INTERACTIVE EFFECTS OF BAZEDOXIFENE ACETATE AND CONJUGATED EQUINE
ESTROGENS ON THE BREAST AND REPRODUCTIVE TRACT OF
POSTMENOPAUSAL MONKEYS

BY

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- Submitted to *Clinical Cancer Research*, May 2012

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<th>Description</th>
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<tr>
<td>AAALAC</td>
<td>Association for Assessment and Accreditation of Laboratory Animal Care</td>
</tr>
<tr>
<td>ABI</td>
<td>Applied Biosystems, Inc.</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-24&lt;/sub&gt;</td>
<td>Area under the curve from time 0 to 24 hours</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-associated agonist of cell death</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
</tr>
<tr>
<td>BAG-1</td>
<td>BCL2-associated athanogene</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>Bel-2</td>
<td>B-cell CLL/lymphoma</td>
</tr>
<tr>
<td>Bel-X&lt;sub&gt;L&lt;/sub&gt;</td>
<td>BCL-2 like 1 (isomeric form L)</td>
</tr>
<tr>
<td>Bel-X&lt;sub&gt;S&lt;/sub&gt;</td>
<td>BCL-2 like 1 (isomeric form S)</td>
</tr>
<tr>
<td>BZA</td>
<td>Bazedoxifene acetate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CEE</td>
<td>Conjugated equine estrogens</td>
</tr>
<tr>
<td>CYP 19</td>
<td>Aromatase (cytochrome P450 enzyme)</td>
</tr>
<tr>
<td>Def β1</td>
<td>Defensin beta 1</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
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<td>DHEA-S</td>
<td>Dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DMBA</td>
<td>Dimethylbenzanthracene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E1</td>
<td>Estrone</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>Estrogen receptor beta</td>
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<tr>
<td>ET</td>
<td>Estrogen-alone therapy</td>
</tr>
<tr>
<td>EPT</td>
<td>Estrogen + progestin therapy</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GREB1</td>
<td>Gene regulated by estrogen in breast cancer 1</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
</tr>
<tr>
<td>HSD17B1</td>
<td>17-beta hydroxysteroid dehydrogenase type 1</td>
</tr>
<tr>
<td>HSD17B2</td>
<td>17-beta hydroxysteroid dehydrogenase type 2</td>
</tr>
<tr>
<td>HT</td>
<td>Hormone therapy</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>Insulin-like growth factor-binding protein 1</td>
</tr>
<tr>
<td>IGSF1</td>
<td>Immunoglobulin superfamily member 1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
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<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>Ki67</td>
<td>MIB1 proliferation marker</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid cell leukemia sequence 1 (BCL2-related)</td>
</tr>
<tr>
<td>MPA</td>
<td>Medroxyprogesterone acetate</td>
</tr>
<tr>
<td>NHP</td>
<td>Nonhuman primate</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>P450c17 (17-20 lyase)</td>
<td>Cytochrome P450 enzyme, 17-20 lyase</td>
</tr>
<tr>
<td>PGR (A or B)</td>
<td>Progesterone receptor A or B</td>
</tr>
<tr>
<td>PMW</td>
<td>Postmenopausal women</td>
</tr>
<tr>
<td>PPM1K</td>
<td>Protein phosphatase 1K, mitochondrial</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>RIN</td>
<td>Ribonucleic acid (RNA) integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RMA</td>
<td>Robust microchip average</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SMART Trials</td>
<td>Selective estrogen, menopause, and response to therapy</td>
</tr>
<tr>
<td>STC2</td>
<td>Stanniocalcin-2</td>
</tr>
<tr>
<td>STS</td>
<td>Estrogen sulfatase</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>Sulfotransferase family 1E, estrogen-preferring, member 1</td>
</tr>
<tr>
<td>TDLU</td>
<td>Terminal ductal lobular units</td>
</tr>
<tr>
<td>TFF1</td>
<td>Trefoil factor 1</td>
</tr>
<tr>
<td>TFF3</td>
<td>Trefoil factor 3</td>
</tr>
<tr>
<td>Tukey HSD</td>
<td>Tukey honestly significant difference post hoc test</td>
</tr>
<tr>
<td>WFUPC</td>
<td>Wake Forest University Primate Center</td>
</tr>
<tr>
<td>WHI</td>
<td>Women’s Health Initiative clinical trial</td>
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ABSTRACT

Kelly F. Ethun, D.V.M.

INTERACTIVE EFFECTS OF BAZEDOXIFENE ACETATE AND CONJUGATED EQUINE ESTROGENS ON THE BREAST AND REPRODUCTIVE TRACT OF POSTMENOPAUSAL MONKEYS

Dissertation under the direction of
Susan E. Appt, D.V.M., Assistant Professor of Pathology / Comparative Medicine

Concerns of breast cancer risk with conjugated equine estrogens (CEE) and medroxyprogesterone acetate have generated interest in alternatives for the progestin component in postmenopausal estrogen co-therapy. Bazedoxifene acetate (BZA) is a third-generation selective estrogen receptor modulator (SERM) currently under investigation as a possible choice for this role. The primary objective of this project was to determine if BZA given at the clinical target dose of 20 mg/day would inhibit the proliferative effects of CEE (0.45 mg/day) on the breast and endometrium without attenuating the maturation effects of CEE on the vaginal mucosa. As part of a nonhuman primate translational trial evaluating the global benefit to risk profile of BZA+CEE and BZA alone, we found that the addition of BZA to CEE significantly antagonized the stimulatory actions of CEE on breast and endometrial Ki67 immunolabeling, epithelial area, and specific markers of ERα activation, while BZA alone had no effect. Global gene expression profiles in the breast showed that BZA+CEE more closely resembled BZA than CEE alone. BZA+CEE and BZA did not significantly upregulate pathways or genes related to cell cycle progression or proliferation in the breast. The addition of BZA to CEE also inhibited CEE-induced changes in breast and endometrial morphology. For instance, BZA+CEE treatment resulted in less lobular enlargement in the breast and reduced the prevalence of simple endometrial hyperplasia, stromal fibrosis, and cystic dilation compared to CEE. Similarly, BZA alone had no effect on these measures, indicating that BZA lacks an estrogen agonist profile in the breast and endometrium. In the vagina, BZA completely inhibited CEE effects on vaginal
proliferation, maturation, and keratinization, while having no estrogenic activity when given alone. A comparable pattern was seen in the endocervical glands, but BZA only partially antagonized CEE effects on epithelial cell height. Interestingly, BZA with and without CEE resulted in lower ERα immunolabeling in the breast, endometrium, vagina, and endocervical glands compared to control and CEE. Collectively, these findings demonstrate that the target dose of BZA given alone and with CEE (0.45 mg/day) lacks an estrogenic profile in the breast, endometrium, and vaginal epithelium of the postmenopausal macaque model.
INTRODUCTION

Loss of ovarian hormone production after menopause is associated with an increased risk of osteoporosis, atherosclerosis, and a range of symptoms that negatively affect the quality of life of aging women (1). Two of the most common symptoms are hot flushes and vaginal atrophy, which affect up to 80% and 50% of postmenopausal women, respectively (2,3). Traditional menopausal hormone therapies (HT) including estrogen-alone therapy (ET) and estrogen + progestin therapy (EPT) effectively alleviate these conditions (4-6), but the effects of these therapies on the breast and endometrium have raised concerns about cancer risk.

Estrogen therapy when given alone has been associated with an increased risk of endometrial hyperplasia and cancer (7-10). When a progestin is added to ET, estrogen-induced epithelial proliferation is prevented and cancer risk is reduced (9-11). For these reasons, progestin co-therapy is required for non-hysterectomized women seeking treatment for menopausal symptoms (12). Historically, the most widely prescribed EPT in the United States has been conjugated equine estrogens (CEE) given with medroxyprogesterone acetate (MPA) (13). However, findings from the Women’s Health Initiative (WHI) Estrogen+Progestin clinical trial (14-15) and several observational studies (16-17) have associated long-term CEE+MPA co-therapy with an increased risk of invasive breast cancer, which contributed to a marked reduction in all forms of HT use by postmenopausal women (13). In recent years, concern of breast cancer remains an important factor in the decision to initiate HT for many women (2,18). Consequently, considerable research has been dedicated to finding an alternative to the progestin component of combined HT that will act in a tissue-specific manner to allow for the treatment of menopausal-related conditions without increasing the risk of breast and endometrial cancer.

The purpose of this project was to investigate the effects of bazedoxifene acetate (BZA) with and without CEE on the breast and reproductive tract of surgically postmenopausal macaques in the context of an investigator-initiated, multisystem, preclinical trial. The following
sections will provide an overview of published preclinical and clinical data pertaining to BZA given alone and in combination with CEE. Important gaps in knowledge and the need for further studies to better define the estrogen-modulating properties of BZA in the breast, uterus, and vagina will also be discussed.

Combined SERM and estrogen therapies: an alternative approach to menopausal therapy

Selective estrogen receptor modulators (SERMs) have been proposed as candidate agents to replace progestins in traditional menopausal EPT. SERMs are a class of non-steroidal compounds that bind to estrogen receptors (ER) alpha and beta and induce a mixed pattern of ER agonist and antagonist activity depending on the target tissue and particular SERM (19). Similar to estrogens, most SERMs increase bone mineral density and improve lipid profiles, but contrary to estrogens, current SERMs given alone do not treat vasomotor symptoms or vaginal atrophy and in some cases worsen these conditions (20-22). The clinical goal of co-administering a SERM with one or more estrogens would be to selectively retain the benefits of both agents while reducing the adverse effects of either agent alone. Ideally, the estrogens in the combination would mitigate hot flushes, vaginal atrophy, and bone loss, while the SERM would help maintain bone mass and prevent proliferative effects on the breast and endometrium (23,24).

Clinical profiles of currently available SERMs

SERMs are a structurally diverse group of compounds which are characterized by their complex pharmacologic effects on ER-mediated signaling in various tissues rather than a common chemical structure (25). As a result, each SERM induces unique tissue-specific phenotypes that determine its overall clinical profile. Current Food and Drug Administration (FDA)-approved SERMs include tamoxifen and raloxifene. Tamoxifen is a first-generation SERM which has anti-estrogen activity in the breast and is recognized as an effective endocrine treatment for early and advanced ER-positive breast cancer in both premenopausal and
postmenopausal women (26-28). In a prevention setting, tamoxifen has also been shown to reduce the incidence of non-invasive and invasive ER-positive breast cancers in women at increased risk for this disease (22,29). Data from clinical trials suggest that tamoxifen is a weak estrogen agonist for bone and lipid profiles, leading to modest increases in bone mineral density and a reduction in low density lipoprotein cholesterol levels (30,31). Raloxifene is a second generation SERM widely used for the prevention and treatment of postmenopausal osteoporosis (32-34). Similar to tamoxifen, raloxifene is an also an ER antagonist in the breast and has comparable effects in reducing the risk of ER-positive invasive breast cancer (35). Tamoxifen and raloxifene are the only agents currently approved by the FDA for the prevention of breast cancer in postmenopausal women.

Despite these benefits, tamoxifen, raloxifene, and other recently developed SERMs have been associated with side effects related to estrogen deficiency, most notably hot flushes and urogenital atrophy, making them suboptimal stand-alone alternatives to traditional HTs (36-38). In addition, tamoxifen and raloxifene have been shown to increase the incidence of venous thromboembolic events and stroke, and tamoxifen increases the risk of endometrial cancer (34,39-44). Data from large clinical trials have indicated that raloxifene does not increase endometrial cancer risk (41,45); however, prolonged tamoxifen treatment confers a 2- to 3-fold increase in endometrial cancer risk (22,39). Tamoxifen also induces other unfavorable endometrial morphologic changes including polyp formation, stromal fibrosis, and cystic dilation which increase endometrial thickness in postmenopausal women (22,39,46-49).

Consequently, in recent years considerable research has been dedicated to developing a new generation of SERMs with improved tissue-specific effects in postmenopausal women. Clinical indications for these third-generation SERMs include the treatment of osteoporosis and, in combination with one or more estrogens, relief of menopausal symptoms and prevention of osteoporosis (24). The “ideal” third-generation SERM, if achievable, would prevent bone loss
and fractures; have estrogen-antagonizing effects in the breast and endometrium; have no adverse tamoxifen-like uterotropin effects when given alone; have neutral or non-estrogen antagonizing effects in the urogenital tract and brain (vasomotor symptoms); and have no increased risk of coronary heart disease, stroke, deep vein thrombosis, or other cancers when administered alone or with ET (50).

**Bazedoxifene acetate: alone and in combination with one or more estrogens**

Bazedoxifene acetate (WAY-140424, TSE-424) is a new third-generation SERM originally synthesized and developed by Ligand Pharmaceuticals and Wyeth Pharmaceuticals (now Pfizer, Inc) (51). Currently, BZA is approved in the European Union and under regulatory review in the United States for the prevention and treatment of postmenopausal osteoporosis. Evaluations of BZA combined with CEE for the reduction of menopausal symptoms and prevention of osteoporosis are on-going in Phase III clinical trials (52,53).

**Structural characteristics:** BZA is a non-steroidal, indole-based ER-ligand with different structural characteristics compared to other non-steroidal ER-ligands such as raloxifene and tamoxifen (Figure 1). BZA was assembled using raloxifene as the template, substituting the benzothiophene (stilbene-like) core for an indole core (51). Unique structural characteristics of BZA include a hexamethylenamine ring at the end of the side chain and a methylene hinge which connects the side chain to the indole core. Structural differences in the side chain such as these are thought to be responsible for inducing unique conformational changes in the ER-ligand complex and differential recruitment of cell-specific coregulator proteins which influence transcription (51,54).
Ligand binding and ERα transcriptional activity in vitro: The binding affinity of BZA to ERα and ERβ have been evaluated in a solid phase competitive radioligand binding assay in which BZA displaced 17β-estradiol (E2) from the ligand binding domain constructs of both human ERα and ERβ (54,55). Results showed that BZA has high affinity for both ERα and ERβ, with a slight preference for ERα. The dissociation constants (K_i) were 0.1 nM for ERα and 0.3 nM for ERβ, while the IC_{50} values were 23 ± 15 nM and 85 ± 59 nM, respectively. BZA has an affinity for ERα that is approximately 10-fold lower than E2 and raloxifene. In transcription assays using an estrogen response element (ERE) combined with a weak promoter and luciferase reporter sequence, BZA was a potent estrogen-antagonist on ERα-mediated transcription (IC_{50} ~5 nM) (54,56).

Preclinical studies of BZA alone and in combination with CEE or E2: Results from preclinical cell culture and rodent studies have suggested that BZA may be an estrogen-agonist in bone and liver but an estrogen-antagonist in the endometrium and breast. For example, BZA given alone at 0.3 mg/kg for 6 weeks significantly increased bone mineral density and bone strength and decreased total plasma cholesterol without increasing uterine wet weight in a mature ovariectomized rat model (55). Similarly, uterine wet weight was not significantly increased with
BZA at a higher dose of 5 mg/kg compared to vehicle in a 3-day immature rat model. Histologic evaluation of these rat uteri revealed no increase in luminal epithelial cell height and no evidence of luminal epithelial cell hyperplasia or hypertrophy, myometrial hypertrophy, or luminal distention (55). When BZA was added to either CEE or E2, BZA significantly inhibited estrogen-induced uterine wet weight gain in rodents receiving up to 3 mg/kg of BZA (57-59). In cell culture studies, BZA did not stimulate proliferation of ERα-positive human breast cancer cells (MCF-7) when given alone and antagonized proliferation when given with E2 (55). Similarly, a study using an ovariectomized sexually immature mouse model found that the addition of BZA to CEE significantly inhibited CEE actions on mammary gland ductal growth and a specific gene marker of ERα activity, while treatment effects of BZA alone were similar to vehicle (58). In this same study, BZA was more effective at antagonizing CEE effects on uterine wet gain and mammary gland ductal growth than raloxifene and lasofoxifene (58).

The aforementioned studies have several notable caveats. First, the rodent uterotrophic assay is a commonly used assay to determine the estrogenicity of new pharmaceutical agents (60), but gain in uterine wet weight may reflect accumulation of uterine fluid and not actual hyperplasia (61). Since uterine histology results in rats treated with BZA and CEE/E2 co-therapies were not reported (57-59), it is not known whether BZA will inhibit estrogen-induced endometrial proliferation. Additionally, due to distinct pathophysiological differences between rodents and humans, rodents may differ in their tissue response to BZA alone or with one of more estrogens (see animal model considerations section). Other important caveats of these studies include the use of non-clinically relevant doses of BZA and CEE and unconventional markers of ERα activation in the mammary gland (e.g. defensin β1, Defβ1) (59). Lastly, cell culture studies do not recapitulate the endocrine, metabolic, and cellular interactions observed in a living organism; therefore, it is not known whether the effects of BZA on MCF-7 cells in vitro will translate to the normal postmenopausal breast in an in vivo model with a high degree of similarity to women (see animal model considerations section).
Clinical trials of BZA alone: As previously mentioned, BZA is currently under regulatory review by the FDA for the prevention and treatment of postmenopausal osteoporosis. Data included in this application were from two randomized (phase III) clinical trials investigating the treatment effects of various doses of BZA (10, 20, and 40 mg/day) in postmenopausal women at high and low risk for osteoporosis (62,63). In the 3-year study of postmenopausal women at high-risk for osteoporosis, BZA given alone significantly increased lumbar spine and hip bone mineral density, reduced bone turnover, and significantly decreased the risk of new vertebral fractures compared to the placebo group. In addition, a post hoc analysis of a subgroup of women at higher risk for fracture showed that BZA given at 20 mg significantly reduced the risk of non-vertebral fractures relative to placebo (62). Retrospective analysis of the mammographic density data from this study revealed that BZA at 20 mg did not significantly alter mammographic density after 2-years of treatment compared to baseline (data after 3-years of treatment have not yet been published) (64). These findings are consistent with breast safety data reported from this trial and a 2-year extension study indicating no significant differences in breast cancer incidence or other breast-related adverse events (breast pain, breast cyst, and fibrocystic breast disease) between the BZA and placebo groups (65,66). Other reproductive and gynecologic data reported in young (67) and old osteoporotic postmenopausal women (66) enrolled in these studies showed that all doses of BZA had no significant adverse effects on endometrial thickness changes and incidences of endometrial hyperplasia, endometrial carcinoma, endometrial polyps, uterine bleeding, or ovarian cysts compared to placebo. Incidences of hot flushes, leg cramps, and venous thromboembolic events, primarily deep vein thrombosis, were higher over 5 years in the BZA groups compared to placebo, while the number of cardiac disorders and cerebrovascular events were low and evenly distributed among the groups (65).
<table>
<thead>
<tr>
<th>SMART Trial</th>
<th>Duration</th>
<th>Population</th>
<th>Interventions</th>
<th>Primary outcome measures</th>
<th>Secondary outcome measures</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 years</td>
<td>PMW (n=3,397)</td>
<td>BZA (10, 20, or 40 mg) + CEE (0.45 or 0.625 mg); Raloxifene 60 mg; Placebo</td>
<td>Endometrial hyperplasia (at 1 year); BMD (osteoporosis prevention at 2 years)</td>
<td>Endometrial hyperplasia; breast density; vaginal atrophy; metabolic parameters; uterine bleeding; vasomotor symptoms; quality of life indices (at 2 years)</td>
<td>Completed; most results published</td>
</tr>
<tr>
<td>2</td>
<td>12 weeks</td>
<td>PMW with hot flushes (n=318)</td>
<td>BZA 20 mg + CEE (0.45 or 0.625 mg); placebo</td>
<td>Moderate or severe hot flushes at week 4 or 12</td>
<td>Breast pain; sleep quality</td>
<td>Completed; results published</td>
</tr>
<tr>
<td>3</td>
<td>12 weeks</td>
<td>PMW with vaginal atrophy (n=652)</td>
<td>BZA 20 mg + CEE (0.45 or 0.625 mg); BZA 20 mg; placebo</td>
<td>Vaginal atrophy</td>
<td>Sexual function; menopause related quality of life</td>
<td>Completed; results published</td>
</tr>
<tr>
<td>4</td>
<td>2 years</td>
<td>PMW (n=1,083)</td>
<td>BZA 20 mg + CEE (0.45 or 0.625 mg); CEE 0.45 mg + MPA 1.5 mg; placebo</td>
<td>Endometrial hyperplasia; BMD (at year 1)</td>
<td>Endometrial hyperplasia; BMD (at year 2); uterine bleeding; breast pain (at year 1)</td>
<td>On-going</td>
</tr>
<tr>
<td>5</td>
<td>1 year</td>
<td>PMW (n=1,843)</td>
<td>BZA 20 mg + CEE (0.45 or 0.625 mg); BZA 20 mg; CEE 0.45 mg + MPA 1.5 mg; placebo</td>
<td>Endometrial hyperplasia; BMD</td>
<td>Breast density; uterine bleeding; breast tenderness; sleep quality; menopause related quality of life</td>
<td>Completed; some abstracts published</td>
</tr>
</tbody>
</table>

SMART = Selective estrogen menopause and response to therapy; PMW = Postmenopausal women; BZA = bazedoxifene acetate; CEE = conjugated equine estrogens; BMD = bone mineral density. Compiled from Clinicaltrials.gov (Reference No. 69).
Clinical trials of BZA combined with CEE: Following positive observations from rodent studies regarding the anti-estrogenic properties of BZA on the mammary gland and endometrium, Wyeth Pharmaceuticals (Pfizer, Inc.) initiated a series of randomized, placebo-controlled, phase III clinical trials investigating several dose combinations of BZA and CEE (Table 1) (68,69). The first "selective estrogen, menopause, and response to therapy" trial (SMART-1) was a large 2-year study consisting of 3,397 postmenopausal women treated with daily BZA (10, 20 or 40 mg) combined with CEE (0.45 or 0.625 mg) and compared with raloxifene (60 mg) and placebo (70). Primary findings showed that BZA at 20 or 40 mg with either dose of CEE increased bone mineral density, relieved hot flushes, and improved lipid and coagulation parameters, while minimizing endometrial stimulation (70-73). Treatment with BZA (20 or 40 mg) and CEE (0.45 or 0.625 mg) combinations were associated with low rates (<1%) of endometrial hyperplasia, endometrial thickness changes, and uterine/vaginal bleeding that were not significantly different compared with placebo (71,72). However, BZA at 10 mg combined with either dose of CEE had significantly higher incidences of endometrial hyperplasia (2.5% and 7.1%, respectively) and vaginal bleeding (2.1% and 3.7%, respectively) relative to placebo or raloxifene (71,72). Based on these results, Wyeth (Pfizer) selected the dose combination of BZA 20 mg and CEE 0.45 mg as the "lowest effective dose combination" for the prevention of menopausal symptoms and osteoporosis.

Mammographic density data from the first SMART trial have not yet been published in a peer-reviewed journal, but preliminary data were reported at the North American Menopausal Society 21st Annual Meeting, Chicago, IL in October of 2010 (74). Similar to the BZA alone studies, the incidences of abnormal mammograms (< 5%) and breast cancer (< 0.3%) were low and similar to placebo. Preliminary analyses from the 1-year SMART-5 study presented at another national conference suggested that treatment with BZA 20 mg with either CEE 0.45 or 0.625 and placebo resulted in lower mammographic density compared to CEE 0.45 mg + MPA
1.5 mg (75), while the incidence of endometrial hyperplasia was low (< 1%) in all treatment groups (76).

