

Stopping Treatment Can Reverse Acquired Resistance to Letrozole

Gauri J. Sabnis,¹ Luciana F. Macedo,¹ Olga Goloubeva,² Adam Schayowitz,¹
and Angela M.H. Brodie^{1,2}

¹Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine and

²University of Maryland Greenebaum Cancer Center, Baltimore, Maryland

Abstract

Using the intratumoral aromatase xenograft model, we have observed that despite long-lasting growth inhibition, tumors eventually begin to grow during continued letrozole treatment. In cells isolated from these long-term letrozole-treated tumors (LTLT-Ca), estrogen receptor- α (ER α) levels were decreased, whereas signaling proteins in the mitogen-activated protein kinase cascade were up-regulated along with human epidermal growth factor receptor 2 (Her-2). In the current study, we evaluated the effect of discontinuing letrozole treatment on the growth of letrozole-resistant cells and tumors. The cells formed tumors equally well in the absence or presence of letrozole and had similar growth rates. After treatment was discontinued for 6 weeks, letrozole was administered again. Marked tumor regression was observed with this second course of letrozole treatment. Similarly, in MCF-7Ca xenografts, a 6-week break in letrozole treatment prolonged the responsiveness of the tumors to letrozole. To understand the mechanisms of this effect, LTLT-Ca cells were cultured in the absence of letrozole for 16 weeks. The resulting cell line (RLT-Ca) exhibited properties similar to MCF-7Ca cells. The cell growth was inhibited by letrozole and stimulated by estradiol. The expression of phosphorylated mitogen-activated protein kinase (MAPK) was reduced and ER α and aromatase levels increased compared with LTLT-Ca cells and were similar to levels in MCF-7Ca cells. These results indicate that discontinuing treatment can reverse letrozole resistance. This could be a beneficial strategy to prolong responsiveness to aromatase inhibitors for patients with breast cancer. [Cancer Res 2008;68(12):4518–24]

Introduction

Approximately 75% of patients with breast cancer have hormone-dependent tumor cells and are given endocrine therapy. Aromatase inhibitors are proving to be more effective than the antiestrogen tamoxifen. Nevertheless, as with all forms of cancer therapy, not all patients respond and some relapse due to the development of resistance to aromatase inhibitor treatment. To study hormone-responsive breast cancer, we created a model system in which tumors of human estrogen receptor (ER)-positive breast cancer cells stably transfected with the aromatase gene (MCF-7Ca) were grown in immunosuppressed ovariectomized

mice (1, 2). This model system has provided us with results (3) that have predicted the outcomes of several clinical trials (4–7). We have previously reported that during treatment with letrozole, the MCF-7Ca xenografts increased the expression of human epidermal growth factor receptor 2 (Her-2) and proteins in the downstream mitogen-activated protein kinase (MAPK) pathway. In order to investigate the mechanisms in the loss of sensitivity of the tumors to aromatase inhibitors, we developed a cell line isolated from tumors of MCF-7Ca cells following long-term letrozole treatment (8, 9). These cells also exhibited a lower expression of ER α and an increase in Her-2 as well as apparent “estradiol-independent” growth (8). In our previous studies, we found that inhibition of these growth factor pathways leads to down-regulation of Her-2/MAPK activation and restoration of ER α -mediated signaling, suggesting that signaling pathways such as Her-2/MAPK are the key regulators of growth of letrozole-refractory cells and tumors. Furthermore, trastuzumab, an inhibitor of the Her-2 pathway, reverses the resistance of long-term letrozole-treated tumor (LTLT-Ca) cells to letrozole (10). This suggests that cross-talk between the Her-2/MAPK and ER α pathways control the growth of cancer cells.

In this study, we evaluated the effect of discontinuing letrozole treatment on these signaling pathways and whether hormone sensitivity could be restored in LTLT-Ca cells and tumors. Treatment with letrozole shifted the balance towards the growth factor pathway whereas when the treatment is stopped, compensatory signaling via the Her-2/MAPK pathway declined, ER α levels were restored, and the cells regained their original sensitivity to letrozole.

Materials and Methods

Materials. DMEM, IMEM, penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin–1 mmol/L EDTA solution, Dulbecco's PBS, and geneticin (G₄₁₈) were obtained from Invitrogen. Androstenedione was obtained from Sigma Chemical Company.

Cell culture. MCF-7Ca cells were routinely cultured in DMEM supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and 700 μ g/mL of G₄₁₈. LTLT-Ca cells were isolated from the tumors of mice treated with letrozole for 56 weeks as previously described (8). In brief, small pieces of tumors were disrupted by repeatedly drawing into a pipette. The tissue was incubated at 37°C, stirring overnight with collagenase type 1A, hyaluronidase type 1S, amphotericin B, and polymyxin B sulfate. The cells were filtered and plated onto Petri dishes. After a week, phenol red-free trypsin was added to remove fibroblasts and washed with Dulbecco's PBS. Epithelial cells were cultured in medium with 750 μ g/mL of G₄₁₈ and 1 μ mol/L of letrozole. Over the next 2 to 3 weeks, serum levels in the medium were reduced to 5% charcoal-stripped serum and penicillin/streptomycin levels were reduced to 1%. The cells were then propagated in phenol red-free IMEM supplemented with 5% CSS, 1% penicillin/streptomycin, 750 μ g/mL of G₄₁₈, and 1 μ mol/L of letrozole. RLT-Ca cells were derived by culturing LTLT-Ca cells supplemented with androstenedione and in the absence of letrozole for at least 4 months.

