

**Upregulation of UDP-glucuronosyltransferase (UGT) 1A4 by 17 $\beta$ -Estradiol: a  
Potential Mechanism of Increased Lamotrigine Elimination in Pregnancy**

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### **Abbreviations**

UGT: UDP-glucuronosyltransferase

ER: estrogen receptor

Sp1: specificity protein 1

ERE: estrogen response element

E<sub>2</sub>: 17 $\beta$ -estradiol

tk: thymidine kinase

## ABSTRACT

Oral clearance of lamotrigine, an antiepileptic drug commonly used in pregnant women, is increased in pregnancy by unknown mechanisms. In this study, we show that 17 $\beta$ -estradiol (E<sub>2</sub>) upregulates expression of UGT1A4, the major enzyme responsible for elimination of lamotrigine. Endogenous mRNA expression levels of UGT1A4 in estrogen receptor (ER) $\alpha$ -negative HepG2 cells were induced 2.3-fold by E<sub>2</sub> treatment in the presence of ER $\alpha$  expression. E<sub>2</sub> enhanced transcriptional activity of UGT1A4 in a concentration-dependent manner in HepG2 cells when ER $\alpha$  was co-transfected. Induction of UGT1A4 transcriptional activity by E<sub>2</sub> was also observed in ER $\alpha$ -positive MCF7 cells, which was abrogated by pretreatment with antiestrogen ICI 182,780. Analysis of UGT1A4 upstream regions using luciferase reporter assays identified a putative Sp1 binding site (-1906 to -1901 bp) that is critical for the induction of UGT1A4 transcriptional activity by E<sub>2</sub>. Deletion of the Sp1 binding sequence abolished the UGT1A4 upregulation by E<sub>2</sub>, and Sp1 protein bound to the putative Sp1 binding site as determined by electrophoretic mobility shift assay. Analysis of ER $\alpha$  domains using ER $\alpha$  mutants revealed that the AF1 and AF2, but not the DNA binding domain, of ER $\alpha$  are required for UGT1A4 induction by E<sub>2</sub> in HepG2 cells. Finally, E<sub>2</sub> treatment increased lamotrigine glucuronidation in ER $\alpha$ -transfected HepG2 cells. Together, our data indicate that upregulation of UGT1A4 expression by E<sub>2</sub> is mediated by both ER $\alpha$  and Sp1, and is a potential mechanism contributing to the enhanced elimination of lamotrigine in pregnancy.

## **(Introduction)**

Human pregnancy is accompanied by various physiological changes, including a dramatic increase in the production of female hormones, i.e., estrogen and progesterone. Blood levels of these hormones rise up to 100-fold by term (Cunningham, 2005). At this high concentration, female hormones manifest functions different from their conventional role as gonadal hormones. As a result, various clinical symptoms associated with pregnancy occur, e.g., delayed gastric emptying or intrahepatic cholestasis. Clinical evidence suggests that pregnancy also alters rate and extent of hepatic drug metabolism (Anderson, 2005; Hodge and Tracy, 2007). Hepatic metabolism is a major elimination route of drugs, and altered drug metabolism during pregnancy can lead to increased drug toxicity or decreased drug efficacy, adversely affecting both the mother and fetus. However, mechanisms underlying altered hepatic drug metabolism in pregnancy are poorly understood.

Lamotrigine is widely prescribed for seizure control in women of child bearing potential (Sabers et al., 2004; EURAP Study Group, 2006). Of clinical importance to its use during pregnancy, apparent clearance of lamotrigine increases by 50-90% in pregnancy, requiring dosage adjustment to prevent exacerbation of seizures (de Haan et al., 2004; Harden, 2007; Pennell et al., 2008). Lamotrigine is rapidly and completely absorbed from the intestine and undergoes extensive metabolism by UDP-glucuronosyltransferase (UGT)1A4 and UGT2B7, with minimal renal excretion (<10%) (Linnet, 2002; Rowland et al., 2006). As a low hepatic extraction ratio drug, the clearance of lamotrigine is determined by hepatic enzyme activity and plasma protein binding (Rambeck and Wolf, 1993). The intermediate level of plasma protein binding of

lamotrigine (~50%) suggests a minor role of protein binding, but a significant role of intrinsic hepatic enzyme activity, in causing the increase in oral clearance in pregnancy. A recent study reporting an increased ratio of lamotrigine glucuronide metabolite to lamotrigine concentration in pregnancy further supports an increased glucuronidation of lamotrigine in pregnancy (Ohman et al., 2008). Interestingly, elimination of lamotrigine is similarly increased by use of oral contraceptives (Sabers et al., 2003; Christensen et al., 2007), especially estrogen-based contraceptives (Reimers et al., 2005). This suggests that estrogen may be involved in regulating expression or function of UGT1A4 and/or UGT2B7.

17 $\beta$ -Estradiol (E<sub>2</sub>), the major estrogen in human, has been reported to control expression of several drug-metabolizing enzymes. For example, E<sub>2</sub> upregulates expression of CYP2A6 and CYP1B1, as well as UGT2B15 (Tsuchiya et al., 2004; Harrington et al., 2006; Higashi et al., 2007). Also, E<sub>2</sub> upregulates *Cyp2b10* expression by activating constitutive androstane receptor (CAR) in mouse (Kawamoto et al., 2000; Makinen et al., 2003).

The biological effects of estrogen are mediated through two cognate nuclear receptors, estrogen receptor (ER)  $\alpha$  and  $\beta$ . In the liver, ER $\alpha$  is the major subtype expressed (Kuiper et al., 1997). Estrogen binding to ER activates the receptor, leading to interaction with *cis*-regulatory elements of target genes either by direct binding to estrogen response element (ERE) or by tethering to other transcription factors such as activation protein-1 (AP-1) or specificity protein-1 (Sp1) (Bjornstrom and Sjoberg, 2005). The transactivation by AP-1 or Sp1 has been shown to mediate ERE-independent activation of many estrogen target genes (Safe and Kim, 2008).

Previously, we have shown that UGT2B7 mRNA expression is not influenced by E<sub>2</sub> in HepG2 cells (Jeong et al., 2008), ruling out potential upregulation of UGT2B7 expression by E<sub>2</sub>. In the present study we report that E<sub>2</sub> activates UGT1A4 expression and the induction of UGT1A4 is mediated by ER $\alpha$  and Sp1. This study presents a potential mechanistic basis of the enhanced elimination of lamotrigine in pregnancy and oral contraceptive users.

## Materials and Methods

**Chemicals and Reagents.** 17 $\beta$ -Estradiol was purchased from Sigma-Aldrich (St. Louis, MO). ICI 182,780 and mithramycin were purchased from Tocris (Ellisville, MO) and Biomol (Plymouth Meeting, PA), respectively. Lamotrigine and lamotrigine 2N-glucuronide were generous gifts from GlaxoSmithKline (Chapel Hill, NC). Formic acid (ACS grade) and methanol (Optima grade) were purchased from Fisher Scientific (Pittsburgh, PA).

