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Research Article

Cancer Prevention Research

Clinical Trial of Acolbifene in Premenopausal Women at High Risk for Breast Cancer

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Abstract

The purpose of this study was to assess the feasibility of using the selective estrogen receptor modulator (SERM) acolbifene as a breast cancer prevention agent in premenopausal women. To do so, we assessed change in proliferation in benign breast tissue sampled by random periareolar fine-needle aspiration (RPFNA) as a primary endpoint, along with changes in other risk biomarkers and objective and subjective side effects as secondary endpoints. Twenty-five women with cytologic hyperplasia ± atypia and ≥2% of breast epithelial cells staining positive for Ki-67, received 20 mg acolbifene daily for 6–8 months, and then had benign breast tissue and blood risk biomarkers reassessed. Ki-67 decreased from a median of 4.6% [interquartile range (IQR), 3.1%–8.5%] at baseline to 1.4% (IQR, 0.6%–3.5%) after acolbifene ($P < 0.001$; Wilcoxon signed-rank test), despite increases in bioavailable estradiol. There were also significant decreases in expression (RT-qPCR) of estrogen-inducible genes that code for pS2, ERα, and progesterone receptor ($P \leq 0.026$). There was no significant change in serum IGF1, IGFBP3, IGF1:IGFBP3 ratio, or mammographic breast density. Subjective side effects were minimal with no significant increase in hot flashes, muscle cramps, arthralgias, or fatigue. Objective measures showed a clinically insignificant decrease in lumbar spine bone density (DEXA) and an increase in ovarian cysts but no change in endometrial thickness (sonography). In summary, acolbifene was associated with favorable changes in benign breast epithelial cell proliferation and estrogen-inducible gene expression but minimal side effects, suggesting a phase IIB placebo-controlled trial evaluating it further for breast cancer prevention. *Cancer Prev Res; 8(12); 1146–55. ©2015 AACR.*

Introduction

The selective estrogen receptor modulator (SERM) tamoxifen is the only FDA-approved agent for breast cancer risk reduction in premenopausal women 35 years of age or older with a Gail Model estimated 5-year risk of breast cancer of 1.66% or higher and/or atypical hyperplasia or *in situ* cancer (1). A recent meta-analysis suggests that use of tamoxifen in the chemopreventive setting reduced risk about 33% compared with placebo among high-risk women (2). Current ASCO guidelines suggest including tamoxifen in discussion of risk reduction strategies for high-risk women (1), but tamoxifen uptake for risk eligible women remains low (3) primarily due to concerns about side effects and lack of demonstrated survival benefit (1, 3). The benefit:risk ratio for tamoxifen used as primary prevention in high-risk premenopausal women is generally seen as particularly favorable given the lack of significantly increased incidence of serious side effects in women younger than 50 years in the NSABP P-1 trial (4, 5). Yet the rate of tamoxifen uptake for premenopausal women attending high-risk clinics has been cited as only 10% (6). Concerns about induction of menstrual abnormalities and perimenopausal symptoms are the likely reasons that most younger women are reluctant to take tamoxifen. Tamoxifen can also result in an increase in size or number of ovarian cysts as well as bone density loss in premenopausal women (7, 8). Currently, tamoxifen for primary prevention is regarded as a preference-sensitive decision (3, 6, 9). A dramatic increase in uptake of endocrine therapy for primary prevention by premenopausal women is probably dependent on development of an agent with fewer uterine side effects and perimenopausal symptoms.

Acolbifene (EM-652.HCl) is a fourth-generation SERM of the benzopyran class which has been found to have no estrogen agonist effects in either the breast or endometrium (10–13). Acolbifene and its prodrug (EM-800) have been associated with reduction of growth of tumor xenografts (14) as well as the incidence of 7,12–dimethylbenz(*a*)anthracene (DMBA)-induced rat mammary cancer (15). The lack of estrogen agonist activity in the uterus of EM-800 as well as reported activity in tamoxifen-resistant metastatic disease (13) made it an attractive agent for assessment for treatment and prevention. Although efficacy has been reported in treatment trials of postmenopausal women, few premenopausal women have been treated in this setting.

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The overall purpose of this pilot study was to assess the suitability of acolbifene as a prevention drug for breast cancer in premenopausal women as assessed by tolerability and favorable modulation of risk biomarkers for breast cancer. Tolerability encompassed subjective side effects relating to vasomotor symptoms, and quality of life, as well as objectively measured change in ovarian cysts, endometrial thickness, and by pelvic ultrasonography and bone density by dual-energy X-ray absorption (DEXA). Primary biomarker assessment was to estimate the effect of acolbifene on proliferation assessed by Ki-67 in benign breast tissue acquired by random periareolar fine-needle aspiration (RPFNA). Secondary objectives explored modulation of other breast cancer risk biomarkers in blood and breast tissue, including serum insulin-like growth factor 1 (IGF1) and insulin-like growth factor–binding protein 3 (IGFBP3) and mammographic breast density. Exploratory markers included change in mRNA levels of selected estrogen response genes which code for estrogen receptor alpha, pS2, progesterone receptor, growth regulation by estrogen in breast cancer 1 (GREB-1), and splice variants for C-X-C motif chemokine 12 which codes for stromal cell–derived factor 1 (SDF-1).

