Induction of UDP-Glucuronosyltransferase 2B15 Gene Expression by the Major Active Metabolites of Tamoxifen, 4-Hydroxytamoxifen and Endoxifen, in Breast Cancer Cells

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Received December 22, 2014; accepted March 19, 2015

ABSTRACT

We previously reported upregulation of UGT2B15 by 17β-estradiol in breast cancer MCF7 cells via binding of the estrogen receptor α (ERα) to an estrogen response unit (ERU) in the proximal UGT2B15 promoter. In the present study, we show that this ERα-mediated upregulation was significantly reduced by two ER antagonists (fulvestrant and raloxifene) but was not affected by a third ER antagonist, 4-hydroxytamoxifen (4-OHTAM), a major active tamoxifen (TAM) metabolite. Furthermore, we found that, similar to 17β-estradiol, 4-OHTAM and endoxifen (another major TAM metabolite) elevated UGT2B15 mRNA levels, and that this stimulation was significantly abrogated by fulvestrant. Further experiments using 4-OHTAM revealed a critical role for ERα in this regulation. Specifically, knockdown of ERα expression by anti-ERα small interfering RNA reduced the 4-OHTAM-mediated induction of UGT2B15 expression; 4-OHTAM activated the wild-type but not the ERU-mutated UGT2B15 promoter; and chromatin immunoprecipitation assays showed increased ERα occupancy at the UGT2B15 ERU in MCF7 cells upon exposure to 4-OHTAM. Together, these data indicate that both 17β-estradiol and the antiestrogen 4-OHTAM upregulate UGT2B15 in MCF7 cells via the same ERα-signaling pathway. This is consistent with previous observations that both 17β-estradiol and TAM upregulate a common set of genes in MCF7 cells via the ER-signaling pathway. As 4-OHTAM is a UGT2B15 substrate, the upregulation of UGT2B15 by 4-OHTAM in target breast cancer cells is likely to enhance local metabolism and inactivation of 4-OHTAM within the tumor. This represents a potential mechanism that may reduce TAM therapeutic efficacy or even contribute to the development of acquired TAM resistance.

Introduction

Breast cancer is the most common cancer in women and a leading cause of cancer mortality worldwide (Jemal et al., 2011; Youlden et al., 2012). The majority of breast cancers are estrogen receptor (ER)–positive and depend on ER signaling for their proliferation (Miller et al., 2008). Blockage of ER activation by antiestrogens (e.g., tamoxifen) and/or suppression of estrogen synthesis by aromatase inhibitors (e.g., exemestane) represent the principal endocrine therapy for ER-positive breast cancer (Burstein et al., 2014). Five years of tamoxifen therapy has been the gold-standard adjuvant treatment of ER-positive breast cancer for many decades (Burstein et al., 2014). Tamoxifen (TAM) is orally administered (20 mg daily) and is converted in vivo by cytochrome P450s (primarily CYP2D6 and CYP3A4) into several metabolites, including 4-hydroxytamoxifen (4-OHTAM) and 4-hydroxy-N-desmethyltamoxifen (endoxifen) (Jacolot et al., 1991; Dehal and Kupfer, 1997). Both 4-OHTAM and endoxifen exist as geometrical isoforms and have a likely trans-to-cis ratio of 70:30 at physiologic pH (Malet et al., 2002; Zheng et al., 2007). As both trans isomers of 4-OHTAM and endoxifen have similar antiestrogenic activity, which is nearly 100-fold greater than that of TAM, it is believed that TAM elicits its antiestrogenic activity primarily through its active metabolites 4-OHTAM and endoxifen (Jordan et al., 1977; Furr and Jordan, 1984; Katzenellenbogen et al., 1984; Johnson et al., 2004; Lim et al., 2005; Borges et al., 2006). Although evidence exists supporting cis–4-OHTAM as a weak estrogenic compound (Williams et al., 1994), cumulative studies have provided strong evidence that, similar to trans–4-OHTAM, cis–4-OHTAM acts as an estrogen antagonist in normal and cancerous breast cells (Katzenellenbogen et al., 1984, 1985; Malet et al., 2002). A recent study has shown that both trans- and cis–4-OHTAM displayed roughly equipotent antiestrogenic effects on 17β-estradiol–induced progesterone receptor gene expression in MCF7 cells (Zheng et al., 2007).