The primary objective of the SMART trials was to determine if the addition of BZA to CEE co-therapy would antagonize the adverse proliferative effects of CEE on the breast and endometrial epithelium in postmenopausal women. However, none of the SMART trials had a CEE alone group as a positive control (73). The SMART trials were, therefore, not designed to test whether or not BZA is a partial or full antagonist in these estrogen-sensitive tissues. This question has clinical importance since long-term CEE therapy is a major risk factor for the development of type I endometrial cancer (7-9) and breast safety is a major concern for many women considering menopausal HT (ET or EPT) (18). Even though short-term CEE therapy (< 5 years) appears to have little impact on breast cancer risk (12,15), data from some observational studies have suggested that CEE use for > 15-20 years may increase a women’s risk for ER-positive breast cancers (16,77). CEE has also been shown to increase mammographic density, breast epithelial proliferation (Ki67 expression), and benign breast proliferative lesions among postmenopausal women (78-80). For these reasons, more detailed knowledge is needed regarding the effects of BZA alone and with CEE on breast and endometrial proliferation and other risk markers for cancer.

When investigating the efficacy of BZA+CEE co-therapies on vaginal atrophy, authors of the SMART trials concluded that BZA 20 mg combined with CEE 0.45 mg was an effective treatment for moderate to severe vaginal atrophy and vaginal symptoms. However, this conclusion was only weakly supported by data presented in these studies. In the 12-week SMART-3 trial evaluating BZA 20 mg combined with either CEE 0.625 or 0.45 mg in 664 postmenopausal women (ages 40-65 years), both BZA+CEE combinations significantly increased superficial cells and decreased parabasal cells compared to placebo, indicating improved vaginal maturation, but only BZA 20 mg + CEE 0.625 mg markedly improved vaginal acidity, an
important factor in preventing urogenital infections (81). Similarly, women receiving BZA 20 mg + CEE 0.625 mg, but not those receiving BZA 20 mg + CEE 0.45 mg, had significant improvements in their most bothersome symptoms (dyspareunia, vaginal dryness or itching/irritation) (81). When individual vaginal symptoms and sexual function were analyzed compared to placebo, both BZA+CEE combinations significantly improved vaginal dryness and lubrication during sexual arousal, but neither combination relieved vaginal itching/irritation or dyspareunia (81,82). Therapeutic effects of BZA+CEE dose combinations on vaginal atrophy were also evaluated in the larger 2-year SMART-1 trial, but vaginal maturation and relief of related symptoms were not the primary outcomes (73). The primary objective of this study was osteoporosis prevention; therefore, women were recruited based on their risk for bone loss and not severity of vaginal atrophy. Among those women that had less than 5% superficial cells at screening (n=1,867), 2-years of BZA 20 mg + CEE 0.45 mg treatment significantly decreased parabasal cells compared to placebo but did not significantly increase superficial cells, indicating an incomplete or blunted estrogenic response. When BZA 40 mg was paired with either CEE dose, the proportions of superficial and parabasal cells were similar to placebo, indicating no improvement in vaginal maturation (73). Collectively, these findings indicate that BZA is at least a partial estrogen-antagonist on the human vaginal mucosa and that its inhibitory effects are dose-dependent. More information regarding the comparative effects of BZA 20 mg + CEE 0.45 mg and CEE 0.45 mg alone would be helpful in determining the estrogen-antagonizing properties of BZA on the atrophic vaginal mucosa, especially considering that ET is the most effective treatment for vaginal atrophy (83). In addition, no data is available regarding the effects of BZA+CEE co-therapy on the cervix, which produces mucus secretions (84) protective of the vaginal mucosa, and the basal/parabasal layers of the vaginal epithelium which are the primary sites of cell division and ERα expression (85).
In summary, available data suggest that BZA may be an estrogen-antagonist in the breast and endometrium and at least a partial estrogen-antagonist in the atrophic vagina epithelium. However, limited information exists regarding the effects of BZA and BZA+CEE co-therapy on proliferation, ER activity, and morphologic markers in the breast and reproductive tract related to cancer risk. Also, no comparative data are available for BZA, CEE, and BZA+CEE co-therapy when given at target clinical doses. More specifically, it is not known whether BZA (20 mg/day) will completely inhibit the proliferative and transcriptional effects of CEE (0.45 mg/day) on the breast and endometrium without abrogating the beneficial effects of CEE on vaginal maturation. The following project was thus designed to compare the interactive effects of BZA, CEE, and BZA+CEE co-therapy on the reproductive tissues of surgically postmenopausal monkeys.
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SPECIFIC AIMS

The primary aims of this project were as follows:

- To determine the effects of BZA, CEE, and BZA+CEE on epithelial cell proliferation and morphology in the breast, endometrium, and vagina.
- To determine the effects of BZA, CEE, and BZA+CEE on ERα transcriptional activity in the breast, endometrium, and vagina.
- To determine the differential gene expression profiles of BZA, CEE, and BZA+CEE in the breast using gene microarray assays.

The primary research hypotheses were as follows:

- CEE will increase epithelial area and Ki67 expression in the breast and endometrium and epithelial proliferation and maturation in the vagina. The addition of BZA to CEE will inhibit these effects, while BZA alone will have neutral effects.
- CEE will increase the expression of ERα activation markers (PGR, TFF1, or GREB1) in the breast, endometrium, and vagina, and BZA will attenuate these effects. BZA will have no effect on these measures when given alone.
- Global gene expression patterns for BZA+CEE will more closely resemble BZA than CEE (i.e. BZA will dominate the BZA+CEE transcriptional phenotype). CEE will increase the numbers of genes represented in pathways related to proliferation or cell cycle progression, and BZA will antagonize these effects.
- CEE will induce uterotrophic effects, while BZA with and without CEE will have no uterotropic effects.
STUDY DESIGN

Data presented in this project were part of a large, multi-system, randomized, placebo-controlled nonhuman primate (NHP) translational trial. The primary endpoint of this study was coronary artery atherosclerosis. Other assessments included reproductive tract and mammary gland measures along with bone density and lipid profiles. Data related to the cardiovascular system, lipid profiles, and bone density were analyzed and reported separately from the reproductive tract and mammary gland assessments presented here.

Animal subjects and study design: This randomized NHP translational trial followed a parallel-arm study design (Figure 1). The subjects of this study were one-hundred adult female cynomolgus macaques (*Macaca fascicularis*), an Old World monkey species in the Cercopithecidae family. All monkeys originated from Indonesia and were obtained through a collaboration with the Indonesian Primate Center (Pusat Studi Satwa Primata) at the Institut Pertanian Bogor. Based on dentition, the mean estimated age for this study population was 12.7 years (range 9 - 18). As shown in figure 1, all animals were ovariectomized and randomized by body weight (BW) to receive one of four treatments for 20 months: *(i)* no treatment (control, n=23), *(ii)* BZA 20 mg (n=24), *(iii)* CEE 0.45 mg (n=24), or *(iv)* BZA 20 mg + CEE 0.45 mg (n=27). All treatments were administered in an isoflavone-free diet formulated to model diets typically consumed by postmenopausal women in the United States and fed once daily. Each group originally consisted of 25 animals, but 2 animals were added to the BZA and BZA+CEE groups to account for any exclusions or deaths during the study. Subsequently, 2 animals from the control and 3 animals from the BZA groups were excluded due to elevated serum ovarian hormone levels indicating the presence of ectopic ovarian tissue (1). Another monkey from the CEE group was humanely euthanized due to an intussusception.
Sample Size Justification: The sample size for this study (n = ~25/group) was based on statistical power needed for endpoints related to coronary artery atherosclerosis. Nevertheless, a primary outcome of this project was breast epithelial cell proliferation determined by immunolabeling with the proliferation marker, Ki67. Based on a prior macaque study (2), we estimated the difference in lobular Ki67 labeling between CEE and control means to be 4.0% with a common intra-group standard deviation of 3.5%. A sample size of approximately 14 in each treatment group would then provide an 80% chance to detect a statistically significant difference between these two sample means at a significance level of 0.05. When adjusting for multiple comparisons, this sample size increases to approximately 19 monkeys per treatment group.

Drug dose determinations: In order to account for differences in metabolic rates between monkeys and women, the standard clinical dose of CEE (0.45 mg/day) was scaled to 1800 kcal of diet (the estimated daily intake of U.S. women). All monkeys consumed approximately 120 kcal of diet per kg of body weight, providing approximately 0.03 mg/kg/day of CEE.

A pilot study was conducted to determine a BZA dose for monkeys that most closely resembled plasma concentrations measured in postmenopausal women receiving 20 mg/day. Target doses of 2.0 or 2.5 mg/kg were investigated based on information provided by a previous
metabolism study using macaques given a single oral dose of BZA via gavage (3). The pilot study conducted at the Wake Forest University Primate Center (WFUPC) was designed to determine the bioavailability and palatability of BZA when fed once daily in a high-fat cake diet for multiple days. Details of this pilot study are displayed in figure 2. Briefly, eight monkeys were fed cake diets containing either 2.0 or 2.5 mg/kg of BZA for 5 days. Heparinized plasma was then collected at 0, 4, 24, 48, and 72 hours post-prandial and analyzed by Pfizer using high-performance liquid chromatography (HPLC) with fluorescence detection. The area under the curve from time 0 to 24 hours (AUC$_{0-24}$) of BZA was then calculated and compared to the AUC$_{0-24}$ of BZA in postmenopausal women receiving 20 mg/day from a similarly designed pharmacokinetic study (4). As a result, the BZA dose of 2.5 mg/kg was selected and used for the remainder of the study. The metabolic disposition of BZA in monkeys has been reported previously to be similar to women (3,5).

![Figure 2](image-url)  
**Figure 2.** BZA dose finding study: design and results. Plasma concentrations of BZA 2.5 mg in cynomolgus macaques (86 ± 37 AUC$_{0-24}$) most closely resemble BZA 20 mg/day concentrations measured in women (84 ± 38 AUC$_{0-24}$). Error bars indicate standard error of the mean.
To confirm dietary intake, 17β-estradiol (E2), estrone (E1), and BZA concentrations were measured in serum and plasma samples, respectively, at several time points throughout the study. E2 and E1 were measured at the WFUPC Clinical Laboratory using commercially available radioimmunoassay kits (Siemens/DPC, Webster, Texas), while plasma BZA analyzes were performed at Pfizer as previously done in the pilot study.
REFERENCES


ANIMAL MODEL CONSIDERATIONS

The use of animal models has been a fundamental component of all fields of biomedical research, particularly in the diverse field of women’s health research. Animal models have advanced our understanding of the pathogenesis, treatment, and prevention of numerous chronic diseases affecting the health and quality of life of postmenopausal women including (but not limited to) breast cancer, endometrial cancer, cognitive function, osteoporosis, and atherosclerosis (1-5). Cell culture systems have also been an essential component in studies pertaining to women’s health and are used extensively as in vitro models in many areas of research such as breast and endometrial cancer biology (6,7). While cell culture systems have provided a wealth of information regarding the molecular and cellular processes that regulate disease, these models cannot recapitulate the complexities of a whole organism. The initiation, progression, and response to treatment of many diseases, such as hormone-sensitive cancers, depend on the complex interactions of different endocrine systems and steroid metabolism in the gut, liver, and local tissues. Cell culture systems also cannot account for paracrine effects mediated by growth factors, cytokines, and steroidogenic pathways (8). Based on this intricacy, well-characterized and clinically relevant animal models are vital to the process of evaluating new pharmaceutical interventions for the treatment and prevention of conditions affecting postmenopausal women. The following sections briefly review rodent models and their contributions to SERM development and the advantages of the macaque model in SERM and other hormone therapy studies.

Rodent models in early SERM development

Rodents have played an integral role in women’s health research, particularly in the development of SERMs as therapeutic and preventive agents for breast cancer. For example, the dimethylbenzanthracene (DMBA)-induced rat mammary carcinoma model was instrumental in
identifying the antitumor properties of tamoxifen and raloxifene (9-11). These laboratory results were then successfully transferred to clinical trials and subsequent approval of tamoxifen and raloxifene for the treatment and prevention of breast cancer (12-15). Similarly, xenografts of human breast cancer cells implanted into athymic mice have been used to identify mechanisms of drug resistance to tamoxifen and have served as an in vivo model to test new drugs for cross-resistance (16,17).

Recognition that early SERMs may have estrogenic and antiestrogenic effects at different sites was also first established in rodent models. Data from these rodent studies and early human studies (18,19) introduced the concept that SERMs may have multiple clinical applications in postmenopausal women (20). For instance, early rodent studies found that both tamoxifen and raloxifene (formerly keoxifene) maintained bone density in ovariectomized rats in addition to preventing the development of carcinogen-induced rat mammary carcinomas (21-23). Other studies using the ovariectomized mature rat model noted that raloxifene prevented bone loss without inducing a significant uterotrophic effect (24,25), results that were later confirmed in human clinical trials (12,26). The link between chronic tamoxifen treatment and endometrial cancer was also first identified using a rodent model, specifically the xenograft mouse model. Athymic mice transplanted with ERα-positive breast cancer cells on one side and endometrial cancer cells on the other showed that tamoxifen treatment controlled E2-stimulated growth of the breast cancer cells but not the endometrial cells (27).

Collectively, these examples demonstrate the value of rodent models in defining the tissue-specific effects of early SERMs. However, several key reproductive characteristics of the normal rodent limit its applicability in studies evaluating the effectiveness and safety of new menopausal hormone therapies, particularly with respect to modeling breast and endometrial cancer risk in postmenopausal women. In some instances in SERM development, rodents have had different endometrial responses compared to women and have failed to detect adverse
gynecological effects preclinically. For example, the clinical development of levormeloxifene, a triphenylethylene SERM, was discontinued due to significant increases in endometrial thickness and high incidences of endometrial edema, vascularization, cystic change, uterovaginal prolapse, and urinary incontinence among levormeloxifene-treated women in phase II and III clinical trials (28,29). Some preclinical rodent studies detected an increase endometrial thickness, whereas others did not; however none of these studies detected the endometrial morphological changes seen in women treated with levormeloxifene (30).

Advantages of the macaque model in SERM and hormone therapy studies

Due to many reproductive similarities to women, macaques are the most relevant animal model for investigating new menopausal hormone therapies. Like women, macaques have a 28 day menstrual cycle (31), cyclic ovarian hormone profiles (32-33), peripheral aromatization of androgens to estrogens (34), and distinct menarche and menopause events (35-38). In contrast, rodents have a 4-5 day estrus cycle, a nonfunctional corpus luteum which is only activated during pregnancy or pseudopregnancy (39), and no peripheral aromatization of androgens to estrogens (40). Although macaques experience a similar pattern of primordial follicle decline to that of aging women (37), menopause does not occur in these species until near their maximal life expectancy (25 years for macaques) (41). Naturally menopausal macaques are therefore rare, short-lived, and largely unavailable for experimental use. In addition, many of these animals may be geriatric and have chronic health issues, making them unsuitable subjects for long-term studies. As an alternative, the ovariectomized adult macaque has been used extensively as a primate model of menopause. Rhesus macaques (Macaca mulatta) were the original models of female reproductive biology and aging (31,33), however studies evaluating the role of hormone therapy in disease risk have been based in the cynomolgus macaque model. A review of the ovariectomized cynomolgus macaque model and its contributions to our understanding of chronic disease risk has recently been published (42).
Unlike ovariectomized rodents, the sex steroid profile of ovariectomized macaques resembles that of naturally postmenopausal women. Humans and Old World NHP uniquely secrete large quantities of the inactive androgens, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S), from their adrenals which are then converted to active androgens (testosterone and androstenedione) and estrogens in peripheral tissues by the cytochrome P450 enzymes, P450c17 (17\,20 lyase) and aromatase (CYP19), respectively (43). In contrast, rodents only produce androgens and estrogens in their gonads; therefore, removal of the ovaries in a female rodent results in a complete elimination of circulating sex steroid hormones (40,44). This sex steroid deficiency in ovariectomized rodents may sensitize reproductive tissues to exogenous hormonal agents and produce exaggerated tissues responses compared to naturally or surgically postmenopausal women (45). Differences in the androgen profiles between rodents and humans are also important considering that certain types of SERMs, such as tamoxifen and levormeloxifene, have been shown to stimulate adrenocortical production of DHEA-S, androstenedione, and cortisol in postmenopausal women and macaques (46,47).

Rodents also have different expression patterns and catalytic activities of hepatic drug-metabolizing enzymes relative to humans and macaques (48,49). Resulting differences in circulating metabolites are thought to lead to species-specific tissue responses. For example, tamoxifen has been shown to cause hepatocellular carcinoma in several stains of rats (50) due to a lack of detoxification of DNA-adduct forming metabolites via the glucuronidation pathway (51,52). In addition to lower clinical doses of tamoxifen, it is thought that breast cancer patients do not have an increased risk of hepatocellular carcinoma because women detoxify reactive metabolites (e.g. α-hydroxytamoxifen) to stable glucuronides at a rate 100 fold higher than in rats (52).

Several recent studies utilizing cynomolagus macaques for the study of new menopausal hormone therapies, such as the current project, have been in the context of a NHP translational
trial (53-57). The term ‘translational trial’ is used to refer to these studies because NHPs serve as the link between rodent studies and human clinical trials. Whether these NHP translational trials are conducted prior to the initiation of human clinical trials or in parallel as ‘periclinical trials,’ the primary goal of these studies is the same: to test hypotheses derived from preclinical cell culture and rodent models and to provide mechanistic insight into findings from human clinical trials. Considering that menopause and hormonal interventions have a global impact on the health of postmenopausal women, NHP translational trials often have multiple organ-system endpoints in order to make a global benefit-risk assessment of a new intervention. This strategy allows for parallel evaluations of breast and uterine effects, cardiovascular disease risk, bone health, insulin resistance, obesity, behavior, and cognitive function.

Other advantages of NHP translational trials include the ability to control diet, dose, and other environmental variables as well as the utilization of tissues in quantitative gene expression, immunohistochemical, and histopathological studies--evaluations not feasible in clinical trials using women. For example, breast evaluations presented in this project were done on tissues obtained from an interim breast biopsy, a procedure that is not typically done in clinical studies. Also, the current NHP translational trial included a CEE alone group as a positive control which was not ethnically feasible in previous BZA+CEE clinical trials because all participating women did not have a prior hysterectomy (58).

Modeling hormonal carcinogenesis in macaques

In addition to physiological similarities, the female cynomolgus macaque and other members of this genus are well suited for evaluating the effects of hormonal agents on the breast and endometrium due to many histological and molecular similarities to women. The following sections compare and contrast basic histology and biology of the human, macaque, and rodent
mammary gland and endometrium. Strategies to predict breast and endometrial cancer risk associated with estrogens and other hormonal agents are also discussed.

Comparative histology and biology of the normal breast: The macaque and human breast share many anatomical and histological similarities that differ in several important ways from the rodent mammary gland. The nulliparous rodent mammary gland is a simple ductal system with minimal branching and small glandular areas (terminal end/alveolar buds) that terminates into a single lactiferous duct or sinus at each nipple (39,59). The human and primate breast contains multiple lactiferous ducts that extend in a branching radial pattern and lead into terminal ductal lobular units (TDLU), the secretory units of the breast. Each TDLU consists of a terminal (intralobular) duct and surrounding alveoli, encased by myoepithelial cells and stromal support containing fibroblasts and adipocytes (39,59,60).

The female human and macaque breast also have similar mechanisms of glandular secretion (61), cytokeratin phenotypes (62), pathways of hormone metabolism (34,43), and expression patterns of sex steroid receptor expression (63-65). For example, ERα is expressed in the ductal and lobular epithelia of the human and macaque breast, whereas rodents express ERα in both the epithelium and stroma (64,66-68). ERβ is diffusely expressed in the epithelium and stromal compartments of the human breast, macaque, mouse, and rat mammary gland (64-66,69,70). Both progesterone receptor (PGR) subtypes, A and B, can be found in the human breast and macaque, mouse, and rat mammary gland, but their expression levels and distributions differ across species (71). PGR-A is the predominant PGR subtype in the mouse and rat mammary gland, whereas similar levels of PGR-A and PGR-B protein are expressed in the human and macaque breast (68,72). Immunohistochemical studies in the human breast, macaque, mouse, and rat mammary gland have revealed that PGR expression is limited to the epithelial regions and primarily absent from the stroma (64,71). PGR-A expression is confined to the luminal epithelium in the rat mammary gland, while both PGR-A and PGR-B are expressed in the
luminal epithelium of the mouse mammary gland (71). In the primate breast, these receptors tend to be most abundant in the terminal (intralobular) ducts (60).

Macaques and women also share comparable breast tissue responses to endogenous and exogenous hormones (1,60,73). For example, several studies using cynomolgus macaques have shown that CEE+MPA and CEE therapies increase breast epithelial cell proliferation (53,54,56), which is consistent with human studies of the normal postmenopausal breast (74). Macaques and women also develop similar hyperplastic and neoplastic lesions of the breast (75,76). While not well-established, the reported lifetime incidence of spontaneous mammary gland neoplasms in macaques is ~6%, similar to women (75,77).

**Comparative histology and biology of the normal endometrium:** In addition to gross anatomical differences, the pathophysiology of the rodent endometrium differs substantially from that of the macaque and human. Rodents lack cyclic endometrial growth and sloughing (menstruation), whereas the endometrium of macaques undergoes coordinated morphologic and functional changes during the menstrual cycle that are nearly identical to those of the human endometrium (78). These histologic changes are largely driven by ovarian sex steroids and their receptors whose expression patterns are also comparable to those reported in women. In general, the endometrium of macaques and humans is divided into two functional compartments: the superficial (zona functionalis) and the basal (zona basalis) endometrium. The most hormone-responsive compartment is the superficial endometrium (the layer that sheds during menstruation), which contains unbranched portions of endometrial glands embedded in relatively loose stroma. The superficial endometrium compartment also contains luminal epithelium, but, in contrast to rodents, maturation of these cells does not always reflect fluctuations in endogenous or exogenous hormones. In rodents, change in luminal epithelial cell height is one of the primary indicators of estrogenicity in the screening of new SERMs and other hormonal agents (79).
Several long-term studies have shown that the endometrium of macaques and postmenopausal women respond similarly to various exogenous hormonal therapies including SERMs, estrogens, and progestins (4,80). For example, chronic tamoxifen treatment induces endometrial stromal fibrosis, cystic dilation, endometrial polyps, and increased endometrial thickness and glandular PGR expression in macaques as reported in postmenopausal women (2,81,82). Furthermore, macaques receiving unopposed ET (CEE or E2) develop endometrial hyperplasia with increased expression of the PGR and proliferation marker, Ki67, and when a progestin is given concomitantly, these effects are significantly antagonized (2,80,83). In contrast, ET generally decreases epithelial PGR expression in the rodent endometrium (84).

Despite these similarities between macaques and women, interspecies differences exist regarding the level of risk associated with these hormone-induced lesions (2). Prolonged unopposed ET most commonly induces simple endometrial (glandular) hyperplasia in both macaques and women (83,85), but the incidence of high-risk lesions such adenomatous (complex) and atypical hyperplasia is much higher in women (85,86). Adenomatous endometrial hyperplasia is only seldom observed in long-term macaque studies, and no endometrial neoplasms have been reported even after 3 years of CEE (0.625 mg/day) treatment (2,83). Treatment of macaques with higher doses or longer durations may result in an increased incidence of high-risk lesions or neoplasms, but this hypothesis has not been tested (2).

Strategies for predicting cancer risk: Due to the low incidence of breast and endometrial cancers in macaques, a surrogate marker approach has been adopted for modeling cancer risk. The primary biomarkers used relate to cell proliferation and ERα activity, based on the hypothesis that ET is thought to induce its cancer promoting effects in the breast and endometrium primarily by increasing cell proliferation via the ERα and hormonal agents that block ERα activity in these tissues negate this risk (87). Proliferating epithelial cells are prone to DNA repair errors which lead to mutations that are then propagated by continued stimulation (88).
The Ki67 nuclear antigen is a well-established biomarker of cell proliferation, particularly in hormone-sensitive tissues, such as the breast and reproductive tract. Several retrospective histopathological studies in women have found that Ki67 protein expression correlates significantly with higher tumor grade and stage, increased risk of invasion, and lower rates of survival for both breast and endometrial cancers (89-94). In macaques, Ki67 expression has been used extensively to estimate risk associated with postmenopausal hormone therapies (2,60). For instance, several studies in the ovariectomized macaque model have shown that CEE+MPA therapy results in greater Ki67 expression in the breast compared to control (53,54,56) which parallels results from immunohistochemical studies in healthy postmenopausal women (74). Notably, findings from these macaque studies were published prior the release of the WHI findings that long-term CEE+MPA therapy increases the risk of postmenopausal breast cancer (95,96), validating the usefulness of the macaque model and relevance of the biomarker approach for modeling cancer risk.

