Abstract  Purpose: Antiestrogens are used to treat estrogen receptor (ER)-α-positive breast cancers and cause a p27-dependent G1 arrest. Estrogen-bound ER recruits Src to mediate proteolysis of p27 and drive cell proliferation. Here, we tested the antitumor efficacy of combined Src and aromatase inhibition for ER-positive breast cancer.

Experimental Design: Antiproliferative effects of the aromatase inhibitor, anastrozole, and Src inhibitor, AZD0530, alone or in combination were tested in vitro and in vivo on aromatase-transfected MCF-7Arom5 xenografts. Xenografts were analyzed by immunohistochemistry and proteomic analysis to identify potential biomarkers of drug response and resistance.

Results: AZD0530 and anastrozole together increased p27 and caused greater G1 cell cycle arrest than either drug alone. AZD0530 monotherapy initially retarded xenograft growth in vivo, but drug resistance rapidly emerged. Combined anastrozole/AZD0530 reduced drug resistance and showed greater antitumor efficacy in vivo with greater Src and epidermal growth factor receptor inhibition and a greater increase in p27 and reduction of Ki-67 than either drug alone, supporting further evaluation of these putative predictors of response to combined Src/aromatase inhibition in vivo. Anastrozole alone stimulated Src activity both in vitro and in vivo. AZD0530-resistant tumors showed activation of bypass pathways including MEK and phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin, raising the possibility that MEK, mammalian target of rapamycin (mTOR), or PI3K inhibitors may augment Src inhibitor efficacy.

Conclusions: These data support clinical investigation of anastrozole-AZD0530 therapy for postmenopausal ER-positive breast cancer. Loss of p27 and increased Ki-67 may predict response and further clinical studies should evaluate for activation of bypass pathways including MEK and PI3K pathways during Src inhibitor therapy.
Src activation, with 40% at high levels (4). In addition, a gene expression signature of Src activation is seen in up to 40% of human breast cancers and is predictive of response to Src inhibitor drugs in vitro (5).

Src is activated in breast cancers via the ErbB family and insulin-like growth factor-I receptor pathways and other cell surface receptors (3, 6). In addition, estrogen binding to the ER leads to rapid, transient Src activation, formation of Src/Shc/ER and Src/p85/ER complexes, and signaling via phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) 1/2 pathways in drug-resistant tumors. MEK or phosphatidylinositol 3-kinase inhibitors together with AZD0530 may have therapeutic potential in breast cancer. AZD0530/anastrozole showed greater antitumor efficacy, raising intratumoral p27 and lowering Ki-67 and pSrc more than either drug alone. Combined aromatase and Src inhibition may delay or prevent emergence of resistance to either drug alone and our preclinical data support initiation of clinical trials to test this in human breast cancer.

Translational Relevance

Although aromatase inhibitors are used to treat postmenopausal estrogen receptor-positive breast cancer, resistance limits treatment efficacy. The cell cycle inhibitor p27 is a key mediator of the antiproliferative effects of antiestrogens. The Src kinase phosphorylates p27 to trigger p27 proteolysis. Because estrogens activate Src to drive p27 proteolysis, we reasoned that combined Src and aromatase inhibitor therapy might have greater efficacy than either drug alone. Xenografts rapidly developed resistance to AZD0530 alone, with strong activation of both MEK and phosphatidylinositol 3-kinase pathways in drug-resistant tumors. MEK or phosphatidylinositol 3-kinase inhibitors together with AZD0530 may have therapeutic potential in breast cancer. AZD0530/anastrozole showed greater antitumor efficacy, raising intratumoral p27 and lowering Ki-67 and pSrc more than either drug alone. Combined aromatase and Src inhibition may delay or prevent emergence of resistance to either drug alone and our preclinical data support initiation of clinical trials to test this in human breast cancer.

Materials and Methods

Generation of MCF-7Arom5. Full-length human placental aromatase cDNA (17) was introduced into pcDNA3.0 and transfected into MCF-7. Clones were selected and screened for aromatase expression. The MCF-7Arom5 line was used for all studies.

Chemicals. The c-Src inhibitor, AZD0530, and the aromatase inhibitor, anastrozole, were provided by Astra Zeneca, dissolved in DMSO and ethanol, respectively, and diluted in tissue culture medium. Vehicle controls had no effect on analyses done.

