Cooperative action of tamoxifen and c-Src inhibition in preventing the growth of estrogen receptor–positive human breast cancer cells

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Abstract

It has long been appreciated that estrogenic signaling contributes to breast cancer progression. c-Src is also required for a number of processes involved in tumor progression and metastasis. We have previously identified the K303R mutant estrogen receptor α (ERα) that confers hypersensitivity to low levels of estrogen. Because ERα and c-Src have been shown to interact in a number of different systems, we wanted to evaluate the role of c-Src kinase in estrogen-stimulated growth and survival of ERα-positive breast cancer cells. MCF-7 cells stably expressing the mutant receptor showed increased c-Src kinase activity and c-Src tyrosine phosphorylation when compared with wild-type ERα-expressing cells. A c-Src inhibitor, AZD0530, was used to analyze the biological effects of pharmacologically inhibiting c-Src kinase activity. MCF-7 cells showed an anchorage-dependent growth IC50 of 0.47 μmol/L, which was increased 4-fold in the presence of estrogen. In contrast, cells stably expressing the mutant ERα had an elevated IC50 that was only increased 1.4-fold by estrogen stimulation. The c-Src inhibitor effectively inhibited the anchorage-independent growth of both of these cells, and estrogen was able to reverse these effects. When cells were treated with suboptimal concentrations of c-Src inhibitor and tamoxifen, synergistic inhibition was observed, suggesting a cooperative interaction between c-Src and ERα. These data clearly show an important role for ERα and estrogen signaling in c-Src–mediated breast cancer cell growth and survival. Here, we show that c-Src inhibition is blocked by estrogen signaling; thus, the therapeutic use of c-Src inhibitors may require inhibition of ERα in estrogen-dependent breast cancer.

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Introduction

Estrogen signaling has long been known to stimulate a variety of effects, including cell growth and survival, two important mechanisms in the progression of cancer. These effects are mediated through cognate receptors of estrogen, estrogen receptors (ERα and β). Although ERα is required for normal mammary gland development (1), work with knockout mice has not shown a requirement for ERβ in mammary gland development (2). Interestingly, 70% of breast tumors express ERα (3, 4), making the estrogen pathway an effective target for a large number of clinical therapeutics.

We have previously identified an ERα A908G somatic mutation in one third of premalignant breast lesions (5), and others have found the mutation at a low frequency in invasive breast cancers (6). The resulting A908G transition leads to the substitution of the 303 lysine for an arginine (K303R; ref. 5); this lysine is an important acetylation site in ERα (7) and is involved in regulation of the S305 protein kinase A phosphorylation site (8). Furthermore, this mutated receptor has reduced binding to corepressors and increased binding to coactivators, which may play a role in its hypersensitivity to estrogen signaling (5). Although the K303R-ERα mutation is one of many receptor variants that have been identified (for a complete review, see ref. 9), it is the only somatic mutation that has been identified in a large number of patient samples.

The nonreceptor tyrosine kinase c-Src has been implicated in a number of different cancers, including breast, colon, and pancreatic cancers (for a complete review, see ref. 10). A number of different groups have shown that c-Src protein is up-regulated in approximately half of breast cancers versus normal mammary epithelium (11, 12). Additionally, transgenic mice expressing a mouse mammary tumor virus v-Src develop mammary hyperplasias (13), whereas knocking out the c-Src gene delays mammary tumorigenesis of polyoma middle T antigen–induced tumors (14). In contrast, knockout of the related c-Yes proto-oncogene did not affect mammary tumorigenesis in this model (14). Collectively, these data show an important and specific role for c-Src in mammary tumor development.

In 1993, Miggliaccio et al. (15) showed that estrogen stimulation of MCF-7 breast cancer cells led to an immediate tyrosine phosphorylation and activation of the c-Src kinase. It was later shown that estrogen stimulation can activate a number of other classic signal transduction pathways.
pathways, such as phosphatidylinositol 3-kinase and extracellular signal-regulated kinase 1/2 (16, 17, and that ligand-induced ERα activation recruited a c-Src/p85/ERα complex (16, 18). In addition to ERα signaling to c-Src, c-Src can phosphorylate ERα on tyrosine 537 (19). Because of the two-way crosstalk between these two proteins, the aberrant activation of either protein could lead to feedback loops that could significantly influence cellular signaling.

