**Research Article**

**Sensitivity to the Aromatase Inhibitor Letrozole Is Prolonged After a “Break” in Treatment**

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**Abstract**

Using a hormone-dependent xenograft model, we established that loss of response to letrozole was accompanied by upregulation of the Her-2/mitogen-activated protein kinase (MAPK) pathway and downregulation of estrogen receptor α (ER\(α\)) and aromatase activity. In our previous study, we showed that stopping letrozole treatment or adding trastuzumab could reverse acquired resistance. In this study, we compared the effects of intermittent letrozole treatment and switching treatment between letrozole and trastuzumab on tumor growth in an attempt to optimize discontinuous letrozole treatment. The mice were treated with letrozole until the tumors developed resistance and then were divided into three groups: (a) letrozole, (b) trastuzumab, and (c) “off” (\(Δ^4\)A supplement only); tumors were collected every week to examine changes in tumor protein expression and activity. In off group tumors, Her-2/p-MAPK activation gradually decreased and EROs and aromatase protein (and activity) increased. Within the first week of trastuzumab treatment, Her-2 and MAPK were downregulated and ER\(α\) was upregulated. When letrozole-resistant MCF-7Ca tumors were taken off treatment for 4 weeks, the second course of letrozole treatment provided a much longer duration of response (\(P = 0.02\)). However, switching treatment to trastuzumab for 4 weeks did not provide any inhibition of tumor growth. Our studies revealed that the adaptation of cells to a low-estrogen environment by upregulation of Her-2/MAPK and downregulation of ER\(α\)/aromatase was reversed on letrozole withdrawal. The tumors once again became responsive to letrozole for a significant period. These results suggest that response to letrozole can be prolonged by a short “break” in the treatment. *Mol Cancer Ther*; 9(1); 46–56.

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**Introduction**

The knowledge that steroids play a critical role in the growth of hormone-dependent tumors is channeled toward development of endocrine therapy of breast cancer, which includes antiestrogens and aromatase inhibitors (1). Since the development of aromatase inhibitors, the treatment of hormone-responsive postmenopausal breast cancer has made significant advances (1–4). However, not all patients respond and some may eventually acquire resistance and relapse. Our study focuses on the mechanisms of acquisition of resistance to aromatase inhibitors and strategies for reversing the resistance to possibly delay the use of chemotherapy.

To study the effects of aromatase inhibitors, we have developed a mouse model system that uses tumors of human estrogen receptor \(α\) (ER\(α\))–positive breast cancer cells (MCF-7) that are stably transfected with human placental aromatase gene (MCF-7Ca) grown in ovariectomized female nude mice (5–7). This model simulates postmenopausal breast cancer, wherein the nonovarian source of estrogen is through conversion of supplemented androstenedione (\(Δ^4\)A) by the intratumoral aromatase. Using this model, we have established that aromatase inhibitors are more effective than the antiestrogen tamoxifen in the treatment of hormone-responsive postmenopausal breast cancer (8–11). However, the tumors eventually developed resistance despite continued treatment (10–12). To determine the mechanisms of resistance to the aromatase inhibitor letrozole, we developed a novel model, wherein a cell line was isolated from the MCF-7Ca xenografts treated with letrozole (10 \(μ\)g/d) for 56 weeks (12). This cell line was designated LTLT-Ca. We evaluated the changes in protein expression compared with parental MCF-7Ca cells and established that the key adaptive changes in this cell line were upregulation of the Her-2/mitogen-activated protein kinase (MAPK) pathway and downregulation of ER\(α\) (12). We also determined that inhibition of Her-2 using trastuzumab (humanized monoclonal antibody against the extracellular domain

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of Her-2) can reverse this resistant phenotype and restore the response of LTLT-Ca cells to letrozole, in addition to other aromatase inhibitors and estrogen (13). Discontinuing the treatment of mice with resistant tumors for a few weeks also reversed this resistance (14). On discontinuation of treatment, the expression of Her-2 and p-MAPK was downregulated and ERα and aromatase were upregulated. Aromatase activity within the tumors was also upregulated (14).

To determine the optimal treatment plan to be used in the intermittent treatment strategy, we examined changes in protein expression within the tumors as they were taken “off” treatment or switched to trastuzumab.

Materials and Methods

Materials

DMEM, Modified Improved MEM, penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin-1 mmol/L EDTA solution, Dulbecco’s PBS, and genetin (G418) were obtained from Invitrogen. Androstenedione (Δ4A) and Matrigel were obtained from Sigma Chemical Company. Antibodies against Her-2 and p-Her-2 were purchased from Cell Signaling Technology. Antibodies against ERα and aromatase (CYP19) were purchased from Santa Cruz Biotechnology. Radioactive ligand for aromatase assay, 3H-Δ4A (23.5 Ci/mmol), was purchased from Perkin-Elmer.

