

*Featured Article*

# Effects of the Antiestrogen Tamoxifen and the Aromatase Inhibitor Letrozole on Serum Hormones and Bone Characteristics in a Preclinical Tumor Model for Breast Cancer

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## ABSTRACT

**Purpose:** The purpose of this study was to evaluate and compare the effects of the antiestrogen tamoxifen and the aromatase inhibitor letrozole on tumor growth, serum hormones, uterine weight, body composition, and bone characteristics in mice.

**Experimental Design:** Human estrogen-dependent breast cancer cells stably transfected with the aromatase gene (MCF-7CA cells) were inoculated in Matrigel subcutaneously into ovariectomized nude mice. This model represents postmenopausal breast cancer in many respects, including the fact that estrogen is no longer produced by the ovaries and is not under feedback regulation by gonadotropins. Mice that received subcutaneously implanted MCF-7CA cancer cells were then treated with tamoxifen or letrozole for 7 weeks.

**Results:** As reported previously, tumor growth was markedly inhibited by both tamoxifen (100 µg/day) and letrozole (10 µg/day). Tamoxifen treatment led to increased bone mineral density (BMD) and hyperplastic uteri. Mice treated with letrozole had significantly smaller uteri than the controls and tamoxifen-treated mice. Letrozole did not affect BMD. There was no significant difference in systemic leptin and insulin-like growth factor I levels as a result of tamoxifen or letrozole treatment.

**Conclusions:** Tamoxifen treatment inhibited breast cancer cell growth and increased BMD but caused uterine hypertrophy in this preclinical model of postmenopausal breast cancer. Letrozole inhibited tumor growth without inducing uterine hypertrophy. In addition, letrozole had no effect on BMD. These findings provide experimental evidence that letrozole is an effective and safe (in terms of risk of endometrial cancer risk and osteoporosis) alternative or complement to tamoxifen treatment for breast cancer.

## INTRODUCTION

Breast cancer is the most common noncutaneous cancer among women in the United States (1). Although breast cancer mortality has declined in the United States over the last decade, it is the second leading cause of cancer-related deaths in women, after lung cancer (2). Approximately 211,300 women in the United States will be diagnosed with invasive breast cancer in 2004, and about 39,800 women will die from the disease (1). Hormonal therapy has an important place in the treatment of breast cancer. The antiestrogen tamoxifen has been shown to prevent pre- and postmenopausal breast cancer and to be a beneficial adjuvant therapy for women with estrogen receptor-positive tumors (3). However, some breast cancers become resistant to tamoxifen (4), and tamoxifen can increase the risk of endometrial cancer (4).

The aromatase inhibitors represent a new class of agents that appear to be more effective than tamoxifen in the treatment of breast cancer (5). Aromatase is a cytochrome P-450 enzyme that catalyzes the rate-limiting step in the synthesis of estradiol from androgens (6). Recent evidence suggests that aromatase inhibitors have several advantages over tamoxifen for the treatment of breast cancer (7–9). However, there is concern that these estrogen-lowering drugs may promote osteoporosis or decrease bone density and, in turn, increase the risk of bone fractures. Despite the potentially adverse effects of tamoxifen on increasing the incidence of stroke and endometrial problems, a beneficial effect of tamoxifen treatment in postmenopausal women is to increase bone mineral density (BMD) due to the estrogen agonist property of tamoxifen (10).

To date, there is limited experimental evidence contrasting the effects of tamoxifen and aromatase inhibitors on tissues such as bone and the uterus. Herein, we evaluated the effects of tamoxifen and the aromatase inhibitor letrozole on bone density and the uterus. We report the following: (a) tamoxifen promoted BMD and uterine hypertrophy; (b) letrozole did not affect BMD; and (c) letrozole did not stimulate uterine hypertrophy. In fact, letrozole-treated mice had smaller uteri than control mice in this model.

Insulin-like growth factor (IGF)-I is a polypeptide with 70

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amino acids (11) that is involved in proliferation, differentiation, and apoptosis of many cell types, including breast cancer cells (12, 13). IGF-I may promote tumor development and metastasis (14). Leptin, a hormone synthesized by fat cells, acts on the hypothalamus to decrease food intake and increase energy expenditure (15). Leptin can promote proliferation of breast cancer cells (16). Both IGF-I and leptin can promote bone formation (11, 17). Furthermore, some of the anticancer effects of tamoxifen and other antiestrogens such as raloxifene may be mediated by altered IGF-I or leptin levels (18, 19). Therefore, we have investigated the effects of letrozole as well as tamoxifen on serum levels of these hormones.

