Expression of the K303R Estrogen Receptor-α Breast Cancer Mutation Induces Resistance to an Aromatase Inhibitor via Addiction to the PI3K/Akt Kinase Pathway

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Abstract
Aromatase inhibitors (AI) are rapidly becoming the first choice for hormonal treatment of estrogen receptor-α (ERα)–positive breast cancer in postmenopausal women. However, de novo and acquired resistance frequently occurs. We have previously identified a lysine to arginine transition at residue 303 (K303R) in ERα in premalignant breast lesions and invasive breast cancers, which confers estrogen hypersensitivity and resistance to tamoxifen treatment. Thus, we questioned whether resistance to AIs could arise in breast cancer cells expressing the ERα mutation. As preclinical models to directly test this possibility, we generated K303R-overexpressing MCF-7 cells stably transfected with an aromatase expression vector. Cells were stimulated with the aromatase substrate, androstenedione, with or without the AI anastrozole (Ana). We found that Ana decreased androstenedione-stimulated growth of wild-type cells, whereas K303R-expressing cells were resistant to the inhibitory effect of Ana on growth. We propose that a mechanism of resistance involves an increased binding between the mutant receptor and the p85α regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K), leading to increased PI3K activity and activation of protein kinase B/Akt survival pathways. Inhibition of the selective “addiction” to the PI3K/Akt pathway reversed AI resistance associated with the mutant receptor. Our findings suggest that the K303R ERα mutation might be a new predictive marker of response to AIs in mutation-positive breast tumors, and that targeting the PI3K/Akt pathway may be a useful strategy for treating patients with tumors resistant to hormone therapy.

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Introduction
Despite the clinical efficacy of the aromatase inhibitors (AI), many ERα-positive breast tumors initially fail to respond (de novo resistance), or resistance develops during treatment leading to disease progression (acquired resistance). To understand resistance mechanisms, several laboratories have developed in vitro cell line models to study the molecular changes associated with long-term estrogen deprivation (1–5). AI resistance has also been examined using aromatase-overexpressing MCF-7 breast cancer cell line models grown as xenografts in athymic nude mice (6), or breast cancer cells made resistant to AIs via long-term treatment with these drugs (7, 8). These models suggest the hypothesis that resistance to endocrine therapy may be through the acquisition of estrogen hypersensitivity, whereby low subphysiologic levels of estrogens remaining after estrogen deprivation are sufficient for maintenance of tumor growth. One unifying feature which has emerged is a retained mitogenic role for ERα (9). Recently, intracellular cross-talk between ERα and several signal transduction pathways have been shown to be associated with endocrine resistance (10, 11).

We have previously identified a frequent somatic mutation at nucleotide 908 of ERα (A908G) in premalignant breast lesions and invasive breast cancers (12, 13). This mutation results in a lysine to arginine transition at residue 303 (termed K303R), that confers hypersensitivity to estrogen (12, 14). We hypothesized that such a mutant could provide a continuous mitogenic stimulus to the breast even during phases of low circulating hormone, such as menopause, thus affording a proliferative advantage especially during treatment with AIs. Here, we present a new model of resistance to endocrine therapy, whereby the expression of the A908G ERα mutation conferred resistance to the AI anastrozole (Ana). We speculate that inhibition of the phosphatidylinositol-3-OH kinase (PI3K)/Akt pathways may represent a promising therapeutic strategy for hormone-resistant cancers that are “addicted” to these pathways due to mutation of the ERα target.