Cell culture. MCF-7Arom5 and MCF-Ca were cultured in estrogen-deprived medium (with 5% charcoal-stripped fetal bovine serum) with or without 25 μmol/L 4-androstenedione and treated with different concentrations of aromatase, 1 μmol/L AZD0530, or both. AZD0530 did not exceed 1 μmol/L to avoid off target effects.

Aromatase is rapidly metabolized in tissue culture and concentrations required for growth inhibition are considerably higher than the 137 nmol/L trough therapeutic plasma concentration in humans. Growth-inhibitory aromatase concentrations vary with aromatase levels, which differ by up to 20-fold between breast cancer lines (19). Prior work showed that whereas 0.1 μmol/L letrozole arrests aromatase-transfected MCF-Ca, 0.1 μmol/L anastrozole had no cell cycle effect (20). Cell cycle inhibition has been observed with 25 to 3,500 μmol/L aromatase in other lines and 50 μmol/L caused only 50% inhibition of aromatase activity in other studies (19, 21).

Aromatase levels were 5-fold higher in MCF-7Arom5 than MCF-Ca. Aromatase concentrations required for growth arrest of MCF-7Arom5 were titrated. Anastrozole 12.5 to 1,000 μmol/L caused increasing degrees of reversible G1 cell cycle arrest with no apparent toxicity. Subsequent investigations used 100 μmol/L aromatase because it caused partial G1 arrest and permitted detection of further cell cycle inhibition when combined with AZD0530. Asynchronously proliferating untreated and drug-treated cells were collected 48 h later for flow cytometry and Western blotting.

Flow cytometric analysis. Cells were pulse labeled with 10 μmol/L bromodeoxyuridine and stained with anti-bromodeoxyuridine-conjugated FITC (Becton Dickinson) and propidium iodide as described (12).

Growth inhibition of MCF-7Arom5 tumor xenografts. Female ovariectomized athymic BALB/c nude mice, 4 to 6 weeks old (National Cancer Institute), were housed in a pathogen-free environment under controlled conditions and received food and water ad libitum. Animals were acclimatized 3 days after shipment before tumor inoculation (18, 22). Subconfluent MCF-7Arom5 cells were resuspended in Matrigel (10 mg/mL; Becton Dickinson) at 2.5 × 10⁶ cells/mL. Each animal was injected with 0.1 mL cell suspension into one mammary fat pad. Median tumor size at initiation of drug treatment was 70 mm³. Mice received either no treatment (without added androstenedione with vehicle alone) or daily treatment with 4-androstenedione (100 μg/day) subcutaneously alone or with either anastrozole (0.25 mg/kg or ~5 μg/animal) subcutaneously, AZD0530 (50 mg/kg) by oral gavage, or both drugs together. This aromatase dose delayed xenograft growth without causing regression in prior studies (23). Oral AZD0530 50 mg/kg inhibits Src in xenograft target tissues (AstraZeneca


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AZD0530 Investigators Brochure). Tumors were measured weekly with calipers, and volumes calculated with the formula: \( V = \frac{1}{2} \times a \times b^2 \) \((a > b)\).

**Immunoblotting and immunoprecipitation.** Cells were lysed in ice-cold 1% NP-40 lysis buffer as described (12). Western analysis of cyclin E, p27, Cdk2, Cdk4, cyclin D1, MAPK, pMAPK (MAPKpT202/Y204), Akt, pAkt (AktpT473), Src, and pSrc (Src-pY416) used the following antibodies: to MAPK, pMAPK, Akt, pAkt, Src, and pSrc were obtained from Cell Signaling; antibodies to cyclin E (monoclonal antibodies E172 and E12) were from E. Harlow (MGH); and antibodies to β-actin were from Sigma. Other antibodies were from Santa Cruz Biotechnology. Protein levels were assayed for three different enhanced chemiluminescence exposures and quantitated by densitometry using Image Quant software from at least three different biological experiments.

Cyclin E was immunoprecipitated from 200 μg protein lysate with cyclin E monoclonal antibody E172 and associated proteins detected by immunoblotting or cyclin E-Cdk2 kinase assayed using γ-[32P]ATP (Amersham) and histone H1 (Roche) as described (12). Products were resolved by SDS-PAGE. Radioactivity in histone H1 substrate was quantitated by phosphorimagery from four different biological assays and graphed as % maximum activity.

