The Effect of Second-Line Antiestrogen Therapy on Breast Tumor Growth after First-Line Treatment with the Aromatase Inhibitor Letrozole: Long-Term Studies Using the Intratumoral Aromatase Postmenopausal Breast Cancer Model[1]

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ABSTRACT

Purpose: **The aromatase inhibitors letrozole and anastrozole have been approved recently as first-line treatment options for hormone-dependent advanced breast cancer. Although it is established that a proportion of patients who relapse on first-line tamoxifen therapy show additional responses to aromatase inhibitors, it has not been determined whether tumors that acquire resistance to aromatase inhibitors in the first line remain sensitive to second-line therapy with antiestrogens. The aim of this study was to determine whether aromatase-transfected and hormone-dependent MCF-7Ca human breast cancer cells remain sensitive to antiestrogens after: (*a*) long-term growth in steroid-depleted medium *in vitro;* and (*b*) long-term treatment with the aromatase inhibitor letrozole *in vivo*.**

Methods: **In the first approach, a variant of the MCF-7Ca human breast cancer cell line was selected that had acquired the ability to grow in estrogen-depleted medium after 6–8 months of culture. Steroid-deprived UMB-1Ca cells were analyzed for aromatase activity levels, hormone receptor levels, and sensitivity to estrogens and antiestrogens *in vitro* and *in vivo*. In the second approach, established MCF-7Ca breast tumor xenografts were treated with letrozole 10 μg/day for 12 weeks followed by 100 μg/day for 25 weeks until tumors acquired the ability to proliferate in the presence of the drug. Long-term letrozole-treated tumors were then transplanted into new mice, and the effects of antiestrogens and aromatase inhibitors on tumor growth were determined.**

Results: **Steroid-deprived UMB-1Ca breast cancer cells continued to express aromatase activity at levels comparable with the parental cell line. However, compared with MCF-7Ca cells, UMB-1Ca cells expressed elevated levels of functionally active estrogen receptor. The growth of UMB-1Ca cells *in vitro* was inhibited by the antiestrogens tamoxifen and faslodex and tumor growth *in vivo* was inhibited by tamoxifen. In the second approach, the time for MCF-7Ca tumor xenografts to approximately double in volume after being treated sequentially with the increasing doses of letrozole was thirty-seven weeks. Long-term letrozole-treated tumors continued to express functionally active aromatase. When transplanted into new mice, growth of the long-term letrozole-treated tumors was slowed by tamoxifen and inhibited more effectively by faslodex. Tumor growth was refractory to the aromatase inhibitors anastrozole and formestane but, surprisingly, showed sensitivity to letrozole.**

Conclusions: **Steroid-deprived UMB-1Ca human breast cancer cells selected *in vitro* and long-term letrozole-treated MCF-7Ca breast tumor xenografts remain sensitive to second-line therapy with antiestrogens and, in particular, to faslodex. This finding is associated with increased expression of functionally active estrogen receptor after steroid-deprivation of MCF-7Ca human breast cancer cells *in vitro*.**

INTRODUCTION

Adjuvant tamoxifen therapy has been demonstrated to be beneficial for patients with advanced breast cancer and as an adjuvant therapy for primary breast cancer (1, 2). Tamoxifen also protects against contralateral breast cancer (3) and prevents the development of breast cancer in high-risk women (4). Many patients who are initially responsive to tamoxifen therapy ultimately acquire drug resistance, and a proportion of these patients remain sensitive to second-line endocrine therapies. The aromatase inhibitors anastrozole, letrozole, and exemestane have established themselves recently as the second-line therapies of choice for tamoxifen-relapsed breast cancer. Anastrozole, letrozole, and exemestane are superior to megestrol acetate (5–7), and letrozole is also better than aminoglutethimide (8). These aromatase inhibitors are very specific and selective for the aromatase enzyme and are well tolerated by patients. One of the reasons why aromatase inhibitors are effective second-line therapies is that their mechanism of action differs from tamoxifen. Tamoxifen blocks the action of estrogen at the receptor level, whereas aromatase inhibitors block the synthesis of estrogen in peripheral tissues including the breast. However, tamoxifen is a partial estrogen agonist in the breast, which may result in less than optimal antitumor activity. Aromatase inhibitors, on the other hand, do not have estrogenic effects and may be a more effective first-line treatment option.

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Anastrozole, letrozole, and exemestane are proving to be effective first-line treatment options for patients with hormone-dependent advanced breast cancer. In two separate trials, anastrozole was shown to be either equivalent to (9) or superior to tamoxifen (10). A combined analysis indicated that anastrozole was a superior therapy to tamoxifen (11). Anastrozole is also beneficial in the neoadjuvant setting (12). Similar encouraging results have been reported for the steroidal aromatase inhibitor exemestane (13, 14). Letrozole has also been shown to be superior to tamoxifen for the first-line treatment of hormone-dependent advanced breast cancer (15) and is also better than tamoxifen in the neoadjuvant setting (16, 17). Anastrozole and letrozole are presently being compared with tamoxifen as adjuvant therapies for early breast cancer. The data reported to date suggest that aromatase inhibitors may become the preferred first-line therapy for postmenopausal patients with hormone-responsive breast cancer (18). As with all treatments for cancer, patients treated first-line with an aromatase inhibitor are likely to eventually acquire drug-resistance, and the most appropriate second-line therapy has not been established. There is preliminary data suggesting that tamoxifen may be an effective second-line therapy in advanced breast cancer patients refractory to anastrozole (19). However, the optimal second-line therapy for patients who progress after aromatase inhibitor treatment has not been determined.

As a guide for optimizing treatment strategies for postmenopausal breast cancer patients, we developed the intratumoral nude mouse breast cancer model that is sensitive to both antiestrogens and aromatase inhibitors (20, 21). In this model, hormone-responsive MCF-7 human breast cancer cells are stably transfected with the human aromatase gene (*MCF-7Ca*) and serve as the source of estrogens in female ovariectomized athymic nude mice. This model is similar to the situation in postmenopausal breast cancer patients where the major sources of estrogens are in nonovarian tissues, including the breast (22, 23). Treating postmenopausal breast cancer patients with an aromatase inhibitor such as letrozole abolishes ~99% of estrogen production (24). In the present study, we attempted to mimic this situation using two separate approaches. The first was to culture MCF-7Ca cells in steroid-depleted medium for an extended period of time. The resulting estrogen-deprived UMB-1Ca variant cell line was then tested for sensitivity to antiestrogens *in vitro* and *in vivo*. The second approach was to treat established MCF-7Ca tumor xenografts growing in ovariectomized nude mice with letrozole until tumors acquired the ability to proliferate in the presence of the drug. The long-term letrozole-treated tumors were then transplanted into new animals, and the effects of second-line hormonal manipulations were investigated.