**Plasmids.** ER $\alpha$  expression plasmid (pcDNA3-ER) was previously constructed in our laboratory (Jeong et al., 2008).  $\beta$ -Galactosidase expression plasmid was kindly provided by Dr. William T. Beck (Ee et al., 2004). pGL3-ERE3 is an E<sub>2</sub>-responsive luciferase reporter plasmid and contains three copies of the estrogen response element (ERE) from *Xenopus vitellogenin A2*, located immediately upstream of thymidine kinase (tk) promoter fused to the luciferase gene (Catherino and Jordan, 1995).

To construct pGL3-UGT1A4 plasmid, the upstream region of UGT1A4 (-2399 to +28) was PCR-amplified using human genomic DNA (Biochain, Hayward, CA) as

template and a pair of primers: forward and reverse primers of 5'-TGCCTACCACAGACTAAG-3' and 5'-TCAGCAGAAGCCACCGAC-3'. The PCR product and NcoI-digested pGL3-basic (Promega, Madison, WI) was blunt ended by treatment with T4 DNA polymerase and were further digested by HindIII restriction enzyme. The resulting PCR product was cloned into the pGL3-basic vector, yielding pGL3-UGT1A4.

To construct luciferase vectors for deletion assays, 3 different 5'-flanking regions of UGT1A4 (-2399 to -1643, -1667 to -863, or -862 to +28) were PCR-amplified using pGL3-UGT1A4 as template and cloned into pGL3tk plasmid that contains tk promoter fused to the luciferase gene. Additional luciferase reporter plasmids were constructed as follows. Upstream regions of UGT1A4 (-1922 to +28, -1886 to +28, -1835 to +28, -1645 to +28, -1130 to +28, -1074 to +28, and -976 to +28) were PCR-amplified using pGL3-UGT1A4 as template and each PCR product was cloned into pGL3-basic plasmid that contains promoterless luciferase gene. Primer sequences are available upon request.

Four plasmids expressing different types of mutant ER $\alpha$  were kindly provided by Dr. Doug Harnish (Harnish et al., 1998): (1) a mutant with deletion of activation function (AF)1, (2) a mutant containing nonfunctional AF2 by point mutations, (3) a mutant containing nonfunctional DNA binding domain by point mutations, and (4) a mutant ER $\alpha$  with both deletion of AF1 and point mutations in AF2.

pGL3-UGT1A4-Sp1 Del, where -1905 to -1901 of UGT1A4 was deleted, was constructed by using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following manufacturer's protocol, using pGL3-UGT1A4 as template. All sequences were confirmed by sequencing.

**Cell Culture.** HepG2 cells from ATCC (Manassas, VA) were cultured in complete DMEM (MediaTech) supplemented with 10% fetal bovine serum (Gemini, Woodland, CA), 2 mM L-glutamine, 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml, and 1% MEM nonessential amino acids. MCF7 cells from ATCC were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 6 ng bovine insulin/ml, 100 units penicillin/ml, 100  $\mu$ g streptomycin/ml, and 1% MEM nonessential amino acids. The media were changed 3 days before each experiment to estrogen-free media, i.e., complete DMEM containing charcoal/dextran-stripped FBS (Gemini, Woodland, CA) and no phenol red.

**Luciferase Reporter Assays.** HepG2 cells were seeded in 12-well plates at a density of  $2 \times 10^5$  cells/ml (day 0) and transfected on the next day (day 1) with 0.3  $\mu$ g of a luciferase construct, 0.3  $\mu$ g of pcDNA3-ER or control vector (pcDNA3), and 0.1  $\mu$ g of  $\beta$ -galactosidase expression plasmid using Fugene 6 transfection reagent (Roche Applied Sciences) according to the manufacturer's protocol. After 24 hr (day 2), the cells were treated with  $E_2$  (1  $\mu$ M) or the ethanol vehicle (0.1%). Following 24 hr incubation (day 3), cells were harvested for determination of both luciferase and  $\beta$ -galactosidase activities using assay kits from Promega (Madison, WI). MCF7 cells were seeded in 12-well plates at a density of  $2 \times 10^5$  cells/ml (day 0) and transfected on the next day (day 1) with a luciferase construct and  $\beta$ -galactosidase expression plasmid using Fugene 6 transfection reagent. At 3 to 8 hr post-transfection, the cells were treated with ICI 182,780 (50  $\mu$ M) or ethanol vehicle. After 24 hr (day 2), the cells were treated with  $E_2$  (1  $\mu$ M) or ethanol vehicle. On day 3, the cells were harvested and analyzed for both luciferase and  $\beta$ -galactosidase activities. In all cases, the luciferase activity was normalized to the  $\beta$ -



galactosidase activity. Each experiment was performed in triplicate (unless indicated otherwise) and repeated on at least two separate occasions. Statistical analysis was performed by Student's *t*-test.

**Quantitative Real-time PCR (qRT-PCR).** Total RNAs were isolated using Trizol (Invitrogen, Carlsbad, CA) and used as template for cDNA synthesis using Superscript II (Invitrogen). Using the cDNA as template, qRT-PCR was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA). The following primers were used: 5'-GAGAGAGGTGTCAGTGGTGGATCT-3' and 5'-AACAGCCACACGGATGCATA-3' for UGT1A4, 5'-GTCACGCCCTCCCAGTGT-3' and 5'-CGAACGGTGTCTCGTAAAC-3' for pS2, and 5'-ATCCTGGCCTCGCTGTCC-3' and 5'-CTCCTGCTTGCTGATCCACAT-3' for  $\beta$ -actin. The PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Amplified products were monitored by measuring the increase of fluorescence intensity from the SYBR green dye that binds to double-strand (ds) DNA amplification product. The dissociation curves for each reaction were examined to ensure amplification of a single PCR product in the reaction. The fold change in mRNA levels by drug treatment was determined after normalizing the gene expression levels by those of  $\beta$ -actin ( $2^{-\Delta\Delta C_t}$  method) (Schmittgen and Livak, 2008). Statistical analysis was performed by Student's *t*-test.

**Electrophoretic Mobility Shift Assays (EMSA).** 5'-Biotinylated sense and antisense oligonucleotides (-1918 to -1888; CTGTGCAGCCCAGGCCCTCCTCATCTCCA) harboring the putative Sp1 binding sequence of UGT1A4 (underlined) were annealed to

generate dsDNA probe. The labeled dsDNA probe (0.5 pmol) was incubated with 430 ng recombinant Sp1 protein (Promega) in 10  $\mu$ L of reaction buffer [12.5 mM HEPES-KOH (pH 7.5), 6.25 mM MgCl<sub>2</sub>, 10% glycerol, 0.05% NP-40, 5  $\mu$ M ZnSO<sub>4</sub>, 50 mM KCl, 50  $\mu$ g/ml BSA] (Pascal and Tjian, 1991). To determine the specificity of the binding to the DNA, competition experiments were conducted by co-incubation with unlabeled competitors or mithramycin. After 1 hr incubation at room temperature, protein-DNA complexes were separated on 6% nondenaturing polyacrylamide gel at 4 °C, transferred onto a nylon membrane, and visualized using streptavidin-conjugated horseradish peroxidase and chemiluminescence reagent (Amersham).

