Combination of Anastrozole with Fulvestrant in the Intratumoral Aromatase Xenograft Model

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Abstract

Although the aromatase inhibitor anastrozole has been shown to be very effective in the treatment of hormone-dependent postmenopausal breast cancer, some patients with advanced disease will develop resistance to treatment. To investigate therapeutic strategies to overcome resistance to anastrozole treatment, we have used an intratumoral aromatase model that simulates postmenopausal breast cancer patients with estrogen-dependent tumors. Growth of the tumors in the mice was inhibited by both anastrozole and fulvestrant compared with the control tumors. Nevertheless, tumors had doubled in size at 5 weeks of treatment. We therefore investigated whether switching the original treatments to anastrozole or fulvestrant alone or the combination of anastrozole plus fulvestrant would reduce tumor growth. The results showed that the best strategy to reverse the insensitivity to anastrozole or fulvestrant is to combine the two agents. Additionally, the tumors treated with anastrozole plus fulvestrant from the beginning had only just doubled their size after 14 weeks of treatment, whereas the anastrozole and fulvestrant treatments alone resulted in 9- and 12-fold increases in tumor size, respectively, in the same time period. Anastrozole plus fulvestrant from the beginning or in sequence was associated with down-regulation of signaling proteins involved in the development of hormonal resistance such as insulin-like growth factor type I receptor β, mitogen-activated protein kinase (MAPK), p-MAPK, AKT, mammalian target of rapamycin (mTOR), p-mTOR, and estrogen receptor α compared with tumors treated with anastrozole or fulvestrant alone. These results suggest that blocking the estrogen receptor and aromatase may delay or reverse the development of resistance to aromatase inhibitors in advanced breast cancer patients.

Introduction

Breast cancer is one of the leading causes of cancer-related deaths among women in the United States and is by far the most common cancer in women worldwide (1). It occurs more frequently in postmenopausal than in younger women and is a highly estrogen-dependent disease. In postmenopausal women, estrogen is produced in a number of extragonadal sites (2). These include the adipose tissue of the breast where estrogen acts locally in a paracrine or intracrine fashion to promote breast tumor development and progression (3, 4). Estrogen levels in the normal breast tissue of postmenopausal women are generally much higher than those in plasma, and levels in malignant tissue are higher than those in normal breast tissue (5). It has been observed that two thirds of breast carcinomas contain aromatase (the enzyme mediating the rate limiting step of estrogen biosynthesis) and its activity is higher in tumor than in normal areas of the breast (6–10).

Current first-line or adjuvant treatment options for postmenopausal hormone-dependent breast cancer patients involve either blocking estrogen from binding to the estrogen receptor (ER) with antiestrogens or inhibiting estrogen synthesis with aromatase inhibitors such as the nonsteroidal agents anastrozole and letrozole or the steroidal agent exemestane. Until recently, the endocrine therapy of choice for ER+ breast cancer was tamoxifen. Unfortunately, due to its estrogen-like effect, the use of tamoxifen significantly increases the risk of endometrial cancer and stroke (11). Additionally, not all patients with advanced disease respond to tamoxifen, and many of those that do respond eventually progress with resistant disease. The search for a more effective antiestrogen without agonist activity ultimately resulted in the development of the pure antiestrogen fulvestrant. This well-tolerated drug binds to ER competitively and causes its degradation and down-regulation (12, 13). Fulvestrant has been approved for the treatment of postmenopausal women with hormone receptor–positive advanced breast cancer with disease progression following prior endocrine therapy (12, 14, 15).

The aromatase inhibitors have been approved as first-line treatment option for hormone-dependent advanced breast cancer in postmenopausal patients. When anastrozole was compared with tamoxifen in several clinical trials (The North American and TARGET trials), the aromatase inhibitor provided significant efficacy and tolerability advantages in postmenopausal patients with advanced breast cancer and a delay in the emergence of endocrine resistance (16, 17). This is supported by the result of preclinical studies showing that estrogen deprivation with aromatase inhibitor is superior to tamoxifen, resulting in greater tumor regressions and prolonged time to regrowth (18). On the other hand, as with any cancer treatment, some patients eventually develop resistance to aromatase inhibitors and experience disease progression. Data from preclinical studies have suggested that acquired aromatase inhibitor resistance may be due to up-regulation of growth factor receptors such as HER2 and insulin-like growth factor type I receptor (IGF-IR; refs. 19, 20). Increased expression of these receptors may promote activation of downstream protein kinases such as mitogen-activated protein kinase (MAPK) and AKT, which, in turn, could result in increased ER phosphorylation and activation and sensitization of tumor cells to estrogen. The mammalian target of rapamycin (mTOR) is one of the most evolutionarily conserved downstream effectors of AKT and executes the most critical functions of AKT with regard to...
increased cell proliferation (21). Some authors also consider mTOR as a nodal point connecting different signaling pathways (22).