## MATERIALS AND METHODS

**Intratumoral Aromatase Model.** As described previously (20, 21), subconfluent, human estrogen receptor-positive MCF-7 cells stably transfected with the human aromatase gene (MCF-7CA cells) were grown in Dulbecco's modified minimal essential medium (pH 7.4; Life Technologies, Inc., Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (Hyclone, Salt Lake City, UT). The cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. MCF-7CA cells used for inoculation were scraped into Hank's solution and centrifuged at 1000 × *g* for 10 min at 4°C, washed twice with calcium-free and magnesium-free PBS, and then resuspended in Matrigel (10 mg/ml; BD Biosciences, Bedford, MA).

Ovariectomized female BALB/c nude mice (body weight, approximately 20 g) were obtained from the animal production area at the National Cancer Institute (Frederick, MD). The animals received food and water *ad libitum* and were housed in a pathogen-free environment under controlled conditions of light and humidity in the Association for Assessment and Accreditation for Animal Care-approved Central Animal Facility of the University of Maryland at Baltimore under the care and management of full-time veterinarians and veterinary staff. All procedures involving animals were approved by the Institutional Animal Care and Use Committee. Mice received subcutaneous inoculation into two sites with 3 × 10<sup>7</sup> MCF-7CA breast cancer cells in 0.1 ml of Matrigel. Because the mice have a diminished production of adrenal steroids, the animals were supplemented with androstenedione, the substrate for aromatase, throughout the experiment at a dose of 0.1 mg/mouse/day, administered subcutaneously. Androstenedione was shown previously not to affect tumor growth directly (20). Tumor size was measured weekly using calipers. The volume of the tumor was calculated using the following formula:  $\frac{4}{3} \pi r_1^2 r_2$ , where  $r_1$  is the smaller radius.

Animals were assigned to groups of five or eight mice when at least one tumor per mouse had reached a measurable size (~500 mm<sup>3</sup>), approximately 35 days after inoculation. Total tumor volumes were similar for each group at the start of treatment. Mice then received daily subcutaneous injections for 7 weeks with (a) letrozole, 10 μg/day (CGS 20,267; kindly provided by Dr. Dean Evans; Novartis, Basel, Switzerland) or (b) tamoxifen, 100 μg/day (Sigma, St. Louis, MO). The compounds were prepared for injection in 0.3% hydroxypropyl cellulose. Control animals received vehicle (0.3% hydroxypropyl

cellulose, 0.1 ml/mouse/day) subcutaneously on a daily basis.

At the end of treatment, blood samples were drawn from the retro-orbital venous plexus of lightly anesthetized mice (metafane; Mallinckrodt Veterinary Inc., Mundelein, IL) and transferred into 1.5-ml microcentrifuge tubes. Serum was separated by centrifugation at 14,000 rpm for 5 min (Marathon 13k/12; Fisher Scientific, Pittsburgh, PA) and stored at -70°C until analysis. At necropsy, animals were decapitated under metafane anesthesia, and tumors and uteri were removed. Carcasses were stored at -20°C for body composition analyses.

**Body Composition.** Fat weight, lean weight, BMD, and bone mineral content (BMC) were determined using dual-energy X-ray absorptiometry (GE Lunar Piximus II, Madison, WI). Three replicate measurements were made on each individual animal. Methods described by Nagy and Clair (22) were followed. In brief, necropsied carcasses were placed on the specimen tray and repositioned after each scan. After scanning, GE-supplied software (version 1.46) was used to exclude heads from the image area. Necropsied carcasses were weighed before scanning. Lean weight was calculated by subtracting fat weight from carcass weight. Our past studies (23) have validated estimates of bone and body composition characteristics based on measurements of necropsied carcasses; thus, these estimates are proportional to measurements of bone and fat characteristics in whole animals.