UDP-glucuronosyltransferases (UGTs), an enzyme superfamily, are responsible for the glucuronidation of numerous therapeutic drugs, including TAM, 4-OHTAM, and endoxifen. N-glucuronidation of TAM and 4-OHTAM at the aminoethoxy side chain has been recently reported to be exclusively carried out by UGT1A4, and the resulting N-glucuronides were found to have ER binding affinities similar to those of their parent counterparts, suggesting that these N-glucuronides might

AABBREVIATIONS: ChIP, chromatin immunoprecipitation; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; ERU, estrogen response unit; FBS, fetal bovine serum; FUL, fulvestrant; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LBD, ligand-binding domain; MT, mutated construct; 4-OHTAM, 4-hydroxytamoxifen; PCR, polymerase chain reaction; siRNA, small interfering RNA; SULT, sulfotransferase; TAM, tamoxifen; UGT, UDP-glucuronosyltransferase; WT, wild-type.
contribute to antiestrogenic activity of TAM in vivo (Kaku et al., 2004; Ogura et al., 2006; Sun et al., 2007; Zheng et al., 2007). By contrast, O-glucuronidation of 4-OHTAM and endoxifen at the 4-hydroxy position has been shown to be carried out by multiple UGTs (Nishiyama et al., 2002; Ogura et al., 2006; Sun et al., 2007; Zheng et al., 2007). This reaction greatly decreases the ER binding affinity of the resultant O-glucuronides and thus represents an inactivation pathway (Ogura et al., 2006; Zheng et al., 2007). Studies using recombinant human UGT isoforms expressed in insect cells have shown O-glucuronidation of both 4-OHTAM isomers by six UGTs (1A1, 1A3, 1A8, 1A9, 2B7, and 2B15), with UGT2B7 and UGT2B15 having the highest activity toward trans-4-OHTAM and cis-4-OHTAM, respectively (Nishiyama et al., 2002; Ogura et al., 2006). A later study using homogenates of individual UGT-overexpressing cell lines showed similar results and further revealed O-glucuronidation activity of 4-OHTAM by three other UGTs (1A7, 1A10, and 2B17) (Sun et al., 2007). Functional polymorphisms in the regulatory and coding regions of three UGT genes (1A4, 1A8, and 2B7) have been shown to be related to differing glucuronidation activity toward TAM and/or its metabolites (Sun et al., 2006; Blevins-Primeau et al., 2009; Greer et al., 2014).

The majority of ER-positive breast cancers respond to TAM but often develop resistance following prolonged TAM administration. This acquired resistance remains a challenge for treating ER-positive breast cancer; however, the underlying molecular mechanisms are not completely known but are thought to be multifactorial, including loss of ER expression and function, altered expression of ER coregulators, activation of ER-independent signaling pathways, and altered metabolism of tamoxifen in the tumor (Chang, 2012; Viedma-Rodriguez et al., 2014). With regard to metabolic changes, it has been reported that tamoxifen upregulates sulfotransferase 1A1 (SULT1A1) in breast cancer cells (Seth et al., 2002). As 4-OHTAM is a SULT1A1 substrate (Nishiyama et al., 2002), this upregulation promotes metabolism and clearance of 4-OHTAM through sulfation. In the present study, we demonstrate that both 4-OHTAM and endoxifen upregulate UGT2B15 in breast cancer cells, and we define the underlying molecular mechanisms of this regulation. Induction of UGT2B15 could facilitate metabolism and clearance of 4-OHTAM through glucuronidation and may contribute to acquired tamoxifen resistance.

Materials and Methods

Materials. Chemical compounds of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO), including 17β-estradiol (E2), Z-4-hydroxytamoxifen (4-OHTAM), raloxifene hydrochloride, fulvestrant ((7α,17β)-7-{9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl}estra-1,3,5(10)-triene-3,17-diol), and...
(EZ)-endoxifen hydrochloride hydrate. Primers were synthesized by Sigma-Genosys (Castle Hill, NSW, Australia).

**Cell Culture, Drug Treatment, RNA Extraction, and Reverse-Transcriptase Quantitative Real-Time Polymerase Chain Reaction.** The human breast cancer MCF7 cell line was routinely maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum (FBS) at 37°C and 5% CO₂. For ligand treatment, cells were plated into six-well plates and cultured for 3–4 days to reach approximately 70% confluence. Cells were further cultured in phenol red–free RPMI media supplemented with 5% dextran charcoal–stripped FBS for 2–3 days and then treated with 17β-estradiol and/or antiestrogens in triplicate at concentrations and lengths of time as indicated in the figures. Total RNA was isolated using the RNeasy MiniKit (Qiagen, Valencia, CA) or TRIzol reagent (Life Technologies) and converted to cDNA using reverse-transcription reagents (Invitrogen, Mulgrave, VIC, Australia) as previously reported (Hu and Mackenzie, 2009). Quantitative real-time polymerase chain reaction (PCR) was performed using a RotorGene 3000 (Corbett Research, NSW, Australia) and either QuantiTect SYBR Green PCR master mix (Qiagen) or GoTaqqPCR master Mix (Promega, Madison, WI) in a 20-μl reaction containing ~60 ng of cDNA sample and previously designed primers for target genes, including UGT2B15, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pS2, and GREB1 (Hu and Mackenzie, 2009, 2010; Hu et al., 2010). The target gene mRNA levels relative to those of 18S ribosomal RNA were quantified using the delta delta cycle threshold method (Livak and Schmittgen, 2001).

