Effects of Various Selective Estrogen Receptor Modulators with or without Conjugated Estrogens on Mouse Mammary Gland

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Selective estrogen receptor modulators (SERMs) are small molecules that, depending on the end point measured, may either function as estrogen receptor (ER) agonists or antagonize estrogens’ agonist activity. A key feature of SERMs is the inhibition of ER agonist action on the uterus and mammary gland, but the degree of antagonism varies among compounds and end points. Bazedoxifene is a SERM that is being clinically evaluated both as a monotherapy for the prevention and treatment of osteoporosis and in combination with conjugated estrogens (CEs) for the treatment of menopausal symptoms and prevention of osteoporosis. The studies reported here compare the relative ER agonist and antagonist effects of three pharmacologically distinct SERMs (bazedoxifene, raloxifene, and lasofoxifene) on the ovariectomized mouse when administered alone or as a tissue-selective estrogen complex, a term used to describe the partnering of a SERM and one or more estrogens. At the minimum dose required to maximally reduce CE-stimulated uterine wet weight increase for each SERM, the degree of inhibition varied among the SERMs, with a rank order of bazedoxifene > raloxifene > lasofoxifene, in which only bazedoxifene was statistically similar to vehicle. In the mammary gland, in which amphiregulin mRNA and morphological effects were measured, bazedoxifene generally exhibited less agonist activity and was a more effective antagonist of CE than raloxifene or lasofoxifene. In summary, in an animal model evaluating estrogen-modulated uterine effects and mammary gland development, bazedoxifene/CE demonstrated less mammary gland stimulation than raloxifene/CE and lasofoxifene/CE. (Endocrinology 150: 1897–1903, 2009)

The estrogens estrone, estriol, and 17β-estradiol (E2) are steroidal hormones produced primarily by the ovaries. Estrogens exert their effects by binding to high-affinity receptors, of which two have been described: estrogen receptor (ER)-α and ERβ. These receptors are most well characterized as ligand-activated transcription factors (1), but there is strong evidence supporting involvement in extranuclear signaling as well (2–6).

Our understanding of estrogen biology has been significantly advanced by the design and discovery of an immense number of small molecule modulators of ER function (7–9). Some of these compounds are approved drugs and generally fall into three functional categories: full agonists (e.g. 17α-ethinyl estradiol used in oral contraceptives), full antagonists (e.g. fulvestrant used to treat breast cancer), and selective estrogen receptor modulators (SERMs; e.g. raloxifene used to treat and prevent postmenopausal osteoporosis as well as reduce the risk of invasive breast cancer in postmenopausal women).

SERMs are compounds that have the seemingly paradoxical ability to mimic or inhibit the activity of estrogens, depending on the end point measured (10). Whereas the term SERM might literally apply to selective agonists of ERα or ERβ, these isoform-selective compounds do not fit the traditional definition of a SERM, and thus, use of this term to describe them should be avoided. The classic profile of a SERM is a compound that antagonizes estrogens’ effects on the uterus and mammary gland and mimics estrogenic effects on lipids and the skeleton. SERMs

Abbreviations: AREG, Amphiregulin; CE, conjugated estrogen; E2, 17β-estradiol; ER, estrogen receptor; SERM, selective estrogen receptor modulator; TSEC, tissue-selective estrogen complex.
also tend to be antagonists of estrogen action in the brain. The precise molecular mechanisms by which a single compound can achieve this range of context-dependent end points remain an active area of investigation, but evidence suggests that subtle conformational changes in the ER induced upon ligand binding lead to differential comodulatory protein recruitment. Effector genes in different tissues will thus be variably modulated. Whereas this explanation requires further substantiation and may not explain all tissue-selective effects, it is the primary working hypothesis in the field (for review, see Refs. 11 and 12).