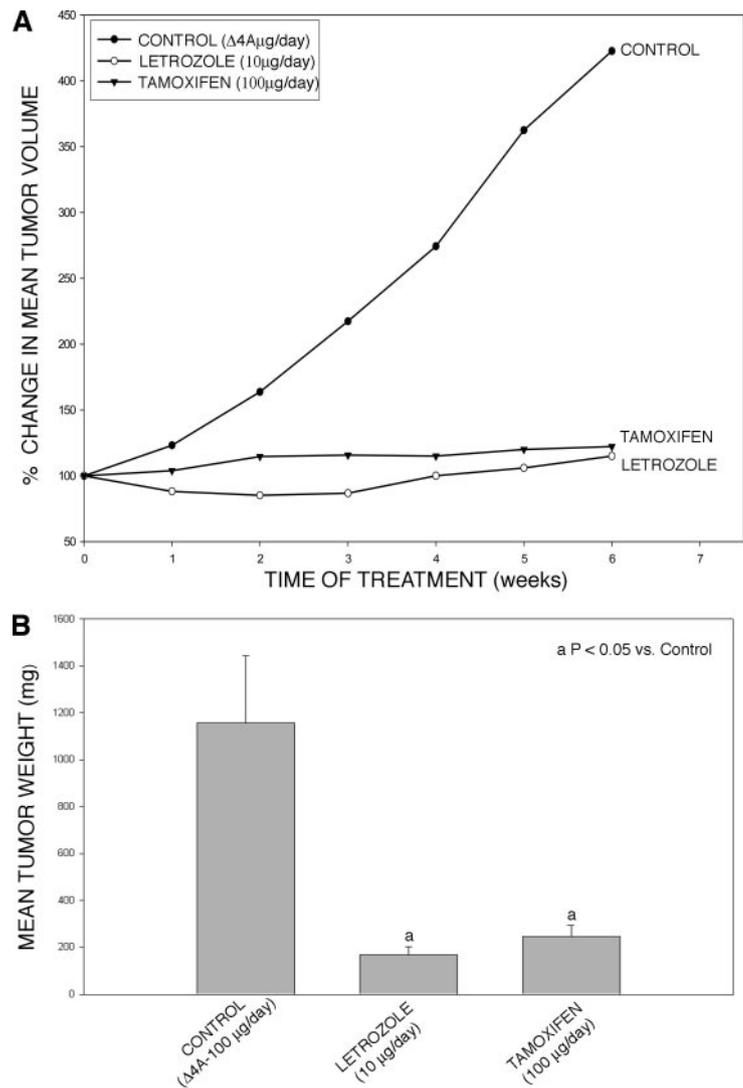
**Serum Hormone Analyses.** IGF-I levels were determined using a radioimmunoassay kit from DSL (Webster, TX) as described previously (24). Leptin levels were analyzed with a mouse leptin radioimmunoassay kit from Linco. One extracted aliquot of serum from each mouse was analyzed in duplicate.

**Data Analysis.** Analysis of variance and analysis of covariance were computed to estimate treatment effects on body composition or bone characteristics, with analysis of covariance to adjust for body weight in the analysis (25). All analyses were computed using SAS JMP Version 5.0 (SAS Institute Inc., Cary, NC). We report effect tests for treatment and body weight estimates where appropriate, as well as *a posteriori* means comparisons using Tukey's Honestly Significant Difference procedure (SAS Institute Inc.).

## RESULTS

**Tumor Growth Rate.** Human estrogen receptor-positive breast cancer cells transfected with the aromatase gene (MCF-7CA cells) were inoculated in Matrigel subcutaneously into ovariectomized nude mice. Because nude mice have a reduced production of adrenal steroids, they were supplemented with androstenedione, the substrate for aromatase, throughout the experiment at a dose of 0.1 mg/mouse/day, administered subcutaneously. Androstenedione does not affect tumor growth directly (20). Mice bearing MCF-7CA tumors were treated with tamoxifen or letrozole for 7 weeks. Previous studies using this tumor model have shown that letrozole at 10 μg/day was superior to tamoxifen at 60 μg/day at inhibiting tumor growth (9). However, in the present study, letrozole at 10 μg/day and tamoxifen at 100 μg/day inhibited tumor growth at a similar rate over time (Fig. 1A). Nevertheless, consistent with previous studies, a smaller amount of letrozole (10 μg/day) was needed

**Fig. 1** The effects of letrozole (10  $\mu\text{g}/\text{day}$ ) and tamoxifen (100  $\mu\text{g}/\text{day}$ ) treatment on the growth of MCF-7CA breast tumor xenografts in female ovariectomized athymic nude mice. Mice were inoculated with MCF-7CA human breast cancer cells at 2 sites/flank and supplemented daily with  $\Delta 4$ -androstenedione (100  $\mu\text{g}/\text{day}$ ) until mean tumor volumes were approximately 300  $\text{mm}^3$ . Mice were then divided into groups ( $n = 5$ ) and received daily subcutaneous injection with letrozole (10  $\mu\text{g}/\text{day}$ ) and/or tamoxifen (100  $\mu\text{g}/\text{day}$ ) in addition to the  $\Delta 4$ -androstenedione supplement. **A**, tumor volumes were measured weekly and are expressed as the percentage change relative to the initial total tumor volume. All groups were compared in one experiment. **B**, tumor weights were measured at autopsy.