Estrogen signaling stimulates growth and survival in ERα-positive breast cancer cells, and these mechanisms may involve the non-receptor tyrosine kinase c-Src. We thus sought to determine if blockade of c-Src kinase activity could inhibit estrogen-stimulated growth and survival of ERα-positive breast cancer cells. AZD0530 is a novel, potent, and highly selective Src/Abl kinase inhibitor (20). Here, we show that MCF-7 breast cancer cells respond to c-Src inhibition with reduced anchorage-dependent growth and reduced ability to form colonies in soft agar. Additionally, in the presence of estrogen, cells are less sensitive to c-Src inhibition. We also show that cells expressing the hypersensitive K303R-ERα mutation have elevated c-Src kinase activity and are less sensitive to c-Src inhibition both in the presence and absence of estrogen when compared with cells expressing only the wild-type (WT) ERα. When suboptimal concentrations of AZD0530 and tamoxifen were tested, synergistic inhibition of anchorage-independent growth was observed. Collectively, these data show that inhibition of c-Src may be an effective therapeutic strategy for ERα-positive breast cancer; however, estrogen signaling may alter the ability of c-Src inhibitors to block cell growth.

Materials and Methods

Chemicals

The c-Src inhibitor AZD0530 was provided by AstraZeneca and dissolved in DMSO (20). AZD0530 was diluted in tissue culture medium, and the corresponding DMSO controls had no effect on the analyses done. G418 and all cell culture reagents were purchased from Life Technologies (Grand Island, NY). Tamoxifen (4-hydroxytamoxifen) was purchased from Sigma (St. Louis, MO) and the stock solution was dissolved in ethanol at 10^-8 mol/L.

Cell Culture Conditions

MCF-7 breast cancer cells were maintained on plastic in MEM supplemented with 10% fetal bovine serum (FBS), 0.1 mmol/L nonessential amino acids, 2 mmol/L/LL-glutamine, 50 units/mL penicillin/streptomycin (Life Technologies), at 37°C with 5% CO2/95% air. Unless otherwise noted, cells were passaged and removed from flasks when 70% to 80% confluent. For cell passage, cells were rinsed with PBS and trypsinized in 0.05% trypsin and 0.02% EDTA for 2 min at 37°C. Trypsin activity was quenched with the addition of medium containing 10% FBS.

Generation of Stable WT ERα and K303R-ERα–Expressing Cells

Generation of the yellow-fluorescent protein (YFP)-tagged expression constructs, YFP-WT ERα and YFP-K303R-ERα, has been previously described (8). MCF-7 cells were stably transfected using Fugene according to the manufacturer’s instructions (Roche, Indianapolis, IN), and individual clones were isolated and expanded with G418 selection. Stably transfected clones were screened for expression of the exogenous ERα by immunoblot analysis. One clone stably expressing YFP-WT ERα and one clone stably expressing YFP-K303R-ERα with similar levels of the exogenous YFP-ERα and endogenous ERα were chosen for further analysis.

Immunoprecipitation and Immunoblot Analysis

Cells were plated in six-well dishes for immunoblot analysis (3 × 10^5 per plate) or in 10-cm plates for immunoprecipitation (3 × 10^6 per plate) in MEM supplemented with 10% FBS. Cells were then incubated overnight at 37°C in 5% CO2, followed by 48 h starvation in phenol red–free MEM with 5% charcoal-stripped FBS. Moreover, cells were pretreated with varying concentrations of AZD0530 for 30 min and 10^-9 mol/L estrogen for 10 min as indicated before lysis (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2% NP40, 0.25% deoxycholic acid, 1 mmol/L EDTA, 1 mmol/L Na2VO4, and 1:200 protease inhibitor cocktail tablet; Roche). c-Src immunoprecipitation was done using 300 μg of total cellular protein with 1 μg anti-Src antisera (clone 327, Oncogene Research Products, Cambridge, MA) and protein A/G (Oncogene Research Products) with rotation at 4°C for 3 h. For coimmunoprecipitation experiments, we used 2 μg of total cellular protein and 2 μg of anti-Src antisera (clone 327) or 2 μg of anti-ERα (H-184, Santa Cruz Biotechnology; San Diego, CA) and protein A/G with rotation at 4°C for 2 h. Immunoprecipitated proteins were washed thrice with lysis buffer.

