Tamoxifen Regulation of Bone Growth and Endocrine Function in the Ovariectomized Rat: Discrimination of Responses Involving Estrogen Receptor $\alpha$/Estrogen Receptor $\beta$, G Protein-Coupled Estrogen Receptor, or Estrogen-Related Receptor $\gamma$ Using Fulvestrant (ICI 182780)

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Received August 10, 2010; accepted March 21, 2011

ABSTRACT

Tamoxifen is a selective estrogen receptor (ER) modulator, but it is also a deactivating ligand for estrogen-related receptor-$\gamma$ (ERR$\gamma$) and a full agonist for the G protein-coupled estrogen receptor (GPER). Fulvestrant is a selective ER down-regulator that lacks agonist effects on ER$\alpha$/ER$\beta$, is inactive on ERR$\gamma$, but acts as a full agonist on GPER. Fulvestrant effects on tamoxifen actions on uterine and somatic growth, bone, the growth hormone (GH)-insulin-like growth factor I (IGF-I) axis, and pituitary prolactin were analyzed to pharmacologically discriminate tamoxifen effects that may be mediated by ER$\alpha$/ER$\beta$ versus ERR$\gamma$ versus GPER. Ovariectomized rats received tamoxifen (0.6 mg/kg/daily) plus fulvestrant at 0, 3, 6, or 12 mg/kg/daily for 5 weeks; controls received vehicle or 6 mg/kg fulvestrant daily. Tamoxifen effects to increase uterine weight, decrease serum IGF-I, increase pituitary prolactin, and increase bone mineral density could be fully blocked by fulvestrant, indicating mediation by ER$\alpha$/ER$\beta$. Tamoxifen effects to decrease pituitary GH, tibia length, and body weight were only partially blocked by fulvestrant, indicating involvement of mechanisms unrelated to ER$\alpha$/ER$\beta$. Fulvestrant did not inhibit tamoxifen actions to reduce total pituitary protein, again indicating effects not mediated by ER$\alpha$/ER$\beta$. Tamoxifen actions to reduce serum GH were mimicked rather than inhibited by fulvestrant, pharmacological features consistent with GPER involvement. However, fulvestrant alone increased IGF-I and also blocked tamoxifen-evoked IGF-I decreases; thus fulvestrant effects on serum GH might reflect increased IGF-I feedback inhibition. Fulvestrant alone had no effect on the other parameters. The findings indicate that mechanisms unrelated to ER$\alpha$/ER$\beta$ contribute to tamoxifen effects on body weight, bone growth, and pituitary function.

Introduction

Tamoxifen is an estrogen receptor (ER) ligand that is characterized as a selective ER modulator (SERM); it acts as estrogen agonist on some targets while acting as an antagonist or partial agonist on others. This is not attributed to differing pharmacological actions on ER$\alpha$ versus ER$\beta$, but reflects the diversity of mechanisms mediating ER actions at different targets.

In classic models of ER function, estrogen binding to the ligand-binding domain (LBD) transforms ER conformation and enhances ER binding to specific target gene DNA sequences (McDonnell et al., 2002; Smith and O’Malley, 2004). One or both of two ER transactivation domains (AF-1 and AF-2) then bind coactivator proteins that alter gene transcription via the recruitment of chromatin-remodeling complexes and other factors. AF-1 is located in the ER N terminus and is ligand-independent, whereas AF-2 in the LBD is ligand-dependent. Involvement of AF-1 or AF-2 in estrogen responses varies from target to target depending on the genes, cell types, coactivators, and other signaling systems involved.

SERMs transform ER conformation in a manner that activates DNA binding and AF-1-dependent gene expression in classic models of ER function. However, SERMs fail to activate coactivator binding to the LBD and AF-2-mediated tran-
cription and can elicit atypical ER interactions with corepressor proteins. Thus, the agonist or antagonist character of tamoxifen depends on the relative role of AF-1 and AF-2 in ER actions at the target (McDonnell et al., 2002; Smith and O’Malley, 2004).

