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Research Article

Molecular Cancer Therapeutics

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 (ERα) 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^4A 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 ERα and aromatase protein (and activity) increased. Within the first week of trastuzumab treatment, Her-2 and MAPK were downregulated and ERα 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 ERα/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.* ©*2010 AACR.*

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 (ERα)–positive beast 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^4A) 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 μg/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 ERα (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 geneticin (G_{418}) were obtained from Invitrogen. Androstenedione (Δ^4A) and Matrigel were obtained from Sigma Chemical Company. Antibodies against Her-2 and p-Her-2 were purchased from Upstate (now Millipore); antibodies against p-MAPK, MAPK, p-Elk-1, and p-p90RSK were purchased from Cell Signaling Technology. Antibodies against ERα and aromatase (CYP19) were purchased from Santa Cruz Biotechnology. Radioactive ligand for aromatase assay, ^{3}H-Δ^4A (23.5 Ci/mmol), was purchased from Perkin-Elmer.

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). Letrozole was kindly provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland).

Cell Culture

MCF-7Ca cells were routinely cultured in DMEM supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and 700 μg/mL G_{418}. LTLT-Ca cells were developed from MCF-7Ca cells as described earlier (12, 14) from tumors of mice treated with letrozole for 56 wk and cultured in steroid-depleted medium containing 1 μmol/L letrozole. Cell proliferation assays were done using MTT assay as described earlier (13, 14). The results were expressed as a percentage of the cell number in the Δ^4A-treated control wells. IC_{50} values for inhibitors were calculated from the linear regression line of the plot of percentage inhibition versus log inhibitor concentration.

Tumor Growth in Ovariectomized Female Athymic Nude Mice

All animal studies were done according to the guidelines and with the approval of the Animal Care Committee of the University of Maryland, Baltimore. Female ovariectomized athymic nude mice, 4 to 6 wk of age, were obtained from the National Cancer Institute-Frederick Cancer Research and Development Center (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 the mice as previously described (6, 9, 12–14). Each mouse received s.c. inoculations in one site per flank with 100 μL of cell suspension containing ~2.5×10^7 cells. The mice were injected daily with supplemental Δ^4A (100 μg/d). Weekly tumor measurements and treatments began when the tumors reached ~300 mm^3. 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. Letrozole and Δ^4A for injection were prepared using 0.3% hydroxypropylcellulose in 0.9% NaCl solution. Trastuzumab for injection was prepared as a 20 mg/mL stock solution in bacteriostatic water for injection, which was then diluted in 0.9% NaCl solution to obtain the required concentration. Mice were then injected s.c. five times weekly with the indicated drugs (except trastuzumab was injected i.p. twice a week). The doses of trastuzumab (5 mg/kg/wk divided into two doses), letrozole (10 μg/d), and Δ^4A (100 μg/d) used are as previously determined and reported (13).

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.

^3H_2O 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 ^3H_2O release assay was done as described previously (13, 14) using [1-β^{3}H]Δ^4A 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_i) 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

