Differential Role of Liver X Receptor (LXR) $\alpha$ and LXR$\beta$ in the Regulation of UDP-Glucuronosyltransferase 1A1 in Humanized UGT1 Mice

Eva Hansmann, Elvira Mennillo, Emiko Yoda, Mélanie Verreault, Olivier Barbier, Shujuan Chen, and Robert H. Tukey

ABSTRACT
Liver X receptors (LXRs), LXR$\alpha$ and LXR$\beta$, are nuclear receptors that regulate the metabolism of cholesterol and bile acids and are activated by oxysterols. Humanized UGT1 (hUGT1) mice express the 9-human UGT1A genes associated with the UGT1 locus in a Ugt1-null background. The expression of UGT1A1 is developmentally delayed in the liver and intestines, resulting in the accumulation of serum bilirubin during the neonatal period. Induction of UGT1A1 in newborn hUGT1 mice leads to rapid reduction in total serum bilirubin (TSB) levels, a phenotype measurement that allows for an accurate prediction on UGT1A1 expression. When neonatal hUGT1 mice were treated by oral gavage with the LXR agonist T0901317, TSB levels were dramatically reduced. To determine the LXR contribution to the induction of UGT1A1 and the lowering of TSB levels, experiments were conducted in neonatal hUGT1/Lxr$\alpha^{-/-}$, hUGT1/Lxr$\beta^{-/-}$, and hUGT1/Lxr$\alpha^{+/-}$ and Lxr$\beta^{-/-}$ mice treated with T0901317. Induction of liver UGT1A1 was dependent upon LXR$\alpha$, with the induction pattern paralleling induction of LXR$\alpha$-specific stearoyl CoA desaturase 1. However, the actions of T0901317 were also shown to display a lack of specificity for LXR, with the induction of liver UGT1A1 in hUGT1/Lxr$\alpha^{+/-}$ mice, a result associated with activation of both preganane X receptor and constitutive androstane receptor. However, the LXR agonist GW3965 was highly selective toward LXR$\alpha$, showing no impact on lowering TSB values or inducing UGT1A1 in hUGT1/Lxr$\alpha^{-/-}$ mice. An LXR-specific enhancer site on the UGT1A1 gene was identified, along with convincing evidence that LXR$\alpha$ is crucial in maintaining constitutive expression of UGT1A1 in adult hUGT1 mice.

SIGNIFICANCE STATEMENT
It has been established that activation of LXR$\alpha$, and not LXR$\beta$, is responsible for the induction of liver UGT1A1 and metabolism of serum bilirubin in neonatal hUGT1 mice. Although induction of the human UGT1A1 gene is initiated at a newly characterized LXR enhancer site, allelic deletion of the Lxr$\alpha$ gene drastically reduces the constitutive expression of liver UGT1A1 in adult hUGT1 mice. Combined, these findings indicate that LXR$\alpha$ is critical for the developmental expression of UGT1A1.

Introduction
UDP-glucuronosyltransferases (UGTs) catalyze the transformation of xeno- and endobiotics into excretable glucuronides and thus participate in biologic inactivation and clearance of these compounds (Hu et al., 2014). Variations in UGT expression in addition to the induction or inhibition of these proteins can result in adverse drug effects as well as hormone imbalance (Yang et al., 2017). The UGT1 gene family encodes nine transferases (UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10) (Ritter et al., 1992), which exhibit unique substrate specificity and are expressed in a tissue-specific as well as inducible manner (Tukey and Strassburg, 2000). Humanized UGT1 (hUGT1) mice express the UGT1A genes in a tissue-specific pattern that was similar to expression patterns of the same genes in human tissues (Fujiwara et al., 2010). Interestingly, UGT1A1, which is the sole transferase responsible for the metabolism of serum bilirubin (Rosma et al., 1994), is developmentally regulated in newborn hUGT1 mice and has been a valuable tool in examining the contribution of UGT1A1 toward the control of neonatal hyperbilirubinemia. (Fujiwara et al., 2010, 2012; Yueh et al., 2014; Chen and Tukey, 2018). Neonatal hUGT1 mice develop near-fatal levels of total serum bilirubin (TSB) during the first 2 weeks after birth (Fujiwara et al., 2010). The

ABBREVIATIONS: CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; hUGT1, humanized UGT1; LXR, liver X receptor; LXRE, LXR response element; NCoR1, nuclear corepressor 1; PCR, polymerase chain reaction; PPAR$\alpha$, peroxisome proliferator–activated receptor $\alpha$; pRL renilla luciferase (pRL-NULL)PXR, pregnant X receptor; pPCR, quantitative polymerase chain reaction; SCD, stearoyl CoA desaturase; TSB, total serum bilirubinTK-pGL3thymidine kinase luciferase reporter vector; UCSD, University of California, San Diego; UGT, UDP-glucuronosyltransferase.
administration of ligands known to activate either liver or intestinal constitutive androstane receptor (CAR) (Cai et al., 2010; Fujiwara et al., 2012), the pregnane X receptor (PXR) (Chen et al., 2005, 2012), or the peroxisome proliferator–activated receptor α (PPARα) (Seneko-Effenberger et al., 2007) result in the induction of UGT1A1 and the simultaneous metabolism and elimination of circulating TSB.