Proteins, either immunoprecipitated or whole-cell lysate, were heated in Laemmli’s sample buffer for 5 min, separated by 7.5% SDS-PAGE, transferred to nitrocellulose membrane (Whatman, Inc., Sanford ME), and probed with the following antisera, as indicated: anti-Src antisera (clone GD11, 1:1,000) and anti-phosphotyrosine antisera (4G10, 1:5,000) from Upstate Biotechnology Inc. (Lake Placid, NY); anti–phospho-Fak antisera (pY861, 1:1,000) and anti–phospho-c-Src antisera (pY418, 1:1,000) from Biosource (Camarillo, CA); anti-Fak antisera (clone 77, 1:1,000) from BD Biosciences (San Jose, CA); anti-tubulin antisera (clone D66, 1:1,000) from Sigma-Aldrich (St. Louis, MO); and anti-ERα (clone 6F1, 1:1,000) from SantaCruz Biotechnology (Santa Cruz, CA) with rotation at 4°C for 2 h. Immunoprecipitated proteins were visualized with ECL chemiluminescence (Piscataway, NJ). Antibody binding was quantified in the linear range of the film by scanning the image using a Canon LicoScan scanner and analyzing with the Scion Image...
software program (Scion Corp., Frederick, MD). Each sample measured was calculated as the ratio of the average area of the phosphorylated protein over the average area of the respective total protein levels.

**Cell Proliferation as Measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Analysis**

Cells were serum-starved for 48 h in phenol red–free MEM with 5% charcoal-stripped FBS before plating. Cells (3,000 per well) were plated into each well of a 96-well plate and allowed to adhere for 8 h before the addition of increasing concentrations of AZD0530 and estrogen (10⁻⁹ mol/L). Seventy-two hours later, 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; thiazolyl blue) was added to each well and incubated at 37°C and 5% CO₂ for 2 h followed by medium removal and solubilization in 100 μL DMSO. The resulting color change was read at 570 nm and calculated as absorbance above background. Each experiment contained 12 different doses of AZD0530 in quadruplicate. A minimum of three experiments was combined for IC₅₀ calculations. The absorbance readings were used to determine the IC₅₀ using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). Briefly, values were log-transformed, then normalized, and nonlinear regression analysis was used to generate a sigmoidal dose-response curve to calculate IC₅₀ values for each cell line.

**Agarose Colony-Forming Assay**

Cells (5,000 per well) were plated in 1.5 mL of 0.3% agarose, 5% charcoal-stripped FBS in phenol red-free MEM, with a 0.6% agarose base in six-well plates, with various concentrations, as indicated, of AZD0530, tamoxifen, and estrogen. Cells were then incubated at 37°C and 5% CO₂ with fresh medium added every 3 days. After 14 days, 150 μL of MTT were added to each well and left to incubate at 37°C for 4 h. Plates were then placed in 4°C overnight and colonies >50 μm in diameter were quantitated.

**Statistical Analysis Tests for Synergy**

To determine if suboptimal concentrations of tamoxifen and AZD0530 synergistically inhibit cell growth, a model-free test for synergy was used based on two simultaneous one-sided t tests comparing each of the single-agent doses of AZD0530 (1 μmol/L) and tamoxifen (10⁻⁸ mol/L) with a combined regimen of the two drugs at suboptimal doses (21). Additionally, dose levels of AZD0530 (0, 0.1, and 1 μmol/L) and tamoxifen (0, 10⁻⁹, and 10⁻⁸ mol/L) were used in an ANOVA model to assess the effect of AZD0530 or tamoxifen alone and the interaction between the two drugs.

**c-Src Immunocomplex Kinase Assay**

Cells (3 × 10⁶) were plated in 10-cm plates with MEM supplemented with 10% FBS and incubated for 24 h at 37°C and 5% CO₂. Cells were then serum-starved for 48 h in phenol red–free MEM supplemented with 5% charcoal-stripped FBS followed by treatment with 10⁻⁹ mol/L estrogen for 10 min before lysis in 50 mmol/L HEPES (pH 7.0), 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10 mmol/L NaPPI, 1% glycerol (v/v), 0.1% Triton X-100 (v/v), 1 mmol/L Na₂VO₄, and 1:200 protease inhibitor cocktail tablet (22). Immunocomplex kinase assays were done on c-Src immunoprecipitated from 500 μg of total cellular protein with 1 μg anti-Src antisera (clone 327) and 50 μL of 10% (w/v) Pansorbin cells (Calbiochem, San Diego, CA) with rotation at 4°C for 2 h. Immunoprecipitated pellets were washed thrice with immunocomplex kinase assay wash buffer [0.1% Triton X-100, 150 mmol/L NaCl, and 10 mmol/L NaPO₄ (pH 7.0); ref. 22]. Ten microliters of 100 μg/mL acid–denatured rabbit muscle enolase (Sigma) was added as an exogenous phosphorylation substrate before the addition of kinase reaction buffer containing 20 mmol/L HEPES (pH 7.0), 6 mmol/L magnesium chloride, 20 mmol/L sodium orthovanadate, and 10 μCi per reaction of [γ-³²P]ATP (3,000 Ci/mmol; NEN, Boston, MA). Reactions were initiated by addition of 50 μL of kinase reaction buffer and allowed to proceed for 10 min at 23°C. The reaction was stopped by the addition of 3× Laemmli’s sample buffer. For analysis of c-Src after kinase assay, samples were subjected to 8% SDS-PAGE followed by 1 h fixation in methanol/water/acytic acid (5:5:1). The gel was dried and radioactive bands were detected by autoradiography and quantitated on a Bio-Rad Personal FX PhosphorImager (Bio-Rad, Hercules, CA).