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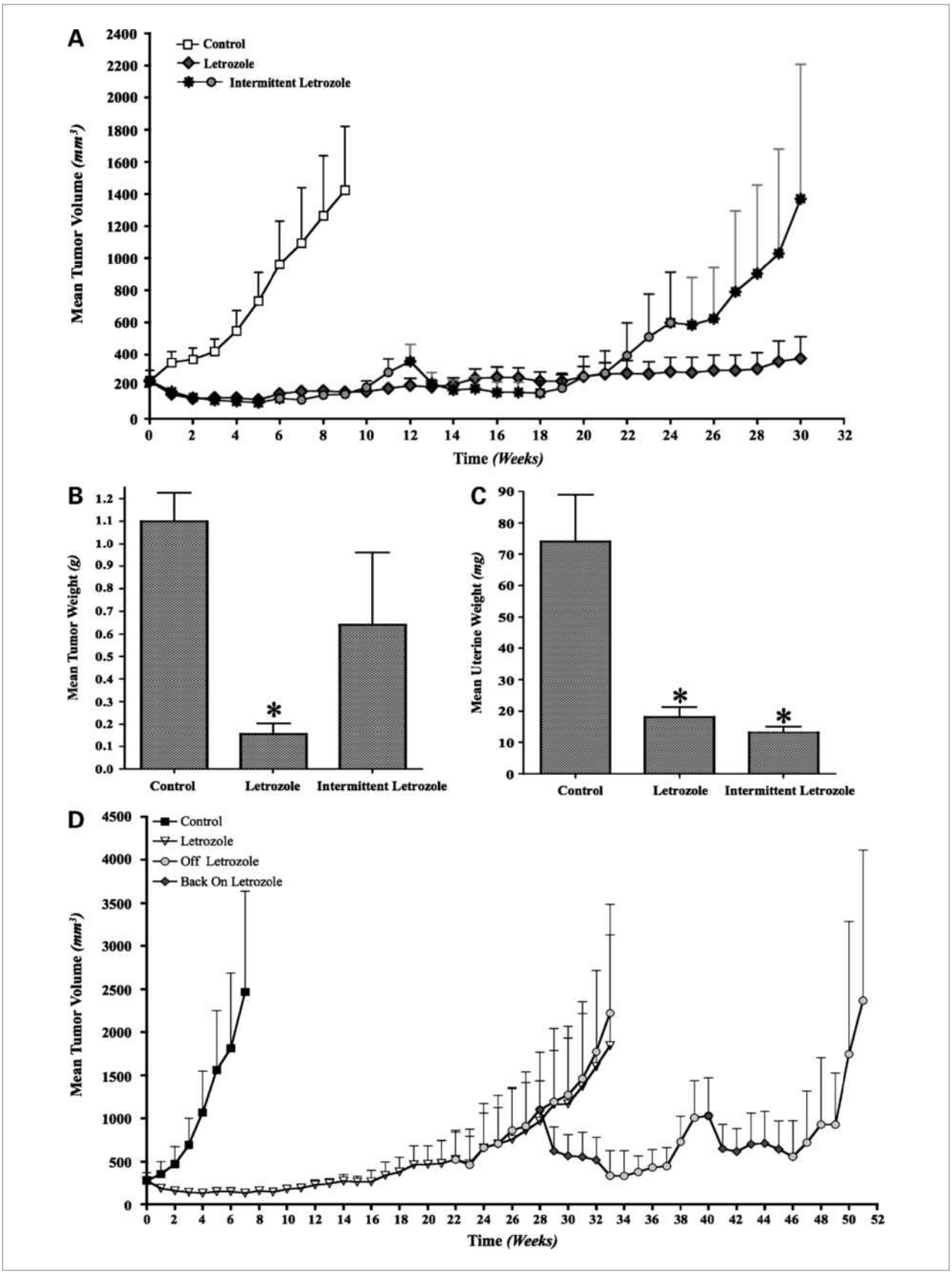

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were considered statistically significant. The graphs are represented as mean ± SEM.

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-p90RSK, 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-p90RSK, 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^4A-treated

Figure 1. **A,** effect of intermittent (6 wk on, 6 wk off) letrozole treatment on the growth of MCF-7Ca xenografts. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice were treated with either continuous letrozole or intermittent (6 wk on, 6 wk off) letrozole. The tumor volumes were measured weekly. **B,** effect of intermittent (6 wk on, 6 wk off) letrozole treatment on the mean tumor weight of mice bearing MCF-7Ca xenografts. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice were treated with either continuous letrozole or intermittent (6 wk on, 6 wk off) letrozole. The tumor weights were measured at autopsy. The continuous letrozole group had significantly lower mean tumor weight (*, $P < 0.01$). **C,** effect of intermittent (6 wk on, 6 wk off) letrozole treatment on the mean uterine weight of mice bearing MCF-7Ca xenografts. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice were treated with either continuous letrozole or intermittent (6 wk on, 6 wk off) letrozole. The uterine weights were measured at autopsy. The mean uterine weights of both continuous and intermittent letrozole mice were significantly lower than that of control mice (*, $P < 0.001$). **D,** effect of intermittent (6 wk on, 6 wk off) letrozole treatment on the growth of MCF-7Ca xenografts that have acquired resistance to letrozole. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were divided into two groups: one group received continuous letrozole and the other received intermittent (6 wk on, 6 wk off) letrozole. The tumor volumes were measured weekly.