**Immunohistochemistry.** Xenograft tumor tissues were formalin-fixed and embedded and deparaffinized samples were incubated with p27Kip1 antibody (Transduction Laboratories; 1:1,000), Ki-67 (DAKO; 1:100), and pSrc (Src-pY416; Stressgen; 1:100), respectively, as in refs. 4, 24 and processed in a DAKO autostainer using DAKO LSAB+-peroxidase detection kit. p27 and Ki-67 were scored as % tumor nuclei positive (4, 24). The predominant activated Src staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining as in ref. 4. Each antigen was scored independently by two pathologists (M.J. and N.G.) in at least 20 high-power fields and the mean score for each was used in statistical analysis.

**Reverse-phase protein lysate array.** Reverse-phase protein lysate array (RPPA) as described previously (25, 26) was used to quantify expression of 86 proteins, including MEK, extracellular signal-regulated kinase 2, c-Jun NH2-terminal kinase, Akt, glycogen synthase kinase 3, mammalian target of rapamycin (mTOR), S6, p110α, β-catenin, ER-α, and cyclin D1, and phosphorylation of MEK at Ser217/Ser218, MAPK at Thr202 and Tyr204, c-Jun NH2-terminal kinase at Thr183 and Tyr185, AKT at Thr308 and Ser473, glycogen synthase kinase-3 at Ser21, mTOR at Ser2448, and S6 at Ser235/Ser236 using antibodies from Cell Signaling, Epitomics (total MEK and p110α antibodies), Lab Vision (total ER-α antibody), and Santa Cruz Biotechnology (total extracellular signal-regulated kinase 2, c-Jun NH2-terminal kinase, glycogen synthase kinase-3, and cyclin D1 antibodies). For a complete list of proteins assayed, see Supplementary Table S1.

**Statistical analysis.** Mean cyclin E-Cdk2 assays and immunohistochemistry scores for tumors in each treatment group were graphed as histograms and error bars show SE. Differences were considered significant when error bars do not overlap and when the two tailed Student’s t test showed differences at P < 0.05.

Analysis of potential synergy between anastrozole and AZD0530 on tumor xenograft growth used the combination ratio as in ref. 27. Individual relative tumor volume (RTV) was calculated as (tumor volume on day of tumor measurement after treatment) / (tumor volume at initial treatment). The inhibition rate was calculated as (1.0 - final RTV / initial treatment). The combination ratio was calculated as (1.0 - final RTV after treatment / final RTV with androstenedione alone) x 100% (27).

The calculated combination ratio (27) evaluated combined effects. Fractional tumor volume (FTV) was defined as the ratio of mean final tumor volume of experimental drug-treated animals divided by the mean final tumor volume of the untreated control animals that received only androstenedione. The combination ratio compared the FTV expected if there were no synergy with the observed FTV. The combination ratio was calculated as (FTV of anastrozole x FTV of AZD0530) / observed FTV of combination. Observed and expected FTV are described as follows:

\[
\text{Expected FTV} = (\text{mean FTV of anastrozole}) \times (\text{mean FTV of AZD0530})
\]

\[
\text{Observed FTV} = \text{final tumor volume combined therapy / final tumor volume androstenedione alone}
\]

Combination ratio = expected FTV / observed FTV.

A combination ratio of >1 indicates a synergistic effect, whereas a ratio of <1 indicates a less than additive effect.

For statistical evaluation of RPPA data, serial dilution curves were constructed for each protein in each sample and relative quantification values were subsequently assigned to each protein in each sample as described (28). Each protein and phosphoprotein measurement was subsequently corrected for loading using the average expression of 86 measured proteins (Supplementary Table S1) in each sample.
AZD0530 and Anastrozole Inhibit Breast Cancer Growth