The liver X receptor (LXR) was first identified in the 1990s as an orphan member of the nuclear receptor superfamily and later found to be activated by endogenous oxysterols (Peet et al., 1998a). LXR consists of two isoforms, LXRα and LXRβ. LXRα is expressed in all tissues (Song et al., 1994), in contrast, the expression of LXRβ is restricted to the liver, intestine, kidney, spleen, macrophages, and adipose tissue (Willy et al., 1995). LXR is largely involved in the regulation of cholesterol homeostasis protecting the cells from cholesterol overload by stimulating reverse cholesterol transport (Millatt et al., 2003). Furthermore, LXR has been linked to other major physiologic functions, including lipid metabolism (Ulven et al., 2005; Cha and Repa, 2007), glucose homeostasis (Greffhorst et al., 2005; Oosterveer et al., 2010), steroidogenesis, immunity, and inflammation (Zelcer and Tontonoz, 2006).

LXRα and LXRβ were cloned in 1995 (Mangelsdorf and Evans, 1995; Willy et al., 1995), and later confirmed that the ligand-binding and DNA-binding domains of the receptors were 77% homologous (Alberti et al., 2000). Following confirmation that expression of the LXRs displayed differential tissue specificity (Shinar et al., 1994; Willy et al., 1995) yet both bound to similar LXR enhancer sequences, it had been speculated and later demonstrated that activation of the LXRα/RXR and LXRβ/RXR complexes have different roles in gene expression (Annicotte et al., 2004; Hong and Willy, 1995) yet both bound to similar LXR enhancer sequences, it had been speculated and later demonstrated that activation of the LXRα/RXR and LXRβ/RXR complexes have different roles in gene expression (Annicotte et al., 2004; Hong and Willy, 1995). The LXRβ-enriched diet had a profound effect on LXRα−/− mice, inducing fatty liver, increased liver mass, and increased cholesterol levels, eventually leading to impaired liver function. The Lxrβ−/− mice were resistant to cholesterol-induced fatty liver formation. There are not highly selective LXRs or LXRβ ligands, making it difficult to isolate the actions of the different LXRs receptors on gene expression or function. Yet, activation of LXR by nonselective LXR agonists in Lxrα−/− or Lxrβ−/− mice can isolate the direct actions of these receptors on gene expression. For example, the LXR agonist T0901317-induced hepatic stearoyl CoA desaturase (SCD1) expression only in wild-type or Lxrβ−/− mice and not Lxrα−/− mice, demonstrating that LXRα controls SCD1 expression (Zhang et al., 2014). There is little information on the direct impact of LXR toward the induction and regulation of the UGT1A1 gene. We have now taken a direct approach to evaluate the direct impact of LXR toward the induction and regulation of the UGT1A1 gene by deleting LXRα, LXRβ, or LXRα and LXRβ in hUGT1 mice.

Materials and Methods

Materials. UDP-glucuronic acid and bilirubin were obtained from Sigma (St. Louis, MO). SN-38 and T0901317 were obtained from Cayman Chemical (Ann Arbor, MI). SN-38 glucuronide was from Santa Cruz Biotechnology (Santa Cruz, CA). Internal standards deuterated d10-CPT-11 and d3-SN-38 were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Rabbit anti-UGT1A1 monoclonal antibody was purchased from Abcam (Cambridge, UK). Mouse anti-GAPDH monoclonal antibody was obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-mouse IgG horseradish peroxidase (HRP) conjugated antibody and anti-rabbit IgG HRP conjugated antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Human hepatoma (HepG2) and human embryonic kidney (HK293) cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured and passaged per standard protocol.

Animal Studies. Humanized UGT1 mice were previously generated by introducing a human bacterial artificial chromosome (BAC) clone encoding the entire UGT1 locus into mice (transgenic(Tg)/UGT1/ mice) (Chen et al., 2005) and crossing TgUGT1 mice with Ugt1−/− mice (Nguyen et al., 2008) until TgUGT1/Ugt1−/− mice were created (Fujiwara et al., 2010). A breeding strategy between hUGT1, Lxra−/−, Lxrb−/−, and Lxrb−/− mice was developed that resulted in the generation of hUGT1/Lxra−/−, hUGT1/Lxrb−/−, and hUGT1/Lxra/Lxrb−/− mice. The Lxr-null mice were originally developed by the David Mangelsdorf laboratory (Repa et al., 2000) and were generously obtained from Christopher Glass at the University of California, San Diego (UCSD). hUGT1/Lxra−/− mice were developed previously (Fujiwara et al., 2012). All mouse strains were housed in a pathogen-free UCSD Animal Care Facility and received fresh food and water ad libitum. All animal protocols were reviewed and approved by the UCSD Animal Care and Use Committee. Neonatal male and female 10-day-old mice were treated orally with either propylene glycol/tween 4:1 (vehicle) or 50 mg/kg T0901317 dissolved in vehicle or GW3965 (75 mg/kg), and tissues were collected 48 hours after treatment.

Bilirubin Measurements. Blood was obtained from the submandibular vein and centrifuged at 16,000g for 2 minutes. Serum samples were immediately measured for TSB levels using a Unistat Bilirubinometer (Reichert, Depew, NY).

Liver Tissue Sections. Entire livers were dissected from mice, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen tissues were pulverized and the powder aliquoted for further RNA and protein extraction.

Total RNA Preparation and Real-Time Reverse Transcription Polymerase Chain Reaction. Total RNA was isolated from liver tissue of treated and control mice according to the TRIzol reagent protocol as specified by the supplier (Thermo Fisher Scientific, Waltham, MA). The samples were prepared for reverse transcription by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative polymerase chain reaction (qPCR) using newly synthesized cDNA was carried out using a CFX96 QPCR system (Bio-Rad) by employing Sybr Advanced SYBR Green reagent (Bio-Rad). Primers used in these studies are shown in Table 1.