Our laboratory has developed an intratumoral aromatase model in rodents that mimics, to some extent, postmenopausal ER+ breast cancer patients (23). Because the mouse has no significant peripheral production of estrogen and no adrenal androgen production (24, 25), MCF-7 cells engineered to express aromatase (26) were inoculated into ovariectomized athymic mice (23). To date, the intratumoral aromatase xenograft model has proved accurate in predicting the outcome of clinical trials such as ATAC and allowed us to show that the aromatase inhibitor letrozole was more effective than tamoxifen in suppressing breast tumor growth without causing endometrial proliferation (27).

Currently, there is a great interest in second-line treatments for patients that received aromatase inhibitors as first-line therapy for advanced disease. Fulvestrant is now being investigated as an appropriate next step in the treatment of refractory breast tumors (28, 29). In the present study, we have used the intratumoral aromatase model to investigate strategies to delay resistance in anastrozole-treated breast tumors and the optimal treatment sequence following anastrozole failure.

Materials and Methods

Materials. Phenol red–free modified IMEM, DMEM, penicillin/streptomycin solution, 0.25% trypsin-EDTA solution (1 mmol/L), Dulbecco’s PBS, and geneticin (G418) were obtained from Life Technologies. Trypsin-verse was purchased from Biosource Biologics Cell Culture Products. Fetal bovine serum (FBS) and charcoal/dextran–treated FBS were obtained from Hyclone. Androstenedione and tamoxifen were obtained from Sigma Chemical Co. Matrigel was purchased from BD Biosciences. Fulvestrant (Faslodex) and anastrozole (Arimidex) were supplied by Dr. E. Anderson (AstraZeneica Pharmaceuticals). Enhanced chemiluminescence (ECL) kits and Hybrid-ECL nitrocellulose membranes were purchased from Abersham Biosciences. Antibodies against HER2, p-HER2, and p-HER3 were purchased from Upstate. Antibodies against p-MAPK, MAPK, Akt, IGF-IR, mTOR, p-mTOR, and β-actin were purchased from Cell Signaling Technology. Horseradish peroxidase–conjugated antiauxine and antirabbit antibodies were purchased Form Bio-Rad. Antibody against ERα was purchased from Santa Cruz Biotechnology. Radioligand for the aromatase assay, 1H-androstenedione (specific activity, 23.5 Ci/mmol), was purchased from Perkin-Elmer. MCF-7 human breast cancer cells stably transfected with the human aromatase gene were provided by Dr. Shiuan Chen (Beckman Research Institute of City of Hope, Duarte, California) as previously reported (26).

Postmenopausal intratumoral aromatase model. All animal studies were carried out according to the guidelines and with the approval of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Female nude athymic ovariectomized mice, 4 to 6 weeks old, were obtained from National Cancer Institute-Frederick Cancer Research and Development Center. The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water ad libitum. Animals were allowed to acclimatize for 48 h after shipment before tumor inoculation was carried out. MCF-7Ca cells were inoculated s.c. into the mice at one site on each flank with 100 μL of cell suspension (4.4 × 10^6 cells per site). Because athymic mice were deficient in adrenal androgens (24, 25), they were supplemented with s.c. injections of the aromatase substrate androstenedione (100 μg/d) for the duration of the experiment. The treatment groups were composed of anastrozole alone (200 μg/d; s.c.; n = 30), anastrozole (200 μg/d s.c.) plus fulvestrant (1,000 μg/d s.c.; n = 10), and fulvestrant alone (1,000 μg/d s.c.; n = 20). The doses of fulvestrant and anastrozole used had previously been determined to be maximally effective in reducing tumor growth (30, 31). All drugs and androstenedione were prepared in vehicle (0.3% hydropropylcellulose in 0.9% NaCl). Tumors were measured weekly with calipers and volumes were calculated using the formula 4/3πr^2h, where r1 ≤ r2. Animals were treated for the times indicated in the figures, after which they were sacrificed by decapitation and tumors and uteri were excised, cleaned, and stored at −80°C.