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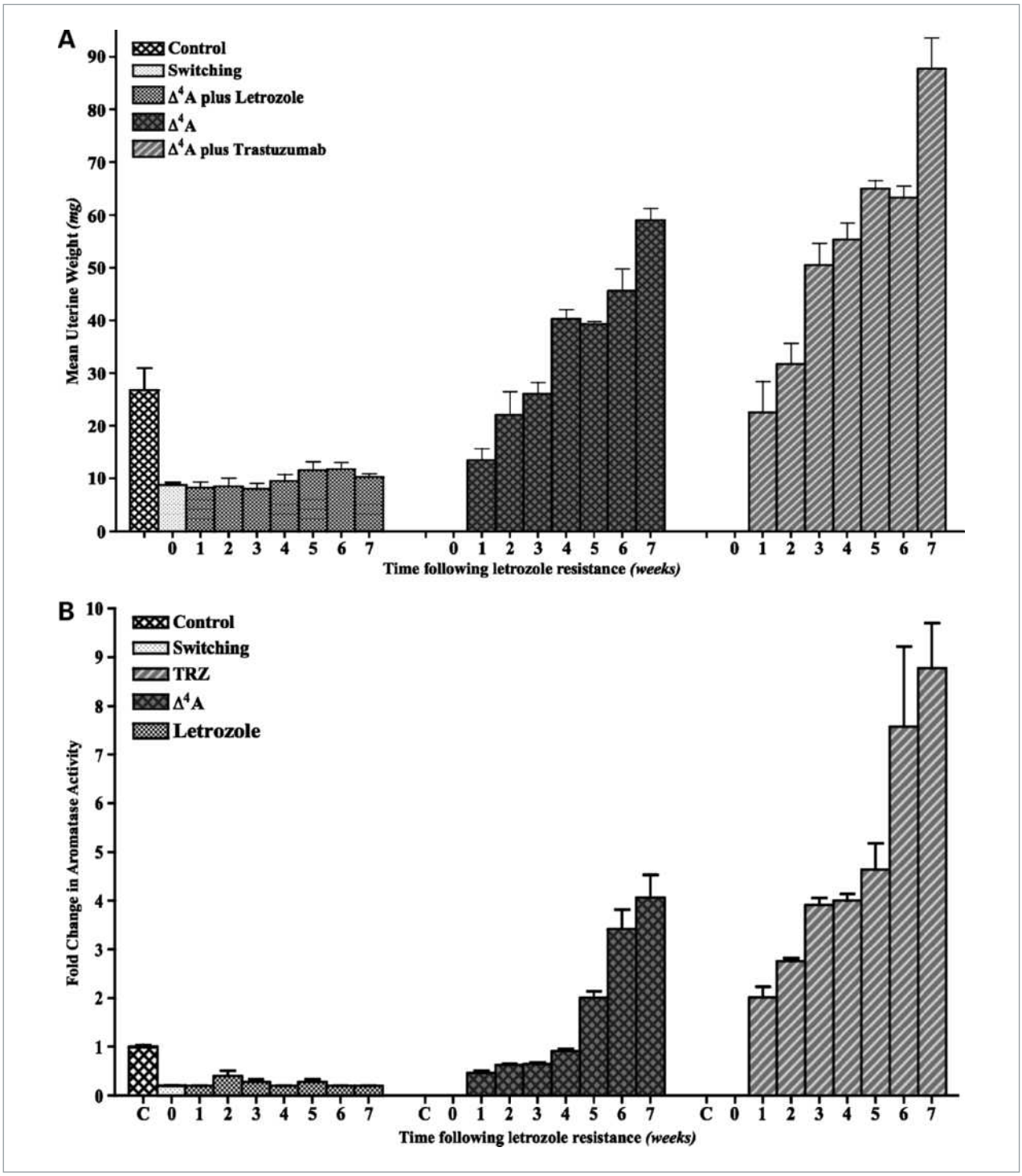

Figure 2. **A,** mean uterine weights of mice bearing MCF-7Ca xenografts that were switched to Δ^4A or trastuzumab (*TRZ*) treatment after acquisition of letrozole resistance. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice 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 Δ^4A, 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 Δ^4A 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 mm^3, 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 Δ^4A, 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 ^3H_2O release assay.