Western Blot Analysis. Tissues were homogenized in RIPA lysis buffer (EMD Milipore Corporation, Temecula, CA) supplemented with protease and phosphatase inhibitor cocktail (1:100 each). Protein solutions were pooled to have a total of four samples for each genotype and condition. Western blots were performed as previously described (Mangelsdorf et al., 1995). Briefly, liver tissue homogenates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk in TBS-T (1× Tris-buffered saline containing 0.05% Tween 20) for 1 hour at room temperature (RT). Membranes were incubated with specific primary antibodies overnight at 4°C in TBS-T, washed, and incubated with horseradish peroxidase-conjugated secondary antibodies. Membranes were developed by using ECL detection reagent (Amersham) and exposed to film. For quantitative determination, bands were quantified by densitometric analysis using NIH Image J software.

Table 1. Sequence of primers used in qPCR analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Oligonucleotide Sequence (5′-3′)</th>
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<tbody>
<tr>
<td>Human UGT1A1</td>
<td>Forward: CCATCATGCGCAATATGTT</td>
</tr>
<tr>
<td>Human UGT1A3</td>
<td>Reverse: CCAACAATCTCATGCTCCCA</td>
</tr>
<tr>
<td>Human UGT1A4</td>
<td>Forward: AGGGACAGCAGGAGACCTCTC</td>
</tr>
<tr>
<td>Human UGT1A6</td>
<td>Reverse: CAAATTCCTGGGATGTTGATT</td>
</tr>
<tr>
<td>Human UGT1A9</td>
<td>Forward: CATACGAGTTGACTGCTGG</td>
</tr>
<tr>
<td>Scd-1</td>
<td>Reverse: CTGCTACACCTGGTTCCTGC</td>
</tr>
<tr>
<td>Scd-2</td>
<td>Forward: CCAGCCGGCCTTGAATGTC</td>
</tr>
<tr>
<td>LXRα</td>
<td>Reverse: TGCCCTTTGATCTGCTGG</td>
</tr>
<tr>
<td>LXRβ</td>
<td>Forward: TACACCCGGGAAGACCTTGC</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>Reverse: CAGAACTGATGCTGAGCCA</td>
</tr>
<tr>
<td>Cyp3a11</td>
<td>Forward: CTGGTGAGCTGAGGTTA</td>
</tr>
<tr>
<td>Cyp4a10</td>
<td>Reverse: AAAGTCCGGTGCAACTTCC</td>
</tr>
<tr>
<td>Cph</td>
<td>Forward: CATCCCAAACTCCTACGCC</td>
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performed by using NuPAGE 4%-12% Bis-Tris-polyacrylamide gels (Thermo Fisher Scientific), with the protocols described by the manufacturer. Protein (30 μg) was electrophoresed at 170 V for 50 minutes and transferred at 20 V for 60 minutes to PVDF membranes (EMD Millipore Corporation). Membranes were blocked with 5% skim milk at room temperature for 1 hour and incubated with primary antibodies [rabbit anti-human UGT1A1 (ab170858), mouse anti-GAPDH (sc-23233)] at 4°C overnight. Membranes were washed and exposed to HRP-conjugated secondary antibodies (anti-mouse IgG or anti-rabbit IgG) for 1 hour at room temperature. Protein was detected by Clarity Western ECL Substrate system (Bio-Rad) and was visualized by the Bio-Rad gel documentation system. All Western blots are cropped from the full-length blots that have been included in the Supplemental Material.

**Glucuronidation Assays.** Microsomal proteins were purified from tissues (50 mg), and 100 μg of liver homogenate was incubated in a previously reported glucuronidation assay buffer (Verreault et al., 2006) in the presence of bilirubin (20 μM), SN-38 (50 μM) for 30 minutes, or imipramine (500 μM) for 2 hours at 37°C. The formation of bilirubin monoglucuronide, SN-38 glucuronide, and imipramine glucuronide was quantified by liquid chromatography coupled to electrospray ionization - tandem mass spectrometry (LC/ESI-MS/MS) as previously reported (Lu et al., 2017). The formation of glucuronide conjugates is expressed as area under the curve or peak area ratio.

**Plasmid Cloning and Transient Transfection Assays.** The various TK-pGL3 plasmids were obtained by cloning three copies of the corresponding dimerized oligonucleotides (Table 2) in the thymidine kinase promoter-driven luciferase reporter (TK-pGL3) vector. For transfection experiments, HepG2 cells previously reported (Lu et al., 2017). The formation of glucuronide conjugates is expressed as area under the curve or peak area ratio.

**Electrophoretic Mobility Shift Assays.** Electrophoretic mobility shift assays (EMSA) using in vitro-produced LXRα and RXRα were performed as described (Johansson et al., 2003) using the radiolabeled probes (100,000 cpm) as indicated in Supplemental Fig. 1. Oligonucleotides used in these studies are shown in Table 2.

