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) is an adjuvant and prophylactic therapy prescribed for estrogen receptor α (ER α , 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*-desmethyltamoxifen (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, Kwekel *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 17 β -estradiol (E₂) and 17 α -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, Kwekel *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

Figure 1 Study design: (A) differential gene expression changes between all treatments were examined using a 2×2 factorial hybridization design to minimize the number of arrays required per biological replicate. Each arrow represents an array with Cy3 as the head and Cy5 as the tail. Microarray analysis was completed in triplicate for 2, 4, 12, 24, and 72 h time points for a total of 90 arrays. (B) Dosing and killing schedule for the study. Mice (*n*=5) were treated with vehicle, 30 µg/kg EE, 1000 µg/kg TAM, or 30 µg/kg plus 1000 µg/kg TAM (MIX). Animals were killed at 2, 4, 8, 12, 18, or 24 h after a single oral gavage or at 72 h with three daily doses at 0, 24, and 48 h. TAM was dosed 8 h before EE to compensate for the delayed TAM-elicited responses associated with metabolism and distribution to facilitate equal competition for estrogen receptor availability.

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

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 samples were quantified spectrophotometrically (A_{260}) and assessed for quality by A_{260}/A_{280} ratio as well as inspected using denaturing agarose gel electrophoresis.

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 Nexterion, 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 v.5.1 (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 ($P(t)$ 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

generated using posterior probability ($P(t) > 0.9999$) and fold-change cutoff ($|\text{fold change}| > 1.5$) criteria and then compared to identify differential expression between EV and EE versus MIX (EM) where $P(t) > 0.9999$. All raw and analyzed data were stored in dbZach (<http://dbzach.fst.msu.edu>), a Minimum Information About Microarray Experiments (MIAME)-supportive relational database (Burgeon *et al.* 2006).

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 ED₅₀ for the uterotrophic response in the immature, ovariectomized C57BL/6 mice has been previously determined to be 22.1 and 33.7 µg/kg for EE and TAM respectively, and that 100 µg/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 µg/kg TAM was significantly repressed after cotreatment with 30 and 60 µg/kg EE (Fig. 2). Consequently, 30 µg/kg EE plus 1000 µg/kg TAM (1:33 ratio) was used to further investigate the additive, synergistic, and antagonistic uterine responses following cotreatment (MIX).

Figure 3 Treatment-induced uterotrophy: uterine wet weight (UWW) was measured at 2, 4, 8, 12, 18, 24, and 72 h after treatment ($n=5$); 'a' indicates a significant increase in UWW compared with the time-matched vehicle control; 'ab' indicates a significant difference in UWW compared with the time-matched vehicle control and the EE-treated sample.

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,

Figure 4 Morphometric changes elicited by EE, TAM, and mixture: morphometric measurements of (A) luminal epithelial cell height (LECH) and (B) luminal circumference were made on all uteri sections; 'a' indicates a significant increase in LECH or luminal circumference compared with the time-matched vehicle control; 'ab' indicates a significant difference in luminal circumference compared with the time-matched vehicle control and the EE-treated sample. TAM inhibited EE-induced luminal circumference only after three daily treatments. There were no significant differences in LECH between treatments at any time point examined.

and 8.0- 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

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

	Treatment group	Stromal edema	Stromal nuclei hypertrophy	Epithelial hyperplasia	Myometrial hypertrophy
Time (h)					
2	V	—	—	—	—
	E	—	—	—	—
	T	Mild	—	—	—
	M	Mild–moderate	None–mild	—	—
4	V	—	—	—	—
	E	Mild	None–mild	—	—
	T	Moderate	Mild	—	—
	M	Mild–moderate	Moderate	—	—
8	V	—	—	—	—
	E	Mild–moderate	Mild	—	—
	T	Marked	Mild	Mild–moderate	—
	M	Marked–severe	Mild	Mild–moderate	—
12	V	—	—	—	—
	E	Mild–moderate	Moderate	Mild–moderate	—
	T	Moderate	Mild	Moderate	—
	M	Moderate	Moderate	Moderate	—
18	V	—	—	—	—
	E	Mild–moderate	Mild–moderate	Mild–moderate	—
	T	Moderate–marked	Mild	Moderate	—
	M	Moderate	Mild	Moderate	—
24	V	—	—	—	—
	E	Moderate	Moderate	Marked	Mild
	T	Moderate	Moderate	Marked	Mild
	M	Mild–moderate	Mild–moderate	Moderate–marked	Mild
72	V	—	—	—	—
	E	Moderate–marked	Marked	Severe	Mild
	T	Moderate	Marked	Severe	Mild
	M	Moderate	Moderate	Marked–severe	Mild

Figure 5 Histological assessment: hematoxylin and eosin staining of uteri treated three times daily with vehicle (V), 30 $\mu\text{g/kg}$ ethynylestradiol (EE), 1000 $\mu\text{g/kg}$ tamoxifen (TAM), and 30 $\mu\text{g/kg}$ ethynylestradiol plus 1000 $\mu\text{g/kg}$ tamoxifen (MIX). All treatments elicited a uterotrophic effect, although MIX attenuated the proliferative effect compared with EE- and TAM-induced responses. Images are representative of five biological replicates; bar represents 30 μm . Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0158>.