Second-line treatment after anastrozole and fulvestrant. Tumors of animals (n = 30) treated with anastrozole alone (200 μg/d) were growing by week 5. These mice were then assigned to the following treatment groups: one group continued on anastrozole treatment (200 μg/d; n = 10); another group was given a combination of anastrozole (200 μg/d) and fulvestrant (1,000 μg/d; n = 12); and a third group was switched to fulvestrant (1,000 μg/d; n = 8). The animals (n = 20) treated with fulvestrant (1,000 μg/d) were randomly assigned to three treatment groups: one group continued treatment on fulvestrant (1,000 μg/d; n = 6); another group was given a combination of anastrozole (200 μg/d) and fulvestrant (1,000 μg/d; n = 7); and a third group was switched to anastrozole (200 μg/d; n = 7). As before, tumors were measured weekly and volumes calculated. The experiment was terminated at week 14 when tumors and uteri were excised and weighed.

Radiometric assay of tumor aromatase activity. This assay was done in a similar manner that was already described (32). Briefly, the tumors were homogenized in ice-cold PBS and aliquots (500 μL) of the homogenates were incubated with cofactors (n-glucose-6-phosphate, β-NADP+, and glucose-6-phosphate dehydrogenase; Sigma) and 0.5 μCi of [1H-1]-androstenedione (specific activity, 23.5 Ci/mmol) for 1 h at 37°C. The reaction mixtures were extracted with chloroform and further treated with a 2.5% charcoal suspension. The 1H2O in the supernatant was measured using a scintillation counter and corrected for protein concentration to give enzyme activity in femtomoles per microgram of protein per hour. The results were expressed as percent of control.

Western blotting. Protein extracts from tumor tissues were prepared by homogenizing the tissue in PBS and centrifuging at 2,500 rpm for 20 min. The cells lysates were obtained by sonicating the cells in RIPA buffer. The protein concentration was measured using the Bio-Rad method. Sixty micrograms of proteins were subjected to SDS-PAGE and then transferred onto a nitrocellulose membrane. The membrane was probed for specific primary antibody as specified in the manufacturer’s protocol. Immunoreactive bands were visualized with ECL detection reagents and quantified by densitometry using Molecular Dynamics software (ImageQuant).

Statistical analysis. Random coefficient models (S-PLUS, 7.0, Insightful Corp.) were used to assess patterns of tumor response to treatment. Data on tumor volume are longitudinal (i.e., tumor volume was observed over time). The model of exponential growth was appropriate to the data. Therefore, tumor volumes were log transformed. We estimated an exponential parameter controlling the tumor growth rate for each of the treatment groups. The resulting model had random effects for the intercept at the tumor level and for the slope at the mouse (ID) level. The unstructured covariance matrix was selected via model diagnostics. There was no site (flank) effect on tumor growth. Average uterine weight was estimated for each treatment group and compared between these groups using the general linear model approach. Appropriate contrasts were used to compare the groups’ mean uterine weight. The differences between the treatment groups in the aromatase activity assay were analyzed by ANOVA. The Tukey-Kramer method was applied for multiple comparisons. All tests were two sided and P < 0.05 was considered statistically significant.

Results

Effects of anastrozole, fulvestrant, or anastrozole plus fulvestrant on tumor growth in vivo. MCF-7Ca cells were
and fulvestrant had a marginal statistical significance (ANA + FUL (\(P = 0.011\)) versus ANA (\(P = 0.013\))). The effect of fulvestrant at 9 weeks in that tumor volume was marginally statistically significant compared with the control (\(P = 0.056\)). The combination of anastrozole plus fulvestrant was the most effective therapy in reducing tumor growth when compared with control (\(P < 0.0001\)).

When the experiment was terminated after 14 weeks of treatment, the combination of anastrozole plus fulvestrant was clearly superior in controlling tumor growth compared with either anastrozole (\(P = 0.0003\)) or fulvestrant (\(P = 0.009\)) given alone.