**Glucuronidation Assay.** MCF7 cells at approximately 80% confluence were cultured in phenol red–free RPMI media containing 5% dextran charcoal–stripped FBS for 3 days and then treated in triplicate with vehicle (0.1% ethanol), 10 nM E2, or 1 μM 4-OHTAM for 24 hours. Cells were then harvested for preparation of whole-cell lysates using TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). Protein concentrations of the resultant lysates were determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Testosterone glucuronidation assays were conducted at 37°C for 4 hours in a 100-μl reaction containing 200 μg of protein of each lysate, 2 mM UDP-glucuronic acid, 15 pmol/μl [14C]testosterone, 100 mM potassium phosphate (pH 7.5), and 4 mM of MgCl₂. Reactions were terminated by addition of 300 μl of chloroform. Free testosterone was then extracted by vortex and centrifugation at 12,000 rpm for 5 minutes. The aqueous phase containing glucuronide was transferred to a fresh tube and diluted with an equal volume of ethanol (50% v/v). Eighty microliters of the resultant solution was applied to a thin layer chromatography (TLC) plate (250 μm silica gel; TLC Uniplates; Analtech, Newark, DE). Chromatography was conducted in solvent containing chloroform, methanol, water, and acetic acid (65:25:4:2, v/v/v/v) for 1 hour. The dried TLC plate was exposed to a phosphor screen (Molecular Dynamics; GE Healthcare Life Sciences) for 4 days and then scanned using a Typhoon 9000 detector (GE Healthcare, Chalfont St. Giles, UK). Quantitation of band intensity and background subtraction were conducted using ImageQuant version 5.2 (Molecular Dynamics; GE Healthcare Life Sciences). The testosterone glucuronidation activity in the E2- or 4-OHTAM–treated cells was presented as fold changes relative to the activity in vehicle-treated cells (set as a value of 1).

**Knockdown of Gene Expression Using Small Interfering RNA.** ON-TARGETplus nontargeting pool small interfering RNA (siRNA; control siRNA, negative control-siRNA) and ON-TARGETplus SMARTpool against ERα (ERα-siRNA) were purchased from Dharmacon RNAi Technologies (Lafayette, CO). MCF7 cells were transfected in quadruplicate in six-well plates with either negative control-siRNA or ERα-siRNA at 100 nM using Lipofectamine 2000 (Invitrogen) as previously reported (Hu and Mackenzie, 2009). Forty-eight hours posttransfection, one well was harvested to prepare whole-cell lysates for Western blotting as described later, and the remaining wells were treated for 24 hours with vehicle, 1 nM E2, or 1 μM 4-OHTAM. After treatment, wells were harvested for total RNA, followed by reverse-transcriptase quantitative real-time PCR to quantify the mRNA levels of three target genes (UGT2B15, pS2, and GAPDH) as described earlier.

**Western Blotting.** Whole-cell lysates were prepared from drug-treated and siRNA-transfected MCF7 cells in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 2 mM EDTA, 0.5% sodium deoxycholate, and 0.1% SDS]. Protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Testosterone glucuronidation assays were conducted at 37°C for 4 hours in a 100-μl reaction containing 200 μg of protein of each lysate, 2 mM UDP-glucuronic acid, 15 pmol/μl [14C]testosterone, 100 mM potassium phosphate (pH 7.5), and 4 mM of MgCl₂. Reactions were terminated by addition of 300 μl of chloroform. Free testosterone was then extracted by vortex and centrifugation at 12,000 rpm for 5 minutes. The aqueous phase containing glucuronide was transferred to a fresh tube and diluted with an equal volume of ethanol (50% v/v). Eighty microliters of the resultant solution was applied to a thin layer chromatography (TLC) plate (250 μm silica gel; TLC Uniplates; Analtech, Newark, DE). Chromatography was conducted in solvent containing chloroform, methanol, water, and acetic acid (65:25:4:2, v/v/v/v) for 1 hour. The dried TLC plate was exposed to a phosphor screen (Molecular Dynamics; GE Healthcare Life Sciences) for 4 days and then scanned using a Typhoon 9000 detector (GE Healthcare, Chalfont St. Giles, UK). Quantitation of band intensity and background subtraction were conducted using ImageQuant version 5.2 (Molecular Dynamics; GE Healthcare Life Sciences). The testosterone glucuronidation activity in the E2- or 4-OHTAM–treated cells was presented as fold changes relative to the activity in vehicle-treated cells (set as a value of 1).