Epithelial density and prevalence of proliferative lesions are also used to predict risk associated with postmenopausal hormone therapies in macaque studies. Specifically, morphometric measurement of breast epithelial density in macaques is used to model mammographic density (60), an independent risk factor for breast cancer in women (97).

Markers of ERα activity used in this project include PGR, TFF1, and GREB1. The gene and/or protein expression of PGR, TFF1, and GREB1 serve as reliable markers of ERα activation in breast and endometrial cancer cells (98-102) and have been shown previously to be induced by ET in the ovariectomized primate model (53,54,56,75,103).

Another strategy to help estimate hormone-associated cancer risk in the macaque model is the application of gene microarray technology to define the unique global gene expression profile of a drug or combination of drugs. This method is particularly useful in the study of SERMs and estrogens which mediate their effects through the ERs (104,105). It is thought that each ligand induces a distinct conformational change in the ER-ligand complex that promotes the
binding and biochemical modification of a tissue-specific set of coregulators which subsequently influence transcriptional events (106). Based on these molecular interactions, a SERM combined with one or more estrogens should result in a different gene expression profile than each individual component. This theory is supported by in vitro studies investigating the effects of BZA, raloxifene, lasofoxifene, and a mixture of unsulfated equine estrogens on MCF-7 breast cancer cells (107). Results from this study showed that each SERM alone and in combination with unsulfated estrogens altered the expression of a unique set of genes. Information gained from gene microarray data aid in the understanding how transcriptional profiles relate to histologic responses. For instance, if a hormonal agent increases Ki67 expression and epithelial density, gene microarray analysis may show an upregulation of specific pathways or genes related to cell proliferation or cell cycle progression. Gene microarray results can also provide useful context for the expression of individual markers of ERα activation, proliferation, apoptosis, growth factor signaling, and steroid metabolism as quantified by RT-PCR or immunohistochemistry.

Assessing vaginal effects of hormonal therapies using macaques

Female macaques are also a valuable animal model for studying vaginal responses to new menopausal treatments. Although rodent models are typically used in such studies (108), the pathophysiology and hormonal responses of the rodent vagina differs in several ways from that of the macaque and human (109-113). In regards to basic histology, the rodent vagina is lined with a keratinized stratified squamous epithelium that undergoes substantial changes during the estrous cycle (114). In contrast, less dramatic changes occur during the menstrual cycle in the human and macaque vagina with respect to maturation, glycogen production (follicular phase), and mucus granule formation (luteal phase) (109,113-115). Also, macaque and human vaginal epithelial cells undergoing mucification do not differentiate into goblet cells as in the rodent (116). These distinct changes allow for reliable staging of the estrous cycle via vaginal cytology in the rodent,
whereas serum ovarian and gonadotropin hormone levels and morphometric measures are more reliable indicators of cycle phase in macaques (109,111,112,115,117). Vaginal cytology, however, is widely used in both macaques and women to assess estrogenic responses to exogenous hormones (80,118). For example, several studies using macaques have shown that CEE treatment significantly improves the vaginal maturation value (index) (83,110) as observed in women (119).

An important model consideration is the difference between the macaque and human vagina in response to exogenous estrogens. Baseline (estrogen-deficient) maturation values and epithelial cell proportions are similar between macaques and women (120-122); however, superficial keratinization of the vagina following ET is generally more pronounced in macaques than in women (123). For example, in postmenopausal women, oral CEE will increase the percentage of superficial cells in vaginal smears by 10-30% from baseline (119), while the same treatment will induce an 80-90% increase in superficial cells among macaques (83).

In summary, animal models are an essential component in the development of new hormonal therapies for postmenopausal women. Female macaques are the most clinically relevant model for such studies due to many shared reproductive characteristics and tissues responses with women. Previous macaque studies have utilized biomarkers to estimate tissue responses relevant to clinical outcomes, including cancer risk, in the breast and reproductive tract. Results of these studies have been shown to parallel similar findings in humans and predict outcomes of human clinical trials.
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CHAPTER 1
EFFECTS OF BAZEDOXIFENE ACETATE WITH AND WITHOUT CONJUGATED EQUINE ESTROGENS ON THE BREAST OF POSTMENOPAUSAL MONKEYS

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The following manuscript has been accepted for publication in *Menopause*. Stylistic variations result from the demands of the journal. Kelly F. Ethun performed or directed the experiments, completed the statistical analyses, and prepared the manuscript. Charles E. Wood assisted with the molecular studies and performed the histopathological review. Thomas C. Register provided guidance on the analysis of the molecular endpoints. Susan E. Appt and J. Mark Cline acted in an advisory and editorial capacity. Thomas B. Clarkson was the principal investigator of the parent nonhuman primate study for this project and provided significant guidance throughout this project.
**Full Title:** Effects of Bazedoxifene Acetate with and without Conjugated Equine Estrogens on the Breast of Postmenopausal Monkeys

Running Title: Breast Effects of Bazedoxifene with CEE

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Abstract

Objective: Concerns about increased breast cancer risk with estrogen and progestin therapy have led to an increased interest in progestin alternatives. The main objective of this study was to determine if bazedoxifene acetate (BZA), a new selective estrogen receptor modulator (SERM), would antagonize the proliferative and transcriptional effects of conjugated equine estrogens (CEE) in the breast.

Methods: As part of a 20 month preclinical trial, ninety-five ovariectomized cynomolgus macaques (Macaca fascicularis) were randomized to receive no treatment or treatment with BZA (20 mg/d), CEE (0.45 mg/d), or BZA and CEE in combination (women’s daily equivalent doses). Data presented here include breast effects following 6 months of treatment. Endpoints included histomorphometry, histopathologic evaluations, gene microarray assays, PCR quantification of specific ERα activity markers, and immunohistochemical detection of sex steroid receptors, and the proliferation marker Ki67.

Results: BZA+CEE and BZA resulted in significantly less total epithelial density, lobular enlargement, and Ki67 immunolabeling in the terminal ducts compared to CEE alone (P < 0.05 for all). The addition of BZA to CEE antagonized the expression of ERα-regulated genes such as GREB1 and TFF1 (P < 0.01 for both), while BZA alone had minimal effects on ERα-mediated transcriptional activity. BZA and BZA+CEE did not significantly up-regulate genes related to cell cycle progression and proliferation. BZA with and without CEE also resulted in less lobular and terminal duct ERα immunolabeling compared to control and CEE (P < 0.0001 for all).

Conclusions: These findings demonstrate that BZA given at a clinically relevant dose is an estrogen antagonist in the breast, supporting the idea that CEE + BZA may provide a lower breast cancer risk profile compared to traditional estrogen + progestin therapies.

Key words: Menopause – Hormone Therapy – Estrogen – Selective Estrogen Receptor Modulator – Estrogen Receptor – Breast
INTRODUCTION

Menopause is associated with an increased risk of osteoporosis and a range of adverse symptoms that decrease the quality of life of postmenopausal women.\textsuperscript{1} Traditional menopausal hormone therapy (HT) regimens including estrogen-alone therapy (ET) and estrogen + progestin therapy (EPT) are commonly used to treat these conditions,\textsuperscript{2-4} but the effects of these therapies on the breast and endometrium have raised concerns about cancer risk. While the proliferative cancer-promoting actions of estrogens on the endometrium can be opposed by progestin co-therapy,\textsuperscript{5,6} results from the Women’s Health Initiative (WHI) clinical trials\textsuperscript{7,8} and several observational studies\textsuperscript{9,10} have associated long-term use of EPT with an increased risk of invasive breast cancer. Consequently, considerable recent research has been dedicated to finding an alternative to the progestin component of EPT that will act in a tissue-specific manner to allow treatment of menopausal-related conditions without increasing risk for breast and endometrial cancer.

Possible candidates for this role are selective estrogen receptor modulators (SERMs), which bind to estrogen receptors alpha (ERα) and beta (ERβ) with high affinity and elicit either an estrogen agonistic or antagonistic transcriptional response depending on the target tissue.\textsuperscript{11} Current SERMs do not relieve vasomotor and vaginal atrophy symptoms when administered alone,\textsuperscript{12-14} leading to a new approach to menopausal HT in which a SERM is given in combination with one or more estrogens.\textsuperscript{15} It has been proposed that this combination may provide a safe and therapeutic balance of tissue-selective ER agonism and antagonism for postmenopausal women, including those at high risk for breast cancer.\textsuperscript{16,17} Ideally, the estrogens in this combination therapy would relieve vasomotor symptoms, improve vaginal maturation, and provide protective effects against bone loss and atherosclerosis progression, while the SERM would provide anti-proliferative effects in the breast and endometrium.
Bazedoxifene acetate (BZA) is a newly developed SERM currently under regulatory review for the prevention and treatment of osteoporosis in postmenopausal women. In a 3-year study of osteoporotic postmenopausal women, BZA given alone significantly increased lumbar spine and hip bone mineral density, reduced bone turnover, and significantly decreased the risk of new vertebral fractures compared to the placebo group. In addition, BZA given at 20 mg/day significantly reduced the risk of non-vertebral fractures in a sub-group of women at high risk for osteoporosis. In a series of clinical trials (SMART: Selective estrogens, Menopause, And Response to Therapy) investigating the efficacy and risk profile of several BZA and CEE dose regimens, BZA at 20 mg/day combined with CEE at 0.45 mg/day was the lowest effective dose regimen to prevent endometrial hyperplasia, relieve hot flushes, improve lipid profiles, and maintain bone mass in healthy postmenopausal women. Following 2 years of treatment, the first SMART trial (SMART-1) reported a low incidence of abnormal mammograms (< 5%) and breast cancer (< 0.3%) with BZA 20 mg + CEE 0.45 mg among 3,397 postmenopausal women; however, further studies were needed to fully determine the risk profile of BZA+CEE in the breast.

The primary objective of this study was to investigate the breast profile of BZA alone and in combination with conjugated equine estrogens (CEE), the most widely prescribed ET in the United States. We hypothesized that BZA would inhibit the proliferative and transcriptional effects of CEE on the breast epithelium, while BZA would lack estrogenic activity when administered alone. This report is the first in a series evaluating the effects of BZA with and without CEE on atherosclerosis progression, serum lipids, bone mineral density, vaginal maturation, and breast/endometrial proliferation in the context of a 20-month, randomized, nonhuman primate preclinical trial. Data presented here include interim assessments on breast biopsies obtained after 6 months of treatment.
METHODS

Animal Model and Study Design

The animal model for this study was the female cynomolgus macaque (*Macaca fascicularis*). The female human and macaque breast share many histological and physiological features which result in comparable tissue and transcriptional responses to exogenous sex hormones. For these reasons, this model has been used in many prior studies to evaluate the hormone-associated risk profile of menopausal HTs and SERMs. Other advantages of this model are the ability to perform repeat breast biopsies without sacrifice and assess the effects of new HTs on multiple organ systems in the same subject in an effort to determine a global benefit to risk profile.

One-hundred adult female cynomolgus macaques were imported from the Indonesian Primate Center (Pusat Studi Satwa Primata) at the Institut Pertanian Bogor in West Java, Indonesia. Estimated mean age determined by dentition was 12 years for this study population with no differences between experimental groups. All animals were monoparous or multiparous based on clinical records from the original breeding colony. Following quarantine, all animals were ovariectomized and randomized by body weight into social groups consisting of two to five animals. Social groups were then assigned to one of four experimental groups to receive no treatment (control, n=23) or treatment with BZA 20 mg (n=22), CEE 0.45 mg (n=25), or the combination of BZA 20 mg + CEE 0.45 mg (n=25). Each experimental group originally consisted of 25 animals, but 2 animals from the control and 3 animals from the BZA groups were excluded due to elevated serum ovarian hormone concentrations post-ovariectomy indicating the presence of ectopic ovarian tissue, a spontaneous condition previously reported in cynomolgus macaques. Treatments were administered in the diet and given once daily for 20 months. As previously mentioned, interim measurements presented here came from breast biopsies taken
after 6 months of treatment. Further assessments of BZA+CEE and BZA effects on the breast, uterus, vagina, as well as bone and the cardiovascular system following 20 months of treatment are on-going and will be reported separately.

All procedures using these animals were approved by the Institutional Animal Care and Use Committee of Wake Forest University and conducted in accordance with federal, state and institutional guidelines. The facilities and animal resources program of Wake Forest University are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Diet and Drug Dose Determinations

All monkeys were fed an isoflavone-free casein/lactalbumin-based cake diet prepared by the Wake Forest University Primate Center (WFUPC) diet laboratory and formulated to be equivalent in cholesterol, macronutrients (fat, protein, carbohydrates), and vitamin and mineral content. The amount of macronutrients and supplemental cholesterol (0.29 mg/Cal) were formulated to model diets typically consumed by women in the United States (U.S.). In order to account for differences in metabolic rates between monkeys and women, the standard clinical dose of CEE (0.45 mg/day) was scaled to 1800 Cal of diet (the estimated daily intake of U.S. women). All monkeys consumed approximately 120 Cal of diet per kg of body weight, providing approximately 0.03 mg/kg/day of CEE.

A pilot study was conducted to determine a BZA dose for monkeys that most closely resembled plasma concentrations measured in postmenopausal women receiving 20 mg/day. Target doses of 2.0 or 2.5 mg/kg were investigated based on information provided by a previous metabolic study using macaques given a single oral dose of BZA via gavage. The pilot study conducted at the WFUPC was designed to determine the bioavailability and palatability of BZA when fed once daily in a high-fat cake diet for multiple days. Eight monkeys were fed cake diets
containing either 2.0 or 2.5 mg/kg of BZA for 5 days. Blood was then collected at 0, 4, 24, 48, and 72 hours post-prandial and analyzed by Pfizer using high-performance liquid chromatography (HPLC) with fluorescence detection. The area under the curve from time 0 to 24 hours (AUC$_{0-24}$) of BZA was then calculated and compared to the AUC$_{0-24}$ of BZA measured in postmenopausal women receiving 20 mg/day from a similarly designed pharmacokinetic study.$^{32}$ As a result, the BZA dose of 2.5 mg/kg was selected and used for the reminder of the study. The metabolic disposition of BZA in monkeys has been reported previously to be similar to women.$^{31,33}$

**Estrogens and Bazedoxifene Acetate Measurement**

Serum hormone and drug concentrations along with body weights were measured to confirm adequate dosing and dietary intake among the treatment groups. Since E1 is the major estrogen component of CEE,$^{34}$ E1 levels were used as the primary indicator of equivalent estrogen exposure between the CEE and BZA+CEE treatment groups. Blood samples to measure estrone (E1), 17β-estradiol (E2), and BZA concentrations were obtained at 4 hours post-prandial and following an overnight fast (18-24 hours). Serum E1 and E2 concentrations were measured at the WFUPC Clinical Laboratory using commercially available radioimmunoassay kits (Siemens/DPC, Webster, Texas). Serum obtained for E2 concentrations was first extracted using ethyl ether, and extracts were then dried and reconstituted with zero-standard serum. For E2 values below the lowest standard in the kit (2.5 pg/mL), a predetermined surrogate value of 2.49 pg/mL was used for statistical analyses. Plasma BZA concentrations were measured at Pfizer using HPLC with fluorescence detection. For BZA values below the lowest standard (1.00 ng/mL), a predetermined surrogate value of 0.99 ng/mL was used for statistical analysis.

**Breast Biopsies**
Following 6 months of treatment, all monkeys were sedated with ketamine HCl (15 mg/kg) and atropine (0.03 mg/kg), intubated, surgically prepped, and maintained on isoflurane (1-2%). A 2.0 cm incision was made and 0.6 grams of breast tissue from the upper lateral breast quadrant was removed, bisected, and prepared for histology and gene expression studies as previously described. Adjacent tissue sections from each animal were used for histomorphometry and immunostaining.

**Histomorphometry & Histopathology**

Fixed breast tissue sections were stained with hematoxylin and eosin (H&E) and digitized using an Infinity 3 digital camera (Lumenera, Lawrenceville, GA). As a surrogate marker for mammographic density in women, the total epithelial area (lobuloalveolar units and extralobular ducts) in each biopsy sample was quantified by histomorphometry using techniques previously described. H&E-stained breast biopsy tissues were also evaluated qualitatively for morphological changes related to epithelial proliferation and exogenous estrogen exposure by a board-certified veterinary pathologist (C.E.W.). Lobular enlargement (a lobule containing > 50 acini) is a physiologic change in the breast seen following exogenous estrogen exposure. Columnar cell change and hyperplasia with or without atypia are benign proliferative lesions in the breast and potential risk markers for human breast cancer. All histomorphometry and histopathological evaluations were completed by persons blinded to the treatment groups.

**Immunohistochemistry**

Breast tissue sections were immunostained using commercially-available primary monoclonal antibodies for the proliferation marker Ki67 (Ki67SP6; Thermo Scientific, Fremont, CA; 1:100 dilution) and the sex steroid receptors ERα (NCL- ER-6F11, Novocastra Reagents, Leica Microsystems Inc., Buffalo, NY; 1:100 dilution) and progesterone receptor (NCL-PGR-312, Novocastra Reagents, Leica Microsystems Inc., Buffalo, NY; 1:100 dilution) using methods
similar to those described elsewhere.\textsuperscript{35} Nuclear immunolabeling was then quantified using a computer-assisted manual counting technique with a grid filter to select cells for counting (Image-Pro Plus software, Media Cybernetics, Silver Spring, MD).\textsuperscript{25} The number of positively stained cells was expressed as a percentage of the total number examined (100 cells) for each breast epithelial structure (lobular, extralobular ductal, and terminal ductal epithelium). All immunohistochemistry (IHC) counting was performed by a technician blinded to the treatment groups.

**Quantitative Real-time PCR**

Transcript levels for key genes associated with breast proliferation (\textit{MKI67}, Ki67 antigen), ER activity (\textit{ESR1}, ER\textalpha; \textit{ESR2}, ER\textbeta; \textit{PGR}, progesterone receptor; \textit{TFF1}, trefoil factor 1 [pS2]; and \textit{GREB1}, gene regulated by estrogen in breast cancer 1), apoptosis (\textit{BCL-2}, B-cell CLL/lymphoma 2), and estrogen metabolism (\textit{CYP19}, aromatase; \textit{HSD17B1}, 17-beta hydroxysteroid dehydrogenase (HSD) type 1; \textit{HSD17B2}, 17-beta HSD type 2; \textit{STS}, estrogen sulfatase; \textit{SULT1E1}, sulfotransferase family 1E, estrogen-preferring, member 1) were measured using quantitative real-time reverse transcription-PCR (qRT-PCR). All primer-probe sets for specific gene targets were generated through the ABI Taqman service and validated by prior macaque studies in our laboratory.\textsuperscript{29, 35, 36, 39} Both custom macaque and commercially available human assays were used. Total RNA was extracted from frozen samples of breast tissue using Tri Reagent (Molecular Research Center, Cincinnati, OH), purified using RNeasy Mini kits (QIAGEN, Valencia, CA), and quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop, Thermo Scientific, Fremont, CA). One animal in the control and one animal in the CEE group were excluded due to low RNA content. Group numbers for the remaining 93 samples available for gene expression studies were \( n = 22 \) (control), \( n = 22 \) (BZA), \( n = 24 \) (CEE), and \( n = 25 \) (BZA+CEE). RNA aliquots (5 \( \mu \)g per sample) were then reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems,
Carlsbad, CA). qRT-PCR reactions (10 µl volume) were performed on an Applied Biosystems 7500 Fast Real-Time PCR System using standard Taqman reagents and thermocycling protocol. β-actin was used as the endogenous control while reference breast tissue cDNA was run in parallel for plate-to-plate calibration. Relative expression of each target gene was calculated using ABI Relative Quantification 7500 Software v2.0.1.

**Gene Microarrays**

Four samples of total RNA from each treatment group (n=16) were selected randomly and submitted to Beckman Coulter Genomics (formerly Cogenics, Morrisville, NC) for gene microarray assays. RNA integrity was confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) and only samples with an RNA integrity number (RIN) greater than 6.0 were used to generate biotin-labeled cRNA. Biotinylated cRNA from each sample was hybridized to an Affymetrix GeneChip Rhesus Macaque Genome Array.  

**Statistical Analyses**

All variables were evaluated for their distribution and equality of variance. Data not normally distributed were transformed (log10 or square root) to improve the normality for analysis and then reverse transformed to the original scale for display in the results. Histomorphometry, IHC for ERα and Ki67, and all qRT-PCR data except for MKI67 and TFF1 had equality of variance; therefore, these data were assessed using analysis of variance (ANOVA). If a significant overall treatment effect was detected, the Tukey HSD post hoc test was used for multiple pair-wise comparisons. The IHC data for PGR and the relative gene expression of TFF1 and MKI67 violated the Levene’s test for equality of variance therefore these data were analyzed using the nonparametric Kruskal-Wallis and the post hoc Wilcoxon (rank sums) tests. P values were then adjusted for multiple pair-wise comparisons using a Bonferroni correction (4 comparisons, each treatment group vs. control and CEE vs. BZA+CEE). Body
weights among the treatment groups were compared using a mixed model approach with baseline body weight as a covariate. This model allowed for the comparison of baseline and 6 months post-treatment body weights within each treatment as well as the comparison of body weights among the treatment groups within the baseline and 6 months post-treatment time periods. A similar mixed model approach was used to determine E1, E2, and BZA concentrations at 4 and 18-24 hours post-prandial, except baseline measures as covariates were not necessary in these models. A two-tailed Fisher’s exact test was used to evaluate treatment group differences in the prevalence of histopathological findings. A two-tailed significance level of 0.05 was selected for all comparisons, and all aforementioned analyses were done using JMP statistical software (version 8.0.2; SAS Institute, Inc, Cary, NC).

Global gene expression profiling was done using the Genesifter software program (Geospiza) and Ingenuity Pathway Analysis (IPA) software version 8.8 (Ingenuity System). Intensity data were uploaded into the Genesifter software program, RMA normalized, converted to a log 2 scale, screened for homogeneity among samples and treatment groups, and evaluated by a supervised ANOVA and pair-wise comparisons. Filter criteria for global profiling included a fold change > 2.0, quality > 2, and corrected P values of 0.05 using the Benjamini and Hochberg method. Overrepresented pathways or terms related to epithelial cell proliferation, cell cycle progression, and apoptosis were identified using z-scores generated in KEGG pathway and ontology analyses in Genesifter and other pathway analyses in IPA. An absolute z-score of > 2.0 in Genesifter was considered significant, while a significant overrepresented pathway in IPA was determined using Fisher’s exact test with a Benjamini and Hochberg correction. Significant differences in gene number altered by each treatment group compared to the control group were determined using \( \chi^2 \) test.

RESULTS
Body Weights and Serum Hormone and Drug Concentrations

Body weights at 6 months post-treatment and serum concentrations of E1 at 4 and 18-24 hours post-feeding were not significantly different between the BZA+CEE and CEE treatment groups ($P > 0.05$ for all, Table 1). Similarly, plasma concentrations of BZA were not significantly different between the BZA+CEE and BZA treatment groups at 4 and 18-24 hours post-feeding ($P > 0.05$ for all). Serum E2 concentrations were significantly lower in the BZA+CEE group relative to the CEE group at 4 hours ($P < 0.05$), but not at 18-24 hours post-feeding. All groups showed a small gain in body weight from baseline to 6 months of treatment, but this increase in body weight only reached significance in the control and BZA groups ($P < 0.01$ for both).

