Effects of tamoxifen and ethynylestradiol cotreatment on uterine gene expression in immature, ovariectomized mice

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Abstract

Tamoxifen (TAM), the primary treatment for estrogen receptor (ER)-positive breast cancer, has been associated with an increased incidence of endometrial cancer in postmenopausal, but not premenopausal women. TAM elicits a partial ER-mediated uterotrophic response in immature rodents when compared with ethynylestradiol (EE), a potent ER agonist. However, cotreatment with 1000 μg/kg TAM antagonizes the uterotrophic effect induced by 30 μg/kg EE. To further investigate the anti-uterotrophic activity of TAM, immature, ovariectomized C57BL/6 mice were treated with a single oral dose of EE, TAM, EE + TAM, or vehicle, and harvested at 2, 4, 8, 12, 18, and 24 h or after three daily treatments at 72 h. Significant increases in uterine wet weight (UWW) were observed at 18 h for EE, TAM, and the mixture. However, mixture induction of UWW was significantly lower when compared with EE-induced uterotrophy at 72 h. This inhibitory effect is also reflected in decreases in luminal circumference, yet EE-induced luminal epithelial cell height was unaffected by cotreatment with TAM. Gene expression analysis using a 2×2 factorial cDNA microarray study design identified 2518 differentially expressed genes following EE treatment alone. However, only 290 EE-elicited gene expression changes were affected by TAM cotreatment, in a manner consistent with the anti-estrogenic response. These data suggest that TAM antagonism of EE-induced UWW increase involves the selective inhibition of EE-induced genes.

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Introduction

Tamoxifen (TAM), an adjuvant and prophylactic therapy prescribed for estrogen receptor α (ERα, listed as ESR1 in the HGNC database)-positive breast cancers. Adjuvant therapy suppresses breast cancer recurrence by 50% and prophylactically reduces incidence in high-risk populations (Lewis & Jordan 2005). TAM and its active metabolites, 4-hydroxytamoxifen (4OH-TAM), N-desmethyloxamoxifen (DMT), and 4-hydroxy-DMT (endoxifene), are thought to act by competing with potent agonists for binding to the ER and inhibiting their proliferative effects (Jordan 1993, Massarweh et al. 2008).

Despite its high therapeutic index, TAM has been associated with a twofold risk increase in endometrial cancer (Houssami et al. 2006) and can stimulate endometrial growth and hyperplasia in postmenopausal women (Kedar et al. 1994, Vosse et al. 2002). TAM induces uterotrophy in immature and ovariectomized rodents and elicits a gene expression profile similar to estrogen in uterine tissue, albeit with lower efficacy (Hodges et al. 2003, Frasor et al. 2004, Fong et al. 2007, Kwikel et al. 2009). Despite TAM exhibiting partial ER-agonist activity in the uterus, there are significant differences between TAM and full agonists. Comparisons between TAM and estrogen have shown that TAM causes a high induction of adenomyosis characterized by disordered location of endometrial glands in the myometrium of neonatal mice (Parrott et al. 2001), not observed with estrogen. TAM lacks the stimulation of water imbibition that is clearly induced in the presence of 17β-estradiol (E2) and 17α-ethynylestradiol (EE; Reel et al. 1996). Moreover, numerous studies report unique gene expression changes elicited by TAM (Pole et al. 2005, Fong et al. 2007, Kwikel et al. 2009).

Although TAM and estrogen independently elicit a uterotrophic response, cotreatment at appropriate ratios results in an antagonistic effect. For example, TAM significantly repressed uterine weight after 28 days in intact adult mice (Sourla et al. 1997). In addition, it antagonizes E2-induced uterotrophy (Robertson et al. 1982), as well as E2 induction of progesterone receptor (Castellano-Diaz et al. 1989) and Fos (Kirkland et al. 1993) expression. Reporter gene (McInerney & Katzenellenbogen 1996) and peroxidase activity (Robertson et al. 1982) assays have also exhibited similar antagonistic effects.

ER conformational changes after ligand binding affect its subsequent activities. The ligand-binding
domain occupied with E2 takes on a ligand-trapping conformation involving helix-12. In contrast, selective antagonists, such as raloxifene, position helix-12 in an orientation where the C-terminal domain of the ER interferes with subsequent transactivation (Brzozowski et al. 1997, Pike et al. 1999). Ligand-induced conformations have been implicated in coactivator protein recruitment. For example, GRIP1 is recruited to E2-bound ER, but not 4OH-TAM-bound receptor (Shiau et al. 1998). Moreover, coactivator recruitment may influence the activated receptors that bind to specific promoter sequences. This was demonstrated through DNA footprinting to show that high-mobility group B coactivator proteins enhance ER binding to estrogen response elements (EREs) (Das et al. 2004). Collectively, these studies indicate that ligand-induced topology can influence gene-specific transcriptional activation (reviewed in Edwards (2000)), and suggest that TAM may elicit its tissue-specific agonist and antagonist activities through a unique ER complex conformation. However, elucidating the influence of ligand structure on receptor conformation and transcriptional activity warrants further investigation.