MATERIALS AND METHODS

Materials. MCF-7 human breast cancer cells stably transfected with the human aromatase gene (*MCF-7Ca*) were kindly provided by Dr. S. Chen (City of Hope, Duarte, CA; Ref. 25). DMEM, penicillin/streptomycin solution, trypsin-EDTA solution, DPBS,[3] and G418 were from Life Technologies, Inc. (Grand Island, NY). Phenol red-free improved minimum essential medium and phenol red-free trypsin-EDTA were from Biofluids Inc. (Rockville, MD). Fetal bovine serum and dextran-coated charcoal-treated serum were from Hyclone (Logan, UT). Matrigel was obtained from BD Biosciences (Bedford, MA). [1β-^{3}H]Δ4A (specific activity 24.9 Ci/mmol, [^{3}H]Δ4A), [2,4,6,7,16,17-^{3}H]estradiol (specific activity 118 Ci/mmol, [^{3}H]E_2), [17α-methyl-^{3}H]R5020 (specific activity 84 Ci/mmol, [^{3}H]R5020), and cold R5020 were from NEN (Boston, MA). Δ4A, E_2, tamoxifen, hydroxypropyl cellulose, NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, were from Sigma Chemical Co. (St. Louis, MO). Letrozole (CGS 20267) and anastrozole (CGP 63606) were kindly provided by Dr. D. Evans (Novartis Pharma A.G., Basel, Switzerland). The pure antiestrogen faslodex (ICI 182,780) was generously supplied by Dr. A. Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, England). Formestane (4-hydroxyandrostenedione) was synthesized in our laboratory as described previously (26).

Cell Culture. MCF-7Ca cells were routinely maintained in DMEM with 5% fetal bovine serum, 1% penicillin/streptomycin solution, and 750 μg/ml G418. For selection of estrogen-deprived UMB-1Ca cells, early passage (passage 6) MCF-7Ca cells were transferred into steroid-depleted medium, which consisted of phenol red-free improved minimum essential medium supplemented with 5% dextran-coated charcoal-treated serum, 1% penicillin/streptomycin, and 750 μg/ml G418. Cells were maintained in this medium for at least 6 months before any experiments. After 6 months of estrogen deprivation, UMB-1Ca cells had acquired the ability to proliferate in estrogen-depleted medium. For growth studies, MCF-7Ca cells were transferred into steroid-free medium for 3 days before plating (2×10^4 cells/well) into 24-well plates. The next day, cells were washed with DPBS and treated with steroid-free medium containing vehicle or the indicated concentrations of estrogens, antiestrogens, Δ4A, and aromatase inhibitor. The medium was changed every 3 days, and the cells were counted 9 days later using a Coulter Counter model Z-1 (Coulter Electronics, Hialeah, FL). The results were expressed as a percentage of the cell number in the vehicle-treated control wells.

Aromatase Activity Assays. The radiometric aromatase (^3H_2O) release assay was performed on MCF-7Ca and UMB-1Ca cells as described previously (27). Briefly, 3×10^5 MCF-7Ca cells or UMB-1Ca cells were plated into six-well plates. The following day the cells were incubated with 0.5 μCi of [^{3}H]Δ4A in 1 ml of medium for 2 h. Medium was then transferred to a glass tube and 300 μl of trichloroacetic acid was added to precipitate proteins. One ml of the mixture was extracted and mixed with 2 ml of chloroform to extract unconverted substrate and other steroids. A 0.7-ml aliquot of the aqueous phase was removed and mixed with 0.7 ml of a 2.5% activated charcoal suspension to remove residual steroids. Tri-

[3] The abbreviations used are: DPBS, Dulbecco's phosphate buffered saline; G418, geneticin; Δ4A, androstenedione; E_2, 17β-estradiol; R5020, promegestone; ER, estrogen receptor; PGR, progesterone receptor.

tiated water (^3H_2O) formed during the aromatization of [^{3}H]Δ4A to estrogen was measured by counting radioactivity in the supernatant. Nonspecific conversion was determined by performing the assays in the presence of a 100-fold excess of cold Δ4A and by performing the assay in wells that contained no cells.

Tumor aromatase activity levels were measured after homogenization of tumors in 0.5 ml PBS (pH 7.4) as described previously (20). The homogenate was incubated with 1 μCi of [^{3}H]Δ4A and an NADPH-generating system (NADP, 6.7 mM; glucose-6-phosphate, 70 mM; and glucose-6-phosphate dehydrogenase, 12.5 IU) at 37°C for 2 h under an atmosphere of oxygen. Unconverted steroids were removed as described above, and the ^3H_2O formed was measured by counting the radioactivity. The protein concentration of the homogenate was measured using the Bradford method (Bio-Rad, Hercules, CA).

Hormone Receptor Assays. ER and PGR levels were measured by whole cell binding assays essentially as described previously (28, 29). For PGR assays, MCF-7Ca cells were transferred into steroid-depleted medium for 3 days before experiments. Cells (1×10^5) were plated into 24-well dishes. Cells were then treated with ethanol vehicle or E_2 (1 nM) for 3 days. Specific binding to receptors was determined after incubation with increasing concentrations of [^{3}H]R5020 in the presence or absence of a 1000-fold excess of cold R5020 to determine nonspecific binding and dexamethasone (1 μM) to saturate glucocorticoid receptors. The data were analyzed using Graphpad Prizm software (San Diego, CA), which determined K_D and B_{max}. ER assays were performed in the same manner using a 1000-fold excess of cold E_2 to determine nonspecific binding.

Tumor Growth in Ovariectomized Female Athymic Nude Mice. All of the animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland School of Medicine. Ovariectomized female BALB/c athymic nude mice 4–6 weeks of age were obtained from Charles River Laboratories (Boston, MA). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity, and received food and water *ad libitum*. MCF-7Ca and UMB-1Ca tumors were grown in the animals as described previously (20, 21, 30, 31). Subconfluent cells were scraped into DPBS, collected by centrifugation, and resuspended in Matrigel (10 mg/ml) at 2.5×10^7 cells/ml. Each animal received s.c. inoculations in either one or two sites per flank (depending on experiment) with 100 μl of cell suspension. Unless noted, the animals were then injected daily with Δ4A (100 μg/day) for the duration of the experiment. Tumor volumes were measured weekly with calipers and were calculated by the formula $4/3\pi \times r_1^2 \times r_2$ ($r_1 < r_2$). Treatments began when the tumors reached a measurable size (~50 mm^3), 3–6 weeks after cell inoculation. Mice were then injected s.c. daily with the indicated drugs in addition to the Δ4A supplement. Drugs were prepared as suspensions in 0.3% hydroxypropyl cellulose. Animals were treated for the indicated times, after which time they were sacrificed by decapitation and the blood collected. Tumors and uteri were excised, cleaned, weighed, and stored in liquid nitrogen for additional analysis. For inoculation of tumor samples into new mice, the tumors were excised and chopped into small pieces in steroid-free medium. Tumor pieces were then incubated, stirring overnight with 1 mg/ml collagenase and hyaluronidase (Sigma Chemical Company) in steroid-free medium at 37°C. The next day the cells were washed extensively with DPBS, suspended in Matrigel, and inoculated into the new animals as described above.

Measurement of Tumor and Serum Estrogen Levels. As described previously (31), tumor and serum samples from each group were pooled and homogenized in PBS at 4°C. The steroids were extracted with diethyl ether, and E_2 was isolated by celite chromatography. E_2 concentrations were measured in the homogenates by RIA. The assay was performed using an E_2 antibody and iodinated E_2 (ICN, Boston, MA).

Statistical Analysis. Tumor volumes are expressed as either mean and SE or as a percentage change in tumor volume per group from the start of treatment. Final tumor weights at sacrifice of the animals were used to determine statistical differences from the control-treated animals. One-way ANOVA on SigmaStat for Windows version 2.0 was used to compare the different treatment groups at the 95% confidence level. All of the statistical tests were two sided, and differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Characterization of Estrogen-deprived UMB-1Ca Cells *in Vitro*. Early passage MCF-7Ca cells were transferred to steroid-depleted medium and examined twice weekly. For the first 2 weeks, cell growth was slower than that of parent MCF-7Ca cells. Cell growth then slowed dramatically for 4 weeks before the cells entered a period of proliferative quiescence. This lasted for ~4 weeks, after which time the cells began to proliferate slowly. Six months after the cells were transferred to steroid-depleted medium, normal growth had resumed and the resulting cell line, designated UMB-1Ca, proliferated at a rate comparable with the parent cell line. There were no morphological changes observed between estrogen-deprived UMB-1Ca cells and estrogen-dependent MCF-7Ca cells.