Cell proliferation assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described

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Requests for reprints: Angela M.H. Brodie, Department of Pharmacology and Experimental Therapeutics, University of Maryland, School of Medicine, Health Science Facility I, Room 580G, 685 West Baltimore Street, Baltimore, MD 21201. Phone: 410-706-3137; Fax: 410-706-0032; E-mail: abrodie@umaryland.edu.

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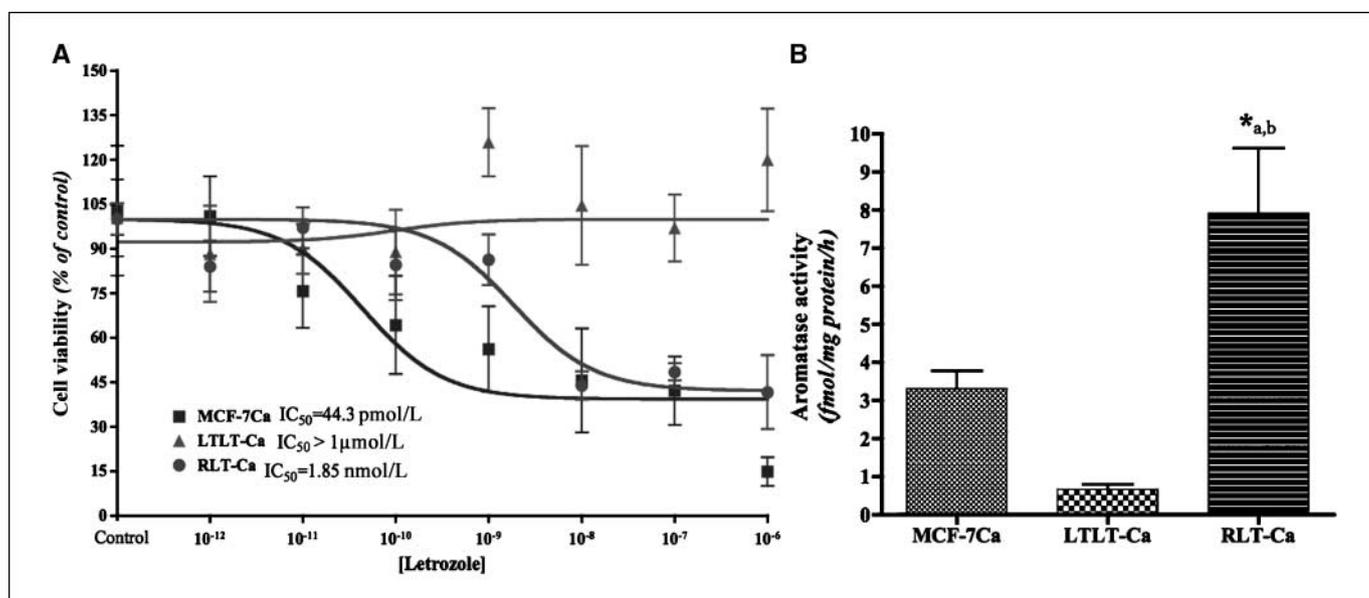


Figure 1. A, effect of letrozole on the proliferation of MCF-7Ca, LTLT-Ca, and RLT-Ca cells *in vitro*: the viability of cells was measured by MTT assay after 6 d of treatment with letrozole as described in the Materials and Methods. The treatment with letrozole was significantly more effective in MCF-7Ca and RLT-Ca cells compared with LTLT-Ca cells ($P = 0.0003$). B, aromatase activity of MCF-7Ca, LTLT-Ca, and RLT-Ca cells: aromatase activity was measured by $^3\text{H}_2\text{O}$ release assay as described in Materials and Methods. RLT-Ca cells exhibited significantly higher aromatase activity compared with MCF-7Ca ($^*a, P < 0.05$) and LTLT-Ca cells ($^*b, P < 0.001$).

earlier (8, 11). The results were expressed as a percentage of the absorbance of the controls. IC_{50} values for inhibitors were calculated from the nonlinear regression line of the plot of cell viability (percentage of control) versus inhibitor concentration.

Tumor growth in ovariectomized female athymic nude mice. All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland School of Medicine. Female ovariectomized athymic nude mice, 4 to 6 weeks of age, were obtained from the National Cancer Institute (Frederick, MD). The mice were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*.

The tumor xenografts of MCF-7Ca cells were grown in mice as previously described (3, 9). Mice were assigned to groups for treatment so that there was no statistically significant difference in tumor volume among the groups at the beginning of treatment ($P = 0.97$). Tumors were measured weekly with calipers and volumes were calculated using the formula $(4/3) \pi r_1^2 r_2$ ($r_1 \leq r_2$). The doses of letrozole and androstenedione used were previously determined and reported. Mice in the androstenedione group ($n = 6$) were treated for 7 weeks, after which they were sacrificed due to large tumor volumes. The mice in the letrozole group ($n = 30$) continued to receive treatment. When the tumor volumes of these mice reached double their starting size, the mice were assigned into two groups. One group ($n = 10$) stayed on continued letrozole treatment and the other group received only androstenedione ($n = 20$). This group was assigned as "off letrozole." Out of these 20 mice, 10 mice were switched back to letrozole after 6 weeks of discontinuation of treatment. This group was designated "back on."