**Chromatin Immunoprecipitation Assays.** Chromatin immunoprecipitation (ChIP) assays were performed according to the method of Forsberg et al. (2000) in a previously modified manner (Verreault et al., 2006). Briefly, 20 × 10^6 HepG2 cells were treated with either vehicle or T0901317 (1 μM) for 4 hours. Protein-DNA cross-linking, nuclear extract preparation, and sonication were performed as reported (Forsberg et al., 2000). A volume of lysate equivalent to 40 × 10^6 cells was immunoprecipitated using 4 μg of anti-LXR (sc1202; Santa-Cruz), anti-PXR (sc9690) antibodies, or with 4 μg of an anti-UGT2B (sc23479) antibody as a negative control. A separate volume of lysate was kept with the primary antibodies as a negative control. A separate volume of lysate was kept with the lytic antibody as a negative control. DNA or was incubated in the presence of protein A-sepharose alone. One-tenth of the immunoprecipitated DNA was PCR amplified as described in Supplemental Fig. 1.

**Statistical Analysis.** Statistically significant differences between vehicle- and T0901317-treated animals were evaluated using unpaired t test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001), and between different genotypes we performed Tukey’s multiple comparison test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

**Results**

**Induction of the UGT1A1 Gene in Neonatal hUGT1 Mice.** The LXR agonist T090137 has an affinity for both LXRα and LXRβ (Schultz et al., 2000), with a binding affinity that displayed an EC₅₀ of approximately 20 nM. It is also capable of activating the PXR (Shenoy et al., 2004) and farnesoid X receptor (Houck et al., 2004) but at a higher concentration than for LXR (EC₅₀ = 4-7 μM). There are five human UGT1 isoforms expressed in the liver (Strassburg et al., 1997) (UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9), with UGT1A1 controlling the metabolism and clearance of serum bilirubin.

To investigate the potential of LXR to induce the UGT1A1 gene, 10-day-old neonatal hUGT1A1 mice were treated once with vehicle (propylene glycol/tween 80, 40:1) or with the LXR agonist T090137 (50 mg/kg) by oral gavage. After 48 hours, serum was collected for TBG analysis and the levels collected for RNA extraction. Neonatal hUGT1 mice develop severe levels of serum bilirubin. T090137 treatment of 48 hours reduced TSB levels to normal adult levels (Fig. 1A). This drop in TSB levels resulted from a significant induction of liver UGT1A1 gene expression (300-fold) as quantitated by qPCR (Fig. 1B). Also, analysis of RNA expression demonstrated there was substantial induction of liver UGT1A3 (200-fold) and UGT1A4 (400-fold), with UGT1A6 and UGT1A9 genes being unaffected (Fig. 1C). We have shown previously that T090137 treatment to transgenic UGT1 mice induced the UGT1A3 gene in liver and intestinal tissue, along with induction of UGT1A3 directed glucuronidation activity toward chemodeoxycholic acid (CDCA) and lithocholic acid (LCA) (Verreault et al., 2006).

**The LXR Receptors Display Gene Specificity.** To examine the specificity of the LXR receptors toward induction of liver UGT1A1, hUGT1, hUGT1/Lxrα⁻/⁻, hUGT1/Lxrβ⁻/⁻, and hUGT1/Lxrα⁻/⁻ hUGT1/Lxrβ⁻/⁻ mice were developed. To confirm the knockout conditions in these mice, qPCR analysis was performed to verify the elimination of LXRα mRNA expression in hUGT1/Lxrα⁻/⁻ and hUGT1/Lxrβ⁻/⁻ mice and the elimination of LXRβ mRNA
expression in hUGT1/Lxrβ−/− and hUGT1/Lxraβ−/− mice (Fig. 2, A and B). Interestingly, there was a consistent reduction in Lxra gene expression in Lxrb−/− mice treated with T090137, yet the values were not statistically significant. Knockout of the Lxr genes leads to a loss of function, as demonstrated in gene expression patterns of the LXRα target genes stearoyl CoA desaturase-1 (Scd1) and Scd2 (Chu et al., 2006; Caputo et al., 2014; Zhang et al., 2014). These proteins catalyze the conversion of saturated fatty acids into monounsaturated fatty acids and have previously been described to be activated by LXR agonists in either an sterol regulatory element-binding-1c (SREBP-1c) dependent or independent manner. The oral treatment of neonatal hUGT1 and hUGT1/Lxrb−/− mice with T090137 resulted in transcriptional activation of Scd1 and Scd2 genes (Fig. 2, C and D). Mice lacking the Lxrα gene did not show increases in SCD1 or SCD2 RNA (Fig. 2, C and D).

**Induction of UGT1A1 by LXR.** Using these same tissues, the induction profile of the UGT1A1 gene was like that of Scd1, with greater induction in hUGT1 and hUGT1/Lxrb−/− mice and significantly reduced induction in hUGT1/Lxra−/− and hUGT1/Lxraβ−/− mice (Fig. 3B). However, the induction of the UGT1A1 gene in hUGT1/Lxra and hUGT1/Lxrb−/− was still considerable with approximately 30% of those values detected in the liver from hUGT1 mice. Surprisingly, the TSB values in the hUGT1 mice and the other LXR variants were all greatly reduced when compared with vehicle-treated mice (Fig. 3A). The phenotype changes in serum TSB values in hUGT1, hUGT1/Lxra−/−, hUGT1/Lxrb−/−, and hUGT1/Lxraβ−/− mice suggests that UGT1A1 was induced in each of these strains following T090137 treatment. This was confirmed by Western blot analysis showing that induction of liver UGT1A1 occurs in all strains treated, with liver abundance reflecting that of gene expression (Fig. 3C). Also, glucuronidation analysis using liver extracts has confirmed that bilirubin (Fig. 3D) and SN-38 (Fig. 3E), substrates that are conjugated by UGT1A1, show a functional induction pattern like UGT1A1 gene expression. Because the induction levels of UGT1A1 in hUGT1/Lxrb−/− mice, which do not express functional LXR, are like those observed in hUGT1/Lxrb−/− mice, this finding indicates that LXRβ plays a negligible role in the induction of UGT1A1 in neonatal mice.