The current clinical utility of the SERMs tamoxifen and raloxifene is limited to postmenopausal women due to both an increased risk of developing ovarian cysts when these compounds are used in premenopausal women and the adverse effects that these compounds may have on pregnancy and fetal development (13–15). In the arena of postmenopausal hormone therapy, SERMs have the potential to treat all of the symptoms of menopause that are responsive to estrogens, but current marketed or late-stage development compounds are lacking a key feature: not only do they fail to reduce vasomotor symptoms (i.e., hot flushes and night sweats), but they may also actually exacerbate them in some instances (http://www.eviesta.com/ pat/index.jsp). Because vasomotor symptoms are the primary reason that women seek treatment at menopause, there remains an unmet medical need to provide an alternative therapy for symptomatic women.

Accordingly, the concept of partnering a SERM with one or more estrogens, referred to as a tissue-selective estrogen complex (TSEC), has evolved and may provide the opportunity for a fully functional progestin-free hormone therapy. Bazedoxifene, a SERM in development for the prevention and treatment of osteoporosis (17), is being studied clinically in combination with conjugated estrogens (CEs) as a TSEC for the treatment of menopausal symptoms and the prevention of postmenopausal osteoporosis (18). Recent work in rats has demonstrated that bazedoxifene/CE prevented ovariectomy-induced bone loss, did not lead to uterine stimulation, reduced total cholesterol, and alleviated vasomotor instability (19).

To be a viable clinical candidate, the coadministered SERM must effectively inhibit the pro-proliferative activity of CE on the mammary gland as well as the uterus. In the present study, we examined the relative agonist activities of bazedoxifene compared with two other SERMs, raloxifene and lasofoxifene, when dosed alone and their antagonist activities when coadministered with CE. The primary end points of interest were mammary gland morphological and estrogen-responsive marker gene expression [amphiregulin (AREG)] in the ovariectomized sexually immature mouse.

Materials and Methods

Animal care and dosing and tissue removal and processing

All animal experiments described here were conducted in accordance with the U.S. Department of Agriculture and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and approved by the Institutional Animal Care and Use Committee of Wyeth Research (Collegeville, PA).

Four-week-old, ovariectomized C57BL/6 female mice (Taconic, Hudson, NY) were fed a casein-based diet and acclimated for at least 4 d before dosing. For the indicated number of days, mice were dosed daily with 0.1 ml of test compound. All compounds were obtained from the Wyeth compound library and were administered orally (by gavage) in a vehicle of 2% Tween 80/0.5% methylcellulose with the exception of E2, which was delivered sc at 5 μg/kg in a vehicle of 50% dimethylsulfoxide/50% 1× Dulbecco’s PBS. Approximately 6 h after the last dose of compound was administered, mice were euthanized by an overdose of CO2, followed by pneumothorax. One inguinal mammary gland was excised and frozen on dry ice for subsequent RNA isolation, whereas the other was fixed in ethanol-acetic acid (6:1) and stained for morphological analysis as previously reported (20). The uterus was excised, trimmed of adherent fat, and weighed after expressing any luminal fluid.

RNA isolation and quantitative real-time PCR

Total RNA was prepared individually from a whole mammary gland from each mouse. The mammary gland was homogenized in about 1.8 ml QIAzol lysis reagent (QIAGEN, Valencia, CA) at about 14,000 rpm for about 15–20 sec using a Polytron homogenizer PT3100 (Brinkmann, Westbury, NY). After a 0.2-ml chloroform extraction of 1 ml homogenate and centrifugation at 4 °C for 15 min at 20,000 × g, 0.5 ml aqueous phase was collected. The RNA from the aqueous phase was then purified using an RNeasy lipid tissue minikit (QIAGEN) according to the manufacturer’s protocol. Residual genomic DNA was removed by on-column ribonuclease-free deoxyribonuclease treatment during RNA purification, and the RNA concentration was adjusted to 0.05 mg/ml. AREG mRNA expression was analyzed using quantitative real-time PCR on an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City CA) and an AREG TaqMan gene expression assay (Applied Biosystems; no. Mm00437583_m1). A comparative cycle threshold method was used to analyze the data, and differences in AREG expression are expressed as a fold change to the mean of the vehicle group.

