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## Stopping treatment can reverse acquired resistance to letrozole

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### Abstract

Using the intra tumoral 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), ER $\alpha$  levels were decreased whereas signaling proteins in the MAPK cascade were upregulated along with Her-2. In the current study we evaluated the effect of discontinuing the 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 six weeks, letrozole was administered again. Marked tumor regression was observed with this second course of letrozole treatment. Similarly in MCF-7Ca xenografts, a six-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 p-MAPK was reduced and ER $\alpha$  and aromatase increased compared to levels in LTLT-Ca cells and were similar to the 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 AIs for breast cancer patients.

### Keywords

aromatase inhibitors; estrogen; letrozole; ER $\alpha$ ; Her-2

### Introduction

About 75% of the breast cancer patients have hormone dependent breast cancer and are treated with 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 development of resistance to aromatase inhibitor treatment. To study the hormone responsive breast cancer, we created a model system in which tumors of human ER positive breast cancer cells stably transfected with the aromatase gene (MCF-7Ca) are grown in immune suppressed 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 expression of Her-2 and proteins in the downstream MAPK pathway. In

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order to investigate the mechanisms in the loss of sensitivity of the tumors to AIs, we developed a cell line isolated from tumors of MCF-7Ca cells treated long term with letrozole (8,9). These cells also exhibited lower expression of ER $\alpha$  and 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 $\alpha$  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 Her-2 pathway reverses the resistance of LTLT-Ca cells to letrozole (10). This suggests cross-talk between the Her-2/MAPK and the ER $\alpha$  pathway is controlling 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 $\alpha$  levels were restored and the cells regain their original sensitivity to letrozole.

## Materials and Methods

### Materials

Dulbecco's Modified Eagle Medium (DMEM), Modified Improved Minimum Essential medium (IMEM), penicillin/streptomycin solution (10,000IU each), 0.25% trypsin–1 mM EDTA solution, Dulbecco's phosphate-buffered saline (DPBS), and geneticin (G<sub>418</sub>) were obtained from Invitrogen (Carlsbad, CA). Androstenedione ( $\Delta^4$ A) was obtained from Sigma Chemical Company (St. Louis, MO).

### Cell culture

MCF-7Ca cells were routinely cultured in DMEM supplemented with 5% FBS, 1% Penicillin/Streptomycin, 700 $\mu$ g/mL G<sub>418</sub>. 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 1-A, hyaluronidase type 1-S, amphotericin B and polymixin B sulfate. The cells were filtered and plated into Petri dishes. After a week, phenol red–free trypsin was added to remove fibroblast and washed with DPBS. Epithelial cells were cultured in medium with 750  $\mu$ g/mL G<sub>418</sub> and 1  $\mu$ M of letrozole. Over the next 2 to 3 weeks, serum levels in the medium were reduced to 5% charcoal stripped serum and penicillin/streptomycin level to 1%. The cells were then propagated in phenol red–free IMEM supplemented with 5% CSS, 1% penicillin/streptomycin, 750  $\mu$ g/mL G<sub>418</sub> and 1  $\mu$ M of letrozole. LTLT-Ca cells were derived by culturing LTLT-Ca cells supplementing with  $\Delta^4$ A and in absence of letrozole for at least 4 months.

Cell proliferation assays were performed using the MTT assay as described earlier (8,11). The results were expressed as a percentage of the absorbance of the controls. IC<sub>50</sub> values for inhibitors were calculated from the non-linear 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 4–6 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 the 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  $(\frac{4}{3}) \pi r_1^2 r_2$  ( $r_1 \leq r_2$ ). The doses of letrozole and  $\Delta^4A$  used are as previously determined and reported. Mice in the  $\Delta^4A$  group ( $n = 6$ ) were treated for 7 weeks after which they were sacrificed due to large tumor volumes. The mice in letrozole group ( $n = 30$ ) were continued on treatment. When the tumor volumes of these mice reached double their starting size, the mice were regrouped into 2 groups. One group ( $n = 10$ ) stayed on continued letrozole treatment and the other group received only  $\Delta^4A$  ( $n = 20$ ). 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 assigned “**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 the tumor volumes ( $p = 0.56$ ).

### Western blotting

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

### $^3H_2O$ release assay for aromatase activity measurement

The radiometric  $^3H_2O$  release assay was performed as described previously (12). The activity of the enzyme is 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 parameter ( $\beta_i$ ) controlling the growth rate for each treatment groups as described previously (13). All  $p$  values less than 0.05 were considered statistically significant.