**Results**

AZD0530 increases $G_1$ arrest by anastrozole in MCF-7Arom5 cells. To provide a model in which to test antitumor efficacy of aromatase inhibitors, MCF-7 cells were transfected with the human aromatase cDNA (17). MCF-7Arom5 expresses high aromatase levels (Fig. 1A). Estrogen deprivation impaired MCF-7Arom5 proliferation. Incubation of MCF-7Arom5 for 48 h in estrogen-depleted medium caused partial $G_{0} - G_1$ arrest, with a decrease in the $\% S$ phase from 43% to 14% (Fig. 1B). Addition of 25 nmol/L androstenedione stimulated proliferation, increasing the $\% S$-phase cells to 52% by 48 h (Fig. 1B). Treatment with 100 nmol/L anastrozole inhibited the cell cycle progression, reducing the $\% S$-phase cells from 52% to 22% and increasing the $\% G_{0} - G_1$. The Src inhibitor, AZD0530, 1 nmol/L alone, had little effect on cell cycle, and the $\% S$-phase cells fell only modestly from 52% to 45%. Both drugs together caused $G_1$ arrest within 48 h, with % S-phase cells falling to 12%, significantly lower than with either drug alone (see error bars in Fig. 1B).

Cell cycle effects of increasing anastrozole concentrations were assayed with and without 1 nmol/L AZD0530, an AZD0530 dose that on its own had little cell cycle effect (Fig. 1B). In cells grown with androstenedione as the only estrogen source, anastrozole caused a dose-dependent $G_1$ arrest over a 100-fold concentration range (Fig. 1C). Anastrozole 12.5 nmol/L for 48 h decreased the $\% S$ phase from 52% to 43%, and considerably higher doses were required to further inhibit cell cycle progression. With 1000 nmol/L anastrozole alone, the $\% S$ phase fell to 14% (Fig. 1C). Although 1 nmol/L AZD0530 had little effect on cell proliferation (Fig. 1B), this dose of AZD0530 increased the cell cycle inhibitory effect of anastrozole by 1 log. At each anastrozole concentration used between 12.5 and 1,000 nmol/L, addition of a noninhibitory dose of AZD0530 caused a more profound increase in $\% G_1$ and decrease in $\% S$-phase cells than observed with either drug alone (see Fig. 1B and C). Thus, although anastrozole alone was a relatively weak cell cycle inhibitor, requiring high doses to cause $G_{0} - G_1$ arrest, it took 10-fold less anastrozole to arrest these cells when anastrozole was used together with the 1 nmol/L dose of AZD0530, which on its own had little effect on the cell cycle. Similar results were observed in another aromatase transfected cell line, MCF-7Ca (data not shown).

Effects of anastrozole and AZD0530 on signaling and cell cycle regulators. Effects of AZD0530 and anastrozole on mitogenic signaling were assayed. These assays used 100 nmol/L anastrozole because this caused a partial $G_1$ arrest that was increased by addition of 1 nmol/L AZD0530 (Fig. 1B and C). When added to cells grown with androstenedione as the only estrogen source, anastrozole 100 nmol/L alone and AZD0530 1 nmol/L alone each modestly reduced pAkt. pAkt in dual-treated cells was 3-fold lower than in untreated cells and 2-fold lower than with either drug alone. Anastrozole alone had little effect on MAPK phosphorylation, whereas AZD0530 caused an unexpected increase in pMAPK that was inhibited by coadministration with anastrozole. AZD0530 decreased pSrc as expected, but anastrozole paradoxically increased pSrc within 48 h, whereas AZD0530 and anastrozole together caused Src inhibition. Denisitometric analysis of band intensities reveals that combined treatment with anastrozole and AZD0530 significantly inhibited Src, Akt, and MAPK activities (Fig. 2; Supplementary Fig. S1) without affecting total kinase levels.

Assays of $G_1$ cell cycle regulators showed p27 was increased by 1.1-fold with AZD0530 and by 1.5-fold with anastrozole alone, whereas both drugs together increased p27 by 3.5-fold over levels in asynchronous androstenedione-treated cells.
Densitometric analysis showed that the increase in p27 was statistically significant in all treatment groups but most marked with both drugs together (see Supplementary Fig. S1). Cyclin D1, cyclin E, and Cdk2 levels were modestly decreased, whereas Cdk4 was unchanged by anastrozole plus AZD0530 (Fig. 2B).