LTLT-Ca xenografts were grown similarly as the MCF-7Ca xenografts (8). However, after inoculating LTLT-Ca cells into the mice, they were not supplemented with any steroidal agent. After 4 months off treatment, the mice were grouped so that there was no statistically significant difference in their tumor volumes ($P = 0.56$).

Western blotting. The protein extracts from tumor tissues were prepared by homogenizing the tissue in ice-cold Dulbecco's PBS containing protease inhibitors, and lysates from cells were prepared as described previously and separated by SDS-PAGE (11).

$^3\text{H}_2\text{O}$ release assay for aromatase activity measurement. The radiometric $^3\text{H}_2\text{O}$ release assay was performed as described previously

(12). The activity of the enzyme was corrected for protein concentration in the tumor homogenates and cells.

Statistics. The tumor volumes were analyzed with S-PLUS (7.0, Insightful Corp.) to estimate and compare an exponential variable (β_i) controlling the growth rate for each treatment groups as described previously (13). $P < 0.05$ was considered statistically significant.

Results

Antiestrogens and aromatase inhibitors produce inhibitory effects and estradiol induces mitogenic effects on RLT-Ca cells.

LTLT-Ca cells were cultured without letrozole for 4 months. These cells were designated as RLT-Ca cells. As shown in Fig. 1A, the IC_{50} of letrozole was 44.3 pmol/L in the parental MCF-7Ca cells and 1.85 nmol/L in RLT-Ca cells, whereas LTLT-Ca cells were resistant

Table 1. IC_{50} values of fulvestrant, tamoxifen, anastrozole, letrozole, and exemestane in MCF-7Ca, LTLT-Ca, and RLT-Ca cells

IC_{50} (nmol/L)	MCF-7Ca	LTLT-Ca	RLT-Ca
Letrozole	0.0443	>1 $\mu\text{mol/L}$	1.85
Anastrozole	28	>1 $\mu\text{mol/L}$	58
Exemestane	59	>1 $\mu\text{mol/L}$	71
Tamoxifen	128	>1 $\mu\text{mol/L}$	19.8 $\mu\text{mol/L}$
Fulvestrant	5.4	>1 $\mu\text{mol/L}$	28

NOTE: Cell viability of cells was measured by MTT assay after 6 d of treatment as described in Materials and Methods. IC_{50} values were calculated from the nonlinear regression of the plot of cell viability (% of control) versus log inhibitor concentration.

to the growth-inhibitory effects of letrozole. Similar effects were observed with other aromatase inhibitors, anastrozole, exemestane and antiestrogen fulvestrant, and tamoxifen. The IC_{50} values of these agents in RLT-Ca cells are comparable to the parental MCF-7Ca cells (Table 1). However, tamoxifen was found to be the least effective compound of all studied ($IC_{50} > 19.8 \mu\text{mol/L}$). Our findings indicate that when letrozole-resistant cells (LTLT-Ca) are no longer exposed to letrozole for several weeks (RLT-Ca), they regain their original sensitivity to aromatase inhibitors and antiestrogens, whereas LTLT-Ca cells exhibit complete cross-resistance to all endocrine agents (data not shown). The reversal of response to letrozole was accompanied by increased aromatase activity in RLT-Ca cells (Fig. 1B) compared with LTLT-Ca cells ($P < 0.01$), which was also higher than the activity in MCF-7Ca cells ($P < 0.05$).

In addition, RLT-Ca cells respond to the mitogenic effects of androstenedione and estradiol. Figure 2A shows that androstenedione stimulated the growth of RLT-Ca cells at concentrations of 10^{-11} and 10^{-10} mol/L ($P < 0.01$), a response similar to that of MCF-7Ca cells. Interestingly, the growth of LTLT-Ca cells was decreased at all androstenedione concentrations (Fig. 2A). This effect may be

due to a specific androgenic effect of androstenedione as it was not converted to estrogen owing to the low levels of aromatase activity in LTLT-Ca cells (Fig. 1B; ref. 14). Estradiol was ineffective in stimulating the growth of LTLT-Ca cells, as shown in Fig. 2B. However, the growth of RLT-Ca cells was significantly stimulated by estradiol at concentrations of 10^{-12} to 10^{-10} mol/L ($P < 0.01$). The biphasic dose-response of androstenedione and estradiol observed in RLT-Ca cells is similar to the response of MCF-7Ca cells. RLT-Ca cells show changes in the activation and expression of several signal transduction proteins. Thus, compared with LTLT-Ca cells, Her-2 and phosphorylated MAPK levels were decreased, whereas ER α increased to levels approaching those of MCF-7Ca cells (Fig. 2C).