Materials and Methods
Reagents, hormones, and antibodies. 17β-Estradiol, 4-androstene-3,17-dione and heregulin were obtained from Sigma. Anastrozole and ICI182,780 were provided by AstraZeneca. PD98059, PI-103, Akt inhibitor VIII isozyme-selective (Akti1/2), and LY294002 were from Calbiochem. Exemestane (Exe) was obtained from Pfizer. Antibodies used for immunoblotting were ERα (clone 6F11) from Vector Laboratories and 4,6-diamidino-2-phenylindole (DAPI) was from Ambion. Total ERK1,2/MAPK, total Akt, phosphorylated p42/44 ERK1,2/MAPK (Thr202/Tyr204), Akt (Ser473), ERα (Ser786), and poly(ADP ribose)polymerase (PARP) were from Cell Signaling Technology. Bax and Bcl-2 were from Calbiochem; cytochrome P450 aromatase was from Serotec; and p85 was from Upstate Biotechnology. Secondary antibodies goat anti-mouse or goat anti-rabbit were obtained from Amersham Biosciences.

Plasmids. Full-length human aromatase cDNA was amplified from the pCMV6-Arom plasmid (OriGene Technologies) by PCR using the following primers: forward 5′-ACACTAGTATGGTTTTGGAAATGCTGAACCC-3′ and reverse 5′-AGCGGCGCGCTAGTGTCCAGACACCTGTCT. This PCR
product was subcloned into the Spe I/Not I sites of the pZeoSV2-vector (Invitrogen). The resulting pZeoSV2-aroam expression vector (pZeo-Arom) sequence was confirmed by DNA sequencing. Generation of yellow fluorescent protein (YFP)-tagged expression constructs, YFP-WT and K303R-Erα, has been previously described (14).

**Cell culture.** MCF-7 parental breast cancer cells were cultured as described (14). MCF-7 wild-type (WT) and K303R Erα-expressing cells were generated as described (15). MCF-7 parental and YFP-K303R Erα clones were stably transfected with the pZeo-Arom expression vector using FuGene 6 reagent according to the manufacturer (Roche). Chinese hamster ovary (CHO) or MCF-7 Arom-expressing pools, stably transfected with YFP-WT Erα and K303R-Erα expression vectors, were also used.

**Aromatase activity assay.** Aromatase activity was evaluated using a "H-water release assay using 0.5 μmol/L of [1H]-androst-4-ene-3,17-dione as substrate (16). The incubations were performed at 37°C for 1 h. The results were expressed as fmol-pmol/h/mg protein.

**Tumor xenografts.** All animal studies were carried out according to the guidelines and with the approval of the Baylor College of Medicine Animal Care and Use Committee. Female nude ovariectomized athymic mice were injected with MCF-7 YFP-WT and YFP-K303R Erα-expressing cells or MCF-7 Arom 1 and K303R Arom 1-expressing cells as described (17). Animals were supplemented with estrogen tubing releasing −80 pg/mL of estradiol, representing low premenopausal levels (Fig. 1C). K303R Erα tumors grew faster than the WT Erα tumors (P = 0.0466). Thus, expression of the mutant generated an estrogen hypersensitive phenotype in vitro and in vivo.

**Expression of the mutation and AI resistance.** Estrogen-deprived MCF-7 cell lines (1–3, 19), and breast cancer cells resistant to aromatase inhibitors (AI) have been generated (6, 7); one hypothesis emerging is that resistance may result from adaptive estrogen hypersensitivity or estrogen-independent Erα activation. This hypothesis led us to question if the K303R Erα mutant might play a role in AI due to its estrogen-hypersensitive phenotype.

To test for AI, cells were stably transfected with an aromatase cDNA expression vector. Figure 1D shows aromatase protein levels in a vector control clone (MCF-7 V), one clone stably expressing aromatase (MCF-7 Arom 1), and two clones coexpressing the YFP-K303R mutant Erα along with aromatase (K303R Arom 1 and Arom 2). These cells overexpressed aromatase protein and activity at ~1,000 times more than vector control cells (0.032 versus 29–65 pmol/h/mg protein).

We next evaluated proliferative responses in anchorage-independent assays (Fig. 1E). K303R Arom 1–expressing cells exhibited significantly enhanced control growth compared with WT Erα MCF-7 Arom 1–expressing cells, and growth was further increased by AD treatment. MCF-7 Arom 1 and K303R Arom 1 cells were also injected into mice to monitor xenograft growth; a significant increase in the growth of AD-treated mutant tumors was seen (Fig. 1F).