## Results

### AEs, AIs produce inhibitory effects and $E_2$ 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 Figure 1A, the  $IC_{50}$  of letrozole was 44.3pM in the parental MCF-7Ca cells and 1.85nM in RLT-Ca cells, whereas LTLT-Ca cells were resistant to growth inhibitory effects of letrozole. Similar effects were observed with other AIs, anastrozole, exemestane and AE 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 ( $IC_{50} > 19.8 \mu M$ ). 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 AIs and AEs whereas LTLT-Ca cells exhibit complete cross-resistance to all the endocrine agents (*data not shown*). The reversal of response to letrozole was accompanied by increased aromatase activity in RLT-Ca cells (Figure 1B) compared to LTLT-Ca cells ( $p < 0.01$ ) and which was higher than in MCF-7Ca cells ( $p < 0.05$ ).

In addition, RLT-Ca cells respond to mitogenic effects of  $\Delta^4A$  and  $E_2$ . Figure 2A shows that  $\Delta^4A$  stimulated the growth of RLT-Ca cells at concentrations of  $10^{-11}$  and  $10^{-9}M$  ( $p < 0.01$ ) a response similar to that of MCF-7Ca cells. Interestingly, the growth of LTLT-Ca cells was decreased at all  $\Delta^4A$  concentrations (Figure 2A). This effect may be due to specific androgenic

effect of  $\Delta^4\text{A}$  as it was not converted to estrogen owing to low level of aromatase activity in LTLT-Ca cells (Figure 1B) (14).  $\text{E}_2$  was ineffective in stimulating the growth of LTLT-Ca cells, as shown in Figure. 2B. However, the growth of RLT-Ca cells was significantly stimulated by  $\text{E}_2$  at concentrations  $10^{-12}$  -  $10^{-10}\text{M}$  ( $p < 0.01$ ). The biphasic dose response of  $\Delta^4\text{A}$  and  $\text{E}_2$  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 to LTLT-Ca cells, Her-2, p-MAPK levels were decreased and ER $\alpha$  increased to levels approaching those of MCF-7Ca cells. (Figure 2C).

### Reversal of letrozole resistance in LTLT-Ca xenografts

As shown in Figure 3A, when LTLT-Ca xenografts were grown in nude mice in 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 ( $\beta_i$ ) of tumors treated with letrozole ( $6.5 \pm 7.1$ ) was significantly ( $p = 0.04$ ) lower compared to control tumors ( $48.8 \pm 16.8$ ). The mean tumor volume of letrozole treated mice at week 24 was found to be  $196 \pm 169.4 \text{ mm}^3$  whereas without letrozole treatment the mice had mean tumor volume of  $1261.9 \pm 409.3 \text{ mm}^3$  ( $p = 0.03$ ). As shown in Figure 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 synthesis of estrogen production (Figure. 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  $\Delta^4\text{A}$  (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 of these mice had a similar growth rate to that of the group continued on letrozole over weeks 22–34. The difference in the exponential parameter governing growth rate ( $\beta_i$ ) was  $0.002 \pm 0.12$ ,  $p = 0.99$ . 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 parameter governing growth rate was  $0.436 \pm 0.12$  ( $p = 0.0005$ ). Also, the “back on” group had a significantly lower growth rate compared to the “letrozole” group. The difference in the exponential parameter governing growth rate was  $0.434 \pm 0.13$  ( $p = 0.0009$ ). (Figure 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  $-80^\circ\text{C}$ . The tumor weights of the mice in control, letrozole and “off” group were not statistically different ( $p = 0.46$ ) (Figure. 4A).

However, the uterine weights exhibit significant differences (Figure. 4B). As the mouse uterus is very sensitive to estrogens, the measurement of uterine weight is a useful bioassay that indicates the level of circulating estrogens in the mouse. The mice in the “letrozole” group had significantly lower uterine weights compared to the control group ( $p = 0.0006$ ) and “off” ( $p = 0.02$ ) group indicating that aromatase was effectively inhibited by letrozole in this group. However, the mice in control and “off” group had similar uterine weights ( $p = 1$ ). Furthermore, the uterine weights of mice in the control and “off” group 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 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 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 the aromatase activity after treatment with letrozole. Tumors used for this assay were collected at different time points during the course of treatment (Figure 4C). When treated with letrozole, aromatase activity of the tumors was significantly lower compared to control ( $p < 0.01$ , week 22) and ( $p < 0.001$ , week 33). However, after discontinuing the treatment (“Off” – 22 weeks), aromatase activity increased and was similar to the control level ( $p < 0.001$  compared to letrozole at week 22 and 33). 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 control ( $p < 0.01$ ).