Reversal of letrozole resistance in LTLT-Ca xenografts. As shown in Fig. 3A, when LTLT-Ca xenografts were grown in nude mice in the absence of letrozole for a period of 4 months, they responded to a second course of letrozole treatment for up to 12 weeks. The growth rate (β_i) of tumors treated with letrozole (6.5 ± 7.1) was significantly ($P = 0.04$) lower compared with control tumors (48.8 ± 16.8). The mean tumor volume of letrozole-treated

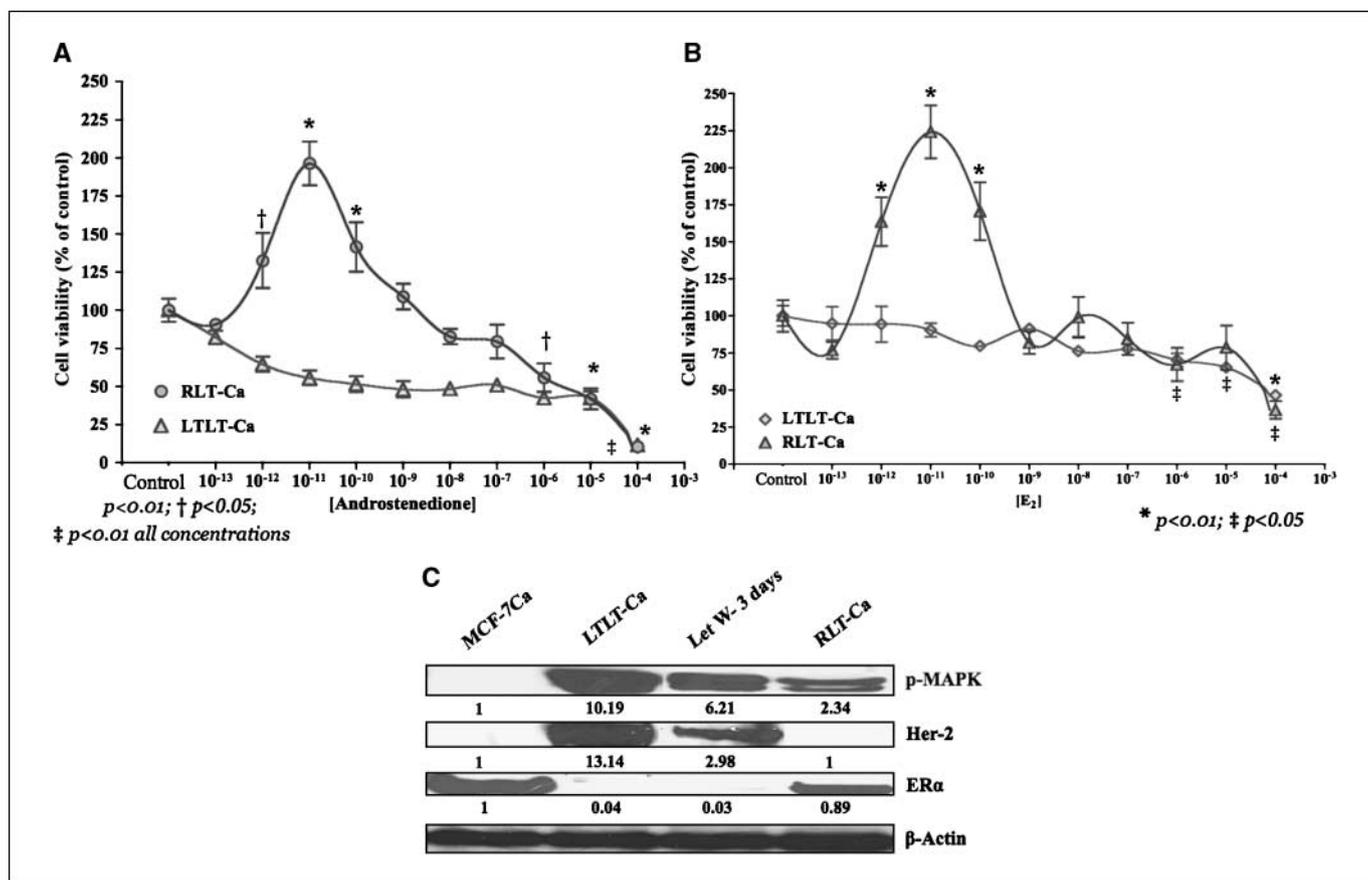


Figure 2. A, effect of androstenedione on proliferation of LTLT-Ca and RLT-Ca cells: the viability of cells was measured by MTT assay after 6 d of treatment as described in Materials and Methods. Compared with LTLT-Ca cells, RLT-Ca cells exhibit a significantly marked stimulation of proliferation in response to androstenedione at concentrations of 10^{-11} mol/L and 10^{-10} mol/L (* , $P < 0.001$). LTLT-Ca cells are markedly inhibited by androstenedione at all concentrations († , $P < 0.01$), but only at a concentration of 10^{-6} mol/L of androstenedione in RLT-Ca cells († , $P < 0.05$) and 10^{-5} mol/L and 10^{-4} mol/L (* , $P < 0.001$). B, effect of estradiol on proliferation of LTLT-Ca and RLT-Ca cells: the viability of cells was measured by MTT assay after 6 d of treatment as described in Materials and Methods. Compared with LTLT-Ca cells, RLT-Ca cells exhibit a significantly marked stimulation of proliferation in response to estradiol at concentrations of 10^{-12} mol/L to 10^{-10} mol/L (* , $P < 0.001$). Estradiol markedly inhibited the growth of LTLT-Ca cells at concentrations of 10^{-6} mol/L to 10^{-4} mol/L († , $P < 0.01$), but only at 10^{-4} mol/L of estradiol in RLT-Ca cells ($P < 0.01$). C, protein expression profile of RLT-Ca cells compared with MCF-7Ca and LTLT-Ca cells: Expression of proteins was examined using Western immunoblotting as described in Materials and Methods. Phosphorylated MAPK at 42 to 44 kDa, ER α at 66 kDa, Her-2 at 44 kDa, and β -actin at 45 kDa. The blots were stripped and reprobed for β -actin to verify equal loading. The blots show a single representative of three independent experiments.