MCF-7Ca and UMB-1Ca cells were assayed for aromatase activity, and levels were similar in both cell lines. Aromatase activity levels in the MCF-7Ca cells were 50 fmol/100,000 cells/h and levels in the UMB-1Ca cells were 55 fmol/100,000 cells/h. To determine whether long-term steroid deprivation was associated with a change in expression of steroid hormone receptors, levels of ER and PGR binding sites were determined in both cell lines by whole-cell ligand-binding assays (Table 1). Compared with the parent MCF-7Ca cell line, estrogen-deprived UMB-1Ca cells expressed significantly higher numbers of ER ($P < 0.0001$). ER levels in the UMB-1Ca cells were approximately five times higher than in the MCF-7Ca cell line. ER functionality was accessed by determining PGR levels in the two cell lines in the presence and absence of E_2 (1 nM). Constitutive expression of PGR was similar in both cell lines, and in agreement with results published previously for wild-type MCF-7 human breast cancer cells (32, 33) and MCF-7Ca tumors (21). In both cell lines, PGR expression was induced by treatment with E_2 indicating that ER was functional. However, treatment with E_2 increased PGR levels 3.5-fold in MCF-7Ca cells and 13.1-fold in UMB-Ca cells. Estrogen-deprived UMB-1Ca cells responded to E_2 (1 nM) with a significantly higher ($P < 0.001$) induction of PGR than MCF-7Ca cells.

Table 1 ER and PGR expression levels in estrogen-dependent MCF-7Ca cells and estrogen-independent UMB-1Ca cells as determined by whole cell ligand binding assays

	ER		PGR ($-E_2$)		PGR ($+E_2$)	
Cell Line	K_D (nM)	B_{max} (receptors/cell)	K_D (nM)	B_{max} (receptors/cell)	K_D (nM)	B_{max} (receptors/cell)
MCF-7Ca	0.19 ± 0.05[a]	55,534 ± 4,318	0.62 ± 0.04	24,493 ± 1,070	0.58 ± 0.09	86,142 ± 9,176
UMB-1Ca	0.12 ± 0.01	249,520 ± 11,015[b]	0.42 ± 0.06	18,434 ± 3,062	0.63 ± 0.03	241,580 ± 17,469[c]

[a] Values represent the mean and SE of at least three independent experiments.
[b] $P < 0.0001$ versus MCF-7Ca cells.
[c] $P < 0.001$ versus MCF-7Ca cells treated with E_2 (1 nM).

Growth Response to Estrogen, Antiestrogens, and Aromatase Inhibitor *in Vitro* and *in Vivo*. MCF-7Ca cells were transferred to steroid-depleted medium 3 days before plating for growth curves *in vitro*. When grown in an estrogen-deprived environment, MCF-7Ca cell growth slows, and the cells show an elevated growth response to E_2 (Fig. 1*A*). Cells were treated with E_2 in the range 1 fM to 1 nM. MCF-7Ca cells responded in a dose-dependent manner to E_2, and maximum growth stimulation was observed in the range of 1 pM to 1 nM. MCF-7Ca cell proliferation was stimulated 10-fold, 9-fold, 5.5-fold, and 2-fold by E_2 at concentrations of 1 nM, 0.1 nM, 10 pM, and 1 pM, respectively. Lower concentrations of E_2 (1 fM to 1 pM) had no effect on the proliferation of MCF-7Ca cells. In contrast to these results, UMB-1Ca cells, which have acquired the ability to grow in an estrogen-deprived environment, did not respond to treatment with E_2 with such a dramatic increase in cell number. Maximum growth stimulation was observed at 10 pM E_2 (1.9-fold), and concentrations of 1 fM to 0.1 pM E_2 had no effect on cellular proliferation. Thus, despite the significantly higher levels of ER, UMB-1Ca cells did not show an increased growth response to E_2 and were less sensitive to its effects than MCF-7Ca cells.

Growth of both MCF-7Ca cells and UMB-1Ca cells were significantly inhibited by the antiestrogens tamoxifen (1 μM) and faslodex (1 μM; Fig. 1*B*). UMB-1Ca cells were more sensitive to the growth inhibitory effects of the antiestrogens than MCF-7Ca cells. Faslodex (1 μM) inhibited the growth of MCF-7Ca cells by 50%, but the growth of UMB-1Ca cells was inhibited by 80%. In MCF-7Ca cells, the growth inhibitory effects of faslodex (1 μM) were reversed by E_2 (1 nM). However, in the presence of E_2 (1 nM) faslodex continued to inhibit the growth of UMB-1Ca cells by 40%. A dose-response study was performed to determine the concentration of E_2 that was required to reverse the growth inhibitory effects of a lower dose of faslodex (1 nM) in both cell lines (Fig. 1*C*). Cells were cotreated with faslodex (1 nM) and E_2 in the range 0.1 pM to 10 nM. The antiproliferative effect of faslodex on MCF-7Ca cell growth was not affected by the lower concentrations of E_2 (0.1 pM to 10 pM). However, the effects of faslodex were reversed at 0.1 nM E_2, and at a concentration of 10 nM, E_2 stimulated MCF-7Ca cell proliferation even in the presence of faslodex. In comparison, the faslodex-induced growth inhibition of UMB-1Ca cells was reversed by 10 nM E_2. Therefore, compared with MCF-7Ca cells, UMB-1Ca cells required a 100-fold higher concentration of E_2 to reverse the growth inhibitory effects of faslodex (1 nM).

Growth studies were also performed using Δ4A (25 nM) as the aromatizable source of estrogens (Fig. 1*D*). In agreement with the results obtained with E_2 (Fig. 1*A*), MCF-7Ca responded to Δ4A with significant growth stimulation ($P < 0.01$). Stimulation of MCF-7Ca cell growth by Δ4A was inhibited by letrozole at each of the concentrations tested (1 nM to 1 μM). In contrast, UMB-1Ca cells, which had acquired the ability to proliferate in a steroid-depleted environment, showed only a moderate and not significant growth response to Δ4A. Consequently, compared with MCF-7Ca cells, letrozole (1 nM to 1 μM) appeared not be as effective at inhibiting Δ4A-induced UMB-1Ca cell proliferation.

The effect of endocrine manipulation on UMB-1Ca tumor growth was also determined *in vivo*. Female ovariectomized athymic nude mice were inoculated with two MCF-7Ca cell suspensions on the right flank and two UMB-1Ca cell suspensions on the left flank. In the first experiment the animals were then treated daily from the day of cell inoculation with 0.3% hydroxypropyl cellulose vehicle, Δ4A (100 μg/day), or Δ4A (100 μg/day) plus letrozole (10 μg/day). In agreement with our results published previously (20), MCF-7Ca cells formed very small tumors without administration of the aromatase substrate Δ4A because adrenal androgen production in nude mice is weak (Fig. 2*A*). In addition, MCF-7Ca cells were not tumorigenic when the animals were cotreated with Δ4A and letrozole. In contrast to these results, UMB-1Ca cells were tumorigenic without Δ4A and in the presence of letrozole. However, UMB-1Ca tumor growth was significantly higher when the animals were treated with Δ4A ($P < 0.01$; Fig. 2*B*).