Tamoxifen and its active metabolite (4-hydroxytamoxifen) can also alter gene expression by actions on estrogen-related receptors (ERRα, ERRβ, ERRγ). The DNA-binding domains of ERRs and ERs are closely related and interact with overlapping sets of DNA sequences and target genes (Giguère, 2008). ERRs and ERs also have related LBDs, but ERRs cannot bind 17β-estradiol or other steroids and are constitutively active in the absence of ligand binding (Greschik et al., 2002). However, binding of tamoxifen and 4-hydroxytamoxifen (triphenylethylene SERMs) to ERRγ and ERRβ evokes conformational changes that disrupt coactivator binding and transcriptional activity (Coward et al., 2001; Tremblay et al., 2001). Thus, fulvestrant coverage efforts have not identified fulvestrant as an ERR agonist, and high-throughput ligand discrimination of tamoxifen effects that may be mediated via ERα/ERγ versus GPER versus ERRγ. The present study used ovarioectomized rats and a 5-week dosing protocol to assess the ability of fulvestrant to mimic or antagonize tamoxifen actions on uterine and somatic growth, bone metabolism, pituitary prolactin (PRL), and the GH-IGF-I axis.

**Materials and Methods**

**Animals.** All protocols followed National Institutes of Health guidelines for the use and care of animals and were approved by our institutional Animal Care and Use Committee. Young adult female Sprague-Dawley rats were used (Taconic Farms, Germantown, NY). Ovariectomy was performed using halothane-O2 anesthesia 4 days after arrival (79–84 days old), and study treatments were started 5 days after surgery (mean starting weight = 234 g). Five rats received only sham surgery to provide ovary-intact controls (mean starting weight = 233 g).

The young adult female rats in this study were 85 to 90 days old at the start of hormone treatment and 120 to 125 days old when euthanized for collection of biological samples. Male and female rats in the same age range were also used in our previous studies of hormone/drug interactions (DiPippo et al., 1995; Fitts et al., 1998, 2001, 2004). Such rats still have active epiphyseal growth plates and growth-related bone modeling and respond to gonadectomy and hormone treatment with robust changes in body weight gain, longitudinal growth, and cancellous bone mass. Moreover, young rats are vigorous with respect to gonadal function and the operation of the GH-IGF-I axis and are well suited for studies assessing the physiological interplay of these hormonal systems.

Young, sexually mature rats in the age range studied have been shown to respond to gonadectomy and sex hormone therapy with changes in cancellous bone mass that are clearly evident within 2 weeks and unequivocal within 4 weeks (Kalu, 1991). Thus, a 5-week treatment period was used to establish robust effects on somatic growth and bone mass.

**Experimental Design and Drug Treatments.** Six groups of ovarioectomized rats received the following drug and hormone treatments: 1) vehicle solution alone (n = 7); 2) tamoxifen (trans isomer, free base; Sigma-Aldrich, St. Louis, MO) at 0.6 mg/kg via subcutaneous injection, once daily (n = 8); 3) fulvestrant at 6 mg/kg via subcutaneous injection once daily (n = 6); 4) 0.6 mg/kg tamoxifen plus 3 mg/kg fulvestrant via subcutaneous injection, once daily (n = 7); 5) 0.6 mg/kg tamoxifen plus 6 mg/kg fulvestrant via subcutaneous injection, once daily (n = 6); and 6) 0.6 mg/kg tamoxifen plus 12 mg/kg fulvestrant via subcutaneous injection, once daily (n = 6). The group of five ovary-intact rats received daily injections of vehicle solution. Rats were caged in groups of three to four rats per cage, and body weights were recorded three times per week.

Fulvestrant and tamoxifen were dissolved in ethanol and then mixed with sesame oil followed by benzyl alcohol (a pharmaceutical solvent with bacteriostatic and local anesthetic activity) to form stock solutions. The resulting vehicle was composed of 10% benzyl alcohol, 20% ethanol, and 70% sesame oil. Drug stocks and the corresponding vehicle were mixed as needed to achieve desired drug concentrations for injection; dosing volumes were 50 μl per 100 g of body weight. It should be noted that tamoxifen and fulvestrant were used at doses that greatly exceed (>50-fold) the amount needed to significantly block the effects of exogenous doses of estradiol benzoate (Wakeling et al., 1991; Powers et al., 2009).
Tissue Processing. After 5 weeks of treatment, rats were euthanized in random order by intraperitoneal injections of sodium pentobarbital at a dose of 100 mg/kg between 10:30 AM and 3:30 PM. Blood, anterior pituitaries, and right tibias were collected within 5 min of pentobarbital injection as described previously (DiPippo et al., 1995; Fitts et al., 1998). Blood samples were allowed to clot for 5 min at room temperature, cooled on ice, and then refrigerated at 5°C; serum was collected the next day, separated into aliquots, and stored at −80°C until assay. Blood samples from two rats did not yield serum suitable for analysis.