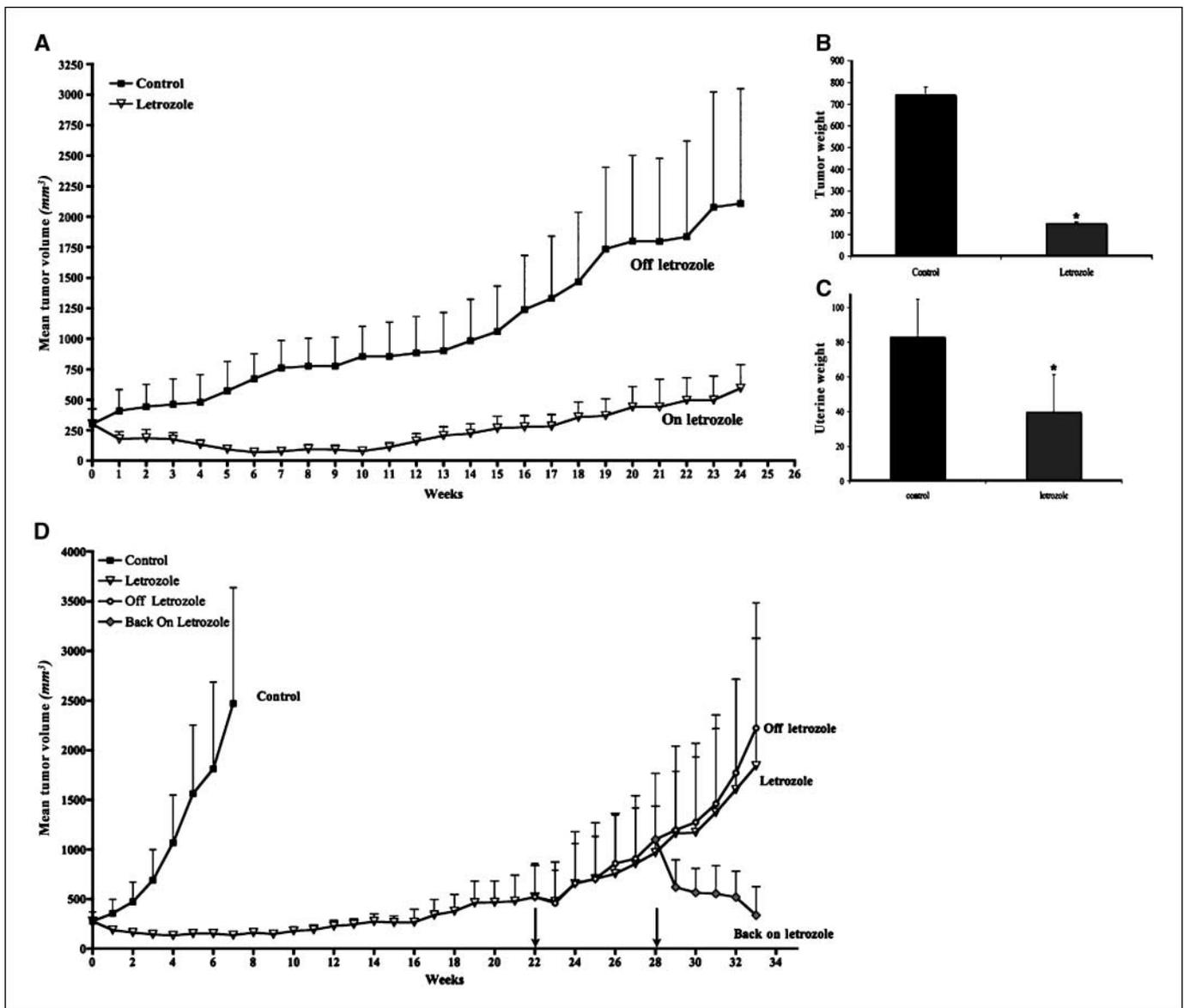


Figure 3. A, effect of letrozole treatment on the growth of LTLT-Ca xenografts after 4 mo off letrozole: LTLT-Ca xenografts were grown in female OVX nude mice as described in Materials and Methods. The mice were kept off letrozole treatment for a period of 4 mo. After which they were assigned to two groups (on and off letrozole). The mice in the control and letrozole-treated groups exhibited significantly different growth rates. The difference in the exponential variable governing growth was 42.3 ($P = 0.04$). B, effect of letrozole on the tumor weight of the LTLT-Ca xenografts: the mean tumor weight of control mice was 607.5 ± 225.5 mg, which was significantly different from those of the letrozole-treated mice (112.5 ± 84.77 mg; $P = 0.024$). Columns, mean; bars, SE. C, effect of letrozole on the uterine weight of LTLT-Ca xenografts: the mean uterine weight of control mice was 83 ± 21.45 mg, which was significantly different from those of the letrozole-treated mice (39.67 ± 9.14 mg; $P < 0.01$). Columns, mean; bars, SE. D, discontinuous treatment prolongs responsiveness of MCF-7Ca xenografts to letrozole: the tumors of MCF-7Ca cells were grown as described in Materials and Methods. The letrozole and off groups were sacrificed on week 34. Columns, mean tumor volume; bars, SE.

mice at week 24 was found to be 196 ± 169.4 mm³, whereas without letrozole treatment, the mice had a mean tumor volume of $1,261.9 \pm 409.3$ mm³ ($P = 0.03$). As shown in Fig. 3B, the tumor weights of these two groups were also significantly different. Furthermore, the low uterine weight in the letrozole-treated group suggests that letrozole was effective in inhibiting the synthesis of estrogen production (Fig. 3C).

Reversal of letrozole resistance in MCF-7Ca xenografts. Consistent with our earlier report, letrozole treatment in MCF-7Ca xenografts caused marked tumor regression, which was maintained for a prolonged period. The mice treated with letrozole versus androstenedione (control) had significantly lower growth rates

($P < 0.0001$) and tumor volumes ($P < 0.0001$). After 22 weeks, letrozole was discontinued in one group of mice (the "off" group). Tumors in these mice had a similar growth rate to that of the group continued on letrozole over weeks 22 to 34. The difference in the exponential variable governing growth rate (β_i) was 0.002 ± 0.12 ($P = 0.99$). This suggests that when resistance to letrozole develops, discontinuing treatment does not affect the growth rate of tumors significantly. When the mice in the "off" group were put "back on" letrozole, the tumor growth was significantly reduced. The difference in the exponential variable governing growth rate was 0.436 ± 0.12 ($P = 0.0005$). Also, the "back on" group had a significantly lower growth rate compared with the "letrozole"

group. The difference in the exponential variable governing growth rate was 0.434 ± 0.13 ($P = 0.0009$; Fig. 3D).

On week 34, the mice were sacrificed due to large tumor volumes of "letrozole" and "off" groups. Tumors and uteri were excised, cleaned, weighed, and stored at -80°C . The tumor weights of the

mice in the control, letrozole, and "off" groups were not statistically different ($P = 0.46$; Fig. 4A).

However, the uterine weights exhibit significant differences (Fig. 4B). As the mouse uterus is very sensitive to estrogens, the measurement of uterine weight is a useful bioassay that indicates