To investigate whether the mutant hypersensitive phenotype could cause AI, we examined the effects of the nonsteroidal aromatase inhibitor Ana on cell growth (Fig. 2A). Growth of MCF-7 vector (V) cells was significantly enhanced by E2, but as expected, they did not respond to AD treatment. AD treatment enhanced growth to the same extent as E2 in MCF-7 Arom 1 cells, as well as K303R Arom 1–expressing cells. Ana treatment decreased AD-stimulated growth at ~40% in MCF-7 Arom 1–expressing cells, but had no effect on the growth of mutant cells.

To extend the MTT data, we performed anchorage-independent soft agar growth assays (Fig. 2B). E2 and AD treatments enhanced colony numbers, and treatment with Ana completely abrogated AD-stimulated growth of MCF-7 Arom 1 cells. In contrast, basal colony number was increased ~10-fold in K303R Arom 1 and Arom 2 clones, and Ana was unable to inhibit AD-induced colony growth.

Because it was possible that overexpression of exogenous Erα alone might stimulate cell growth and contribute to the AI phenotype, we also transfected MCF-7 Arom 1–expressing cells with an expression vector for YFP-WT Erα. Aromatase and exogenous YFP-Erα levels are shown (Fig. 2C, top).
expressing exogenous WT or mutant receptor were evaluated in soft agar assays (Fig. 2C, bottom). The basal growth of K303R ERα–Arom pools (P) was significantly higher than WT pools. Inhibition of aromatase activity by Ana caused a significant reduction in AD-stimulated growth in WT ERα Arom cells, but Ana was unable to reduce AD-stimulated mutant cell growth, confirming our stably transfected clones results. We also generated stable pools of YFP-tagged ERα in ER-negative, aromatase-positive CHO cells (Fig. 2D). Ana did not reverse AD-stimulated growth compared with AD treatment alone, which may be a variable clonal effect. We also generated stable pools of YFP-tagged ERα in ER-negative, aromatase-positive CHO cells (Fig. 2D). Ana did not reverse AD-stimulated growth compared with AD treatment alone, which may be a variable clonal effect. We also generated stable pools of YFP-tagged ERα in ER-negative, aromatase-positive CHO cells (Fig. 2D). 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ICI treatment resulted in a decrease in pAkt levels in mutant cells, concomitant with a reduction in YFP-ERα levels (Fig. 3F). This suggests that activation of pAkt may be related to nongenomic activities of the mutant receptor, as short-term treatments did not block Akt phosphorylation, but are known to block ERα’s genomic activities (21).

ERα can bind to the p85α regulatory subunit of PI3K and activate PI3K/Akt signaling in cells (22). We have previously shown that the mutant ERα receptor exhibits altered binding with several nuclear receptor coregulatory proteins, such as the TIF-2 coactivator, and for the ERBB2 (HER2) oncogene when compared with WT ERα (12, 18). We next examined whether the mutation might alter binding with the PI3K subunit p85, and alter PI3K activity. ER-negative CHO cells were transiently transfected with YFP-tagged receptors, and coimmunoprecipitation studies were performed. Enhanced binding of the p85 subunit to mutant receptor in the absence of ligand was observed (Fig. 4A). Equal amounts of lysates of CHO transfected cells (CHO + WT or K303R) or stable pools (CHO WT or K303R P; Fig. 4B) were immunoprecipitated with an anti-p85 antibody, and kinase activity was determined. Mutant ERα either transiently expressed or in stable pools significantly induced PI3K activity. We also performed an in vitro PI3K assay in our stably transfected MCF-7 clones, and found constitutively increased PI3K activity in the mutant cells, which was abrogated by PI3K inhibitor LY294002 treatment (Fig. 4C). We also detected elevated constitutively pAkt in CHO cells transiently or stably expressing the mutant receptor (Fig. 4D). These data support a mechanism whereby enhanced binding of the regulatory subunit of PI3K results in enhanced downstream Akt signaling in the mutant cells.