Materials and Methods

Cohort and trial eligibility

Potential participants were required to be at increased risk for breast cancer as defined by any one or more of the following criteria: (i) 5-year Gail predicted probability of breast cancer >1.67% or 5× that of an average woman of the same age (16); (ii) known deleterious BRCA1/2 mutation carrier or a family history consistent with hereditary breast cancer; (iii) prior diagnosis of atypical hyperplasia, lobular or ductal carcinoma *in situ*; or (iv) a prior RPFNA showing hyperplasia with atypia (17). A normal mammogram performed within 3 months of the baseline RPFNA on days 1 to 10 of the menstrual cycle was required with at least 5% estimated visual mammographic density. Participants were required to use birth control (hormonal, intrauterine device, or double barrier) for the duration of the study.

Protocols for screening RPFNA (HSC 4601; NCT00291096) and for the acolbifene intervention (HSC 10588; NCT00853996) were approved by the University of Kansas Medical Center Human Subjects Committee. Separate consents were utilized for the screening and interventional protocols. For entry into study, the RPFNA specimen must have exhibited cytologic evidence of hyperplasia with or without atypia (18) with a Masood cytology index score of ≥14 (19) and have ≥2% positive staining for Ki-67.

RPFNA and cytomorphology

RPFNA was performed (C.J. Fabian) on two sites per breast under local anesthesia during the follicular phase (days 1–10) of the menstrual cycle. The first aspiration pass per site (four sites total) was placed in a 2-mL cryovial containing 0.5-mL PBS, immediately immersed in liquid nitrogen and transferred to a −80°C freezer within 12 hours for later use in gene expression assays. Remaining specimens were pooled from both breasts in a single 15-cc tube containing 9 cc of CytoLyt and 1 cc of 10% neutral-buffered formalin. Cells were spun, washed, and resuspended in PreservCyt after at least 24 hours in CytoLyt. Aliquots were processed to slides using a ThinPrep (Hologic LP) Non-Gyn standard protocol. Slides for cytomorphology and Ki-67 were Papanicolaou-stained using an RNase-free technique.

All slides were assessed by a single cytopathologist (C.M. Zalles) who assigned a categorical assessment of nonproliferative, hyperplasia, borderline hyperplasia with atypia, or hyperplasia with atypia (18); as well as a Masood semiquantitative index score (19).

Ki-67 immunocytochemistry

Only slides having more than 500 epithelial cells visible by Papanicolaou staining were further processed for Ki-67. After destaining, antigen retrieval was performed with a 10 mmol/L citrate buffer (pH 6) in a Biocare decloaking chamber for 2 minutes at 120°C. Slides were stained with a MIB-1 monoclonal antibody (M7240 Dako Cytomation) at a 1:20 dilution using a Dako autostainer (20). Hyperplastic clusters were preferentially assessed, and the number of cells with unequivocal nuclear staining of a total of 500 cells was assessed manually by two technicians and a consensus score recorded (20).

Gene expression by RT-qPCR

Total RNA was extracted from frozen RPFNA samples using TRIzol LS according to the manufacturer's instructions. The RNA collected was thus reflective of adipocytes, stroma, and epithelial cells. RNA was amplified using MessageAmpII aRNA amplification kit (Life Technologies) and reverse transcribed to cDNA using SMARTScribe Reverse Transcriptase (Clontech Laboratories, Inc.) and random nonamer primers. Real-time PCR (qPCR) was performed in the Breast Cancer Prevention Laboratory via 5′ nuclease assay using hydrolysis probes as previously described (21).

Reference transcripts were β-glucuronidase (*GUSB*), β-actin (*ACTB*), cyclophilin A (peptidylprolyl isomerase A, *PPIA*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), cytokeratin 19 (*KRT19*), and E-cadherin (*CDH1*). Tested transcripts were estrogen receptor 1 (*ESR1*) for ERα, trefoil factor 1 (*TFF1*) for pS2, two splice variants of C-X-C motif chemokine 12 (*CXCL12*) which code for SDF1α and SDF-1β, growth regulation by estrogen in breast cancer 1 (*GREB1*), steroid sulfatase (*STS*), progesterone receptor (*PGR*), cyclin D1 (*CCND1*), *IGF1R*, and keratin 5 (*KRT5*). Baseline and postintervention specimens were assessed together. PCR reactions were run on an Applied Biosystems Prism 7000 Sequence Detection System. The quantity of each biomarker transcript in a sample is expressed relative to the level of the reference transcript *HPRT1* which showed the least change between paired specimens. Further normalization by epithelial cell markers (cytokeratin 19 and E-cadherin) was not indicated on the basis of lack of significant directional change in these markers; but if done, the results of statistical analysis for the tested transcripts were not materially altered.

Hormones, IGF1, and IGFBP3

Blood was obtained for analysis of estradiol and sex hormone–binding globulin (SHBG) during the follicular phase (days 1–10) of the menstrual cycle, at the time of RPFNA. Fasting blood for progesterone, SHBG, testosterone, IGF1, and IGFBP3 was obtained at days 20 to 24 of the menstrual cycle. Samples were stored frozen at −80°C until analysis. Commercial kits from R&D Systems, Inc. were used for ELISA of IGF1 (DG100) and IGFBP3 (DGB300). Commercial kits from Diagnostics Biochem Canada were used for enzyme immunoassay of estradiol (CAN-E-430), progesterone (CAN-P-305), testosterone (CAN-TE-250), and ELISA of SHBG (CAN-SHBG-4010). Baseline and postintervention specimens were run together with pooled serum controls to assess batch

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variation. Bioavailable estradiol and testosterone were calculated according to standard formulae (22, 23).