***Determination of Lamotrigine Glucuronide Concentration.*** Concentrations of lamotrigine 2N-glucuronide in cell culture media were determined by using LC/MS/MS (Applied Biosystems, 3200 Qtrap) equipped with electrospray ion source. Separation was performed with a Zorbax Eclipse XDB-C8 column (4.6  $\times$  50 mm, 3.5  $\mu$ m; Agilent Technologies) at a flow rate of 0.4 ml/min. The following linear gradient of the mobile phase, consisted of water [0.1% (v/v) formic acid] and methanol, was used for separation: 15% methanol at time 0 increased to 90% at 7 min. Lamotrigine glucuronide was detected in the positive ion mode by examining an ion pair of 432.2/256.0. Midazolam 4-hydroxide was used as an internal standard (ion pair of 341.9/324.0). The limit of quantification was approximately 1 ng/ml.

## Results

**Induction of UGT1A4 expression by E<sub>2</sub>.** To examine the effect of E<sub>2</sub> on the expression of UGT1A4, we initially used ER $\alpha$ -negative HepG2 cells transfected with a plasmid expressing ER $\alpha$  (pcDNA3-ER) or an empty plasmid (pcDNA3). Upon treatment of cells with E<sub>2</sub>, mRNA levels of UGT1A4 were determined by qRT-PCR. Expression level of pS2, a known estrogen-responsive gene (Barkhem et al., 2002), was determined as a positive control. In ER $\alpha$ -transfected HepG2 cells (called HepG2/pcDNA3-ER), pS2 expression was increased 7-fold by E<sub>2</sub> treatment as compared to vehicle treatment, confirming that our model system is responsive to E<sub>2</sub> (Fig. 1A). In the same cells, UGT1A4 expression increased significantly by E<sub>2</sub> treatment (>2 fold) as compared to vehicle-treated cells (Fig. 1A) whereas no induction was observed in cells transfected with pcDNA3. These results indicate that E<sub>2</sub> induces UGT1A4 expression through an ER $\alpha$ -mediated mechanism.

Transcriptional activation of UGT1A4 by E<sub>2</sub> was further examined by using a luciferase reporter system. We constructed a reporter plasmid pGL3-UGT1A4, which carries upstream region of UGT1A4 (from -2399 to +28) fused to a luciferase reporter gene. ER $\alpha$ -negative HepG2 cells were co-transfected with pGL3-UGT1A4 and pcDNA3-ER (or pcDNA3), and ER $\alpha$ -positive MCF7 cells were transfected with pGL3-UGT1A4. Each cell line was also transfected with pGL3-ERE3 as a positive control for estrogen responsiveness (see *Materials and Methods*) and pGL3-basic empty vector as a negative control. The transfected cells were then treated with either E<sub>2</sub> or vehicle control, and luciferase activity was determined.

The HepG2 and MCF7 cells transfected with control plasmids, pGL3-basic and pGL3-ERE3, respectively, displayed the expected response to E<sub>2</sub> (Fig. 1B). Upon treatment with E<sub>2</sub>, HepG2/pcDNA3-ER and MCF7 cells transfected with pGL3-UGT1A4 exhibited increased luciferase activity (Fig. 1B): 13-fold increase in HepG2/pcDNA3-ER cells and 2.5-fold increase in MCF7 cells as compared to vehicle-treated cells. Induction of UGT1A4 transcriptional activity in MCF7 cells was abrogated by treatment with ER $\alpha$ -degrading antiestrogen, ICI 182,780 (Osborne et al., 2004) (Fig. 1B), further supporting the notion that ER $\alpha$  mediates the upregulation of UGT1A4 expression by E<sub>2</sub>. In both HepG2/pcDNA3-ER and MCF7 cells, E<sub>2</sub> increased UGT1A4 transcriptional activity in a concentration-dependent manner (Fig. 1C). No significant induction by E<sub>2</sub> was observed in HepG2 cells transfected with pcDNA3 and MCF7 cells treated with antiestrogen. Taken together, these results demonstrate that E<sub>2</sub> upregulates UGT1A4 expression, and this induction requires ER $\alpha$ .

### **Identification of a *cis*-regulatory element required for UGT1A4 induction by E<sub>2</sub>.**

Because induction of UGT1A4 expression by E<sub>2</sub> required the presence of ER $\alpha$  (Fig. 1), we analyzed the transcriptional regulatory region of UGT1A4 for existence of potential EREs using Dragon ERE Finder and Possum programs that can also identify putative AP-1 and Sp1 binding sites (Bajic et al., 2003; Tang et al., 2004). Indeed, this analysis retrieved multiple putative EREs as well as AP-1 and Sp1 binding sites within the ~2.4-kb transcriptional regulatory region of UGT1A4.

To approximately map E<sub>2</sub>-responsive regulatory regions of UGT1A4, we analyzed the 2.4-kb upstream region of UGT1A4 by using luciferase reporter system. A

DNA fragment (2399 to -1643, -1667 to -863, or -862 to +28) that contains varying numbers of ERE or binding sites for AP-1 or Sp1 was fused to a constitutive thymidine kinase (tk) promoter linked to the luciferase gene (Fig. 2A). These luciferase constructs were transiently transfected into HepG2 (along with pcDNA3-ER) and MCF7 cells, and E<sub>2</sub>-responsiveness was determined. As shown in Fig. 2A, the -2399 to -863 of UGT1A4 transcription regulatory region was found to be responsible for the induction of UGT1A4 expression by E<sub>2</sub>. This region contains four putative EREs, one AP-1 and two Sp1 binding sites (Fig. 2B).

To specifically identify E<sub>2</sub>-responsive *cis*-element within -2399 to -863 region of UGT1A4, we systematically deleted potential binding sites of ER, AP-1 or Sp1 from the pGL3-UGT1A4 and examined for a loss of E<sub>2</sub>-responsiveness (Fig. 2B). A 5'-nested deletion construct of pGL3-UGT1A4 was transiently transfected into HepG2 cells along with pcDNA3-ER, and E<sub>2</sub>-responsiveness was determined. Deletion of a region containing the most distal ERE, -2399 to -1923, caused almost no change in E<sub>2</sub>-mediated induction of luciferase activity. However, subsequent deletion of a region carrying a putative Sp1-binding site, -1922 to -1887, led to a complete loss of E<sub>2</sub>-responsiveness (Fig. 2B). The essential role of the putative Sp1 binding site in UGT1A4 regulation by E<sub>2</sub> was further verified by using another luciferase reporter, pGL3-UGT1A4-Sp1 Del. This plasmid harbors the UGT1A4 transcriptional regulatory region (-2399 to +28) that lacks the Sp1 binding site (Fig. 2C). HepG2 cells transfected with pGL3-UGT1A4-Sp1 Del exhibited no response to E<sub>2</sub>, in contrast to those transfected with pGL3-UGT1A4 that contains the intact transcriptional regulatory region. Taken together, these results suggest

that the DNA sequence between -1922 and -1887, containing a putative Sp1 binding site, is required for the induction of UGT1A4 transcriptional activity by E<sub>2</sub>.