The right tibia was stripped of most muscle and connective tissue and stored in 70% ethanol for subsequent measurements of tibia length (an index of longitudinal growth) and bone mineral density. Pituitaries were sonicated in 400 μl of homogenization buffer (10 mM sodium phosphate, 150 mM NaCl, 0.1% Triton X-100, pH 7.5) and stored at −20°C until assay.

The uterus was removed, drained of luminal contents, and dissected to remove adherent parametrial fat tissue. The uterus was then spread out on sheets of paraffin wax (Parafilm) to dry at room temperature, and dry uterine weights were obtained 24 h later.

Analysis of Bone Mineral Density. Tibia bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry with a QDR-1000 (Hologic, Waltham, MA) as described previously (DiPippo et al., 1995; Fitts et al., 1998). Image analysis software (Hologic) calculated bone mineral content (g), cross-sectional area (cm²), and BMD (g/cm²) in two regions: the proximal tibia and the tibia diaphysis. The proximal tibia (upper third of tibia length) has a higher ratio of cancellous to cortical bone than the diaphysis (middle third of tibia length) (Kalu, 1991).

Radioimmunoassay of Serum GH and Insulin-Like Growth Factor-I. Serum GH and IGF-I levels were used to assess changes in the GH-IGF-I axis. Total serum levels of IGF-I were determined using a RIA kit from Nichols Diagnostics (San Juan Capistrano, CA) after extraction of IGF-I from serum. IGF-I in rat serum was separated from IGF binding proteins using the acid-ethanol extraction method described by Crawford et al. (1992). Serum samples (100 μl) were mixed with 900 μl of acid-ethanol solution (12.5% 2 M HCl/87.5% ethanol, v/v), incubated 30 min at room temperature, and then centrifuged at 1500 g for 30 min at 5°C (IGF-binding protein precipitation 1). A 200-μl sample of the supernatant was then mixed with 100 μl of 0.855 M Tris base, pH 11, incubated 30 min at room temperature, and then centrifuged at 1500 g for 30 min at 5°C (IGF-binding protein precipitation 2). A 100-μl sample of the final supernatant was mixed with 1.4 ml of phosphate buffer, pH 7.5, and used for RIA. This acid-ethanol extraction method has previously been validated for RIA of total IGF-I in male and female rat serum by comparison with results using high-performance liquid chromatography methodology (Crawford et al., 1992).

Anterior Pituitary Analyses. PRL levels in the anterior pituitary were determined using a rat PRL RIA kit from GE Healthcare Life Sciences (Piscataway, NJ). Relative differences in pituitary GH content were assessed by polyacrylamide gel electrophoresis of aliquots of pooled pituitary homogenates from each group. Electrophoresis and protein staining was performed as described previously (DiPippo et al., 1995), and differences in GH content were measured by image analysis with ImageJ 1.42q software (National Institutes of Health, Bethesda, MD). Pituitary protein was measured to provide an index of changes in pituitary mass (Fitts et al., 2004).

Statistical Analysis of Grouped Data. Data were subjected to one-way analysis of variance followed by a post hoc analysis with Fisher’s least significant difference test; P < 0.05 was the criterion of significance. Serum GH values were log-transformed, and serum IGF-I values were in-transformed for statistical analyses to equalize group variances.

Data in the tamoxifen + fulvestrant groups was also transformed to express the percentage of inhibition of tamoxifen effect by fulvestrant: percentage of inhibition = 100 × (1 − (observed value − vehicle group mean) ÷ (tamoxifen group mean − vehicle group mean)). This enabled a formal statistical comparison of the fulvestrant sensitivity of different tamoxifen actions. For the tamoxifen groups given 6 or 12 mg/kg fulvestrant, the percentage of inhibition of tamoxifen effects was also calculated with a formula that adjusted for effects of fulvestrant alone: adjusted percentage of inhibition = 100 × (1 − (observed value − 6 mg/kg fulvestrant alone group mean) ÷ (tamoxifen group mean − vehicle group mean)).

Results

Uterine Weight. Uterine growth is the classic in vivo bioassay for estrogen agonist effects and is exquisitely sensitive to estrogens. Ovariectomy significantly decreased uterine weight, and tamoxifen treatment of ovariectomized rats produced a significant increase that was equivalent to 30% of the estrogenic effect of ovarian secretions in ovari-intact rats (Fig. 1). Fulvestrant lacked estrogen agonist activity on the uterus, and all dose levels produced >90% inhibition of the tamoxifen effect.