**Statistical Analysis.** After treatment, wells were harvested for total RNA, followed by reverse-transcriptase quantitative real-time PCR of three target genes (UGT2B15, pS2, and GAPDH) as described earlier. Data shown are the fold induction (mean ± S.E.M.) in target gene mRNA levels in cells treated with E2 or 4-OHTAM over those in vehicle-treated cells (set as a value of 1) from three independent experiments performed in triplicate. (B) Cells were treated with vehicle (0.1% ethanol), 10 nM E2, or 1 μM 4-OHTAM for 24 hours. Cell lysates were prepared and then subjected to testosterone glucuronidation assays as described in Materials and Methods. Data shown are the fold induction (mean ± S.E.M.) of testosterone glucuronidation activity in cells treated with E2 or 4-OHTAM compared with vehicle-treated cells in two independent experiments, carried out in triplicate. Statistical analyses used one-way analysis of variance followed by Tukey’s post-hoc multiple comparison test. *P < 0.001.

**Fig. 2.** 4-Hydroxytamoxifen elevates UGT2B15 mRNA levels and enzymatic activity in breast cancer MCF7 cells. (A) MCF7 cells were treated in triplicate with vehicle (0.1% ethanol), 1 nM E2, or 1 μM 4-OHTAM for 24 hours. Total RNA was extracted and then subjected to reverse-transcriptase quantitative PCR of three target genes (UGT2B15, pS2, and GAPDH) as described in Materials and Methods. Data shown are the fold induction (mean ± S.E.M.) in target gene mRNA levels in cells treated with E2 or 4-OHTAM over those in vehicle-treated cells (set as a value of 1) from three independent experiments performed in triplicate. (B) Cells were treated with vehicle (0.1% ethanol), 10 nM E2, or 1 μM 4-OHTAM for 24 hours. Cell lysates were prepared and then subjected to testosterone glucuronidation assays as described in Materials and Methods. Data shown are the fold induction (mean ± S.E.M.) of testosterone glucuronidation activity in cells treated with E2 or 4-OHTAM compared with vehicle-treated cells in two independent experiments, carried out in triplicate. Statistical analyses used one-way analysis of variance followed by Tukey’s post-hoc multiple comparison test. *P < 0.001.
the Bradford protein assay (Bio-Rad). Fifty micrograms of protein in each sample was separated on SDS-polyacrylamide gels (10%) and transferred to nitrocellulose membranes for immunodetection. Membranes were probed first with an anti-ERα antibody (HC-20; Santa Cruz Biotechnology, Dallas, TX), and then with a horseradish peroxidase–conjugated donkey anti-rabbit secondary antibody (Neomarkers; Fremont, CA). The same membranes were reprobed with an antiactin antibody (Sigma-Aldrich) followed by the previously described anti-rabbit secondary antibody. Immunosignals were detected with the SuperSignalWest Pico Chemiluminescent kit (Thermo Fisher Scientific, Waltham, MA) and an ImageQuant LAS 4000 luminescent image analyzer (GE Healthcare, Chalfont St. Giles, UK). Quantitation of band intensity and background subtraction were conducted using MultiGauge version 3.0 (Fujifilm Corporation, Tokyo, Japan).

Transcript Transfection and Luciferase Reporter Assay. The three pGL3 luciferase UGT2B15 promoter constructs containing the region between nucleotides −458 and −3 relative to the translation start site used in this study were reported previously (Hu and Mackenzie, 2009). As shown in Fig. 4A, the UGT2B15-458 wild-type (WT) construct had a functional wild-type estrogen response element (ERE) half sites, and an imperfect ERE full site (Hu and Mackenzie, 2009). This ERE was mutated at three sites in two other constructs as indicated [UGT2B15-458-mutated construct 15 (MT15) and UGT2B15-458-MT16]. MCF7 cells (3 × 104 cells/well) were seeded into 96-well plates 24 hours prior to transfection. Each well was transfected with the transfection mixture in 50 μl of phenol red-free RPMI containing pRL-null vector (2 ng), a UGT2B15 promoter construct (100 ng), and with or without an ERα expression vector (2.5 ng) using Lipofectamin 2000 (Invitrogen). The ERα expression vector was the same as we previously reported (Hu and Mackenzie, 2009). Six hours posttransfection, the transfection medium was removed, and cells were then treated in triplicate in stripped FBS containing RPMI media with vehicle (0.1% ethanol), 10 nM E2, or 1 μM 4-OHTAM for 48 hours. After treatment, cells were lysed and analyzed using the Dual-Luciferase Reporter Assay System (Promega) as previously reported.

