Inhibition of the Phosphatidylinositol 3-Kinase/Akt Pathway Improves Response of Long-term Estrogen-Deprived Breast Cancer Xenografts to Antiestrogens

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Abstract

Purpose: Aromatase inhibitors that block the synthesis of estrogen are proving to be superior to antiestrogens and may replace tamoxifen as first-line treatment for postmenopausal estrogen receptor (ER)-positive breast cancer patients. However, acquisition of resistance to all forms of treatments is inevitable and a major clinical concern. In this study, we have investigated the effects of long-term estrogen deprivation in the breast cancer xenograft model and whether sensitivity to antiestrogens can be restored in vivo. We also compared whether combining wortmannin with tamoxifen or fulvestrant inhibited tumor growth better than either drug alone.

Experimental Design: Long-term estrogen-deprived aromatase-transfected human ER-positive breast cancer cells (UMB-1Ca) were grown as tumors in ovariectomized athymic nude mice. Twelve weeks after inoculation, when tumors reached 300 mm³, animals were grouped and injected with vehicle, Δ4A, letrozole, tamoxifen, fulvestrant, wortmannin, tamoxifen plus wortmannin, and wortmannin plus fulvestrant. Tumor volumes were measured weekly.

Results: Tumors of UMB-1Ca cells grew equally well with and without androstenedione, indicating the ability of the cells to proliferate in the absence of estrogen. The combination of wortmannin with tamoxifen or fulvestrant inhibited tumor growth better than either drug alone. The combination of wortmannin plus fulvestrant was the most effective treatment that maintained tumor regression for a prolonged time.

Conclusion: These results suggest that blocking both ER and growth factor receptor pathways could provide effective control over tumor growth of long-term estrogen-deprived human breast cancers.

Estrogen is the major stimulus to breast cancer progression in both premenopausal and postmenopausal patients. In young women, the main source of estrogen is the ovary. After menopause, ovarian production declines and extragonadal sites such as adipose tissue are the main source of circulating estrogen (1). However, tissue concentrations within the breast are comparable with those of premenopausal women (2, 3). The actions of estrogen on the tumor are mediated by estrogen receptor (ER). In breast cancer patients, tumor ER concentrations are higher after menopause, resulting in cancers that are sensitive to even low levels of circulating estrogens (4).

A large proportion of breast cancer patients are postmenopausal women with ER-positive tumors, and treatment strategies that inhibit the growth stimulus of estrogen are effective in providing long-term benefit. Aromatase inhibitors that reduce estrogen production have now been shown to be effective treatment for breast cancer (3, 6). The triazole compound letrozole has been found to be superior to tamoxifen (7, 8). Anastrozole, a similar triazole compound, was more effective than tamoxifen in ER-positive patients and had fewer side effects (9). These clinical findings are consistent with results from previous investigations in our tumor model (10–12). The model mimics the postmenopausal breast cancer patient because the source of estrogen is from nonovarian tissue and is not under gonadotropin regulation (13). However, because the mouse has no significant production of peripherally formed estrogen, the MCF-7 human breast cancer cells stably transfected with the human aromatase gene serve as an autocrine source of estrogen to stimulate tumor growth in the ovariectomized, immunosuppressed mice. The resulting tumors synthesize estrogen from supplemented androstenedione (Δ4A) and proliferate in response to estrogen acting via the ER. These tumors are therefore useful for studying the effects of both antiestrogens and aromatase inhibitors in the same model system (13, 14). This model has provided valuable data that have predicted the efficacy of aromatase inhibitors in breast cancer patients. We have reported a number of studies on growth effects of aromatase inhibitors and antiestrogens used in...
Materials and Methods

Materials. Improved MEM, penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin-1 mL/L EDTA solution, Dulbecco’s PBS, and geneticin (G418) were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from HyClone (Logan, UT). Akt inhibitor wortmannin, antrostenedione, tamoxifen, Matrigel, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against p-Akt (Ser473 and Thr308), p-PDK-1, Akt, and progesterone receptor were purchased from Cell Signaling Technology (Beverly, MA).

MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca) were provided by Dr. S. Chen (City of Hope, Duarte, CA; ref. 13). Letrozole (Femara, CGS 20267) was provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland). The pure antiestrogen fulvestrant (Faslodex, ICI 182,780) was supplied by Dr. A. Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom). ECL chemiluminescent detection and exposure to ECL X-ray film. The membranes were then incubated with respective primary anti-

In vitro protein kinase assay. The Akt kinase assay was carried out according to the manufacturer’s protocol. Briefly, Akt protein in 200-μL tumor lysates, which contain 200 μg total proteins, was immunoprecipitated with immobilized monoclonal antibody for Akt (Cell Signaling Technology). The next day, the reaction was carried out in 50 μL of IP kinase buffer supplemented with 200 μmol/L ATP and 2 μg of glycogen synthase kinase-3α/β fusion protein and incubated for 30 min at 30°C. The reaction is terminated by addition of 25-μL SDS sample buffer. The samples were boiled for 5 min, vortexed, and then microcentrifuged for 2 min. The sample (30 μL) was then loaded on to a SDS-PAGE gel and analyzed by Western blotting. The gel was probed with phospho antibody for glycogen synthase kinase-3α/β. The bands were quantitated using ImageQuant (Molecular Dynamics).

Statistics. Data on tumor volume and weight, as well as uterine weight, were analyzed separately. Growth curve models were used to summarize the pattern of response over treatment duration. For tumor growth, diagnostic plots suggested that the model of exponential growth was appropriate to the data. Therefore, tumor volumes were transformed to ln(volume + 1), adding 1 to all volume measurements,
including zeros. We used linear mixed-effects models to estimate an exponential variable controlling the tumor growth rate for each of the six treatment groups. The tumor growth rate (the slope of the regression line) was compared between prespecified treatment groups. Responses from different animals were assumed to be statistically independent whereas those within a subject were correlated. The mean tumor volume was modeled as a combination of fixed effects shared by all subjects and random effects unique to a particular animal. We used random effects for both intercept and slope at animal level. In the longitudinal assessment of tumor growth, we have chosen not to assume any specific form for the random effects covariance matrix. The simple covariance structure was selected for measurement within a mouse. The baseline or start of treatment tumor volume was included as a covariate in the resulting regression model. The results of treatment group comparisons are presented as differences in exponential variables controlling the growth rate with the corresponding 95% confidence intervals. For the analyses of uterine weight data, we used logarithmic transformation to stabilize variance. General linear models were used for the uterine data. The proportions of tumors that shrunk below a threshold during treatment were compared using Fisher’s exact test. Mixed-effects models were used to compare average tumor volume on prespecified time points. The Tukey-Kramer method was used to adjust for multiple comparisons.

For in vitro studies, comparison of all treatment was made with vehicle-treated control using one-way ANOVA (Tukey’s Test) on Sigma Plot 9.0 software (Point Richmond, CA). *P* < 0.05 was considered statistically significant.

### Results

**Growth response of UMB-1Ca cells to treatment with Δ4A and Δ4A plus letrozole in vivo.** Long-term estrogen-deprived UMB-1Ca cells were developed as reported earlier (16). Briefly, low-passage MCF-7Ca cells were cultured in steroid-depleted medium for 6 to 8 months. After a brief period of quiescence, the growth of the cells resumed and the cell line was designated as UMB-1Ca. These cells were insensitive to the effect of Δ4A or E2 and significantly less sensitive to letrozole *in vitro*, whereas in parental MCF-7Ca cells, proliferation was increased after treatment with Δ4A or E2 and markedly inhibited by treatment with letrozole (19).

Furthermore, when UMB-1Ca cells were inoculated into groups of ovariectomized nude mice and injected s.c. daily with either vehicle, Δ4A (100 μg/d), or Δ4A plus aromatase inhibitor letrozole (10 μg/d), tumors grew equally well in the presence and absence of Δ4A (Fig. 1A), suggesting that the tumors no longer require estrogens for growth. This finding is consistent with our previous report (16, 19) that UMB-1Ca cells have adapted to grow in estrogen-deprived medium and did not require the aromatase substrate Δ4A for proliferation, regardless of high aromatase activity (16). In addition, UMB-1Ca xenografts also grew equally well in the presence and absence of letrozole (10 μg/d), indicating loss of sensitivity of the tumors to aromatase inhibitor therapy with letrozole (Fig. 1A).