**Results**

**c-Src Binds Similar Amounts of the K303R Mutant ERα and WT ERα**

Previous reports have shown that c-Src and the ERα interact in a protein complex (16, 18). Therefore, we first sought to determine if the K303R-ERα mutation alters the ability of ERα to bind with c-Src. MCF-7 cells were treated with or without 10⁻⁹ mol/L estrogen for 10 min and then lysed. ERα (Fig. 1A and B, lanes 1 and 2) or c-Src (Fig. 1A and B, lanes 3 and 4) was subjected to immunoprecipitation followed by immunoblot for c-Src (Fig. 1A) and ERα (Fig. 1B). In the absence of estrogen, ERα and c-Src are in a protein complex (Fig. 1A and B, lanes 1 and 3). Treatment with estrogen did not increase the amount of protein in the complex (Fig. 1A and B, lanes 2 and 4). Similar amounts of starting protein are shown in the whole-cell lysates in Fig. 1A and B (lanes 5 and 6). These data show that ERα and c-Src are in a hormone-independent complex that was not altered by short-term treatment with estrogen. Figure 1C compares cells stably expressing YFP-WT ERα or YFP-K303R-ERα vector. Shown in Fig. 1C (top) is the immunoprecipitation with anti-ERα antisera followed by immunoblot for c-Src (lanes 1–4) compared with whole-cell lysate input (lanes 5–8). Basal levels of c-Src and ERα binding were similar in WT ERα–expressing cells when compared with K303R-ERα–expressing cells (Fig. 1C; lane 1 versus lane 3). Additionally, short-term estrogen treatment did not alter the binding of either WT ERα or K303R-ERα with c-Src (Fig. 1C; lane 2 versus lane 4). The bottom panel shows that similar amounts of ERα protein were immunoprecipitated under all conditions tested. Cells expressing the YFP-ERα express a 66 kDa (endogenous ERα) and no...
c-Src Kinase Activity Is Elevated in K303R-ERα–Expressing Cells

To determine if cells expressing WT ERα or the mutant ERα have similar levels of c-Src kinase activity, we next analyzed the c-Src kinase activity and tyrosine phosphorylation of the c-Src 416 residue. A c-Src–specific in vitro immunocomplex kinase assay was done as previously described (22) and a representative assay is shown in Fig. 2A. In the absence of estrogen, WT ERα–expressing cells showed a low basal level of c-Src kinase activity as shown by phosphorylation of the exogenous substrate enolase (Fig. 2A, lane 1). When cells were treated with estrogen for 10 min, kinase activity was increased by 15% (Fig. 2A, lane 2). In contrast, K303R-ERα–expressing cells showed an elevated basal level of c-Src activity that was 30% higher (Fig. 2A, lane 3) when compared with WT ERα–expressing cells. This elevated kinase activity was not further increased by estrogen treatment. To confirm the elevated c-Src kinase activity in K303R-ERα versus WT ERα–expressing cells, c-Src was immunoprecipitated and the levels of pY416 and total pY content of c-Src was examined using immunoblot (Fig. 2B). Treatment with estrogen resulted in a 1.7-fold increase in phosphorylation at Y416, and a 1.7-fold increase in total tyrosine phosphorylation of c-Src in WT ERα–expressing cells (Fig. 2B; lane 1 versus lane 2). Consistent with its observed increased basal kinase activity, K303R-ERα–expressing cells exhibited a 1.4-fold increase in basal c-Src pY416 and a 1.7-fold increase in total phosphorylation (Fig. 2B, lane 3). The elevated basal c-Src phosphorylation in mutant cells was not further increased by estrogen treatment (Fig. 2B; lane 3 versus lane 4). Thus, whereas WT ERα–expressing cells increased c-Src kinase activity following estrogen treatment, K303R-ERα–expressing cells showed constitutive c-Src kinase activity and phosphorylation.