**T090137 and LXR Elicit Cross-Talk with Other Nuclear Receptors.** The dramatic induction of UGT1A1 by T090137 in all LXR mouse lines led us to examine in greater detail this unusual property of T090137. In the liver, the Cyp2b10, Cyp3a11, and Cyp4a10 genes are significantly induced in hUGT1 mice (Fig. 4, A–C) by T090137. It is interesting to note that in the absence of LXRα, T090137 elicits a highly synergistic Cyp3a11 induction response. This observation indicates that activation of PXR by T090137, which can induce Cyp3a11, may be repressed by activated LXRα. In contrast, induction of Cyp4a10, which can be induced by PPARα, is not induced above control values in LXRα deficient mice, indicating that induction of Cyp4a10 by PPARα requires functional LXRα. In both of these examples, activation of either PXR or PPARα by T090137 is interconnected with LXRα and not LXRβ. Different from these examples, however, is the induction pattern by CAR activation of the Cyp2b10 gene in hUGT1, hUGT1/Lxra−/−, hUGT1/Lxrb−/−, hUGT1/Lxraβ−/−, and hUGT1/Lxrb−/−, and hUGT1/Car−/− mice (Fig. 4C). Western blot analysis of liver extracts from these treated mice demonstrates that the induction of Cyp2b10 by T090137 is CAR dependent because induction is significantly reduced in hUGT1/Car−/− mice (Fig. 4D). Thus, the human UGT1A1 gene can be induced by T090137 through activation of PXR, PPARα, and CAR, in addition to the more dominant action of LXRα.

**Induction of Liver UGT1A1 by GW3965.** In addition to T090137, the synthetic LXR agonist GW3965 has been used as an alternative agent to examine LXR-activated target genes. We treated 10-day-old neonatal hUGT1, hUGT1/Lxrb−/−, and hUGT1/Lxra−/− mice with GW3965 (75 mg/kg) and examined TSB values and induction of Cyp4a10 (Fig. 5B), and second, Western blot analysis shows induction of UGT1A1 in hUGT1 and hUGT1/Lxrb−/− mice only (Fig. 5C). Based upon current knowledge of the specificity of T090137 and GW3695
to activate both LXR\(\alpha\) and LXR\(\beta\), the induction of liver UGT1A1 is driven selectively by activated LXR\(\alpha\). It can be noted that the TSB levels following oral GW3965 treatment are not as low as noted for T0901317 treatment (Fig. 1). It is possible that T0901317, which is able to activate not only LXR but also PXR and CAR, is also inducing UGT1A1 in intestinal tissue, a site that contributes toward bilirubin clearance (Chen et al., 2017).

**Identification of a Functional LXR Response Elements within the UGT1A1 Gene Promoter.** A computer-assisted analysis (Quandt et al., 1995) of the UGT1A1 promoter gene sequence revealed the

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![Fig. 2](image2.png)

**Fig. 2.** Gene expression of Lxr\(\alpha\) and Lxr\(\beta\) and LXR downstream target genes Scd1 and Scd2 in LXR-deficient mice. Following the treatment of neonatal hUGT1, hUGT1/Lxr\(\alpha\)\(^{-}\) (Lxr\(\alpha\)\(^{-}\)), hUGT1/Lxr\(\beta\)\(^{-}\) (Lxr\(\beta\)\(^{-}\)), and hUGT1/Lxr\(\alpha\beta\)\(^{-}\) (Lxr\(\alpha\beta\)\(^{-}\)) mice with 50 mg/kg T0901317 for 48 hours (see Fig. 1), Lxr\(\alpha\) (A), Lxr\(\beta\) (B), Scd1 (C), and Scd2 (D) gene expression was determined by real-time PCR and expressed relative to mouse Cph gene expression. Values are the means \(\pm\) S.E.M. (\(n > 4\)). Statistically significant differences are outlined in Materials and Methods.

![Fig. 3](image3.png)