Breast Epithelial Proliferation: Histomorphometry and Ki67

As depicted in Fig. 1, groups treated with BZA+CEE and BZA had significantly less breast epithelial density relative to CEE ($P < 0.05$ for both) and similar breast epithelial density compared to control. Similarly, BZA+CEE and BZA treatment resulted in significantly less immunolabeling for the proliferation marker Ki67 in the terminal ducts than with CEE (Fig. 2A, $P < 0.05$ for all). Ki67 immunolabeling within the lobules showed a similar pattern (Fig. 2B, $P < 0.05$ for BZA and $P = 0.08$ for BZA+CEE compared to CEE), while Ki67 expression within the extralobular ducts was modestly lower in the BZA group compared to CEE and BZA+CEE (Fig. 2C, $P < 0.05$ for both). No significant group differences were seen for Ki67 mRNA expression (see graph A, Supplemental Figure 1).

Breast Histopathology

Histopathological findings are summarized in Table 2. Lobular enlargement was most prevalent in the CEE group, while prevalence in the BZA+CEE group was not different from control ($P = 1.0$) and significantly less than that observed in the CEE group ($P = 0.02$). Similarly,
the prevalence of lobular enlargement in the BZA group was not significantly different from control ($P = 0.17$). Mild columnar cell change was observed in one subject receiving BZA and three subjects receiving CEE. Mild to moderate columnar cell hyperplasia was evident in one breast biopsy among the BZA-treated animals and two breast biopsies among the CEE-treated animals. Atypical ductal hyperplasia was present in one breast biopsy among the BZA and CEE-treated animals; however, no lobular enlargement was observed in these cases. No cases of atypical lobular hyperplasia or neoplasia were observed among any breast tissues examined.

**ERα Expression and Transcriptional Activation**

Treatment with BZA, CEE, and BZA+CEE altered ERα immunoreactivity in the breast epithelium, but had no significant affect on ERα mRNA levels (Fig. 3A-D). Groups treated with BZA and BZA+CEE had significantly less ERα immunolabeling in the terminal ducts and lobules than the control and CEE groups (Fig. 3A and 3B, $P < 0.0001$ for all). In the extralobular ducts, CEE treatment increased ERα immunolabeling compared to control (Fig. 3C, $P < 0.05$), but this effect was completely blocked by the addition of BZA to CEE ($P < 0.05$, CEE compared to BZA+CEE; $P > 0.05$, BZA+CEE compared to control). Similar to ERα gene expression, ERβ mRNA expression in the breast was not affected by HT (Fig. 3E, $P = 0.80$).

As expected, treatment with CEE significantly increased GREB1 and TFF1 expression compared to the control group (Fig. 4A and 4B, $P < 0.0001$ for both). In BZA+CEE co-therapy, BZA inhibited CEE-stimulated GREB1 expression by ~5 fold and TFF1 expression by ~75 fold compared to treatment with CEE ($P < 0.01$ for GREB1 and $P < 0.0001$ for TFF1). Similar to the histology results, treatment with BZA had minimal stimulatory effects on these ERα activation markers however TFF1 expression with BZA and BZA+CEE treatment was slightly higher compared to control ($P < 0.05$ for both).
Treatment with BZA, CEE, and the combination significantly increased the mRNA expression of PGR relative to the control group ($P < 0.01$ for all; see graph B, Supplemental Figure 1). As shown in Fig. 4C-E, IHC expression of PGR showed a comparable pattern in which CEE increased PGR expression compared to control in the terminal ducts and lobules as well as in the extralobular ducts ($P < 0.01$ for all). Protein expression of PGR in the BZA+CEE-treated group was less than that seen with CEE, but this attenuation did not reach statistical significance in all epithelial regions and PGR protein expression was significantly induced by BZA+CEE co-therapy in the lobules and extralobular ducts compared to control ($P < 0.05$). In contrast to PGR mRNA expression, treatment with BZA did not result in a significant increase in PGR protein expression ($P > 0.05$ for all breast epithelial regions vs. control).

**Global Transcriptional Profiles**

Compared to control, treatment with CEE significantly altered a greater number of transcripts than BZA+CEE and BZA alone ($P < 0.0001$ by $\chi^2$ for both). For instance, CEE treatment uniquely changed the expression of 36 (named) genes compared to one gene altered individually by BZA+CEE and BZA treatment (Fig. 5A, Venn diagram). These specific genes are provided in Supplemental Table 1. The divergent pattern of CEE from BZA+CEE and BZA was also apparent in the global expression analyses as shown in Fig. 5B and C. The principle component analysis showed divergent vectors for CEE and BZA+CEE, but similar directional profiles for BZA+CEE and BZA (Fig. 5B). Similarly, the corresponding hierarchical dendrogram clustered BZA+CEE with BZA instead of CEE (Fig. 5C), indicating that the transcriptional profile of BZA+CEE more closely resembles BZA than CEE. The heatmap for these significantly altered genes identified a large group of genes up-regulated by CEE and antagonized by BZA in BZA+CEE co-therapy, but not significantly altered by BZA compared to control (Fig. 5D).
Using pattern navigation (ANOVA) and pair-wise comparisons at a fold change >2 (adjusted $P < 0.05$, Benjamini & Hochberg), a complete list of 23 (named) genes antagonized by BZA in BZA+CEE co-therapy was generated. As shown in Table 3, several of the genes identified were well-known estrogen-driven genes including TFF1, GREB1, IGFBP1, TFF3, IGSF1, STC2, and PPM1K. Among these BZA-antagonized genes, no specific pathways related to epithelial cell proliferation or cell cycle progression were identified. On the contrary, a pair-wise comparison between BZA+CEE and CEE showed that genes assigned to the ontology terms ‘immune system process’ and ‘cell death’ were significantly up-regulated by BZA+CEE (z scores of 4.81 and 3.16, respectively). Notable BZA-agonized genes within these functional categories included major histocompatibility complex II DP alpha 1, Granzyme B, Chemokine (C-C motif) ligand 5, and ubiquitin D (see Table, Supplemental Table 2). However, classic genes related to apoptosis of epithelial cells including inhibitors (bcl-2, bcl-X1, BAG-1, and mcl-1) and inducers of cell death (bax, bad, and bcl-X3) were not significantly regulated by BZA+CEE and BZA therapy in these datasets (data not shown). No significant between treatment differences or trends toward significance were observed in estrogen metabolizing enzymes (see Supplemental Table 3).

DISCUSSION

Bazedoxifene acetate (20 mg/day) is a novel SERM currently being considered as a new menopausal therapy for the treatment of osteoporosis and, in combination with conjugated equine estrogen (0.45 mg/day), for menopausal symptoms and the prevention of osteoporosis. Since concern of breast cancer is an important factor in the decision to initiate HT for many women, here we investigated the effect of BZA with and without CEE on several biomarkers of cancer promotion in the breast. The addition of BZA to CEE significantly antagonized the stimulatory effects of CEE on total breast epithelial density, lobular size, Ki67 immunolabeling, and specific gene markers of ERα activity, while treatment effects of BZA alone were
comparable to control. Similarly, BZA and BZA+CEE had no effect on gene markers of cell proliferation or cell cycle progression, indicating that both treatments lack an estrogen agonist profile in the breast. ERα protein immunolabeling was significantly lower with BZA and BZA+CEE compared to control and CEE treatments, while ERα mRNA expression was not significantly different, suggesting that increased ERα protein degradation may contribute to the estrogen inhibitory effects of BZA.

Endogenous estrogens and ERs are well-known for their critical role in the development and progression of many breast cancers. Many of the established risk factors of breast cancer (e.g., early menarche and late menopause) relate to a lifetime exposure to estrogens, and high levels of endogenous estrogens have been associated with increased breast cancer risk in both premenopausal and postmenopausal women.\textsuperscript{47, 48} Estrogens may contribute to breast cancer risk by increasing epithelial cell proliferation and possibly inducing DNA mutations through genotoxic metabolites.\textsuperscript{49} Many successful strategies for the prevention and treatment of breast cancer have focused on blocking estrogen exposure and actions in the breast. For example, large chemoprevention trials have shown that SERMs such as tamoxifen and raloxifene reduce the incidence of ER-positive breast cancers by 50-75\% in both high-risk\textsuperscript{14, 50} and normal-risk women,\textsuperscript{51} whereas aromatase inhibitors, which block estrogen biosynthesis, reduce recurrence and prevent contralateral tumors during adjuvant therapy.\textsuperscript{52}

Exogenous ET in the form of CEE increases mammographic density and benign proliferative lesions in the normal postmenopausal breast,\textsuperscript{8, 53, 54} but whether these changes contribute to an increase in breast cancer risk with long-term use is complex and not completely understood. In the Nurses’ Health Study, a large prospective U.S. cohort study in which most participating women took CEE at a standard dose of 0.625 mg/day, the relative risk (RR) of ER+/PGR+ breast cancers was not significantly elevated until after 20 years of use (RR 1.42; 95\% CI 1.13 - 1.77).\textsuperscript{55} Similarly, another U.S. cohort study reported that ET, consisting primarily
of CEE (0.625 mg/day), did not significantly increase the RR of breast cancer among normal weight women until after 15 years of use (RR 1.6; 95% CI 1.2 - 2.2).9 In the WHI Estrogen-alone Trial, oral CEE (0.625 mg/day) did not increase the risk for invasive breast cancer over a mean follow-up period of 7.1 years (hazard ratio (HR) = 0.80; 95% CI 0.62 - 1.04)8 and resulted in a significant reduction in the incidence of invasive breast cancer among adherent women (HR 0.67; 95% 0.47 - 0.97; P = 0.03)8 which continued for at least four years beyond the end of the study.56 The biological mechanisms related to these effects are currently unclear.

In the current study, BZA fully inhibited the estrogenic effects of CEE on total breast epithelial density, lobular size, and Ki67 protein expression in the terminal ductal epithelium, while having neutral effects when administered alone. These results support a small body of prior evidence from cell culture, preclinical, and clinical studies demonstrating that BZA is an estrogen antagonist in the breast.23, 57-60 Results from in vitro studies showed that BZA did not stimulate proliferation of ERα-positive human breast cancer cells when given alone and antagonized proliferation when given with E2.57 Similarly, a study in an ovariectomized sexually immature mouse model found that the addition of BZA to CEE completely blocked CEE actions on mammary ductal growth and a specific gene marker of ERα activity, while treatment effects of BZA alone were comparable to vehicle.59 A randomized (phase III) clinical trial investigating the treatment effects of various BZA doses in osteoporotic postmenopausal women reported that BZA 20 mg did not significantly alter mammographic density after 24 months of treatment compared to baseline.60 Safety data from this trial revealed no significant difference in breast cancer incidence or other breast-related adverse events (breast pain, breast cyst, and fibrocystic breast disease) between the BZA and placebo groups, which persisted for an additional 3 years.61 Mammographic density and breast safety data for the BZA 20 mg + CEE 0.45 mg combination have only been reported after 24-months of treatment in a randomized, phase III clinical trial.
consisting of 3,397 osteoporotic postmenopausal women (SMART-1) and the findings were similar to the BZA 20 mg alone results.\textsuperscript{23}

Although data from prior reports and the present study have shown that BZA is an estrogen-antagonist in the breast, the inhibitory effects of BZA on ER-activity are highly dependent on the BZA to estrogen dose ratio and, possibly, the type of estrogen used in the BZA + estrogen regimen. In human breast cancer cell culture studies, a BZA dose of 10 nM completely antagonized the proliferative effects of co-administered E2, but a smaller BZA dose of 1.0 pM resulted in negligible inhibition.\textsuperscript{57} This dose-dependent effect is also apparent in other estrogen-sensitive tissue such as the endometrium. For instance, in the 24-month SMART-1 trial, the uterotrophic effects of CEE (0.45 mg/d or 0.625 mg/d) on the occurrence of endometrial hyperplasia were effectively antagonized with 20 mg/d but not 10 mg/d of BZA.\textsuperscript{21} Thus far, the effects of BZA on the normal postmenopausal breast have only been evaluated with oral CEE as the primary estrogen therapy\textsuperscript{23} and it is not known whether BZA 20 mg would provide protective effects in the breast and endometrium if co-administered with standard doses of oral or transdermal E2. Based on evidence from studies of oral estrogen effects in macaques, a standard dose of CEE may have less stimulatory effects on breast epithelial proliferation than a standard 1.0 mg/day dose of E2,\textsuperscript{62} suggesting that the dose of BZA needed for complete antagonism may vary with type of ET.

In the current study, ER$\alpha$ protein levels in lobular and terminal ductal epithelium were significantly lower with BZA and BZA+CEE compared to control and CEE treatments, while ER$\alpha$ mRNA levels remained unchanged. This unexpected finding suggests that BZA may increase degradation of ER$\alpha$ post-translationally. Proteolysis of ER$\alpha$ in breast epithelial cells has been shown to be mediated by the ubiquitin-proteasome pathway\textsuperscript{63} and it is possible that BZA binding to the ER$\alpha$ may facilitate ubiquitination and proteasome-mediated degradation. This idea is supported by a recent \textit{in vitro} study that showed proteasome-mediated degradation of the ER$\alpha$
by BZA (without the co-administration of one or more estrogens) in hormone-resistant breast cancer cells. In this study, MCF-7:5C cells were treated with a proteasome inhibitor which completely blocked ERα degradation by BZA, while treatment with a protein synthesis inhibitor had minimal effects on BZA-induced ERα protein degradation. Collectively, these data support the idea that ERα degradation may contribute to the estrogen antagonist effects of BZA in the breast.

Other notable findings in this study are: 1) the antagonism of Ki67 immunoexpression in the terminal ducts by BZA+CEE treatment compared to CEE; 2) the induction of PGR expression by BZA with and without CEE; and 3) the up-regulation of genes related to immune-mediated apoptosis, specifically cytotoxic T lymphocyte-mediated apoptosis, by BZA+CEE compared to CEE treatment. The terminal ducts are part of the terminal ductal lobular units (TDLUs) of the breast which is the epithelial unit at the end of an arborizing network of (extralobular) ducts. Marked inhibition of Ki67 expression in this region by BZA+CEE is of clinical importance considering this is the site from which many breast cancers originate. The biological significance of the increased mRNA expression of PGR with BZA and BZA+CEE therapy is not known; however increased levels of PGR protein have been reported previously in the postmenopausal breast of macaques treated with tamoxifen. Equally of interest is the finding that the addition of BZA to CEE significantly up-regulated the expression of genes related to cytotoxic T lymphocyte-mediated apoptosis, particularly Granzyme B. Previous in vitro studies have shown that E2 increases breast cancer cell survival by inducing the expression of a Granzyme B inhibitor in these cells (proteinase inhibitor 9) and treatment with a SERM (tamoxifen) antagonizes these effects. Whether BZA has a similar role in the normal postmenopausal breast is not known. Qualitative assessments of the breast biopsy tissues revealed mild lobular lymphocytic infiltration in approximately 40% of the samples with no between group differences.
Strengths of this study include the randomized placebo-controlled study design, inclusion of a CEE alone study group, and the ability to control diet, dose, and other environmental variables. The phase III human clinical trials investigating BZA alone and BZA+CEE did not have a CEE alone group because all participating women did not have a prior hysterectomy.\textsuperscript{18,19} In addition, these trials were designed to measure changes in bone mineral density as the primary endpoint among osteoporotic women and not changes in mammographic density or breast cancer incidence.\textsuperscript{18,19} Breast assessments among these trials were retrospective analyses and therefore may have been subject to selection bias.\textsuperscript{23,60,61} Notable in the same regard, participants in a BZA vs. placebo trial who had previously taken HT (> 8 week before the study) were included in the mammogram analyses.\textsuperscript{60} A potential weakness of the current macaque study is the lack of individual dosing. Due to the large size of the study, hormone therapies were administered in the diet and animals were fed in social groups.

**CONCLUSIONS**

In this preclinical trial, BZA antagonized the proliferative and transcriptional effects of CEE in the normal postmenopausal nonhuman primate breast, while BZA had neutral effects. ER$\alpha$ protein levels were significantly lower with BZA and BZA+CEE treatment compared to control and CEE, suggesting that BZA may promote ER$\alpha$ protein degradation in addition to blocking the binding of estrogens. These findings support the idea that BZA may be a safe alternative to the progestin-component in combined HT for symptomatic postmenopausal women.

**ACKNOWLEDGEMENTS**

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FIG. 1. Breast epithelial density measurement in postmenopausal macaques treated with BZA, CEE, BZA+CEE, or no treatment (CTL = control) for 6 months. (A) BZA significantly inhibited the stimulatory effects of CEE on breast epithelial density, while having no effect when given alone. Values represent means ± 95% confidence intervals (CI). Treatment groups not connected by the same letter are significantly different ($P < 0.05$, ANOVA). (B) Representative photomicrographs demonstrate increased lobular enlargement with CEE, but not with BZA+CEE and BZA. Open arrows indicate terminal ducts centrally located within the lobules, the functional secretory units of the breast. Closed arrows represent less differentiated extralobular (large) ducts. Hematoxylin and eosin (H&E) stain.
FIG. 2. Immunohistochemical detection of the proliferation marker, Ki67, in the breast of postmenopausal macaques given BZA, CEE, BZA+CEE, or no treatment (CTL, control) for 6 months. (A) BZA and BZA+CEE treatment resulted in significantly less immunostaining for Ki67 in the terminal ducts than with CEE. (B) A trend for lower Ki67 immunostaining with BZA+CEE treatment compared to CEE was seen in the lobules ($P = 0.08$). (C) Immunolabeling for Ki67 in the extralobular ducts was significantly less with BZA than with CEE and BZA+CEE treatment. Values represent means ± 95% CI. For each epithelial region (A-C), treatment groups not connected by the same letter are significantly different ($P < 0.05$, ANOVA).
FIG. 3. Immunohistochemical and gene expression of estrogen receptors in the breast of surgically postmenopausal monkeys treated with BZA, CEE, BZA+CEE, or no treatment (CTL, control) for 6 months. BZA and CEE altered ERα protein levels without affecting mRNA levels in the breast. BZA with and without CEE decreased ERα protein levels in the terminal ducts (A) and lobules (B) compared to control and CEE. In the extralobular ducts (C), CEE treatment induced ERα protein levels compared to control, but this agonist effect was completely blocked by the addition of BZA to CEE co-therapy. (D and E) No significant group differences were seen for ERα and ERβ mRNA expressions \((P > 0.1)\). Gene expression values for ERα and ERβ were measured by qRT-PCR, corrected for endogenous β-actin gene expression, and expressed relative to control group values. Values represent means ± 95% CI. For all analyses (A-E), treatment groups not connected by the same letter are significantly different \((P < 0.05, ANOVA)\).
**FIG. 4.** BZA attenuated the expression of ERα activity markers in the breast of postmenopausal macaques. (A and B) BZA given with CEE significantly inhibited CEE-induced expression of *GREB1* and *TFF1*, while BZA alone had minimal stimulatory effects. (C-E) Treatment with BZA+CEE had less immunolabeling for PGR, but this decrease in protein expression did not reach statistical significance in all epithelial regions examined. Gene expression values were measured by qRT-PCR, corrected for endogenous β-actin gene expression, and expressed relative to control group values. Values represent means ± 95% CI. For all analyses (A-E), treatment groups not connected by the same letter are significantly different (*P* < 0.05). *TFF1* and PGR expressions were analyzed using non-parametric tests, while *GREB1* expression was evaluated using a parametric ANOVA (see methods).
FIG. 5. Global gene expression analyses indicate that BZA+CEE treatment lacks an estrogenic profile in the macaque breast (n = 4 for all groups). (A, Venn diagram) Compared to control, treatment with CEE significantly altered a greater number of transcripts than BZA+CEE and BZA alone (P < 0.0001 by χ² for both). (B) Principle component analysis showed divergent vectors for CEE and BZA+CEE, but similar directional profiles for BZA+CEE and BZA. (C) Similarly, the corresponding hierarchical dendrogram clustered BZA+CEE with BZA instead of CEE, indicating that the transcriptional profile of BZA+CEE more closely resembles BZA than CEE alone. (D) The heatmap for these significantly altered genes suggested that a large group of genes up-regulated by CEE were antagonized by BZA in BZA+CEE co-therapy but not significantly altered by BZA alone compared to control.
SUPPLEMENTAL FIG. 1. Relative gene expression of progesterone receptor (PGR) and the proliferation marker, MKI67. (A) No significant difference in MKI67 expression was observed among treatment groups (non-parametric Kruskal-Wallis). (B) Treatment with BZA, CEE, and BZA+CEE significantly increased PGR expression relative to control ($P < 0.01$ for all). A trend for a significant difference in PGR expression was observed between CEE and BZA+CEE ($P = 0.051$, ANOVA). Values represent means ± 95% CI. Treatment groups not connected by the same letter are significantly different ($P < 0.05$).
**TABLE 1. Body Weights and Hormone/Drug Concentrations**

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<tr>
<th></th>
<th>Control</th>
<th>BZA 20 mg/d</th>
<th>CEE 0.45 mg/d</th>
<th>BZA+CEE 20 mg/d + 0.45 mg/d</th>
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<td><strong>Body Weight, Kg</strong></td>
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<td>2.88</td>
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<td>(2.80 - 2.94)</td>
<td>(2.81 - 2.94)</td>
<td>(2.81 - 2.95)</td>
<td></td>
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<tr>
<td>6 months</td>
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<td>3.08</td>
<td>2.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.02&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>(3.12 - 3.28)</td>
<td>(3.00 - 3.15)</td>
<td>(2.90 - 3.04)</td>
<td>(2.95 - 3.10)</td>
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</tr>
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<td><strong>N</strong></td>
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<td>22</td>
<td>25</td>
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<td>(26.3 - 33.5)</td>
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<td>(136.7 - 172.8)</td>
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<td>18-24 hr PP</td>
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<td>42.2</td>
<td>103.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>(38.3 - 48.9)</td>
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<td>(92.4 - 116.7)</td>
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<td>22</td>
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<td><strong>17β-estradiol, pg/ml</strong></td>
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<tr>
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<td>16.9</td>
<td>11.9&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>(14.6 - 19.5)</td>
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<td>NS</td>
<td>3.8</td>
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<td>(2.9 - 4.3)</td>
<td>(3.2 - 4.5)</td>
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<td>18-24 hr PP</td>
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<td>1.7</td>
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<td>(0.8 - 2.2)</td>
<td>(1.0 - 2.4)</td>
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<tr>
<td><strong>N</strong></td>
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NS = not sampled; PP = post-prandial. Values represent means with 95% CI. For conversion to SI units, multiply by the following conversion factors: 3.70 for estrone (picomoles per liter) and 3.67 for estradiol (picomoles per liter). For the 17β-estradiol concentrations, control and BZA alone group values are provided for reference only. Serum used to measure 17β-estradiol concentrations were first extracted with ethyl ether (see methods). *<sup>a</sup> P < 0.001- 0.05 compared to control; *<sup>b</sup> P < 0.0001 compared to respective control and BZA groups; *<sup>c</sup> P < 0.05 compared to CEE.
TABLE 2. Histopathological Findings

<table>
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<tr>
<th></th>
<th>Control</th>
<th>BZA 20 mg/d</th>
<th>CEE 0.45 mg/d</th>
<th>BZA+CEE 20 mg/d + 0.45 mg/d</th>
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<tr>
<td>Lobular Enlargement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>3 (13%)</td>
<td>7 (32%)</td>
<td>13 (52%)</td>
<td>4 (16%)</td>
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<tr>
<td>Moderate</td>
<td>0</td>
<td>3</td>
<td>5</td>
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<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
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<tr>
<td>P value vs. Control</td>
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<td>0.17</td>
<td>0.01</td>
<td>1.0</td>
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<tr>
<td>(Fisher’s Exact Test)</td>
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<td>Benign Proliferative Lesions</td>
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<td>Number Examined</td>
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TABLE 3. Bazedoxifene-antagonized Genes in BZA+CEE Co-therapy