This report extends our previous studies examining ER-mediated changes in uterine gene expression elicited by EE, a full ER agonist with enhanced oral bioavailability (Bolt 1979), and TAM (Fertuck et al. 2003, Kwekel et al. 2005, Fong et al. 2007), by examining their effects after cotreatment. A temporal 2×2 factorial microarray hybridization design (Fig. 1A; Yang & Speed 2002) was used to comprehensively examine differential gene expression associated with the antagonism of EE-induced uterine wet weight (UWW) by TAM cotreatment, with complementary histopathology. Interestingly, global repression of gene expression changes was not observed in only a select subset of EE-induced genes affected by cotreatment. Moreover, the responses were associated with cell growth and proliferation pathways, consistent with the anti-uterotrophic effect.

Materials and methods

Animal husbandry and treatment

Female C57BL/6 mice, ovariectomized by the vendor on postnatal day (PND) 20, were obtained from Charles River Laboratories (Raleigh, NC, USA) on PND 25. Animals (n=5) were housed in polycarbonate cages bedded with cellulose fiber chips (Aspen Chip Laboratory Bedding, Northeastern Products, Warrensburg, NY, USA) in a 23 °C environment with 30–40% humidity and a 12 h light:12 h darkness cycle (0700–1900 h). Animals had access to deionized water and Harlan Teklad 22/5 Rodent Diet 8640 (Madison, WI, USA) and were allowed to feed ad libitum and acclimatized for 4 days before treatment. TAM (Sigma) and EE (Sigma) were dissolved in sesame oil (Sigma) to the desired concentrations. To account for the delayed gene expression responses (Fong et al. 2007), animals (n=5 per group) were primed at −8 h with 1000 μg/kg TAM (TAM and mixture (MIX) groups) or sesame oil (vehicle and EE groups; Fig. 1B). At 0 h, animals were dosed with 30 μg/kg EE (EE and MIX groups) or sesame oil (TAM and vehicle groups). Four groups (n=5) of mice were also treated with sesame oil, 30 μg/kg EE (Sigma), 1000 μg/kg TAM (Sigma), or 30 μg/kg EE and 1000 μg/kg TAM (MIX) at 24 and 48 h to represent the 3×24 h treatment group. Doses were prepared based on average animal weight. Animals were killed by cervical dislocation, and body weights were recorded. The uterus was transected at the border of the cervix, and stripped of extraneous connective tissue and fat. Whole uterine weights were recorded before (wet weight) and after blotting (blotted weight) with absorbent tissue. A 6–8 mm section of unblotted uterine horn was placed in 10% neutral-buffered formalin (NBF) for histology. The remainder was snap frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Liver sections were prepared based on average animal weight. Animals were killed by cervical dislocation, and body weights were recorded. The uterus was transected at the border of the cervix, and stripped of extraneous connective tissue and fat. Whole uterine weights were recorded before (wet weight) and after blotting (blotted weight) with absorbent tissue. A 6–8 mm section of unblotted uterine horn was placed in 10% neutral-buffered formalin (NBF) for histology. The remainder was snap frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Liver sections.
from the left lobe were snap frozen for LC/MS/MS analysis. All procedures were performed with the approval of the Michigan State University All-University Committee on Animal Use and Care.

**Histological processing, morphometric, and pathological analysis**

Samples in 10% NBF were allowed to fix for at least 24 h at room temperature, then placed into tissue cassettes, and stored in 30% ethanol holding solution at 4 °C. Paraffin embedding, sectioning (5 μm), mounting, and hematoxylin and eosin staining were completed by the Michigan State University Laboratory for Anatomical Histology and Molecular Sciences (East Lansing, MI, USA) using standard techniques. Assessments were evaluated according to standardized National Toxicology Program pathology codes.

Morphometric analysis was performed on midhorn uterine cross sections (n=5 per treatment group) using Scion Image analysis software (Scion Corp., Frederick, MD, USA). Histological markers of uterotrophy, including luminal epithelial cell height (LECH), luminal circumference, and a number of endometrial glands, were quantified. Statistical analysis of morphometry data was assessed by Dunnett’s or two-way ANOVA followed by Tukey’s honestly significant difference (HSD) post hoc analysis to examine dose-dependent and temporal effects respectively (SAS version 9.1; SAS Institute Inc., Cary, NJ, USA).