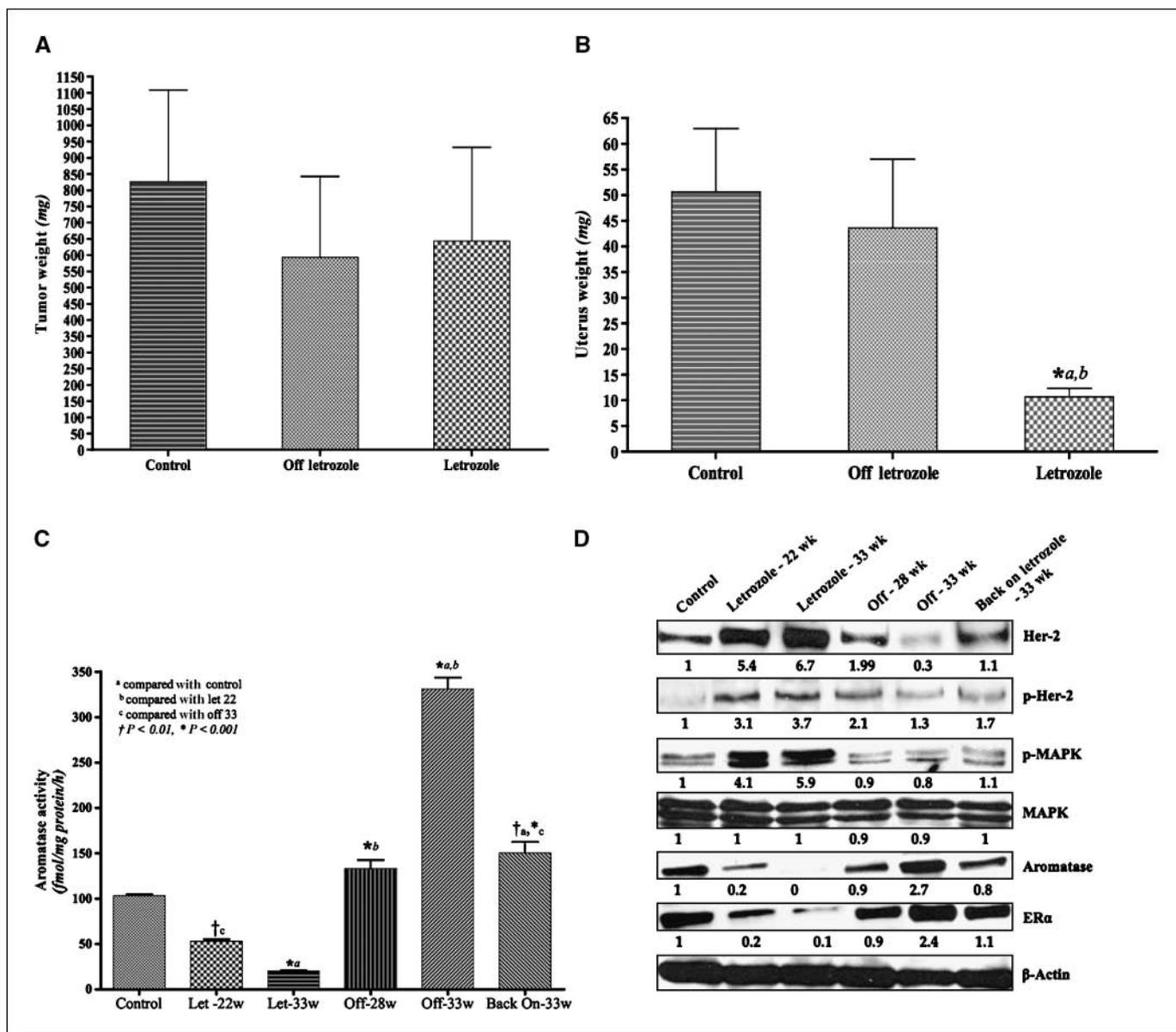


Figure 4. A, effect of letrozole on/off treatment on the tumor weight of the MCF-7Ca xenografts: The mean tumor weight of control mice was 1.38 ± 0.39 g, letrozole-treated mice was 0.92 ± 0.36 g, and for "off" letrozole mice was 0.74 ± 0.34 g. Columns, mean; bars, SE. The mean tumor weights were not significantly different across the groups ($P = 0.46$). B, effect of letrozole on/off treatment on the uterus weight of the MCF-7Ca xenografts: the mean uterus weight of letrozole-treated mice was 10.71 ± 1.658 mg, which was significantly different from those of the control ($*a$) mice (50.67 ± 12.28 mg; $P = 0.0006$) and "off" letrozole ($*b$) mice (43.63 ± 13.44 mg; $P = 0.02$). The mean uterine weights of control and "off" letrozole mice were not significantly different ($P = 1$). Columns, mean; bars, SE. Pearson correlation coefficient for uterine weight and tumors in letrozole group was -0.19 and 0.14 (left and right tumors, respectively; $P = 0.68$ and 0.76 —no correlation). Pearson correlation coefficient for uterine weight and tumors in the control group was 0.96 and 0.96 (left and right tumors, respectively; $P = 0.003$ and 0.002 —very strong positive correlation is present). Pearson correlation coefficient for uterine weight and tumors in the "off" group was 0.95 and 0.84 (left and right tumor, respectively; $P = 0.0002$ and 0.009 —very strong positive correlation is present). C, aromatase activity was measured using $^3\text{H}_2\text{O}$ release assay as described in Materials and Methods. Control tumors were collected at week 7; letrozole-treated tumors were collected at week 22 (when the group was split into "on" and "off" letrozole) and week 33 (when the experiment was terminated); "off" letrozole tumors at week 28 (when the group was split into "off" and "back on" letrozole) and week 33 (when the experiment was terminated); "back on" letrozole at week 33. Columns, mean; bars, SE. One-way ANOVA with post hoc Tukey multiple comparison test was performed to examine statistical significance. D, protein expression profile of the tumors of MCF-7Ca cells treated with letrozole: expression of proteins was examined using Western immunoblotting as described in Materials and Methods. Lane 1, androstenedione-treated controls; lane 2, letrozole-treated tumors at week 22; lane 3, letrozole-treated tumors at week 33; lane 4, tumor from the "off" group at week 28; lane 5, "off" tumors at week 33; and lane 6, tumor from the "back on" group at week 33. Blot shows Her-2 and phosphorylated Her-2 at 185 kDa, phosphorylated MAPK and MAPK at 42 kDa, aromatase at 55 kDa, ER α at 66 kDa, and β -actin at 45 kDa. The blots were stripped and reprobed for β -actin to verify equal loading. The blots show a single representative of three independent experiments.

the level of circulating estrogens in the mouse. The mice in the "letrozole" group had significantly lower uterine weights compared with the control group ($P = 0.0006$) and the "off" ($P = 0.02$) group, indicating that aromatase was effectively inhibited by letrozole in this group. However, the mice in the control and "off" groups had similar uterine weights ($P = 1$). Furthermore, the uterine weights of mice in the control and "off" groups correlated well with their tumor weights. In contrast, the uterine weights of mice in the letrozole group were markedly reduced and did not correlate with the tumor weights. This suggests that estrogen production in the control and "off" groups was sufficient to stimulate tumor growth. Although estrogen synthesis was suppressed as evidenced by low uterine weight in the letrozole group, the growth of tumors in the letrozole group was independent of estrogen.