In week 10 of treatment, these mice were sacrificed due to large tumor size. The mean tumor weight was found to be 936.5 ± 232.2 mg in Δ4A group and was not statistically different from the mean tumor weight of Δ4A plus letrozole group, 867.0 ± 143.0 mg (Fig. 2A). The mean uterine weight of vehicle-treated mice was 18.8 ± 1.3 mg. The mean uterine weight of mice receiving Δ4A was 38.9 ± 1.8 mg (Fig. 2B). This suggests that aromatase activity in the UMB-1Ca cells was sufficient to produce enough estrogen from Δ4A to maintain the uterine weight (16). The mean uterine weight in the letrozole-treated group was 11.0 ± 1.4 mg, significantly less than in the control and Δ4A treated groups (*P* < 0.001), suggesting that estrogen synthesis is inhibited by letrozole in these mice compared with vehicle-treated mice. The low uterine weight suggests that letrozole effectively inhibited the...
conversion of Δ^3A to estrogens although the suppression of estrogen did not result in tumor growth inhibition.

**Treatment of UMB-1Ca xenografts with PI3K inhibitor wortmannin alone or in combination with antiestrogen tamoxifen or fulvestrant.** After ~12 weeks, mice were randomized in various treatment groups (week 0). The mean tumor volume is plotted in Fig. 1B. Tumor volume doubled in vehicle-treated controls in 2 weeks and showed a 10-fold increase in volume by 14 weeks. These mice were sacrificed at this time due to large tumor size. The mean tumor weight was found to be 977.8 ± 180.0 mg.

Tumor growth was not inhibited in UMB-1Ca xenografts by tamoxifen (100 μg/d) treatment. The mean tumor volume doubled at 1.5 weeks and these mice were also sacrificed after 14 weeks (Fig. 1B). As shown in Fig. 2A and B, the mean tumor weight was 810.0 ± 93.1 mg and mean uterine weight was 73 ± 3.3 mg. The high uterine weight in the tamoxifen-treated group is consistent with the estrogenic effects of tamoxifen on the uterus as previously reported (16). Tumors of mice treated with tamoxifen continued to proliferate and had a similar estimated growth rate (exponential variable $\beta = 0.036 ± 0.007$) as tumors of mice in the control group ($\beta = 0.027 ± 0.007$).

Wortmannin (350 μg/kg/d) was not a potent inhibitor of tumor growth. The tumor growth rate ($\beta = 0.019 ± 0.007$) was similar to vehicle-treated mice ($P = 0.23$). However, wortmannin-treated group had a significantly lower mean tumor volume than tamoxifen-treated group ($P = 0.0004$) in week 4. Tumor volume doubled in 5.5 weeks and increased 7-fold by week 14. At this time, these mice were sacrificed and tumors and uteri were collected and stored for analysis. The mean tumor weight of wortmannin-treated mice was 782.5 ± 231.5 mg. The mean uterine weight was 23.1 ± 4.4 mg, which was not significantly different from the control group. This suggests that wortmannin lacks any estrogenic or antiestrogenic properties.

The combination of wortmannin plus tamoxifen was found to be better than single agent tamoxifen ($P = 0.04$), showing that wortmannin was, to some extent, able to restore the growth inhibitory effects of tamoxifen (Fig. 1B). Regardless, the effect of this combination was of limited duration because the mean tumor volumes increased 2-fold in 2.5 weeks. However, tumors were static from week 3 through week 11. The addition of wortmannin to tamoxifen prolonged the time to progression and suggests partial restoration of responsiveness of UMB-1Ca xenografts to tamoxifen therapy.

Fulvestrant (1,000 μg/d) alone did not cause any significant regression of the tumors; however, growth was static until week 9 ($\beta = -0.0004 ± 0.006$), at which point growth resumed and final tumor volume was increased 3-fold at termination of the experiment. The mean tumor weight at this time was 518.7 ± 145.7 mg and mean uterine weight was 6.0 ± 0.8 mg. Lower uterine weight compared with the vehicle control group suggests that there is residual estrogen in the ovariecotomized mice, which is antagonized by fulvestrant. Fulvestrant was considerably more effective than tamoxifen in suppressing tumor growth. The mean tumor weight at the termination was significantly lower ($P < 0.001$) than all the other groups, although this group was sacrificed at the latest time point.