**RNA isolation**

Briefly, 1·0 ml Trizol (Invitrogen) was added to the frozen uterine tissue in a 2·0 ml microfuge tube and homogenized in the presence of steel beads by a Mixer Mill 300 homogenizer (Retsch, Haan, Germany). Total RNA was isolated and extracted according to the manufacturer’s protocol and resuspended in the RNA Storage Solution (Ambion, Austin, TX, USA). RNA quality was assessed by A₂₆₀/A₂₈₀ ratio as well as intensity values recorded using a GenePix v5.1 (Molecular Devices).

**Microarray hybridization and analysis**

Custom in-house cDNA arrays consisting of 13 361 features, representing 7952 unique genes (Unigene Build 152), were spotted on epoxy-coated glass slides (SCHOTT Nextrion, Louisville, KY, USA) using an Omnigrid arrayer (GeneMachines, San Carlos, CA, USA) and Telechem Chipmaker 3 pins in a TeleChem CHP3 printhead head (Telechem International Inc., Sunnyvale, CA, USA) at the DNA Sequencing and Gene Expression Analysis facility at Michigan State University (East Lansing, MI, USA). Selected clones were obtained from the US Environmental Protection Agency Micro-Array Consortium (EPAMAC; Rockett & Dix 1999), Research Genetics, the National Institute of Aging and Lion Biosciences. Detailed protocols for processing of microarrays are available at http://dbzach.fst.msu.edu.

A 2×2 factorial hybridization design was used to assess treatment effects (Yang & Speed 2002) such that each group could be compared with the other (Fig. 1A). Four time-matched samples, of each treatment group, were hybridized to six slides to generate a single replicate of data. Three biological replicates were completed for 2, 4, 12, 24, and 3×24 h time points for a total of 90 arrays. The Genisphere 900 3DNA Array Detection (Genisphere Inc., Hatfield, PA, USA) indirect incorporation kit was used to generate cDNA samples for hybridization. Briefly, 1 μg of RNA was reverse transcribed in the presence of an oligo-tagged primer specifically targeted for Cy3- or Cy5-conjugated dendrimers. The cDNA was resuspended in 58 μl of 2× formamide-based hybridization buffer and hybridized overnight on arrays sealed in a light-shielded, humid chamber submerged in a 42 °C water bath. Slides were then washed in SSC containing decreasing concentrations of SDS, spin dried, and re-hybridized with a Cy3:Cy5 (1:1) dendrimer mixture in formamide-based buffer to indirectly incorporate dyes at the Cy3- and Cy5-dendrimer-tagged cDNA hybridized on the first day. Slides were washed and dried as described earlier, and scanned at 635 nm (Cy3) and 532 nm (Cy5) using a Molecular Devices Genepix 4100A scanner (Sunnyvale, CA, USA). Images were examined, features were identified, and intensity values were recorded using GenePix v5.1 (Molecular Devices).

**Microarray quality control, statistical analysis, and gene list filtering**

All arrays were compared with a historical dataset of high-quality arrays. Parameters assessed included background signal intensity, feature signal intensity, feature versus background signal intensity ratios, the number of features with background intensities greater than the feature intensity for each array, and relationships between feature and background signal intensities (Burgoon et al. 2005).

Data were normalized using a semi-parametric approach (Eckel et al. 2005). Model-based t values were calculated comparing all time-matched treated and vehicle samples. Posterior probabilities of activity (Pl(t) value) were then calculated on a per-gene and per-time point basis using an Empirical Bayes analysis (Eckel et al. 2004). Gene lists were filtered to identify genes that demonstrate differential expression between EE and MIX treatment. At each time point, both EE versus V (EV) and MIX versus V (MV) lists were
Quantitative real-time-PCR

Aliquots of RNA isolated from each of the five biological replicates were set aside for SYBR Green quantitative real-time PCR (QRT-PCR) verification. EE-treated, temporal mouse uteri RNA were isolated earlier (Kwekel et al. 2005). An oligo-dT-anchored Superscript II (Invitrogen) reverse transcriptase reaction was carried out on 1 µg RNA, in a 20 µl reaction, from each biological sample as per the manufacturer’s instructions. Samples were diluted fourfold, and 3 µl were used in a 30 µl real-time reaction mix containing 1× SYBR Green PCR buffer, 3 mM MgCl₂, 0.33 mM dNTPs, 0.5 IU AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), and 0.15 mM forward and reverse primers. All primers were designed by submitting cDNA microarray clone sequences into Primer3 (http://frodo.wi.mit.edu/primer3) to obtain an amplicon of ~125 bp.