Protein expression and activity after letrozole withdrawal in MCF-7Ca xenografts. MCF-7Ca xenografts were examined for changes in aromatase activity after treatment with letrozole. Tumors used for this assay were collected at different time points during the course of treatment (Fig. 4C). When treated with letrozole, the aromatase activity of the tumors was significantly lower compared with the control groups ($P < 0.01$ at 22 weeks and $P < 0.001$ at 33 weeks). However, after discontinuing treatment (off treatment for 22 weeks), aromatase activity increased and was similar to the control levels ($P < 0.001$ compared with letrozole at 22 and 33 weeks). The aromatase activity, however, had increased further by week 33, and it was significantly higher than the control level ($P < 0.001$). When the mice were put "back on" letrozole, the aromatase activity ceased to increase and remained at the same level as the "off" group at week 28 but it was still higher than the levels in the control group ($P < 0.01$).

When protein expression was examined (Fig. 4D), tumors treated with letrozole (22 weeks) exhibited a higher expression of Her-2, phosphorylated Her-2, and phosphorylated MAPK compared with controls. The levels increased further after 33 weeks. In contrast, the tumors from the "off" group showed decreased levels of Her-2, phosphorylated Her-2, and phosphorylated MAPK compared with letrozole-treated tumors and were similar to control tumors. On the other hand, ER α and aromatase expression decreased with letrozole treatment and was restored in the "off" groups (28 and 33 weeks). These results suggest that tumors may adapt using the Her-2/MAPK signaling pathway during letrozole treatment but revert to the ER α pathway when letrozole is withdrawn.

Discussions

Despite significant advances in the treatment of breast cancer since the development of aromatase inhibitors (15–17), some patients eventually become resistant to treatment. Thus, it is important to understand the mechanisms of this resistance and determine how resistance to treatment can be overcome. We have developed a xenograft model in order to investigate how cells adapt and survive the effects of treatment. Although letrozole caused marked inhibition of tumor growth initially, tumors eventually acquired the ability to grow in the presence of letrozole and were refractory to estrogen stimulation and second-line therapy with antiestrogens (3, 8, 9).

Previous studies of cells isolated from the long-term letrozole-treated tumors (LTLT-Ca) revealed that overexpression of Her-2 and activation of the MAPK signaling pathway accompanied the resistance to antiestrogens, aromatase inhibitors, androstenedione, and estradiol (8, 18). In these cells, ER levels were reduced and cell

growth was independent of estrogen, indicating cross-talk between ER α and Her-2 (10). These results suggest that acquisition of resistance and progression to hormone independence represents a shift in the balance that leans towards growth factor signaling in the presence of letrozole and back to hormonal signaling upon withdrawal of the aromatase inhibitor. These results led to the hypothesis that the Her-2/MAPK pathway provides a compensatory signaling mechanism for the cells when estrogen levels are extremely low (10).

Analysis of the protein expression and aromatase activity in RLT-Ca cells revealed that Her-2 and the downstream signaling proteins as well as ER α had returned to the levels in the original phenotype (MCF-7Ca cells). Thus, RLT-Ca cells respond to estradiol, androstenedione, aromatase inhibitors, and fulvestrant. The EC₅₀ values for these RLT-Ca cells were similar to those previously reported for MCF-7Ca cells (19). The response to tamoxifen, however, was not completely restored in RLT-Ca cells.

The *in vivo* findings were consistent with the *in vitro* results. When mice were inoculated with MCF-7Ca cells and treated with letrozole, tumor growth was suppressed for several weeks, but eventually, tumors began to grow despite continued treatment. By 22 weeks, the tumor volume reached twice the initial volume. The mice were then divided into two groups. One group continued on letrozole whereas treatment was then stopped in the other group for 6 weeks. At week 28, half of the animals in the latter group began treatment with letrozole again while the others continued on androstenedione until week 33. When letrozole treatment was discontinued, aromatase activity returned to MCF-7Ca control levels by 28 weeks. However, at 33 weeks, aromatase activity was more than 3-fold higher than control levels. Although the mechanism of this increase is unclear, previous studies have reported increased aromatase activity after letrozole withdrawal, which has been explained by stabilization and reduced degradation of the aromatase protein. Interestingly, growth of tumors in the "off" group was not significantly different from those of the animals continuing on letrozole. The increase in aromatase activity may contribute to an increase in tumor growth in the "off" group and may also contribute to increased sensitivity of the tumors to letrozole in the "back on" group. The further increase in Her-2/MAPK signaling could drive the growth of tumors on continuous letrozole treatment.

In conclusion, our results suggest that resistance to letrozole can be reversed by discontinuing treatment for 6 weeks. These findings suggest that alternating "on/off" letrozole treatment may be an effective way to delay resistance and prolong sensitivity to aromatase inhibitors. This strategy could have advantages for patients relapsing from aromatase inhibitor treatment.

Disclosure of Potential Conflicts of Interest

A.M.H. Brodie received commercial research support from AstraZeneca, honoraria from Novartis, and is a consultant for Pharmacia. The other authors disclosed no potential conflicts of interest.

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