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<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change CEE vs. BZA+CEE</th>
<th>Fold Change CEE vs. Control</th>
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<td>TFF1</td>
<td>Trefoil factor 1</td>
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<td>CYP2A13</td>
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<td>8.28&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>GREB1</td>
<td>GREB1 protein</td>
<td>9.99&lt;sup&gt;e&lt;/sup&gt;</td>
<td>11.19&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGFBP1</td>
<td>Insulin-like growth factor binding protein 1</td>
<td>9.71&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.21&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>TFF3</td>
<td>Trefoil factor 3 (intestinal)</td>
<td>9.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.28&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGSF1</td>
<td>Immunoglobulin superfamily, member 1</td>
<td>5.82&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.80&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C1orf173</td>
<td>Chromosome 1 open reading frame 173</td>
<td>5.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13.28&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>STC2</td>
<td>Stanniocalcin 2</td>
<td>4.30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.36&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLK11</td>
<td>Kallikrein-related peptidase 11</td>
<td>4.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.86&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>SYT13</td>
<td>Synaptotagmin XIII</td>
<td>3.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.22&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SGK493</td>
<td>Protein kinase-like protein SgK493</td>
<td>2.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.37&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CLGN</td>
<td>Calmegin</td>
<td>2.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.36&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>NTNG1</td>
<td>Netrin G1</td>
<td>2.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.81&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TTC36</td>
<td>Tetratricopeptide repeat domain 36</td>
<td>2.56&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.53&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TPRG1</td>
<td>Tumor protein p63 regulated 1</td>
<td>2.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.02&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPM1K</td>
<td>Protein phosphatase, Mg2+/Mn2+ dependent, 1K</td>
<td>2.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.55&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>P4CRG</td>
<td>PARK2 co-regulated</td>
<td>2.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.92&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCC2</td>
<td>Methylcrotonoyl-Coenzyme A carboxylase 2 (beta)</td>
<td>2.42&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>DDX4</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 4</td>
<td>2.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SUSD3</td>
<td>Sushi domain containing 3</td>
<td>2.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.98&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLK12</td>
<td>Kallikrein-related peptidase 12</td>
<td>2.19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.65&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAGED2</td>
<td>Melanoma antigen family D, 2</td>
<td>2.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ASPN</td>
<td>Asporin</td>
<td>1.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.16&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Filtered dataset generated in Genesifter software program using pattern navigation (ANOVA) and pair-wise comparisons (fold change > 2, Benjamini & Hochberg correction). Adjusted \( P < 0.05 \); \( P < 0.01 \); \( P < 0.001 \); \( P < 0.0001 \) (post-hoc Tukey HSD). All genes were not significantly regulated by BZA and BZA+CEE compared to control. \( N = 4 \) for each treatment group.
**SUPPLEMENTAL TABLE 1.**
*Genes Regulated by 0.45 mg/d CEE treatment in the Breast*^a^  

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>CEE-regulated Direction vs. Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFBP1</td>
<td>Insulin-like growth factor binding protein 1</td>
<td>Up</td>
</tr>
<tr>
<td>C1orf173</td>
<td>Chromosome 1 open reading frame 173</td>
<td>Up</td>
</tr>
<tr>
<td>TFF1</td>
<td>Trefoil factor 1</td>
<td>Up</td>
</tr>
<tr>
<td>MCCC2</td>
<td>Methylcrotonyl-Coenzyme A carboxylase 2 (beta)</td>
<td>Up</td>
</tr>
<tr>
<td>TFF3</td>
<td>Trefoil factor 3 (intestinal)</td>
<td>Up</td>
</tr>
<tr>
<td>PGR</td>
<td>Progesterone receptor^b^</td>
<td>Up</td>
</tr>
<tr>
<td>HIST3H2A</td>
<td>Histone cluster 3, H2a</td>
<td>Up</td>
</tr>
<tr>
<td>ASPN</td>
<td>Asporin</td>
<td>Up</td>
</tr>
<tr>
<td>SGK493</td>
<td>Protein kinase-like protein SgK493</td>
<td>Up</td>
</tr>
<tr>
<td>KLK11</td>
<td>Kallikrein-related peptidase 11</td>
<td>Up</td>
</tr>
<tr>
<td>GREB1</td>
<td>GREB1 protein</td>
<td>Up</td>
</tr>
<tr>
<td>IGSF1</td>
<td>Immunoglobulin superfamily, member 1</td>
<td>Up</td>
</tr>
<tr>
<td>KLK12</td>
<td>Kallikrein-related peptidase 12</td>
<td>Up</td>
</tr>
<tr>
<td>PPM1K</td>
<td>Protein phosphatase, Mg2+/Mn2+ dependent, 1K</td>
<td>Up</td>
</tr>
<tr>
<td>SYT13</td>
<td>Synaptotagmin XIII</td>
<td>Up</td>
</tr>
<tr>
<td>PACRG</td>
<td>PARK2 co-regulated</td>
<td>Up</td>
</tr>
<tr>
<td>MAGED2</td>
<td>Melanoma antigen family D, 2</td>
<td>Up</td>
</tr>
<tr>
<td>C2orf80</td>
<td>Chromosome 2 open reading frame 80</td>
<td>Up</td>
</tr>
<tr>
<td>CLGN</td>
<td>Calmegin</td>
<td>Up</td>
</tr>
<tr>
<td>STC2</td>
<td>Stanniocalcin 2</td>
<td>Up</td>
</tr>
<tr>
<td>CACNA1D</td>
<td>Ca++ channel, voltage-dependent, L type, alpha 1D subunit</td>
<td>Up</td>
</tr>
<tr>
<td>RET</td>
<td>Ret proto-oncogene</td>
<td>Up</td>
</tr>
<tr>
<td>NTNG1</td>
<td>Netrin G1</td>
<td>Up</td>
</tr>
<tr>
<td>CSRP3</td>
<td>Cysteine-serine-rich nuclear protein 3</td>
<td>Up</td>
</tr>
<tr>
<td>NR2C2</td>
<td>Nuclear receptor subfamily 2, group C, member 2</td>
<td>Down</td>
</tr>
<tr>
<td>SLC4A8</td>
<td>Solute carrier family 4, sodium bicarbonate cotransporter, member 8</td>
<td>Up</td>
</tr>
<tr>
<td>DACH1</td>
<td>Dachshund homolog 1 (Drosophila)</td>
<td>Up</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
<td>Up</td>
</tr>
<tr>
<td>FXYD3</td>
<td>FXYD domain containing ion transport regulator 3</td>
<td>Up</td>
</tr>
<tr>
<td>RNASAE1</td>
<td>Ribonuclease, RNase A family, 1 (pancreatic)</td>
<td>Up</td>
</tr>
<tr>
<td>DDX4</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 4</td>
<td>Up</td>
</tr>
<tr>
<td>MYB</td>
<td>V-myb myeloblastosis viral oncogene homolog (avian)</td>
<td>Up</td>
</tr>
<tr>
<td>TFCP2L1</td>
<td>Transcription factor CP2-like 1</td>
<td>Down</td>
</tr>
<tr>
<td>AGR2</td>
<td>Anterior gradient homolog 2 (Xenopus laevis)</td>
<td>Up</td>
</tr>
<tr>
<td>C4orf30</td>
<td>Chromosome 4 open reading frame 30</td>
<td>Down</td>
</tr>
<tr>
<td>UQRC2</td>
<td>Ubiquinol-cytochrome c reductase core protein II^c^</td>
<td>Up</td>
</tr>
<tr>
<td>NELL1</td>
<td>NEL-like 1 (chicken)</td>
<td>Up</td>
</tr>
<tr>
<td>IL22RA2</td>
<td>Interleukin 22 receptor, alpha 2</td>
<td>Down</td>
</tr>
</tbody>
</table>

^a^ Gene names are listed in descending order of significance level (pair-wise comparison between control and CEE-alone, fold change > 2, adjusted P values 0.01 – 0.05).

^b^ Commonly up-regulated by BZA treatment compared to control.

^c^ Contrarily down-regulated by BZA+CEE treatment compared to control.

Bold terms represent genes well-known to be up-regulated by estrogen therapy in human breast cancer cells.\(^40-42\) N = 4 for each treatment group.
**SUPPLEMENTAL TABLE 2.**
_Bazedoxifene-agonized Genes in BZA+CEE Co-therapy*

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Fold Change CEE vs. BZA/CEE</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGHA1</td>
<td>Immunoglobulin heavy constant alpha 1</td>
<td>3.28</td>
</tr>
<tr>
<td>CCL5</td>
<td>Chemokine (C-C motif) ligand 5</td>
<td>2.79</td>
</tr>
<tr>
<td>MDM4</td>
<td>Mdm4 p53 binding protein homolog (mouse)</td>
<td>2.67</td>
</tr>
<tr>
<td>GZMK</td>
<td>Granzyme K (granzyme 3, tryptase III)</td>
<td>2.49</td>
</tr>
<tr>
<td>UBD</td>
<td>Ubiquitin D</td>
<td>2.31</td>
</tr>
<tr>
<td>HLA-DPA1</td>
<td>Major histocompatibility complex, class II, DP alpha 1</td>
<td>2.21</td>
</tr>
<tr>
<td>GZMB</td>
<td>Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)</td>
<td>2.10</td>
</tr>
</tbody>
</table>

*a Filtered dataset generated in Genesifter software program using a pair-wise comparison between CEE and BZA+CEE at a fold change (FC) > 2, adjusted $P < 0.05$, Benjamini & Hochberg correction. All genes were not significantly regulated by BZA, CEE, and BZA+CEE compared to control, except for IGHA1 (CEE down-regulated compared to control, FC 3.58). N = 4 for each treatment group.
**SUPPLEMENTAL TABLE 3.** Treatment Effects of Bazedoxifene and CEE on Genes Related to Estrogen Metabolism

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>BZA 20 mg/d</th>
<th>CEE 0.45 mg/d</th>
<th>BZA + CEE 20 mg/d + 0.45 mg/d</th>
<th>P value (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19</td>
<td>1.00</td>
<td>0.78</td>
<td>0.65</td>
<td>0.98</td>
<td>0.105</td>
</tr>
<tr>
<td></td>
<td>(0.71 - 1.33)</td>
<td>(0.59 - 0.99)</td>
<td>(0.45 - 0.87)</td>
<td>(0.73 - 1.27)</td>
<td></td>
</tr>
<tr>
<td>HSD17B1</td>
<td>1.00</td>
<td>1.25</td>
<td>1.05</td>
<td>1.32</td>
<td>0.498</td>
</tr>
<tr>
<td></td>
<td>(0.77 - 1.25)</td>
<td>(0.79 - 1.79)</td>
<td>(0.81 - 1.32)</td>
<td>(0.94 - 1.75)</td>
<td></td>
</tr>
<tr>
<td>HSD17B2</td>
<td>1.00</td>
<td>1.70</td>
<td>1.65</td>
<td>1.88</td>
<td>0.102</td>
</tr>
<tr>
<td></td>
<td>(0.71 - 1.33)</td>
<td>(1.09 - 2.48)</td>
<td>(1.09 - 2.33)</td>
<td>(1.28 - 2.63)</td>
<td></td>
</tr>
<tr>
<td>STS</td>
<td>1.00</td>
<td>1.03</td>
<td>0.92</td>
<td>1.15</td>
<td>0.665</td>
</tr>
<tr>
<td></td>
<td>(0.73 - 1.31)</td>
<td>(0.77 - 1.32)</td>
<td>(0.70 - 1.16)</td>
<td>(0.84 - 1.51)</td>
<td></td>
</tr>
<tr>
<td>SULT1E1</td>
<td>1.00</td>
<td>0.97</td>
<td>0.90</td>
<td>1.21</td>
<td>0.492</td>
</tr>
<tr>
<td></td>
<td>(0.72 - 1.31)</td>
<td>(0.71 - 1.27)</td>
<td>(0.64 - 1.21)</td>
<td>(0.87 - 1.60)</td>
<td></td>
</tr>
</tbody>
</table>

N 22 22 24 25

*Values represented as means (95% CI); expressed relative to control (1.0)*
REFERENCES


CHAPTER 2

THE ENDOMETRIAL PROFILE OF BAZEDOXIFENE ACETATE ALONE AND IN COMBINATION WITH CONJUGATED EQUINE ESTROGENS

Kelly F. Ethun, Charles E. Wood, Thomas C. Register, J. Mark Cline, Susan E. Appt, Thomas B. Clarkson

The following manuscript has been submitted to Clinical Cancer Research. Stylistic variations result from the demands of the journal. Kelly F. Ethun collected or directed the collection of the data, completed the statistical analyses, and prepared the manuscript. Charles E. Wood performed the histopathological review and provided guidance in manuscript preparation. Thomas C. Register assisted with the molecular endpoints. Susan E. Appt and J. Mark Cline acted in an advisory and editorial capacity. Thomas B. Clarkson was the principal investigator of the parent nonhuman primate study for this project and provided significant guidance throughout this project.
Full Title: The endometrial profile of bazedoxifene acetate alone and in combination with conjugated equine estrogens

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Charles E. Wood
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Susan E. Appt
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Running Title: Endometrial Profile of Bazedoxifene and Conjugated Equine Estrogens

Key Words: Estrogens, menopause, hormone therapy, selective estrogen receptor modulator, endometrial cancer.

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ABSTRACT

Purpose: Concerns of breast cancer risk in postmenopausal women taking combined estrogen+progestin therapy have generated interest in the use of selective estrogen receptor modulators (SERMs) as potential progestin alternatives. Endometrial proliferation and cancer risk are major concerns, however, for estrogens and certain types of SERMs when given alone. The primary aim of this study was to evaluate the endometrial profile of bazedoxifene acetate (BZA), a third-generation SERM, alone and in combination with conjugated equine estrogens (CEE) in a postmenopausal primate model.

Experimental Design: Ninety-eight adult female cynomolgus monkeys (Macaca fascicularis) were ovariectomized and randomized by social groups to receive no hormone treatment (control), BZA 20 mg, CEE 0.45 mg, or the combination of BZA 20 mg + CEE 0.45 mg once daily for 20 months in a parallel-arm study design.

Results: BZA+CEE and BZA treatment resulted in significantly less endometrial epithelial area and proliferation compared to CEE (P < 0.0001 for all). The prevalence of endometrial hyperplasia and other estrogen-induced morphologic changes in the BZA+CEE and BZA groups were not significantly different from control. The addition of BZA to CEE completely inhibited the expression of ERα-regulated genes (TFF1 and PGR), while BZA alone had no effect. BZA+CEE and BZA treatment also resulted in lower ERα protein expression in the endometrium compared to control and CEE (P < 0.05 for all).

Conclusion: BZA given at a clinically relevant dose inhibits estrogen effects on the endometrium and lacks uterotrophic effects when given alone.
STATEMENT OF TRANSLATIONAL RELEVANCE

Exposure to estrogens is a key risk factor for endometrial cancer. The addition of the selective estrogen receptor modulator bazedoxifene acetate (BZA) to conjugated equine estrogens (CEE) therapy is currently being evaluated as a treatment for menopausal symptoms and osteoporosis. However, limited data are available regarding the endometrial safety of BZA alone and in combination with CEE. The purpose of this study was to investigate the endometrial profile of BZA alone and the combination of BZA+CEE in a randomized preclinical trial using cynomolgus monkeys. Our results show that BZA at the target human equivalent dose fully antagonized the proliferative and transcriptional effects of CEE on the endometrium. This information should be useful in the planning of future SERM+estrogen clinical trials and to symptomatic postmenopausal women seeking alternatives to traditional estrogen + progestin therapies.
INTRODUCTION

Estrogen-alone therapy (ET) alleviates menopausal symptoms and reduces osteoporosis and fracture risk in aging women (1-3). However, long-term use of ET has been associated with an increased risk of endometrial hyperplasia and endometrial cancer, even when given at low doses (4-7). To date, progestin co-therapy has been the only clinical strategy to effectively prevent estrogen-induced endometrial proliferation and lower cancer risk (6, 8). Historically, the most commonly prescribed estrogen + progestin co-therapy (EPT) in the United States has been conjugated equine estrogens (CEE) given with medroxyprogesterone acetate (MPA) (9). However, primary results from the Women’s Health Initiative (WHI) randomized clinical trial (10) and prior observational studies (11) have associated long-term CEE+MPA co-therapy with an increased risk of breast cancer as well as a higher incidence of gynecological surgeries (e.g. hysterectomies) due to increased uterine bleeding (12). Consequently, there has been increased interest in new menopausal therapies that provide comparable efficacy to ET without adverse proliferative effects on the breast and endometrium.

Recently, selective estrogen receptor modulators (SERMs) have been proposed as an alternative to the progestin component in EPT (13). As a class of non-steroidal compounds, SERMs bind to estrogen receptors (ERs) alpha and beta and induce a mixed pattern of ER agonist and antagonist responses depending on the particular SERM and the target tissue (14). Current SERMs include tamoxifen and raloxifene, which are widely used in the prevention of breast cancer (15). Similar to ET, most SERMs increase bone mineral density and improve lipid profiles, but in contrast to ET, current SERMs given alone do not treat menopausal symptoms and in some cases worsen them (16, 17, 18). The ideal clinical goal of combining a SERM with ET would be to selectively retain the benefits of both agents while reducing the adverse effects of either agent alone. The estrogen component of such a combination would relieve hot flushes, improve urogenital atrophy, and prevent bone loss, while the SERM would help maintain bone
mass and provide anti-estrogenic effects in the breast and endometrium. Information regarding the uterotropin effects of the SERM component is critical considering that SERMs such as tamoxifen have been associated with an increased incidence of endometrial cancer and other adverse morphologic changes such as stromal fibrosis, cystic change, and polyp formation (16, 19-22).

Bazedoxifene acetate (BZA; 20 mg/day) is an indole-based SERM currently under evaluation for the treatment of osteoporosis and, in combination with CEE (0.45 and 0.625 mg/day), for the reduction of menopausal symptoms and the prevention of osteoporosis (23-26). The purpose of this study was to investigate the endometrial safety profile of BZA alone and in combination with CEE in a randomized multisystem nonhuman primate translational trial. We hypothesized that BZA would antagonize the proliferative and transcriptional effects of CEE on the endometrium, while BZA would have minimal estrogen agonist effects when administered alone. Effects on other systems including breast, bone, and the cardiovascular system will be reported elsewhere.

MATERIALS AND METHODS

Study Design and Treatments

This randomized preclinical trial followed a parallel-arm study design in which one-hundred ovariectomized adult cynomolgus macaques (Macaca fascicularis) were randomized by body weight to receive one of the following four treatments for 20 months: (i) no treatment (control (CTL), n=23), (ii) BZA 20 mg (n=24), (iii) CEE 0.45 mg (n=24), or (iv) the combination of BZA 20 mg + CEE 0.45 mg (n=27). An important feature of this design is the inclusion of a CEE-alone group as a positive control, which was not feasible in previous clinical trials since all participating women did not have a prior hysterectomy (26). Each group originally consisted of 25 animals, but 2 animals were added to the BZA and BZA+CEE groups to account for any
exclusions or deaths during the study. Subsequently, 2 animals from the control and 3 animals from the BZA groups were excluded due to elevated serum ovarian hormone levels indicating the presence of ectopic ovarian tissue (27). Another monkey from the CEE group was euthanized due to an intussusception.

An important rationale for the use of the macaque model in this trial is the high degree of similarity in pathophysiology (28) and responses to hormonal agents (29) between the human and macaque endometrium. All monkeys were imported from the Indonesian Primate Center (Pusat Studi Satwa Primata) at the Institut Pertanian Bogor in Bogor, Indonesia, and housed in stable social groups consisting of two to five animals. All monkeys were considered monoparous or multiparous based on clinical records from the original breeding colony. Procedures involving these animals were approved by the Institutional Animal Care and Use Committee of Wake Forest University and conducted in accordance with federal, state, and institutional guidelines. The facilities and animal resources program of Wake Forest University are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Hormone treatments were administered in a standard isoflavone-free casein + lactalbumin control diet and fed once daily for 20 months. The standard control diet was formulated to model the high cholesterol (0.29 mg/Cal) and high fat (35% of calories from fat) diets typically consumed by postmenopausal women in the United States. Studies conducted to determine CEE and BZA doses for female macaques that were equivalent to a woman’s daily dose of CEE 0.45 mg and BZA 20 mg are published elsewhere (30). Briefly, drug doses were determined based on caloric intake to account for differences in metabolic rates between female monkeys and women as well as plasma concentrations measured in postmenopausal women receiving BZA 20 mg/day (31). Animals in the CEE-treated groups were given 0.03 mg/kg body weight/day of CEE while those animals in the BZA-treated groups received 2.5 mg/kg body weight/day of BZA.
Circulating Estrogen and BZA Concentrations

As part of the main study, plasma estrone (E1), 17β-estradiol (E2), and BZA concentrations were obtained at 4 hours post-prandial and following an overnight fast to confirm adequate dosing and dietary intake. These results are reported elsewhere (30). Repeat measurements of E1 and E2 were also done in a subset of animals (n=47) following an 18 hour fast on the day of necropsy, and these values are reported here. E1 and E2 concentrations were measured at the Wake Forest University Primate (WFUPC) Center Clinical Laboratory using commercially available radioimmunoassay kits (Siemens/DPC), while plasma BZA concentrations were measured at Pfizer using high-performance liquid chromatography (HPLC) with fluorescence detection. Serum used to measure E2 concentrations were first extracted with ethyl ether, and extracts were then dried and reconstituted with zero-standard serum.

Uterine Area via Uterine Ultrasounds

Trans-abdominal ultrasounds were conducted using a Sonosite MicroMaxx portable ultrasound machine with a 6.0-MHz linear transducer (Sonosite, Bothell, WA) at baseline (4 weeks after ovariectomy) and following 6, 12, and 20 months of treatment. Static digital images were captured at the maximal transverse cross-sectional area of the uterus. Measurements of uterine area (cm²) were made using public domain software (National Institutes of Health ImageJ 1.34, available at http://rsb.info.nih.gov/ij/upgrade/).

Histomorphometry and Histopathology

At necropsy, uteri were collected, weighed, and prepared for histology (32). Percent epithelial area and mean endometrial thickness were quantified using hematoxylin and eosin (H&E) stained slides and morphometry techniques similar to those described previously (22, 33). Briefly, epithelial area was determined by manual tracing of glandular units (minus the luminal areas) within the superficial and basal endometrium and expressed as a percentage of the total
area examined (Image-Pro Plus software, Media Cybernetics, Silver Spring, MD). Mean endometrial thickness (superficial + basal) was measured in a similar manner. H&E-stained slides were also evaluated for evidence of glandular hyperplasia (simple and complex), stromal hyperplasia, stromal edema, cystic dilation, and other histological lesions by a board-certified veterinary pathologist (CEW). Stromal edema and cystic dilation are considered low-risk morphologic changes associated with exogenous estrogen exposure (29). Endometrial stromal fibrosis (collagen content) was quantified from slides stained with Masson's trichrome (containing Weigert's iron hematoxylin, Crocein Scarlet MOO, 5% aqueous phosphomolybdic acid, and aniline blue; Fisher Scientific and Sigma) using a selective color-based analysis in the Image Pro-Plus Software (22). Blue-stained areas in the superficial endometrial stroma represented collagen, while red area represented stromal cell nuclei and cytoplasm. Area of stromal edema and undefined (pale blue) ground substance was estimated by subtracting total area examined by the blue and red-stained areas.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on fixed endometrial sections using commercially-available primary monoclonal antibodies for the proliferation marker Ki67 (Ki67SP6; Thermo Scientific, Fremont, CA) and the sex steroid receptors ERα (NCL-ER-6F11, Novocastra Reagents, Leica Microsystems Inc., Buffalo, NY) and progesterone receptor (PGR) (NCL-PGR-312, Novocastra Reagents, Leica Microsystems Inc., Buffalo, NY) as described previously (30). Nuclear immunolabeling within the superficial and basal endometrium was quantified using a computer-assisted manual counting technique with a grid filter to select cells for counting (Image-Pro Plus software, Media Cybernetics, Silver Spring, MD). An H score was then calculated using the equation (3 x % of strongly stained nuclei) + (2 x % of moderately stained nuclei) + (% of weakly stained nuclei) (34).
Quantitative Real-time PCR

Expression of genes related to cell proliferation (MKI67, Ki67 antigen) and estrogen receptor activity (ESR1, ERα; ESR2, ERβ; PGR, progesterone receptor; TFF1, trefoil factor 1 [pS2]) was determined by quantitative real-time reverse transcription-PCR (qRT-PCR) using techniques described elsewhere (30). Briefly, all qRT-PCR reactions were run with Applied Biosystems (ABI) Taqman primer-probe sets and performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). β-actin was used as the endogenous control while reference endometrial tissue cDNA was run in parallel for plate-to-plate calibration. Relative expression of each target gene was calculated using ABI Relative Quantification 7500 Software v2.0.1.