**Sp1 protein binds to the putative Sp1 binding site.** We next investigated whether Sp1 binds to the putative Sp1 binding site of UGT1A4 (called UGT1A4/Sp1). EMSA were performed using recombinant Sp1 and a DNA fragment (-1918 to -1888) containing UGT1A4/Sp1 as a probe. As previously reported (Pascal and Tjian, 1991), we observed multiple shifted bands that appear to represent homomultimeric complexes of Sp1 (Fig. 3). Direct binding of Sp1 to the UGT1A4/Sp1 was indicated by: (1) the appearance of mobility-shifted bands (lane 2), (2) disappearance of the bands upon competition by a unlabeled UGT1A4/Sp1 probe (lane 3-5) or a DNA probe containing a known consensus Sp1-binding site (lane 9), (3) inability of M1 and M2 probes (each containing mutated version of UGT1A4/Sp1 sequence) to compete for Sp1 binding (lane 6-7), and (4) partial blockade of the interaction by mithramycin (lane 10)—a known inhibitor of Sp1 binding to DNA.

M3 probe differed from the sequence of original UGT1A4/Sp1 probe at a single nucleotide position and bound Sp1 with lower affinity (lane 8). These observations, together with results obtained from luciferase reporter assay (Fig. 2C), suggest that direct binding of Sp1 is required for upregulation of UGT1A4 expression by E<sub>2</sub>.

**DNA binding domain of ER $\alpha$  is not required for UGT1A4 regulation by E<sub>2</sub>.** To investigate the role of ER $\alpha$  in upregulation of UGT1A4 transcription by E<sub>2</sub>, we used plasmid constructs containing one of the following mutated versions of ER $\alpha$ : (1) ER $\alpha$

with deletion of AF1, (2) ER $\alpha$  with point mutations in the DNA binding domain, (3) ER $\alpha$  with point mutations in the C-terminal AF2, and (4) ER $\alpha$  with deletion of AF1 and point mutations in AF2. The point mutations result in loss of functionality in the relevant domains (Harnish et al., 1998). HepG2 cells co-transfected with pGL3-UGT1A4 and one of the ER $\alpha$  mutants were examined for E<sub>2</sub>-responsiveness. HepG2 cells co-transfected with pGL3-ERE3 and one of the ER $\alpha$  mutants served as controls.

Deletion of the AF1 domain and/or mutation of the AF2 domain completely abolished the E<sub>2</sub>-responsiveness of UGT1A4 (Fig. 4A). In contrast, the E<sub>2</sub>-responsiveness of UGT1A4 was retained when the DNA-binding domain of ER $\alpha$  was nonfunctional (Fig. 4A). The same mutation led to a complete loss of E<sub>2</sub>-responsiveness in the cells transfected with pGL3-ERE3 (Fig. 4B). These results suggest that AF1 and AF2 domains of ER $\alpha$ , but not DNA binding domain, play a critical role in upregulation of UG1A4 expression by E<sub>2</sub>.

**Correlation between UGT1A4 transcription and enzymatic activity.** The E<sub>2</sub>-mediated upregulation of UGT1A4 expression was examined using lamotrigine as a UGT1A4 substrate. HepG2 cells transfected with pcDNA3-ER (or pcDNA3) were treated with E<sub>2</sub> (or ethanol) for 48 hr, and then lamotrigine was added to the culture media. After 24 hr incubation, the concentration of lamotrigine 2N-glucuronide in the media was determined. As shown in Fig. 5, in HepG2/pcDNA3-ER cells, E<sub>2</sub> treatment increased production of the glucuronide metabolite 3-fold in comparison to vehicle-treated cells. This result shows that the upregulation of UGT1A4 transcription by E<sub>2</sub> leads to increased UGT1A4 enzyme activity.

## Discussion

Clinical data show that oral clearance of lamotrigine increases in pregnancy by 50 to 90% (de Haan et al., 2004; Harden, 2007; Pennell et al., 2008). This may be attributed partially to fetal or placental expression of drug metabolizing enzymes. Substantial enzyme activity has been observed in fetal liver microsomes for certain UGTs (de Wildt et al., 1999). However, the similar degree of increase in lamotrigine clearance in women during pregnancy and oral contraceptive use (Sabers et al., 2003; Christensen et al., 2007) suggests a prominent role of UGT1A4 and/or UGT2B7 activity in the maternal liver in contributing to the pregnancy related changes.

Previously, we have shown that UGT2B7 mRNA expression is not influenced by E<sub>2</sub> in HepG2 cells (Jeong et al., 2008), ruling out potential upregulation of UGT2B7 expression by E<sub>2</sub>. In the present study, using HepG2 and MCF7 cells as our model systems, we show that E<sub>2</sub> upregulates UGT1A4 expression. We also attempted to use a more physiologically relevant system, freshly isolated human hepatocytes. However, we did not observe significant induction of UGT1A4 expression by E<sub>2</sub> in primary human hepatocytes (data not shown). This discrepancy between results obtained in HepG2 cells and primary human hepatocyte is not uncommon. In fact, transcription of most UGT1A genes in human hepatocytes is not readily inducible by typical enzyme inducers, including rifampin (Soars et al., 2004). For example, although clinical findings indicate upregulation of both CYP3A4 and UGT1A1 activities by rifampin, the fold increase in UGT1A1 activities by rifampin was negligible (0.5 to 1.4-fold) in comparison to the significant increase in CYP3A4 activity (2 to 17-fold) in human hepatocytes (Soars et al.,



2004). These results appear to suggest that to study upregulation of UGT1A expression, there is a need for a different model system than primary hepatocytes.

To elicit E<sub>2</sub> response in HepG2 or MCF7 cells, we used a relatively high concentration of E<sub>2</sub>, 1 μM (Fig. 1). This high concentration reflects physiological E<sub>2</sub> levels attained in pregnancy and potential accumulation of E<sub>2</sub> in the liver (Schleicher et al., 1998), the major site of E<sub>2</sub> metabolism. Interestingly, even at this high concentration, maximal effects of E<sub>2</sub> on ERα-mediated transactivation were not obtained in our experimental system (Fig. 1C). This is somewhat contrary to previous reports where maximal response to E<sub>2</sub> of its target genes was shown at 1-10 nM in MCF7 cells (Wijayarathne et al., 1999). Although the underlying mechanism for this discrepancy is unclear, differences in experimental conditions, e.g., use of different batches of charcoal-stripped FBS, may be responsible. Notably, our parallel study using pGL3-ERE3 exhibited a pattern of concentration dependency similar to that of pGL3-UGT1A4 (i.e., saturation not reached at 1 μM; data not shown) in HepG2/pcDNA3-ER cells, suggesting that the transcriptional regulatory region of UGT1A4 responds to E<sub>2</sub> in a similar manner as the consensus ERE does at least in our experimental system.

Our luciferase assay results obtained from tk promoter were slightly different from those from intact UGT1A4 promoter (Fig. 2A and 2B). UGT1A4 upstream region, -1666 to -863, exhibited E<sub>2</sub>-mediated induction in a tk promoter-based luciferase construct; an effect not verified using the native UGT1A4 promoter-based luciferase construct. This discrepancy likely arises from differential recruitment of transcription factors in two different promoters, which can subsequently influence interaction with transcription factors bound to upstream enhancers (Thomas and Chiang, 2006). This

observation emphasizes the importance of cross-validation of promoter assay results using native promoters.

