Therapeutic Observations in MCF-7 Aromatase Xenografts

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ABSTRACT

In previous studies using a xenograft model with tumors of human estrogen receptor (ER)–positive breast cancer cells transfected with aromatase (MCF-7$_{Ca}$), we explored the antitumor efficacy of treatment combining the nonsteroidal aromatase inhibitor letrozole with tamoxifen. However, treatment with this combination resulted in tumor suppression similar to tamoxifen alone but was less effective than letrozole alone. Clinical findings with the nonsteroidal inhibitor anastrozole in combination with tamoxifen (ATAC trial) were consistent with our results. Although letrozole was the most effective single agent in the model, tumors ultimately began to grow during continued treatment. To investigate the mechanisms by which tumors adapted to growth during letrozole treatment, we determined the expression of proteins in tumors during letrozole treatment compared with the tumors of control mice. We found that tumors initially up-regulated the ER, but subsequently receptor levels decreased in tumors unresponsive to letrozole. Adapter proteins (p-Shc and Grb-2) as well as all of the signaling proteins in the mitogen-activated protein kinase cascade (p-Raf, p-MEK1/2, and p-MAPK) but not Akt were increased in tumors no longer responsive to letrozole. The results suggest that tumor cells adapt to estrogen deprivation during letrozole treatment by activation of alternate signaling pathways. When letrozole was combined with the pure antiestrogen fulvestrant, which down-regulates ER, the combination was extremely effective. Tumors regressed by 45% and were maintained without growth for the duration of the experiment (29 weeks). Thus, achieving more complete estrogen blockade may delay development of hormone-independent signaling pathways regulating proliferation.

INTRODUCTION

Two strategies that ameliorate the effects of estrogen in promoting breast cancer are currently being compared in clinical trials of postmenopausal patients with estrogen receptor (ER)–positive breast cancer. Antiestrogens (e.g., tamoxifen and fulvestrant) block binding of estrogen to its receptor, whereas aromatase inhibitors (e.g., letrozole, anastrozole, and exemestane) block estrogen synthesis. Of the three aromatase inhibitors currently approved for breast cancer treatment, letrozole and anastrozole are nonsteroidal triazole compounds, whereas exemestane is a steroidal analogue of androstenedione. In addition to tamoxifen, the pure antiestrogen fulvestrant, which down-regulates the ER, has recently been approved for treatment of advanced breast cancer.

To investigate the effectiveness of aromatase inhibitors, our laboratory developed a xenograft tumor model using human hormone-responsive (ER-positive) breast cancer cells stably transfected with the human aromatase gene (MCF-7$_{Ca}$). In this model, MCF-7$_{Ca}$ cells (1) are grown as tumors and serve as an autocrine source of estrogen in the ovariectomized, immune-suppressed mouse (2, 3). The resulting tumor xenografts are sensitive to both the antiproliferative effects of antiestrogens and aromatase inhibitors (2–5). The model simulates the postmenopausal breast cancer patient in that the source of estrogen after menopause is from nonovarian tissue, including normal and malignant breast tissue (6, 7), and where estrogen synthesis is not under gonadotropin regulation.

We have used the model to study several strategies of treatment with aromatase inhibitors and antiestrogens. We have investigated whether the antitumor effects of these agents can be extended by using them in sequence. In addition, we have investigated whether combining the two types of agents could be more effective than either aromatase inhibitor or antiestrogen alone.

MCF-7 AROMATASE XENOGRAFTS

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

MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7$_{Ca}$) were kindly provided by Dr. S. Chen (City of Hope, Duarte, CA; ref. 3). MCF-7$_{Ca}$ cells were routinely maintained in DMEM with 5% fetal bovine serum, 1% penicillin/streptomycin solution, and 750 μg/mL G418. Subconfluent cells were resuspended in Matrigel (10 mg/mL) at 2.5×10^7 cells/mL. Each animal received s.c. inoculations in two sites per flank with 100 μL of cell suspension. All animals were then injected daily with δ4-androstenedione (100 μg/d; aromatase substrate) for the duration of the experiment. Tumor volumes were measured weekly as reported previously (3–5).

Treatments began when the tumors reached a measurable size (~300 mm^3). Letrozole (CGS 20267) was kindly provided by Dr. D. Evans (Novartis Pharma A.G., Basel, Switzerland). The pure antiestrogen fulvestrant (ICI 182,780) was generously supplied by Dr. A. Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom). Exemestane was provided by

Pharmacia (Pfizer Pharmaceutical, Groton, CT). Tamoxifen was purchased from Sigma Co. (St. Louis, MO). 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. Mice were injected s.c. daily with the drugs in 0.3% hydroxypropyl cellulose. At the times indicated in the figures, mice were killed by decapitation and the trunk blood was collected. Tumors and uteri were excised, cleaned, weighed, and stored in liquid nitrogen for analysis later.