Inhibition of c-Src Tyrosine Phosphorylation

The pharmacologic inhibitor of c-Src, AZD0530 (20), was used to inhibit c-Src phosphorylation and activity. To determine an effective dose of the c-Src inhibitor, MCF-7 cells were treated with increasing amounts of AZD0530 for 30 min because we wanted to analyze short-term signaling events associated with c-Src inhibition. Whole-cell lysates were analyzed for phosphorylation of Y416 of c-Src and total c-Src. In the absence of estrogen, AZD0530 caused a 30% reduction of phosphorylation at Y416 of c-Src versus controls even at concentrations as low as 10 nmol/L (Fig. 3A; lane 1 versus lane 2). Higher doses of

Figure 1. c-Src binds similar amounts of WT and K303R-ERα. A and B, MCF-7 cells were serum-starved, then treated with or without 10^{-9} mol/L estrogen (E2) for 10 min before lysis. ERα (lanes 1 and 2) or c-Src (lanes 3 and 4) was immunoprecipitated (IP) and immunoblotted (IB) for c-Src (A) or ERα (B). One representative experiment of three. C, MCF-7 cells stably overexpressing WT ERα (YFP-WT ERα vector) or the mutant K303R-ERα (YFP-K303R-ERα vector) were serum-starved, then treated with or without 10^{-9} mol/L estrogen for 10 min before lysis. Immunoprecipitated ERα (lanes 1–4) and whole-cell lysates (lanes 5–8) that have been immunoblotted for c-Src (top) and ERα (bottom). One representative experiment of two.

~96 kDa ERα representing the exogenously added YFP-ERα as shown in Fig. 1C (bottom). These results confirm that ERα forms a complex with c-Src that is independent of hormone treatment and that the K303R-ERα mutation does not seem to alter this complex.

c-Src Kinase Activity Is Elevated in K303R-ERα–Expressing Cells

Figure 2. c-Src kinase activity and phosphorylation in WT and K303R-ERα–expressing MCF-7 cells. WT ERα or K303R-ERα–expressing cells were serum-starved, then treated with or without 10^{-9} mol/L estrogen for 10 min before analysis. A, c-Src was immunoprecipitated and an immunocomplex kinase assay was done to analyze c-Src–induced phosphorylation of the exogenous substrate enolase. Quantitation of 32P-labeled enolase was done on a Bio-Rad Personal FX PhosphorImager. One representative experiment of three. B, c-Src was immunoprecipitated and immunoblotted for pY416, total tyrosine phosphorylation, and c-Src. Numbers, fold change relative to control (lane 1) as described in Materials and Methods. One representative experiment of two.
AZD0530 (μM) 0 10−9 E2 10−7 E2 10−5 E2 10−3 E2 0 10−9 E2 10−7 E2 10−5 E2 10−3 E2
pY416 Src
Total-Src
pYFak
Fak
Tubulin

K303R-ERα–expressing cells showed constitutively activated c-Src kinase; thus, we wanted to determine if increased c-Src inhibitor would be required to block c-Src signaling in WT ERα versus K303R-ERα–expressing cells. WT ERα– and WT ERα–expressing cells were pretreated with 0.01 or 0.1 μmol/L of AZD0530 for 30 min followed by 10 min of estrogen treatment. Cells were lysed, separated by SDS-PAGE, and whole-cell lysates were analyzed by immunoblot. Phosphorylation of c-Src at Y416 was partially blocked with 0.01 μmol/L and completely inhibited with 0.1 μmol/L AZD0530 (Fig. 3B, lanes 1 versus lanes 2 and 3). Even with estrogen treatment, 0.1 μmol/L of the inhibitor blocked 40% of the c-Src phosphorylation (Fig. 3B, lanes 4 versus lanes 5 and 6). K303R-ERα–expressing cells also showed reduced c-Src pY416 in the presence of AZD0530 (Fig. 3B, lanes 7 versus lanes 8 and 9) that was not affected by estrogen treatment (Fig. 3B, lanes 10 versus lanes 11 and 12). The estrogen-induced increase in phosphorylation of c-Src shown by immunoblot of whole-cell lysates confirms the phosphorylation increase shown by immunoprecipitation and immunoblot analysis in Fig. 2B. Immunoblot of whole-cell lysates allowed us to correlate downstream signaling of c-Src kinase with c-Src tyrosine phosphorylation. Immunoblot of Fak showed reduced tyrosine phosphorylation following treatment with AZD0530 (Fig. 3B) and correlated with reduced pY416 levels showing reduced c-Src kinase activity in WT ERα– and K303R-ERα–expressing cells. These data show that c-Src inhibitors can effectively block constitutive c-Src phosphorylation and kinase activity in WT and mutant ERα–expressing breast cancer cells.