Our data suggest that the UGT1A4-inducing activity of E<sub>2</sub> is likely mediated by ER $\alpha$  and Sp1. Previous studies report the role of E<sub>2</sub> as an inducer of expression of drug-metabolizing enzymes, including CYP1B1 and CYP2A6 (Tsuchiya et al., 2004; Higashi et al., 2007). Upregulation of CYP1B1 and CYP2A6 by E<sub>2</sub> is mediated by the classic mechanism of ER $\alpha$  action (Tsuchiya et al., 2004; Higashi et al., 2007); ER $\alpha$ , activated upon E<sub>2</sub> binding, translocates into the nucleus and, subsequently, induces the expression of target genes by binding to ERE in their transcriptional regulatory regions. On the other hand, in this study, the E<sub>2</sub> effect on UGT1A4 expression was found to be mediated by a non-classic mechanism of ER $\alpha$  action. Our results show that the induction of UGT1A4 by E<sub>2</sub> requires binding of Sp1 to a putative binding site within the transcriptional regulatory region of UGT1A4. Thus, Sp1, rather than ER $\alpha$ , is the likely factor directly binding to the UGT1A4 upstream sequence and activating UGT1A4 expression. Furthermore, the DNA binding domains of ER $\alpha$  is not required for induction of the UGT1A4 transcriptional activity by E<sub>2</sub> (Fig. 4) whereas the AF1 and AF2 domains are necessary (Fig. 4). The AF1 and AF2 domains are known to be critical for ER $\alpha$  dimerization and ligand binding, as well as ER $\alpha$  interaction with other transcriptional regulators, such as Sp1, to form a transcriptional complex (Sun et al., 1998; Castro-Rivera et al., 2001). In fact, ER $\alpha$  was previously shown to enhance formation and stability of the Sp1-DNA complex, although the direct formation of ternary ER $\alpha$ -Sp1-DNA complexes could not be detected (Safe and Kim, 2008). Similarly, in our study, it remains to be characterized how E<sub>2</sub>-activated ER $\alpha$  interacts

with Sp1 in the cells and whether factors additional to ER $\alpha$  are involved in ER $\alpha$ -Sp1 interaction for regulation of UGT1A4 expression.

The transcription factor, Sp1, plays a critical role in cell differentiation and proliferation. Sp1 also mediates hormonal regulation of gene expression (Safe and Kim, 2008). Interestingly, many drug-metabolizing enzyme genes contain putative Sp1 binding sites in their transcriptional regulatory regions (unpublished data, Yang and Jeong), suggesting that possibly Sp1 is broadly involved in controlling expression of drug metabolizing enzymes.

In conclusion, we show that UGT1A4 is upregulated by E<sub>2</sub> in a manner requiring both ER $\alpha$  and Sp1. The results suggest that increased expression of hepatic UGT1A4 resulting from elevated E<sub>2</sub> contributes to altered metabolism of UGT1A4 substrate drugs in pregnancy, and provide a potential mechanistic basis for the increased lamotrigine clearance in pregnancy and in oral contraceptive users.

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## References

- Anderson GD (2005) Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach. *Clin Pharmacokinet* **44**:989-1008.
- Bajic VB, Tan SL, Chong A, Tang S, Strom A, Gustafsson JA, Lin CY and Liu ET (2003) Dragon ERE Finder version 2: A tool for accurate detection and analysis of estrogen response elements in vertebrate genomes. *Nucleic Acids Res* **31**:3605-3607.
- Barkhem T, Haldosen LA, Gustafsson JA and Nilsson S (2002) pS2 Gene expression in HepG2 cells: complex regulation through crosstalk between the estrogen receptor alpha, an estrogen-responsive element, and the activator protein 1 response element. *Mol Pharmacol* **61**:1273-1283.
- Bjornstrom L and Sjoberg M (2005) Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* **19**:833-842.
- Castro-Rivera E, Samudio I and Safe S (2001) Estrogen regulation of cyclin D1 gene expression in ZR-75 breast cancer cells involves multiple enhancer elements. *J Biol Chem* **276**:30853-30861.
- Catherino WH and Jordan VC (1995) Increasing the number of tandem estrogen response elements increases the estrogenic activity of a tamoxifen analogue. *Cancer Lett* **92**:39-47.
- Christensen J, Petrenaite V, Atterman J, Sidenius P, Ohman I, Tomson T and Sabers A (2007) Oral contraceptives induce lamotrigine metabolism: evidence from a double-blind, placebo-controlled trial. *Epilepsia* **48**:484-489.

- Cunningham FG (2005) Implantation, Embryogenesis, and Placental Development, in *Williams obstetrics* pp 70-82, McGraw-Hill Medical Publishing Division, New York.
- de Haan GJ, Edelbroek P, Segers J, Engelsman M, Lindhout D, Devile-Notschaele M and Augustijn P (2004) Gestation-induced changes in lamotrigine pharmacokinetics: a monotherapy study. *Neurology* **63**:571-573.
- de Wildt SN, Kearns GL, Leeder JS and van den Anker JN (1999) Glucuronidation in humans. Pharmacogenetic and developmental aspects. *Clin Pharmacokinet* **36**:439-452.
- Ee PL, Kamalakaran S, Tonetti D, He X, Ross DD and Beck WT (2004) Identification of a novel estrogen response element in the breast cancer resistance protein (ABCG2) gene. *Cancer Res* **64**:1247-1251.
- EURAP Study Group (2006) Seizure control and treatment in pregnancy: observations from the EURAP epilepsy pregnancy registry. *Neurology* **66**:354-360.
- Harden CL (2007) Pregnancy and epilepsy. *Semin Neurol* **27**:453-459.
- Harnish DC, Evans MJ, Scicchitano MS, Bhat RA and Karathanasis SK (1998) Estrogen regulation of the apolipoprotein AI gene promoter through transcription cofactor sharing. *J Biol Chem* **273**:9270-9278.
- Harrington WR, Sengupta S and Katzenellenbogen BS (2006) Estrogen regulation of the glucuronidation enzyme UGT2B15 in estrogen receptor-positive breast cancer cells. *Endocrinology* **147**:3843-3850.

- Higashi E, Fukami T, Itoh M, Kyo S, Inoue M, Yokoi T and Nakajima M (2007) Human CYP2A6 is induced by estrogen via estrogen receptor. *Drug Metab Dispos* **35**:1935-1941.
- Hodge LS and Tracy TS (2007) Alterations in drug disposition during pregnancy: implications for drug therapy. *Expert Opin Drug Metab Toxicol* **3**:557-571.
- Jeong H, Choi S, Song JW, Chen H and Fischer JH (2008) Regulation of UDP-glucuronosyltransferase (UGT) 1A1 by progesterone and its impact on labetalol elimination. *Xenobiotica* **38**:62-75.
- Kawamoto T, Kakizaki S, Yoshinari K and Negishi M (2000) Estrogen activation of the nuclear orphan receptor CAR (constitutive active receptor) in induction of the mouse Cyp2b10 gene. *Mol Endocrinol* **14**:1897-1905.
- Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S and Gustafsson JA (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**:863-870.
- Linnet K (2002) Glucuronidation of olanzapine by cDNA-expressed human UDP-glucuronosyltransferases and human liver microsomes. *Hum Psychopharmacol* **17**:233-238.
- Makinen J, Reinisalo M, Niemi K, Viitala P, Jyrkkarinne J, Chung H, Pelkonen O and Honkakoski P (2003) Dual action of oestrogens on the mouse constitutive androstane receptor. *Biochem J* **376**:465-472.
- Ohman I, Beck O, Vitols S and Tomson T (2008) Plasma concentrations of lamotrigine and its 2-N-glucuronide metabolite during pregnancy in women with epilepsy. *Epilepsia* **49**:1075-1080.