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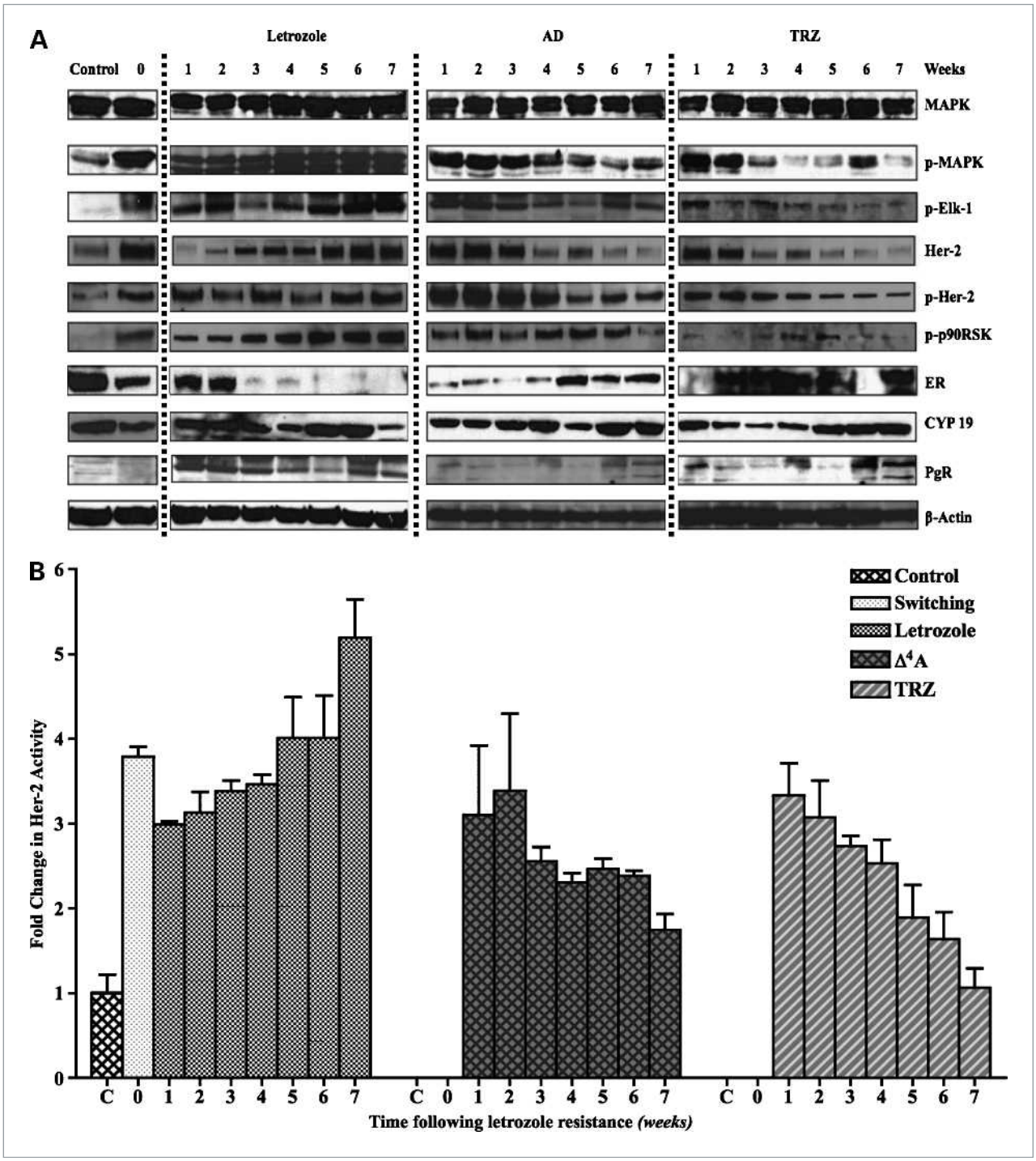

Figure 3. **A,** Western blot analysis of MCF-7Ca xenografts that were switched to Δ^4A or trastuzumab treatment after acquisition of letrozole resistance. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice were treated with letrozole 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 Δ^4A, 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 Δ^4A 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 mm^3, 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 Δ^4A, 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.