PRL, GH, and Total Protein Contents of the Anterior Pituitary. PRL and GH are the most abundant proteins in the rat pituitary (Fig. 2). Ovariectomy produced a 36% decrease in PRL content (μg/pituitary) and a 38% decrease in concentration (μg/mg protein) without changing the total protein content of the anterior pituitary (Figs. 2 and 3). In ovariectomized rats, fulvestrant alone had no effect on PRL content (μg/pituitary) or concentration (μg/mg protein) or total pituitary protein content. In contrast, tamoxifen treatment of ovariectomized rats caused a 5% increase PRL content that was coupled with a 24% decrease in pituitary protein content, resulting in a significant 39% increase in PRL concentration (μg/mg protein) (Fig. 3). Fulvestrant completely inhibited the increase in PRL concentration evoked by tamoxifen, but did not inhibit tamoxifen’s effect to decrease pituitary protein (Fig. 3). All fulvestrant doses completely blocked tamoxifen effects to increase PRL concentration and failed to block the tamoxifen-evoked decrease in total pituitary protein.

It should be noted that changes in anterior pituitary total protein content were poorly correlated with changes in body weight.
weight during the study. Thus, ovariectomy caused no change in total pituitary protein, while increasing body weight gain, and fulvestrant had no effect on total pituitary protein while blocking up to 60% of tamoxifen's effect to suppress growth.

Pituitary GH levels were assessed semiquantitatively using gel electrophoresis (Fig. 2). Ovariectomy produced a 25% increase in pituitary GH content. Tamoxifen treatment of ovariectomized rats reduced GH content by $\frac{3}{10}35\%$, and this tamoxifen effect was unaffected by 3 mg/kg fulvestrant and only modestly blocked by 6 or 12 mg/kg doses of fulvestrant ($\frac{2}{10}20$ and $\frac{3}{10}35\%$, respectively).

Somatic Growth. Ovarian estrogens contribute to the sexual dimorphism in somatic growth, and body weight gain and final tibia lengths were measured as indices of growth. Ovary-intact female rats gained 40 g during the 5-week treatment period; ovariectomy approximately doubled weight gain during this period and tamoxifen completely suppressed weight gain (Fig. 4). Fulvestrant alone did not significantly affect weight gain, but 3 mg/kg fulvestrant inhibited 34% of the tamoxifen effect and larger fulvestrant doses caused greater inhibition. Fulvestrant inhibition of tamoxifen effects on weight gain were evident within 1 week and continued throughout the study period (data not shown). Nonetheless, $\sim 40\%$ of the tamoxifen effect on weight gain persisted with 12 mg/kg fulvestrant.

Similar results were seen with tibia length, an index of longitudinal growth. Tibia length was modestly longer in ovariectomized rats than in intact females, but the difference was not significant (Fig. 4). However, tamoxifen-treated rats had tibias that were significantly shorter than those in intact females or ovariectomized rats. Fulvestrant alone had no effect on tibia length, but 3 mg/kg inhibited 35% of the tamoxifen effect; larger fulvestrant doses caused greater inhibition, but $\sim 40\%$ of the tamoxifen effect on tibia length persisted with 12 mg/kg fulvestrant.

**Fig. 2.** Polyacrylamide gel showing the effects of tamoxifen, fulvestrant (ICI), and their combination on GH and PRL levels in the anterior pituitary. The gel was loaded with equal amounts of pooled pituitary homogenate from each group and stained with Coomassie blue R-250; PRL and GH bands are indicated. Lane 1, ovary-intact group; lanes 2 to 7, ovariectomized groups; lane 2, vehicle control; lane 3, TM; lane 4, 6 mg/kg ICI; lane 5, TM + 3 mg/kg ICI; lane 6, TM + 6 mg/kg ICI; lane 7 = TM + 12 mg/kg ICI.

**Fig. 3.** The effect of fulvestrant on tamoxifen actions to increase pituitary PRL concentration and decrease total anterior pituitary protein content. Left, PRL concentration in the pituitary measured using RIA. Right, total pituitary protein content. *, $P < 0.05$ versus V.