Phosphorylation of ERα by a number of kinases is an important mechanism by which ERα activity can be regulated (23–25). Akt kinase phosphorylates ERα at serine (S) 167. Increased levels of pS167 YFP-ERα were found in K303R mutant cells (Fig. 4D), and the mutant increased ERE-transcriptional activity ~ 8-fold (Fig. 4E). ERE activity was inhibited by treatment with either the PI3K inhibitor LY294002, or the antiestroogen ICI182,780. Thus, Akt may serve as a functional link between nongenomic and genomic ERα activities, engaging in a bidirectional cross-talk that may set up a vicious cycle between these two growth-regulatory pathways and augment signaling between them.

K303R ERα-aromatase cells exhibited altered apoptotic responses. Akt/protein kinase B signaling is involved in the control of survival and apoptosis (26, 27), and AIs induce cell death by apoptosis (28). This suggestion led us to question if the K303R ERα mutation could protect cells from apoptosis induced by Ana, providing these cells with a potential survival advantage. We first evaluated PARP proteolysis, and found a reduction in the basal levels of the proteolytic form of PARP (85 kDa) in mutant cells.
under control conditions (Fig. 5A). AD treatment reduced its levels in a time-dependent fashion in WT and mutant cells, but this reduction was less pronounced in WT cells. $E_0$ was able to reduce PARP-cleavage levels only in mutant cells, suggesting that reduced apoptosis may underlie their hypersensitivity. Ana treatment of MCF-7 Arom 1-expressing cells increased proteolysis compared with AD treatment; cleavage was unchanged in mutant cells (Fig. 5B). In addition, we found an increase in the Bcl-2/Bax ratio in K303R Arom 1-expressing cells, which was further increased with AD and Ana treatments (Fig. 5C and D).

To determine the levels of cellular apoptosis, we also used ELISA cell death detection assays (Fig. 5E), and found that AD-stimulated mutant cells exhibited a lower apoptosis compared with MCF-7 Arom 1-expressing cells, suggesting that the proliferative advantage provided by the mutation may be achieved by a decreased apoptotic response of these cells. In addition, Ana treatment induced an increase in cell apoptosis only in MCF-7 Arom 1 cells. We hypothesize that mutant-expressing cells are resistant to AI-induced cellular apoptosis.

**The AI$^R$ phenotype is dependent on the PI3K/Akt pathway.**

We next addressed whether activated PI3K/Akt signaling may be a functional mechanism of resistance to AI therapies using PI3K/Akt inhibitors. To define the effective dose, mutant cells were treated with different doses of these agents and analyzed for pAkt. LY (10 μmol/L), PI-103 (1 μmol/L), Akt1/2 (1 μmol/L) effectively blocked basal Akt phosphorylation (Fig. 6A). We then performed soft agar growth assays. LY completely inhibited mutant proliferation, whereas it induced a slight reduction in the growth of MCF-7 Arom 1 cells (Fig. 6B). Similar results were obtained using the PI-103 inhibitor in another mutant clone (K303R Arom 2; data not shown). Because PI3K inhibitors affect not just all three Akt isoforms, but also other PH domain-containing molecules (29), we tested a specific Akt inhibitor, Akt1/2 (Fig. 6C), and found that basal, AD-stimulated and Ana-stimulated growth was inhibited by ~30% in MCF-7 Arom 1-expressing cells, whereas growth was inhibited by >90% in mutant cells. These results suggest that Akt signaling is essential for the growth of mutant cells, but may not play an important role in the growth of WT cells.

We also used a MEK1 inhibitor (PD98059) in soft agar assays (Fig. 6D); antiproliferative effects were not observed and PD treatment was unable to reverse the AI$^R$ mutant phenotype. These results confirm that the p44/42 Erk1,2/MAPK pathway was not involved in the AI$^R$ mutant phenotype. We also used ICI182,780, and it suppressed the colony growth of both cell lines, indicating...