**Anastrozole and AZD0530 inhibit cyclin E-Cdk2 activity.** After 48 h, p27 bound to cyclin E-Cdk2 increased by 1.2-fold following AZD0530 alone and by 2.4-fold following anastrozole alone (Fig. 2C) and by 4.8-fold with AZD0530 and anastrozole together. Although AZD0530 alone had little effect on cyclin E-Cdk2 activity, both drugs together caused a statistically significant kinase inhibition compared with that observed with either alone (Fig. 2D). Data show the mean radioactivity incorporated into the substrate of four repeat assays, graphed as a histogram showing % maximal activity ± SE (Fig. 2D).

**Effects of anastrozole and AZD0530 on breast cancer xenograft growth.** To test the antitumor efficacy of aromatase inhibitors with or without AZD0530 in *vivo*, MCF-7-Arom5 xenografts were grown in ovariectomized mice. Nude mice are deficient in adrenal androgen precursor production and animals were supplemented with androstenedione. In these xenografts, intratumoral aromatase-mediated conversion of exogenous androstenedione to estrogen provides the only estrogen source. Thus, the tumor serves as an autocrine source of estrogen that stimulates tumor growth and is responsive to aromatase inhibitors (18). Androstenedione on its own, in the absence of transfected aromatase, does not affect MCF-7 xenograft growth (22). MCF-7-Arom5 xenografts were estrogen dependent for proliferation. In the absence of androstenedione, tumors grew very little over the 14-week experiment. In contrast, xenografts grew rapidly in androstenedione-treated animals. AZD0530 administration initially showed an antitumor effect, but within 12 to 14 weeks, resistance developed and final tumor volumes were similar to the androstenedione-alone controls (Fig. 3A). The experiment could not be extended beyond 14 weeks because tumors reached maximum size permitted. Anastrozole alone suppressed tumor growth significantly compared with androstenedione alone, with 0.25 mg/kg anastrozole yielding an inhibition rate [1 - (final RTV after treatment / final RTV with androstenedione alone) x 100] of 37.9% (see Table 1A and Fig. 3A). Combined anastrozole and AZD0530 therapy more potently inhibited tumor growth, yielding an inhibition rate of 53.3% (Fig. 3A).

To test for interaction between anastrozole and AZD0530, the effect of dual therapy was evaluated by the combination ratio of expected over observed FTV (27). FTV is defined by the ratio of mean final tumor volume of experimental drug-treated animals divided by the mean final tumor volume of the untreated control animals (27). The combination ratio = (FTV of anastrozole × FTV of AZD0530) / (observed FTV of combination). A combination ratio of >1 indicates a synergistic effect between the two drugs. In the initial few weeks of the experiment, before the emergence of resistance to AZD0530, the effect of the two drugs was not synergistic. However, as resistance to AZD0530 monotherapy emerged, by 14 weeks, the ratio was 1.57 (see Table 1B), indicative of synergy between anastrozole and AZD0530. In all treatment groups, the animals showed no signs of toxicity or weight loss throughout the experiment (Fig. 3B). Coadministration of anastrozole with AZD0530 prevented the development of resistance to AZD0530 monotherapy and augmented the effect of anastrozole alone.

**Effects of anastrozole and AZD0530 on proliferation and pSrc in *vivo.** In an effort to identify predictors of antitumor response, p27, pY416 phosphorylated activated Src (Src-pY416), and Ki-67 were assayed by immunohistochemistry in xenografts recovered at the end of the experiment. Scores were reached by consensus of two different pathologists. As observed in cell culture, xenograft p27 levels increased more in tumors treated with anastrozole and AZD0530 together than with each drug alone. Ki-67, a proliferation indicator (29), was not reduced by AZD0530, was significantly decreased by anastrozole alone, and was lowest after combination therapy compared with controls. pSrc was highest in androstenedione-alone control tumors. Src was not significantly inhibited in the resistant tumors recovered at the end of AZD0530 therapy. As observed in tissue culture, anastrozole alone modestly increased Src phosphorylation. Anastrozole and AZD0530 combination therapy significantly decreased Src-pY416 (Fig. 4).

**MEK and PI3K pathways are activated in AZD0530-treated xenografts.** Proteomic analysis of 86 proteins using RPPA including apoptotic markers, X-linked inhibitor of apoptosis, cleaved caspase, and cleaved poly (ADP-ribose) polymerase showed no evidence for apoptosis in any treatment groups. Consistent with results in tissue culture (Fig. 2B) and effects on p27 and Ki-67 by immunohistochemistry (Fig. 4), xenograft cyclin D1 levels were reduced by each drug alone and most...
notably by AZD0530/anastrozole treatment compared with androstenedione-alone controls ($P = 0.01$). Dual therapy also reduced levels of phospho-activated epidermal growth factor receptor p922 ($P = 0.07$).