In the second experiment, the effects of tamoxifen and letrozole on tumor growth were determined (Fig. 3*A*). Animals bearing two MCF-7Ca tumors on the right flank and two UMB-1Ca tumors on the left flank were supplemented with Δ4A for 3 weeks until tumor volume was ~50 mm^3. The animals were then divided into three groups for continued supplementation with Δ4A (100 μg/day), for treatment with Δ4A plus letrozole (10 μg/day), or for Δ4A plus tamoxifen (100 μg/day). MCF-7Ca tumors in the animals receiving Δ4A increased 4.6-fold over the duration of the experiment. As reported previously (30, 31), letrozole induced marked tumor regression in the MCF-7Ca tumors (Fig. 3*A*). Here, the initial tumor volume was reduced by 45% after a 6-week treatment with letrozole. MCF-7Ca tumors in the mice treated with tamoxifen did not regress, but tumor growth rate was significantly reduced compared with the control (Δ4A) tumors. Tamoxifen-treated MCF-7Ca tumors increased by only 0.45-fold over the duration of the experiment (Fig. 3*A*). In the same animals, tumors on the opposite flank grown from

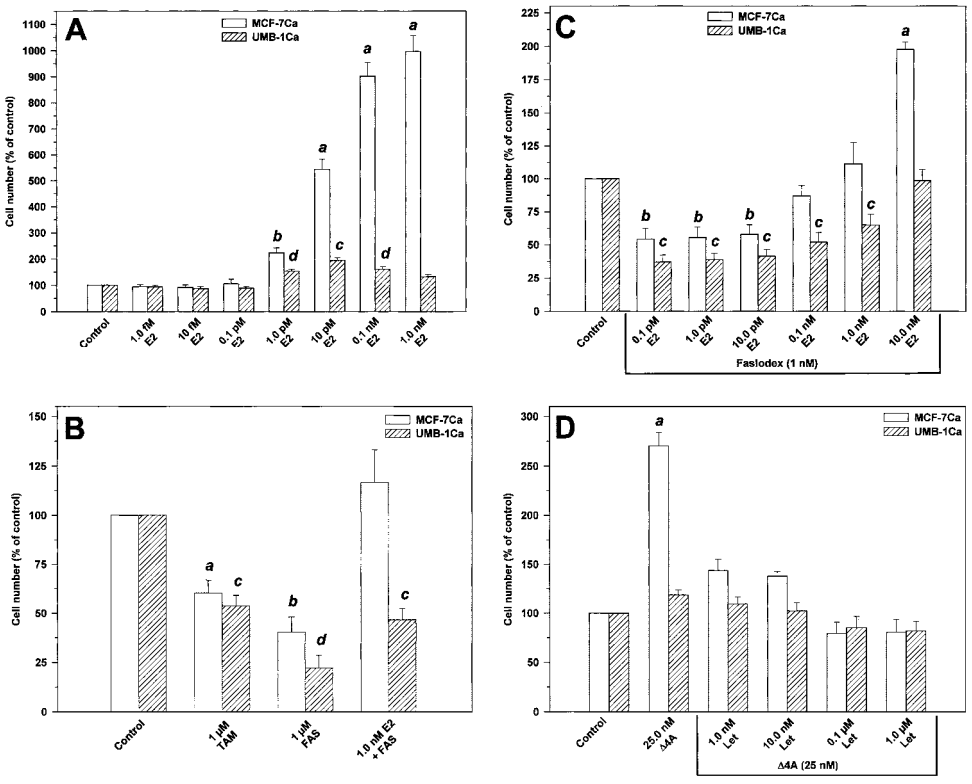

Fig. 1 The effect of (*A*) E_2 and (*B*) tamoxifen (*TAM*), faslodex (*FAS*), Δ4A, and letrozole (*LET*) on the growth of MCF-7Ca and UMB-1Ca human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating. Triplicate wells were then treated with the indicated concentrations of E2, antiestrogens, Δ4A, or aromatase inhibitor for 9 days, and the medium was refreshed every 3 days. Cell numbers are expressed as a percentage of the mean number of cells in the control (vehicle-treated) wells. The results show the mean from triplicate experiments; *bars*, ±SE. *A: a*, $P < 0.0001$ *versus* MCF-7Ca control; *b*, $P < 0.01$ *versus* MCF-7Ca control; *c*, $P < 0.01$ *versus* UMB-1Ca control; *d*, $P < 0.02$ *versus* UMB-1Ca control. *B: a*, $P < 0.05$ *versus* MCF-7Ca control; *b*, $P < 0.02$ *versus* MCF-7Ca control; *c*, $P < 0.02$ *versus* UMB-1Ca control; *d*, $P < 0.01$ *versus* UMB-1Ca control. *C: a*, $P < 0.01$ *versus* MCF-7Ca control; *b*, $P < 0.02$ *versus* MCF-7Ca control; *c*, $P < 0.01$ *versus* UMB-1Ca control. *D: a*, $P = 0.01$ *versus* MCF-7Ca control.

UMB-1Ca cells increased 3.8-fold in the animals supplemented with the aromatase substrate Δ4A. In contrast to the results obtained with the MCF-7Ca tumors, letrozole did not cause regression of UMB-1Ca tumor growth indicating that the tumors had the ability to grow in the presence of the aromatase inhibitor. UMB-1Ca tumor volumes increased 2-fold in the animals treated with letrozole. Nevertheless, UMB-1Ca tumors retained sensitivity to the antiestrogen tamoxifen *in vivo*. UMB-1Ca tumors in the animals treated with tamoxifen increased only 0.45-fold during the 6 weeks of treatment, and tumor weights were significantly lower ($P < 0.001$) compared with the animals receiving Δ4A only (Fig. 3*B*).

Three MCF-7Ca and UMB-1Ca tumors from the animals receiving the Δ4A supplement were assayed for aromatase activity levels (Table 2). Both MCF-7Ca and UMB-1Ca tumors expressed aromatase activity, and there was no significant difference in the levels of activity. In addition, the uteri of the animals treated with letrozole weighed significantly less ($P < 0.001$) than those receiving only the Δ4A supplement (Fig. 3*B*). The reduction in uterine weights in the letrozole-treated animals correlated with a decrease in serum E_2 levels to 10 pg/ml compared with 62 pg/ml in the control, Δ4A-supplemented animals. In contrast, the uteri of the animals treated with tamoxifen were hyperplasic, and their weight was significantly higher ($P < 0.001$) than the Δ4A-supplemented animals. There were no appreciable differences in the serum levels of E_2 in the Δ4A-supplemented (62 pg/ml) and tamoxifen-treated mice (74 pg/ml).