**Fig. 3.** The loss of LXR\(\alpha\) and/or LXR\(\beta\) impacts T0901317-dependent modulation of UGT1A1 expression and activity. Ten-day-old neonatal hUGT1, hUGT1/Lxr\(\alpha\)\(^{-}\) (Lxr\(\alpha\)\(^{-}\)), hUGT1/Lxr\(\beta\)\(^{-}\) (Lxr\(\beta\)\(^{-}\)), and hUGT1/Lxr\(\alpha\beta\)\(^{-}\) (Lxr\(\alpha\beta\)\(^{-}\)) mice were treated with 50 mg/kg T0901317 or vehicle (propylene glycol/Tween 80 4:1) by oral gavage. After 48 hours, serum was collected, and the mice were sacrificed to collect liver tissue. The liver was pulverized under liquid nitrogen and used for the preparation of total RNA, whole-cell extract, and glucuronidation assays. (A) Before and 48 hours after the treatment TSB levels were taken. (B) UGT1A1 gene expression was determined by real-time PCR and expressed relative to Cph gene expression. (C) Whole-liver tissue extracts were used to perform Western blot analysis to examine UGT1A1 expression. The bands have been cropped from the full-length blots but not enhanced in any way. Densitometric values of UGT1A1 protein bands obtained using the GAPDH signal as normalizing control are shown. 100 \(\mu\)g liver homogenate was incubated in the presence of bilirubin (20 \(\mu\)M) (D) or SN-38 (50 \(\mu\)M) (E) for 30 minutes at 37°C. The formation of bilirubin monoglucuronide and SN-38 glucuronide was quantified by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). The formation of glucuronide conjugates is expressed as area under the curve or peak area ratio. Values are the means \(\pm\) S.E.M. (\(n > 4\)). Statistically significant differences were performed as outlined in Materials and Methods.
presence of five degenerated LXR response element ( LXRE) sequences (Willy et al., 1995) localized at positions —9903, —9855, —7632, —4076, and —888 bp of the promoter (Supplemental Fig. 1). Occupancy of these response elements by LXRα in living cells was analyzed through a ChIP assay performed on DNA from vehicle- or T0901317-treated HepG2 cells (Fig. 6). Only the DNA sequences encompassing the —9855 bp LXRE and an LXRE previously identified within the human UGT1A3 promoter (positive control) (Verreault et al., 2006) were precipitated by the anti-LXRα antibody in T0901317-treated cells (Fig. 6, A and C, Lane 8) but not in untreated cells (Lane 3). None of the other LXRE-like sequences found in the UGT1A1 gene promoter were PCR amplified in any of the precipitated DNA preparations, suggesting that they are not occupied by LXRα/β. Because T0901317 was also reported as an activator of PXR (Mitro et al., 2007), DNA was also immunoprecipitated with an anti-PXR antibody (Lanes 4 and 9). No amplifications were observed.

Transient transfection with a —9855 bp LXRE-TK-pGL3 construct revealed that coexpression of LXRα and RXRα increased activity of the LXRE in human hepatoma HepG2 cells, an effect that was dose-dependently enhanced in the presence of T0901317 and GW3965 (0.1–10 μM) (Fig. 6B). In EMSA, a clear binding was observed when a radiolabeled probe encompassing the —9855 bp LXRE sequence was incubated in the presence of both LXRα and RXRα (Fig. 6C, Lane 4). Introducing mutated bases within the 3’-half-site of the LXRE (Table 1) completely abolished the formation of this binding (Fig. 6, lane 5). In competition experiments, LXRE/RXRα binding to the WT probe was efficiently competed by itself and the consensus LXRE (Fig. 6, Lanes 6–8 and 12–14, respectively). In contrast, the mutated response elements failed to significantly compete for LXRα binding (lanes 9–11). Overall, these data identify the LXRE motif at position —9855 bp in the human UGT1A1 gene promoter as a functionally active LXR response element.

The Role of LXRα and Expression of UGT1A1 in Adult hUGT1 Liver. There is a limited expression of human UGT1A1 in the liver tissue of neonatal hUGT1 mice, but in adult hUGT1 mice there is adequate expression. When we examined the constitutive expression pattern of UGT1A1 by qPCR and Western blot analysis, there was an excellent agreement with gene expression and protein detection (Fig. 7). However, it is clear from this analysis that LXRα plays a key role in basal or constitutive expression of liver UGT1A1, with greatly reduced expression in hUGT1/Lxrα−/− and hUGT1/Lxrαβ−/− mice.

**Fig. 4.** The impact of the LXR agonist T0901317 and activation of additional nuclear receptors. Ten-day-old neonatal hUGT1, hUGT1/Lxrα−/− (Lxrα−/−), hUGT1/Lxrαβ−/− (Lxrβ−/−), and hUGT1/Lxrαβ−/− (Lxrβ−/−) mice were treated with 50 mg/kg T0901317 or vehicle (propylene glycol/Tween 80 4:1) by oral gavage. RNA was isolated from liver tissue after 48 hours and gene expression was evaluated by real-time PCR for Cyp3a11 (A), Cyp4a10 (B), and Cyp2b10 (C). Samples of liver tissue were used for analysis of CYP2B10 expression by Western blot analysis (D). Statistically significant differences were performed as outlined in Materials and Methods.

**Fig. 5.** Induction of UGT1A1 by the LXR agonist GW3965. Ten-day-old neonatal hUGT1, hUGT1/Lxrα−/− (Lxrα−/−), hUGT1/Lxrαβ−/− (Lxrβ−/−), and hUGT1/Lxrαβ−/− (Lxrβ−/−) mice were treated with 75 mg/kg T0901317 or vehicle (propylene glycol/Tween 80 4:1) by oral gavage. After 48 hours, serum was collected along with liver tissue. TSB values (A) were determined along with UGT1A1 gene expression (B). Liver tissue was prepared as outlined and used in Western Blot analysis to examine UGT1A1 expression (C).
The LXRs bind directly to these genes at LXR enhancer sequences to bile acids (Peet et al., 1998a; Zhang et al., 2012). Under these conditions, cholesterol absorption, transport, efflux, excretion, and conversion to regulate the expression of genes that are linked to functional control of Wagner et al., 2003; Wang and Tontonoz, 2018). Activated LXRs transcriptional control of lipid metabolism (Edwards et al., 2002; Edwards et al., 2002; Wagner et al., 2003) that in turn regulates genes that encode proteins involved in fatty acid elongation and desaturation (Schultz et al., 2000) that in turn regulates genes that encode proteins involved in fatty acid elongation and desaturation (Schultz et al., 2000). Along with these important regulatory events, LXRs also drive the incorporation of polyunsaturated fatty acids into phospholipids. Each of these regulatory events leads to the proper homeostasis of the membrane environment and lipid composition, which if disrupted can impact diseases such as atherosclerosis, diabetes, fatty liver disease, and cancer.