- Osborne CK, Wakeling A and Nicholson RI (2004) Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *Br J Cancer* **90 Suppl 1**:S2-6.
- Pascal E and Tjian R (1991) Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism. *Genes Dev* **5**:1646-1656.
- Pennell PB, Peng L, Newport DJ, Ritchie JC, Koganti A, Holley DK, Newman M and Stowe ZN (2008) Lamotrigine in pregnancy: clearance, therapeutic drug monitoring, and seizure frequency. *Neurology* **70**:2130-2136.
- Rambeck B and Wolf P (1993) Lamotrigine clinical pharmacokinetics. *Clin Pharmacokinet* **25**:433-443.
- Reimers A, Helde G and Brodtkorb E (2005) Ethinyl estradiol, not progestogens, reduces lamotrigine serum concentrations. *Epilepsia* **46**:1414-1417.
- Rowland A, Elliot DJ, Williams JA, Mackenzie PI, Dickinson RG and Miners JO (2006) In vitro characterization of lamotrigine N2-glucuronidation and the lamotrigine-valproic acid interaction. *Drug Metab Dispos* **34**:1055-1062.
- Sabers A, Dam M, B AR-H, Boas J, Sidenius P, Laue Friis M, Alving J, Dahl M, Ankerhus J and Mouritzen Dam A (2004) Epilepsy and pregnancy: lamotrigine as main drug used. *Acta Neurol Scand* **109**:9-13.
- Sabers A, Ohman I, Christensen J and Tomson T (2003) Oral contraceptives reduce lamotrigine plasma levels. *Neurology* **61**:570-571.
- Safe S and Kim K (2008) Non-classical genomic estrogen receptor (ER)/specificity protein and ER/activating protein-1 signaling pathways. *J Mol Endocrinol* **41**:263-275.

- Schleicher F, Tauber U, Louton T and Schunack W (1998) Tissue distribution of sex steroids: concentration of 17beta-oestradiol and cyproterone acetate in selected organs of female Wistar rats. *Pharmacol Toxicol* **82**:34-39.
- Schmittgen TD and Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* **3**:1101-1108.
- Soars MG, Petullo DM, Eckstein JA, Kasper SC and Wrighton SA (2004) An assessment of udp-glucuronosyltransferase induction using primary human hepatocytes. *Drug Metab Dispos* **32**:140-148.
- Sun G, Porter W and Safe S (1998) Estrogen-induced retinoic acid receptor alpha 1 gene expression: role of estrogen receptor-Sp1 complex. *Mol Endocrinol* **12**:882-890.
- Tang S, Han H and Bajic VB (2004) ERGDB: Estrogen Responsive Genes Database. *Nucleic Acids Res* **32**:D533-536.
- Thomas MC and Chiang CM (2006) The general transcription machinery and general cofactors. *Crit Rev Biochem Mol Biol* **41**:105-178.
- Tsuchiya Y, Nakajima M, Kyo S, Kanaya T, Inoue M and Yokoi T (2004) Human CYP1B1 is regulated by estradiol via estrogen receptor. *Cancer Res* **64**:3119-3125.
- Wijayarathne AL, Nagel SC, Paige LA, Christensen DJ, Norris JD, Fowlkes DM and McDonnell DP (1999) Comparative analyses of mechanistic differences among antiestrogens. *Endocrinology* **140**:5828-5840.



## Footnotes

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## Figure legends

**Fig. 1.** Effect of E<sub>2</sub> on UGT1A4 expression. A. HepG2 cells were seeded onto a 12-well plate at  $1.5 \times 10^5$  cells/ml, and on the next day they were transfected with 0.6  $\mu$ g of pcDNA3-ER or control vector (pcDNA3) using Fugene 6 transfection reagent. After 16-18 hr, the cells were treated with E<sub>2</sub> (1  $\mu$ M) or vehicle control (ethanol). Following 72 hr incubation, mRNA levels of pS2 and UGT1A4 were determined by qRT-PCR and normalized by those of  $\beta$ -actin. Results represent fold change relative to vehicle control (mean  $\pm$  S.D.; n = 3). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . B. HepG2 cells were transfected with luciferase constructs (pGL3-basic, pGL3-ERE3 or pGL3-UGT1A4) and pcDNA3-ER (or pcDNA3), along with  $\beta$ -galactosidase expression plasmid (for normalization of transfection efficiency). MCF7 cells were transfected with the luciferase constructs and  $\beta$ -galactosidase expression plasmid, and subsequently treated with antiestrogen ICI 182,780 (or ethanol). The transfected HepG2 or MCF7 cells were treated with 1  $\mu$ M E<sub>2</sub> (or ethanol) for 24 hr, and luciferase assay were performed (see *Materials and Methods*). Results represent fold change relative to vehicle control (mean  $\pm$  S.D.; n = 3). \*\*,  $p < 0.01$ . C. HepG2 cells were co-transfected with pGL3-UGT1A4, pcDNA3-ER (or pcDNA3), and  $\beta$ -galactosidase expression plasmid. MCF7 cells were transfected with pGL3-UGT1A4 and  $\beta$ -galactosidase expression plasmid, and subsequently treated with ICI 182,780 (or ethanol). The transfected cells were treated with E<sub>2</sub> in different concentrations, and luciferase assay was performed. Data presented are the mean of results obtained from a duplicate experiment.

**Fig. 2.** Transcriptional regulatory region mediating UGT1A4 induction by E<sub>2</sub>. A. Luciferase constructs containing different segments of UGT1A4 5'-flanking region proximal to tk promoter were co-transfected into HepG2 cells with pcDNA3-ER (or pcDNA3) and β-galactosidase expression plasmid. pGL3tk is a luciferase vector containing tk promoter only. MCF7 cells were transfected with the luciferase constructs and β-galactosidase expression plasmid, and treated with ICI 182,780 (or ethanol). The transfected HepG2 or MCF7 cells were treated with 1 μM E<sub>2</sub> (or ethanol) for 24 hr, and luciferase assay was performed. Results represent fold change relative to vehicle control (mean ± S.D.; n = 3). \*, *p* < 0.05; \*\*, *p* < 0.01. B. HepG2 cells were co-transfected with pcDNA3-ER (or pcDNA3), 5'-nested deletion constructs of pGL3-UGT1A4, and β-galactosidase expression plasmid. The transfected HepG2 cells were treated with 1 μM E<sub>2</sub> (or ethanol) for 24 hr, and luciferase assay was performed. Results represent fold change relative to vehicle control (mean ± S.D.; n = 3). \*\*, *p* < 0.01 vs. ethanol treated group. C. HepG2 cells were co-transfected with pcDNA3-ER (or pcDNA3), β-galactosidase expression plasmid, and pGL3-UGT1A4-Sp1 Del where the putative Sp1 binding site (underlined) of pGL3-UGT1A4 was deleted. The transfected cells were treated with 1 μM E<sub>2</sub> (or ethanol) for 24 hr, and luciferase assay was performed. Results represent fold change relative to vehicle control (mean ± S.D.; n = 3). \*\*, *p* < 0.01