The combination of fulvestrant plus wortmannin treatment showed a synergistic effect and tumors responded for a longer duration. Before termination, tumor volume had reached the initial size. Importantly, the mean tumor volume in the wortmannin plus fulvestrant group was significantly lower than with wortmannin ($P < 0.0001$) or fulvestrant alone ($P < 0.0023$) at weeks 4, 10, 14, and 17. When compared with
Akt and Estrogen Receptor Pathway Cross-talk after Long-term Estrogen Deprivation

Modulation in the expression of ERα and proteins in the PI3K/Akt pathway after treatment with wortmannin and/or antiestrogens. At the end of the tumor growth study, the mice were sacrificed and tumors and uteri excised and weighed for further analysis of protein expression. The tumor lysates were examined for p-Akt (Ser^{473} and Thr^{308}) expression (Fig. 3). The tumors treated with wortmannin alone or in combination with tamoxifen or fulvestrant showed reduction in the expression of p-Akt (densitometric values are corrected for loading control with expression of total Akt; Fig. 3). Significantly, fulvestrant alone was also found to be effective in reducing p-Akt (Ser^{473} and Thr^{308}) expression. However, p-PDK-1 (upstream of Akt) was not affected by fulvestrant treatment (Fig. 3C). This suggests activation of Akt may not only occur through PI3K activation, but could be through ER, further implicating cross-talk between the two signaling pathways. However, tamoxifen alone did not affect the expression of any of these proteins. The inability of tamoxifen in inhibiting the cross-talk between the PI3K and ERα pathways may be due to the partial agonistic effects of tamoxifen or to the agonistic action of tamoxifen in cells with high AIB-1 levels.3

The tumor lysates were examined for ERα expression. ERα protein expression was slightly reduced in wortmannin-treated tumors (Fig. 3). Fulvestrant, known as a selective ER down-regulator, was more effective than wortmannin in reducing the ERα levels in UMB-1Ca xenografts. Treatment of UMB-1Ca xenografts with wortmannin showed a slight reduction in the expression of progesterone receptor, suggesting little inhibition of ERα-mediated transcription. However, treatment with fulvestrant alone or wortmannin plus fulvestrant showed ~50% reduction in progesterone receptor levels (Fig. 3), suggesting inhibition of ERα-mediated transcription by fulvestrant but not by wortmannin.

When Akt kinase activity was measured in these tumor lysates, all the treatments, except tamoxifen alone, were found to inhibit the kinase activity to some extent compared with control samples (Fig. 4A). The combination of fulvestrant with wortmannin was most effective in reducing Akt kinase activity. Furthermore, interaction between AKT and ERα was examined when tumor lysates from UMB-1Ca xenografts were first immunoprecipitated with anti-AKT antibody and then immunoblotted with the antibody for ERα. UMB-1Ca xenografts treated with the combination of fulvestrant plus wortmannin significantly (P < 0.01) inhibited this association compared with other treatment groups (Fig. 4B). This finding again correlates with the significant tumor growth inhibition. Similarly, single agent fulvestrant was moderately effective in inhibiting the association and tumor growth. Additionally, as shown in Fig. 4A, fulvestrant decreased ERα expression. However, the combination of fulvestrant plus wortmannin reduced ERα expression and the association to a much greater extent, the effect being significantly different from that of fulvestrant alone. Thus, the complete down-regulation of this association in wortmannin plus fulvestrant treatment group indicates the necessity of deactivation or down-regulation of both Akt and ERα. Thus, it is clear from the study that inhibition of both pathways and the cross-talk between the two pathways are essential for better control over tumor growth.

Discussion

There has been significant improvement in the efficacy of hormone therapy of breast cancer after the introduction of aromatase inhibitors (18, 23). Despite reduced estrogen production in postmenopausal breast cancer patients, a major clinical concern is the inevitable acquisition of resistance to treatment as the cancer cells adapt to the treatment environment. Our recent in vitro studies involving the long-term estrogen-deprived aromatase-transfected MCF-7 cells (UMB-1Ca; ref. 19) have shown activation of growth factor receptor signaling cascades that lead to growth stimulation and loss of

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3 Tilghman S, Gauri S, and Brodie A: unpublished data.