Although there is approximately 78% amino acid similarity between LXRα and LXRβ, the genes encoding these proteins in mice exist on different chromosomes, with Lrxa residing on chromosome 2 and Lrxb on chromosome 7. The evolutionary split of these genes indicates that their expression may differ in selective tissues coupled with changes in receptor function. The LXRs are activated by naturally occurring oxysterols and by synthetic nonsteroidal compounds such as T0901317 and GW3965. However, LXRα and LXRβ are expressed differently with regards to abundance and tissue specificity. Annicotte et al. (2004) employed selectively in situ hybridization in embryonic and adult tissue to demonstrate that LXRα is highly expressed in metabolically active tissues such as the liver, intestine, and adipose tissue while LXRβ is ubiquitously expressed. Although there is a commonality in the ability of ligands to activate the LXRs, there are several key examples that the LXRs elicit unique functional roles. For example, mice lacking LXRα accumulate massive amounts of cholesterol in the liver when fed a high-cholesterol diet (Peet et al., 1998b). This results from the inability of cytochrome P450 7A1, a key LXRα target gene, to metabolize cholesterol to bile acids. This does not occur when cholesterol is fed to LXRβ-deficient mice. Numerous other examples have been reported favoring a selective advantage in controlling physiologic functions or gene regulation for one of the receptors over the other (Alberti et al., 2001; Jakobsson et al., 2014; Zhang et al., 2014; Whitfield et al., 2016; Endo-Umeda et al., 2018).

In the absence of ligand, the LXR/RXR complexes bind to transcriptional corepressors, such as the nuclear corepressor 1 (NCOR1), and repress target gene expression (Chen and Evans, 1995; Horlein et al., 2001; Jakobsson et al., 2014; Zhang et al., 2014; Whitfield et al., 2016; Endo-Umeda et al., 2018). Upon ligand binding, LXRs dissociate from NCOR1 and assemble with coactivators, leading to modulation of histone acetylation or chromatin remodeling, facilitating transcriptional

When the Lrxa allele is either null or exists in the heterozygous state (hUGT1/Lrxa+/-), the expression of liver UGT1A1 is greatly reduced. This indicates that both Lrxa alleles are necessary to maintain the full expression of UGT1A1.

Discussion

Liver X receptors α and β have been shown to play pivotal roles in the transcriptional control of lipid metabolism (Edwards et al., 2002; Wagner et al., 2003; Wang and Tontonoz, 2018). Activated LXRs regulate the expression of genes that are linked to functional control of cholesterol absorption, transport, efflux, excretion, and conversion to bile acids (Peet et al., 1998a; Zhang et al., 2012). Under these conditions, the LXRs bind directly to these genes at LXR enhancer sequences to induce transcription (Sabol et al., 2005). Also, LXRs regulate fatty acid metabolism by controlling the lipogenic transcriptional factor sterol regulatory element-binding protein 1c (SREBP-1c) (Repá et al., 2000; Wagner et al., 2003) that in turn regulates genes that encode proteins involved in fatty acid elongation and desaturation (Schultz et al., 2000). Along with these important regulatory events, LXRs also drive the incorporation of polyunsaturated fatty acids into phospholipids. Each of these regulatory events leads to the proper homeostasis of the membrane environment and lipid composition, which if disrupted can impact diseases such as atherosclerosis, diabetes, fatty liver disease, and cancer.

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Fig. 6. Ligand-activated LXRα activates a positive LXRE at position –9855 in the UGT1A1 promoter. (A) Soluble chromatin was prepared from HepG2 cells treated with vehicle (ethanol-lanes 1–5), T0901317 (1 μM-lanes 6–10) for 4 hours and immunoprecipitated with antibodies directed against LXRα (lanes 3 and 8), PXR (lanes 4 and 9), or with an anti-UGT2B antibody as a negative control (lanes 5 and 10). The final DNA extracts were amplified using pairs of primers covering the five degenerated DR4 motifs identified within the UGT1A1 gene promoter or a previously reported LXRE from the human UGT1A3 gene promoter (Verreault et al., 2006) as a positive control (a. 1:50 (input) or 1:10 (precipitated DNA) of PCR products were separated on an ethidium-bromide stained 2% agarose gel. (B) HepG2 cells were transfected with the indicated LXRE-driven luciferase (Luc) reporter plasmids (TK-pGL3 constructs: 100 ng) in the absence or presence of LXRα or RXRα plasmids (10 ng) and a pRL-NULL expression plasmid (30 ng). Cells were treated for 24 hours with vehicle (ethanol) or increasing concentrations of T0901317 or GW3695 (0.1; 0.5; 1; 2.5; 5; and 10 μM) as indicated. Values are expressed as fold induction over the control (TKpGL3, set at 1), normalized to internal renilla activity. (C) EMSA were performed with the end-labeled –9855 bp LXRE wild-type (wt) or mutated (mt) probes, in the presence of in vitro produced RXRα, LXRα, or both, as indicated. Competition of the wild-type probe was performed by adding 5-, 25-, or 50-fold molar excess of the indicated cold competitor probe in EMSA with RXRα and LXRα. n.s., nonspecific binding.