Statistical Analyses

All variables were evaluated for their distribution and equality of variance. Data not normally distributed were transformed (log10 or square root) to improve normality for analysis and then reverse transformed to the original scale for display in the results. The following data violated the Levene’s test for equality of variance and were analyzed using the nonparametric Kruskal-Wallis and post hoc Wilcoxon (rank sums) tests: uterine weights, E2 concentrations, (superficial) epithelial area, (superficial) luminal area, all qRT-PCR assays except for ERα and ERβ, and IHC for Ki67 (superficial glands and stroma) and ERα (superficial glands). Significance levels were then adjusted for multiple pair-wise comparisons using a Bonferroni correction. Differences in uterine area and body weight among the treatment groups were determined using a mixed model approach with baseline values as a covariate. For each variable, this model allowed for a within-group comparison at each post-treatment time period and a between time point comparison within each group (e.g. 20 months post-treatment values compared to baseline). A two-tailed Fisher’s exact test was used to evaluate treatment group
differences in the prevalence of histopathological findings. The remaining data were assessed using analysis of variance (ANOVA) and the Tukey HSD *post hoc* test for multiple pair-wise comparisons. A two-tailed significance level of 0.05 was selected for all comparisons and all analyses were done using JMP statistical software (version 9.0.2; SAS Institute, Inc, Cary, NC).

RESULTS

*Treatment Group Characteristics*

Treatment group characteristics including age, body weights, and hormone concentrations are summarized in Supplemental Table 1. At baseline, the mean estimated age of all animals was 12.7 (range 9 - 18) years with no between-group differences (*P* > 0.1). All groups showed a small increase in body weight from baseline to 20 months post-treatment, but this gain in body weight reached significance only in the control group (*P* < 0.001). After 20 months of treatment, the BZA+CEE group weighed significantly less than the control group (*P* < 0.01); however, no significant differences in body weight and plasma estrogen (E1 and E2) concentrations were noted between the BZA+CEE and CEE groups (*P* > 0.1 for all).

*Uterine Area via Trans-abdominal Ultrasound*

Uterine area among CEE-treated animals increased progressively from baseline to 20 months post-treatment (*P* < 0.0001) and was significantly greater than the control group at 6, 12, and 20 months of treatment (*P* < 0.0001 for all, Fig. 1). The BZA+CEE and BZA groups had significantly smaller uterine areas relative to CEE (*P* < 0.0001 for all) and similar uterine areas compared to control at each post-treatment time point (Fig. 1).

*Endometrial Thickness, Epithelial Area, and Proliferation*

Following 20 months of treatment, uterine weight and endometrial thickness among the control, BZA, and BZA+CEE groups were comparable and 2.5 to 3.5-fold lower than the CEE
group ($P < 0.0001$ for all compared to CEE, Fig. 2A). Similarly, epithelial area within the superficial endometrium was not significantly different among the control, BZA, and BZA+CEE groups and measured 1.5 to 2.0-fold less than the CEE group ($P < 0.0001$ for all compared to CEE, Fig. 2B). Epithelial area specifically within the basal endometrium was also significantly less in the BZA+CEE and BZA groups compared to the CEE group ($P < 0.0001$ for both) but 44% higher than control ($P < 0.01$ for both).

The addition of BZA to CEE significantly inhibited CEE-induced endometrial proliferation, indicated by lower $MKI67$ gene expression and Ki67 immunolabeling in the superficial glands and stroma ($P < 0.001$ BZA+CEE compared to CEE for all) (Fig. 2C and 2D). Proliferation in the superficial glands was also lower for BZA+CEE compared to control ($P < 0.01$) (Fig. 2C). Treatment with BZA alone did not induce $MKI67$ expression (Fig. 2D) or Ki67 immunolabeling in the superficial or basal endometrial glands (Fig. 2C and 2E) but did result in 3-fold higher Ki67 immunoreactivity in the basal stroma compared to control ($P = 0.04$) (Fig. 2E).

**Endometrial Morphology**

The addition of BZA to CEE also inhibited estrogen-induced changes in endometrial morphology. The prevalence of simple glandular and stromal hyperplasia was high in the CEE group ($P < 0.0001$ compared to control and BZA+CEE) but not in the BZA and BZA+CEE groups (Table 1). Similarly, the prevalence of stromal edema and cystic dilation in the endometrial glands was most evident in the CEE group. These findings were confirmed using quantitative morphometric measurements, shown in Figs. 3A and 3B. Treatment with BZA+CEE and BZA had no significant effect on endometrial collagen content compared to control, but treatment with CEE resulted in significantly more collagen content compared to the other groups ($P < 0.01$ for all) (Fig. 3C). Incidental histological findings included vascular remodeling
(adventitial expansion) associated with prior pregnancy. No evidence of endometrial polyps, complex or atypical hyperplasia, or neoplasia was observed.

**ERα Expression and Transcriptional Activation**

Treatment with BZA, CEE, and BZA+CEE altered endometrial ERα immunolabeling (Fig. 4A) but not gene expression (Supplemental Fig. 1A). Groups treated with BZA+CEE and BZA had significantly less glandular and stromal ERα immunolabeling in the superficial and basal endometrium compared to the control and CEE groups ($P < 0.0001$ to $P < 0.05$ for all compared to control and CEE). Treatment with CEE resulted in significantly lower ERα immunolabeling in the basal stroma compared to control ($P < 0.05$) but greater ERα immunoreactivity relative to BZA+CEE ($P < 0.05$). Unlike ERα gene expression, ERβ mRNA expression was significantly decreased by CEE treatment ($P < 0.0001$ compared to control, Supplemental Fig. 1B).

As expected, CEE treatment significantly induced the expression of the estrogen response genes *TFF1* and *PGR* compared to control ($P < 0.001$ for both, Fig. 4B). The addition of BZA to CEE significantly attenuated these effects ($P < 0.001$ for both), while BZA alone had no effect. Glandular and stromal PGR immunolabeling within the endometrium showed a similar pattern (Fig. 4C).

**DISCUSSION**

Traditional hormone therapies are associated with increased cancer risk in the endometrium (ET) and breast (EPT) in postmenopausal women. Estrogen + SERM co-therapies are emerging as potential alternatives to these traditional therapies. Endometrial stimulation and cancer risk are major concerns, however, for both estrogens and certain types of SERMs when given alone and limited data exist regarding the endometrial safety of estrogen + SERM co-therapies. In this preclinical trial, we investigated the endometrial risk profile of a new SERM,
BZA, given alone and with CEE, the most widely prescribed ET in the United States (9). Treatment with CEE increased uterine size, endometrial thickness, epithelial area, proliferation, and gene markers of ERα activity, while the addition of BZA to CEE significantly antagonized these effects. Treatment effects of BZA alone were comparable to control. These findings show for the first time that BZA is a clear estrogen antagonist in the endometrium at a clinically relevant dose.

The full inhibition of CEE effects by BZA shown here supports data from phase III clinical trials suggesting that BZA+CEE co-therapy does not have tamoxifen-like uterotrophic effects in postmenopausal women (35, 36). In a 2-year study of osteoporotic postmenopausal women (SMART-1: Selective estrogens, Menopause, And Response to Therapy), endometrial hyperplasia (evaluated via biopsy) and uterine bleeding incidences with BZA 20 mg combined with either CEE 0.45 mg or 0.625 mg were not significantly different from placebo (35, 36). Similarly, preliminary data from another smaller 1-year trial (SMART-5) reported comparable incidences of endometrial hyperplasia among those women receiving BZA 20 mg + CEE 0.45 mg (0.3%) and BZA 20 mg + CEE 0.625 mg (0.27%) relative to MPA 1.5 mg + CEE 0.45 mg (0%), BZA 20 mg monotherapy (0%), and placebo (0%) (37).

Evidence from prior reports, however, suggests that the inhibitory effects of BZA on ER-activity may differ according to the dose and/or type of estrogen used in BZA+estrogen therapies. For example, cell culture studies showed that 10 nM of BZA completely inhibits the growth of E2-stimulated MCF-7 breast cancer cells, while 1.0 pM of BZA results in negligible inhibition (38). Similarly, in the 2-year SMART-1 trial, the uterotrophic effects of CEE (0.45 mg/d or 0.625 mg/d) were effectively antagonized with 20 mg/d but not 10 mg/d of BZA (35). To date, the interactive effects of estrogens and BZA on the breast and endometrium have only been evaluated with oral CEE as the primary ET (30, 35-37, 39); therefore, it is not known whether the target
dose of BZA (20 mg/d) would prevent endometrial and breast proliferation if co-administered with standard doses of other ETs such as oral and transdermal E2.

Few prior studies have evaluated the endometrial effects of estrogen + SERM therapies. In one small study evaluating the combination of raloxifene (60 mg/d) and oral E2 (1 mg/d) in postmenopausal women transitioning from EPT, the frequency of hot flushes and night sweats were significantly reduced compared to baseline and raloxifene monotherapy (40). However, this combination was associated with increased endometrial thickness and two cases of atypical endometrial hyperplasia. Similarly, another pilot study found significantly less vasomotor events but increased endometrial thickness with the concomitant use of oral raloxifene and a transdermal E2 patch (41). Results from a rodent study have suggested that BZA has greater ER-antagonist activity than raloxifene in the endometrium and may inhibit proliferation if co-administered with E2 (42), but this observation has not been tested in a randomized clinical trial or at clinically relevant doses of BZA and E2. It is also worth noting that some estrogen + SERM combinations have shown mixed agonist and antagonist effects in the endometrium. In a prior macaque study, the addition of tamoxifen to low-dose E2 therapy inhibited E2-induced proliferation and expression of genes related to cell cycle progression, but still induced stromal fibrosis, cystic change, and increases in endometrial thickness similar to tamoxifen-alone therapy (22).

We have previously reported that BZA with and without CEE reduces ERα immunolabeling in the breast (30). As reported here, a similar observation was noted in the endometrium in which both glandular and stromal ERα immunolabeling were significantly less with BZA and BZA+CEE treatments compared to control and CEE, while ERα gene expression was not affected. These unanticipated results suggest that BZA may increase ERα turnover in addition to blocking estrogen binding. This hypothesis is supported by a recent breast cancer cell study that showed proteasome-mediated degradation of the ERα by BZA (43). However, further studies should be conducted to determine if BZA increases ERα ubiquititation and degradation in normal endometrial cells.
Many studies have demonstrated that estrogen-induced uterine proliferation is mediated primarily by ligand-dependent ERα activity (44-48). For example, mutant mice deficient of ERα within all uterine compartments (luminal and glandular epithelium, stroma, and myometrium) lack uterine stimulation and mitotic growth responses to exogenous E2, while ERβ knockout mice have wild-type-like uterotrophic responses (44). Another recent study using an epithelial-specific ERα knockout mouse strain demonstrated that stromal ERα is a key mediator of estrogen-induced epithelial proliferation in the murine uterus, while epithelial ERα is important in preventing epithelial apoptosis (45). Results from the current study provide additional information on the role of ERα in uterine proliferation, showing that ERα blockade by a SERM clearly negates estrogen-induced proliferation but that responses may differ between epithelial and stromal compartments.

Estrogen exposure is a central determinant of endometrial cancer risk. In postmenopausal women, exogenous ET leads to increased endometrial proliferation, hyperplasia, and up to a 5-fold higher incidence of cancer (1, 49, 50). Findings from the current study show that the SERM, BZA, given at a clinically relevant dose effectively inhibits estrogenic responses in the postmenopausal endometrium while having no estrogen agonist activity when given alone. These results add to the available information on the safety of third generation SERMs and estrogen+SERM co-therapies. This information should be useful in the design of future clinical trials and for clinicians and postmenopausal women considering available hormone therapy options.
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Figure 1. Ultrasonographic measurements of uterine area in postmenopausal macaques. Uterine area values among the control (CTL), BZA, and BZA+CEE groups were comparable and significantly smaller than the CEE group at 6, 12, and 20 months of treatment. *P < 0.0001 compared to respective control and BZA+CEE groups. **P < 0.0001 compared to baseline values. Values represent means±95% confidence interval (CI).
Figure 2. Effects of CEE, BZA, and BZA+CEE on uterine weight, endometrial thickness, epithelial area, and endometrial proliferation in macaques (A-E). The addition of BZA to CEE significantly inhibited the agonistic effects of CEE on uterine weight (A upper panel), endometrial thickness (A lower panel), epithelial area (B), and protein expression of the proliferation marker Ki67 in the superficial endometrium to the level of control or beyond (C) ($P < 0.0001$ to $P < 0.05$ for all). Gene expression of $MKI67$ within the endometrium showed a similar pattern (D). Treatment groups not connected by the same letter are significantly different. Values represent means ± 95% CI. CTL = control (no hormone treatment).
Figure 3. Effects of CEE, BZA, and BZA+CEE on estrogen-induced morphologic changes in the macaque endometrium (A-C). Within the superficial endometrium, only treatment with CEE induced change in luminal space (cystic dilation) (A), stromal edema (B), and collagen content (C) compared to control ($P < 0.01$ for all). In the basal endometrium, treatment with BZA+CEE induced a modest but significant increase in luminal space of the endometrial glands ($P < 0.05$). Treatment groups not connected by the same letter are significantly different. Values represent means ± 95% CI.
Figure 4. Effects of CEE, BZA, and BZA+CEE on ERα expression and markers of ERα activity in the macaque endometrium (A-C). BZA treatment with and without CEE decreased ERα immunolabeling in the superficial and basal endometrium (A) compared to control and CEE alone ($P < 0.0001$ to $P < 0.05$ for all). The concomitant use of BZA and CEE significantly attenuated ERα-mediated expression of TFF1 and PGR (B and C) compared to CEE alone ($P < 0.0001$ to $P < 0.05$ for all). Treatment groups not connected by the same letter are significantly different. Values represent means ± 95% CI.
Supplemental Figure 1. ERα and ERβ gene expression in the macaque endometrium. Treatment with BZA, CEE, and BZA+CEE did not affect ERα gene expression (A), while treatment with CEE decreased ERβ gene expression (B) relative to other treatment groups ($P < 0.05$). Values represent means ± 95% confidence interval (CI).
TABLE 1. Histopathological Findings

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Control</th>
<th>BZA 20 mg/d</th>
<th>CEE 0.45 mg/d</th>
<th>BZA+CEE 20 mg/d + 0.45 mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple Glandular Hyperplasia</td>
<td>1 (4%)</td>
<td>4 (17%)</td>
<td>23 (96%)</td>
<td>2 (7%)</td>
</tr>
<tr>
<td>Mild</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Marked</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>P value vs. Control</td>
<td>NA</td>
<td>0.35</td>
<td>&lt;0.0001</td>
<td>1.0</td>
</tr>
<tr>
<td>Stromal Hyperplasia</td>
<td>1 (4%)</td>
<td>4 (17%)</td>
<td>22 (92%)</td>
<td>3 (11%)</td>
</tr>
<tr>
<td>Mild</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Marked</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>P value vs. Control</td>
<td>NA</td>
<td>0.35</td>
<td>&lt;0.0001</td>
<td>0.61</td>
</tr>
<tr>
<td>Stromal Edema</td>
<td>3 (13%)</td>
<td>1 (4%)</td>
<td>23 (96%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mild</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Marked</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>P value vs. Control</td>
<td>NA</td>
<td>0.35</td>
<td>&lt;0.0001</td>
<td>0.09</td>
</tr>
<tr>
<td>Cystic Dilation</td>
<td>0 (0%)</td>
<td>1 (4%)</td>
<td>9 (38%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Marked</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>P value vs. Control</td>
<td>NA</td>
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<tr>
<td>Number Examined</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>27</td>
</tr>
</tbody>
</table>

NOTE: All lesions demonstrate evidence of an estrogenic effect but are not considered high-risk. Neither complex nor atypical endometrial hyperplasia was observed. All cases had evidence of vascular remodeling (adventitial expansion) associated with past pregnancy. P values were determined using a two-tailed Fisher’s Exact Test.
### SUPPLEMENTAL TABLE 1. Treatment Group Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BZA 20 mg/d</th>
<th>CEE 0.45 mg/d</th>
<th>BZA+CEE 20 mg/d + 0.45 mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years(^a)</td>
<td>12.6</td>
<td>12.8</td>
<td>12.6</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>(11.7 - 13.6)</td>
<td>(11.8 - 13.8)</td>
<td>(11.9 - 13.5)</td>
<td>(12.0 - 13.9)</td>
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<tr>
<td>N</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td>Body Weight, kg(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.90</td>
<td>2.90</td>
<td>2.90</td>
<td>2.90</td>
</tr>
<tr>
<td></td>
<td>(2.79 - 3.00)</td>
<td>(2.79 - 3.00)</td>
<td>(2.79 - 3.01)</td>
<td>(2.90 - 3.00)</td>
</tr>
<tr>
<td>20 months</td>
<td>3.31</td>
<td>3.14</td>
<td>3.12</td>
<td>3.00(^c)</td>
</tr>
<tr>
<td></td>
<td>(3.19 - 3.44)</td>
<td>(3.02 - 3.26)</td>
<td>(3.00 - 3.23)</td>
<td>(2.90 - 3.11)</td>
</tr>
<tr>
<td>P value compared to baseline values</td>
<td>&lt; 0.001</td>
<td>0.06</td>
<td>0.12</td>
<td>0.87</td>
</tr>
<tr>
<td>N</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td>Estrone, pg/ml(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 hr PP</td>
<td>45.5</td>
<td>50.4</td>
<td>142.2(^c)</td>
<td>128.2(^c)</td>
</tr>
<tr>
<td></td>
<td>(36.6 - 56.6)</td>
<td>(39.7 - 64.0)</td>
<td>(118.9 - 170.1)</td>
<td>(101.5 - 162.0)</td>
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<td>N</td>
<td>10</td>
<td>12</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>17β-estradiol, pg/ml(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 hr PP</td>
<td>&lt; 2.5</td>
<td>&lt; 2.5</td>
<td>6.79(^c)</td>
<td>4.96(^c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(4.81 - 9.59)</td>
<td>(3.34 - 7.38)</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>12</td>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

\(^a\) Analysis of variance (ANOVA).
\(^b\) Mixed-effect model with baseline values as a covariate.
\(^c\) P < 0.01 compared to the control group at 20 months post-treatment.

NOTE: Values represent means with 95% CI. Serum samples used to measure 17β-estradiol concentrations were first extracted with ethyl ether. PP = post-prandial.
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30. Ethun KF, Wood CE, Register TC, Cline JM, Appt SE, Clarkson TB. Effects of bazedoxifene acetate with and without conjugated equine estrogens on the breast of postmenopausal monkeys. Accepted for publication in *Menopause* (DOI: 10.1097/GME.0b013e318252e46d).


CHAPTER 3

CERVICOVAGINAL EFFECTS OF BAZEDOXIFENE ACETATE, CONJUGATED EQUINE
ESTROGENS, AND THE COMBINATION IN POSTMENOPAUSAL MACAQUES

Kelly F. Ethun, Charles E. Wood, Thomas C. Register, J. Mark Cline,
Susan E. Appt, Thomas B. Clarkson

The following manuscript has been submitted to American Journal of Obstetrics and Gynecology.
Stylistic variations result from the demands of the journal. Kelly F. Ethun collected or directed
the collection of the data, completed the statistical analyses, and prepared the manuscript.
Charles E. Wood performed the histopathological review and provided guidance in manuscript
preparation. Thomas C. Register assisted with the molecular endpoints. Susan E. Appt and J.
Mark Cline acted in an advisory and editorial capacity. Thomas B. Clarkson was the principal
investigator of the parent nonhuman primate study for this project and provided significant
guidance throughout this project.
Title: Cervicovaginal effects of bazedoxifene acetate, conjugated equine estrogens, and the combination in postmenopausal macaques.

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**Condensation:** Bazedoxifene acetate (BZA), a third-generation selective estrogen receptor modulator, inhibited effects of oral conjugated equine estrogens on vaginal maturation, while BZA-alone had no estrogenic effects.

**Short Version of the Title:** Cervicovaginal Effects of Bazedoxifene with CEE
ABSTRACT

Objective: The purpose of this study was to evaluate the effects of bazedoxifene acetate (BZA), oral conjugated equine estrogens (CEE), and the combination of BZA and CEE on vaginal maturation and other estrogen-related measures in ovariectomized macaques.

Study Design: As part of a 20-month multisystem preclinical trial, ninety-eight cynomolgus monkeys were given no treatment, BZA 20 mg, CEE 0.45 mg, or BZA and CEE in combination (women’s daily equivalent doses).

Results: BZA completely inhibited CEE effects on vaginal epithelial maturation, proliferation, and keratinization, while BZA alone had no estrogenic effects. ERα immunolabeling within the vaginal mucosa and endocervical glands were significantly lower with BZA and BZA+CEE treatment compared to CEE and control.

Conclusion: BZA given at a target clinical dose antagonizes estrogen effects in the lower reproductive tract of the macaque and, when given alone, does not provide comparable effects to CEE monotherapy for the treatment of vaginal atrophy.

Key Words: menopause, hormone therapy, selective estrogen receptor modulator, vagina, cervix
INTRODUCTION

Loss of ovarian estrogen production after menopause is associated with changes in the lower reproductive tract that negatively impact the quality of life of aging women. In a hypoestrogenic state, the vaginal epithelium becomes thin, dry, and less elastic, which can cause vaginal discomfort, pruritus, dyspareunia, and urinary incontinence. Diminished circulating estrogens can also lead to changes in vaginal acidity and normal flora that can predispose postmenopausal women to urogenital tract infections. Unlike vasomotor symptoms, which often resolve within 8 years of menopause, vaginal atrophy worsens over time and remains a bothersome issue for many women for several decades.

Estrogen-based hormone therapies (HTs) are the most effective treatment for vaginal atrophy related to estrogen-deficiency. However, long-term safety of traditional HTs remains a major concern for many postmenopausal women. Estrogen-alone therapy (ET) has been associated with an increased risk of endometrial cancer and stroke, while combined estrogen+progestin therapy (EPT) has been associated with increased risk of breast cancer, coronary heart disease, stroke, and venous thromboembolism. For these reasons, there is continued interest in alternative therapies for postmenopausal women suffering from vaginal atrophy.