Uterine wet weight and AREG data analysis and statistics

Uterine wet weight and AREG data were analyzed using an in-house SAS (SAS Institute, Cary, NC)/Excel (Microsoft, Seattle, WA) program and one-way ANOVA. Uterine weight data were not transformed before analysis, but mRNA expression data were log transformed to determine statistical significance (P < 0.05). All graphs are presented on a linear scale.

Whole-mount analyses and statistics

To quantify the morphological effects of the compounds on the mammary gland, two parameters were measured. First, as a measure of ductal complexity, three reviewers (who were unaware of what treatment the animals received) counted the total number of branch points in each mammary gland. When consistency of scoring was compared among the reviewers, it was found that there was a systematic difference such that, for each of the animals, one reviewer tended to consistently score a number of points higher than the others. Separate ANOVA analysis (not shown) for each reviewer showed that, in terms of group comparisons, the results were quite consistent among the reviewers (e.g., the overall F test statistics and the pair-wise comparison t statistics were all in very good agreement). Based on these observations, the data from three reviewers were normalized to remove scorer effect. There were four animals that appeared to be outliers in that all three reviewers consistently scored them very differently. None of the animals were in the same treatment group. To verify these animals were outliers and justified eliminating them from analysis, a quartile analysis was applied as outlined elsewhere (21). The raw data were square root transformed because variability among the samples increased with the mean score. The adjusted score for each animal was taken to be the average of the three normalized values, and then ANOVA was applied to the averaged scores. The significance of the difference between each group pair was based on
the t statistics for the pair-wise comparison, and significance level $\alpha = 0.05$ was used. No multiplicity adjustment was applied.

The second measurement of mammary gland morphology assessed the degree to which the ductal tree had invaded the fat pad. These measurements were made by one person who was unaware of the treatment the animals received. Using a digital ruler and similarly enlarged pictures oriented so that the nipple was to the right of the lymph node (see as arranged in Fig. 5), distance was measured from the rightmost edge of the lymph node to the tip of the leftmost point of the ductal tree, which invaded the fat pad from right (nipple) to left (lymph node). The lymph node was used as a reference point because, unlike the nipple, it was clearly visible in all of the samples. Positive numbers represent the distance (in mm) to the left of the rightmost edge of the lymph node to which the ductal tree had invaded the fat pad, whereas negative numbers indicate distance measurements from mammary glands for which the ductal tree failed to extend to the lymph node. Because the ruler was present in each photo, the numbers presented represent accurate millimeter measurements of ductal tree growth relative to the rightmost edge of the lymph node. The distances were analyzed using an in-house SAS/Excel program and one-way ANOVA, and a threshold of $P < 0.05$ was employed to determine statistical significance between groups.

### Results

**Determination of CE dose and dosing duration and gene marker validation**

An initial study was conducted to determine the appropriate dose of CE to elicit an increase in uterine wet weight as well as an appreciable invasion of the mammary gland ductal tree into the inguinal fat pad. Mice were dosed with 1, 3, or 10 mg/kg of CE for 7, 10, or 14 d, and E2 (5 $\mu$g/kg) was included as a positive control. As shown in supplemental Fig. 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org, compound-induced uterine wet weight increase was similar after 10 or 14 d of dosing, whereas 7-d dosing resulted in a lower and/or more variable response at 1 and 3 mg/kg, thereby rendering 7-d dosing suboptimal for the uterine wet weight end point. Qualitative mammary gland whole-mount analysis (data not shown) demonstrated that 3 and 10 mg/kg of CE administered for 10 or 14 d resulted in equivalent gland development as assessed by primary branching complexity and fat pad invasion. Based on the uterine wet weight and qualitative mammary morphology data from this study, we chose to proceed with 3 mg/kg of CE given for 14 d as our reference estrogenic dose for future combination studies with SERMs.