**Fig. 4.** The effect of fulvestrant on tamoxifen actions to suppress indices of somatic growth (body weight gain and tibia length). Left, body weight gain during the treatment period. Right, tibia length. *, $P < 0.05$ versus V; **, $P < 0.05$ versus V and TM.
Although food intake was not measured in this study, it should be noted that prior work indicates that tamoxifen effects on body weight and longitudinal growth cannot be attributed to decreased food intake because food intake per kilogram of body weight is unchanged by tamoxifen (Fitts et al., 1998, 2001; Wallen et al., 2001).

Serum GH and IGF-I Levels. Serum GH was 30% lower in ovariectomized rats than in intact females but the difference was not significant. Tamoxifen produced a 65% decrease in serum GH in ovariectomized rats, and fulvestrant alone (6 mg/kg) produced a 60% decrease (Fig. 5). Combined treatment with tamoxifen and fulvestrant caused ~90% reductions in serum GH at all dose levels of fulvestrant, indicating an additive interaction.

Ovariectomy had little effect on serum IGF-I levels, but tamoxifen treatment of ovariectomized rats produced a significant 38% decrease; in contrast, fulvestrant alone (6 mg/kg) produced a 37% increase in serum IGF-I. In tamoxifen-treated rats, 3 mg/kg fulvestrant restored IGF-I to the level of vehicle-treated controls, and 6 or 12 mg/kg yielded IGF-I levels equivalent to those in rats given fulvestrant alone.

BMD in the Proximal Tibia and the Tibia Diaphysis. BMD was measured in both the proximal tibia (enriched in cancellous bone) and the tibia diaphysis, (predominantly cortical bone). Ovariectomy significantly decreased proximal tibia BMD, and tamoxifen prevented >80% of the ovariectomy effect. Fulvestrant alone had no effect on proximal tibia BMD in ovariectomized rats, but antagonized the protective effects of tamoxifen; ~60% blockade was produced with fulvestrant doses of 3 mg/kg, and 80% blockade was achieved with 12 mg/kg dosing (Fig. 6).

BMD in the tibia diaphysis was unaffected by ovariectomy or any of the drug treatments used (Fig. 6). This is consistent with the slower metabolic rate and turnover of cortical bone compared with cancellous bone and the greater effect of sex hormone treatments on cancellous bone.

Comparison of the Fulvestrant Sensitivity of Different Tamoxifen Actions. The fulvestrant sensitivity of different tamoxifen actions was statistically compared by using data transformed to express the percentage of inhibition of tamoxifen effects by fulvestrant. Serum GH was not included in such analyses because it was subject to an apparent additive interaction between fulvestrant and tamoxifen. Analysis of variance of the transformed data set indicated that fulvestrant effects were dose- and target-dependent ($P < 0.01$). In subsequent post hoc multiple comparison analyses, the uterine weight data were used as the index for discriminating tamoxifen actions that exhibited high, low, or no sensitivity to fulvestrant. The results showed that tamoxifen effects on

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**Fig. 5.** The effect of fulvestrant on tamoxifen actions to decrease serum levels of GH and IGF-I. Left, serum GH. Right, serum IGF-I. *, $P < 0.05$ versus V; **, $P < 0.05$ versus TM.

**Fig. 6.** The effect of fulvestrant on tamoxifen actions to increase bone mineral density in the tibia. Left, bone mineral density in the proximal tibia (upper third of tibia length), which is enriched in cancellous bone. Right, bone mineral density in the tibia diaphysis (middle third of tibia length) which has little cancellous bone. *, $P < 0.05$ versus V; **, $P < 0.05$ versus TM.
uterine weight were significantly more sensitive to 3 mg/kg fulvestrant than tamoxifen actions on body weight gain, tibia length, and total pituitary protein (Table 1). Tamoxifen actions on PRL, IGF-I, proximal tibia BMD, and uterine weight did not significantly differ in their sensitivity to 3 mg/kg fulvestrant. With larger fulvestrant dosages (6 or 12 mg/kg), pituitary protein seemed to be the only parameter that exhibited significantly less fulvestrant sensitivity than the uterus (Table 1). Indeed, the effect of tamoxifen to decrease total pituitary protein was insensitive to all fulvestrant doses.