Selective estrogen receptor modulators (SERMs) are a class of non-steroidal compounds that bind to estrogen receptors (ERs) and act as either ER-agonists or ER-antagonists depending on the tissue and particular SERM. Current SERMs include tamoxifen and raloxifene, which are estrogen agonists in bone and estrogen antagonists in the breast. Notably, these SERMs lack estrogenic effects in the lower reproductive tract and thus are not suitable alternatives to standard HTs for treatment of vaginal atrophy. In addition, these SERMs may worsen other menopausal conditions such as vasomotor symptoms. The lack of an ideal tissue-specific
profile in current HTs or SERMs has led a new approach to menopausal hormone therapy in which a SERM is given concurrently with ET.\textsuperscript{18} It has been proposed that the estrogens in this combination would mitigate hot flushes, vaginal atrophy, and bone loss, while the SERM would provide anti-proliferative effects on the endometrium and breast.\textsuperscript{19} In order to adequately relieve symptoms related to vaginal atrophy, the SERM component of an ideal co-therapy must also lack ER antagonist effects on vaginal maturation when given with one or more estrogens.

Bazedoxifene acetate (BZA) is a third-generation SERM currently under evaluation for the treatment of osteoporosis when given alone and for the reduction of menopausal symptoms and prevention of osteoporosis when given in combination with oral CEE (0.45 and 0.625 mg/day).\textsuperscript{20-25} Preclinical and clinical studies have shown that BZA has estrogen-antagonist effects on the breast and endometrium;\textsuperscript{26-29} however, little information exists regarding the effects of BZA+CEE co-therapy on the lower reproductive tract compared to CEE alone. The primary objective of this study was to investigate the comparative effects of BZA, CEE, and BZA+CEE on vaginal epithelial maturation and related estrogen-responsive measures in a randomized, multisystem, nonhuman primate preclinical trial.

**MATERIALS AND METHODS**

**Study Design**

One-hundred adult female cynomolgus macaques (Macaca fascicularis) were imported from Indonesia. Based on dentition, the mean estimated age for the cohort was 12.7 years. Following quarantine, all animals were placed in social groups, ovariectomized, and randomized by body weight to receive one of four treatments for 20 months: (i) no treatment (control, n=23), (ii) BZA 20 mg (n=24), (iii) CEE 0.45 mg (n=24), or (iv) BZA 20 mg + CEE 0.45 mg (n=27). All treatments were administered in an isoflavone-free diet formulated to model diets typically consumed by postmenopausal women in the United States. As previously described, studies were
conducted to determine CEE and BZA doses for macaques that were equivalent to women’s daily doses of CEE 0.45 mg and BZA 20 mg. Based on caloric intake and plasma concentrations in postmenopausal women receiving BZA 20 mg/day, animals in the CEE-treated groups were given 0.03 mg/kg body weight/day of CEE while those animals in the BZA-treated groups received 2.5 mg/kg body weight/day of BZA. Body weights and plasma hormone/drug concentrations were measured to confirm adequate dosing and dietary intake among the treatment groups. Blood samples were collected at 4 hours post-prandial and following an overnight fast (18-24 hours) at multiple time points. Plasma concentrations of BZA and estrone (E1), the primary estrogen component of CEE, were then measured using techniques described elsewhere. All procedures were approved by the Institutional Animal Care and Use Committee of Wake Forest University and conducted in accordance with Federal and State laws and standards of the U.S. Department of Health and Human Services. The facilities and animal resources program of Wake Forest University are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

**Vaginal cytology**

Following 6, 12, and 20 months of treatment, vaginal epithelial cells were collected from the anterior portion of the vaginal mucosa with a cotton swab, rolled onto a glass slide, fixed immediately, and stained using a modified Papanicolaou method. Cells were scored using established criteria. Maturation value was calculated as follows: (0.2 x % parabasal cells) + (0.6 x % intermediate cells) + (% superficial cells).

**Pathologic evaluations**

Following 20 months of treatment, the reproductive tract was collected and prepared for histology and gene expression studies using methods similar to those described previously. Epithelial tissue from the anterior vagina was trimmed and frozen in liquid nitrogen for gene
expression studies. Vaginal epithelial thickness, vaginal keratin layer thickness, and endocervical gland cell height were measured by histomorphometry on hematoxylin and eosin (H&E) stained slides using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). For each parameter, 3 to 4 measurements were taken then a mean was calculated. Cervicovaginal sections were also qualitatively assessed for any histological abnormalities by a board certified veterinary pathologist (CEW).

Immunohistochemistry

A subset of fixed cervicovaginal sections (n = 8/group) were immunostained for ERα using methods described previously. ERα nuclear staining within the vaginal lamina propria, epithelial basal cells, and endocervical glands was quantified using a computer-assisted manual counting technique with a grid filter to select cells for counting (Image-Pro Plus software). An H score was then calculated using the equation: (3 x % of strongly stained nuclei) + (2 x % of moderately stained nuclei) + (% of weakly stained nuclei).

Real-time PCR

Transcript levels of genes related to vaginal epithelial proliferation (MKI67, Ki67), vaginal keratinization (KRT1, Keratin 1; KRT13, Keratin 13), and ERα expression (ESR1, ERα; ESR2, ERβ) and activity (PGR, progesterone receptor) were quantified by real-time reverse transcription-PCR using standard techniques described elsewhere. Expression of KRT1 and KRT13 is limited to the more superficial layers of stratified squamous epithelia, and these genes thus provide reliable markers of estrogen-induced differentiation.

Statistics

Statistical analyses were done using JMP statistical software (version 9.0.2; SAS Institute, Inc, Cary, NC). All variables were evaluated for their distribution and equality of
variance. Data not normally distributed were transformed to improve homogeneity for analysis and then reverse transformed to the original scale for display in the results. The histomorphometry data for keratin layer thickness and endocervical gland cell height and the gene expression data for MKI67 and KRT13 violated the Levene’s test for equality of variance; therefore, these data were analyzed using the nonparametric Kruskal-Wallis and the post hoc Wilcoxon (rank sum) tests. P values were then adjusted for multiple pair-wise comparisons using a Bonferroni correction. Differences in vaginal maturation values, percentage of epithelial cells, and body weights among the treatment groups were determined using a mixed model approach with baseline values as a covariate. For each variable, this model allowed for a within-group comparison at each post-treatment time period and a between time point comparison within each group. A similar model was used to determine E1 and BZA concentrations without baseline values. A two-tailed Fisher’s exact test was used to determine group differences in the prevalence of pathologic findings. The remaining data were evaluated using analysis of variance (ANOVA) and the Tukey HSD post hoc test.

RESULTS

Vaginal cytology

Following 6, 12, and 20 months of treatment, vaginal maturation values in the CEE group were markedly increased from baseline ($P < 0.0001$ for all) and significantly higher than the control group at every post-treatment time point ($P < 0.0001$ for all, Fig. 1A). The BZA+CEE and BZA groups had significantly lower vaginal maturation values relative to CEE ($P < 0.0001$ for all) and similar vaginal maturation values compared to control (Fig. 1A). At every treatment time point, CEE significantly increased the percentage of superficial cells and reduced the percentage of intermediate cells and parabasal cells in vaginal smears compared to baseline and control, indicating improved maturation ($P < 0.0001$ for all, Fig. 1B-D). Treatment with BZA
alone had no effect on the proportion of any epithelial cell type but completely diminished CEE effects when given concurrently ($P < 0.0001$ for BZA+CEE compared to CEE at 6m, 12m, and 20m).

**Histology and morphometry**

Following 20 months of treatment, CEE markedly increased vaginal epithelial thickness, vaginal keratin thickness, and endocervical gland cell height ($P < 0.001$ compared to control for all, Table 1 and Fig. 2). BZA alone had no effect on these measures. The addition of BZA to CEE completely antagonized CEE effects on vaginal epithelial maturation ($P < 0.001$ BZA+CEE compared to CEE for all) and partially antagonized CEE effects on endocervical gland cell height ($P < 0.001$ BZA+CEE compared to control and CEE).

Animals in the CEE group showed histologic evidence of vaginal keratinization (100%) and cervical gland squamous metaplasia (75%), while all animals in the control, BZA, and BZA+CEE groups had cervicovaginal atrophy. Vaginal keratinization was not present in the control or BZA alone groups. One case in the BZA+CEE group had focal cervical squamous metaplasia at the squamocolumnar junction with multifocal keratinization of the cervicovaginal squamous epithelium. No papillomavirus-related dysplastic or invasive intraepithelial lesions were present in this case, but low-grade focal vaginal or cervical intraepithelial lesions (grade 1, mild dysplasia) were noted in 3 CEE-treated animals and 2 BZA+CEE-treated animals. These latter changes are considered incidental and not related to treatment.39

**Vaginal epithelial proliferation and keratinization gene markers**

CEE treatment resulted in significantly greater gene expression of the proliferation marker *MKI67* compared to control ($P < 0.05$), while treatment with BZA and BZA+CEE had no effects ($P > 0.1$ compared to control for both, Fig. 3A). Similarly, CEE treatment significantly induced the expression of keratin genes *KRT1* and *KRT13* ($P < 0.05$ compared to control), and the
addition of BZA to CEE completely inhibited these effects ($P < 0.0001$ compared to CEE for both, Fig. 3B and 3C). When given alone, BZA had no effect on the gene expression of $KRT1$ but suppressed $KRT13$ expression relative to control ($P < 0.05$).

**ERα expression and transcriptional activity**

Groups treated with BZA and BZA+CEE had significantly lower ERα immunolabeling in the basal cells of the vaginal epithelium, stroma, and endocervical glands than the control and CEE groups ($P < 0.05$ for all, Fig. 4). On the contrary, vaginal gene expression of ERα and ERβ were not significantly affected by BZA, CEE, or BZA+CEE treatments ($P > 0.1$ for both, supplemental Fig.1). In regards to ERα transcriptional activity in the vaginal epithelium, CEE treatment significantly increased the expression of $PGR$ compared to control and BZA+CEE ($P < 0.05$ for both), while $PGR$ expression in the BZA group was not significantly different from control ($P > 0.1$, Fig. 3D).

**Body weights and hormone/drug concentrations**

Body weights and serum concentrations of E1 at 4 and 18-24 hours post-feeding were not significantly different between the BZA+CEE and CEE groups ($P > 0.1$ for all, Supplemental Table 1). Similarly, plasma concentrations of BZA were not significantly different between the BZA+CEE and BZA groups at 4 and 18-24 hours post-feeding ($P > 0.1$ for all).

**COMMENT**

Due to safety concerns associated with ETs and combined estrogen+progestin therapies (EPT) among postmenopausal women, ET + SERM combination therapies are emerging as potential treatments for many menopause-related conditions, including vaginal atrophy. In this preclinical study, we compared the effects of BZA (20 mg), oral CEE (0.45 mg), and the combination on vaginal epithelial maturation and other estrogen-related measures. Our findings
showed that BZA inhibited the estrogenic effects of CEE on vaginal atrophy in ovariectomized macaques, while BZA alone had neutral effects. Treatment with BZA alone also had no effect on the cervical squamous and glandular epithelium but partially inhibited CEE effects on glandular endocervical cell height. Treatment with BZA+CEE and BZA also resulted in lower ERα immunolabeling in the basal cells of the vaginal epithelium, lamina propria, and endocervical glands relative to CEE and control. Together, these findings demonstrate that BZA given at 20 mg/day is an estrogen antagonist in the lower reproductive tract of the macaque model and may not be an effective treatment for vaginal atrophy when given alone or in combination with CEE.

Limited data are available regarding the efficacy of different BZA+CEE dose combinations in postmenopausal women with severe vaginal atrophy. In a 12-week study evaluating BZA 20 mg combined with either CEE 0.625 or 0.45 mg, both BZA+CEE combinations significantly increased superficial cells and decreased parabasal cells compared to placebo, but only BZA 20 mg + CEE 0.625 mg markedly improved vaginal acidity. Similarly, women receiving BZA 20 mg + CEE 0.625 mg, but not those receiving BZA 20 mg + CEE 0.45 mg, had significant improvements in their most bothersome symptoms (dyspareunia, vaginal dryness or itching/irritation). When individual vaginal symptoms and sexual function were analyzed compared to placebo, both BZA+CEE combinations significantly improved vaginal dryness and lubrication during sexual arousal, but neither combination relieved vaginal itching/irritation or dyspareunia. Other dose combinations of BZA and CEE have been studied in a 24-month clinical trial, but improvements in vaginal atrophy and symptoms were not the primary endpoints. The primary objective of this study was osteoporosis prevention therefore women were recruited based on their risk for bone loss and not severity of vaginal atrophy. Among those women that had less than 5% superficial cells at screening, 24-months of BZA 20 mg + CEE 0.45 mg treatment significantly decreased parabasal cells compared to placebo, but did not significantly increase superficial cells, indicating an incomplete estrogenic
response. In contrast, CEE combinations (0.625 or 0.45 mg) with BZA 10 mg induced a full estrogenic response characterized by a significant increase in superficial cells and decrease in parabasal cells relative to placebo. Collectively, these results suggest that BZA is a partial estrogen antagonist on the human vaginal epithelium and that estrogen-antagonist effects are dose-dependent.

Improvements in vaginal dryness and lubrication in these studies may reflect changes in vaginal blood flow and endocervical mucus secretion rather than vaginal maturation. Vaginal secretions primarily consist of vascular transudate and mucus from endocervical glands. After menopause, vascular support within the vagina is reduced, leading to diminished blood flow and vaginal secretions. Mucus secretion from the endocervical glands is also hormone dependent, and loss of ovarian hormones after menopause is associated with a decrease in mucus production that can be reversed with exogenous ET or EPT. In addition to causing vaginal dryness, loss of lubricating vaginal secretions and blood flow also impairs genital arousal in postmenopausal women. In the current study, morphometric measures indicated that endocervical glands were significantly larger in the BZA+CEE group compared to control. Whether this small increase in endocervical gland size translates to an increase in mucus production is not known, but such a finding would help explain the perceived improvements in vaginal dryness and lubrication in the BZA+CEE human studies. Further studies are needed to refine BZA+CEE dose effects on cervicovaginal measures and to better relate vaginal blood flow, transudation, and endocervical mucus production to clinical symptoms.

Previous studies have shown that approximately 10-25% of women taking systemic ET will continue to experience symptoms such as vaginal dryness and dyspareunia despite improved vaginal maturation. Subsequently, vaginal ET formulations are commonly prescribed for this subset of patients. It is currently unclear whether postmenopausal women showing signs of vaginal atrophy, and taking BZA for osteoporosis, may benefit more from concurrent local
vaginal ET. A few short-term studies support this idea, showing that the concomitant use of vaginal estrogens help improve signs and symptoms of vaginal atrophy in postmenopausal women taking the SERM raloxifene.45-47

In the current study, BZA and BZA+CEE treatment significantly reduced ERα immunolabeling within the cervicovaginal epithelium and stroma. The clinical significance of this finding is not known, but previous studies have shown similar effects in the primate mammary gland26 and endometrium (unpublished data). In addition, a recent breast cancer cell study showed that BZA increased proteasome-mediated degradation of the ERα.48 Together, these findings suggest that BZA may increase ERα turnover in addition to blocking the ER binding of estrogens in certain target tissues.

While macaques provide strong surrogates for human reproductive tract effects,49,50 a model consideration for this study is the difference between the macaque and human vaginal mucosa in response to exogenous estrogens. Baseline (estrogen-deficient) maturation values and epithelial cell proportions are similar between macaques and women.14,51,52 However, superficial keratinization of the vagina following ET is generally more pronounced in macaques than in women.49 For example, in postmenopausal women, oral CEE will increase the percentage of superficial cells in vaginal smears approximately 10-30% from baseline,53 while the same treatment will induce an 80-90% increase in superficial cells among macaques. It is not clear whether this enhanced response in the macaque may alter the balance of estrogen/SERM effects in the cervicovaginal mucosa. Strengths of the current study include the assessment of tissue measures beyond routine cytology and the evaluation of a CEE-alone group, which was not present in phase III human clinical trials investigating BZA+CEE because all participating women did not have a prior hysterectomy.24
In summary, a standard target dose of BZA completely inhibited oral CEE effects on vaginal epithelial proliferation, maturation, and superficial keratinization, while BZA had no estrogenic effect when administered alone. A similar pattern was seen in the endocervical glands, where BZA partially antagonized CEE effects. Further studies are needed to better define dose effects of oral CEE and BZA and determine whether local vaginal ET will improve vaginal atrophy and symptoms with the concomitant use of BZA.

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Figure 1. Vaginal cytology. The addition of BZA to CEE completely inhibited the estrogic effects of CEE on vaginal maturation, while BZA alone did not improve vaginal maturation. \( ^a \) \( P < 0.0001 \) compared to respective control and BZA+CEE groups. \( ^b \) \( P < 0.0001 \) compared to baseline values. Values represent means ± 95% confidence interval (CI).
Figure 2. Morphology of the vaginal epithelium and endocervical glands.  (A) Groups treated with placebo (control, CTL), BZA, and BZA+CEE had diffuse vaginal epithelial atrophy with minimal to no superficial keratinization, in contrast to the CEE group.  (B) BZA treatment had no estrogenic effect on endocervical gland maturation when given alone and partially attenuated estrogen effects on epithelial cell height when given with CEE. Images taken of H&E-stained slides at 20x objective magnification.
**Figure 3.** Vaginal epithelial proliferation and keratinization. The addition of BZA to CEE completely antagonized CEE effects on the gene expression of the proliferation marker *MKI67* (Ki67 antigen) (A), keratinization markers *KRT1* (keratin 1) (B) and *KRT13* (keratin 13) (C), and the estrogen-response gene *PGR* (progesterone receptor), while BZA had no effects on these markers when given alone. Treatment groups not connected by the same letter are significantly different at *P* < 0.05. BZA+CEE was different from CEE at *P* < 0.001 for all. Values represent means ± 95% CI.
Figure 4. Estrogen receptor alpha (ERα) protein expression. Treatment with BZA and BZA+CEE resulted in less ERα immunolabeling in the basal cells of the vaginal epithelium, lamina propria, and endocervical glands than control and CEE ($P < 0.05$ for all). Treatment groups not connected by the same letter are significantly different at $P < 0.05$. Values represent means ± 95% CI.
Supplemental Figure 1. Gene expression of ERα and ERβ in the macaque vagina. The gene expression of ERα and ERβ were not significantly affected by treatment. Treatment groups not connected by the same letter are significantly different at $P < 0.05$. Values represent means ± 95% CI.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>BZA</th>
<th>CEE</th>
<th>BZA + CEE</th>
</tr>
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<tbody>
<tr>
<td><strong>Histomorphometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratin layer thickness (µm)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0 - 0)</td>
<td>0 (0 - 0)</td>
<td>209.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.2 (0 - 0.8)</td>
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<tr>
<td>Vaginal epithelial thickness (µm)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.9 (30.8 - 50.4)</td>
<td>41.5 (35.5 - 49.2)</td>
<td>191.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.4 (36.9 - 54.2)</td>
</tr>
<tr>
<td>Endocervical gland cell height (µm)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6 (8.44 - 11.0)</td>
<td>11.6 (10.1 - 13.7)</td>
<td>38.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.0&lt;sup&gt;c&lt;/sup&gt; (14.0 - 18.6)</td>
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<tr>
<td><strong>Pathologic findings</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cervicovaginal atrophy&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23 (100%)</td>
<td>24 (100%)</td>
<td>0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>27 (100%)</td>
</tr>
<tr>
<td>Vaginal keratinization&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0 (0%)</td>
<td>0 (%)</td>
<td>24 (100%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>Marked cervicovaginitis&lt;sup&gt;ae&lt;/sup&gt;</td>
<td>0 (0%)</td>
<td>1 (4%)</td>
<td>2 (8%)</td>
<td>0 (0%)</td>
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<tr>
<td>Cervical squamous metaplasia&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (4%)</td>
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<tr>
<td>Number examined</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>27</td>
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<sup>a</sup> Values represented as means (95% CI); non-parametric Kruskal-Wallis and post hoc Wilcoxon tests with Bonferroni correction (keratin layer thickness and cervical epithelial cell height) and ANOVA with post hoc Tukey HSD (vaginal epithelial thickness).

<sup>b</sup> P < 0.001 compared to respective control and BZA + CEE groups

<sup>c</sup> P < 0.001 compared to control group.

<sup>d</sup> Values represented as sum of the number of animals with these changes (percentage of total animals examined); Fisher’s exact test.

<sup>e</sup> Minimal to mild cervicovaginitis was noted in most sections.
### SUPPLEMENTAL TABLE 1.
Body weights and hormone and drug concentrations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>BZA</th>
<th>CEE</th>
<th>BZA + CEE</th>
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<tr>
<td><strong>Body weight, kg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.90 (2.79 - 3.00)</td>
<td>2.90 (2.79 - 3.00)</td>
<td>2.90 (2.79 - 3.01)</td>
<td>2.90 (2.80 - 3.00)</td>
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<tr>
<td>20 months</td>
<td>3.31 (3.19 - 3.44)</td>
<td>3.14 (3.02 - 3.26)</td>
<td>3.12 (3.00 - 3.23)</td>
<td>3.00 (2.90 - 3.11)</td>
</tr>
<tr>
<td><strong>P value compared to baseline values</strong></td>
<td>&lt; 0.001</td>
<td>0.06</td>
<td>0.12</td>
<td>0.87</td>
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<tr>
<td><strong>Estrone, pg/ml</strong></td>
<td></td>
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<tr>
<td>4 hr PP</td>
<td>29.7 (26.4 - 33.4)</td>
<td>31.5 (27.9 - 35.5)</td>
<td>153.7 (137.3 - 172.1)</td>
<td>132.1 (118.0 - 147.9)</td>
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<tr>
<td>18-24 hr PP</td>
<td>44.3 (39.4 - 49.8)</td>
<td>45.1 (40.0 - 50.8)</td>
<td>118.3 (105.7 - 132.4)</td>
<td>108.5 (96.9 - 121.5)</td>
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<td><strong>Bazedoxifene, ng/ml</strong></td>
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<td></td>
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<tr>
<td>4 hr PP</td>
<td>NS</td>
<td>3.1 (2.5 - 3.7)</td>
<td>NS (2.9 - 4.2)</td>
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<td>18-24 hr PP</td>
<td>NS</td>
<td>1.5 (1.1 - 1.9)</td>
<td>NS (1.3 - 2.0)</td>
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</table>

a Values represent means with 95% CI; Mixed-effect model.
b $P < 0.01$ compared to the control group at 20 months post-treatment.
c $P < 0.0001$ compared to respective control and BZA groups.
NS = not sampled; PP = post-prandial.
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26. Ethun KF, Wood CE, Register TC, Cline JM, Appt SE, Clarkson TB. Effects of bazedoxifene acetate with and without conjugated equine estrogens on the breast of postmenopausal monkeys. Accepted for publication in *Menopause* (DOI: 10.1097/GME.0b013e318252e46d).


SUMMARY AND DISCUSSION

The primary objective of this project was to determine if BZA given at the clinical target dose of 20 mg/day would inhibit the proliferative effects of CEE (0.45 mg/day) on the breast and endometrium without attenuating the maturation effects of CEE on the vaginal mucosa. Using the same cohort of cynomolgus monkeys, all reproductive endpoints were conducted in parallel as part of a NHP translational trial investigating the global benefit to risk profile of BZA alone and in combination with CEE. We found that the addition of BZA to CEE significantly antagonized the stimulatory actions of CEE on breast and endometrial Ki67 immunolabeling, epithelial area, and specific markers of ERα activation, while BZA alone had no effect. Global gene expression profiles in the breast showed that BZA+CEE more closely resembled BZA than CEE alone. BZA+CEE and BZA did not significantly upregulate pathways or genes related to cell cycle progression or proliferation in the breast. The addition of BZA to CEE also inhibited CEE-induced changes in breast and endometrial morphology. For instance, BZA+CEE treatment resulted in less lobular enlargement in the breast and reduced the prevalence of simple endometrial hyperplasia, stromal fibrosis, and cystic dilation compared to CEE. Similarly, BZA alone had no effect on these measures, indicating that BZA lacks an estrogen agonist profile in the breast and endometrium. In the vaginal epithelium, BZA completely inhibited CEE effects on vaginal proliferation, maturation, and keratinization, while having no estrogenic activity when given alone. A comparable pattern was seen in the endocervical glands, but BZA only partially antagonized CEE effects on epithelial cell height. Interestingly, BZA with and without CEE resulted in lower ERα immunolabeling in the breast, endometrium, vagina, and endocervical glands compared to control and CEE. Taken together, these findings demonstrate that the target dose of BZA given alone and with CEE (0.45 mg/day) lacks an estrogenic profile in the breast, endometrium, and vaginal epithelium of the postmenopausal macaque model. This information
should be useful to symptomatic postmenopausal women seeking alternatives to traditional estrogen + progestin therapies.