AREG is an estrogen-responsive gene in the mouse mammary gland that mediates estrogen stimulated puberal gland development, specifically proliferation of the ductal epithelium into the mammary fat pad (22). To confirm that AREG mRNA regulation was a relevant gene marker for our studies, expression was analyzed in the glands taken from mice dosed for 14 d. As shown in supplemental Fig. 2, expression of AREG mRNA was induced dose dependently by CE, and these data further confirmed that 3 mg/kg of CE would be suitable for future studies.

**Determination of the uterine minimum fully antagonist dose of each SERM**

To determine the minimum fully effective antagonist dose on the end point of uterine weight for bazedoxifene, raloxifene, and lasofoxifene, four doses of each SERM were coadministered with 3 mg/kg of CE for 14 d. The minimum fully antagonist dose of each SERM would then be tested in subsequent studies for which the end point of interest was the mammary gland. This study was needed to choose pharmacologically equivalent doses of these pharmacokinetically dissimilar SERMs on the end point of uterine ER antagonist activity so that mammary gland effects could be fairly compared. Figure 1 shows that 2 and 10 mg/kg bazedoxifene and 10 mg/kg raloxifene fully antagonized the CE-induced increase in uterine wet weight, lowering uterine wet weight to levels statistically similar to vehicle. As a result, 2 mg/kg bazedoxifene and 10 mg/kg raloxifene were chosen for future studies. Lasofoxifene, however, did not antagonize CE at any of the four doses tested, so a fully effective antagonist dose could not be chosen based on this study. A dose of 2 mg/kg was selected for future studies based on our previous experience with this compound.

**SERM effects on uterine wet weight and AREG expression**

To determine the relative estrogenic and antiestrogenic effects of bazedoxifene, raloxifene, and lasofoxifene on the mammary gland, we assessed the end points of gene expression and morphology at doses at which bazedoxifene and raloxifene had equivalent uterine activity, in addition to 2 mg/kg lasofoxifene. The uterine wet weight data shown in Fig. 2 demonstrate that bazedoxifene was the only compound that was indistinguishable from vehicle when tested alone or in combination with CE as a TSEC. The agonist and antagonist effects of raloxifene were statistically similar to bazedoxifene but different from vehicle. More specifically, raloxifene’s agonist activity was not as consistently low as bazedoxifene (compare Figs. 1 and 2) in that it was statistically similar to vehicle on some, but not all, occasions. Lasofoxifene had the greatest agonist activity when tested alone and correspondingly, the least CE antagonist activity.

![FIG. 1](https://academic.oup.com/endo/article-abstract/150/4/1897/245852/1899)

**FIG. 1.** Dose-dependent effects of bazedoxifene (BZA), raloxifene (RAL), and lasofoxifene (LAS) on CE-induced (3 mg/kg) increase in uterine wet weight after 14 d of compound treatment (n = 6/group). Vertical arrows indicate the antagonist dose of each SERM on uterine wet weight endpoint used in subsequent studies. *, Groups significantly greater ($P < 0.05$) than vehicle (V) group.
FIG. 2. Uterine wet weight after treatment with bazedoxifene (BZA; 2 mg/kg), raloxifene (RAL; 10 mg/kg), and lasofoxifene (LAS; 2 mg/kg) alone and in combination with CE (3 mg/kg) for 14 d (n = 7–10/group). Groups labeled with the same letter are statistically similar (P > 0.05). V, Vehicle.

Figure 3 presents mammary gland AREG mRNA expression data from this study showing that bazedoxifene demonstrated less agonist and more effective antagonist activity compared with raloxifene. Lasofoxifene was consistently the most agonistic of the three SERMs and was a correspondingly less effective antagonist. These results were confirmed in a duplicate study (data not shown). Similar results were obtained from this replicate set of RNA using a second estrogen-regulated gene, indolamine pyrrole 2,3 dioxygenase (supplemental Fig. 3), although in this case, the agonist activity of bazedoxifene and raloxifene were statistically equivalent.