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

### Discussions

Despite significant advances in the treatment of breast cancer since the development of AIs, (15-17) some patients eventually become resistance to treatment. Thus, it is important to understand the mechanisms of the 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 to 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 AEs, AIs,  $\Delta^4$ A and E<sub>2</sub> (8,18). In these cells, ER levels were reduced and cell growth was independent of estrogen indicating, cross-talk between ER $\alpha$  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 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 $\alpha$  had returned to the levels in the original phenotype, MCF-7Ca cells. Thus, RLT-Ca cells respond to E<sub>2</sub>,  $\Delta^4$ A, AIs and fulvestrant. The EC<sub>50</sub> 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  $\Delta^4$ A 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. Although the mechanism of this increase is unclear, previous studies have reported increased aromatase activity after letrozole withdrawal that has been explained by stabilization and reduced degradation of the aromatase protein. Interestingly, growth of tumors in “off” group was not significantly different from those of the animals continuing on letrozole. Although, the increase in aromatase activity may subsequently lead to increase in tumor growth in the “Off” group, 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 AIs. This strategy could have advantages for the patients relapsing from aromatase inhibitor treatment.

## Acknowledgements

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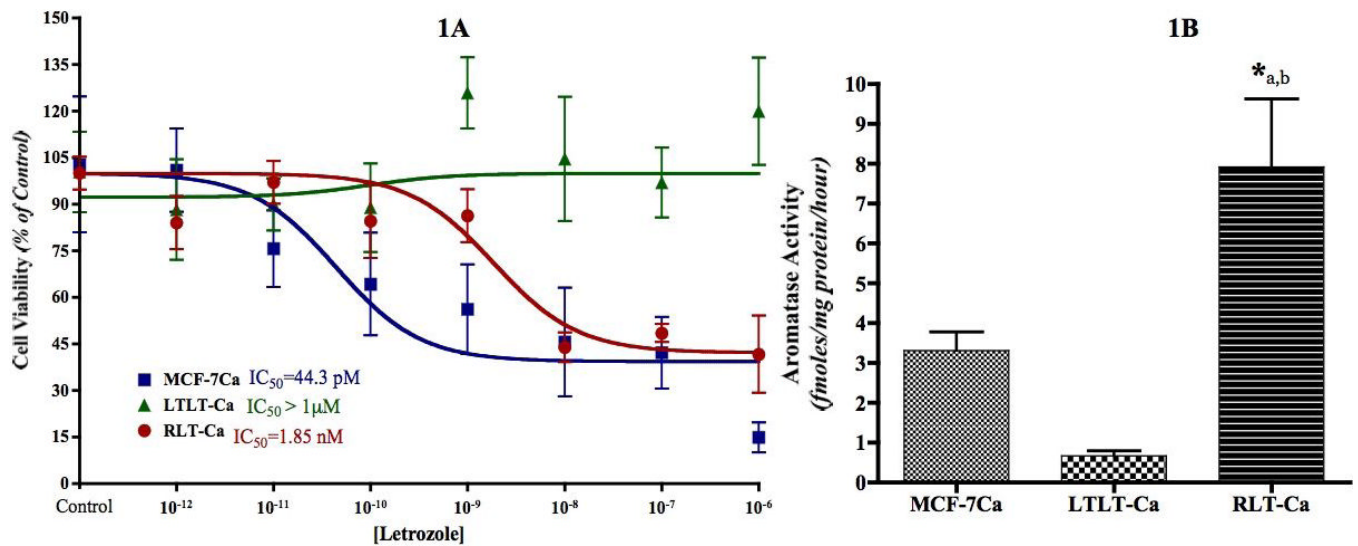
## Abbreviations used

ER $\alpha$ , Estrogen Receptor  $\alpha$ ;  $\Delta^4$ A, Androstenedione; E<sub>2</sub>, Estradiol; Her-2, Human Epidermal Growth factor Receptor- 2; MAPK, Mitogen Activated Protein KInase; AIs, Aromatase Inhibitors; AEs, Antiestrogens.