SEQUENTIAL TREATMENT WITH AROMATASE INHIBITORS AND ANTIESTROGENS

We have previously investigated the effects of switching mice treated first with tamoxifen to second-line treatment with letrozole (8). This sequence was effective in slowing tumor growth compared with tamoxifen, but overall this strategy was inferior to treatment with letrozole as first-line treatment. Tumors of mice treated with letrozole (10 μg/d) initially regressed but gradually resumed growth and had doubled in volume by ~21 weeks (9, 10). Mice were then assigned to second-line treatment with tamoxifen or with a higher dose of letrozole (100 μg/d).[2] However, although the higher dose of letrozole slowed tumor growth, tumor volumes were not significantly different from those continued on letrozole (10 μg/d) treatment. Tamoxifen was ineffective as second-line therapy as reported previously (8). Similarly, fulvestrant was also ineffective (8). These data indicate that switching from letrozole to tamoxifen, or increasing the dose of letrozole, might not be the optimal treatment choice for patients with tumors progressing on a therapeutically effective dose of letrozole.

COMBINATION THERAPY WITH AROMATASE INHIBITORS AND ANTIESTROGENS

In previous studies using this model, we explored the antitumor efficacy of combining the nonsteroidal aromatase inhibitor letrozole with tamoxifen. However, treatment with this combination resulted in tumor suppression similar to treatment with tamoxifen alone but was less effective than letrozole alone. Clinical findings with the nonsteroidal inhibitor anastrozole in combination with tamoxifen (ATAC trial) are consistent with our results (5, 11). In brief, in our model, treatment was not improved by either of the two nonsteroidal inhibitors combined with tamoxifen. More recently, we determined the effects of the steroidal inhibitor exemestane in combination with tamoxifen (9). In addition, we have investigated the effects of letrozole with the ER down-regulator fulvestrant.

Exemestane. As shown in Fig. 1, groups of mice were treated with two doses of exemestane either 100 or 250 μg/d. All mice assigned to tamoxifen treatment were injected with 100 μg/d. The lower dose of exemestane had been determined to be partially effective in reducing tumor growth, whereas the higher dose was the optimally effective dose in these animals. However, in combination with tamoxifen, better inhibition of
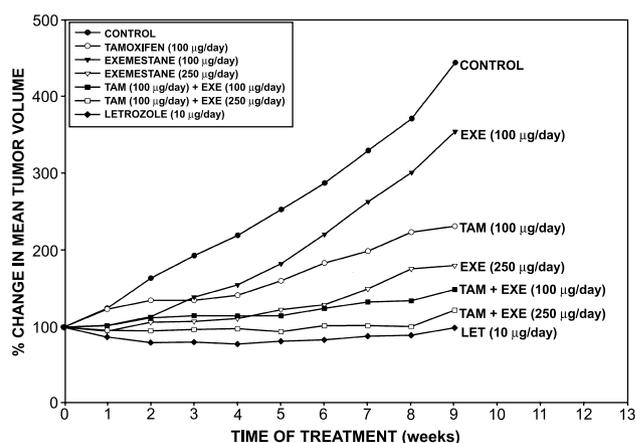
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[2] D. Jelovac et al., submitted for publication.

Fig. 1 Effect of exemestane and tamoxifen on the growth of MCF-7 aromatase tumor xenografts in ovariectomized athymic mice. All animals were inoculated with MCF-7$_{Ca}$ aromatase-transfected human breast cancer cells at two sites per flank and supplemented with androstenedione ($\delta 4A$, 100 μg/d) for the duration of experiment. When tumor volumes reached ~500 mm^3, animals were grouped ($n = 5$) for treatment with vehicle, tamoxifen (100 μg/d), and exemestane at different doses (100 and 250 μg/d). Tumor volumes were measured weekly and expressed as the percentage change from their initial tumor volume (18).

tumor growth was seen with both doses of exemestane, although the higher dose (250 μg/d) was rather more effective (9).