Fig. 7. Contribution of LXR toward the expression of UGT1A1 in adult liver. Adult livers from untreated hUGT1, hUGT1/Lrxa+/- (Lrxa+/+), hUGT1/Lrxa-/- (Lrxa-/-), hUGT1/Lrxb+/- (Lrxb+/+), and hUGT1/Lrxb-/- (Lrxb-/-) mice were collected to examine UGT1A1 gene expression by qPCR (A) and protein expression by Western blot analysis (B).
activation. When NCoR1 is selectively deleted in target tissues, LXR becomes activated in the absence of ligand and stimulates transcription of target genes (Li et al., 2013). In neonatal hUGT1 mice that are deficient in intestinal NCoR1, the UGT1A1 gene was derepressed leading to the metabolism of serum bilirubin (Chen et al., 2017). Gene expression data indicated that LXR target genes were in part activated in hUGT1/Ncor1/−/− mice, leading us to examine in greater detail the role of LXRα and LXRβ in the potential induction of the UGT1A1 gene. Using neonatal hUGT1 mice deficient in LXRα, LXRβ, or both LXRα and LXRβ that were orally treated with synthetic LXR agonists T0901317 or GW3695, induction of hepatic UGT1A1 was dominated by LXRα. However, the induction patterns of UGT1A1 and TSB values in these mice were quite different when we compared the impact of the two agents. The oral administration of GW3695 led to hepatic induction of UGT1A1 in hUGT1/− and hUGT1/Lxrβ/− mice only, which matched the reduction in TSB values. The absence of UGT1A1 induction in hUGT1/Lxrα/− and hUGT1/Lxrαβ/− mice establishes that activated LXRα underlies the induction process.

The most well-characterized synthetic LXR ligand to date is T0901317, but this agent elicited a different pattern of induction. Although the general pattern of UGT1A1 induction in these mice by T0901317 was like that of GW3695, a reduction in TSB was observed in all treatment groups. Also, there was considerable T0901317 was like that of GW3695, a reduction in TSB was observed in all treatment groups. Also, there was considerable T0901317 was like that of GW3695, a reduction in TSB was observed in all treatment groups. Also, there was considerable reduction in TSB values. The absence of UGT1A1 induction in hUGT1/Lxrα/− and hUGT1/Lxrαβ/− mice establishes that activated LXRα underlies the induction process.

With the treatment of hUGT1 neonatal mice with T0901317 or GW3695 we present convincing evidence the LXRα is directly activating the UGT1A1 gene in the liver, an event that leads to the metabolism and clearance of serum bilirubin. This analysis is based upon the induction patterns of UGT1A1 in hUGT1 mice that are deficient either LXRα or LXRβ in hepatic tissue. However, it does not account for the relative abundance of LXRα and LXRβ in this tissue or the relative binding affinities of the ligands to the receptors. Few examples document the abundance of these receptors in different tissues. Using LXRα and LXRβ cDNAs to generate antisense RNA for in situ hybridization experiments, Annicotte et al. (2004) examined the developmental expression of the receptors in postcoitum at different days as well as in adult tissue. The conclusions from this experiment indicated that LXRα and LXRβ were both expressed in liver tissue but the abundance of LXRβ RNA dropped as the pregnancy came closer to term, with LXRβ RNA being reduced in adult liver. However, LXRβ is expressed in liver tissue. Because GW3695 has a greater binding affinity toward LXRβ, we can be confident that both LXRα and LXRβ are being activated in liver tissue following oral administration. With the identification of the LXRβ binding sequence flanking the human UGT1A1 gene and the complete absence of UGT1A1 induction by GW3695 in hUGT1/Lxrβ−/− mice, our findings strongly implicate LXRα as a regulator of liver UGT1A1 gene expression.

The initial discovery that LXRα could be activated by oxysterols was the first of many clues suggesting that LXRα controlled cholesterol homeostasis, eventually implicating the production of bile acids and controlling lipogenesis (Wang and Tontonoz, 2018). Our findings have confirmed that LXRα is a potent regulator of the UGT1A1 gene during development and could serve as a therapeutic target in events that require the regulation of accumulating serum bilirubin. It remains unclear the physiologic advantage of regulating the UGT1A1 gene along with other genes of the UGT1 locus by LXRβ because there is no perceived link between oxysterols and the function of UGT1A1. One might consider, however, that oxysterols play an important role in maintaining adequate LXR-driven constitutive expression of the UGT1A1 gene, which is essential in facilitating metabolism and elimination of the major heme metabolite product, bilirubin. Regardless, these findings suggest that events linking abnormal cholesterol and lipid homeostasis to the onset of lipogenic diseases will have an impact on the control and regulation of UGT1A1 and the functional properties of this important protein in endogenous and exogenous drug metabolism.

Authorship Contributions
Participated in research design: Hansmann, Menillo, Yoda, Verreault, Barbier, Chen, Tukey.
Conducted experiments: Hansmann, Menillo, Yoda, Verreault, Chen.
Performed data analysis: Hansmann, Menillo, Yoda, Tukey.
Wrote or contributed to the writing of the manuscript: Hansmann, Barbier, Tukey.

References
Cell Biochem Funct 32:637–646.
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Chu K, Miyazaki M, Man WC, and Ntambi JM (2006) Stearoyl-coenzyme A desaturase 1 deficiency protects against hyperglycemia and increases plasma high-density lipoprotein cholesterol induced by liver X receptor activation. Mol Cell 26:6786-6789.


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