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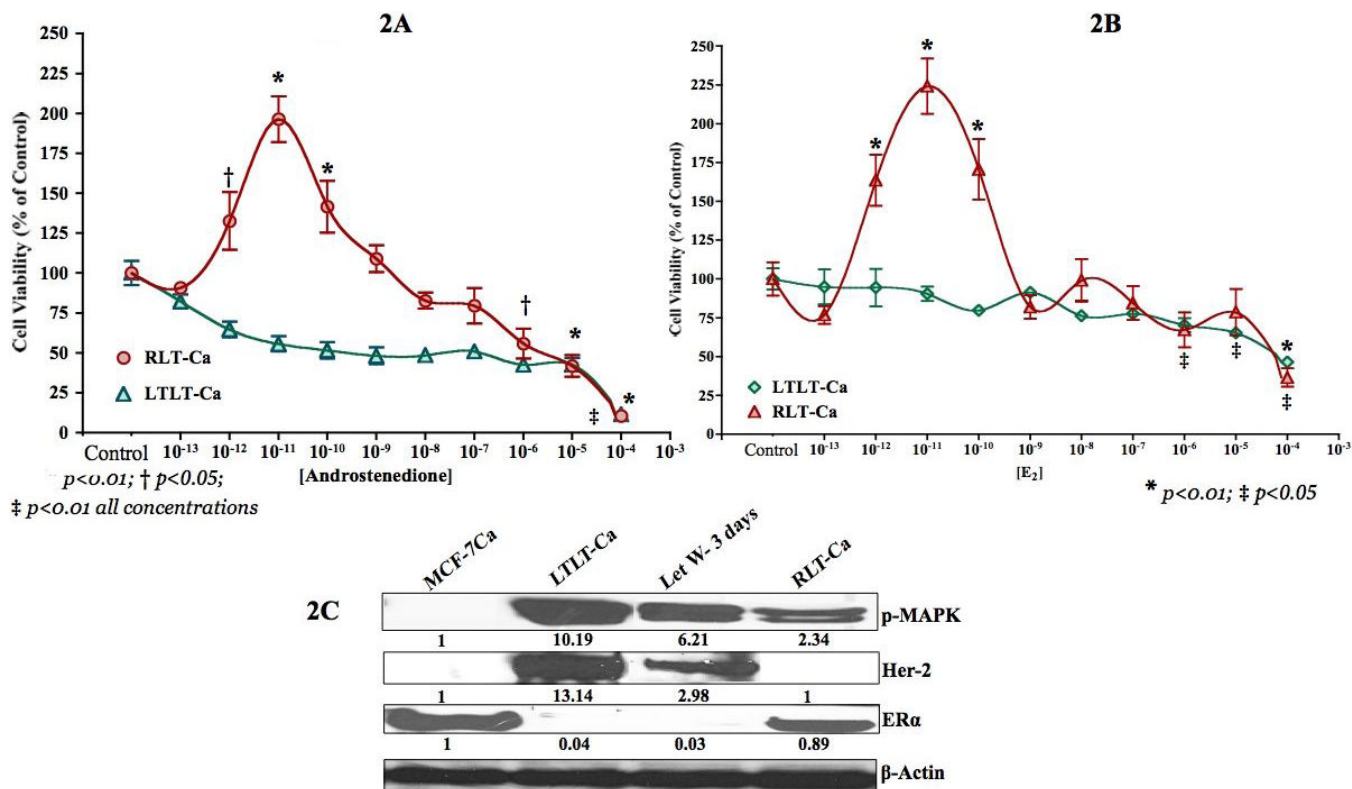
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**Figure 1. A: Effect of letrozole on proliferation of MCF-7Ca, LTLT-Ca and RLT-Ca cells in vitro** Viability of cells was measured by MTT assay after 6 day treatment with letrozole as described in Materials and Methods. The treatment with letrozole is significantly more effective in MCF-7Ca and RLT-Ca cells compared to LTLT-Ca cells ( $p = 0.0003$ ).

Figure 1B: Aromatase activity of MCF-7Ca, LTLT-Ca and RLT-Ca cells: Aromatase activity was measured by <sup>3</sup>H<sub>2</sub>O release assay as described in Materials and Methods. RLT-Ca cells exhibit significantly higher aromatase activity compared to MCF-7Ca (\*<sub>a</sub>,  $p < 0.05$ ) and LTLT-Ca cells (\*<sub>b</sub>,  $p < 0.001$ ).