Tamoxifen actions were also assessed for their sensitivity to 6 and 12 mg/kg fulvestrant using a data transformation that also included an adjustment for any effect associated with 6 mg/kg fulvestrant alone. This was of particular interest with respect to serum IGF-I, which was significantly increased by fulvestrant in the absence of tamoxifen. Such transformed data indicated that tamoxifen effects on IGF-I were inhibited 68 and 87% by 6 and 12 mg/kg fulvestrant, respectively (Table 1). The adjusted analysis continued to indicate that tamoxifen effects on IGF-I and uterine weight did not significantly differ in their inhibition by fulvestrant; tamoxifen effects on PRL and proximal tibia BMD also continued to display fulvestrant sensitivity equivalent to that on the uterus. In contrast, the adjusted analyses revealed that tamoxifen effects on weight gain were less sensitive to 6 or 12 mg/kg fulvestrant than the tamoxifen effect on uterine weight (Table 1).

### Discussion

This study examined the ability of fulvestrant to mimic or antagonize tamoxifen actions in ovariectomized rats in an effort to pharmacologically discriminate tamoxifen effects that may be mediated via ERα/ERβ versus GPER versus ERRγ. Tamoxifen effects mediated by ERα/ERβ would be expected to be blocked by fulvestrant; effects mediated by GPER would be expected to be mimicked by fulvestrant; and tamoxifen effects mediated by ERRγ would be expected to be resistant to fulvestrant. Tamoxifen induced uterine growth and pituitary PRL, suppressed somatic growth (i.e., body weight gain and tibia growth), decreased pituitary GH and total pituitary protein, decreased serum GH and IGF-I, and increased proximal tibia BMD. In contrast, fulvestrant alone had no effect on the above parameters with the exception of serum GH and IGF-I, where it mimicked the GH decrease produced by tamoxifen while paradoxically increasing IGF-I. Tamoxifen was equal to or more efficacious than endogenous estrogen in its effects on BMD, somatic growth, and IGF-I.

Tamoxifen actions could be further discriminated into three classes based on their differing sensitivity to inhibition by fulvestrant (high sensitivity, low sensitivity, and no sensitivity). For comparisons of fulvestrant sensitivity, the effect of tamoxifen to increase uterine weight provided an index for responses exhibiting high fulvestrant sensitivity; uterine weight increases were completely blocked with 3 mg/kg fulvestrant and larger doses produced no further inhibition. In comparisons with the uterine index, tamoxifen effects on PRL, IGF-I, and proximal tibia BMD were also classified as having high fulvestrant sensitivity because 3 mg/kg fulvestrant produced inhibition that was statistically equivalent to that seen on uterine weight. These data indicate that tamoxifen effects on uterine growth, PRL, IGF-I, and BMD can be largely attributed to its actions on ERα and ERβ. Based on studies comparing the phenotypes of mice lacking ERα or ERβ (see Curtis Hewitt et al., 2000) it seems that ERα is the key mediator of tamoxifen effects on uterine growth, PRL, and BMD. At present there are insufficient data to determine whether ERα, ERβ, or both mediate tamoxifen effects to decrease serum IGF-I. It should also be noted the present data are consistent with a recent report showing that fulvestrant can fully block tamoxifen effects to increase cancellous bone volume in the tibia of ovariectomized mice (Sugiyama et al., 2010).

The ability of fulvestrant to increase IGF-I in the absence of estrogens or tamoxifen was puzzling. One possibility is that fulvestrant may have down-regulated hepatic ERs and interfered with basal repression of IGF-I synthesis that might occur even in the absence of estrogens. However, fulvestrant has not been reported to decrease hepatic ER levels in the rat (Stavréus-Evers et al., 2001). Another possibility is that the IGF-I increase evoked by fulvestrant alone may reflect a GPER-mediated response that is not seen with tamoxifen because of an overriding repression of IGF-I evoked by ERα/ERβ activation.

Tamoxifen effects to decrease pituitary GH content, tibia length, and weight gain had low fulvestrant sensitivity. Fulvestrant at 3 mg/kg had no effect on pituitary GH and its effects on tibia length and weight gain were significantly less than seen on the uterine response. Although this difference was reduced by larger doses causing further inhibition, ~40 to 60% of tamoxifen effects on pituitary GH, tibia length, and weight gain persisted with 12 mg/kg fulvestrant. These data match previous findings using low fulvestrant doses (1–3 mg/kg/day) (Wade et al., 1983a,b; DiPippo and Powers, 1997; Alfinito et al., 2008) and show that part of tamoxifen actions to suppress growth are resistant to large fulvestrant doses. Thus, approximately 40 to 60% of tamoxifen effects to sup-