to inhibit tumor growth, compared with the tamoxifen dose of 100  $\mu\text{g}/\text{day}$ . The final tumor weights in the letrozole and tamoxifen groups were significantly lower than those in the control group ( $P < 0.05$ ; Fig. 1B).

**Uterine Weight.** Uterine weight has been extensively used as a bioassay of the effects of estrogenic compounds. In our model, estrogen produced by the tumor is sufficient to maintain the uterine weight of the ovariectomized controls at approximately that of an intact mouse (21). Tamoxifen-treated mice had larger uteri than mice treated with letrozole or those of the control mice (Fig. 2). These findings are consistent with previously reported results of the agonistic effects of tamoxifen on the mouse uterus (26). Letrozole-treated mice had significantly smaller uteri than other animals.

**Body Composition.** There were moderate and statistically significant effects of treatment on weight, lean weight, and BMD ( $n = 29$  for all analyses: weight,  $P = 0.03$ ; % fat,  $P = 0.18$ ; lean weight,  $P = 0.012$ ; fat weight,  $P = 0.15$ ; bone density,  $P = 0.017$ ; and bone contents,  $P = 0.31$ ).

Comparisons of mean values for the four treatments (Table 1) show that ovariectomized control animals (receiving androstenedione only) had the smallest body weight, and animals treated with letrozole had the greatest body weight. Differences in body weight were not associated with tumor volume. Untreated ovariectomized control (not receiving androstenedione) and tamoxifen-treated mice had the highest percentage of body fat, whereas the letrozole-treated and the ovariectomized mice receiving androstenedione had lower percentages of body fat, but these differences were not statistically significant. Treatments also influenced lean weight; animals receiving letrozole had greater lean weights than animals receiving the other treatments. BMD was highest in animals treated with tamoxifen and lowest in untreated ovariectomized mice, as expected. Letrozole did not have a statistically significant effect on bone density or BMCs (Table 1). Overall, bone density and BMC were positively correlated ( $r = 0.80$ ;  $P < 0.001$ ;  $n = 29$ ).

Differences in bone density and fat content between treat-

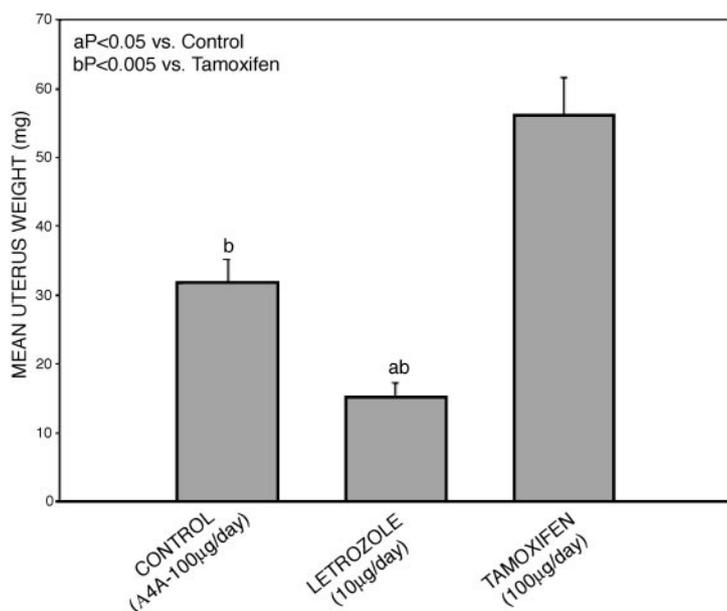


Fig. 2 The effect of letrozole and tamoxifen on uterine weights of mice carrying MCF-7CA breast cancer xenograft tumors. The uteri were removed, cleaned, and weighed from the same mice treated with tamoxifen (100  $\mu\text{g}/\text{day}$ ) or letrozole (10  $\mu\text{g}/\text{day}$ ) shown in Fig. 1. *a*,  $P < 0.05$  compared with control; *b*,  $P < 0.005$  compared with tamoxifen.