Mammographic breast density

Digital mammograms were converted to a common, deidentified format for breast density assessments. The left cranial caudal view was generally used for assessments by a single reviewer (C.J. Fabian) using the Cumulus software program developed by Boyd and Yaffee (24). Breast density was calculated as percent dense area compared with the entire breast area. Baseline and postintervention mammographic images were assessed together in a blinded fashion (25).

Adverse events and quality of life

Subject-reported adverse events were recorded using NCI common toxicity criteria (version 3.0). Subjects were contacted monthly for adverse events reporting. For quantitative assessment of quality-of-life aspects, specific information was collected monthly regarding the frequency and severity of muscle cramps and hot flashes. The Health Assessment Questionnaire II (HAQ-II) and the Brief Fatigue Inventory (BFI) questionnaire were also completed at baseline and postintervention.

Safety assessments by pelvic sonography and DEXA

To monitor for possible side effects that might relate to administration of a SERM, pelvic sonography and DEXA bone density assessments were performed at baseline and postintervention on all subjects. Number and size of ovarian cysts and endometrial thickness were recorded by the evaluating radiologist. From DEXA, the T-score was used to evaluate bone mineral density for both the femur and lumbar spine.

Study agent

Acolbifene was provided by Endorecherche, Inc. as 20-mg capsules. Subjects were instructed to take one capsule orally each day.

Sample size and statistical analysis

Our planned accrual was 44 subjects, anticipating a 10% dropout rate. With 40 evaluable subjects, there would be at least 80% power to detect an effect size (defined as the mean change divided by the SD of change) of 0.45 or greater for change in Ki-67 (as percentage of cells staining positive) at a two-sided level of 0.05 in a one-sample t test. After 25 subjects had been accrued (and 9 completed study), a technical problem with the Ki-67 immunocytochemical staining was identified and accrual was temporarily suspended. By the time the staining problem was resolved (see Supplementary File S1), all 25 subjects had completed study (no dropouts) and had post-study RPFNA specimens evaluable for the primary endpoint. A decision was made to not reopen the study for further accrual but to stop at 25 subjects. With this number of evaluable subjects, there would still be 80% power to detect an effect size of 0.59.

For the primary endpoint of change in Ki-67, which did not appear normally distributed, the nonparametric Wilcoxon signed-rank test was used to assess whether acolbifene had any effect on this marker. Similarly, for the secondary endpoints, serum hormones, IGF1 and IGFBP3 levels, IGF1:IGFBP3 ratio, breast density, and gene expression, the Wilcoxon test was also used. For qualitative dichotomous outcomes, McNemar test was used. Two-sample comparisons were made using nonparametric Mann–Whitney test. All analyses were conducted by IBM, SPSS, version 20. As these secondary analyses were all considered exploratory, no corrections were made for multiple comparisons.

Results

Screening and enrollment

A total of 75 high-risk women were screened by RPFNA, of whom three (4%) were not medically eligible and 39 (52%) were not eligible on the basis of Ki-67 < 2.0% and/or cytomorphology. A total of eight elected not to participate in the intervention, leaving 25 (33%) who enrolled and received acolbifene. The first subject started in March 2009, the last started in December 2009, and the last subject completed study in July 2010.

Demographic and risk information

Demographic and risk information for the 25 premenopausal women enrolled is shown in Table 1. All subjects were Caucasian, with one self-identified as Hispanic. Seven subjects (28%) were taking oral contraceptives.

Retention and compliance

All 25 women enrolled completed the intervention, met the study definition of compliance, had a repeat RPFNA, and provided paired biomarker data for assessment of change over the study period. The minimum value for compliance was 81% of prescribed agent, and the median compliance was 95%, on the basis of subject-maintained logs and returned pill counts. The median duration on study agent was 204 days (range, 182–243). Per protocol, the nominal 6 months of study agent could be extended to 8 months for purposes of scheduling RPFNA with menstrual cycle.

Changes in Ki-67 and cytomorphology in benign breast tissue

The median level of Ki-67 staining at baseline was 4.6% [range, 2.4%–21.8%; interquartile range (IQR), 3.1%–8.5%]; postintervention, the median was 1.4% (range, 0%–6.6%; IQR, 0.6%–3.5%; Table 2). The median change was −3.0% (range, −20.2% to 2.8%; IQR, −7.1% to 2.0%), which corresponded to a relative change of −77% with a range of −100% (zero staining postintervention) to 117% (IQR, −88% to −53%; $P < 0.001$, Wilcoxon). Despite increases in serum bioavailable estradiol (see below), a decrease in Ki-67–positive staining was noted in 23 of 25 (92%) subjects (Fig. 1). There were no differences between the seven oral contraceptive users and 18 non-users for expression of Ki-67: baseline ($P = 0.93$), postintervention ($P = 1.0$), change ($P = 0.88$), or relative change ($P = 0.69$; Mann–Whitney test). Both oral contraceptive users and non-users showed decreases (5 of 7, $P = 0.091$; 18 of 18, $P = 0.001$) with intervention.