Mammary gland whole-mount evaluation

Representative whole mounts illustrating mammary gland morphology are shown in Fig. 4. The effects of compounds on mammary gland morphology were quantified using two end points. First, three reviewers who were blinded to the sample identity counted the total number of branch points seen in the ductal tree. Branch points were chosen as a measure of the ductal tree complexity. A summary of this analysis is shown in Fig. 5A, and the key interpretations are as follows. First, mammary glands from animals treated with CE had significantly more branch points than mice treated with E2. Second, when the SERMs were tested as agonists, bazedoxifene and raloxifene were statistically similar to vehicle. Third, when the SERMs were evaluated for their ability to inhibit CE’s activity, only bazedoxifene completely inhibited the activity of CE.

The second end point used to quantify mammary gland morphological effects was measurement of the degree of invasion of the ductal tree into the fat pad (Fig. 5B). Ductal invasion measurements represent the relative distance (in millimeters) from the rightmost edge of the lymph node to the leftmost point of the ductal tree; positive numbers indicate ductal growth (from right to left in Fig. 5) beyond the rightmost edge of the lymph node, whereas negative numbers indicate ductal invasion failing to extend to the lymph node. Unlike the ductal branching endpoint results, E2 and CE treatments led to equivalent fat pad invasion. The degree of fat pad invasion was similar in mammary glands taken from vehicle- and bazedoxifene-treated mice, and bazedoxifene was also the only SERM to fully antagonize the action of CE in a TSEC. Raloxifene and lasofoxifene behaved similarly to each other when tested as agonists or antagonists.

Discussion

Substantial clinical data on the effects of SERMs in postmenopausal women have accrued over the last few decades and clearly demonstrate their therapeutic value in the prevention and treatment of osteoporosis and, more recently, on the prevention of breast cancer (see Ref. 23 for review). SERM therapy was originally designed with expectations of effectively treating menopausal symptoms and osteoporosis without the unwanted stimulation of the uterus and breast, but to date no SERM has completely demonstrated this desired profile. Although partial success has been achieved with raloxifene, this SERM does not capture the full range of benefits of traditional estrogen (or estrogen plus progestin) therapy. In particular, raloxifene can increase vasomotor symptoms and is not indicated to treat vaginal atrophy (http://www.evista.com/pat/index.jsp). Developing a single molecule that confers the entire spectrum of benefits remains an active area of investigation. Several preclinical reports (24–26) indicate that a SERM can be designed to also treat vasomotor symptoms with minimal uterine stimulation, but to date clinical data are lacking.

Partnering a SERM with one or more estrogens, a combination now defined as a TSEC, is another potential strategy to achieve comparable efficacy to estrogen/progestin hormone therapy regarding menopausal symptoms and the prevention of osteoporosis. Importantly, the TSEC does not require a progestin to negate the endometrial stimulation normally associated with estrogen use in nonhysterectomized women, and thus, endometrial hyperplasia and the risk of endometrial cancer should be eliminated with TSEC therapy. The key to success with this approach will be to achieve a balance of ER agonist and antagonist activity such that the endometrium is protected, breast stimulation is minimal, and ER agonist activity is retained in the brain (as measured by hot flashes), vagina, and skeleton.

One clinical study pairing raloxifene (60 mg/d) with oral E2 (1 mg/d) as a TSEC demonstrated that the combination of raloxifene and E2 significantly reduced the number of vasomotor symptoms
compared with raloxifene alone; however, there was an increase in endometrial hyperplasia in the raloxifene/E2 combination group that was not seen with raloxifene alone (27). The investigators concluded that although effective at reducing vasomotor symptoms, this specific combination therapy yielded an unacceptable incidence of uterine hyperplasia.