### Effects of second-line therapies on tumors proliferating during anastrozole and/or fulvestrant treatment.

At week 5, groups of animals receiving anastrozole or fulvestrant alone were randomized to cross over to fulvestrant or anastrozole, respectively, or anastrozole plus fulvestrant, whereas the others remained on their original treatments (Fig. 1A). After 9 weeks of second-line therapy, we observed that the replacement of anastrozole by fulvestrant or fulvestrant by anastrozole had not reduced growth compared with the tumors that remained in anastrozole or fulvestrant treatment alone (Fig. 1A). However, addition of fulvestrant to the anastrozole treatment or anastrozole to the fulvestrant treatment was very effective in controlling tumor growth. Tumors of mice on anastrozole and fulvestrant treatments continued to proliferate in that their mean volumes increased four and five times, respectively, after the beginning of the second-line therapy (week 5). Although the tumors of mice that switched from 300 mm\(^3\), the tumors in the control group doubled after 5 weeks. The tumors in mice receiving the combination therapy remained stable at 300 mm\(^3\) for >7 weeks when they began to show a slow increase in tumor volume, such that they had doubled at week 14. Statistical analysis of tumor volume after the first 6 weeks of treatment showed that anastrozole (\(P = 0.001\)), fulvestrant (\(P = 0.012\)), and anastrozole plus fulvestrant (\(P < 0.0001\)) caused a significant decrease in tumor growth when compared with control. The combination therapy was superior to anastrozole (\(P = 0.037\)) or fulvestrant (\(P = 0.011\)) treatment alone. Analysis of tumor volume at week 9 showed that tumors in the anastrozole- and fulvestrant-treated groups continued to grow whereas tumors in the anastrozole plus fulvestrant-treated group remained at 300 mm\(^3\). Nevertheless, tumors in the anastrozole-treated group remained significantly smaller than those in the control group (\(P = 0.013\)), and the effect of fulvestrant at 9 weeks in that tumor volume was marginally statistically significant compared with the control (\(P = 0.056\)). The combination of anastrozole plus fulvestrant was the most effective therapy in reducing tumor growth when compared with control (\(P < 0.0001\)).

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### Figure 1. Effect of anastrozole and fulvestrant alone, in sequence, or in combination on the growth of MCF-7Ca xenografts. A, MCF-7Ca xenografts were grown in female ovariectomized nude mice as described in Materials and Methods. When the tumors reached a measurable size (~300 mm\(^3\)), animals were assigned to the treatment groups for the duration of the experiment. The treatment groups were injected with anastrozole (200 mg/d; \(n = 30\); ANA), anastrozole (200 mg/d) plus fulvestrant (1,000 mg/d; \(n = 10\); ANA + FUL) and fulvestrant (1,000 mg/d; \(n = 20\); FUL) in addition to androstenedione (100 mg/d) s.c. wk. The control group received androstenedione (100 mg/d). At week 5, mice in the anastrozole group were assigned to the following treatment groups: one group continued on anastrozole treatment (200 mg/d; \(n = 10\); ANA); another group was given a combination of anastrozole (200 mg/d) and fulvestrant (1,000 mg/d; \(n = 20\); ANA + FUL); and a third group switched to fulvestrant (1,000 mg/d; \(n = 8\); ANA to FUL). Animals (\(n = 20\)) treated with fulvestrant (1,000 mg/d) were assigned to three treatment groups: one group continued treatment on fulvestrant (1,000 mg/d; \(n = 6\); FUL); another group was given a combination of anastrozole (200 mg/d) and fulvestrant (1,000 mg/d; \(n = 7\); FUL to ANA + FUL); and a third group switched to anastrozole (200 mg/d; \(n = 7\); FUL to ANA). Tumors were measured weekly and tumor volumes were calculated. On week 6, the differences in growth rate of control versus ANA (\(P = 0.001\)), ANA + FUL (\(P < 0.0001\)), and FUL (\(P = 0.012\)) were statistically significant. The differences in growth rate of ANA versus ANA + FUL (\(P = 0.037\)) and FUL versus ANA + FUL (\(P = 0.011\)) were statistically significant. On week 9, the differences in growth rate of control versus ANA (\(P = 0.013\)) and ANA + FUL (\(P < 0.0001\)) were statistically significant whereas the difference between control and fulvestrant had a marginal statistical significance (\(P = 0.056\)). At week 14, the differences in growth rate of ANA versus ANA + FUL (\(P = 0.0003\)) and ANA to ANA + FUL (\(P = 0.019\)) were statistically significant. The differences in growth rate between FUL versus ANA + FUL (\(P = 0.009\)) and FUL to ANA + FUL (\(P = 0.047\)) were considered statistically significant. Points, mean tumor volume (mm\(^3\)); bars, SE. A, the effect of anastrozole (ANOVA, ANOVA + fulvestrant, fulvestrant, and anastrozole plus fulvestrant on uterus weights. Columns, mean uterine weight (g); bars, SE. Animals in all treatment groups had significantly lower uterine weights compared with the control group (\(P < 0.0001\)).
Anastrozole or fulvestrant alone to the combination therapy also continued growing, their growth rate was much slower ($P = 0.019$ and $P = 0.047$, respectively) and mean tumor volumes increased by 2.4- and 1.5-fold, respectively.