Cell Culture

MCF-7 human breast cancer cells stably transfectioned with the human aromatase gene (MCF-7Ca) were kindly provided by Dr. S. Chen (City of Hope, Duarte, CA). Letrozole was kindly provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland).

Western Blotting

The protein extracts from tumor tissues were prepared by homogenizing the tissue in ice-cold Dulbecco’s PBS containing protease inhibitors. Cellular protein extracts were made as described earlier. A total of 50 μg of protein from each sample were analyzed by SDS-PAGE as described previously (13, 14). Bands were quantitated by densitometry using Molecular Dynamics Software (ImageQuant). The densitometric values are corrected for loading control.

3H₂O Release Assay for Aromatase Activity Measurement

For measuring aromatase activity in tumor samples, the tumors were homogenized in ice-cold Dulbecco’s PBS. The resulting homogenate was used for aromatase activity assay. The radiometric 3H₂O release assay was done as described previously (13, 14) using [1-μH]Δ4A as substrate. The activity of the enzyme is corrected for protein concentration in the tumor homogenates.

Her-2 Activity ELISA

Her-2 activity was measured by photometric ELISA assay as per manufacturer’s instructions (Cell Signaling Technologies).

Statistics

For in vivo studies, mixed-effects models were used. The tumor volumes were analyzed with S-PLUS (7.0, Insightful Corp.) to estimate and compare an exponential parameter (β) controlling the growth rate for each treatment groups. The original values for tumor volumes were log transformed. The spline model with a single knot at time = week-22 was used to accommodate the nonlinearity with a piece-wise linear model. All P values <0.05
were considered statistically significant. The graphs are represented as mean ± SEM.

Results

Intermittent Letrozole Treatment in Letrozole-Responsive Tumors
Mice with tumors of MCF-7Ca cells were given intermittent letrozole treatment of 6 weeks on and 6 weeks off. In this case, we observed that the tumors acquired resistance quicker than those in the continuous letrozole group (Fig. 1A). All mice were sacrificed on week 30 (intermittent and continuous). The tumor weights were significantly different. The group receiving continuous letrozole treatment had significantly lower mean tumor weight ($P < 0.01$) than control and intermittent letrozole group (Fig. 1B). The uterine weights in the two groups (intermittent and continuous letrozole) were not significantly different ($P = 0.8$), although they were significantly lower than that in the control group ($P < 0.001$; Fig. 1C). This suggests that letrozole was able to maintain suppression of estrogen synthesis, even though the tumors grew. This suggests that intermittent letrozole treatment may be detrimental compared with continuous treatment in hormone-responsive breast cancers.

Intermittent Letrozole Treatment in Letrozole-Resistant Tumors
The tumors of mice treated with letrozole for 22 weeks were withdrawn from letrozole treatment for 6 weeks and put back on letrozole for 6 weeks. This cyclic treatment was continued for two additional cycles. The tumor volume was maintained at 500 mm$^3$ until week 45. However, there was no substantial reduction in tumor volume (Fig. 1D).

Analysis of Changes in Protein Expression and Activity in Response to Discontinuation of Letrozole Treatment
Next, we examined the time-dependent changes that occurred in letrozole-resistant tumors on discontinuation of treatment. The mice were treated with letrozole until the mean tumor volume reached double the initial volume. This was assigned as week 0. At this time, the mice were randomized into three groups: one continued on letrozole, the second group received trastuzumab, and the third group was taken off treatment. Three mice were sacrificed each week (assigned as weeks 1–7); tumors were collected and analyzed by Western blotting. In addition, uteri were weighed and collected.

The uteri of the mice in the letrozole group did not show any change in the mean uterine weight from that at week 0 (Fig. 2A). The mean uterine weights of the mice in the “off” group increased gradually over the next 7 weeks. The mean uterine weights of the mice in the trastuzumab group also increased weekly, but this increase was significantly higher than that of the off group. This suggests that estrogen synthesis is resumed after stopping letrozole in the off group. Trastuzumab, however, enhances estrogen synthesis as confirmed by a rapid increase in uterine weight with trastuzumab treatment. This increase in uterine weight also correlates with an increase in aromatase activity (Fig. 2B).