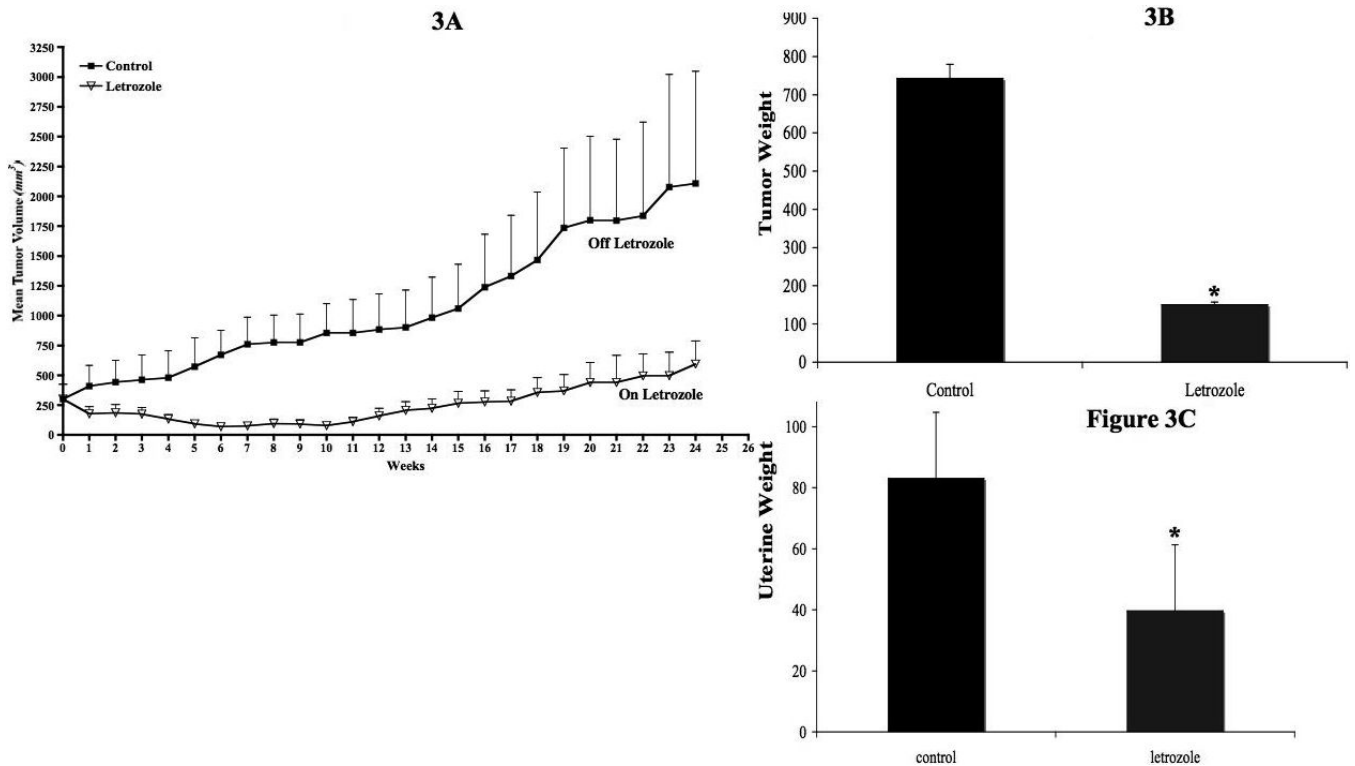


### Figure 2. A: Effect of $\Delta^4$ A on proliferation of LTLT-Ca and RLT-Ca cells

Viability of cells was measured by MTT assay after 6 day treatment as described in Materials and Methods. Compared to LTLT-Ca cells, RLT-Ca cells exhibit a significantly marked stimulation of proliferation in response to  $\Delta^4$ A at concentrations of  $10^{-11}$ M and  $10^{-10}$ M (\*  $p < 0.001$ ). LTLT-Ca cells are markedly inhibited by  $\Delta^4$ A at all concentrations ( $\ddagger p < 0.01$ ), but only at concentration of  $10^{-6}$ M  $\Delta^4$ A in RLT-Ca cells ( $\dagger p < 0.05$ ) and  $10^{-5}$ M and  $10^{-4}$ M (\*  $p < 0.001$ )

Figure 2B: Effect of estradiol on proliferation of LTLT-Ca and RLT-Ca cells: Viability of cells was measured by MTT assay after 6 day treatment as described in Materials and Methods. Compared to LTLT-Ca cells, RLT-Ca cells exhibit a significantly marked stimulation of proliferation in response to E<sub>2</sub> at concentrations of  $10^{-12}$ M through  $10^{-10}$ M (\*  $p < 0.001$ ). E<sub>2</sub> markedly inhibited growth of LTLT-Ca cells at concentrations  $10^{-6}$ M through  $10^4$ M ( $\ddagger p < 0.01$ ), but only at  $10^{-4}$ M E<sub>2</sub> in RLT-Ca cells ( $p < 0.01$ ).