Preclinical observation demonstrated that raloxifene stimulates the rat uterus (17), and this stimulatory effect has been seen clinically as an increase in endometrial thickness but not to an extent considered clinically relevant. The preclinical data indicate that raloxifene may not sufficiently antagonize the uterine stimulation associated with an estrogen, such as E2 or CE, due to its own endogenous uterine stimulatory activity. Furthermore, although comparative SERM data reveal that other SERMs in development demonstrate varying effects on several typical SERM end points, uterine stimulation appears to be the one end point differentiating most SERMs. The SERM bazedoxifene has proven less uterotrophic than raloxifene in preclinical studies (17). Recently published data generated in the rat indicate bazedoxifene can be successfully combined with CE to achieve the desired profile on the uterus, skeleton, and lipid profile and alleviate vasomotor instability (19). Moreover, the initial clinical data have been positive from studies evaluating the efficacy of a bazedoxifene/CE TSEC on vasomotor symptoms and postmenopausal osteoporosis as well as endometrial safety and tolerability (i.e. amenorrhea, breast pain) (18, 28).

The purpose of the studies reported here was to compare the effects of bazedoxifene, raloxifene, and lasofoxifene on estrogen-dependent end points in the mammary gland, both when each SERM was administered alone and as a TSEC partnered with CE. Previously we evaluated the effects of these three SERMs in combination with E2 in a similar model but using older mice (29). In that study, uterine wet weight and expression of an mRNA marker showed that bazedoxifene had less agonist and equivalent or better antagonist activity than the other SERMs, depending on the end point measured. For the present studies, we chose to use sexually immature ovariectomized mice to maximize our ability to detect estrogen-dependent responses in the mammary gland. By limiting previous exposure to estrogens, we were able to more sensitively detect changes in the ductal invasion of the mammary fat pad as well as primary branching patterns, which are two estrogen-dependent processes that are largely complete in older mice (16). In addition to these morphological end points, we measured mammary gland AREG mRNA expression. AREG has recently been demonstrated to be an estrogen-responsive gene responsible for mediating the puberal increase in ductal proliferation and elongation (22). This gene has very low expression in vehicle-treated mice, and thus, the dramatic up-regulation by estrogens makes this end point a very sensitive one.

Assessing the relative effects of SERMs in vivo is complicated by the fact that compound effects can vary by end point. To correctly compare the activities of the three SERMs, we first determined the dose of each compound that maximally antagonized CE-induced uterine wet weight increase. This end point was chosen as a clinically relevant and critical pharmacological

![Figure 4](https://academic.oup.com/endo/article-abstract/150/4/1897/2455852)
feature of a SERM. As expected from previously published work, bazedoxifene consistently displayed minimal or no ER agonist activity compared with raloxifene or lasofoxifene and, in fact, was frequently statistically equivalent to vehicle. The response to raloxifene was more often significantly greater than vehicle, and lasofoxifene was always greater than vehicle. When tested in a TSEC, bazedoxifene demonstrated similar to superior CE antagonism efficacy compared with raloxifene, and both of these SERMs were consistently more effective antagonists of CE than lasofoxifene. Collectively, the AREG and whole-mount data from the mammary gland mirror the data from the uterus in this model. On the end points we measured, bazedoxifene is a more effective antagonist of CE activity on both the uterus and mammary gland compared with raloxifene and lasofoxifene. The SERMs’ ability to effectively inhibit CE’s activity is inversely related to the compound’s agonist effects. These preclinical data suggest that the intrinsic ER agonist properties of the SERM will determine its effectiveness as a component of a TSEC, and the existing clinical data on raloxifene/E2 (27) and bazedoxifene/CE (18) would further suggest that there is very little tolerance for inherent agonist activity in a clinically successful combination. In addition, because the mammary gland data also reveal SERM-specific distinctions among the TSECs, the potential for variation in breast responses with long-term treatment exists as well. Understanding these key preclinical properties may allow for the clinical development of a TSEC for the effective treatment of menopausal symptoms and the prevention of osteoporosis with improved tolerability and safety compared with currently available therapies.

Acknowledgments

We thank Wyeth BioResources for animal care and dosing support, Vargheese Chennathukuzhi and Xiaochun Zhang for assistance with tissue collection, Connie Stephens and Christa Smolenski for help with quantitative whole mount assessment, and Shuguang Huang for statistical analyses of mammary gland whole-mount data.

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Disclosure Summary: All authors are full-time employees of Wyeth Research.

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