There were no significant changes in cytomorphology over the course of the intervention, either by a categorical descriptor or by

Table 1. Demographic variables for 25 enrolled participants

Variable	Mean ± SD	Median (range)
Age, y	42.8 ± 5.2	43 (33–52)
Height, in	65 ± 2	65 (61–70)
Weight, lb	155 ± 29	153 (109–224)
BMI, kg/m^2	25.8 ± 4.8	25.3 (18.9–34.1)
5-year Gail risk, %	3.6 ± 4.4	2.3 (0.4–16.5)
Age first live birth, y (2 nonparous)	28 ± 4	29 (18–37)

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Table 2. Changes in (a) cytomorphology and Ki-67 in RPFNA specimens and (b) mammographic breast density over the course of the study

Biomarker	Baseline	Postintervention	Absolute change	Relative change	*P*
Masood Score	15	14	−1	−7%	0.10
	14–17	9–17	−5–2	−36%–14%	
	[14–15]	[13–16]	[−2–1]	[−14%–7%]	
	14.8 ± 1.0	14.0 ± 2.2	−0.8 ± 2.0	−5% ± 14%	
			14 decrease		
			9 increase		
Atypia	13 (52%)	12 (48%)	4 "gain" atypia		1.00
			5 "lose" atypia		
Estimated epithelial cell number per slide					
1×10^2 to 5×10^2	0 (0%)	4 (16%)	10 decrease		0.012
5×10^2 to 1×10^3	2 (8%)	5 (20%)	2 increase		
1×10^3 to 5×10^3	18 (72%)	12 (48%)			
$>5 \times 10^3$	5 (20%)	4 (16%)			
Ki-67, %	4.6	1.4	−3.0	−77%	**<0.001**
	2.4–21.8	0.0–6.6	−20.2–2.8	−100%–117%	
	[3.1–8.5]	[0.6–3.5]	[−7.1–−2.0]	[−88%–−52%]	
	6.6 ± 4.8	2.1 ± 1.9	−4.5 ± 4.7	−57 ± 52%	
			23 decrease		
			2 increase		
Mammographic breast density (percentage of area at increased density)	35.8	35.0	−3.9	−11%	0.067
	2.9–76.3	3.6–70.8	−31.4–16.2	−49%–71%	
	[21.1–60.8]	[18.5–48.8]	[−10.0–0.9]	[−23%–4%]	
	40.5 ± 22.5	36.3 ± 20.2	−4.2 ± 10.3	−5 ± 30%	
			16 decrease		
			8 increase		

NOTE: Median, range [IQR], and mean, ± SD are shown for the 25 subjects, except mammographic density where only 24 comparisons were available.

Masood score (Table 2). Thirteen of the 25 women exhibited hyperplasia with atypia at baseline versus 12 at study conclusion. Similarly, for the semiquantitative Masood cytomorphology score, median score was 15 at baseline and 14 at second RPFNA, with a median 1 point decrease ($P = 0.10$, Wilcoxon).

Changes in gene expression in benign breast tissue

Seventeen paired specimens (baseline and postintervention) were available for RT-qPCR analysis for levels of mRNA. Specimens from eight women were excluded from analysis because either the baseline or postintervention specimen was grossly bloody. Significant decreases ($P < 0.05$) were noted for transcripts for three estrogen-inducible genes that code for pS2, ERα, and PgR. There were also borderline significant decreases for GREB1 and borderline significant increases for SDF-1α and SDF-1β (Fig. 2). There were no changes noted for keratin 5, IGF1R, cyclin D1, or steroid sulfatase.

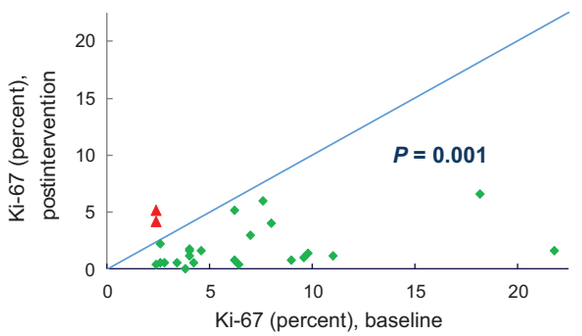

Figure 1.
Ki-67 expression (percentage of cells staining positive) postintervention as a function of baseline value. Baseline aspiration values are shown on the *x*-axis; repeat aspiration on the *y*-axis. The line represents no change in value; triangles above the line denote an increase and diamonds below the line a decrease.

Changes in mammographic breast density

There was no statistically significant change in mammographic breast density, expressed as the percentage of breast area with increased density, from baseline (median, 35.8%) to postintervention (median, 35.0%), with an average of 9 months between mammograms (Table 2). Breast density was statistically significantly ($P < 0.001$) lower at baseline and postintervention for women with higher BMI (dichotomized at the median of 25 kg/m^2); but there was no difference for either absolute or relative change in density.