Molecular mechanisms of SERMs

The molecular mechanisms underlying the tissue-specific actions of SERMs are complex and only partly understood. Several interactive mechanisms have been proposed, including the following: 1) differences in the relative abundance of ER subtypes (ERα and ERβ) in a given tissue or cell type; 2) unique structural features of individual SERMs and varying binding affinities for the ERs; 3) differential changes in the conformation of the ER structure induced by ligand binding; and 4) differential expression and binding of coactivator and corepressor molecules to the ER-ligand complex in various tissues (1, 2).

_Tissue distribution and activity of ER subtypes:_ Both ER subtypes, ERα and ERβ, are widely distributed throughout the body (3). In some organ systems, ERα and ERβ are expressed at comparable levels, whereas in others, one subtype predominates. ERα and ERβ expression can also vary among the different cell types within the same organ. Although species and isoform differences exist, ERα is primarily expressed in the uterus, cervix, vagina, breast (epithelium), prostate (stroma), ovary (theca cells), epididymis, testis, bone, liver, kidney, adipose tissue, and various regions of the brain, while ERβ is predominantly found in the colon, lung, prostate (epithelium), breast (epithelium), ovary (granulosa cells), testis (leydig cells), bladder, bone marrow, salivary gland, vascular endothelium, anterior pituitary, and hypothalamus (3, 4). When co-expressed in the same cell, ERα and ERβ are thought to heterodimerize and modulate each other’s activity (5). ERα and ERβ homodimers are also believed to have distinct functional roles in gene regulation that is both ligand and promoter context-specific (6-8). For example, _in vitro_ transcriptional activation assays using isolated ligand-binding domains (LBDs) have shown that tamoxifen antagonizes ERα and ERβ activation at classic estrogen response element (ERE).
sites but activates transcription on activator protein 1 (AP-1) sites more strongly through ERα than ERβ (7, 9). On the contrary, E2 activates transcription through ERα on AP-1 and ERE and inhibits transcription through ERβ at both sites (7).

**Ligand-specific conformational changes in the ER:** Protein crystallography studies have demonstrated that individual ER ligands induce unique conformational changes in the ER-ligand complex (10-13). Structural differences in the side chain are thought to be the primary determinant of whether a ligand initially induces an agonist, antagonist, or an intermediate shape in the ER complex (14). Like steroid estrogens, SERMs bind to both ERα and ERβ with high binding affinities; however, small variations in ER binding affinities and conformational changes do not exclusively account for differences in transcriptional responses among individual SERMs and cell types (15).

**Coregulator expression and activity:** Ligand-specific conformational changes in the ER structures allow for the recruitment and binding of coactivators and corepressors that either positively or negatively regulate transcription, respectively. Many of these co-regulators have enzymatic activities that can directly modify the chromatin structure to enable gene expression or repression (16). Additionally, post-translational modifications of coregulators, particularly phosphorylation and sumoylation, can also alter interactions between the ERs with which they bind and other transcription factors (17). Differential recruitment and expression of these coregulators vary among ER-containing tissues and are thought to play a critical role in defining the tissue-specific effects of SERMs (2,17). For instance, *in vitro* studies have found that tamoxifen and raloxifene, which are estrogen antagonists in the breast, recruit corepressors to ER target promoters. In contrast, tamoxifen recruits coactivators in uterine cells where it has been shown to have an agonistic profile clinically, whereas raloxifene, which has neutral effects on the endometrium, does not recruit coactivators (18). These studies have also indicated that the agonist effects of tamoxifen may, in part, be dependent on the higher expression levels of SRC-1.
(steroid receptor coactivator) in uterine cells than in breast cells (18). Interestingly, other studies have shown that coactivators, such as SRC-1, can stabilize the ER-ligand complex in an agonist shape, thus enhancing the interaction of the receptor with its ligand and possibly other components of the transcription apparatus (19). Moreover, DNA regulatory elements (e.g. EREs) have also been shown to influence not only ER-DNA binding, but coregulator recruitment to the DNA-bound ER (20).

Current knowledge of the molecular actions of BZA

To date, a few in vitro studies have provided insight into the molecular basis of BZA (21, 22). As the clinical profile of BZA is developed further, more mechanistic studies are expected.

Alternative conformation of ERα-LBD bound to BZA: Using molecular modeling, Lewis-Wambi et al. compared the differential conformations of ERα-LBD bound to BZA, raloxifene, 4-hydroxytamoxifen (a major metabolite of tamoxifen), and the pure antiestrogen fulvestrant (ICI 182, 780) (22). Results revealed that BZA binding to ERα-LBD induced an antagonist shape similar to that induced by raloxifene, but distinct from those induced by 4-hydroxytamoxifen and fulvestrant. BZA and raloxifene formed many of the same ligand-receptor interactions including key hydrogen bonds, hydrophobic interactions, and retention of a highly ordered water molecule. However, a few key differences in the orientation of amino acid residues were observed following BZA binding compared to raloxifene. The most notable difference was for Leu539 of helix 12. The larger (hexamethylenamine) ring on the side chain of BZA (see figure 1, pp. 5) caused the hydrophobic side chain of Leu539 to be displaced by 1 angstrom. The authors hypothesized that this alteration could lead to a change in the overall orientation of helix 12 and possibly differential binding of coactivators to BZA-ERα compared to the raloxifene-ERα complex (22). This concept is based on prior crystallography studies showing that breast antiestrogens, such as 4-hydroxytamoxifen and raloxifene, block the binding of coactivators to
the ERα-LBD complex by inducing a conformational change that causes helix 12 to occupy a coactivator recognition groove (10,13).

*Inhibition of coactivator recruitment by BZA:* Using multiplex biochemical assays, Berrodin and colleagues compared the effects of BZA with two other SERMs (raloxifene and lasoloxifene) on CEE-mediated coactivator recruitment by ERα-LBD (21). Results indicated that BZA significantly inhibited the recruitment of several coactivators onto the ERα-LBD protein, including SRC-1, SRC-2, SRC-3, CBP I, p300 I, NRIP II, and MNAR II. Although BZA appeared to be less potent than raloxifene and lasoloxifene, the set of coactivators inhibited by BZA was different from raloxifene and lasofoxifene. In accordance with these biochemical findings, each SERM+CEE combination antagonized a unique set of CEE-regulated genes in MCF-7 breast cancer cells, as determined by gene microarray analyses. Corepressor recruitment by ERα-LBD was not investigated in this experiment. While these findings offer valuable insight into the molecular actions of BZA *in vitro,* “CEE treatment” used in this study was actually a synthetic mixture including a limited number of unsulfated estrogens found in the clinical preparation of CEE (Premarin) and, therefore, may not mimic the biological effects of the whole preparation when given *in vivo.*

*Downregulation of ERα protein levels by BZA:* In the current study, treatment with BZA and BZA+CEE resulted in less nuclear ERα immunolabeling in the breast, endometrium, vagina, and endocervical glands compared to control and CEE, while mRNA levels remained unchanged. This finding is similar to that of Taylor and coworkers (23) who reported reduced endometrial ERα immunolabeling with BZA treatment in an intact mouse model. Together, these observations suggest that BZA may inhibit estrogen-mediated transcription and proliferation in the breast and reproductive tract by increasing degradation of the ERα post-translationally. Proteolysis of ERα has been shown to be regulated by the ubiquitin-proteasome pathway (24), and it is possible that BZA binding to the ERα may facilitate ubiquitination and proteasome-
mediated degradation. This idea is supported by an *in vitro* study that showed proteasome-mediated ERα degradation by BZA in hormone resistant breast cancer cells (in an estrogen free medium) (22). In this study, MCF-7:5C cells were treated with a proteasome inhibitor completely blocked ERα degradation by BZA, while treatment with a protein synthesis inhibitor had minimal effects on BZA-induced ERα protein degradation (22). Collectively, these data support the idea that ERα degradation may contribute to the estrogen antagonist effects of BZA in the macaque breast and reproductive tract; however further *in vivo* studies should be conducted to confirm that BZA binding increases ERα ubiquitination and degradation via the proteasomal pathway.

In support of this hypothesis, several *in vitro* and *in vivo* experiments have indicated that ERα stability and proteasome-mediated proteolysis is ER ligand specific (24). For example, it is well known that E2 decreases ERα protein and mRNA levels in human breast cancer cell lines (25). The pure antiestrogen, fulvestrant (ICI 182, 780), also rapidly increases ERα turnover in these cells (greater than E2 alone), but has no effect on mRNA levels (25-27). In contrast, tamoxifen and raloxifene has been shown to stabilize the ERα protein *in vitro* and prevent degradation (25,27,28). In accordance with these *in vitro* studies, E2 treatment has been shown to downregulate ERα protein levels in the murine mammary gland and endometrium, while no change is seen with tamoxifen treatment (29). Similarly, in women with ER+ breast cancer, tamoxifen has no effect on ERα expression, while fulvestrant decreases ERα protein levels in breast cancer patients (30). Interestingly, fulvestrant is thought to increase ERα turnover in the breast by inducing an unstable conformational change in the activated dimer which is recognized by the cell as a “misfolded” protein. The ”misfolded” protein is then ubiquitinated and targeted for destruction via the proteasomal pathway (31). Further studies are needed to determine whether a similar response occurs with BZA binding to ERα in the breast or other ER target tissues.
For the current macaque study, two other possible mechanisms exist for the lower nuclear ERα immunoreactivity seen with BZA and BZA+CEE treatment in the breast and reproductive tract. First, BZA may upregulate the expression of ubiquitinating enzymes (ubiquitin E3 ligases) involved in the normal degradation of ERα. Previously described ubiquitin E3 ligases in breast tissue include E6-AP, EFP, MDM2, and BRCA1/BARD1 (32-35). Recently, Ramamoorthy et al. (36) generated transgenic mouse lines that overexpress a wild-type E6-AP (EP-AP wt) or an ubiquitin-protein ligase defective E6-AP (E6-AP C8335) in the mammary gland. Results from this study showed that E6-AP wt overexpression significantly lowers ERα protein levels in the murine mammary gland while overexpression of E6-AP C8335 or E6-AP knockout results in higher levels of ERα compared to non-transgenic and wild-type, respectively (36). To address the question of whether BZA effects E6-AP gene expression in the primate model, gene microarray data from the 6 month post-treatment breast biopsies were reviewed. In a pair-wise comparison between BZA and control, the gene for E6-AP (UBE3A) was identified with filter criteria of > 1.2 fold change and no false-discovery rate correction (P < 0.05). However, subsequent PCR validation showed no significant effect of BZA on UBE3A expression, providing evidence that BZA may lower ERα protein levels by another mechanism. Secondly, nuclear ERα immunoreactivity may be diminished with BZA and BZA+CEE treatment relative to control and CEE due to the differential conformational changes induced by these ERα-ligands. The target epitope may be detectable in the control and CEE-treated tissues, but altered and no longer recognizable by the ERα antibody in the BZA-treated tissues.

Lack of knowledge regarding the effect of BZA on ERβ protein levels: Similar to ERα gene expression, ERβ gene expression in the breast and reproductive tract was not affected by BZA treatment in this project. However, it is currently not known whether BZA with or without CEE downregulates ERβ protein levels in the breast, reproductive tract, or other ERβ-containing tissues such as bone and the cardiovascular system. Information regarding ERβ protein levels
and activity in these tissues is important due to increased interest in developing ERβ selective agonists for menopause-related conditions such as osteoporosis, cardiovascular disease, hot flushes, and depression (37,38). E2 binds ERβ with high affinity and ERβ is widely distributed in bone, brain, and vascular endothelium, but ERβ is not highly expressed in the endometrium. Therefore, it has been proposed that ERβ selective agonists may provide beneficial clinical effects without increasing endometrial proliferation. This idea is supported, in part, by ER knockout mice studies which have indicated that ERβ plays a minor role in mediating the effects of estrogens in the endometrium compared to ERα (38). For instance, ERα knockout mice have defective uterine responses to exogenous estrogens, whereas nonselective estrogens (e.g. E2) induce equivalent or exaggerated uterine responses in ERβ knockout mice compared to wild-type (38,39). In regards to the breast, ERβ selective agonists may have neutral effects on the breast based on data from ER knockout studies showing normal mammary gland development in mice lacking ERβ (40). Moreover, in vitro data have indicated that ERβ can act as a dominant repressor of ERα-mediated transcription and proliferation in breast cancer cells, suggesting that ERβ selective agents may have antiproliferative effects in the breast (41,42).

It is important to note that the role of ERβ in human breast cancer and normal breast physiology is not well established. Based on the observation that ERβ expression is often downregulated in human breast cancer, some investigations have described low ERβ protein levels as a potential marker for a poor prognosis and tamoxifen resistance (43,44). Today, however, ERβ-status is not routinely measured in breast cancers and its value as a diagnostic marker remains unknown (38).

In the present study, ERβ expression was not assessed by immunohistochemical method in the normal macaque breast because ERβ immunolabeling is diffuse (involving > 50% of the breast epithelium) (45) and obtaining a significant difference among treatment groups is often difficult (insufficient statistical power). Immunolabeling for individual ERβ isoforms (e.g ERβ1,
ERβ2 [ERβcx, ERβ5] may yield more reliable results (46,47), but validated antibodies against the different ERβ isoforms are currently not widely available (48).

**CEE: a mixture of weak and potent estrogens**

Although several other estrogen preparations are available, CEE has been the most prescribed ET for the past 70 years (Premarin, Wyeth, Philadelphia, USA) (49). Derived from pregnant mare urine, CEE is a mixture of at least 10 estrogens in their sulfate esters forms. These estrogen components consist of saturated B-ring estrogens (E1 and E2) and unsaturated B-ring estrogens (e.g. equilin, 17β-dihydroequilin, 17α-dihydroequilin, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin) that are not naturally produced by the human ovary (50).

*Relative ER binding affinities and transcriptional potencies:* Studies using human recombinant ERα and ERβ have shown that all estrogen components of CEE bind to ERs with different affinities, compete to varying degrees with E2 for ER binding, and transactivate ERα and ERβ with different efficacies (50,51). Notably, most ring B unsaturated estrogens bind preferentially to ERβ compared to ERα, have lower binding affinity for both ERα and ERβ compared to E2, and are less potent in transcriptional assays relative to E2 (51).

*Differential conformations of ERα-LBD bound to E2 and an equine estrogen analog:* A recent study compared the crystal structure of E2 and 17β-methyl-17α-dihydroequilenin (NCI 122), an analog of the dihydroequilenin estrogens in CEE, complexed with the ERα LBD and coactivator GRIP-1 (52). Structural analysis showed that NCI 122/ERα binding adopted an agonist conformation, but several features of this complex were identified that may weaken the transactivational potency of this compound, including a more rigid unsaturated B-ring, fewer hydrophobic interactions, and the absence of key hydrogen bonds that may influence coactivator recruitment and interaction. This evidence suggests that the unsaturated B-ring equine estrogens in CEE may partially dilute E2 effects in some tissues such as the breast.
Differential effects of CEE and E2 on breast proliferation and considerations for BZA pairing

Some investigators have proposed that CEE has less promotional effects on breast proliferation than E2 (53,54). In a recent retrospective analysis of 9 studies investigating oral estrogen effects in ovariectomized macaques, a standard dose of CEE 0.625 mg/day resulted in less breast epithelial proliferation (Ki67 expression) than a standard 1.0 mg/day dose of E2 (54). Interestingly, endometrial thickness and breast progesterone receptor expression were not differentially regulated by oral E2 and CEE in this analysis, suggesting that dissimilarities in breast proliferation cannot be simply explained by systemic estrogen exposure (54). In support of these findings, epidemiologic studies have shown that the relative risk of invasive breast cancer with CEE use (0.625 mg/day) does not increase until after ≥ 15-20 years of use (55, 56) whereas E2 use (1-2 mg/day) has been associated with an increased risk of breast cancer after only 5 years of use (57). Data from the current study and the SMART clinical trials clearly demonstrate that BZA adequately abrogates the proliferative effects of CEE on the breast and endometrium; however, it is not known whether BZA (20 mg) would provide similar protective effects in these tissues if co-administered with standard doses of oral or transdermal E2. Given the evidence that E2 may have greater proliferative effects in at least the breast than CEE, it is reasonable to hypothesize that the BZA dose needed for complete inhibition may vary with type of ET; specifically, a higher dose may be required when pairing BZA with oral E2. Further preclinical and clinical studies are needed to test this hypothesis.

BZA+CEE co-therapy and the clinical management of postmenopausal vaginal atrophy

It is also currently not clear whether oral BZA (20 mg) + CEE (0.45 mg) will be perceived as an adequate treatment for severe vaginal atrophy among clinicians and patients. Vaginal ET is the first choice among clinicians for the management of menopause-related vaginal atrophy (58) because available clinical data suggests that locally administrated ET does not stimulate the endometrium (59) and local ET tends to be more efficacious than oral ET (60,61).
A meta-analysis of 19 randomized trials found that all formulations of topical therapies (vaginal estrogen creams, ring devices, and tablets) provided better symptom relief than oral estrogen (60). Specifically, findings from one study suggested that topical CEE cream resulted in better vaginal vascularization and relief of vaginal dryness and dyspareunia than oral CEE (0.625 mg/day) (61). Therefore, based on these findings and data from the current macaque study, postmenopausal women showing signs of vaginal atrophy, and taking BZA for osteoporosis, may benefit more from concurrent local vaginal ET. However, to date, this hypothesis has not been tested in a randomized clinical trial.

Additional studies pertaining to the clinical profile of BZA and BZA+CEE

Several important research questions remain regarding the clinical effects and application of BZA+CEE that could not be addressed in the current macaque study. First, the safety of BZA+CEE for postmenopausal women at high risk for breast cancer is presently not known (62). To date, there is no planned chemoprevention study for BZA at 20 mg/day or any other dose. In addition, none of the SMART or BZA-alone trials, which consisted of postmenopausal women with normal risk, were powered to investigate the incidence of ER+ breast cancer (62). Other third-generation SERMs have been investigated as chemoprevention agents with favorable results. For example, the Postmenopausal Evaluation and Risk-Reduction with Lasofoxifene (PEARL) trial showed that 0.5 mg/day of lasofoxifene reduced the risks of total and ER+ invasive breast cancers in osteoporotic postmenopausal women over a 5-year period regardless of Gail score but particularly in women with higher relative E2 levels (>0.35 ng/dL) (63). Secondly, little information is known regarding the long-term risk of venous thromboembolism with BZA monotherapy and BZA+CEE co-therapy (64,65). Similar to breast cancer risk assessments, the BZA-alone and SMART trials were not adequately powered to evaluate the risk of venous thromboembolism (64). Accordingly, participants are currently being enrolled into the BZA Post Approval Safety Study (PASS) study (Phase IV) to further characterize the rate of venous
thromboembolism among osteoporotic women prescribed bazedoxifene, bisphosphonates, or raloxifene (66). The follow-up period for this observational cohort study will be up to 5 years. Secondary endpoints will include cerebrovascular events and cardiac disorders such as ischemic stroke and atrial fibrillation (66). Safety studies investigating the effects of BZA+CEE co-therapy on the incidence of either venous thromboembolism or breast cancer are currently not planned.

Conclusions

In this preclinical trial, BZA inhibited the proliferative effects of CEE in the postmenopausal macaque breast and reproductive tract, while BZA alone had neutral effects. ERα protein levels in these tissues were significantly lower with BZA and BZA+CEE treatment compared to control and CEE, suggesting that BZA may promote ERα protein degradation in addition to blocking estrogen binding. These findings support the idea that the target BZA (20 mg/day) + CEE (0.45 mg/day) combination therapy may have a lower breast and endometrial cancer risk profile than traditional hormone therapies but may be an inferior treatment option for postmenopausal women suffering from severe vaginal atrophy.
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2001 Tri-Beta National Biological Honor Society, Saint Louis University
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2004 – 2006 Advanced Opportunity Fellowship, UW School of Veterinary Medicine
2005 American College of Laboratory Animal Medicine Scholarship
2006 American Veterinary Medical Association Student Career Exploration Scholarship
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2006 Elizabeth R. Griffin Primate Medicine Extern Program Scholarship
2008 Association of Primate Veterinarians, Student Travel and Abstract Award
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“Preparing for your First Management Position” Workshop
“Preparing for an AAALAC International Site Visit” Workshop
2008 American Association of Laboratory Animal Science 59th National Meeting
"Effective Scientific Writing for Veterinary Staff and Scientists: Intermediate Course" Workshop
2008 IACUC Training: Basics and Challenges, NCABR
2008 Professional Grant Development Workshop, Grant Training Center
2008 – 2009 Association of Primate Veterinarians Workshop
2009 Triangle Consortium for Reproductive Biology
2010 North American Menopause Society Annual Meeting
2009 – 2011 Endocrine Society Annual Meeting
Publications:

Journal Articles:


Ethun KF, Wood CE, Register TC, Cline JM, Appt SE, Clarkson TB. Effects of bazedoxifene acetate with and without conjugated equine estrogens on the breast of postmenopausal monkeys. Accepted for publication in *Menopause* (DOI: 10.1097/GME.0b013e318252e46d).


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Schnatz PF, O’Sullivan DM, **Ethun KF**, Appt SE, Clarkson TB. Individual differences in plasma concentrations of vitamin D3 are associated with the beneficial effect of estrogen treatment on bone density of surgically postmenopausal monkeys. *Menopause* 2010;17:1225

**Scientific Posters and Presentations:**

**Ethun K**, Wood CE, Register TC, Cline JM, Appt SE, Clarkson TB. An evaluation of bazedoxifene acetate alone and in combination with conjugated equine estrogens on the breast of surgically postmenopausal monkeys. Endocrine Society 93rd Annual Meeting, Boston, MA, June 2011; Wake Forest School of Medicine 11th Annual Graduate Student Research Day; Wake Forest University 6th Annual Women’s Health Research Day. POSTER

**Ethun K**, Wood CE, Appt SE, Cline JM, Chen H, Clarkson T. Effects of combination therapy with bazedoxifene and conjugated equine estrogens on the endometrial and vaginal epithelium of surgically postmenopausal monkeys. Endocrine Society 92nd Annual Meeting, San Diego, CA, June 2010. POSTER


**Ethun K**, Appt SE, Kaplan JR, Kavanagh K. Chronic consumption of a high fat diet is associated with coagulopathy in african green monkeys (*Chlorocebus aethiops saebeus*). American Association of Laboratory Animal Science 59th National Meeting, Indianapolis, IN, November 2008. POSTER

**Ethun, K.** Primate malaria: Prevalence in cynomolgus macaques imported from southeast asia. Primate Medicine Virtual Grand Rounds, Wake Forest Primate Center, August 8th, 2008. ORAL PRESENTATION
APPENDIX 1
CHEMICAL STRUCTURES OF SERMS REPRODUCED WITH PERMISSION

FIGURE 1, INTRODUCTION

Confirmation Number: 10877053
Order Date: 02/01/2012

Customer Information
Customer: Kelly Ethun
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