**c-Src Inhibition of Anchorage-Dependent Growth**

It is well known that estrogen treatment stimulates mitogenesis and cell growth in ERα–positive breast cancer cells. Because estrogen did not alter the ability of AZD0530 to block c-Src phosphorylation, we sought to examine the effectiveness of c-Src on estrogen-stimulated cell growth. We first tested the ability of AZD0530 to block c-Src phosphorylation, we sought to examine the effectiveness of c-Src on estrogen-stimulated cell growth. We first tested the ability of AZD0530 to block anchorage-dependent growth. The IC50 values of multiple cell lines treated with estrogen are shown in Table 1. All cells showed a dose-dependent growth inhibition induced by AZD0530 (data not shown). MCF-7 cells exhibited an IC50 of 0.47 μmol/L, and the IC50 was significantly increased 4.5-fold (P < 0.001, t test) in the presence of 10−9 mol/L estrogen (Table 1). Additionally, WT ERα–expressing cells also showed an IC50 of 0.45 μmol/L, which was not statistically significant compared with the parental MCF-7 cells (P > 0.1, t test). This IC50 was increased 4-fold in the presence of estrogen (P < 0.001, t test). Thus, estrogen signaling results in the requirement for higher concentrations of the c-Src inhibitor to block cell growth. K303R-ERα–expressing cells exhibited a higher basal IC50 value that was significantly higher than MCF-7 or WT ERα–expressing cells (P < 0.01, t test). The addition of estrogen to mutant expressing cells increased the IC50 value by only 1.4-fold (Table 1). Thus, when cells are expressing the mutant ERα, and hence have increased basal c-Src kinase activity, higher levels of c-Src inhibitor were required to
reduce anchorage-dependent growth. In the ERα-negative MCF-7 subline, C4-12-5 (23), estrogen did not affect the concentration of inhibitor required for growth blockade ($P = 0.45$, t test; Table 1). This result shows that estrogen signaling through the ERα is required to increase the amount of AZD0530 to effect cell growth. Higher concentrations of AZD0530 may be needed to affect the K303R-ERα mutant. These results predict that the presence of estrogen in ERα-positive cells or expression of the K303R-ERα mutant may alter the efficacy of c-Src inhibitors.

**Estrogen Treatment Reduces the Ability of AZD0530 to Inhibit Anchorage-Independent Growth**

Increased signaling through ERα decreases the ability of the AZD0530 c-Src inhibitor to reduce anchorage-dependent growth, and c-Src inhibitors have previously been shown to reduce HER2 and c-Met–mediated anchorage-independent growth (24, 25). We next wanted to determine whether inhibition of c-Src would also reduce estrogen-induced, anchorage-independent growth. Cells were grown in 0.3% soft agar with varying concentrations of AZD0530 added as described in Materials and Methods. Under basal conditions, estrogen treatment stimulated a 1.5-fold increase in the ability of MCF-7 cells to form colonies in soft agar ($P < 0.01$, t test; Fig. 4A). Inhibition of c-Src kinase activity caused a dose-dependent reduction in soft-agar colony formation as shown in Fig. 4A. Although 1 μm was effective at reducing both basal and estrogen-stimulated colony formation ($P < 0.01$, t test), 3 μm of AZD0530 completely abolished the ability of MCF-7 cells to form colonies in soft agar ($P < 0.001$, t test; Fig. 4A). Thus, in the presence of estrogen, more AZD0530 was required to block soft-agar colony-forming ability.

WT ERα− or K303R-ERα-expressing cells were also tested for their ability to form colonies in soft agar (Fig. 4B). In the absence of AZD0530, basal growth was not statistically different between WT ERα− and K303R-ERα−-expressing cells. Estrogen stimulated a 2.3-fold increase in colony formation in WT ERα cells ($P < 0.01$, t test), but a 4-fold increase in K303R-ERα−-expressing cells ($P < 0.01$, t test; Fig. 4). Similar to MCF-7 cells, both WT ERα− and K303R-ERα−-expressing cells showed a dose-dependent reduction in soft-agar colony formation following c-Src inhibition (Fig. 4B), and the presence of estrogen required more inhibitor to block soft-agar colony formation. These data show that c-Src inhibition blocked anchorage-independent growth of breast cancer cells and that more drug was required to block cell growth in the presence of estrogen in K303R-ERα−-expressing breast cancer cells.