Table 1 Mean values for body composition variables  $\pm$  SE

Treatment	N	Weight* (g)	% Fat†	Lean weight (g)	Fat weight (g)	BMD (g/cm <sup>2</sup> )	BMC (g)
OVX	5	19.1 $\pm$ 0.9 <sup>ab</sup>	24.0 $\pm$ 1.2 <sup>a</sup>	14.0 $\pm$ 0.7 <sup>a</sup>	4.8 $\pm$ 0.2 <sup>a</sup>	0.049 $\pm$ 0.001 <sup>a</sup>	0.54 $\pm$ 0.02 <sup>a</sup>
OVX + A	8	18.4 $\pm$ 0.7 <sup>a</sup>	20.9 $\pm$ 1.0 <sup>a</sup>	14.0 $\pm$ 0.6 <sup>a</sup>	4.0 $\pm$ 0.3 <sup>a</sup>	0.053 $\pm$ 0.001 <sup>ab</sup>	0.51 $\pm$ 0.02 <sup>a</sup>
OVX + Let + A	8	21.6 $\pm$ 0.7 <sup>b</sup>	22.3 $\pm$ 1.0 <sup>a</sup>	16.2 $\pm$ 0.6 <sup>b</sup>	4.6 $\pm$ 0.2 <sup>a</sup>	0.052 $\pm$ 0.001 <sup>ab</sup>	0.56 $\pm$ 0.02 <sup>a</sup>
OVX + Tam + A	8	19.0 $\pm$ 0.7 <sup>ab</sup>	23.2 $\pm$ 1.0 <sup>a</sup>	14.1 $\pm$ 0.6 <sup>a</sup>	4.6 $\pm$ 0.2 <sup>a</sup>	0.054 $\pm$ 0.001 <sup>b</sup>	0.56 $\pm$ 0.02 <sup>a</sup>

NOTE. Means sharing a letter superscript are not significantly different based on the Tukey-Kramer HSD test.

Abbreviations: OVX, ovariectomized; A, androstenedione, 100  $\mu\text{g}/\text{day}$ ; Let, letrozole, 10  $\mu\text{g}/\text{day}$ ; Tam, tamoxifen, 100  $\mu\text{g}/\text{day}$ ; HSD, Honestly Significant Difference.

\* All means and SEs are from one-way analysis of variance; weight was obtained after necropsy.

† Percentage of fat reported from GE Lunar Piximus dual-energy X-ray absorptiometer output.

ments could be mediated by changes in fat weight, lean weight, or both. Fat weight was positively correlated with lean body weight ( $r = 0.64$ ;  $P = 0.0002$ ;  $n = 29$ ), indicating that overall changes in weight between treatments were caused by changes in both lean and fat weight. Both fat and lean weight contributed to the increased size of letrozole-treated animals, but changes in lean weight predominated (Table 1). Bone density was positively correlated with body mass ( $r = 0.15$ ;  $n = 29$ ), but the association was not statistically significant ( $P = 0.45$ ). Bone content increased with mouse weight ( $r = 0.52$ ;  $n = 29$ ;  $P = 0.003$ ).

#### Serum Leptin and Insulin-Like Growth Factor I Levels.

Both leptin and IGF-I have been shown to regulate bone metabolism (11, 17). Leptin regulates bone formation via the sympathetic nervous system (17). Evidence suggests that IGF-I is necessary for bone growth and remodeling (11). Therefore, serum IGF-I and leptin levels were measured to determine whether tamoxifen and letrozole affected their circulating levels. The data show that tamoxifen and letrozole did not affect the levels of these hormones in the serum (Table 2). Leptin levels were positively associated with body fat levels ( $r = 0.40$ ).

## DISCUSSION

Our objective was to evaluate the effects of tamoxifen and the aromatase inhibitor letrozole on mammary tumor growth, bone and body composition, and uterine weight. For this purpose, we used human estrogen-dependent breast cancer cells stably transfected with the aromatase gene (MCF-7CA cells). These cells grow as tumors when inoculated in Matrigel subcutaneously into ovariectomized nude mice supplemented with androstenedione. The model simulates postmenopausal breast

Table 2 Serum leptin and IGF-I levels (ng/ml) from control, letrozole-treated, and tamoxifen-treated ovariectomized female nude mice

Treatment	N	Leptin	IGF-I
OVX	5	1.96 $\pm$ 0.41	481 $\pm$ 50
OVX + A	8	1.39 $\pm$ 0.29	449 $\pm$ 22
OVX + Let + A	8	1.23 $\pm$ 0.24	452 $\pm$ 36
OVX + Tam + A	8	1.60 $\pm$ 0.44	526 $\pm$ 16

NOTE. Values represent mean  $\pm$  SE.