PCR amplification was conducted in 96-well MicroAmp Optical plates (Applied Biosystems) on an Applied Biosystems PRISM 7000 Sequence Detection System under the following conditions: 10 min denaturation and enzyme activation at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After amplification, a 30 min dissociation protocol was conducted to assess primer specificity and product uniformity. Each plate contained duplicate standards of purified PCR product of known template concentration over eight orders of magnitude to generate a log template concentration standard curve. No template control samples were included on each plate such that experimental samples within 2 s.d. of the NTC are considered below the limits of detection. Plots were visualized and thresholds were determined using ABI Prism 7000 SDS Software (Applied Biosystems). Results were normalized to RpL7 mRNA levels to control the differences in RNA loading, quality, and cDNA synthesis. Expression differences were assessed using a two-way ANOVA followed by Tukey’s HSD post hoc analysis to examine treatment and treatment over time effects (SAS version 9.1). Correlation analyses of QRT-PCR and microarray data were generated using the correlation function of R v2.1.0.

LC/MS/MS

Liver tissue was homogenized with ddH₂O, in a 1:20 dilution, using a handheld Polytron homogenizer (Kinematica, Bohemia, NY, USA). In all, 1 ml ddH₂O, 200 µl of 1 M NaOH, and 1 ng [15N, 13C₂] TAM (Sigma), as an internal standard, were added to 1 ml homogenate. The mixture was extracted in an ether:methanol (95:5 v/v) solution and evaporated at 55 °C under a stream of N₂. Residue was resuspended in 200 µl acetonitrile:ammonium acetate (65:35 v/v) and stored at −20 °C in amber sample vials until use. Appropriate standards were also prepared for quantitative inter-polation of TAM and 4OH-TAM concentrations.

Extracted samples were analyzed at the MSU Mass Spectrometry Facility (East Lansing, MI, USA). Samples were injected into the LC-20AD (Shimadzu, Columbia, MD, USA) HPLC system with the SIL-5000 Injector (Shimadzu) and separated on an Atlantis dC18 3 mm column (Waters Corporation, Milford, MA, USA) using a 60:40 (v/v) methanol:100 mm ammonium acetate (pH = 3) solution. Electrospray ionization

Figure 2 Dose range-finding study: uterotrophic inhibition. Preliminary dose-finding experiments examined effects on uterine wet weight (UWW) at 72 h after three orally administered daily doses. EE (0, 0-01, 0-03, and 0-06 mg/kg) was cotreated with TAM (0-1, 1, and 10 mg/kg) to determine the optimal doses resulting in inhibition of EE-induced uterotrophy. In all, 0-030 mg/kg EE plus 1 mg/kg TAM (1:33 ratio) was selected for further examination. The asterisk (*) indicates significant (P < 0-05) inhibition relative to EE alone.
mass spectrometry was carried out on a Quattro micro API instrument (Waters Corporation), and data were analyzed using Mass Lynx v4.0 software (Waters Corporation).

Results

Dose-finding studies

Preliminary studies were conducted to determine the optimal EE and TAM doses to investigate possible additive, synergistic, and antagonistic uterotrophic tissue and gene expression responses. The oral ED\textsubscript{50} for the uterotrophic response in the immature, ovariectomized C57BL/6 mice has been previously determined to be 22.1 and 33.7 mg/kg for EE and TAM respectively, and that 100 mg/kg EE induced a maximal UWW response (~10-fold; Kwekel et al. 2005, Fong et al. 2007). There was also a pronounced temporal delay in TAM-elicited gene expression compared with EE (Fong et al. 2007). Hierarchical clustering illustrated that the 4 h EE and 12 h TAM gene expression profiles were most similar (Fong et al. 2007). In order to accommodate this delay, and to ensure TAM and EE had equivalent access to the ER, a modified treatment regimen was used, which predosed animals with TAM 8 h before EE (Fig. 1B).

Preliminary dose range-finding studies at 72 h identified the optimal EE:TAM ratio for the antagonism of EE-induced uterotrophy by TAM. Induction of UWW with 100 and 1000 mg/kg TAM was significantly repressed after cotreatment with 30 and 60 mg/kg EE (Fig. 2). Consequently, 30 mg/kg EE plus 1000 mg/kg TAM (1:33 ratio) was used to further investigate the additive, synergistic, and antagonistic uterine responses following cotreatment (MIX).