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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 μmol/L each) to inhibit new protein synthesis and the half-lives of ERα and Her-2 were measured by Western blotting. As shown in Fig. 4A, when supplemented with 17β-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_2W) 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 ERα protein was then examined (Fig. 4B). The half-life of ERα was not significantly different between MCF-7Ca and LTLT-Ca cells. In the presence of E_2, 50% of the ERα was degraded by 8 hours. In the absence of E_2, ERα protein levels were stabilized and a significant reduction in ERα 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 (Δ^4A + letrozole, 10 μg/d), off (Δ^4A, 100 μg/d), and trastuzumab (Δ^4A + trastuzumab, 5 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

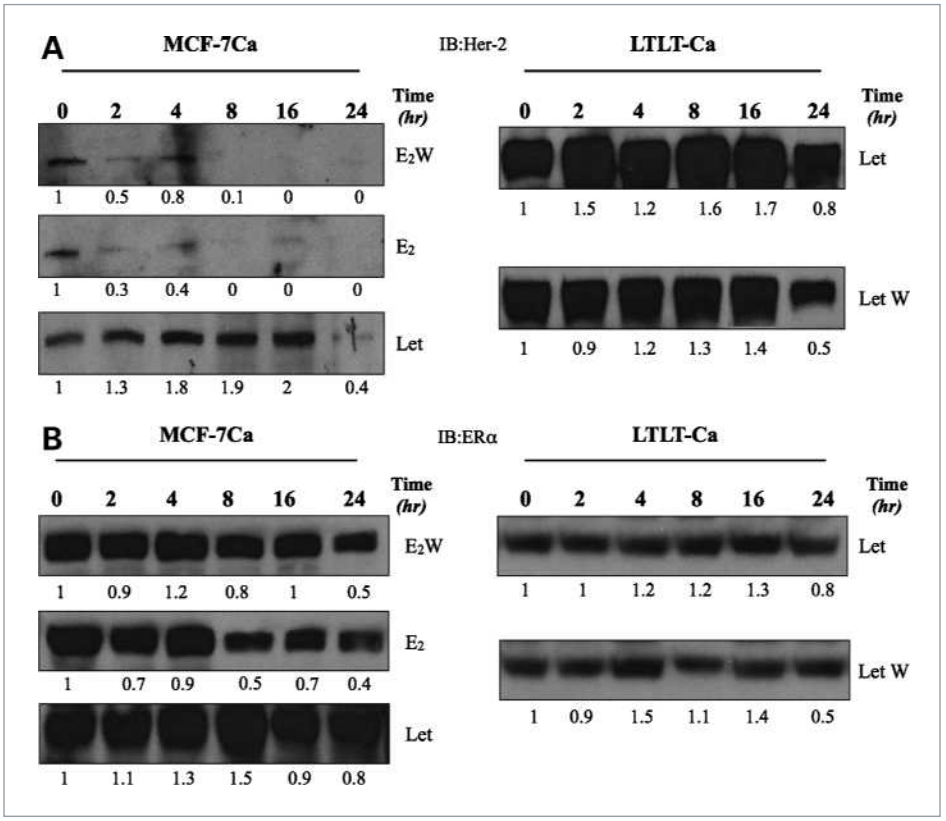

Figure 4. **A,** half-life of Her-2 in MCF-7Ca and LTLT-Ca cells. The cells were treated with actinomycin D and cyclohexamide (5 μmol/L each) to inhibit new protein synthesis. The cells were either subjected to E_2W or treated with E_2 (10 nmol/L) or letrozole (1 μmol/L). Cell lysates were made as described in Materials and Methods. Protein (50 μg) was analyzed by Western blotting to measure relative Her-2 protein levels. *Left*, MCF-7Ca cells; *right*, LTLT-Ca cells. Each lane corresponds to treatment time in hours. **B,** half-life of ERα in MCF-7Ca and LTLT-Ca cells. The cells were treated with actinomycin D and cyclohexamide (5 μmol/L each) to inhibit new protein synthesis. The cells were either subjected to E_2W or treated with E_2 (10 nmol/L) or letrozole (1 μmol/L). Cell lysates were made as described in Materials and Methods. Protein (50 μg) was analyzed by Western blotting to measure relative ERα protein levels. *Left*, MCF-7Ca cells; *right*, LTLT-Ca cells. Each lane corresponds to treatment time in hours.