Figure 2C: Protein expression profile of RLT-Ca cells compared to MCF-7Ca and LTLT-Ca cells: Expression of proteins was examined using western immunoblotting as described in Materials and Methods. Blot shows phospho-MAPK at 42–44 kDa, Her-2 at 185 kDa, ER $\alpha$  at 66 kDa and  $\beta$ -actin at 45 kDa. The blots were stripped and re-probed for  $\beta$ -actin to verify equal loading. The blots show a single representative of three independent experiments.



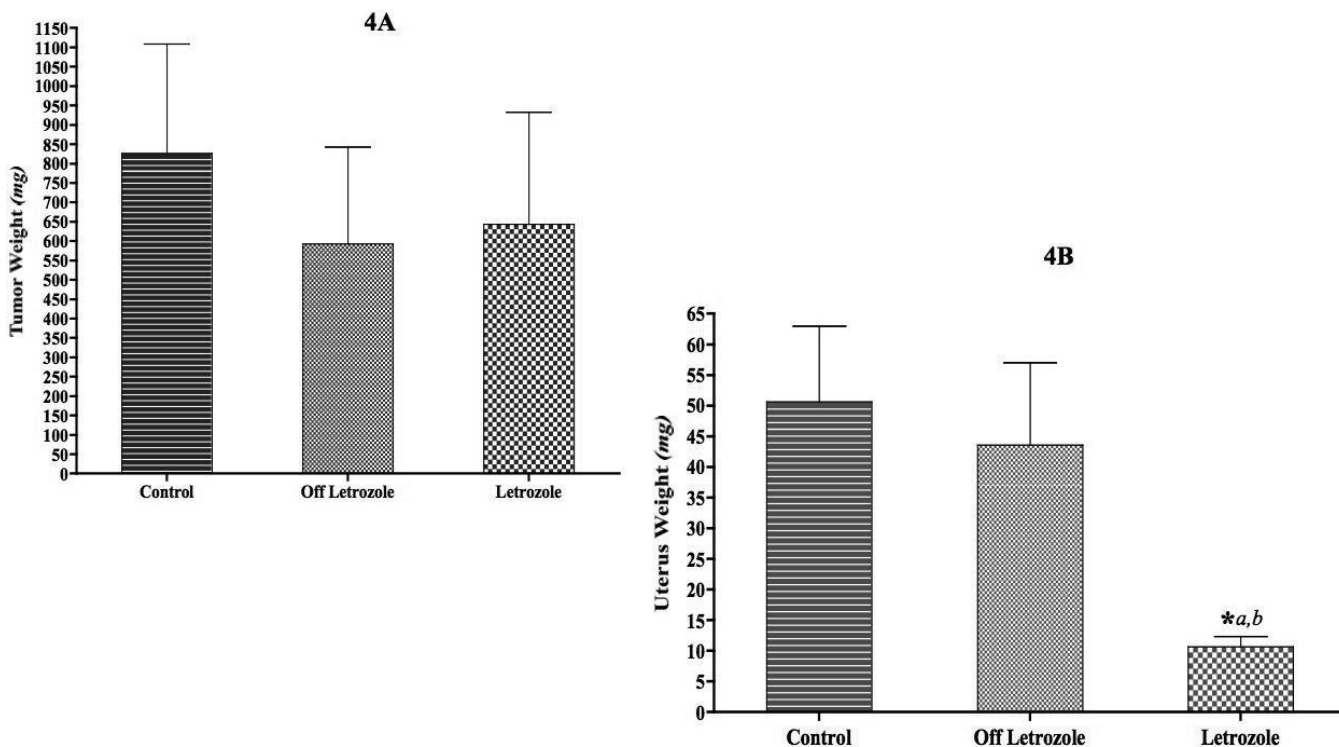
**Figure 3. A: Effect of letrozole treatment at on the growth of LTLT-Ca xenografts after 4 months off letrozole**

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 months. After which they were grouped into two groups, shown as on and off letrozole. The mice in the control and letrozole treated group exhibited significantly different growth rate. The difference in the exponential parameter governing growth was 42.3,  $p = 0.04$ .