As shown in the heat maps and means plots in Fig. 5, RPPA analysis showed tumors treated with Src inhibitor alone (condition C) or in combination with anastrozole (condition D) had increased phosphorylation of components of the MEK and PI3K signaling pathways compared with untreated controls. AZD0530-resistant xenografts showed increased pMEK, pMAPK, and p110, activation of Akt, mTOR, and S6 kinase, and inhibitory phosphorylation of glycogen synthase kinase-3β compared with androstendione- or anastrozole-alone groups. β-Catenin levels were increased, in keeping with the known effect of glycogen synthase kinase-3β inhibition to stabilize β-catenin (30). This may reflect activation of these pathways after long-term Src inhibitor therapy and could contribute to resistance to the antitumor effects of AZD0530.

**Discussion**

Aromatase inhibitors have had a major effect on breast cancer treatment and are more effective than tamoxifen as first-line therapy for metastatic ER-positive breast cancers and as adjuvant therapy for hormone-responsive postmenopausal disease (1, 2). However, *de novo* and acquired resistance limit therapeutic efficacy of aromatase inhibitors (31). Oncogenic activation of various growth factor pathways can lead to antiestrogen resistance (31, 32). Given the complexity of mitogenic signaling in ER-positive breast cancer, monotherapy with signal transduction inhibitors may have only modest antitumor efficacy and prove less effective than endocrine therapy (31). Combined endocrine/signal transduction inhibitor therapy may cause greater growth inhibition and delay emergence of resistance. Several preclinical and clinical trials are now examining signal transduction inhibitors combined with aromatase inhibitors in an effort to block crosstalk between activated ER and signaling pathways and oppose resistance (31–34).

Src is frequently activated in human breast cancers and high pSrc levels were observed in 37% of ER-positive breast cancers (4). Estrogen binding to the ER recruits and activates Src leading to MEK/MAPK and PI3K/Akt activation (7–9). Moreover, oncogenic activation of insulin-like growth factor-I receptor or the ErBb family, through ErBb1/3 overexpression or ErBb2 gene amplification, leads to constitutive Src activation and Src in turn, further activates these receptor tyrosine kinases (6, 35). Activation of these signaling kinases modulates cell cycle regulators to stimulate cell cycle progression (12), and activated kinases phosphorylate ER-α to positively regulate its transcriptional activity (36). Up-regulation of Src mediates antiestrogen resistance in culture models (37).

The cell cycle inhibitor p27 is ubiquitously expressed and regulates cell proliferation (38). Although normal quiescent mammary epithelial tissues express high p27 (24, 39), p27 protein is reduced in up to 60% of primary human breast and this is associated with poor patient outcome (24, 40). Estrogen stimulation of estrogen-deprived cells mediates loss of p27, leading to cyclin E-Cdk2 activation and cell cycle entry (11, 12, 14, 41). p27 and the related Cdk2 inhibitor, p21, are both required for G1 arrest by antiestrogens in human breast cancer lines (12, 15). Tyrosine phosphorylation of p27 by Abl and Src family kinases impairs the Cdk2 inhibitory action of p27 in *vitro* and promotes p27 proteolysis (4, 42). Src-mediated p27 phosphorylation at Y74, Y88, and Y89 led to reduced binding to cyclin E-Cdk2, loss of inhibition of cyclin E-Cdk2 by p27, and facilitated cyclin E-Cdk2-mediated T187 phosphorylation of p27 to drive p27 proteolysis. Src induction reduced the p27 $t_{1/2}$, whereas Src inhibition by AZD0530 increased p27 stability (4). These findings provided the molecular rationale for the current preclinical study. Because p27 is required for the therapeutic growth-inhibitory effects of estrogen deprivation or ER blockade (12), and because Src activates p27 proteolysis (4), we reasoned that Src