**Fig. 3.** Binding of Sp1 to the putative Sp1 binding site in the UGT1A4 upstream region. EMSA was performed using recombinant Sp1 protein and biotinylated probe (-1918 to -1888) containing UGT1A4/Sp1. Upon observing multiple bands on the gel (see *text* for more details), we employed the electrophoresis running time long enough to release

unbound (free) DNA from the gel to better resolve the bands (thus free DNA probes are not visible on the gel). Unlabeled UGT1A4/Sp1 probe was used as competitors at 5-, 10-, and 40-fold molar excess (lanes 3-5). Also, unlabeled UGT1A4/Sp1 probes containing the mutated Sp1 binding sequences (M1, M2, and M3; lanes 6-8) or unlabeled DNA probe containing consensus Sp1 binding sequence (C, lane 9) was used as competitors at 40-fold molar excess. Mithramycin, a known inhibitor of Sp1 binding to DNA, was added to the binding reaction at 100 nM (lane 10). Underlined sequences are putative or consensus Sp1 binding sites of probes. The antisense strand of consensus Sp1 binding sequence was shown for better visualization of sequence homology to UGT1A4/Sp1.

**Fig. 4.** Role of ER $\alpha$  domains in UGT1A4 induction by E<sub>2</sub>. HepG2 cells were co-transfected with pGL3-UGT1A4 (A) or pGL3-ERE3 (B) along with  $\beta$ -galactosidase expression plasmid and one of plasmids containing ER $\alpha$  mutants (see *text* for details). The black box domain represents inactivation by point mutations. The transfected cells were treated with 1  $\mu$ M E<sub>2</sub> (or ethanol), and luciferase assay was performed. Results represent fold change relative to vehicle control (mean  $\pm$  S.D.; n = 3). DBD, DNA-binding domain. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  vs. cells transfected with wild-type ER $\alpha$ .

**Fig. 5.** Effect of E<sub>2</sub> on lamotrigine glucuronidation. HepG2 cells were seeded onto a 12-well plate at  $1.5 \times 10^5$  cells/ml, and on the next day they were transfected with 0.6  $\mu$ g of pcDNA3-ER (or pcDNA3) using Fugene 6. After 3-8 hr, the cells were treated with E<sub>2</sub> (1  $\mu$ M) or ethanol vehicle. Following 48 hr incubation, the media were changed to contain lamotrigine at a final concentration of 40  $\mu$ M. After 24 hr incubation, the media were

collected and concentrations of lamotrigine 2N-glucuronide (LMT-G) in the media were determined by LC/MS/MS. Data presented are concentrations of LMT-G in the media (mean  $\pm$  S.D.; n = 3). \*\*,  $p < 0.01$

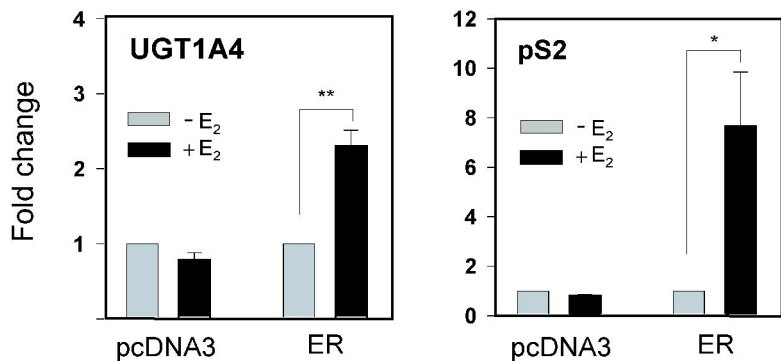
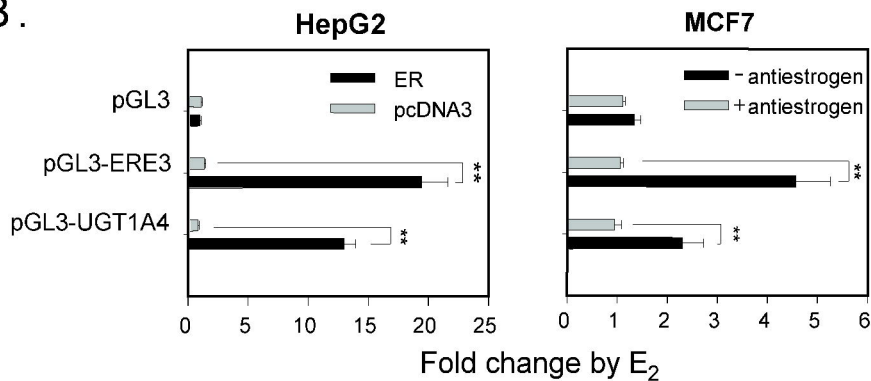
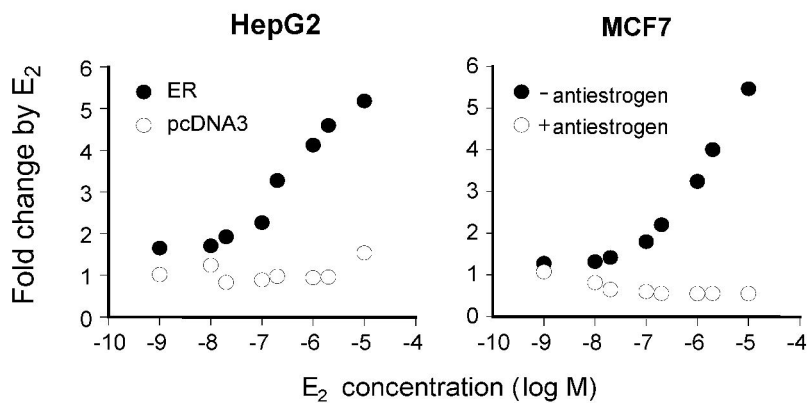
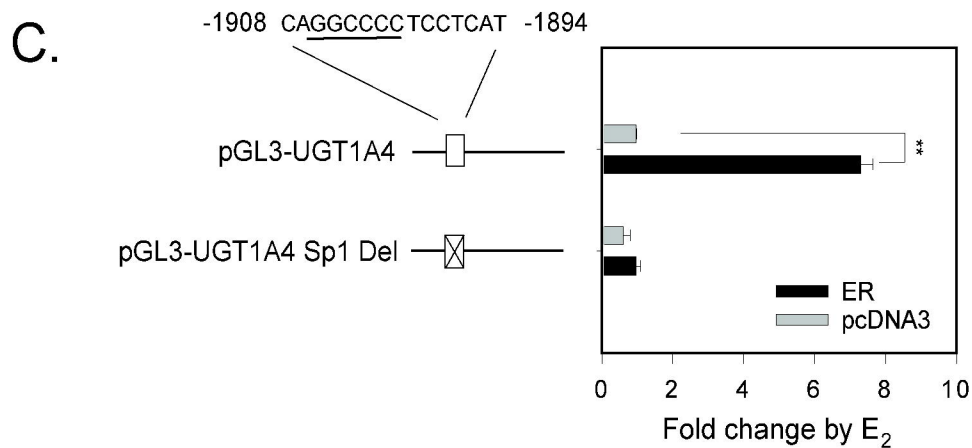
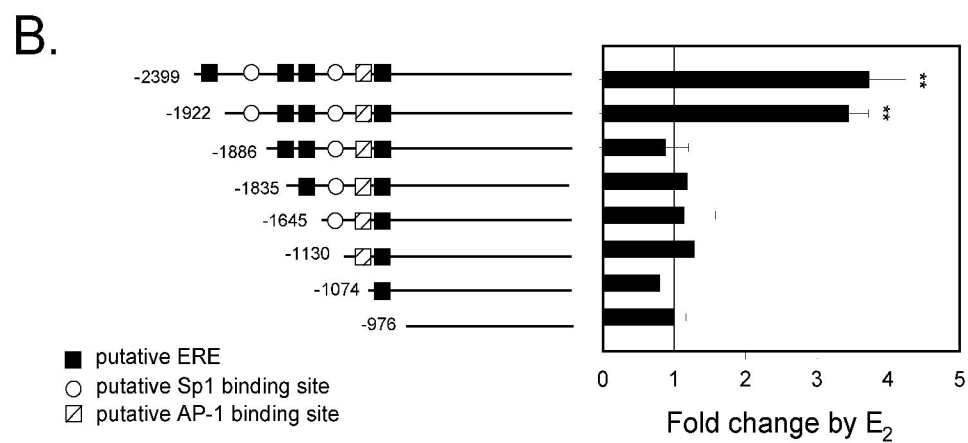
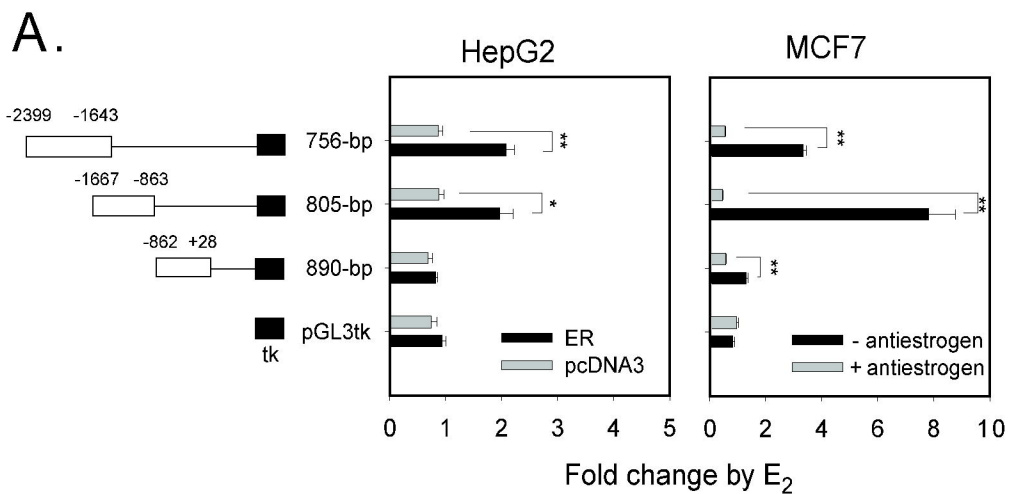
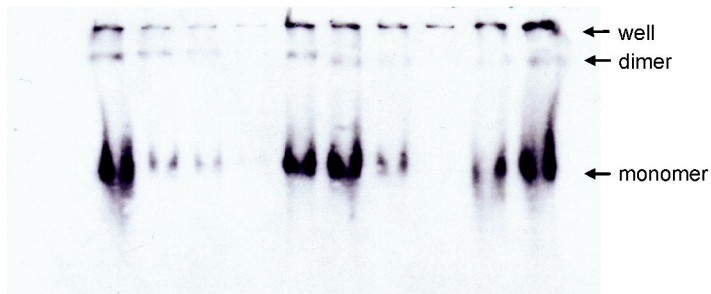
**Fig. 1****A.****B.****C.**