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**Figure 3.** Mutant cells exhibited constitutive pAkt. A to F, immunoblotting showing phosphorylated Akt (pAkt), Akt, and Rho GD1α in cells treated with vehicle or AD 1, 10, 25, and 50 nmol/L (1 h, A), or AD 10, 30, 60, and 120 min (10 nmol/L, B); or heregulin 2 ng/mL (H 5 min, B); in xenograft tumors extracts (C, numbers below blots represent the average fold change in pAkt levels of K303R Arom 1 samples versus MCF-7 Arom 1 samples); in cells treated with vehicle; AD 10 nmol/L + Ana 1 μmol/L (1 h, D); in cells treated with vehicle and ICI 1 μmol/L for 1, 5, 10, and 30 min (E) or 1, 3, 24, and 48 h (F). Quantitative analysis is the fold difference in pAkt/Akt/Rho GD1α ratio relative to vehicle-treated MCF-7 Arom 1 cells.
that ERα expression remained important in their growth regulation (Fig. 6D).

We next evaluated the effects of the PI3K inhibitor LY294002 on apoptosis using an ELISA cell death detection assay (Fig. 6E), and found that LY treatment in MCF-7 Arom 1 cells showed similar apoptosis induction as Ana-treated cells (1.7-fold increase), whereas inhibitor treatment of mutant cells was able to induce a 3.6-fold increase in the apoptotic rate compared with AD, and most significantly, after Ana treatment. We conclude that the mutation provides a selective dependency on the PI3K/Akt survival pathway for sustained proliferation and/or survival. We suggest that inhibition of this potential pathway addiction may provide an effective means to reverse AI R associated with the expression of K303R ERα mutation.

Discussion

Despite significant advances in the treatment of breast cancer following the introduction of Albas (30, 31), de novo and acquired resistance remains a major clinical concern. Here, we report that expression of the K303R ERα mutant conferred resistance to the AI anastrozole, and show that resistance occurs through a constitutive activation of the PI3K/Akt prosurvival signaling pathway to which the mutant cells have become addicted for maintenance of growth.

Breast cancer cells can adapt in response to the selective pressures exerted by exposure to estrogen deprivation therapy by developing enhanced sensitivity to the proliferative effects of low estrogen levels (32). A number of laboratories have developed in vitro models with long-term estrogen deprivation (1–5), and they propose a dynamic interplay between growth factor signaling and ERα action. Letrozole resistance involves cross-talk between the HER2 growth factor receptor pathway and ERα, leading to activation of Erk1,2/MAPK and ERα phosphorylation (6). Herein, we show that a naturally occurring mutation of ERα, termed K303R, conferred hypersensitivity to estradiol, and show that the mutant ERα in aromatase-expressing cells exhibited enhanced growth in basal conditions, as well as with AD treatment. We found that the hypersensitive phenotype associated with mutant expression resulted in the acquisition of resistance to the AI anastrozole. The growth of mutant cells was suppressed by fulvestrant treatment, indicating that ERα remained essential for growth.

There is extensive evidence to support the role of HER2 as a mediator of resistance to endocrine therapy (33), but we did not...
detect increased HER2 levels in our aromatase-overexpressing mutant cells (data not shown), highlighting the uniqueness of our resistance model. K303R ERα-expressing cells exhibited constitutive activation of the PI3K/Akt signaling pathway, and increased binding to the p85α regulatory subunit of PI3K that led to increased PI3K activity and downstream Akt phosphorylation. The increased levels of phosphorylated Akt in mutant-expressing cells were associated with a specific increase in ERα pS167, a known Akt phosphorylation site. In agreement with these data, we found that the mutant had enhanced transcriptional activation, which was inhibited by treatment with either the PI3K inhibitor LY294002, or the antiestrogen ICI182,780. This suggests that Akt may serve as a functional link between both cytoplasmic and nuclear ERα functions, resulting in an integrated bidirectional signaling that promoted and sustained the proliferation of mutant cells. Despite the extensive disruption in the genome of cancer cells (34, 35), there are examples whereby the reversal of only one or a few of these abnormalities can profoundly inhibit the cancer growth (36). PI3K/Akt inhibition drastically inhibited the growth of mutant cells suggesting an exquisite “dependency” of the mutant cells on the PI3K/Akt pathway for maintenance of AIR.