(Fig. 6C and D), 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 ($P(t)$ value) on a per-gene, per-time point basis (Supplementary Table 1, see section on supplementary data given at the end of this article). $P(t)$ 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: $P(t) \geq 0.9999$; 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: $P(t) \geq 0.9999$) 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.

Figure 6 Temporal LC/MS/MS analysis of hepatic TAM and 4OH-TAM levels: hepatic TAM and 4OH-TAM were extracted from a previous study (Fong *et al.* 2007) to determine tissue levels using LC/MS/MS. (A) TAM was detected ($^*P < 0.05$ compared with time-matched vehicle) at 2 h after treatment. (B) 4OH-TAM levels peaked at 4 h ($^*P < 0.05$ compared with time-matched vehicle) and plateaued at 12 h before steadily decreasing over time. TAM and 4OH-TAM were also extracted from liver samples from the current study to determine hepatic tissue levels using LC/MS/MS. TAM and 4OH-TAM were not significantly different between vehicle and EE treatments. (C) TAM levels in TAM- and MIX-treated samples are significantly different from time-matched vehicle and EE controls ($^aP < 0.05$), but not significantly different between TAM and MIX treatments at any time point. (D) 4OH-TAM levels are significantly different from time-matched vehicle controls and EE-treated animals ($^aP < 0.05$). At 2 h, TAM- and MIX-treated animals demonstrate significantly different 4OH-TAM levels ($^bP < 0.05$) but not beyond 4 h after treatment.

Functional categorization of microarray data

The majority of EE-elicited differentially expressed genes affected by TAM cotreatment identified at 2 and 4 h are associated with cell growth and proliferation including oncogenes such as myelocytomatosis 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 (*Ifi30*, category C) and chemokine (C-X-C motif) ligand 12 (*Cxcl12*, 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

	EE-induced genes ^a <i>P1(t)</i> ≥ 0.9999 fold-change ≥ 1.5	EE-induced genes modified by tamoxifen cotreatment ^a <i>P1(t)</i> ≥ 0.9999
Time (h)		
2	49	25
4	336	87
12	1946	128
24	1534	79
72	591	48
Total unique genes ^b	2518	290

^aNumber of unique Entrez gene-annotated genes at indicated time points.
^bNumber of unique Entrez gene-annotated genes across all time points.

Figure 7 EE-mediated gene expression affected by TAM cotreatment: only 209 out of 2518 EE-elicited gene expression changes ($P(1/t) \geq 0.9999$; fold change ≥ 1.5) were affected by TAM cotreatment at doses that significantly inhibited EE-induced uterotrophy. These genes were classified 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 that is further repressed by MIX. The numbers within each panel indicate the number of genes exhibiting the pattern. Note that some genes exhibited different TAM cotreatment expression patterns at different time points, and therefore were placed in more than one category.

In this study, TAM inhibited ER-mediated, EE induction of UWW by $\sim 50\%$ 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 *Glpb4* (Laping *et al.* 2001, Wang *et al.* 2004), uterotrophy-associated *Gnl3* (Beekman *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 *Cirbp* (category C), which exhibits an inverse relationship

Table 3 MIX-modified, ethynylestradiol (EE)-induced gene classifications. Detailed gene information may be found in Supplemental Table 1

	A MIX repression of EE induction	B MIX augmentation of EE induction	C MIX diminution of EE repression	D MIX augmentation of EE repression
Time (h)				
2	9	15	1	0
4	58	1	28	0
12	48	29	50	1
24	0	45	34	0
72	5	20	23	0
Total unique genes ^a	109	87	106	1

^aA 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 .

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.

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 *Atf4* (category B) impairs mammary proliferation and development

(Bagheri-Yarmand *et al.* 2003), and *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). *Crip1* (category C), which is upregulated in proliferating mammary luminal epithelial cells (Mackay *et al.* 2003),

Cdc2l1 (category C; Wilker *et al.* 2007), and endometrium-expressed *Tgfa* (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*, *Asns* (Inoue *et al.* 2002), and *Fos* (Frasor *et al.* 2004), and category C genes *Il1r1*, *Tm4sf1*, and *Ifi30* (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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