We next examined the changes in protein expression in the tumors (Fig. 3A). Consistent with our previous results (12–14), letrozole-resistant tumors (week 0) showed upregulation of the Her-2/MAPK pathway. This was also accompanied by downregulation of ERα and aromatase. In the letrozole group, from week 1 to week 7, p-MAPK expression showed gradual increase, and by week 7, it had increased significantly. A similar pattern was seen with p-Elk, p-90RSK, Her-2, and p-Her-2. Her-2 activity (Fig. 3B) also increased gradually until week 7. ERα expression decreased during letrozole treatment from week 1 to week 7. When the mice were taken off, p-MAPK, p-90RSK, Her-2, and p-Her-2 expression and Her-2 activity decreased, whereas ERα and aromatase increased. A similar effect of trastuzumab was observed; however, a marked difference was seen at week 2. In the off group, by week 4, the protein expression had changed to levels similar to that in control tumors. We next examined the effect of 4-week off treatment and trastuzumab switch on tumor growth.

Mechanism of Increase in Her-2 in Letrozole-Resistant Tumors
To evaluate the mechanism of Her-2 upregulation in letrozole-resistant tumors, we performed fluorescence in situ hybridization analysis on tumors treated with letrozole (Fig. 1D) and compared them with the Δ$^3$A-treated...
Figure 2. A, mean uterine weights of mice bearing MCF-7Ca xenografts that were switched to Δ⁴A or trastuzumab (TRZ) treatment after acquisition of letrozole resistance. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm³, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were randomized into three groups: one group continued on letrozole, the second group received Δ⁴A, and the third group received trastuzumab; after which, three mice were sacrificed each week from each group. The uterine weights were measured at autopsy. B, aromatase activity in MCF-7Ca xenografts that were switched to Δ⁴A or trastuzumab treatment after acquisition of letrozole resistance. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm³, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were randomized into three groups: one group continued on letrozole, the second group received Δ⁴A, and the third group received trastuzumab; after which, three mice were sacrificed each week from each group. The tumors were collected at autopsy. Aromatase activity was measured by radiometric ³H₂O release assay.
Figure 3. A, Western blot analysis of MCF-7Ca xenografts that were switched to ΔA or trastuzumab treatment after acquisition of letrozole resistance. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm³, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were assigned to three groups: one group continued on letrozole, the second group received ΔA, and the third group received trastuzumab; after which, three mice were sacrificed each week from each group. The tumors were collected at autopsy and protein expression was analyzed by Western immunoblotting.

B, Her-2 activity in MCF-7Ca xenografts that were switched to ΔA or trastuzumab treatment after acquisition of letrozole resistance. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm³, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were assigned to three groups: one group continued on letrozole, the second group received ΔA, and the third group received trastuzumab; after which, three mice were sacrificed each week from each group. The tumors were collected at autopsy and Her-2 activity was measured by ELISA.
control and off groups. As shown in Supplementary Fig. S1, letrozole-resistant tumors did not have amplification of the Her-2 gene. We next evaluated the stability and half-life of Her-2 protein in LTLT-Ca cells and compared it to the parental MCF-7Ca cells. The cells were treated with actinomycin D and cyclohexamide (5 \(\mu\)mol/L each) to inhibit new protein synthesis and the half-lives of ER\(\alpha\) and Her-2 were measured by Western blotting. As shown in Fig. 4A, when supplemented with 17\(\beta\)-estradiol (E\(_2\)), Her-2 protein in MCF-7Ca cells has a short half-life. The levels dropped to below 30\% in the first 2 hours, whereas under E2 Withdrawal (E\(_2\)W) condition, the levels stayed higher longer (10\% at 8 hours). In addition, when treated with letrozole, Her-2 protein levels stayed up for 16 hours and was 40\% by 24 hours. In LTLT-Ca cells, Her-2 protein has a long half-life (80\% by 24 hours). When letrozole was withdrawn, Her-2 half-life in LTLT-Ca cells was shorter (50\% in 24 hours) but still longer than that in MCF-7Ca cells. This suggests that low levels of estrogen (as achieved by aromatase inhibitors) inhibit degradation of Her-2, causing increased Her-2 levels.

The stability of ER\(\alpha\) protein was then examined (Fig. 4B). The half-life of ER\(\alpha\) was not significantly different between MCF-7Ca and LTLT-Ca cells. In the presence of E\(_2\), 50\% of the ER\(\alpha\) was degraded by 8 hours. In the absence of E\(_2\), ER\(\alpha\) protein levels were stabilized and a significant reduction in ER\(\alpha\) protein levels was only observed at 24 hours.