Change in serum hormones and IGF1 and IGFBP3

Follicular phase (days 1–10 of cycle) estradiol and bioavailable estradiol increased by medians of 78% and 110%, respectively, relative to baseline ($P \leq 0.002$; Table 3). Both oral contraceptive users and non-users exhibited significant ($P = 0.018$; $P = 0.044$) increases in bioavailable estradiol. For oral contraceptive users, this was due not only to an increase in estradiol but also a significant decrease (7 of 7 subjects; $P = 0.018$) in SHBG. Luteal phase total testosterone increased by a median of about 30% relative to baseline ($P = 0.002$). Bioavailable testosterone did not change for the 18 oral contraceptive non-users ($P = 0.91$) but did increase in each of the seven oral contraceptive users ($P = 0.018$), in part due to significant (7 of 7; $P = 0.018$) decreases in SHBG. Oral contraceptive users also had lower levels of bioavailable testosterone at baseline than non-users (median, 2.3 vs. 17.2 nmol/L; $P = 0.046$). There were no statistically significant changes for progesterone, IGF1, IGFBP3, or the IGF1:IGFBP3 molar ratio (Table 3).

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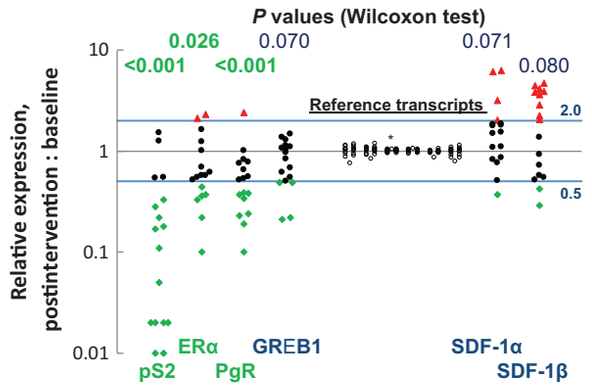

Figure 2.
Effects of acolbifene on relative change (postintervention:baseline) in expression (by RT-qPCR) of genes that code for relevant proteins. There was minimal change for the six reference transcripts assessed (cytokeratin 19, E-cadherin, HPRT1, cyclophilin A, β-actin, β-glucuronidase). HPRT1 (*) was used for normalization purposes.

Self-reported adverse events

Five (20%) of 25 subjects reported no adverse events, 11 (44%) subjects reported only grade 1 events, seven (28%) subjects reported a grade 2 event, and two (8%) subjects reported a grade 3 event. One subject reported at the postintervention visit that grade 3 hot flashes had begun approximately 2 months earlier (after 4 months on study agent). One subject reported a grade 3 dizziness that began approximately 6 weeks after starting study agent; this was considered to be unrelated to study agent. A total of 71 adverse events (50 grade 1, 19 grade 2, 2 grade 3) were self-reported, with only half being attributed by the protocol chair (C.J. Fabian) as possibly or probably related to study agent. Most common reported adverse events (percentage of subjects) included irregular menses (32%), leg/muscle cramps (25%), diarrhea (16%), and hot flashes (16%). No serious adverse events were reported. Nor did any subject drop out of the study due to adverse events.

Quantitative assessment of hot flashes, menstrual irregularities, musculoskeletal symptoms, and general quality of life

Consistent with the low incidence of study-related moderate or severe adverse events, no significant changes were observed for the quantitative assessments of quality of life.

Problems with hot flashes were assessed for average number per day and intensity. Only six women reported mild-to-moderate hot flashes before starting drug and only for two were these as frequent as daily. Five of the six with initial hot flashes did not report hot flashes at their postintervention visit; for the sixth, there was a slight increase in number and intensity. Five other participants with no hot flashes at baseline reported infrequent hot flashes postintervention. Overall, there was no effect of acolbifene use on symptoms associated with hot flashes.

The Health Assessment Questionnaire II (HAQ-II) measures interference in daily activities from arthralgias and joint pain. No woman reported a score above zero at baseline and only one had a non-zero score (1.0) postintervention. Thus, acolbifene use was not associated with joint discomfort or disability. Similar results were obtained for self-reported incidence, frequency, and severity of muscle cramps. Only three women reported mild muscle cramps before starting drug. For two, no muscle cramps were reported postintervention; for the third, there was no change in any aspect of muscle cramp symptoms. For three other women with no muscle cramps at baseline, there were mild muscle cramps reported postintervention. There was thus no adverse effect of acolbifene use on symptoms associated with muscle cramps.

The Brief Fatigue Inventory (BFI) measures intensity of fatigue and interference with daily activities. BFI scores at baseline and postintervention were similar, reflecting no change overall ($P = 0.82$, Wilcoxon test). Medians were 9 and 10; ranges were 0–54 and 0–44; and means/SDs were 12.8 ± 13.2 and 12.9 ± 13.5, respectively. For change over the study, there was a median of 1, range of −13 to 13, and mean of −0.2 ± 7.6.

Gynecologic parameters assessed by pelvic ultrasonography

Endometrial thickness was unchanged over the course of the study (Table 4). In contrast, the number of women in whom ovarian cysts could be visualized increased from 15 (60%) to 23 (82%; $P = 0.011$, McNemar test). The largest diameter of ovarian cysts increased from a median of 12 mm at baseline to 23 mm postintervention ($P < 0.001$).

Bone density assessed by DEXA

From DEXA assessments (Table 4), there was a statistically significant ($P < 0.001$) but minor decrease in lumbar spine bone density measurements. Median changes were −0.04 g/cm^2 (range, −0.11 to +0.03) and −0.40 (range, −1.10 to +0.30) for T-score. Only one participant showed a clinically significant T-score decrease of at least one unit, from −0.1 to −1.2. There was no observable effect on femur bone density or percent body fat measured.