Treatment effects on uterine weight

Significant (P<0.05) increases in UWW were observed at 18, 24, and 72 h after treatment with 30 μg/kg EE, and at 8, 18, 24, and 72 h with 1000 μg/kg TAM (Fig. 3). However, TAM only elicited a 4-0-fold increase compared with the 8-1-fold induction by EE at 72 h. Although cotreatment of 30 μg/kg EE plus 1000 μg/kg TAM still increased UWW from 12 to 72 h compared with vehicle, UWW was inhibited ~50% at 72 h compared with EE alone.

Morphometric analysis and histopathology

Increases in LECH and luminal circumference are uterine hallmarks of estrogenicity (Owens & Ashby 2002). LECH was significantly induced 3.7-, 3.5-, and 3.3-fold by EE, TAM, and MIX treatment respectively compared with time-matched vehicle controls at 72 h (Fig. 4A). There was no significant difference in LECH between EE and TAM at 72 h, and TAM cotreatment did not antagonize EE-induced LECH. Luminal circumference was induced 3.1- and 2.9-fold at 24 h,
and 8.9- and 4.9-fold at 72 h for EE and TAM respectively (Fig. 4B). MIX repressed luminal circumference by 54% compared with EE alone at 72 h, but was not significantly different from TAM alone.

**Temporal histological changes**

Temporal- and dose-dependent uterine histological changes induced by EE and TAM in the immature, ovariectomized C57BL/6 mouse have been described (Kwekel et al. 2005, Fong et al. 2007). The same approach was used to characterize changes elicited by vehicle, EE, TAM, and EE + TAM treatment (Table 1). Mild to moderate stromal edema was observed at 2 h in the TAM and MIX groups, likely because of early priming. All treatments elicited mild to moderate hypertrophy in stromal nuclei by 4 h, with mild to moderate epithelial hyperplasia in the MIX treatment at 8 h. At 12 h, EE induced mild to moderate uterine stromal edema, mild stromal cell hypertrophy, and moderate endometrial hyperplasia, whereas TAM elicited qualitatively similar changes. MIX induced comparable uterine morphology relative to EE and TAM treatments alone. After 24 h, EE and TAM alone elicited marked increases in uterine edema, stromal cell hypertrophy, and endometrial hyperplasia, and were not histologically distinguishable. Comparable changes were also present 24 h after MIX treatment. The severity of the uterotrophic response continued to 72 h after EE and TAM alone. In contrast, MIX attenuated the uterine changes induced by EE and TAM alone, as evident in the areas of stromal hypertrophy and endometrial hyperplasia (Fig. 5). Overall, EE, TAM, and MIX elicited similar histological changes. Only at 72 h, there was evidence of a diminished response elicited by MIX when compared with EE and TAM.

**LC/MS/MS analysis of liver TAM and 4OH-TAM levels**

Liver samples from the same animals were used as a surrogate because of the limited availability of uterine tissue in order to measure TAM and 4OH-TAM levels by LC/MS/MS (Fig. 6). Extracts from our previous study (Fong et al. 2007) detected TAM in hepatic tissues 2 h after treatment, which decreased after 4 h (Fig. 6A), with 4OH-TAM reaching a plateau by 4 h and decreasing after 12 h (Fig. 6B) as expected. In the current cotreatment study, comparable levels of TAM and 4OH-TAM were detected in hepatic liver extracts.

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**Table 1** Histological evaluations of treated uterine sections. Each treatment group represents a biological replicate of five animals.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Stromal edema</th>
<th>Stromal nuclei hypertrophy</th>
<th>Epithelial hyperplasia</th>
<th>Myometrial hypertrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>E</td>
<td>T</td>
<td>M</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>None–mild</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
<td>Mild</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mild–moderate</td>
<td>Mild</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mild–moderate</td>
<td>Mild</td>
<td>Mild</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>Mild</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Mild–moderate</td>
<td>Moderate</td>
<td>Mild</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>–</td>
<td>Mild–moderate</td>
<td>Mild–moderate</td>
<td>–</td>
</tr>
<tr>
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<td>Moderate</td>
<td>Mild</td>
<td>–</td>
</tr>
<tr>
<td>24</td>
<td>–</td>
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<td>Moderate</td>
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</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Mild</td>
</tr>
<tr>
<td>72</td>
<td>–</td>
<td>Moderate</td>
<td>Marked</td>
<td>Mild</td>
</tr>
</tbody>
</table>

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**References**


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Although there was a shift from the earlier studies because of TAM priming. Approximately 70 ng/ml TAM were detected at 2 h in TAM and MIX-treated liver extracts. Peak levels of 130 ng/ml were detected at 8 h, which decreased to 50 ng/ml by 24 h. TAM levels did not significantly differ between TAM and MIX hepatic extracts at any time point. However, 4OH-TAM levels were significantly higher in MIX (208 ng/ml) compared with TAM (92 ng/ml) at 2 h, which converged to 100 ng/ml at 4 h; 4OH-TAM levels were not significantly different between TAM and MIX groups at any other time point. It was not possible to determine EE levels because of the low doses administered and the inefficiency of EE ionization and detection using LC/MS/MS.