**Treatment of Letrozole-Resistant Tumors Using Intermittent Off or Trastuzumab Regimen**

We evaluated the effect of intermittent treatment using the MCF-7Ca xenograft model as described in Materials and Methods (13, 14). Figure 5 (top) shows a schematic of the experimental design. Figure 5 shows the tumor volumes across different groups versus time (weeks). The mice were grouped so that the tumor volumes on week 0 were not different across the groups (\(P = 0.76\)). As expected, the growth rate of letrozole-treated (\(\beta_i = -0.11 \pm 0.019\)) tumors was significantly lower than control (\(\beta_i = 0.24 \pm 0.5\)) tumors (\(P < 0.0001\)) over the first 9 weeks. At this time point, the mice in the control group were sacrificed due to large tumor volumes. However, the tumors of the letrozole-treated mice eventually began to grow and had doubled in volume by week 22. At this time, they were assigned to three groups: letrozole (\(\Delta^4A + \text{letrozole}, 10 \mu\)g/d), off (\(\Delta^4A, 100 \mu\)g/d), and trastuzumab (\(\Delta^4A + \text{trastuzumab}, 5 \text{mg/kg/wk divided into two doses}\)). The tumor volumes were not significantly different across the groups (\(P = 0.86\)). Based on tumor growth rates, we concluded that the groups did not have different rates of growth through week 26 (letrozole versus off, \(P = 0.47\); letrozole versus trastuzumab, \(P = 0.93\); off versus trastuzumab, \(P = 0.2\)).

As a continuation of treatment, the off group was split into two: one continued without letrozole and the other group received letrozole. The growth rates of the letrozole and the off groups were not significantly different.
over weeks 26 to 33 ($P = 0.37$). However, the growth rate of mice switched back to letrozole had significantly lower growth rate compared with the continuous letrozole group ($P = 0.02$).

On the other hand, the mice receiving trastuzumab were assigned to three groups; trastuzumab, trastuzumab plus letrozole, and letrozole. The growth rates across these groups were not significantly different over 32 weeks ($P = 0.42$).

This suggests that trastuzumab, as single agent in letrozole-resistant tumors, does not provide any benefit. In addition, when switched from letrozole to trastuzumab and then back to letrozole or letrozole plus trastuzumab, the tumors continued to grow. In contrast, when given a 4-week “break” in treatment (off) and then switched back to letrozole, the tumors were inhibited for a prolonged period of 18 weeks (weeks 26–44), approximately the same as that of the first course of letrozole.

We also examined the tumors for protein expression and activity. Consistent with previous results (12, 13), tumors in the letrozole-treated group had higher Her-2/MAPK activation and lower ERα and aromatase activity (Fig. 6A–C). We have shown that treatment with MAPK inhibitor decreased MAPK but increased ERα. In contrast, when taken off treatment or switched to trastuzumab, Her-2 and MAPK were downregulated and ERα was upregulated. Aromatase activity also followed ERα expression. Interestingly, tumors of mice on letrozole at 44 weeks had a similar protein expression profile to tumors from mice on letrozole at 15 weeks (Fig. 6B).

**Discussions**

Despite the significant improvement in the outcome of hormone-responsive breast cancer following aromatase...
Figure 6. A, Her-2 activity in the letrozole-resistant MCF-7Ca xenografts in Fig. 5. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm³, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were randomized as described in the legend to Fig. 5. The tumors were collected at autopsy and Her-2 activity was measured by ELISA. B, Western blot analysis of the three letrozole-resistant MCF-7Ca xenografts in Fig. 5. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm³, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were randomized as described in the legend to Fig. 5. The tumors were collected at autopsy and protein expression was analyzed by Western immunoblotting. C, aromatase activity in the letrozole-resistant MCF-7Ca xenografts in Fig. 5. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm³, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were randomized as described in the legend to Fig. 5. The tumors were collected at autopsy and aromatase activity was measured by radiometric ³H₂O release assay.
Within 1 week. ERα expression was detected in the LTLT-Ca cells. Using this new model, we established that activation of growth factor receptor (LTLT-Ca) cells. Following treatment with trastuzumab, the HER-2 activation was downregulated and ERα levels were restored. This result suggests that HER-2 is a negative regulator of ERα. A similar reversal of resistant phenotype was observed on letrozole withdrawal (14). Stopping letrozole treatment for 6 weeks led to restoration of the response of tumors to letrozole in the MCF-7Ca xenograft model (14). Studies with the MAPK inhibitor U0126, however, showed that inhibition of the MAPK pathway leads to activation of the phosphoinositide 3-kinase/Akt pathway (data not shown). As such, MAPK activation results in longer response and disease stabilization in patients. However, detailed clinical studies need to be done to establish correct intermittent scheduling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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