The effect of exemestane on tumor growth in the MCF-7$_{Ca}$ xenograft model suggests that this aromatase inhibitor at optimal doses (250, 500, and 1,000 μg/d) is similar to or slightly better than the maximal dose of tamoxifen (500 μg/d) in reducing tumor weight after 4 weeks of treatment. A maximally effective dose of tamoxifen (100 μg/d) was used in combination with 100 and 250 μg/d exemestane to determine whether the two agents, acting by different mechanisms, have additive effects. There was a significantly greater reduction in tumor growth with both doses of exemestane (100 and 250 μg/d) used in the combined treatment than with either dose of exemestane alone or tamoxifen alone. A similar result was reported previously by Zaccheo et al. (12) who found that growth of carcinogen [7,12-dimethylbenz(*a*)anthrazene]−induced tumors in the rat were inhibited to a greater extent by the combination of exemestane and tamoxifen than with either alone. The combined treatment, which inhibited estrogen action and estrogen synthesis, delayed tumor doubling for nearly 16 weeks, twice as long as with tamoxifen alone. This suggests that combining tamoxifen with this steroidal aromatase inhibitor may be more effective in breast cancer patients than the drugs administered separately. However, although the exemestane/tamoxifen combination was more effective than either agent alone, it was less effective than letrozole only. Thus, after 28 weeks of the combined treatment, tumors were significantly larger than those treated with letrozole.

One possible explanation for the difference in results between the two types of aromatase inhibitors is that letrozole is more potent than exemestane (Fig. 1). Thus, in the combined treatment with the nonsteroidal inhibitors, the agonist effects of tamoxifen on tumor growth are apparent in the presence of very low concentrations of estrogen in the tumor, whereas when

exemestane is combined with tamoxifen, any remaining estrogen is effectively blocked by tamoxifen. The slight reductions reported in serum levels of nonsteroidal aromatase inhibitors [27% in one study (8) and 38% in another (13)] when combined with tamoxifen seem unlikely to influence their activity, because estrogen concentrations remain maximally suppressed. Clearance rates of exemestane and tamoxifen in combination compared with those of the drugs alone seem not to be altered in breast cancer patients (14, 15). Further studies are therefore needed to explore whether these or other mechanisms explain the difference between the two classes of aromatase inhibitors.

Fulvestrant. Because the anti-estrogen fulvestrant causes ER degradation, we hypothesized that the combination of fulvestrant with letrozole may be a more effective treatment than either compound alone. To test this hypothesis, mice with MCF-7 aromatase xenografts were injected s.c. daily with either vehicle (control), fulvestrant (1 mg/d), letrozole (10 μg/d), or letrozole (10 μg/d) plus fulvestrant (1 mg/d).[3] After 3 weeks, tumors in the control group had doubled their initial volume. By 7 weeks, mice were sacrificed due to large tumor size as tumor volumes had increased ~6-fold (Fig. 2). All treatments were effective in suppressing tumor growth compared with the control group ($P < 0.001$). In mice treated with fulvestrant alone, tumors were static for the first 4 weeks of treatment. Thereafter, these tumors began to proliferate and had doubled in volume after 10 weeks of treatment (Fig. 2). By week 17, tumor volumes were significantly larger in the group treated with fulvestrant alone compared with the letrozole-treated group ($P < 0.001$). Tumor volumes were reduced by 40% over the first 8 weeks of treatment with letrozole. These tumors slowly returned to their initial size by 17 weeks and had doubled in volume at 21 weeks of treatment. The effect of letrozole (10 μg/d) on tumor growth in the MCF-7 aromatase xenograft model suggests that this aromatase inhibitor is better than the pure anti-estrogen fulvestrant (1 mg/d) in controlling tumor growth and delaying the time of tumor progression.

When the two drugs, fulvestrant inhibiting estrogen action and letrozole inhibiting estrogen synthesis, were combined, there was a significantly greater effect on tumor suppression than treatment with either letrozole or fulvestrant alone. The combined treatment resulted in tumor regression, which was maintained throughout the 29-week treatment period. These findings suggest that the combination of fulvestrant with letrozole could be more effective in breast cancer patients than these agents administered separately. The combination of letrozole *plus* fulvestrant was significantly more effective in suppressing tumor growth than fulvestrant alone at week 17 or letrozole alone at week 29 ($P < 0.0001$).[4] The additive effect on tumors treated with the combination of these two compounds suggests that some transcription via the ER may occur with fulvestrant treatment alone that is not completely blocked by the anti-estrogen.

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[3] D. Jelovac et al., submitted for publication.
[4] D. Jelovac et al., submitted for publication.

MECHANISMS INVOLVED IN LETROZOLE INSENSITIVITY

To investigate the mechanisms involved in loss of response to letrozole treatment, tumors were collected at several time points from mice during treatment with letrozole (10 μg/mouse/d; ref. 8). Tumor extracts were analyzed by Western immunoblotting for changes in ER levels and several proteins in alternate signaling pathways.