Fig. 2



# Fig. 3

	1	2	3	4	5	6	7	8	9	10	11
Sp1	-	+	+	+	+	+	+	+	+	+	+
UGT1A4 probe	+	+	+	+	+	+	+	+	+	+	+
Competitor	-	-	1A4	1A4	1A4	M1	M2	M3	C	Mithra	-



UGT1A4/Sp1	CTGTGCAGCCCAG <u>G</u> CCCCCTCCTCATCTCCA
M1	CTGTGCAGCCCAGAAACCTCCTCATCTCCA
M2	CTGTGCAGCCCAGAAAAATCCTCATCTCCA
M3	CTGTGCAGCCCAG <u>A</u> CCCCCTCCTCATCTCCA
Consensus	GCTCGCCCCG <u>CC</u> CCGATCGAAT



Fig. 4

A. UGT1A4

B. ERE3

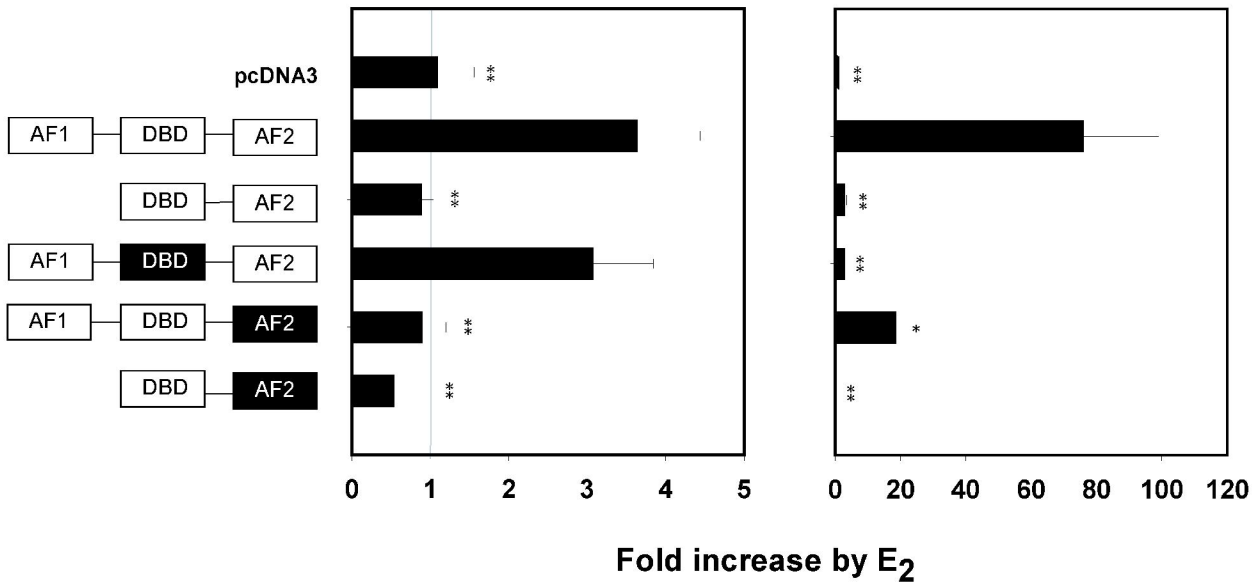


Fig. 5