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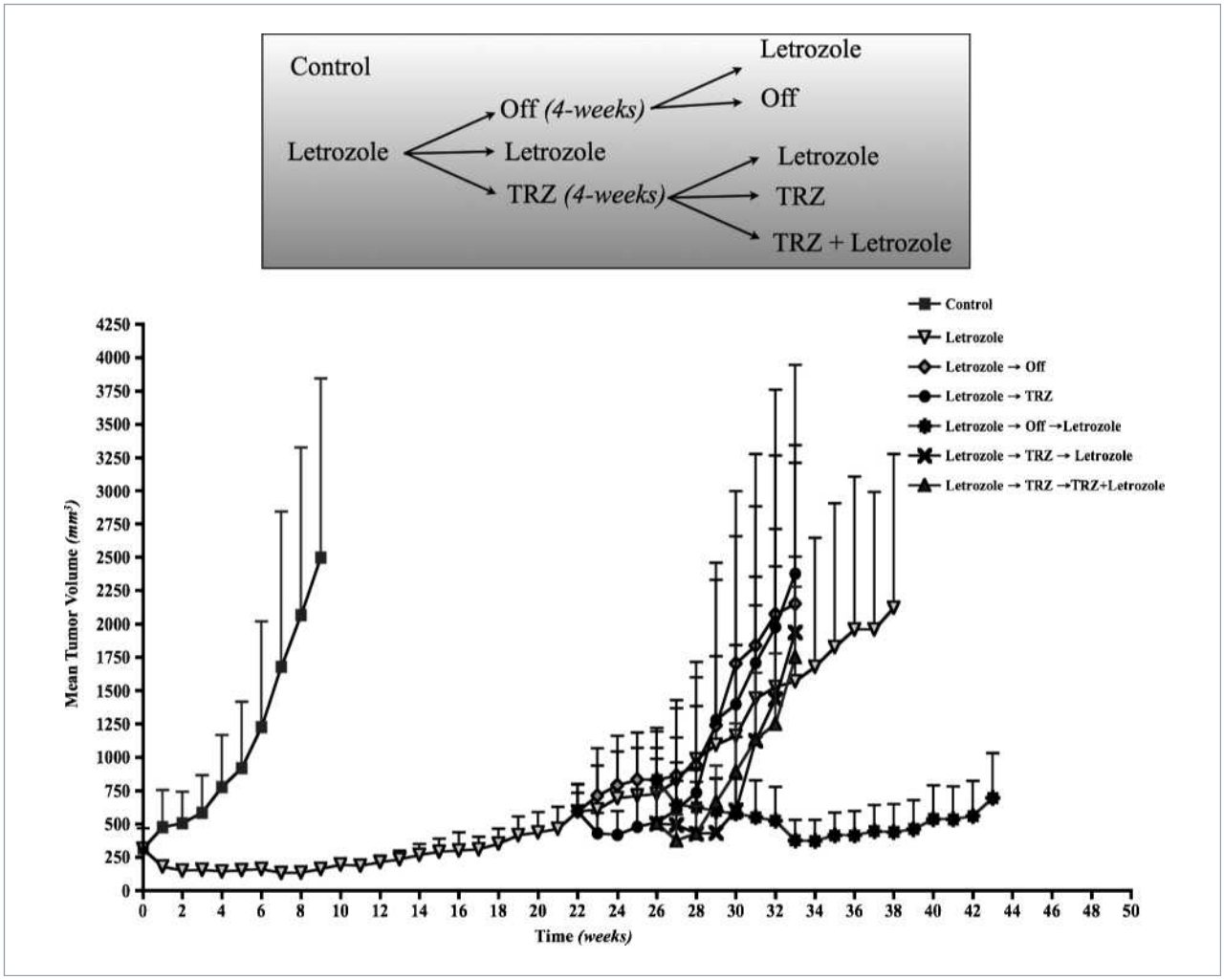

Figure 5. Tumor volumes of MCF-7Ca xenografts. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice were randomized as shown in the schematic. The tumor volumes were measured weekly. *Top*, schematic representation of the treatment schedule.