The ER was initially increased after 4 weeks of letrozole treatment while tumors were regressing. After 56 weeks of letrozole treatment, tumors were growing. ER expression was decreased by 50% compared with control tumors. Nevertheless, despite low ER levels, progesterone receptor expression was modestly increased. Phospho-ER (Ser[167]) was increased 2-fold in tumors only at weeks 28 and 56, suggesting that ligand-independent activation of ER may be occurring in tumors proliferating on letrozole. Expression of tyrosine kinase receptor erbB-2 was increased ~2-fold throughout treatment with letrozole (weeks 4, 28, and 56). In addition, p-Shc protein expression was increased by 2-fold at all time points with letrozole treatment, suggesting that the tumors are adapting to surviving without estrogens by activating hormone-independent pathways. However, expression of adapter protein Grb-2 was increased by 4-fold at weeks 28 and 56 only in tumors that were growing on letrozole treatment. Phospho-MAPK was increased
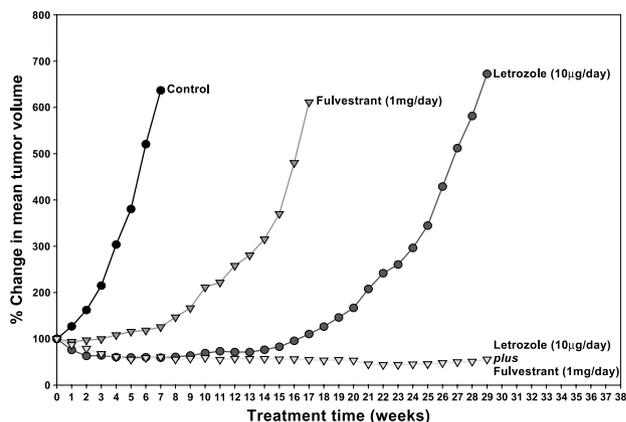

Fig. 2 Effect of letrozole (10 μg/d) and fulvestrant (1 mg/d) alone or the combination on the growth of MCF-7 aromatase tumor xenografts in female ovariectomized athymic nude mice. Animals were inoculated with MCF-7$_{Ca}$ cells at one site on each flank and were supplemented with androstenedione (100 μg/d) for the duration of experiment. When tumors reached ~300 mm^3, animals were divided into four groups and injected s.c. daily with vehicle (control; $n = 6$), fulvestrant (1 mg/d; $n = 7$), letrozole (10 μg/d; $n = 18$), or letrozole (10 μg/d) plus fulvestrant (1 mg/d; $n = 5$). Tumor volumes were measured weekly and expressed as the percentage change in mean tumor volume relative to the initial size at week 0. At week 7, all treatments were significantly better in suppressing tumor growth compared with the control animals ($P < 0.001$), which were sacrificed due to the large tumor size. At week 17, letrozole was superior to fulvestrant in controlling tumor growth ($P < 0.001$). In addition, treatment with letrozole plus fulvestrant was superior to fulvestrant alone ($P < 0.001$). Fulvestrant-treated mice were sacrificed at week 17 due to large tumor size. At week 29, letrozole (10 μg/d) was less effective than letrozole plus fulvestrant in controlling tumor growth ($P = 0.0005$). In addition, tumor volumes were statistically significantly larger in the letrozole treatment group than in the combination ($P < 0.0001$).

2.3-fold in tumors that were responding to letrozole treatment at week 4 compared with vehicle-treated tumors, but expression was further increased up to 6-fold in tumors growing on letrozole at weeks 28 and 56 (16).

Tumor cells were isolated from tumors treated with letrozole for 56 weeks and maintained in the presence of letrozole (1 μmol/L) after isolation (long-term letrozole-treated cells). Signaling protein expression in these cells was compared with the parental MCF-7$_{Ca}$ and also a variant cell line derived from MCF-7$_{Ca}$ by culturing in steroid-free medium for 6 months (UMB-1$_{Ca}$; ref. 17). This cell line had a 2-fold increase in ER expression compared with MCF-7$_{Ca}$, whereas in the long-term letrozole-treated cells, ER expression was diminished consistent with expression in the tumor. Expression of erbB-2 was increased in both cell lines. However, expression of adapter proteins p-Shc and Grb-2 and signaling proteins p-MAPK, p-MEK1/2, p-Raf, p-p90RSK, and p-Elk were all increased in the long-term letrozole-treated cells but not in the UMB-1 cells. These results suggest that increase in Grb-2 expression in tumors proliferating on letrozole may be an important amplifier of the Ras signaling pathway, which leads to a further increase in activated mitogen-activated protein kinase and activation of ER.