Recently, Hiscox et al. (26) reported that AZD0530 blocked invasion and migration of tamoxifen-resistant MCF-7 cells. Therefore, we next determined if c-Src inhibition combined with tamoxifen could synergistically inhibit the anchorage-independent growth of MCF-7 cells. Figure 5 (columns 1–5) again shows a dose-dependent reduction in soft-agar colony-forming ability, confirming our previous results. Additionally, tamoxifen treatment also showed a dose-dependent reduction in soft-agar colony-forming ability (Fig. 5, columns 6–8). Because 3 μmol/L AZD0530 and 10−7 mol/L tamoxifen were each >90% effective at inhibiting the anchorage-independent growth of MCF-7 cells, we next tested suboptimal doses of these drugs in combination to determine if additive or

### Table 1. IC50 of AZD0530 for various breast cancer cell lines on anchorage-dependent growth

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<th>Cell Line</th>
<th>Control</th>
<th>+E2</th>
<th>E2</th>
<th>IC50 (μmol/L)</th>
<th>95% confidence interval</th>
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<td>MCF7</td>
<td>0.47</td>
<td>2.1</td>
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<td>0.62 – 0.91</td>
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<td>1.82</td>
<td>+E2</td>
<td>1.1 – 1.5</td>
<td>1.6 – 2</td>
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<tr>
<td>C4-12-5</td>
<td>0.75</td>
<td>0.61</td>
<td>+E2</td>
<td>0.62 – 0.91</td>
<td>0.48 – 0.76</td>
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**Abbreviation:** E2, estrogen.

*Each experiment contained 12 different doses of AZD0530 in quadruplicate and a minimum of three experiments were used to calculate the IC50 values.

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synergistic effects could be achieved. Growing cells in the presence of $10^{-8}$ mol/L tamoxifen almost completely inhibited basal colony formation but not estrogen-induced colony formation (Fig. 5, column set 7). Tamoxifen ($10^{-9}$ mol/L) alone only inhibited 34% of basal growth, and 24% of estrogen-induced growth (Fig. 5). Low concentrations (0.1 and 1 µmol/L) of AZD0530 alone gave 36% and 70% inhibition, respectively, when used alone (Figs. 4A and 5), and these doses of AZD0530 completely inhibited basal colony formation in combination with low doses ($10^{-8}$ or $10^{-9}$ mol/L) of tamoxifen (Fig. 5, columns 9–12).

A model-free test for synergy was used based on two simultaneous one-sided t tests comparing each of the single-agent doses of AZD0530 and tamoxifen with a combined regimen of the two drugs at suboptimal doses (21). Specifically, the comparison between AZD0530 at 1 µmol/L and AZD0530 at 0.1 µmol/L plus tamoxifen ($10^{-9}$ mol/L) was statistically significant ($P < 0.0001$). Likewise, the second comparison between tamoxifen at $10^{-8}$ mol/L was compared with AZD0530 at 0.1 µmol/L plus tamoxifen at $10^{-9}$ mol/L was also found to be statistically significant ($P = 0.022$), indicating synergistic effects between the two drugs. Finally, estimates of the differences between specific drug combinations versus control groups were calculated based on an ANOVA model with interaction terms between the two drugs. The mean difference in the number of colonies between AZD0530 at 0.1 µmol/L plus tamoxifen at $10^{-9}$ mol/L versus the control group was 139.3 (95% confidence interval, 124.1–154.5, $P < 0.0001$). This effect was more than the combined effect of either drug alone compared with the control group. However, when cells were treated with estrogen, the interactions were not significant. Thus, both AZD0530 and tamoxifen were effective at reducing soft-agar colony formation; but here we show that combinations of these inhibitors were more effective than either inhibitor alone and estrogen was able to block this effect.

**Figure 5.** Synergistic cell growth inhibition with AZD0530 and tamoxifen. MCF-7 cells (5,000 per well) were seeded in 0.3% agarose with increasing concentrations of AZD0530 or tamoxifen (Tam) as controls, or suboptimal concentrations of AZD0530 plus tamoxifen with (black columns) or without (white columns) $10^{-9}$ mol/L estrogen. Cells were allowed to grow for 14 d and the number of colonies >50 μm were quantitated and graphed. This experiment was repeated twice with similar results.