### Table 1.

(A) Synergy between anastrozole and AZD0530 therapy on human breast cancer MCF-7 Arom5 xenograft growth in nude mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage</th>
<th>No. mice</th>
<th>Tumor volume (mm$^3$, mean ± SE)</th>
<th>Inhibition rate (%) *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
<td>End</td>
</tr>
<tr>
<td>Without androstenedione</td>
<td>0 mg/kg/d, s.c.</td>
<td>9</td>
<td>9</td>
<td>74 ± 15</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>5 mg/kg/d, s.c.</td>
<td>9</td>
<td>9</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>AZD0530</td>
<td>50 mg/kg/d, intragastrically</td>
<td>9</td>
<td>9</td>
<td>74 ± 11</td>
</tr>
<tr>
<td>Anastrozole</td>
<td>0.25 mg/kg/d, s.c.</td>
<td>9</td>
<td>9</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>Anastrozole +</td>
<td>0.25 mg/kg/d, s.c. +</td>
<td>9</td>
<td>9</td>
<td>68 ± 6</td>
</tr>
</tbody>
</table>

(B) Anastrozole and AZD0530 FTV relative to untreated control *

<table>
<thead>
<tr>
<th>FTVanastrozole</th>
<th>FTVAZD0530</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expected FTV</td>
</tr>
<tr>
<td>0.66</td>
<td>1.07</td>
<td>0.71</td>
</tr>
</tbody>
</table>

**NOTE:** Student’s t test compared with androstenedione group.

$^1$Inhibition rate = [1.0 - (final RTV after treatment / final RTV with androstenedione alone) × 100]

$^2$P < 0.01.

$^3$P < 0.001.

$^4$P < 0.05.

$^5$P < 0.01.
inhibitors may oppose antiestrogen resistance and could potentially cooperate with antiestrogens to augment antitumor efficacy.

AZD0530 is a highly selective, orally available Abl and Src family kinase inhibitor (43). It showed potent antiproliferative activity (below micromolar IC50) in over 17 human cancer lines and inhibited growth of 6 of 13 xenograft tumor models tested. It is currently in phase II clinical trials for cancer. AZD0530 can cooperate with tamoxifen to arrest proliferation of resistant ER-positive breast cancer lines (4, 44). AZD0530 and tamoxifen synergize to inhibit anchorage-independent growth in vitro (45). The growth-inhibitory effect of AZD0530 is opposed by estrogen, and Src inhibitors may require ER-α blockade for efficacy in estrogen-dependent breast cancer (45).

The present data indicate that, as for ER-blocking drugs (12), the aromatase inhibitor anastrozole up-regulates p27 protein and p27 inhibits cyclin E-Cdk2 to arrest MCF-7Arom5 cells in G1. AZD0530-anastrozole more effectively increased p27 levels and binding and inhibition of cyclin E-Cdk2 by p27 than either drug alone, causing a greater cell cycle arrest. Although the increase in p27 was modest (3.5-fold), this is sufficient for stoichiometric binding and inhibition of cyclin E-Cdk2 (46). The reduction in cyclin D1 levels observed with AZD0530-anastrozole likely reflects a loss of protein stability as cells enter G1 arrest (47) and reduced CCND1 transcription (30).

The present data suggest potential synergy between anastrozole and AZD0530 both in cell culture and in xenograft tumors. In cell culture, addition of 1 μmol/L AZD0530, which on its own had little antiproliferative effect, decreased by 10-fold the anastrozole concentration required for proliferation arrest. Although AZD0530 alone had little antiproliferative effect, this Src inhibitor appears to cooperate with estrogen deprivation by anastrozole to block cell cycle. This may, in part, result from abrogation by AZD0530 of the stimulation of Src seen with anastrozole alone in vitro and in xenografts. In xenografts treated with dual therapy, p27 levels were significantly increased and Src-pY416, cyclin D1, and the proliferation marker Ki-67 were reduced when compared with androstenedione-alone controls, supporting further study of these markers as indicators of treatment response in clinical trials. Ki-67 has been widely used to assess cancer prognosis. A change in the expression of Ki-67 after exposure of patients to therapeutic agents may represent a pharmacodynamic marker of efficacy. Recent neoadjuvant studies suggest that on-treatment measurement of Ki-67 may be a
Fig. 5. RPPA was used to quantify expression of the indicated proteins in the MEK (A) and PI3K (B) pathways in MCF-7Arom5 xenografts treated with androstenedione alone (A) or together with anastrozole (B), AZD0530 (C), or both anastrozole and AZD0530 (D). In the heat map, green, reduced expression levels; red, higher expression levels.
more effective predictor of treatment efficacy for both endo-
crine treatment and chemotherapy (48). It will be of interest
to further evaluate whether changes between pretreatment
and post-treatment p27 and Ki-67 levels are predictive of pa-
tient response in the context of clinical trials.