CONCLUSIONS

Our results suggest that tumor cells adapt to estrogen deprivation initially by up-regulating the estrogen signaling pathway, resulting in activation of the kinase signaling proteins to maintain transcription and cell proliferation. The importance of the ER in this process is evident from the finding that when letrozole treatment is combined with fulvestrant, an ER down-regulator, tumor regression is maintained for an extended period compared with letrozole alone, suggesting that the kinase signaling pathway is not activated during this treatment. Treatment with the combination of letrozole *plus* fulvestrant was able to maintain tumor growth inhibition by 45% over 29 weeks of treatment. This combination was significantly more effective than either letrozole alone or fulvestrant alone and may be the best option to date for treatment of estrogen-dependent breast cancer.

OPEN DISCUSSION

Dr. Kent Osborne: Did you check to see what fulvestrant does to tumor growth in your non–aromatase-transfected MCF-7 cell?

Dr. Angela Brodie: No, we haven't really looked at that.

Dr. Osborne: What you see with letrozole plus fulvestrant is identical to what we see with fulvestrant alone in MCF-7 cells that are not transfected with aromatase. When you inhibit the aromatase-transfected cells with letrozole or with any other aromatase inhibitor, it may be that you are not lowering the estrogen level to the same extent as in non–aromatase-transfected cells in an ovariectomized animal. Do you know to what extent you are inhibiting estrogen in that environment with the aromatase inhibitors?

Dr. Brodie: Yes, we have measured the estrogen levels in the letrozole-only study, not in the combination. The levels are extremely low and it is very tough to measure it. The levels are lower than in vehicle-treated cells.

Dr. Osborne: Of course, it can be at extremely low levels while still stimulating tumors.

Dr. Brodie: Exactly. I think that's the point.

Dr. Richard Santen: In the long-term letrozole-treated tumors, if you retransplant them and do a dose response with estrogen, have those cells become hypersensitive to the proliferative effects of estrogen?

Dr. Brodie: We did do that experiment in culture with MCF-7Ca estrogen-deprived (UMBI) cells, and they weren't. They seemed to be more sensitive to tamoxifen. They did not show that increased sensitivity to estrogen that the long-term estrogen-deprived cells show. Cell growth was relatively unchanged with increasing concentrations of estrogen in UMBI cells, whereas MCF-7 estrogen-deprived cells (LTED) were inhibited by increasing doses of estrogen. In mice that were transplanted with long-term letrozole-treated tumors, the retransplanted tumors grew equally well with and without androstenedione, suggesting that estrogen was not essential for tumor proliferation.

Dr. Stephen Johnston: When you switched the cells off the letrozole to either tamoxifen or fulvestrant and showed there was no response, was that taking the letrozole away? At the time of resistance, have you done the experiment of keeping letrozole with fulvestrant versus taking letrozole away?

Dr. Brodie: Actually, we haven't, though we plan to do it because we have seen an up-regulation of aromatase once we take the mice off letrozole.

Dr. Johnston: When we have done that *in vitro* it appears to make a big difference. In the long-term estrogen-deprived cells, we have shown that fulvestrant is effective, but if you then titrate back estrogen, the growth-inhibitory effects of fulvestrant are blocked. Thus, in the setting of resistance to aromatase inhibitors, fulvestrant may work better in a continued low estrogen environment in combination with the AI rather than on its own following withdrawal of AI and restoration of postmenopausal estrogen levels. Those observations prompted the SoFEA clinical trial where following progression on AI, patients will be randomized to fulvestrant plus continued AI versus fulvestrant alone.

Dr. Douglas Yee: Why do you think that it is, given the action of fulvestrant?

Dr. Johnston: There are issues related to fulvestrant's pharmacokinetics as to whether at the current clinical dose, it has maximal suppressive effect. In the setting of resistance to AIs and supersensitivity to low estrogen levels, the activity of fulvestrant in that setting may be critically dependent on whatever level of estradiol is there providing cross-talk activation of ER. That has prompted us to do the clinical trial, but I would love to see the animal data.

Dr. Per Lønning: How do you think about taking your results with exemestane into the clinic? Will you suggest any clinical studies with exemestane plus tamoxifen to put your hypothesis to the test in the clinical setting?

Dr. James Ingle: We actually had proposed a trial of exemestane plus tamoxifen versus exemestane, and U.S. FDA categorically turned it down. The FDA rejected it because they wanted letrozole as the third arm and it was impossible to accrue sufficient patients. We may go back to the FDA now that Paridaens' study is available [J Clin Oncol 2004;22:6 (abstract 515)].

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