Fig. 3. 4-Hydroxytamoxifen induces UGT2B15 gene expression via the ERα-signaling pathway in MCF7 cells. (A) MCF7 cells were treated in triplicate for 24 hours with vehicle, 1 μM fulvestrant (FUL), 1 nM E2, 1 μM 4-OHTAM, 1 nM E2 plus 1 μM FUL, or 1 μM 4-OHTAM plus 1 μM FUL and then harvested for total RNA, followed by reverse-transcriptase quantitative real-time PCR to quantify the mRNA levels of three target genes (UGT2B15, pS2, and GAPDH). Data shown are the fold induction (mean ± S.E.M.) in target gene mRNA levels in drug-treated cells over those in vehicle (ethanol)-treated cells (set as a value of 1) from two independent experiments performed in triplicate. Statistical analyses used one-way analysis of variance followed by Tukey’s post hoc multiple comparison test. (B and C) MCF7 cells were cultured in six-well plates, and four wells were transfected and treated with NC-siRNA/E2, NC-siRNA/4-OHTAM, or ERα-siRNA/E2, NC-siRNA/4-OHTAM, or ERα-siRNA/4-OHTAM over those in cells transfected and treated with NC-siRNA/vehicle (set as a value of 1) from two experiments performed in triplicate. Statistical analyses used the Kruskal-Wallis test followed by Mann-Whitney U Test. #P < 0.05; $P < 0.01; *P < 0.001.
(Hu and Mackenzie, 2009). The firefly luciferase activity was first normalized to the Renilla activity and then presented as fold changes relative to those observed in cells transfected with the promoterless pGL3 basic vector and treated with the same chemical (set as a value of 1).

**Chromatin Immunoprecipitation (ChIP) – Quantitative Real-Time PCR.** Chromatin immunoprecipitation (ChIP)–quantitative PCR was performed essentially as reported previously (Hu and Mackenzie, 2009). In brief, MCF7 cells were precultured in T175 flasks in phenol red–free RPMI media containing 5% stripped FBS for 2 days and then treated in triplicate with vehicle (0.1% ethanol), 10 nM E2, or 1 μM M 4-OHTAM for 2 hours. Following treatment, cells were cross-linked using 1% formaldehyde at 37°C for 30 minutes, followed by quenching using 125 mM glycine solution at 37°C for 10 minutes. Cells were lysed, sonicated, and then subjected to immunoprecipitation with 8 μg of the rabbit preimmune IgG control (sc-2027; Santa Cruz Biotechnology) or equivalent amounts of the anti-ERα antibody (HC-20). Precipitated chromatin was captured by Protein A Sepharose CL-4B beads (GE Healthcare) and subsequently eluted from the beads as described previously (Hu and Mackenzie, 2009). Cross-linking was reversed by heating the eluates at 65°C overnight. The resulting DNA/protein precipitates were digested with proteinase K, followed by phenol–chloroform extraction and ethanol precipitation. The DNA pellets were resuspended in 50 μl of Tris-EDTA buffer. Using 3 μl of each of the resultant DNA samples as template and previously reported primers (Carroll et al., 2006; Hu and Mackenzie, 2009), real-time quantitative PCR was conducted to quantify the UGT2B15 ERU region and two control loci (ERE negative 1 and 2) that have been shown to have no ERα binding upon E2 exposure in MCF7 cells. Data from ERE negative locus 1 were used to normalize the starting amounts of immunoprecipitated DNA added to each PCR. The ERα binding activity in ligand-treated cells at the UGT2B15 ERU and ERE negative locus 2 was presented as fold changes relative to those observed in the respective vehicle-treated cells using the ΔΔCt method (Livak and Schmittgen, 2001).

**Statistical Analysis.** Statistical analysis was performed using the IBM (Armonk, NY) SPSS program (version 22) as we recently reported (Hu et al., 2015). In brief, log-transformed data met the assumption of equal variance at a significance level of less than 0.01 (Levene’s test) unless otherwise stated. Statistical analyses were conducted by one-way or two-way analysis of variance followed by Tukey’s post-hoc test or the Kruskal-Wallis test (nonparametric alternative for one-way analysis of variance) followed by Mann-Whitney U test as detailed in the legends of the figures. A P value less than 0.05 was considered statistically significant.

