Combination of Anastrozole with Fulvestrant in the Intratumoral Aromatase Xenograft Model

Luciana F. Macedo,1 Gauri J. Sabnis,1 Olga G. Goloubeva,2 and Angela Brodie1,2

1Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine and 2Division of Biostatistics, University of Maryland Greenebaum Cancer Center, Baltimore, Maryland

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 1 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 1 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 aromatase-treated breast tumors and the optimal treatment sequence following aromatase 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 Biosearch Biosciences. Fulvestrant (Faslodex) and anastrozole (Arimidex) were supplied by Dr. E. Anderson (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 (AstraZeneca Pharmaceuticals). Enhanced chemiluminescence (ECL) kits and Hybond-ECL nitrocellulose membranes were purchased from Amersham 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 antimonos and antitubulin antibodies were purchased form Bio-Rad. Antibody against Eκ was purchased from Santa Cruz Biotechnology. Radioactive ligand for the aromatase assay, [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 3H2O 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 cell 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
inoculated s.c. into athymic nude mice supplemented with androstenedione (100 μg/mouse/d s.c.). Once the tumors reached a measurable size of 300 mm³ (after 2 months), the mice were assigned to four treatment groups so that mean tumor volumes were not significantly different at the start of treatment: control (vehicle alone), anastrozole (200 μg/d s.c.), fulvestrant (1,000 μg/d s.c.), and anastrozole (200 μg/d) plus fulvestrant (1,000 μg/d). All animals continued to be supplemented with androstenedione. The mean tumor volumes for each of the groups measured weekly are shown in Fig. 1A. The tumors in the control group doubled in size after 2 weeks whereas the tumors in the anastrozole and fulvestrant groups doubled after 5 weeks. The tumors in mice receiving the combination therapy remained stable at 300 mm³ 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 mm³. 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). 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

![Figure 1](cancerres.aacrjournals.org)
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 uterus 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 at 9 wk as described in Materials and Methods. Blots show IGF-IR 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.

Anastrozole-resistant tumors also showed a decrease in ERα expression compared with control and with single-agent therapy. Anastrozole-resistant tumors also showed a decrease in ERα expression of 50% compared with control similar to that seen with long-term letrozole-treated MCF-7Ca xenografts (19). Treatment with fulvestrant as single agent and the switch from fulvestrant to anastrozole or from anastrozole to fulvestrant caused up-regulation of ERα expression of 2.2-, 4.2-, and 2.4-fold, respectively.

**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.
increased. These proteins are capable of interacting with the ER, 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 increased. These proteins are capable of interacting with the ER, resulting in ER transcription despite reduced estrogen levels (19). Similarly, anastrozole also caused a decrease in tumor growth. Although the tumors became refractory to treatment after 14 weeks, anastrozole was able to inhibit tumor aromatase activity and reduced estrogen-dependent uterine weight. Resistance to anastrozole treatment was associated with an increase in IGF-IRβ, mTOR, and p-mTOR expression. We also found up-regulation of the same proteins in LTLCa cells derived from the letrozole-resistant tumors (20). However, unlike the LTLCa cells (19), no up-regulation or activation of HER2 or MAPK was observed in the anastrozole-resistant tumors. Both letrozole-resistant (19) and anastrozole-resistant tumors showed a decrease in ERα expression and aromatase activity. Other breast cancer models of endocrine resistance developed by long-term estrogen deprivation or prolonged tamoxifen treatment (22, 34) also showed an association between IGF-IR expression and signaling and cross talk with ERα.

**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 increased. These proteins are capable of interacting with the ER.

![Figure 3. Effect of anastrozole and fulvestrant alone, in sequence, or in combination on aromatase activity of MCF-7Ca xenografts. The tumors of mice treated with anastrozole, anastrozole + fulvestrant, fulvestrant, anastrozole to fulvestrant, anastrozole to anastrozole + fulvestrant, fulvestrant to anastrozole, and fulvestrant to fulvestrant + anastrozole were examined for aromatase activity as described in Materials and Methods. The activity of the enzyme after treatment is corrected for total protein amount in the tumors. Columns, mean of aromatase activity as percent of control from two different experiments with at least three replicates each; bars, SE. The differences in aromatase activity of control tumors versus tumors treated with anastrozole (P < 0.01), anastrozole + fulvestrant (P < 0.05), and anastrozole to fulvestrant (P < 0.001) were statistically significant. The difference in aromatase activity of anastrozole-treated tumors versus the anastrozole to fulvestrant group was statistically significant (P < 0.001). The differences in aromatase activity of fulvestrant-treated tumors versus the fulvestrant to anastrozole (P = 0.001) and fulvestrant to anastrozole + fulvestrant (P < 0.01) groups were statistically significant.](image-url)
in combination with fulvestrant resulted in longer growth inhibition. We have also shown that the combination of letrozole and fulvestrant was more effective than either treatment alone in delaying development of resistance (38).

The positive effect of the combination of anastrozole plus fulvestrant in sequence or continuously is associated with down-regulation of a refractory phenotype represented by IGF-IR, AKT, MAPK, m-PAPK, mTOR, p-mTOR, and ERα expression. The combination of letrozole and fulvestrant also repressed HER2 and p-MAPK expression in the tumors treated with the combination therapy compared with tumors treated with letrozole alone (48).

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 SOFEA, FACT, SWOG-S0226, FIRST, and D6997C00057 (36) are ongoing and will contribute further information about the benefits of combining aromatase 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.

Acknowledgments

Received 12/21/2007; revised 2/16/2008; accepted 2/21/2008.

Grant support: AstraZeneca and National Cancer Institute, NIH grant CA-62483 (A. Brodie).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


Combination of Anastrozole with Fulvestrant in the Intratumoral Aromatase Xenograft Model


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/9/3516

Cited articles
This article cites 44 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/9/3516.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/68/9/3516.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.