It has been reported that estrogen deprivation, antiestrogen, or AI treatment induced apoptotic cell death (28, 37). We found a lower frequency of apoptosis in AD-stimulated K303R ERα-expressing cells, suggesting an altered apoptotic response in these cells. In addition, Ana treatment induced a significant increase in cell apoptosis in WT cells, but no changes were observed in the apoptotic response of mutant-expressing cells. It has been proposed that Bcl-2/Bax protein ratios may determine whether a cell will undergo apoptosis (38). We found an increased Bcl-2/Bax ratio in the mutant cells that was further increased after AD and Ana treatments, confirming a potential survival advantage for the mutant receptor cells.

There are relatively few mutations which have been reported in the ERα gene, which is surprising because many clinical resistance mechanisms involve mutation of the target. The K303R ERα mutation was associated with poor outcomes in univariate analyses, and its presence was correlated with older age, larger tumor size, and lymph node–positive disease, all factors associated with worse outcomes (13). Four other studies did not detect the mutation in invasive cancers (39–42), but our studies suggest that the detection method used might be insensitive. However, the K303R mutation has been reported, but at low frequency in invasive breast tumors by Conway and colleagues (43).

Molecular analyses have shown that the mutated arginine at the 303 position allows ERα to be more highly phosphorylated by protein kinase A (14) and Akt kinase signaling. 6 We have

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6 Our unpublished data.
previously shown that overexpression of the K303R ERα mutation in ERα-positive MCF-7 breast cancer cells conferred estrogen hypersensitivity in vitro (12, 14), and decreased sensitivity to tamoxifen treatment when engaged in cross-talk with growth factor receptor signaling (18). Here, we provide a unique model of endocrine resistance, whereby expression of the K303R ERα mutant receptor conferred resistance to the AI anastrozole. The potential mechanisms of resistance include cellular strategies to evade apoptosis, and increased proliferation through an enhanced cross-talk between the mutant receptor and the PI3K/Akt signaling pathway.

There are two major clinical implications of our study. First, because K303R mutant cells may escape from growth inhibition when treated with AIs, genetic assays for the mutation might offer a new predictive marker for hormonal response. Second, these data support the development of treatment strategies utilizing signal transduction inhibitors, which may be used to extend the duration of sensitivity to estrogen deprivation, or to reverse resistance at its time of emergence.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Figure 6. Inhibition of PI3K/Akt pathway reversed AI". A, immunoblotting showing phosphorylated Akt and Akt in cells treated with vehicle, LY 10 μmol/L or PI-103 (1, 5, and 10 μmol/L) or Akt1/2 (1, 5, and 10 μmol/L) for 30 min. B to D, anchorage-independent growth assay in cells treated with vehicle, AD 10 nmol/L, Ana 1 μmol/L ± LY 10 μmol/L (B) or Akt1/2 1 μmol/L (C) or PD98059 (PD 10 μmol/L, D) or ICI 1 μmol/L (D). Bars, SD (***, $P < 0.0005$ LY, Akt1/2 and ICI-treated cells versus control cells and cells treated with AD or Ana + AD). E, cell death detection assay in cells treated with AD 10 nmol/L ± Ana 1 μmol/L ± LY 10 μmol/L. Columns, mean; bars, SD (*, $P < 0.05$ versus AD in MCF-7 Arom 1 and AD and Ana + AD in K303R Arom 1).


43. Conway K, Parrish E, Edmiston SN, et al. The ESR1 variant


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