Long-Term Treatment of MCF-7Ca Tumors with Letrozole. To determine the time for MCF-7Ca tumors to progress on therapy with letrozole, established MCF-7Ca tumor xenografts were treated with the drug until tumors acquired the ability to proliferate. Tumors were determined to be proliferating on letrozole therapy when they had doubled in volume. Mice were inoculated with MCF-7Ca cells at one site per flank. The animals were supplemented daily with Δ4A for 3 weeks to promote tumor growth. Animals were then treated daily with letrozole (10 μg/day) in addition to the Δ4A supplement (Fig. 4). As described above, letrozole caused marked tumor regression. For the first 4 weeks of treatment MCF-7Ca tumor volume regressed to 37% of the initial volume (63% growth inhibition). After this period the tumors began to slowly proliferate in the presence of letrozole. After an additional 8weeks of treatment with 10 μg/day letrozole, the dose was increased to

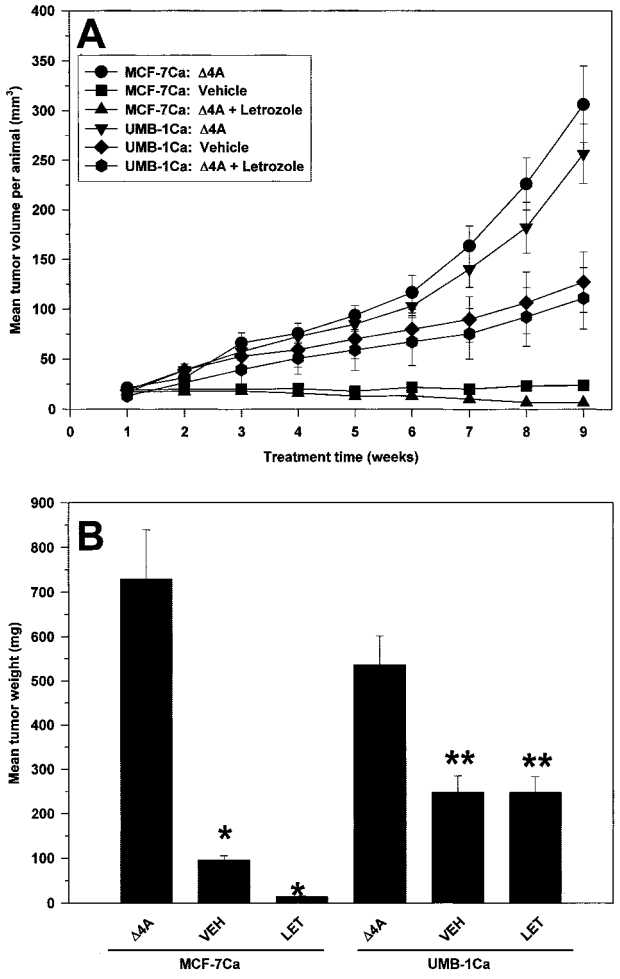

Fig. 2 The effect of vehicle, Δ4A (100 μg/day) and Δ4A plus letrozole (10 μg/day) on the growth of MCF-7Ca and UMB-1Ca breast tumor xenografts in female ovariectomized athymic nude mice. Groups of five mice received two MCF-7Ca cell inoculations on the right flank and two UMB-1Ca cell inoculations on the left flank (2×10^6 cells/inoculation). From the day of cell inoculation, animals were treated daily with vehicle, Δ4A, or Δ4A plus letrozole. *A*, tumor volumes were measured weekly and values are expressed as mean tumor volume per animal; *bars*, ±SE. *B*, at the end of the experiments the animals were sacrificed, and the tumors were removed, cleaned, and weighed. Final tumor weights that were significantly lower than the Δ4A-treated animals are shown. *, $P < 0.0001$ *versus* MCF-7Ca (*Δ4A*); **, $P < 0.001$ *versus* UMB-1Ca (*Δ4A*).

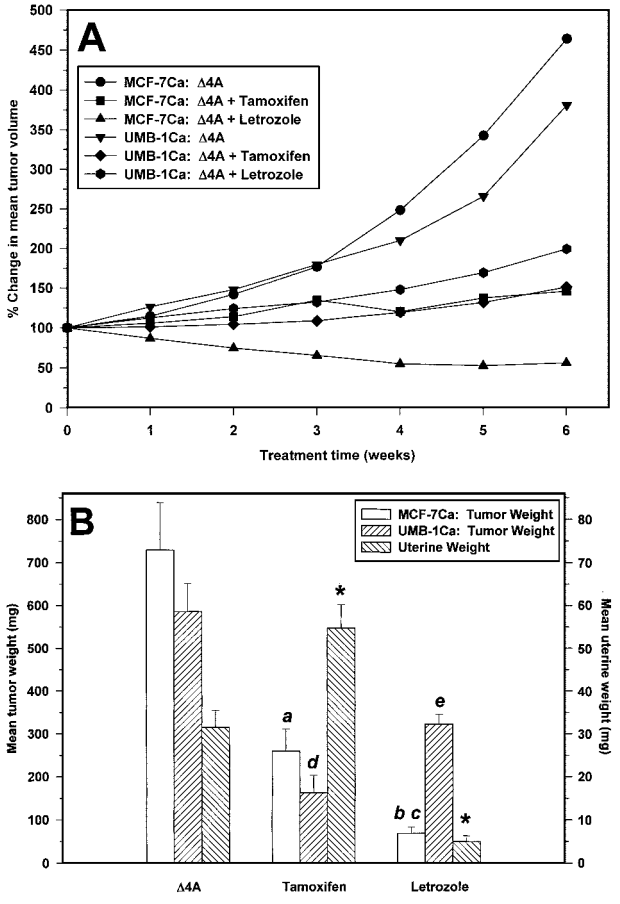

Fig. 3 The effect of tamoxifen (100 μg/day) and letrozole (10 μg/day) on the growth of MCF-7Ca and UMB-1Ca breast tumor xenografts in female ovariectomized athymic nude mice. Mice received two MCF-7Ca cell inoculations on the right flank and two UMB-1Ca cell inoculations on the left flank (2×10^6 cells/inoculation). Animals were treated daily with Δ4A for the duration of the experiment. After 3 weeks of treatment when tumor volume was ~50 mm³, the animals were grouped ($n = 5$) for treatment with tamoxifen or letrozole. *A*, tumor volumes were measured weekly, and values are expressed as the percentage of change in mean tumor volume. *B*, at the end of the experiments the animals were sacrificed, and the tumors and uteri were removed, cleaned, and weighed. Final tumor weights that were significantly lower than the Δ4A-treated animals are shown. *a*, $P < 0.02$ *versus* MCF-7Ca (*Δ4A*); *b*, $P < 0.0001$ *versus* MCF-7Ca (*Δ4A*); *c*, $P < 0.005$ *versus* MCF-7Ca (*tamoxifen*); *d*, $P < 0.001$ *versus* UMB-1Ca (*Δ4A*), e $P < 0.05$ *versus* UMB-1Ca (*Δ4A*). Compared with the Δ4A-treated animals, uterine weights were significantly higher in the animals treated with tamoxifen and significantly lower in the animals treated with letrozole (*, $P < 0.001$); *bars*, ±SE.

100 μg/day. At the 100 μg/day dose of letrozole, tumors regressed again (Fig. 4). After an additional 9 weeks of treatment with 100 μg/day letrozole, tumors had regressed by 43% of the volume measured at week 15. MCF-7Ca tumors then began to slowly proliferate in the presence of this dose of letrozole (100 μg/day). After a total treatment period of 34 weeks, tumor volumes had returned to the initial pretreated levels. In the following 3 weeks of treatment tumor volumes doubled. After 37 weeks of treatment with letrozole, the animals were sacrificed, and the tumors were removed for additional experiments.

Three of the tumors were homogenized and assayed for aromatase activity using the tritiated water release assay (Table 2). Aromatase activity levels in the long-term letrozole-treated tumors were comparable with those in the untreated MCF-7Ca tumors. However, compared with untreated Δ4A-supplemented MCF-7Ca tumors, tumor E_2 concentrations in the long-term letrozole-treated tumors were lowered from 2305 ± 425 pg/g tissue to 240 ± 20 pg/g tissue. The serum E_2 levels in the long-term letrozole-treated mice were also reduced from 62 pg/ml to 32 pg/ml.