**Effects of anastrozole and/or fulvestrant on uterine weight.** The measurement of uterine weight is a useful bioassay because the uterus is very sensitive to the effects of estrogens. The uteri of mice bearing MCF-7Ca xenografts and treated for 14 weeks were removed and weighed. Anastrozole, fulvestrant, and the combination therapy all reduced uterine weight (Fig. 1B). The uterine weights of all seven treatment groups were statistically different from control ($P < 0.0001$). This finding suggests that the treatments were effective in inhibiting the synthesis or action of estrogens even if the tumors did not respond.

**Effects of anastrozole and fulvestrant alone or in combination on the activation of growth factor receptor pathways.** To understand the mechanisms associated with resistance to anastrozole and fulvestrant and the responsiveness to the combination therapy of anastrozole plus fulvestrant, we analyzed tumors collected from mice at the end of the treatment period. Thus, tumors were collected at 14 weeks and submitted to immunoblot analysis (Fig. 2). The results were quantified by densitometric analysis of the bands and compared with vehicle-treated tumors (control) collected after 14 wk, analyzed by Western blot, and compared with vehicle-treated tumors (Control) collected at 9 wk as described in Materials and Methods. Blots show IGF-IRβ at 95 kDa, AKT at 60 kDa, MAPK and p-MAPK at 42 to 44 kDa, mTOR and p-mTOR at 289 kDa, and ERα at 66 kDa. The blots were stripped and reprobed for β-actin (45 kDa) to verify equal loading. Numbers below the blots represent fold change in protein expression compared with control obtained by densitometric analysis. Representative of at least two independent experiments.

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**Effects of anastrozole and fulvestrant alone or in combination on tumor aromatase activity.** To understand whether changes in the intratumoral aromatase activity could be correlated with response or resistance, the aromatase activity in the tumors collected from the treatment groups was determined. Although tumors were insensitive to anastrozole treatment by week 14, there was an 86% reduction in aromatase activity when compared with control ($P < 0.01$; Fig. 3). Fulvestrant, which has previously been shown also to have inhibitory effects on aromatase activity (32), did not cause a significant reduction in aromatase activity in vivo. The combination of anastrozole plus fulvestrant from the beginning was also able to inhibit aromatase by 73% ($P < 0.05$) but was not statistically different from the anastrozole or fulvestrant group. The switch from anastrozole to fulvestrant caused a significant increase in aromatase activity when compared with the anastrozole alone.
group (14-fold; \(P < 0.001\)). On the other hand, the switch from anastrozole to the combination therapy did not result in any change in aromatase activity when compared with the anastrozole alone group. Surprisingly, the switch from fulvestrant to the aromatase inhibitor anastrozole or to the combination therapy caused an increase of 11- and 3-fold, respectively, when compared with fulvestrant alone group (\(P < 0.01\)).

Discussion

Aromatase inhibitors have been shown to be very effective for the treatment of hormone-dependent postmenopausal breast cancer, and they are now used as standard adjuvant and first-line therapy for advanced disease. Although recent clinical trials have shown aromatase inhibitors to be more effective than tamoxifen, most patients with advanced disease ultimately develop resistance to the treatment. Furthermore, some patients are resistant to therapy de novo. Our mouse model of human ER+ breast cancer cells stably transfected with the aromatase gene (MCF-7Ca cells; ref. 27) simulates the postmenopausal breast cancer patient and has predicted the findings of several clinical studies (17, 33). Using this model, we have previously explored the mechanisms involved in the acquisition of resistance to letrozole (19). Tumors that responded initially to treatment with this aromatase inhibitor eventually adapted to the pressure of endocrine treatment (after 56 weeks) by activating growth factor receptor pathways. In particular, proteins in the HER2/Raf/MAPK signaling pathway were in-
In summary, the additive effects of combining aromatase inhibitors such as anastrozole or letrozole with fulvestrant suggest that the complete blockade of estrogen action by down-regulation of ER and inhibition of estrogen synthesis has a greater effect on tumor growth than either treatment strategy alone. The results also suggest that the combination of an aromatase inhibitor and fulvestrant may be an optimal second-line therapy for patients with tumors progressing on a therapeutically effective dose of aromatase inhibitor by preventing activation of growth factor pathways (HER2 or IGF-IR) and a possible cross talk between these pathways and ER. Several clinical trials such as SOFIA, FACT, SWOG-S0226, FIRST, and D6997C00057 (36) are ongoing and will contribute further information about the benefits of combining anastrozole and fulvestrant in patients. Delay in the use of chemotherapy by a sequential use of endocrine therapies offers significant quality-of-life advantages due to good tolerability of the agents, especially in elderly patients or those with advanced disease.

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