**Results**

**4-Hydroxytamoxifen Elevates UGT2B15 mRNA Levels and Enhances Testosterone Glucuronidation Activity in Breast Cancer Cells.** We previously reported that E2 induces UGT2B15 gene expression via binding of the ERα to the UGT2B15 ERU at the proximal promoter in MCF7 breast cancer cells (Hu and Mackenzie, 2009). In the present study, as shown in Fig. 1A, we found that this

![Fig. 4. 17β-Estradiol and 4-hydroxytamoxifen activate the UGT2B15 promoter through the ERU at the proximal promoter. (A) The UGT2B15 promoter between nucleotides -458/-3 relative to the translation start site has a functional ERU that contains two AP-1 binding sites, two half sites of ERE, and an imperfect ERE full site. Shown are three UGT2B15 pGL3-driven promoter constructs containing a wild-type ERU (UGT2B15-458WT) and two mutated ERU (UGT2B15-458-MT15 and UGT2B15-458-MT16), with or without the ERE binding sites. Relative to untreated controls, the wild-type ERU and the imperfect ERE full site are activated by 17β-estradiol and 4-hydroxytamoxifen, respectively.

![Diagram showing the effects of 17β-Estradiol and 4-hydroxytamoxifen on UGT2B15 promoter activity.](https://example.com/diagram.png)
E2-induced UGT2B15 gene expression was significantly abrogated by two ER antagonists (raloxifen and fulvestrant, both at 1 μM) but was not affected by a third ER antagonist, 4-OHTAM (1 μM) in MCF7 cells. In contrast, all three antagonists inhibited E2-mediated induction of GREB1, a well known ER target gene in MCF7 cells (Fig. 1B) (Deschenes et al., 2007). We further found that, similar to E2 at 1 nM, 4-OHTAM at 1 μM significantly elevated UGT2B15 mRNA levels but had no significant effects on the mRNA levels of two known ER target genes (GREB1 and pS2) and the house-keeping gene GAPDH in MCF7 breast cancer cells (Figs. 1B and 2A). Of note, fulvestrant reduced basal UGT2B15 mRNA levels, and both 4-OHTAM and raloxifen decreased basal GREB1 mRNA levels. Consistent with this observation, 4-OHTAM–treated MCF7 cells showed significantly higher testosterone (a UGT2B15 substrate) glucuronidation activity compared with vehicle-treated cells (Fig. 2B). Taken together, these results demonstrate that, similar to E2, 4-OHTAM increases the expression and enzymatic activity of UGT2B15 in breast cancer cells.

4-Hydroxytamoxifen Stimulates UGT2B15 Gene Expression via the ERa-Signaling Pathway in MCF7 Cells. Previous studies have shown that up to 25% of E2-induced genes are also induced by 4-OHTAM via the ERα-signaling pathway in MCF7 cells (Frasor et al., 2004; Lim et al., 2006). The previously described induction of UGT2B15 by both E2 and 4-OHTAM in MCF7 cells prompted us to investigate a potential role of ERα in 4-OHTAM–mediated induction of UGT2B15. As expected, E2 (1 nM) elevated the mRNA levels of both UGT2B15 and pS2, and this elevation was completely abolished by fulvestrant (Fig. 3A). Similarly, fulvestrant also completely abrogated the induction of UGT2B15 by 4-OHTAM, suggesting a role of ER signaling in mediating this induction (Fig. 3A). In support of this role, knockdown of ERα expression using siRNA (ERα-siRNA) completely ablated the induction of UGT2B15 by both E2 and 4-OHTAM (Fig. 3, B and C). As expected, knockdown of ERα expression also abolished the induction of pS2 by E2 but did not alter the control GAPDH mRNA levels (Fig. 3, B and C). Collectively, our results demonstrate that, similar to E2, 4-OHTAM induces UGT2B15 gene expression via the ERα-signaling pathway in MCF7 cells.