(Uterine gene expression changes demonstrating mixture effects)

Differentially expressed genes were identified based on their empirical Bayes posterior probability of activity (PI(t) value) on a per-gene, per-time point basis (Supplementary Table 1, see section on supplementary data given at the end of this article). PI(t) values approaching 1.0 indicate a greater likelihood of treatment-related differential expression. EE-induced gene expression affected by TAM cotreatment was identified using a two-step process. All genes were first filtered (EE versus V: PI(t) ≥ 0.9999; fold change ≥ 1.5) to identify 2518 EE-elicited gene expression changes for at least one time point. These 2518 genes were then screened for modulation by TAM cotreatment (EE versus MIX: PI(t) ≥ 0.9999) to identify only 290 unique, annotated genes exhibiting gene expression differences comparing MIX to EE, representing potential nonadditive interactions (Table 2).

Gene expression changes were further examined by comparing EE versus V and MIX versus V to classify potential nonadditive interactions as A) EE-induced expression repressed by MIX, B) EE-induced expression augmented by MIX, C) EE-repressed expression diminished by MIX, and D) EE-repressed expression further repressed by MIX (Fig. 7 and Table 3). The distribution of gene expression behavior across time shifts from categories A, B, and C (2–12 h) to primarily categories B and C (24 and 72 h). Note that a potential nonadditive interaction may occur at several time points. For example, fos-like antigen 2 (Fosl2) is a category A gene at 2 and 4 h. A gene may also exhibit different nonadditive patterns across time, such as inhibin β-B (Inhbb), which is a category A gene at 2 h but classified as category B at 24 and 72 h.
MIX-treated animals demonstrate significantly different 4OH-TAM levels peaked at 4 h (* compared with time-matched vehicle) at 2 h after treatment. (B) 4OH-TAM levels were significantly different from time-matched vehicle and MIX treatments at any time point. (C) TAM levels in TAM- and MIX-treated samples were also extracted from liver samples from the current study to determine hepatic tissue levels using LC/MS/MS. (A) TAM was detected (* compared with time-matched vehicle) at 2 h after treatment. (B) 4OH-TAM levels were significantly different from time-matched vehicle and EE treatments. (C) TAM levels in TAM- and MIX-treated samples are significantly different from time-matched vehicle and EE controls (*P<0.05), but not significantly different between TAM and MIX treatments at any time point. (D) 4OH-TAM levels were significantly different from time-matched vehicle controls and EE-treated animals (*P<0.05). At 2 h, TAM- and MIX-treated animals demonstrate significantly different 4OH-TAM levels (*P<0.05) but not beyond 4 h after treatment.

Functional categorization of microarray data

The majority of EE-elicted differentially expressed genes affected by TAM cotreatment identified at 2 and 4 h are associated with cell growth and proliferation including oncogenes such as myeloma ubiquitin oncogene (Myc, category A), Jun oncogene (Jun, category A), and FBJ osteosarcoma oncogene (Fos, category A). Genes involved in the cell cycle, cyclin-dependent kinase inhibitor 1A (Cdkn1a, category B) and branched chain aminotransferase 1, cytosolic (Bcat1, category B), as well as guanine nucleotide-binding protein-like 3 (Gnl3, category B) and activating transcription factor 4 (Atf4, category B), which are associated with proliferation, were also affected by TAM cotreatment. Other affected functional categories included lipid metabolism (peroxisomal trans-2-enoyl-CoA reductase (Pecr, category C) and carnitine palmitoyltransferase 2 (Cpt2, category C)), immune response (interferon γ inducible protein 30 (Iif30, category C) and chemokine (C-X-C motif) ligand 12 (Ccl12, category C)), and ion binding and transport (selenoprotein K (Selk, category B) and solute carrier family 23 (nucleobase transporters, member 2 (Slc23a2, category C)).

Eleven of these genes, representing different categories of MIX-mediated changes from Table 3, were verified by QRT-PCR. Each exhibited a strong correlation between the microarray and QRT-PCR data (Fig. 8). In addition, the more sensitive QRT-PCR assay uncovered an intermediate behavior in the gene expression pattern where MIX induced a significantly greater response than TAM alone but less than EE (Fig. 8).