Abbreviations: OVX, ovariectomized; A, androstenedione, 100  $\mu\text{g}/\text{day}$ ; Let, letrozole, 10  $\mu\text{g}/\text{day}$ ; Tam, tamoxifen, 100  $\mu\text{g}/\text{day}$ .

cancer in several respects, including the absence of ovarian function and the lack of feedback regulation of estrogen production by gonadotropins (9). When tumors reached a measurable size, mice were treated with tamoxifen or letrozole for 7 weeks.

A major concern with estrogen-lowering drugs is that they may promote osteoporosis or decrease bone density, which in turn can lead to an increased number of bone fractures in patients (7). In addition, it has been shown that tamoxifen increases the risk of developing invasive endometrial cancer compared with women receiving placebo (27). In this study, bone density and uterine weight were measured in animals receiving treatment with tamoxifen and letrozole in a preclinical model. Consistent with data from others (28, 29), we show that tamoxifen treatment promotes BMD (Table 1) and uterine hypertrophy (Fig. 2). The aromatase inhibitor letrozole did not affect BMD (Table 1). Mice treated with letrozole had significantly smaller uteri than controls receiving androstenedione and tamoxifen-treated mice (Fig. 2), suggesting that this aromatase inhibitor does not have estrogenic effects. In this model, ovariectomized mice were supplemented with androstenedione that is aromatized into estrogen. Thus, control uteri were similar to uteri of intact cycling mice. Results suggest that the effects of tamoxifen and letrozole on the bone and the uteri are independent of alterations in systemic leptin and IGF-I levels because no differences in leptin and IGF-I levels were observed (Table 2).

We also compared the antitumor effects of tamoxifen and letrozole. Tamoxifen has been shown to be beneficial as an adjuvant treatment during chemotherapy for women with estrogen receptor-positive tumors. Furthermore, it has also been shown to prevent breast cancers in women who are at high risk of developing the disease (30). Long-term tamoxifen treatment may eventually lead to resistance of breast cancers to tamoxifen (8). Aromatase inhibitors are effective in treating tamoxifen-resistant cancers as second-line agents (9). Recently, aromatase inhibitors have been found to have several advantages over tamoxifen in the treatment of advanced breast cancer, in adjuvant therapy, and in early-stage breast cancer (8, 9). Similar to previous results in our preclinical model (9), we showed here that a lower dose of letrozole (10  $\mu\text{g}/\text{day}$ ) was as effective as a higher dose of tamoxifen (100  $\mu\text{g}/\text{day}$ ) at inhibiting breast cancer cell growth.

The human breast carcinoma xenograft model in nude mice has been used effectively to model human breast cancer (9). The uterotrophic assay that utilizes estrogen-induced growth of the uterus in either sexually immature rodents (in which the uterus has yet to develop) or ovariectomized rodents (in which the uterus has regressed with the loss of ovarian estrogen drive) is one of the principal assays used to evaluate the estrogenic and antiestrogenic properties of chemicals. In the present study, as expected, tamoxifen increased uterine weight, whereas letrozole reduced uterine weight relative to the controls. These effects of tamoxifen on the uterus and bone are consistent with previous reports (28, 29, 31). However, nude mice are immunodeficient, and others have shown that T-cell-deficient mice are protected against bone loss induced by ovariectomy (32). Thus, one needs to be cautious about interpreting how closely these results with letrozole in the MCF-7CA tumor model in nude mice relate to potential effects in humans. However, BMD was lowest in

ovariectomized mice not supplemented with androstenedione. Here, we showed that tamoxifen increased BMD and that letrozole did not affect BMD in comparison with controls receiving androstenedione.

In summary, this study showed that tamoxifen decreased MCF-7CA tumor cell growth and increased BMD but induced uterine hypertrophy. In contrast, letrozole (which also decreased MCF-7CA tumor growth at a lower concentration than tamoxifen) did not influence bone density or stimulate uterine hypertrophy. Taken together, these results in a preclinical model for breast cancer suggest that letrozole is a safe and effective alternative or complement to tamoxifen therapy for breast cancer.

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# Clinical Cancer Research

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