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**Discussion**

Estrogen-induced activation of ERα has been shown to activate the c-Src signaling pathway (17). ERα and c-Src bind in a protein complex that has been proposed to involve other proteins such as modulator of nongenomic activity of ER and phosphatidylinositol 3-kinase (16, 27, 28). Our studies showed hormone-independent interactions between ERα and c-Src that were not altered by estrogen or the K303R-ERα mutation. Although their interaction did not change, K303R-ERα–expressing cells showed a 30% increase in basal c-Src kinase activity, which was correlated with increased phosphorylation of c-Src. We have previously reported that the K303R-ERα mutant is hypersensitive to very low levels of estrogen (5), and here we showed that expression of the mutant ERα led to higher basal c-Src kinase activity in breast cancer cells. Thus, although this mutation in ERα did not seem to alter the ability of the receptor to bind with c-Src, as has been shown for the TIF-2 coactivator (5), it did alter the ability of the receptor to phosphorylate and activate c-Src.

Although c-Src activity was elevated, this increase was very low, and therefore its effects on the cell could be minimal; thus, a c-Src inhibitor was used to address this question. Anchorage-dependent growth analysis showed that estrogen-stimulated growth required higher concentrations of AZD0530 to achieve the same inhibitory effects seen in the nonstimulated systems. This result was also confirmed in anchorage-independent growth assays. From the anchorage-dependent and anchorage-independent growth data, one would have expected that estrogen stimulation would increase c-Src phosphorylation as it did under basal conditions. However, in our model system, estrogen did not affect inhibitor blockade of c-Src phosphorylation. Estrogen signaling has been shown to activate a number of prosurvival pathways, including Bcl-2 (29), caspase regulation (30), and calcium homeostasis (31) to name a few. Estrogen signaling may be activating alternative survival and growth pathways that can bypass the requirement for c-Src activation, hence an estrogen-induced survival effect. Cells expressing the mutant receptor had an elevated basal IC$_{50}$ thus, more drug may be required to block the basal in vitro growth rate of K303R-ERα–expressing cells. Additionally, estrogen only stimulates a <1.5-fold increase in the IC$_{50}$ required to inhibit the K303R-ERα–expressing cells. Although the moderately increased c-Src activity in K303R-ERα–expressing cells was enough to alter the basal biology of the cells in serum-free conditions as shown by the growth assays, it may also raise the basal level of signaling to a threshold level, thereby serving to “prime” the cell for other activating stimuli such as estrogen or growth factor signaling. One such example has been shown in PC12 cells in which chemotactic migration is not stimulated until a threshold level of extracellular signal-regulated kinase 1/2 signaling has been reached (32). Thus, the increased c-Src activity
observed in the K303R-ERα-expressing cells may not have crossed the threshold level required to cause other biological effects, but it may be at the threshold and thus primed for a stimulus.

It has recently been shown that tamoxifen-resistant MCF-7 cells were more sensitive to AZD0530 than their nonresistant counterparts (26). We therefore tested the effect of combining suboptimal doses of tamoxifen and AZD0530 in soft-agar growth assays. Our data showed that the addition of AZD0530 made the cells more sensitive to growth inhibition by tamoxifen. Hiscox et al. (26) also showed that in tamoxifen-resistant epidermal growth factor receptor overexpressing MCF-7 cells, the combination of AZD0530 and the epithelial growth factor receptor inhibitor gefitinib blocked migration and invasion in an additive manner. A number of studies have shown that inhibiting c-Src activity in breast cancer cells blocks estrogen-induced cell cycle progression and mitogenesis (33, 34). Additionally, c-Src inhibitors have also been shown to block estrogen-induced migration in endometrial cells (35) and disrupt adherans junctions in endothelial cells (36). Thus, multiple biological phenotypes required for tumor progression may be converging and signaling through c-Src. It remains to be determined if c-Src inhibitors can inhibit or reverse tamoxifen resistance in vivo. Although these data support investigating the potential therapeutic benefit of c-Src kinase inhibition in women with ERα-positive breast cancer, a rational approach evaluating the c-Src activity, ERα levels, and perhaps the K303R mutation status is warranted. Although the influence of circulating estrogen still requires further elucidation, the data suggest that strategies combining drugs that lower estrogen signaling with a c-Src kinase inhibitor could provide a rational approach to treating these patients.

References


2. Couse JF, Lindzy J, Grandien K, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology 1997;138:4613–21.


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