4-Hydroxytamoxifen Activates the UGT2B15 Promoter and Enhances ERα Binding to the ERU of the UGT2B15 Proximal Promoter in MCF7 Cells. We previously found that the upregulation of UGT2B15 by E2 is mediated by ERα binding to the UGT2B15 ERU, which contains two AP-1 sites, two ERE half sites, and an imperfect ERE full site (Fig. 4A) (Hu and Mackenzie, 2009). We tested whether 4-OHTAM upregulated UGT2B15 via the same ERU using luciferase reporter assays and ChIP assays. Consistent with our previous report (Hu and Mackenzie, 2009), E2 (10 nM) activated the wild-type ERU-containing UGT2B15 proximal promoter (UGT2B15-458WT), and ERα overexpression further enhanced this activation; however, this activation was completely abolished by mutation of the two AP-1 sites plus either the 3′ ERE half site (UGT2B15-458-5MT15) or the imperfect ERE full site (UGT2B15-458-5MT16) (Fig. 4B). Similarly, 4-OHTAM (1 μM) alone or together with ERα overexpression enhanced the activity of the wild-type UGT2B15 promoter (UGT2B15-458WT) but did not stimulate the activity of the two ERα-mutated constructs (UGT2B15-458-5MT15 and UGT2B15-458-5MT16) (Fig. 4C). Furthermore, ChIP assays showed that treatment of MCF7 cells with either E2 (10 nM) or 4-OHTAM (1 μM) enhanced ERα binding at the UGT2B15 ERU but not at the control negative ERE 2 locus (Fig. 5). Of note, significant basal ERα occupancy at the UGT2B15 ERU was also observed. This is consistent with previous findings that many ER sites have basal ER binding before ligand stimulation, presumably due to the presence of residual levels of active estrogenic molecules in the stripped FBS added to the media or synthesized by MCF7 cells under hormone-depleted culture conditions (Carroll et al., 2006). Taken together, our results indicate that, similar to E2, 4-OHTAM upregulates UGT2B15 via ERα binding to the previously reported UGT2B15 ERU in MCF7 cells.

Endoxifen Stimulates UGT2B15 Gene Expression via the ER-Signaling Pathway in MCF7 Cells. 4-OHTAM and endoxifen are the two major active TAM metabolites, and endoxifen is considered as a new therapeutic agent for breast cancer (Ahmad et al., 2010a,b). Recent studies using MCF7 cells showed that endoxifen and 4-OHTAM have similar effects on global gene expression patterns, and the majority of the affected genes are estrogen-regulated genes (Lim et al., 2006). As such, we treated MCF7 cells with endoxifen to see whether endoxifen could also induce UGT2B15 gene expression via the ER-signal pathway. As shown in Fig. 6, A and B, endoxifen at 1 μM significantly increased UGT2B15 mRNA levels but had no impact on the mRNA levels of pS2 and GAPDH. Endoxifen slightly but significantly reduced E2-elevated UGT2B15 mRNA levels; however, the endoxifen-induced UGT2B15 expression was completely abolished by fulvestrant, confirming a role for ER. Of note, the 3.4-fold induction of UGT2B15 by endoxifen (1 μM) was lower than the 8-fold or 6-fold induction seen with 4-OHTAM (1 μM) alone or 4-OHTAM (1 μM) together with endoxifen (1 μM). Furthermore, we demonstrated that, similar to 4-OHTAM, endoxifen (1 μM) significantly activated the activity of the UGT2B15 promoter carrying a wild-type ERU (UGT2B15-458WT), and this activation was abolished by mutating the ERU (UGT2B15-458-5MT15 or UGT2B15-458-5MT16) (Fig. 6C). Collectively, our results indicate that, similar to 4-OHTAM, endoxifen induces UGT2B15 gene expression via the UGT2B15 ERU.

**Discussion**

We recently reported that 17β-estradiol upregulates UGT2B15 in MCF7 breast cancer cells via ERα binding to an ER in the proximal
promoter of UGT2B15 (Hu and Mackenzie, 2009). In the present study, we showed that, similar to 17β-estradiol, 4-OHTAM and endoxifen, the two major antiestrogenic metabolites of tamoxifen, induce UGT2B15 gene expression in breast cancer MCF7 cells via ERα binding to the same UGT2B15 ERU. This finding is consistent with previous observations that about 74% of the genes regulated by both 4-OHTAM and endoxifen were regulated by 17β-estradiol via the ERα-signaling pathway in MCF7 cells (Lim et al., 2005). 4-OHTAM and raloxifene are selective estrogen receptor modulators; however, they both act primarily as ER antagonists in breast cancer cells, suppressing estrogen-mediated gene transcription and breast cancer cell proliferation (Frasor et al., 2004). Consistent with this notion, we showed in the present study that, similar to the pure ER antagonist fulvestrant, both raloxifene and 4-OHTAM significantly suppressed the 17β-estradiol–induced expression of two ER target genes (i.e., GREB1 and PS2) in MCF7 cells. However, these three agents displayed differing effects at the UGT2B15 ERU in MCF7 cells. Specifically, fulvestrant and raloxifene acted as ER antagonists to repress the 17β-estradiol–induced UGT2B15 expression. 4-OHTAM was not able to reduce this induction, and furthermore, it acted as an ER agonist to stimulate UGT2B15 expression. As discussed in previous studies, whether 4-OHTAM acts as an ER agonist or an ER antagonist can depend on the target promoter structure and dynamic changes of the ligand-bound ERα–ligand-binding domain (LBD) dimer at the promoter. Regarding the conformational changes of the ERα-LBD dimer following ligand binding, Chakraborty and Biswas (2014) recently showed that the binding of 4-OHTAM to the LBD remodels the conformational dynamics of the ERα-LBD dimer such that both the agonist and antagonist conformations are accessible. Results from the present study suggest that an agonist conformation of the 4-OHTAM–bound ERα-LBD dimer is preferentially established at the UGT2B15 ERU, followed by the assembly of an active transcriptional complex that promotes UGT2B15 transcription. This assumption remains to be validated in future studies.

