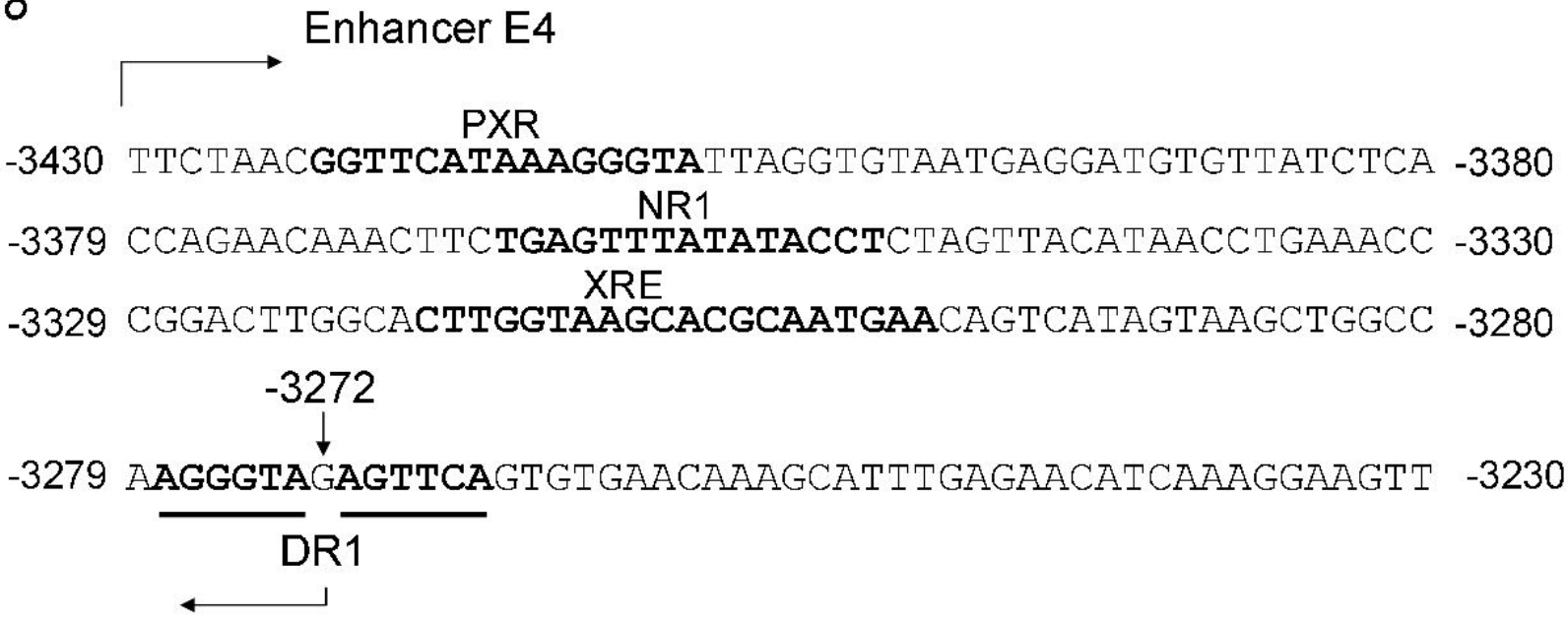
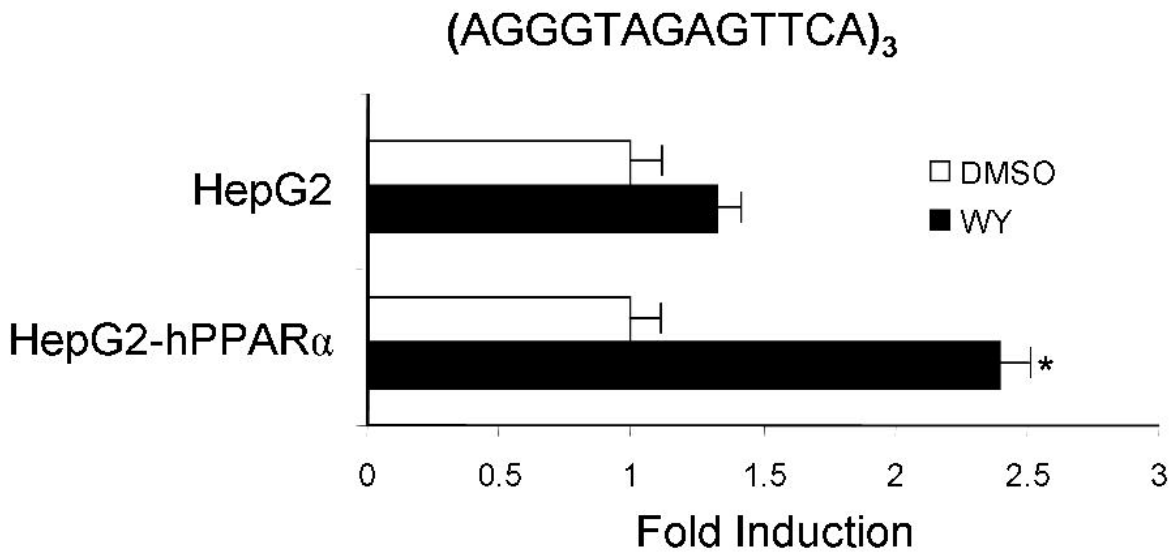

PREM
SXR NR1 XRE DR1
DMS0
WY-14643
HepG2 cells
hPPARα-HepG2 cells
-3714/-7
Luciferase
PR
-3714/-7
Luciferase
PR
-3714/-2068
SV40 Luciferase
E1
-3126/-2068
SV40 Luciferase
E2
-3526/-3143
SV40 Luciferase
E3
DR1mt
-3526/-3143mt
SV40 Luciferase
E3mt
-3430/-3272
SV40 Luciferase
E4
*
*
0.0
1.0
2.0
3.0
4.0
5.0
Fold Induction

Figure 8

A)


Enhancer E4
PXR
-3430 TTCTAACGGTTCATAAAGGGTATTAGGTGTAATGAGGATGTGTTATCTCA -3380
NR1
-3379 CCAGAACAAACTTCTGAGTTTATATACCTCTAGTTACATAACCTGAAACC -3330
XRE
-3329 CGGACTTGGCACTTGGTAAGCACGCAATGAACAGTCATAGTAAGCTGGCC -3280
-3272
-3279 AAGGGTAGAGTTCAGTGTGAACAAAGCATTTGAGAACATCAAAGGAAGTT -3230
DR1

B)


(AGGGTAGAGTTCA)3
HepG2
HepG2-hPPARα
DMSO
WY
*
0
0.5
1
1.5
2
2.5
3
Fold Induction