### Table 1

<table>
<thead>
<tr>
<th>Fulvestrant Dose</th>
<th>Inhibition of Tamoxifen Effect</th>
<th>Uterine Weight</th>
<th>Pituitary PRL</th>
<th>Serum IGF-I</th>
<th>Proximal Tibia BMD</th>
<th>Tibia Length</th>
<th>ΔBody Weight</th>
<th>Pituitary Protein</th>
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<td>3 mg/kg</td>
<td></td>
<td>94 ± 7</td>
<td>96 ± 9</td>
<td>82 ± 21</td>
<td>58 ± 16</td>
<td>35 ± 12**</td>
<td>34 ± 8**</td>
<td>−2 ± 9**</td>
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<tr>
<td>6 mg/kg</td>
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<td>96 ± 10</td>
<td>133 ± 11</td>
<td>136 ± 17</td>
<td>67 ± 13</td>
<td>69 ± 19</td>
<td>40 ± 4</td>
<td>−4 ± 11**</td>
</tr>
<tr>
<td>Adjusted</td>
<td></td>
<td>97 ± 10</td>
<td>129 ± 11</td>
<td>68 ± 17</td>
<td>74 ± 13</td>
<td>73 ± 19</td>
<td>27 ± 4**</td>
<td>−22 ± 11**</td>
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<td>12 mg/kg</td>
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<td>87 ± 10</td>
<td>63 ± 9</td>
<td>47 ± 8</td>
<td>5 ± 14**</td>
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</table>

**P < 0.05 vs. uterine weight at corresponding fulvestrant dose.
press somatic growth seem to be mediated by ERα or ERβ, and the remainder seems to involve other mechanisms. Studies comparing mice lacking ERα or ERβ suggest that ERα is probably responsible for the portion of tamoxifen effects on body weight and bone growth that are blocked by fulvestrant (see Curtis Hewitt et al., 2000).

In the present study, tamoxifen decreased serum GH, which was consistent with its ability to decrease pituitary GH in this and prior studies (DiPippo et al., 1995; DiPippo and Powers, 1997). However, fulvestrant mimicked rather than antagonized tamoxifen effects on serum GH, which was unexpected because fulvestrant alone had no effect on pituitary GH content in this study or prior work (DiPippo and Powers, 1997). A membrane-bound GPER (GPR30) has been postulated to mediate some estrogen effects, and estradiol, tamoxifen and fulvestrant have been identified as full agonist ligands for the GPER by multiple groups (Revankar et al., 2005; Thomas et al., 2005; Li et al., 2010; Lucas et al., 2010). Thus, the ability of fulvestrant to mimic rather than inhibit tamoxifen effects on serum GH seems consistent with involvement of GPER in this response. However, although the physiological features of the serum GH response seem consistent with involvement of GPER, fulvestrant alone increased serum IGF-I, and fulvestrant also blocked tamoxifen actions to lower IGF-I (Fig. 5). Because IGF-I produces negative feedback inhibition of GH release (see Giustina and Veldhuis, 1998), the decreases in serum GH produced by fulvestrant might reflect increased IGF-I feedback inhibition rather than a GPER-mediated effect.

The ability of fulvestrant to only partially block tamoxifen effects on growth was incongruous with its ability to fully block tamoxifen-evoked decreases in serum IGF-I. Such data underscore the complexity of tamoxifen actions in vivo and support the idea that growth suppression evoked by tamoxifen is mediated by multiple mechanisms and includes changes in parameters beyond those examined here, such as IGF-binding proteins or GH and IGF-I signaling cascades, etc. (Leung et al., 2004).

In rats, estrogen induces PRL synthesis and secretion and can cause a selective hypertrophy and hyperplasia of lactotrophs that increases pituitary weight and total pituitary protein (De Nicola et al., 1978; Holtzman et al., 1979). Tamoxifen exerts partial agonist effects to stimulate PRL production, but lacks agonist effects to stimulate lactotroph growth. Indeed, in this and other studies tamoxifen decreased total pituitary protein while modestly increasing PRL to yield a significant increase in PRL concentration (µg/mg protein) (DiPippo et al., 1995; Fitts et al., 2001, 2004). The reduction in total pituitary protein/mass was the only effect of tamoxifen that was not significantly inhibited by any dose of fulvestrant and does not seem to be mediated by ERα or ERβ. It should be noted that lactotrophs and somatotrophs account for the bulk of pituitary mass, and tamoxifen has previously been shown to inhibit the thyroid hormone induction of pituitary GH in the rat (DiPippo et al., 1995; Fitts et al., 2001). Thus, tamoxifen-evoked decreases in pituitary mass involve both cell types.