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

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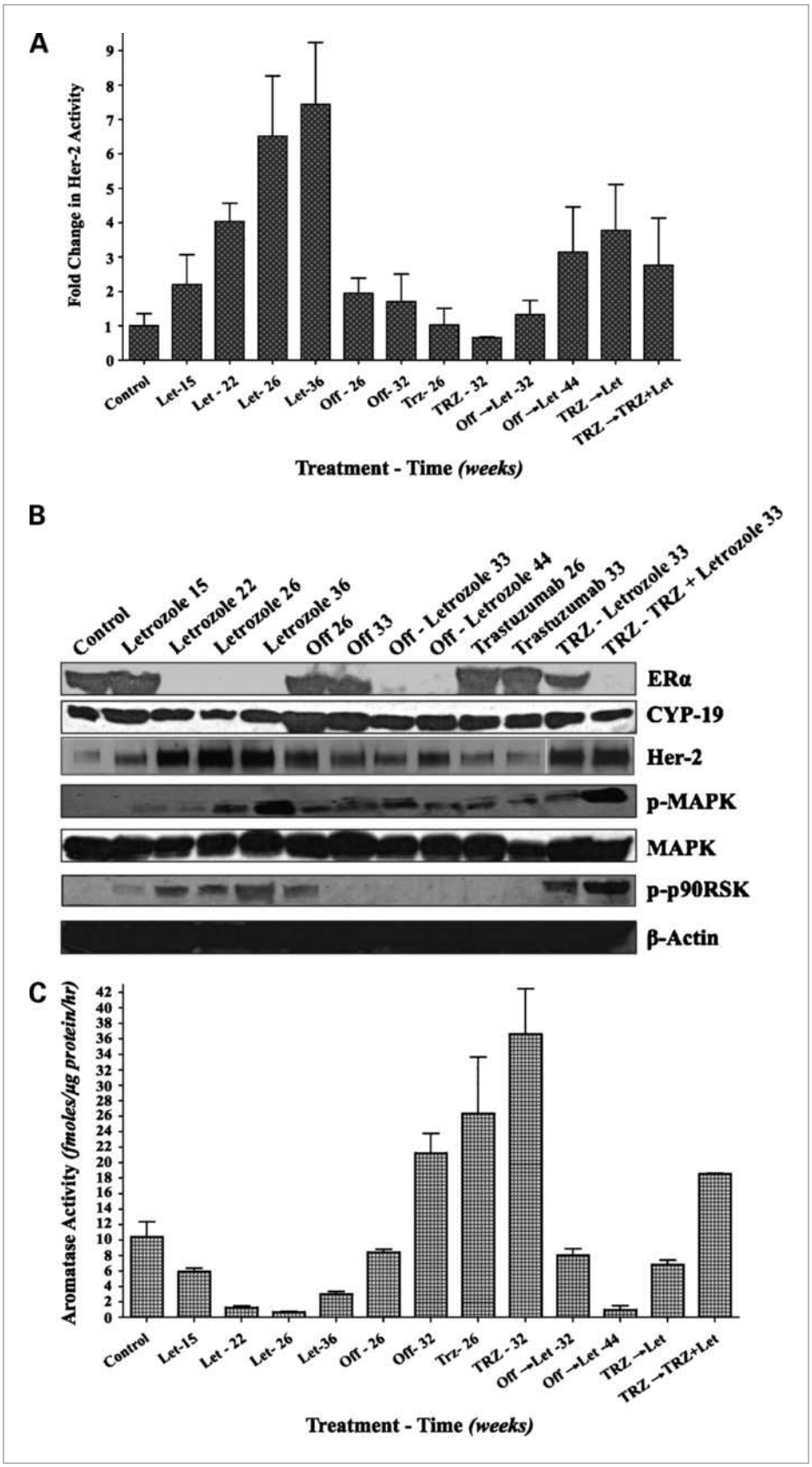