Table 2 Tumor aromatase activity levels, tumor E_2 concentrations, and serum E_2 levels in MCF-7Ca, UMB-1Ca, long-term letrozole-treated (LTLT), and transplanted letrozole-treated (TLT) tumors grown in female ovariectomized athymic nude mice

Tumor	Aromatase activity (fmol/mg protein/h)	Tumor estradiol (pg/g tissue)	Serum estradiol (pg/ml)
MCF-7Ca (Δ4A)	213.55 ± 11.29[a]	2305 ± 425.00[b]	62[c]
UMB-1Ca (Δ4A)	252.24 ± 10.13	2080 ± 60.00	62
LTLT	200.81 ± 16.19	240 ± 20.00	32
TLT (Δ4A)	289.90 ± 23.51	1890 ± 120.00	63
TLT (Letrozole)	322.39 ± 25.02	440 ± 30.00	19
TLT (Anastrozole)	293.11 ± 20.50	651 ± 201.00	11
TLT (Formestane)	423.09 ± 35.86	1084 ± 328.00	8
TLT (Vehicle)	335.75 ± 24.73	420 ± 80.00	7

[a] Values represent the means and SE from three tumors from each group.

[b] Values represent the means and SE from two tumors from each group.

[c] Values represent the means and SE from serum pooled from three to five animals.

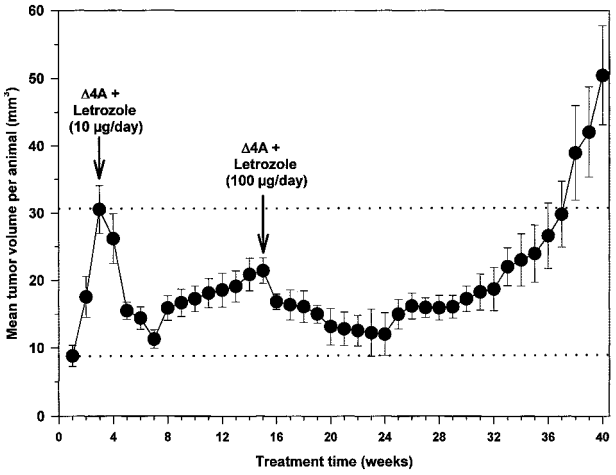

Fig. 4 The effect of long-term letrozole treatment on the growth of MCF-7Ca human breast tumor xenografts in female ovariectomized athymic nude mice supplemented with Δ4A (100 μg/day). A group of five mice were inoculated with 2×10^6 MCF-7Ca cells at one site per flank, and tumor volumes were measured weekly. Animals were treated daily with Δ4A for the duration of the experiment. Three weeks after cell inoculation, animals were treated with Δ4A and letrozole (10 μg/day). After 12 weeks of treatment, the letrozole dose was increased to 100 μg/day. Values are expressed as mean tumor volume per animal; *bars*, ±SE.

Growth of Transplanted Letrozole-treated Tumors *in Vivo*. To determine the effect of sequential treatment on tumors progressing on letrozole treatment, one of the largest tumors was minced into small pieces and dissociated into a single cell suspension that was inoculated into new female ovariectomized athymic nude mice at one site per flank. With the exception of a group of five mice, which were injected daily with the vehicle hydroxypropyl cellulose, all of the other mice were supplemented daily with Δ4A (100 μg/day). Tumor volumes were measured weekly with calipers. Transplanted tumors grew equally well in the presence and absence of Δ4A, indicating that the aromatase substrate was not required for cell proliferation (Fig. 5). After 8 weeks of Δ4A supplementation when mean tumor volume was ~50 mm^3, the animals were grouped (five mice per group) for continued supplementation with Δ4A (100 μg/day) or for treatment with tamoxifen (100 μg/day), faslodex (1 mg/day), letrozole (100 μg/day), anastrozole (100 μg/day), and formestane (1 mg/day) in addition to the Δ4A supplement (Fig. 6*A*).

The control Δ4A-supplemented tumors increased 6.4-fold over the 8 week duration of treatment. Anastrozole- and formestane-treated tumors increased in volume by 5.5-fold and 6.0-fold, respectively. Tumor weights in these treatment groups were not significantly different compared with the Δ4A-supplemented tumors (Fig. 6*B*). After the long-term treatment with letrozole, tumors retained sensitivity to the antiproliferative effects of antiestrogens. Both tamoxifen and faslodex significantly slowed tumor growth. Tumor volumes in the mice treated with tamoxifen increased 4-fold after 8 weeks of treatment. However, between weeks 7 and 8, tumor volume increased considerably. Tumors were very sensitive to the pure antiestrogen faslodex, and volumes increased by only 0.5-fold over the 8 weeks of treatment. Tumor weights were significantly reduced in the animals treated with tamoxifen ($P < 0.05$) and faslodex ($P < 0.01$; Fig. 6*B*). Moreover, tumor weights in the animals treated with faslodex were significantly lower ($P < 0.05$) compared with those of the animals treated with tamoxifen. Most surprisingly, the transplanted tumors remained sensitive to the aromatase inhibitor letrozole at the dose of 100 μg/day. Tumor volumes of the letrozole-treated animals increased by only 2.4-fold after 8 weeks of treatment, and final tumor weights were significantly lower ($P < 0.05$) than the Δ4A-supplemented animals and lower than the tamoxifen-treated animals (Fig. 6*B*).

Tumors were assayed for aromatase activity and levels of E_2. Tumors in the animals treated with vehicle, Δ4A, letrozole, anastrozole, and formestane continued to express aromatase activity at levels comparable with parent MCF-7Ca tumors (Table 2). Moreover, tumor E_2 concentrations in the transplanted Δ4A-treated tumors were comparable with the parental MCF-7Ca breast tumor xenografts (1890 ± 120 pg/ml *versus* 2305 ± 425 pg/ml). In the transplanted tumors treated with the vehicle hydroxypropyl cellulose tumor E_2 concentrations were reduced by 78% from 1890 ± 120 pg/g tissue to 420 ± 80 pg/g tissue. Of the three aromatase inhibitors tested in this experiment, only letrozole reduced tumor E_2 concentrations to levels comparable with the vehicle-treated transplanted tumors. Tumor E_2 concentrations in the mice treated with anastrozole and formestane were higher than the letrozole-treated tumors by 1.5-fold and 2.5-fold, respectively. However, compared with the animals treated with anastrozole and formestane serum E_2 levels in the letrozole-treated animals were higher by 1.7-fold and 2.4-fold, respectively. Wet uterine weight was used as a bioassay for serum E_2 levels. The uteri of the animals supplemented with Δ4A were significantly higher than the uteri in the animals that received the vehicle hydroxypropyl cellulose and the aromatase inhibitors letrozole, anastrozole, and formestane ($P <$

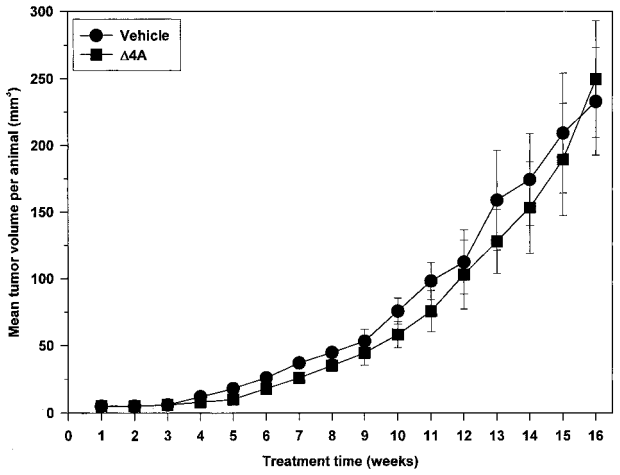

Fig. 5 The effect of vehicle and Δ4A (100 μg/day) on the growth of transplanted letrozole-treated tumors in female ovariectomized athymic mice. One of the long-term letrozole-treated tumors was mixed to a single cell suspension, suspended in Matrigel, and inoculated into animals at one site per flank. From the day of inoculation, animals (five mice per group) were treated daily with vehicle or Δ4A. Tumor volumes were measured weekly and are expressed as mean tumor volume per animal; *bars*, ±SE.