Discussion

This is the first report of the effect of the SERM acolbifene on benign breast tissue of healthy premenopausal women. There was favorable modulation of the risk biomarker Ki-67 as well as expression of several estrogen-responsive genes, including pS2 and PgR, despite dramatic increases in serum estradiol levels. There were no subjects who discontinued use because of side effects and no increase in endometrial thickness. Clinically insignificant decreases in lumbar spine bone density were observed following 6- to 8-month exposure, as well as an asymptomatic increase in ovarian cysts. Overall, acolbifene appears to modulate tissue risk biomarkers in a similar fashion as tamoxifen; but in this single arm study, hot flashes and other perimenopausal symptoms were not increased as would be expected with tamoxifen. Nor was there an increase in endometrial thickness.

Ki-67 was selected as the primary risk biomarker endpoint of this study because proliferation is permissive for cancer development. In observational studies, Ki-67 was higher in foci of hyperplasia and atypical hyperplasia of women who subsequently developed breast cancer than in those who did not (26, 27). Women with ≥2% of cells in atypical foci labeling for Ki-67 had a 4-fold increased risk for breast cancer (27). By eligibility criterion, a minimum baseline Ki-67 of 2% was required in clusters of cells judged to be hyperplastic by cytologic criteria. The reduction in Ki-67 observed was statistically significant and almost universal (23 of 25 paired specimens), consistent with the well-known

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Table 3. Change in serum hormones and growth factors from baseline to postintervention

Variable or biomarker	Baseline	Postintervention	Change	*P*
Collected at time of RPFNA (day 1–10 of menstrual cycle)				
SHBG (with E2), nmol/L	82 18–221 [38–124] 90 ± 56	68 25–154 [41–92] 73 ± 37	1 −140–43 [−54–18] −18 ± 51	0.46
Estradiol, pg/mL	88 <20[a]–615 [56–131] 112 ± 115	190 <20[a]–312 [120–224] 174 ± 76	80 −481–247 [35–129] 62 ± 136	**0.001**
Estradiol, nmol/L	0.32 <0.07[a]–2.28 [0.21–0.49] 0.41 ± 0.43	0.70 <0.07[a]–1.15 [0.45–0.83] 0.64 ± 0.28	0.29 −1.79–0.92 [0.14–0.48] 0.23 ± 0.51	**0.001**
Bioavailable (free) estradiol, pmol/L	4.5 0.7[a]–31.1 [1.4–6.4] 5.3 ± 6.2	8.0 1.2[a]–15.7 [5.3–10.8] 7.8 ± 3.4	3.5 −25.5–10.1 [1.0–7.0] 2.6 ± 7.0	**0.002**
Collected at day 20–24 of menstrual cycle				
IGF1, ng/mL	129 72–223 [107–167] 139 ± 42	146 71–220 [103–173] 141 ± 41	3 −41–66 [−15–18] 2 ± 27	0.74
IGF1, nmol/L	16.8 9.4–29.0 [13.9–21.7] 18.1 ± 5.4	19.0 9.3–28.6 [13.4–22.6] 18.3 ± 5.3	0.4 −5.4–8.6 [−1.9–2.3] 0.3 ± 3.5	0.74
IGFBP3, ng/mL	2,350 1,505–3,683 [2,061–3,025] 2,504 ± 609	2,667 1,497–3,556 [2,249–3,006] 2,618 ± 539	111 −307–1,239 [−62–246] 114 ± 267	0.051
IGFBP3, nmol/L	82.3 52.7–128.9 [72.1–105.9] 87.6 ± 21.3	93.4 52–124 [78.7–105.2] 92 ± 19	3.9 −10.7–32.6 [−2.2–8.6] 4.0 ± 9.3	0.051
IGF1:IGFBP3 molar ratio	0.21 0.10–0.37 [0.16–0.25] 0.21 ± 0.06	0.19 0.10–0.42 [0.16–0.24] 0.21 ± 0.07	−0.01 −0.12–0.11 [−0.03–0.02] −0.01 ± 0.05	0.41
Progesterone, ng/mL	3.5 0.4–26.2 [0.9–3.5] 4.4 ± 5.5	2.8 0.5–36.3 [0.7–9.7] 7.1 ± 10.0	0.0 −10.7–30.1 [−2.6–1.9] 2.7 ± 9.7	0.78
Progesterone, nmol/L	11.0 1.1–83.3 [2.7–18.8] 14.0 ± 17.3	8.9 1.5–115.3 [2.1–30.7] 22.6 ± 31.7	−0.1 −34.1–95.8 [−8.3–6.1] 8.6 ± 30.7	0.78
SHBG, nmol/L	87 22–276 [59–193] 117 ± 80	85 34–179 [48–135] 93 ± 46	6 −227–60 [−45–18] −24 ± 73	0.53
Testosterone, ng/mL	0.40 <0.08[b]–2.59 [0.18–0.83] 0.63 ± 0.64	0.58 0.19–3.52 [0.29–0.83] 0.85 ± 0.91	0.16 −0.21–2.33 [0.02–0.24] 0.22 ± 0.48	**0.002**
Testosterone, nmol/L	1.37 <0.28[b]–9.00 [0.63–2.87] 2.18 ± 2.21	2.0 0.64–12.21 [1.00–2.86] 2.93 ± 3.15	0.53 −0.74–8.09 [0.06–0.80] 0.75 ± 1.66	**0.002**
Bioavailable (free) testosterone, pmol/L	13.4 1.1–120.2 [4.6–35.1] 25.1 ± 30.7	17.3 3.8–194.4 [9.5–38.3] 32.4 ± 41.6	4.1 −26.9–118.2 [−2.1–8.5] 7.3 ± 26.8	0.13

NOTE: Median, range, [IQR], and mean ± SD are shown for the 25 subjects.