![Fig. 3. Western immunoblotting analysis of tumor lysates from UMB-1Ca xenografts. Blot shows phospho-Akt (Ser^{473}) and phospho-Akt (Thr^{308}) at 60 kDa, p-PDK-1 (Ser^{445}) at 58 to 62 kDa, total-Akt at 60 kDa, ERα at 66 kDa, progesterone receptor-A and progesterone receptor-B (PgR) at 90 and 118 kDa, and β-actin at 45 kDa. Experimental protocol was as described in Materials and Methods. Lane 1, control; lane 2, tamoxifen (100 μg/d); lane 3, wortmannin (0.35 mg/kg/d); lane 4, fulvestrant (1 mg/d); lane 5, wortmannin plus tamoxifen; and lane 6, wortmannin plus fulvestrant. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Representative of three independent experiments; densitometric measurements are corrected for β-actin.](www.aacrjournals.org)
responsiveness to endocrine therapy involving aromatase inhibitors (19). The key growth factor receptor pathways that regulate the cell cycle and transcription of genes involved in proliferation include Her-2 and the downstream kinase cascade. In the UMB-1Ca estrogen-deprived cells, PI3K and Akt are up-regulated compared with parental MCF-7Ca cells. ER also seems to be crucial in these resistant cells as cross-talk between ER and the signal transduction pathways seems to be responsible for the ability of the cells to adapt to a low-estrogen environment (19) and grow in the presence of aromatase inhibitors (24). Our experimental data suggest that both fulvestrant, which reduces ER levels, and a signal transduction inhibitor that blocks the PI3K/Akt pathway in these cells can inhibit tumor growth more effectively compared with each treatment alone (21).

This article focuses on the synergistic effect of combining an inhibitor of the PI3K/Akt pathway, such as wortmannin, with fulvestrant on tumor growth inhibition in breast cancer cells deprived of estrogen for an extended period of time. The pure estrogen receptor antagonist fulvestrant, which reduces ER levels, and as signal transduction inhibitors (24). Our experimental data suggest that both fulvestrant, which reduces ER levels, and a signal transduction inhibitor that blocks the PI3K/Akt pathway in these cells can inhibit tumor growth more effectively compared with each treatment alone (21).

Although wortmannin alone was not effective in suppressing tumor growth, wortmannin treatment was found to be significantly more effective than any other treatment because tumor growth was inhibited for a prolonged period (>10 weeks). Furthermore, at the end of the experiment, the tumor volume was less than twice the initial volume. In animals treated with fulvestrant plus wortmannin, tumors had a lower growth rate than animals treated with either wortmannin or animals that received fulvestrant. Tumors from the combined treatment group expressed both ERα and p-Akt at a very low level. The Akt kinase activity was lowest and the association between ERα and Akt was also significantly reduced. Although wortmannin is not a specific PI3K inhibitor and not used clinically, our studies show the importance of inhibiting the PI3K/Akt pathway in overcoming resistance to antiestrogen treatment. It has been suggested that an increase in growth factor and cellular kinase signaling in breast cancer potentiates the ER pathway, which in turn reactivates growth factor signaling via genomic and nongenomic actions. This is believed to result in a stimulatory cycle that intensifies the activities of both ER and growth factor receptors (25).

Our results show that ER signaling can also be regulated by the complex growth factor signaling pathways. Bidirectional cross-talk is evident in many in vitro and in vivo model systems as well as some breast cancer patients (26). Our results suggest that tumor cells adapt to estrogen deprivation by up-regulating the estrogen signaling pathway and activating kinase signaling proteins to maintain transcription and cell proliferation. The involvement of ERα is supported by inhibition of growth of hormone refractory UMB-1Ca xenografts sensitive to fulvestrant. The combination of wortmannin plus fulvestrant had a significantly lower growth rate and also showed more pronounced inhibition of kinase signaling and ERα-mediated transcription than either compound alone. Thus, molecular communications from several intracellular kinases can alter the function of ER and contribute to tumor growth. This necessitates dual inhibition of ER-mediated transcription and kinase-mediated growth stimulation for better control of tumor growth (23).

In previous studies involving long-term letrozole-treated tumors and cell lines (LTLT-Ca; ref. 24), ER and aromatase expression were reduced and the mitogen-activated protein kinase signaling pathway was activated. Although fulvestrant did not have a significant effect on the growth of these tumors, ER and growth response to aromatase inhibitors and anti-estrogens could be restored in the LTLT-Ca cells by inhibiting the kinase pathway (24). This suggests that combined inhibition of the ERα and the kinase signaling pathways may provide better control over growth of tumors proliferating in estrogen-deprived conditions.

References


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