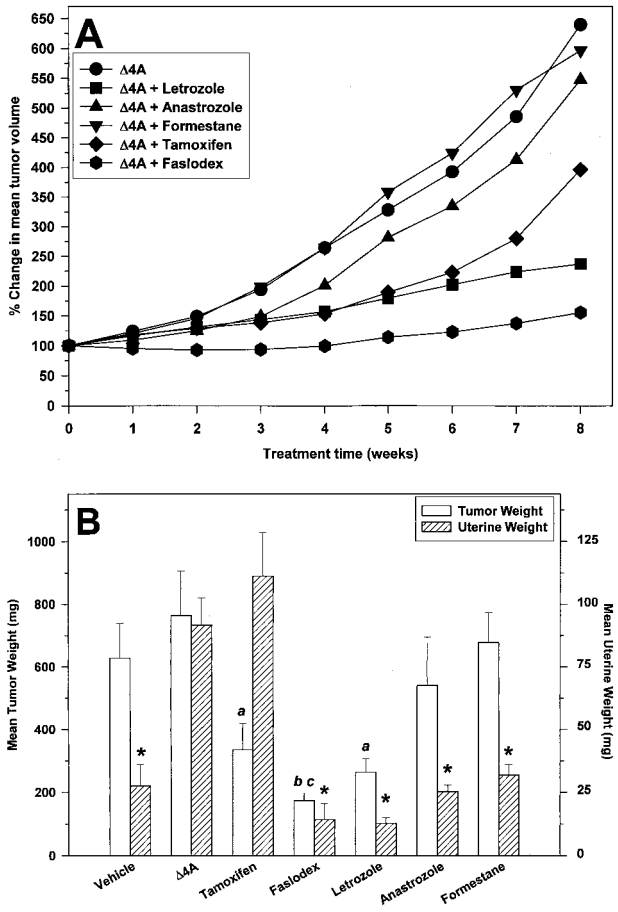

Fig. 6 The effect of antiestrogens and aromatase inhibitors on the growth of transplanted letrozole-treated tumors in female ovariectomized athymic nude mice supplemented with Δ4A (100 μg/day). One of the long-term letrozole-treated tumors was mixed to a single cell suspension, suspended in Matrigel, and inoculated into the animals at one site per flank. Animals were then treated daily with Δ4A for the duration of the experiment, and tumor volumes were measured weekly. When mean tumor volume was ~50 mm^3, animals were grouped ($n = 5$) for treatment daily with control Δ4A, letrozole (100 μg/day), anastrozole (100 μg/day), formestane (1 mg/day), tamoxifen (100 μg/day), and faslodex (1 mg/day). *A*, tumor volumes were measured weekly and are expressed as the percentage change in initial tumor volume. *B*, after 8 weeks of treatment, the mice were sacrificed, and the tumors and uteri were removed, cleaned, and weighed. Final tumor weights that were significantly lower than the Δ4A-treated animals are shown. *a*, $P < 0.05$ *versus* Δ4A; *b*, $P < 0.01$ *versus* Δ4A; *c*, $P < 0.05$ *versus* tamoxifen. Compared with the Δ4A-treated animals, uterine weights were significantly lower in the animals treated with the aromatase inhibitors letrozole, anastrozole, and formestane, and the pure antiestrogen faslodex (∗, $P < 0.001$).

0.001; Fig. 6*B*). Although serum E_2 levels were higher in the animals treated with letrozole compared with the other aromatase inhibitors, there were no significant differences between the weights of the uteri in these groups. Wet uterine weight was also significantly lower in the animals treated with the pure antiestrogen faslodex ($P < 0.001$; Fig. 6*B*), and serum E_2 levels were reduced from 63 pg/ml to 32 pg/ml. In contrast, the weights of the uteri of the tamoxifen-treated animals were not decreased compared with the Δ4A-supplemented animals.

DISCUSSION

The aim of this study was to use a preclinical model of hormone-dependent breast cancer to determine the effects of second-line antiestrogen therapy after tumor progression after treatment with the aromatase inhibitor letrozole. The MCF-7Ca model of hormone-dependent breast cancer correlates well with the clinical situation observed in postmenopausal breast cancer patients. In postmenopausal breast cancer patients, estrogen levels are higher in tumor tissues compared with normal mammary tissue, and this is believed to be, at least in part, a consequence of intratumoral expression of aromatase (23, 34–36). The MCF-7Ca postmenopausal breast cancer model is also unique because it provides us with the opportunity to compare directly the antiproliferative effects of antiestrogens and aromatase inhibitors on human breast cancer xenografts. We have used this model previously and have shown that aromatase inhibitors are more effective than the antiestrogens tamoxifen and faslodex at inhibiting the growth of hormone-responsive human breast tumors (30, 31). In comparisons with tamoxifen, these predictions have been verified recently in clinical settings (9–18).

Treating breast cancer patients with a triazole aromatase inhibitor such as letrozole deprives the tumors of mitogenic estrogens (24). In this study, we determined the long-term effects of estrogen deprivation on human breast cancer cells using two approaches. The first approach was to culture MCF-7Ca cells in an estrogen-depleted medium for an extended period of time. This resulted in the selection of the variant UMB-1Ca cell line that had adapted to growth in steroid-depleted medium with a significant increase in expression of ER (Table 1). This finding has been reported for other variants of

the MCF-7 human breast cancer cell line that had acquired the ability to proliferate in an estrogen-depleted environment (33, 37–39). In addition, we have reported previously that ER levels in MCF-7Ca human breast tumor xenografts are significantly increased after short-term treatment with letrozole (21). Masamura *et al.* (39) have reported that the increased ER levels in long-term estrogen-deprived MCF-7 cells sensitizes them to low concentrations of E_2 and to the antiproliferative effects of antiestrogens. However, it should be noted that other investigators who established an MCF-7 variant cell line from tumor xenografts that had acquired the ability to proliferate in untreated female ovariectomized athymic nude mice did not observe estrogen hypersensitivity (32). Our results are similar to those reported by Brünner *et al.* (32) because in this study the estrogen-deprived UMB-1Ca cells were less sensitive to E_2 than the MCF-7Ca cells, which responded to E_2 treatment with a higher rate of proliferation. Growth of UMB-1Ca cell was stimulated 1.9-fold by 10 pM E_2, whereas MCF-7Ca cell growth was increased 5.5-fold by the same concentration of steroid. Our results suggest that compared with MCF-7Ca cells, UMB-1Ca cells have a reduced sensitivity to E_2. This conclusion is supported by the finding that the concentration of E_2 required to reverse the antiproliferative of faslodex (1 nM) was only 0.1 nM for MCF-7Ca cells but was 10 nM for UMB-1Ca cells. However, it should be noted that MCF-7Ca cell growth was stimulated maximally (10-fold) by 1 nM E_2, whereas UMB-1Ca cell growth was stimulated maximally 1.9-fold by 10 pM E_2. This suggests that UMB-1Ca cells are less sensitive to physiological concentrations of E_2.