[a]One woman had estradiol levels below limit of detection at both times and was imputed to have no change.

[b]Another woman had testosterone levels below limit of detection only at baseline and was considered to have exhibited an increase.

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Table 4. Change in quantitative measures assessed by pelvic sonography and DEXA from baseline to postintervention

Variable or biomarker	Baseline	Postintervention	Change	*P*
Pelvic ultrasonography				
Endometrial thickness, mm	6 2–26 [4–9] 7.6 ± 5.5	6 1–17 [3.5–8] 6.3 ± 4.0	0.0 −11–5 [−3–2] −0.7 ± 3.9	0.40
Endometrial evaluation as abnormal	0%	0%	0%	1.00
Ovarian cyst largest diameter, mm	12 0–26 [0–19] 10.5 ± 9.5	23 0–54 [16–35.5] 25.6 ± 14.4	14 −19–49 [3.5–24.5] 15.1 ± 15.4	<**0.001**
Ovarian cysts present				
Any	15 (60%)	23 (92%)	8 (32%)	**0.011**
>30 mm	0 (0%)	9 (36%)	9 (36%)	**0.003**
Fibroid largest diameter, mm	0 0–16 [0–0] 2.3 ± 5.5	0 0–25 [0–0] 1.8 ± 6.3	0 −15–9 [0–0] −0.52 ± 4.4	0.47
Fibroids present	4 (16%)	2 (8%)	2 (8%)	0.16
DEXA				
Lumbar spine bone mineral density, g/cm^2	1.28 1.09–1.48 [1.19–1.37] 1.28 ± 0.11	1.24 1.07–1.42 [1.17–1.30] 1.24 ± 0.09	−0.05 −0.11–0.03 [−0.07–−0.02] −0.05 ± 0.04	<**0.001**
Lumbar spine T-score	0.70 −0.8–2.3 [0–1.4] 0.69 ± 0.84	0.30 −1.2–1.9 [−0.25–0.9] 0.30 ± 0.78	−0.40 −1.1–0.3 [−0.6–−0.2] −0.39 ± 0.30	<**0.001**
Femur bone mineral density, g/cm^2	0.99 0.80–1.28 [0.94–1.15] 1.04 ± 0.13	1.03 0.88–1.35 [0.95–1.18] 1.05 ± 0.14	−0.01 −0.06–0.36 [−0.02–0.01] 0.016 ± 0.08	0.48
Femur T-score	−0.2 −1.7–2.2 [−0.6–1.1] 0.25 ± 1.03	−0.1 −1.1–2.7 [−0.5–1.4] 0.36 ± 1.10	−0.1 −0.60–2.90 [−0.2–0.1] 0.11 ± 0.69	0.68
Percent body fat	35.8 16.7–54.2 [31.3–46.1] 37.0 ± 9.4	37.5 19.3–54.7 [31.2–46.7] 37.8 ± 9.9	0.3 −4.0–5.8 [−1.7–2.4] 0.3 ± 2.6	0.76

NOTE: For quantitative measures, median, range, [IQR], and mean ± SD are shown for the 25 subjects. For categorical indices, the number and percentage are shown.

effects induced by tamoxifen in early breast cancer in short-term window of opportunity trials and premenopausal benign breast tissue (28–30). In neoadjuvant cancer treatment studies, reduction of or low post-tamoxifen Ki-67 in tumor tissue is associated with superior recurrence-free survival (31). However, a serial biopsy study reported by Moshin and Allred in 2005 did not show an effect of 1 year of tamoxifen versus control on Ki-67 in a small number of women with benign hyperplastic foci generally without atypia (32).

We did not see any reduction in cytologic evidence of atypia after 6 months of acolbifene use. Cytologic evidence of atypia by RPFNA, like atypical hyperplasia in diagnostic biopsies, is associated with increased risk (17), but there is no evidence that short-term use of a SERM, including tamoxifen, will significantly change morphology in benign breast tissue (32, 33).

Reduction observed at the transcript (mRNA) level of the estrogen-inducible genes for pS2 and PgR is qualitatively similar for acolbifene as that observed with tamoxifen (34, 35). Expression of the gene for ERα was reduced dramatically and *GREB1* which is an estrogen response gene associated with proliferation (34, 36) was slightly reduced. There was no clear effect on the chemokine SDF-1 which is important for viability of stem cells and has been implicated in ligand-independent phosphorylation of the estrogen receptor and tamoxifen resistance (37–39) Tamoxifen has been variably associated with increases in SDF-1 (34, 40, 41). A recent report assessing the short-term effects of several SERMs and fulvestrant on a large number of genes in mammary cancer from ovariectomized mice suggests that acolbifene reverses the effect of estradiol on more estrogen-inducible genes than tamoxifen, raloxifene, or fulvestrant (42).