Although AZD0530 initially delayed xenograft growth com-
pared with androstenedione-only controls, tumors rapidly ac-
quired AZD0530 resistance. Resistance to AZD0530 was also
observed in breast cancer lines after prolonged (>10 weeks)
treatment in vitro (44). In contrast to AZD0530 resistance seen
in cell culture, in which Src remained inhibited (44), resistant
xenografts showed little Src inhibition after 14 weeks of
AZD0530. Although increased AZD0530 metabolism or accel-
erated drug expulsion from xenografts could contribute to resis-
tance, our proteomic analysis showed that prolonged AZD0530
therapy was associated with activation of bypass pathways not
seen in untreated (androstendione-alone) controls. AZD0530
treatment alone increased pMAPK within 48 h in tissue culture
and AZD0530-resistant xenografts recovered after prolonged
therapy showed both MEK/MAPK and PI3K/Akt/mTOR path-
way activation. RPPA analysis of xenograft tumors showed that
HER-2 and epidermal growth factor receptor activities (which
would activate MEK and PI3K) were also increased by
AZD0530 alone but were reduced by the drug combination.
Both PI3K/Akt/mTOR and MEK/MAPK pathways could mediate
constitutive Src activation, competing with the effect of
AZD0530 on its target. Further investigation is warranted to test
if MEK or PI3K/AKT/mTOR pathway inhibitors could abort de-
velopment of resistance to AZD0530 monotherapy.

In human cancers, both MEK/MAPK and PI3K/Akt alter
p27 phosphorylation and oppose the Cdk2 inhibitory effects
of p27 (30). Transfection of activated MEK reduces the cyclin
E-Cdk2 inhibitory activity of p27 (49, 50) and, in some cell
types, activates p27 proteolysis (49). Moreover, constitutive
Akt activation shifts p27 into the cytoplasm away from nucle-
lar Cdk targets, leading to loss of p27 binding to cyclin E-
Cdk2 (38, 51). Thus, both MEK and PI3K bypass pathway
activation could abort p27 function causing resistance to
coadministered anastrozole during prolonged AI-AZD0530
therapy in humans.

Because estrogen/ER stimulates Src and MAPK, the estrogen
depression resulting from anastrozole treatment was expected
to block MAPK and Src activation in our studies. Used alone,
anastrozole had little inhibitory effect on pMAPK in culture
and but caused a more important MAPK inhibition in xeno-
grafts. Although anastrozole monotherapy caused a partial G1
arrest in cell culture and had an antiproliferative effect with re-
duced Ki-67 and increased p27 in treated tumors, paradoxically,
anastrozole appears to activate Src in MCF-7/Arom5 cells both
in cell culture and after prolonged monotherapy in xenografts.
AZD0530 combined with anastrozole effectively inhibited Src,
MEK/MAPK, and Akt activation in cell culture and in xenografts
and caused a sustained inhibition of xenograft growth. Src acti-
vation by anastrozole may constitute one mechanism leading
to anastrozole resistance in vitro and supports further investiga-
tion of the therapeutic potential of combined aromatase and
Src inhibitor therapy. The combination of the two drugs togeth-
er appears to overcome the activation of the bypass pathways
observed with either treatment alone in these models.

In summary, the combination of AZD0530 and anastrozole
more effectively inhibits proliferation of ER-positive MCF-
7/Arom5 breast cancer cells than either drug alone both in vitro
and in vivo. These data support further clinical investigation of
the therapeutic potential of AZD0530 in combination with ana-
drozole for treatment of postmenopausal ER-positive breast
cancer. Both PI3K and MEK pathways were activated following
prolonged AZD0530 treatment but not by androstendione
alone in xenograft tumors. Proteomic analysis of xenografts
may permit the preclinical identification of putative predictive
markers and reveal potential resistance mechanisms that can be
further evaluated in the context of clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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