Our study adds UGT2B15 regulation to the likely mechanisms underlying acquired TAM resistance. Although both trans- and cis–4-OHTAM isomers are substrates of multiple UGTs, UGTs other than UGT2B15 are expressed at low levels in breast cells or are not induced by 4-OHTAM (data not shown). In contrast, UGT2B15 is highly upregulated by 4-Hydroxytamoxifen and Endoxifen.
expressed in breast cancer tissues and cell lines (Nakamura et al., 2008; Ohno and Nakajin, 2009) and has the highest activity toward cis-4-OHTAM (Nishiyama et al., 2002; Ogura et al., 2006). Hence, the upregulation of UGT2B15 by 4-OHTAM and endoxifen in breast cancer cells, as reported in the present study, would promote 4-OHTAM inactivation through glucuronidation. Consistent with this idea, a comparison of gene expression profiles using DNA microarrays between a TAM-sensitive breast cancer xenograft (MaCa 3366) and its TAM-resistant subline (MaCa 3366/TAM) showed significantly higher UGT2B15 mRNA levels in the resistant tumor compared with the sensitive tumor (Naundorf et al., 2000; Becker et al., 2005). Collectively, these results suggest a role of 4-OHTAM in autoinduction of its own metabolism through upregulation of UGT2B15 in breast cancer cells. UGT2B15 D85Y is a prevalent polymorphism (rs1902023), and the Y85 allele confers a 2-fold higher catalytic activity compared with the D85 allele (Levesque et al., 1997). The Y85 allele has been linked to an increased risk of developing TAM resistance in a subset of genotype-phenotype association studies (Nowell et al., 2005; Dezentje et al., 2013; Markievicz et al., 2013), although some other similar studies did not find an association (Wegman et al., 2007; Ahern et al., 2011; Brooks et al., 2013). A larger study using more patients will be required to clarify this link. In addition to UGT2B15, other enzymes (i.e., SULT1A1 and CYP3A4) and the efflux transporter P-glycoprotein (also termed multidrug resistance 1) that are involved in either metabolism or disposition of TAM and/or its metabolites are also induced by 4-OHTAM in MCF7 cells (Nishiyama et al., 2002; Seth et al., 2002; Nagako et al., 2006; Tfelt et al., 2011). Our new findings, together with the previous studies described earlier, provide a possible explanation for previous observations from studies of MCF7-derived breast cancer xenografts in nude mice that intratumoral concentrations of TAM and 4-OHTAM in TAM-resistant tumors are significantly lower compared with concentrations in TAM-sensitive tumors (Osborne et al., 1991). Collectively, these studies reaffirm the early hypothesis that reduced intratumoral concentrations of TAM and its active metabolites likely contribute to the development of acquired tamoxifen resistance (Katznenellenbogen, 1991; Osborne et al., 1991; Johnston et al., 1993). In conclusion, we showed that, similar to 17beta-estradiol, 4-OHTAM and endoxifen, the two major antiestrogenic metabolites of tamoxifen, upregulate UGT2B15 in breast cancer cells via ER binding to the previously reported UGT2B15 ERE. This upregulation, together with the previously reported upregulation of SULT1A1 and CYP3A4 by tamoxifen and/or 4-OHTAM, demonstrates induction of metabolism of tamoxifen and its active metabolites in breast cancer cells upon exposure to tamoxifen. If a similar induction occurs in vivo, this would likely lead to reduced tamoxifen therapeutic efficacy and could contribute to the development of acquired resistance to tamoxifen treatment.

**Authorship Contributions**

*Participated in research design: Chanawong, Hu, Mackenzie, McKinnon.*

*Conducted experiments: Chanawong.*

*Performed data analysis: Chanawong, Hu, Mackenzie.*

*Wrote or contributed to the writing of the manuscript: Chanawong, Hu, Mackenzie, Meech, McKinnon.*

**References**


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