The mechanisms underlying the fulvestrant-resistant effects of tamoxifen remain to be determined. CNS effects of E2 were found to be resistant to modest systemic doses of fulvestrant (1 mg/kg/day) in some early short-term experiments (Wakeling et al., 1991; Wade et al., 1993a). This suggested that fulvestrant could not cross the blood-brain barrier (BBB) and led to the idea that fulvestrant resistance indicated a CNS-mediated estrogen effect (Wade et al., 1993a). Because tamoxifen readily penetrates the BBB, fulvestrant resistance might thus indicate an ER-mediated effect arising in the CNS. However, fulvestrant is a highly lipophilic drug that would be expected to traverse membranes composing the BBB, and others have reported that systemic doses of fulvestrant inhibit diverse estradiol responses attributed to CNS actions. This includes effects on body weight, sexual behavior, and induction of luteinizing hormone surges (Wade et al., 1993a,b; Donath and Nishino, 1998; Gardener and Clark, 2001; Yin et al., 2002). Moreover, hot flashes attributed to hypothalamic actions are one of the most common adverse effects of fulvestrant in women receiving the drug for advanced breast cancer (Bross et al., 2002). The ability of fulvestrant to cross the BBB of the rat was directly assessed in a study by Alfinito et al. (2008). Pharmacokinetic analyses showed that systemic doses of fulvestrant (1–3 mg/kg/day) readily crossed the BBB; brain and hypothalamic concentrations were dose-dependent and approximated plasma levels within 3 days. Alfinito et al. (2008) also showed that systemic doses of fulvestrant (1–3 mg/kg) inhibited ethinyl estradiol effects on thermoregulation and body weight, which are attributed to CNS actions. Thus, lack of drug entry into the CNS is unlikely to explain fulvestrant resistance of some tamoxifen actions.

The ability of tamoxifen and its active metabolite (4-hydroxytamoxifen) to block the constitutive activity of ERRγ provides a novel, biologically plausible mechanism through which tamoxifen may exert actions that are not mediated by ERα or ERβ (Coward et al., 2001; Tremblay et al., 2001). Although the present study does not address the molecular mechanism mediating the fulvestrant-resistant actions of tamoxifen, the data suggest that parameters related to pituitary function and somatic growth may be relevant to future in vivo analyses of drugs specifically targeting ERRγ.

In this study, no tamoxifen effects other than decreases in serum GH were mimicked by fulvestrant in a manner suggesting involvement of the GPER. Indeed, no changes in estrogen effects on uterine growth were reported in the two GPER knockout models where this was investigated (Otto et al., 2009; Windahl et al., 2009). In addition, estrogen and ovarioectomy effects on cancellous BMD in the tibia were unaffected by GPER knockout in the single study where such data were reported (Windahl et al., 2009). Such findings are consistent with results of the present study and diverse investigations by others that indicate a predominant role of ERα in estrogen and tamoxifen effects on the uterus and BMD. Indeed, fulvestrant alone (i.e., a GPER agonist) did not significantly affect weight gain, bone length, BMD, or cancellous bone volume in ovarioctomized rodents in this or other studies (e.g., Wakeling et al., 1991; Gallagher et al., 1993; Sibonga et al., 1998; Sugiyama et al., 2010). Nonetheless, fulvestrant only partially blocked tamoxifen effects to suppress weight gain and tibia growth in the present study, implying that mechanisms unrelated to ERα and ERβ also contribute to such effects. In this regard, it is noteworthy that altered somatic growth phenotypes have been reported in two of the four GPER knockout mouse models. Decreased body weight and bone length in females but not males was found in the model created by Mårtensson et al. (2008), and
increased body weight and bone length in both sexes have been reported in the model developed by Wang et al. (2008).

At this point it should be noted that chronic in vivo dosing models designed to detect robust changes elicited by long-term manipulation of nuclear receptors (such as in this study), may be ill-suited for detecting more subtle modulatory effects arising from rapid signaling events mediated by GPER. For example, it is now clear that fulvestrant lacks estrogen agonist effect to induce uterine weight and can fully block tamoxifen and estrogen induction of uterine weight. Nonetheless, multiple groups have reported data indicating that GPER activation can stimulate the proliferation of normal or malignant endometrial cells (Vivacqua et al., 2006; Dennis et al., 2009; Ignatov et al., 2010). Short-term acute dosing models examining more transient and refined responses may be better suited for clarifying the role of the GPER.

**References**


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