Figure 6. **A,** Her-2 activity in the letrozole-resistant MCF-7Ca xenografts in Fig. 5. Xenografts of MCF-7Ca cells were grown in mice as described in Materials and Methods. After the tumors reached 300 mm^3, mice were treated with letrozole at 10 μg/d for 22 wk. At this time, the mice were randomized 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 mm^3, 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 mm^3, 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 ^3H_2O release assay.

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inhibitor treatment, acquisition of resistance remains a major concern. In our model system that mimics the postmenopausal hormone-dependent breast cancer patient, the aromatase inhibitor letrozole was more effective than tamoxifen in controlling tumor growth. However, tumors eventually began to regrow. To understand the mechanisms of this acquired resistance, we developed a cell line from tumors treated with letrozole for a prolonged period. These were designated as long-term letrozole-treated (LTLT-Ca) cells. Using this new model, we established that activation of growth factor receptor–mediated pathways such as Her-2 and MAPK was associated with letrozole resistance. Furthermore, inhibition of these pathways with inhibitor of Her-2 (trastuzumab; ref. 13) or MAPK (PD98059; ref. 12) resulted in reversal of resistance. Thus, we concluded that resistance to letrozole was a result of adaptation of tumor cells to a low-estrogen environment through upregulation of Her-2 and downregulation of ERα. Following treatment with trastuzumab, 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 seems to be one of the effectors in the Her-2 pathway.

In tumors of mice in the trastuzumab group, these changes occurred at a faster rate. Our previous studies have shown that inhibition of Her-2 (with trastuzumab) can activate ERα and increase aromatase activity in an ERα-dependent manner (13). As such, inhibition of both the ERα and Her-2 pathways is essential to overcome acquired resistance to letrozole. This study also shows that trastuzumab treatment can reverse resistant phenotype within 1 week. ERα and aromatase are increased to the same levels as in hormone-responsive tumors, and hence, inhibition of ER-mediated pathways would be necessary within the first week. This suggests that Her-2 is a negative regulator of ERα. In patient tissue samples, a strong inverse correlation is observed before any endocrine treatment. However, on resistance, ERα was lost in 17% of samples and Her-2 was increased in 11% of the patients (15, 16). Acquired Her-2 amplification was also observed in patients' circulating tumor cells (17), but was not evidenced here. However, letrozole seemed to inhibit Her-2 degradation up to 16 hours, which may account for the increase in the levels of Her-2. Similarly, conversion of serum Her-2 from negative to positive has been observed in patients with advanced cancer that has progressed on endocrine therapy (16).

Several reports have suggested a role of Her-2 in mediating resistance to hormonal therapy such as the antiestrogen tamoxifen and aromatase inhibitors. A bidirectional cross talk between ERα and Her-2 and/or other members of the epidermal growth factor receptor family has been shown to be a key phenomenon in the resistant model systems (18–23). Intracellular kinases such as Akt and MAPK can phosphorylate the serine residues (such as S118 and S167) in the AF-1 domain of ERα and activate transcription (24–26). This is consistent with our LTLT-Ca model, wherein inhibition of Her-2 with trastuzumab restored responsiveness to letrozole (13).

Studies involving tamoxifen resistance have also revealed that cyclic treatment with tamoxifen and E_2 leads to longer duration of response to tamoxifen. Jordan and colleagues have shown that tamoxifen stimulates the growth of tumors on acquisition of resistance as tamoxifen exerts more agonistic effects (27). In these tumors, E_2 can inhibit tumor growth (27, 28). After a few days on E_2, tumors once again became sensitive to the growth inhibitory effects of tamoxifen (27, 28). The results presented here indicate that once letrozole treatment is stopped, aromatization of Δ^4A is resumed and E_2 is synthesized. This suggests that off treatment slowly reverses resistance, whereas switching to trastuzumab forces an increase in ERα, allowing response to endogenous estrogen production. The strategy presented here could result 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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