When tested in the intratumoral aromatase postmenopausal breast cancer model, tumors formed from UMB-1Ca cells but not from MCF-7Ca cells proliferated in the absence of the Δ4A supplement. Both tumor types proliferated equally well in the presence of Δ4A. However, at sacrifice the Δ4A-supplemented MCF-7Ca tumors weighed seven times more than the vehicle-treated tumors, whereas the Δ4A-supplemented UMB-1Ca tumors weighed only 1.6-times more than their corresponding vehicle-treated tumors. This parallels the results obtained *in vitro*, where compared with UMB-1Ca cells, MCF-7Ca cells responded to E_2 and Δ4A with an increased rate of proliferation. However, there was no difference in the sensitivity of MCF-7Ca tumors and UMB-1Ca tumors to the antiestrogen tamoxifen. One explanation for this finding is that the high tumor estrogen levels may result in decreased expression of ER in the UMB-1Ca tumor cells that would again diminish the antiproliferative effects of tamoxifen. This was suggested by studies with long-term estrogen-deprived MCF-7 cells (39). The authors reported that estrogen hypersensitivity in these cells is reversed when the cells are re-exposed to estrogens *in vitro*. It would be of interest to determine whether UMB-1Ca tumors show increased sensitivity to antiestrogens when the animals are supplemented with a much lower dose of Δ4A that would maintain high levels of ER expression.

To reflect more accurately the clinical situation of treating postmenopausal breast cancer patients with an aromatase inhibitor, established MCF-7Ca breast cancer xenografts were treated with letrozole until tumors acquired the ability to proliferate in the presence of the drug. We have reported previously that letrozole induces marked tumor regression in the first 4 weeks of treatment (30, 31, 40), and it is worthwhile noting that this response is not observed when MCF-7Ca tumor xenografts are treated with formestane, anastrozole, tamoxifen, or faslodex. In this long-term experiment it was particularly interesting to find that when the dose of letrozole was increased from 10 μg/day to 100 μg/day, the tumors underwent a second regression. A dose-response effect for letrozole has been reported from several clinical trials (6, 8). Smith (41, 42) has suggested that this may be attributable to the suppression of intratumoral aromatase activity, which plays an important role in the autocrine/paracrine production of mitogenic estrogens. However, in another second-line clinical study letrozole did not show dose-dependent antitumor activity (43). This raises the possibility that there may be an induction of liver cytochrome P450 enzymes in the animals that metabolize letrozole to a less active form. Thus, when the dose of letrozole was increased from 10 μg/day to 100 μg/day, the tumors may have undergone a second regression because of the higher circulating levels of unmetabolized drug.

In this report, the time for letrozole-treated MCF-7Ca tumors to approximately double in size from the initial (untreated) tumor volume was 37 weeks. We have since determined that the time for tamoxifen-treated MCF-7Ca breast tumor xenografts to approximately double in size is 16 weeks (44). Therefore, in our model, letrozole is superior to tamoxifen because it extends time to disease progression. This finding has also been reported in a recent clinical trial that compared the efficacy of letrozole to tamoxifen in the first-line setting for advanced breast cancer (15). The mechanisms associated with the ability of MCF-7Ca tumors to proliferate in the presence of letrozole (100 μg/day) have not been determined. The data presented in this report indicate that although the long-term letrozole-treated tumors continue to express aromatase activity at levels comparable with parental MCF-7Ca tumors, letrozole therapy reduces tumor E_2 concentrations by ~90% (Table 2). Therefore, MCF-7Ca tumors proliferating in the presence of letrozole after 37 weeks of treatment have adapted to grow in an estrogen-depleted environment. To our knowledge, this is the first report of a human breast tumor xenograft that has acquired the ability to proliferate in the presence of the aromatase inhibitor letrozole *in vivo*. The molecular mechanisms associated with the acquisition of letrozole-resistant growth are presently being examined.

The presence of functionally active aromatase in the long-term letrozole-treated tumors may help explain why the transplanted tumors remained sensitive to letrozole but not to anastrozole or formestane. The lack of effect of formestane was particularly interesting because it has been reported that tumors progressing on nonsteroidal aromatase inhibitors may show a response to the steroidal aromatase inhibitor exemestane (45). The reverse scenario has also been described for tumors progressing first on the steroidal aromatase inhibitor formestane (46). The data in this report suggest that tumors progressing on letrozole may not be sensitive to additional therapy with a steroidal or nonsteroidal aromatase inhibitor. One explanation for this finding may be the potent ability of letrozole to inhibit aromatase. We have reported previously that in a panel of aromatase-expressing cell lines (including MCF-7Ca), letrozole is more effective than formestane and anastrozole at inhibiting aromatase activity (27). Consequently, MCF-7Ca tumors that had been sensitized to letrozole may not respond to drugs that

are less efficacious at inhibiting the aromatase enzyme. This is reflected in the fact that compared with the transplanted tumors treated with letrozole, E_2 concentrations in the tumors treated with anastrozole and formestane are 1.5-fold and 2.5-fold higher, respectively. Although serum E_2 levels were lower in the animals treated with anastrozole and formestane compared with the animals treated with letrozole, our results suggest that tissue E_2 concentrations may be more important, because this is a reflection of the amount of E_2 available in the tumor microenvironment. The half-life of anastrozole in rodents has been reported to be considerably shorter than the half-life of letrozole (47). Therefore, the circulating levels of anastrozole may not approach those of letrozole. Thus, anastrozole may not be completely inhibiting tumor aromatase activity and, as a consequence, tumor growth. However, it is important to note that in our previous studies (30, 31) we reported that anastrozole had potent antitumor activity at a daily dose of 10 μg/day, and in this study anastrozole was administered at ten times that dose.

After transplantation of the long-term letrozole-treated tumors, letrozole slowed but did not regress tumor growth, and tumor volumes actually increased slowly over the duration of the experiment. Thus, the transplanted tumors appear to have acquired at least partial but not complete resistance to letrozole. This suggests that there is a possibility of returning to letrozole therapy after initial relapse and second-line therapy with an antiestrogen. Nonetheless, in these experiments the transplanted long-term letrozole-treated tumors retained sensitivity to second-line treatment with the antiestrogens tamoxifen and faslodex. The pure antiestrogen faslodex was very effective at slowing tumor growth. In fact, over the 8-week duration of the experiment the volumes of the tumors treated with faslodex increased by only 0.55-fold.

In summary, we have used the aromatase-transfected, estrogen-dependent MCF-7Ca human breast cancer cell line, and shown that when cells and tumor xenografts acquire the ability to proliferate in an estrogen-depleted environment *in vitro* they remain sensitive to second-line therapy with tamoxifen. Moreover, after a long-term treatment with the aromatase inhibitor letrozole *in vivo*, transplanted MCF-7Ca tumor xenografts are sensitive in the second-line to tamoxifen and to a greater extent to faslodex. This is most likely because of the increased cellular ER levels that enhance the sensitivity of estrogen-deprived cells to the antiproliferative effects of antiestrogens.

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