The risk biomarkers of serum IGF1:IGFBP3 ratio (43) and mammographic breast density (24) are known to be modulated by tamoxifen (33, 44, 45). In IBIS-1, tamoxifen reduced mammographic breast density in premenopausal women with baseline density of >10% by a mean of 13% compared with placebo (45). The median absolute decrease in mammographic density of 3.9% after 6 months of acolbifene was not statistically significant, although a greater numerical effect is likely had the drug been given longer (46) and might have reached significance had more subjects been entered into the trial.

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Although acolbifene did not significantly modulate either IGF1 or breast density, this does not necessarily mean that acolbifene is a less effective anti-estrogen than tamoxifen. Aromatase inhibitors modulate neither IGF1 nor mammographic breast density but have demonstrated efficacy in prevention and are generally viewed as more effective than tamoxifen in a low-estrogen environment (46, 47).

Acolbifene was associated with an increase in serum estradiol levels and ovarian cysts. Increased mid-cycle and luteal levels of estrogen and increased ovarian cysts have been observed for tamoxifen where the prevalence in asymptomatic premenopausal women undergoing regular pelvic ultrasound monitoring has been reported as up to 80% without regard to cyst size and approximately 30% for cysts of 30 mm or greater diameter (7, 48–51). In the absence of symptoms, these cysts are probably of little clinical significance and are likely due to prolonged elevation of follicle-stimulating hormone (FSH) in the follicular phase combined with elevated mid-cycle or luteal estradiol for both tamoxifen and acolbifene (7). Increase in ovarian cyst formation after acolbifene tended to resolve shortly after the drug was stopped (data not shown). We found no change in SHBG or free testosterone. Information on SHBG and free testosterone in premenopausal women without breast cancer is limited, but SHBG is generally increased and free testosterone generally reduced in postmenopausal women after tamoxifen (52). There was a minimal reduction in premenopausal bone density similar to that observed with tamoxifen (53). Finally, there was a statistically significant, but clinically insignificant, decrease in white blood cells and platelets (data not shown), similar to what has been observed with tamoxifen (33).

Importantly, from the standpoint of uptake of a prevention agent by premenopausal women, there was no evidence of worsening of hot flashes, other perimenopausal or musculoskeletal symptoms, or overall quality of life; nor was there any evidence of endometrial thickening. In contrast in the NSABP P-1 trial, hot flashes were reported by 81% of individuals randomized to 5 years of tamoxifen versus 65% of those randomized to placebo (54) and endometrial thickening is commonly observed in premenopausal women taking tamoxifen in the absence of concomitant Goserelin (55, 56).

The ability of a SERM to act as an agonist or antagonist depends on hormone levels and the specific tissue as activator/repressor levels vary by tissue type. Acolbifene differs from tamoxifen in that it blocks the coactivator SRC-1 expressed in high amounts in uterine but not breast tissue (12), thus explaining the lack of agonist effect of acolbifene on the uterus. Acolbifene has the potential to be more effective than tamoxifen, as it inhibits both the AF-1 and AF-2 functions of both ERα and ERβ, whereas the inhibitory action of tamoxifen is limited to AF-2. SERMs which block only AF-2 are likely to have partial estrogen agonist activity (reviewed in ref. 57). The relative potency of SERM depends on many factors, including its bioavailability, serum and tissue half-life, affinity for the estrogen receptor, and rate of ubiquination of the ligand–ER complex (58). The plasma half-life of tamoxifen is about 7 days (59) whereas that of acolbifene is about 24 hours (F. Labrie; personal communication).

Limitations of this study include the small number of subjects and lack of a control (placebo) arm. Because of the eligibility criterion of Ki-67 $\geq$ 2% and the resulting baseline mean Ki-67 of our cohort being higher than the population mean, there is a risk that the apparent decrease in Ki-67 was the result of a regression to the mean artifact. Without a parallel placebo arm, one cannot conclusively distinguish between this possibility and a true effect of acolbifene. In addition, the large number of variables considered, without correction for multiple comparisons, increases the risk of type I error for the exploratory biomarkers. Nonetheless, a number of factors were identified as potential pharmacodynamic effect markers or that might assist in elucidation of mechanisms of action; these can be evaluated further in future trials.

In conclusion, acolbifene was associated with a favorable side effect profile, and an apparent favorable modulation of risk biomarkers including proliferation as well as the estrogen response genes for pS2, ERα, and PgR. Given the lack of demonstrated increase in hot flashes and other subjective symptoms, acolbifene should be compared with placebo (2 arms) or placebo and tamoxifen (3 arms) in a phase IIB trial for premenopausal women with modulation of benign breast tissue proliferation and vasomotor symptoms as co-primary endpoints.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: C.J. Fabian, B.F. Kimler
Development of methodology: C.J. Fabian
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.J. Fabian, T. Metheny, B.K. Petroff
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.F. Kimler, T.A. Phillips, B.K. Petroff, T.C. Havighurst, K.M. Kim, H.H. Bailey
Writing, review, and/or revision of the manuscript: C.J. Fabian, B.F. Kimler, C.M. Zalles, T.A. Phillips, T.C. Havighurst, K.M. Kim, H.H. Bailey, B.M. Heckman-Stoddard
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Study supervision: C.J. Fabian, H.H. Bailey, B.M. Heckman-Stoddard

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Cancer Prevention Research

Clinical Trial of Acolbifene in Premenopausal Women at High Risk for Breast Cancer

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