Discussion

The uterotrophic assay is a well-established model to study the physiological and morphological effects elicited by estrogenic compounds (Diel et al. 2002). We have used it to examine differential uterine gene expression elicited by EE (Fertuck et al. 2003, Moggs et al. 2004, Kwekel et al. 2005, Naciff et al. 2007) and, more recently, the partial agonist effects of TAM (Fong et al. 2007). Although TAM inhibits ER-mediated estrogen-induced increases in UWW (Jordan et al. 1978a, Jordan & Gosden 1983), the effects of cotreatment on gene expression have not been comprehensively examined. In this study, the same model, study design, and analysis methods reported earlier for EE and TAM differential gene expression investigations (Fertuck et al. 2003, Kwekel et al. 2005, Fong et al. 2007) were used to examine the inhibition of EE-induced uterotrophy by TAM cotreatment in order to re-examine several hypotheses regarding the mechanisms involved in the anti-estrogenicity of TAM.

Table 2 MIX-modified, ethynylestradiol (EE)-induced gene list generation. Genes may be active across multiple time points; thus, the sum of each column is greater than total unique genes in each category. Detailed gene information may be found in Supplementary Table 1.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>EE-induced genes P(t) ≥ 0.9999</th>
<th>EE-induced genes modified by tamoxifen cotreatment P(t) ≥ 0.9999</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>49</td>
<td>25</td>
</tr>
<tr>
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<td>Total</td>
<td>2518</td>
<td>290</td>
</tr>
</tbody>
</table>

*Number of unique Entrez gene-annotated genes at indicated time points.

**Number of unique Entrez gene-annotated genes across all time points.
In this study, TAM inhibited ER-mediated, EE induction of UWW by 50% in immature, ovariectomized C57BL6 mice, comparable to other published reports (Jordan et al. 1978a, b, Black & Goode 1980). Although the model system limited our ability to directly examine TAM levels in the uterus, orally dosed TAM was absorbed and metabolized to 4OH-TAM, consistent with the physiological effects. Histologically, TAM cotreatment inhibited EE induction of luminal circumference but did not antagonize LECH induction, suggesting that the effect is cell-type specific.

Differential gene expression data also indicate that the nonadditive effects of TAM are not global since a majority of EE-elicited responses were not affected by TAM cotreatment. Of the 2518 EE-elicited differential gene responses, only 290 were affected by cotreatment, with 214 being repressed and 76 enhanced, relative to EE alone. Consequently, only a subset of EE-elicited gene expression changes are affected by TAM at doses required to inhibit uterotrophy. In addition, further examination identified 229 differentially expressed genes that showed comparable changes in the EE and MIX groups but were not affected by TAM. Therefore, competition for ER binding (Robertson et al. 1982, Mnif et al. 2007) and downregulation of ER expression (Koibuchi et al. 2000) do not sufficiently explain the gene expression effects resulting in the inhibition of ER-mediated, EE-induced UWW increases.

Examination of the functions of ER-mediated, EE-elicited gene expression affected by TAM is consistent with the inhibition of EE-induced UWW. Several genes associated with growth and proliferation were repressed (category A) by TAM at early time points, including Myc, Jun, and Fos. The proliferation-regulating, uterine-expressed transcription factors, Fosl2 (Bamberger et al. 2001), Ets1, and Ets2 (Kilpatrick et al. 1999), as well as estrogen-responsive proliferation-associated Gtpbp4 (Laping et al. 2001, Wang et al. 2004), uterotrophy-associated Gnl3 (Beckman et al. 2006), and stromal cell differentiation regulator Socs3 (Dimitriadis et al. 2006) were also repressed and found in category A. Other category A proliferation-related genes including Stx2 (Wang et al. 2006), estrogen-responsive Clu, mouse uterus-expressed Popdc2 (Andree et al. 2000), and Gja1 found in human myometrium (Cluff et al. 2006) were also repressed at later time points. EE-elicited repression of some genes was also attenuated such as Gas1 (category C). Gas1 is repressed by Myc (Lee et al. 1997), consistent with the repression observed in EE-induced Myc. In addition, the endometrial expression of C11bp (category C), which exhibits an inverse relationship

### Table 3 MIX-modified, ethynylestradiol (EE)-induced gene classifications.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>A MIX repression of EE induction</th>
<th>B MIX augmentation of EE induction</th>
<th>C MIX diminution of EE repression</th>
<th>D MIX augmentation of EE repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>9</td>
<td>15</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>1</td>
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<td>12</td>
<td>48</td>
<td>29</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>45</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>5</td>
<td>20</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Total unique genes</td>
<td>109</td>
<td>87</td>
<td>106</td>
<td>1</td>
</tr>
</tbody>
</table>

*a A total of 290 EE-induced genes modified by tamoxifen cotreatment were identified. Some genes demonstrated different expression patterns at different time points; thus, the sum of total unique genes across all four categories is > 290.
with proliferation (Hamid et al. 2003), was de-repressed by TAM, also consistent with the antagonism of EE-induced UWW.