Figure 3B: Effect of letrozole on the tumor weight of the LTLT-Ca xenografts: The mean tumor weight of control mice was  $607.5 \pm 225.5$  mg which was significantly different from those of the letrozole treated mice ( $112.5 \pm 84.77$  mg);  $p = 0.024$ . The graph shows mean  $\pm$  SEM.

Figure 3C: Effect of letrozole on the uterine weight of the LTLT-Ca xenografts: The mean uterine weight of control mice was  $83 \pm 21.45$  mg which was significantly different from those of the letrozole treated mice ( $39.67 \pm 9.14$  mg);  $p < 0.01$ . The graph shows mean  $\pm$  SEM.

Figure 3D: 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. The graph shows mean tumor volume  $\pm$  SEM versus time (weeks).



**Figure 4. A: Effect of letrozole on/off treatment the tumor weight of the MCF-7Ca xenografts**

The mean tumor weight of control mice was  $1.38 \pm 0.39$  g, letrozole treated mice was  $0.92 \pm 0.36$  g and off letrozole mice was  $0.74 \pm 0.34$ g. The graph shows mean  $\pm$  SEM. The mean tumor weights were not significantly across the groups ( $p = 0.46$ ).

Figure 4B: Effect of letrozole on/off treatment the uterus weight of the MCF-7Ca xenografts: The mean uterus weight of letrozole treated mice was  $10.71 \pm 1.658$  mg which was significantly different from those of the control mice ( $50.67 \pm 12.28$  mg);  $p = 0.0006$  (\*a) and “off letrozole” mice ( $43.63 \pm 13.44$  mg);  $p = 0.02$  (\*b). The mean uterine weight of control and off letrozole mice was not significantly different ( $p = 1$ ). The graph shows mean  $\pm$  SEM.

Pearson correlation coefficient for uterine weight and tumors in **letrozole** group was  $-0.19$  and  $0.14$  (left and right tumor respectively);  $p = 0.68$  and  $0.76$ - no correlation.

Pearson correlation coefficient for uterine weight and tumors in **control** group was  $0.96$  and  $0.96$  (left and right tumor respectively);  $p = 0.003$  and  $0.002$  – very strong positive correlation is present.

Pearson correlation coefficient for uterine weight and tumors in **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.

Figure 4C: Aromatase activity in the tumors of MCF-7Ca cells treated with letrozole:

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 experiment was terminated); off letrozole tumors at week 28 (when group was split into off and back on letrozole) and week 33 (when experiment was terminated); back on letrozole at week 33. The graph represents mean  $\pm$  SEM. One Way ANOVA with post-hoc Tukey multiple comparison test was performed to examine statistical significance.

Figure 4D: 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  $\Delta^4\text{A}$  treated control, lane 2 letrozole treated tumor at week 22, lane 3

letrozole treated tumors at week 33, lane 4 tumor from “off” group at week 28, lane 5 “off” tumors at week 33 and lane 6 tumor from “back on” group at week 33. Blot shows Her-2 and p-Her-2 at 185 kDa, phospho-MAPK and MAPK at 42–44 kDa, aromatase at 55kDa, ER $\alpha$  at 66 kDa and  $\beta$ -actin at 45 kDa. The blots were stripped and re-probed for  $\beta$ -actin to verify equal loading. The blots show a single representative of three independent experiments.

**Table 1****IC<sub>50</sub> values of fulvestrant, tamoxifen anastrozole, letrozole and exemestane in MCF-MCF-7Ca, LTLT-Ca and RLT-Ca cells**

Cell viability of cells was measured by MTT assay after 6 day treatment with described in Materials and Methods. IC<sub>50</sub> values were calculated from the non-linear regression of the plot of cell viability (% of control) versus log inhibitor concentration.

IC <sub>50</sub> (nM)	MCF-7Ca	LTLT-Ca	RLT-Ca
Letrozole	0.0443	> 1μM	1.85
Anastrozole	28	> 1μM	58
Exemestane	59	> 1μM	71
Tamoxifen	128	> 1μM	19.8 μM
Fulvestrant	5.4	> 1μM	28