Furthermore, TAM enhanced the induction or repression of some gene expression changes (categories B and D). Although these responses appear counter intuitive, several are consistent with the inhibition of the EE-induced uterotrophic effect. For example, overexpression of \textit{Atf4} (category B) impairs mammary proliferation and development (Bagheri-Yarmand et al. 2003), and \textit{Cdkn1a} (category B) is known to promote growth arrest and apoptotic pathways (Qiao et al. 2002). These responses provide further support for a gene expression role in MIX repression of ER-mediated, EE-induced uterine weight. However, there were also late differential responses elicited by EE that were enhanced by TAM cotreatment, and are consistent with proliferation (category B). \textit{Crip1} (category C), which is upregulated in proliferating mammary luminal epithelial cells (Mackay et al. 2003),

\textbf{Figure 8} Quantitative real-time PCR verification: microarray results were verified using QRT-PCR with the genes representing various affected pathways and demonstrating different patterns of MIX-modified, EE-mediated changes. Overall, there was good agreement between microarray (left) and QRT-PCR (right) data; ‘a’ denotes a statistically significant QRT-PCR difference ($P<0.05$, $n=4$) because of treatment, ‘b’ indicates a significant difference from the time-matched EE-treated sample, and * denotes a difference between TAM and MIX-treated samples.
Cdc2l1 (category C; Wilker et al. 2007), and endometrium-expressed Tgfα (category C; Ejskjaer et al. 2005) all exhibited enhanced differential expression at later time points. This may be an attempt to over compensate for the limited ER-mediated induction of UWW in response to the majority of gene expression changes that were otherwise unaffected under TAM cotreatment or, as is the case with Gas1 and Myc, may be due to changes in the levels of early response genes.

Cytoskeletal reorganization is integral to estrogen-mediated restructuring of proliferating tissue (Moggs et al. 2004). Several genes associated with the cytoskeleton including Bicd2 (Hoogenraad et al. 2003), Dctn2 (Uetake et al. 2004), and Mfap5 (Lemaire et al. 2007) were induced by EE and repressed after TAM cotreatment (category A). Although these genes have not been identified to be ER regulated, their differential expression serves to prepare the tissue for proliferation, and therefore, their inhibition is consistent with a compromised uterotrophic response.

Selective ER modulator activity is based on the ability to differentially affect various tissues (Shang & Brown 2002, Jordan 2007). This study is the first to demonstrate that TAM also elicits selective in vivo gene expression responses within the uterus. Estrogen and 4OH-TAM cotreatment studies in MCF-7 cells have also identified genes that exhibit comparable patterns of antagonism. For example, category A genes Fosl2, Aims (Inoue et al. 2002), and Fos (Frasor et al. 2004), and category C genes Il1r1, Tm4sf1, and If30 (Frasor et al. 2004) exhibited similar gene expression behavior in MCF-7 cells and C57BL/6 uterine tissue. Differences in models, study design, microarray platforms, gene representation on the arrays, and data analysis limit comparative analysis to identify additional genes affected by TAM cotreatment in both models. Several factors confound further comparisons between human breast cancer MCF-7 cells and mouse uterine gene expression profiles including differences in ER protein levels (Shang & Brown 2002), tissue-specific co-regulating factor availability (McDonnell et al. 1995, Webb et al. 2003), and gene-specific thresholds of activation (Geum et al. 1997).

Conclusions
This study represents the first comprehensive in vivo investigation of the anti-estrogenic effects of TAM on uterine gene expression. Repression of ER-mediated, EE-induced uterotrophy, by TAM cotreatment, did not globally repress all EE-mediated gene expression. In contrast, only a selected subset was affected, which included genes associated with cellular growth and proliferation, consistent with an anti-uterotrophic effect. However, comparative studies in the rat or more sophisticated transgenic approaches are required to conclusively demonstrate the importance of these potential targets in uterine proliferation and growth and as critical TAM targets for the inhibition of ER-mediated, EE-induced increases in UWW.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1677/JME-09-0158.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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