Src Inhibition with Saracatinib Reverses Fulvestrant Resistance in ER-Positive Ovarian Cancer Models In Vitro and In Vivo

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Abstract

Purpose: More effective, less toxic treatments for recurrent ovarian cancer are needed. Although more than 60% of ovarian cancers express the estrogen receptor (ER), ER-targeted drugs have been disappointing due to drug resistance. In other estrogen-sensitive cancers, estrogen activates Src to phosphorylate p27 promoting its degradation and increasing cell-cycle progression. Because Src is activated in most ovarian cancers, we investigated whether combined Src and ER blockade by saracatinib and fulvestrant would circumvent antiestrogen resistance.

Experimental Design: ER and Src were assayed in 338 primary ovarian cancers. Dual ER and Src blockade effects on cell cycle, ER target gene expression, and survival were assayed in ERα+ ovarian cancer lines, a primary human ovarian cancer culture in vitro, and on xenograft growth.

Results: Most primary ovarian cancers express ER. Src activity was greater in ovarian cancer lines than normal epithelial lines. Estrogen activated Src, ER-Src binding, and ER translocation from cytoplasm to nucleus. Estrogen-mediated mitogenesis was via ERβ, not ERβ. While each alone had little effect, combined saracatinib and fulvestrant increased p27 and inhibited cyclin E-Cdk2 and cell-cycle progression. Saracatinib also impaired induction of ER-target genes c-Myc and FOSL1; this was greatest with dual therapy. Combined therapy induced autophagy and more effectively inhibited ovarian cancer xenograft growth than monotherapy.

Conclusions: Saracatinib augments effects of fulvestrant by opposing estrogen-mediated Src activation and target gene expression, increasing cell-cycle arrest, and impairing survival, all of which would oppose antiestrogen resistance in these ERα+ ovarian cancer models. These data support further preclinical and clinical evaluation of combined fulvestrant and saracatinib in ovarian cancer.

Cancer Therapy: Preclinical

Introduction

Ovarian cancer is the most lethal gynecologic malignancy. About 80% of patients are diagnosed with stage III/IV disease and 80% suffer incurable relapse within 2 years (1). ER blocking drugs are well tolerated and thus appealing in this setting, but are frequently attended by drug resistance. Estrogens (E2) are implicated in the etiology of ovarian cancer. E2 drives proliferation via estrogen receptor α (ERα) and antiestrogens inhibit ovarian cancer growth in vitro and in vivo (2–5). Data on ERβ in ovarian cancer are limited. Ovarian cancer lines and primary ovarian cancers express lower ERβ than normal ovary and its loss correlates with malignant transformation (5, 6). ERα, hereafter ER, is expressed in ovarian cancers at levels similar to those in breast cancer (7). A meta-analysis (n = 2,500 patients) showed 67% of primary ovarian cancers expressed ER protein (8). Despite high ER expression in ovarian cancers, numerous small trials of ER-blockers have been disappointing (9, 10). Tamoxifen, used as second-fourth line treatment for recurrent ovarian cancer (n = 648 patients/18 trials), yielded low response rates (mean 13%) with 38% disease stabilization. Most trials (15/18) failed to correlate ER expression with response (9). The pure ER-antagonist, fulvestrant (Fulv) showed 8% overall response and 35% disease stabilization in advanced,

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heavily pretreated recurrent ovarian cancer ($n = 26$) (10). To date, 9 aromatase-inhibitor trials (total 229 patients) have yielded response rates from 0 to 35.7% and recurrent disease stabilization in 20% to 42% (11). Ovarian cancer ER expression was not routinely assayed in these trials.

In summary, therapies that block protumorigenic estrogen effects in ovarian cancer have not been studied in well-designed trials that consider tumor ER status. Ovarian cancers manifest frequent de novo antiestrogen resistance and the 11% to 35% that do initially respond invariably develop resistance.

Estrogens, in ER-sensitive cells, promote exit from quiescence and G1 progression (12). Cell-cycle progression is governed by cyclin-bound cyclin-dependent kinases (Cdk) and Cdk inhibitors (13). ER blockade causes G1 arrest in breast cancer cells by increasing the Cdk inhibitor, p27 (12). Importantly, p27 levels are frequently reduced in ovarian cancer (13). This may result from constitutive activation of Src, which phosphorylates p27 to promote its degradation (14, 15). Cross-talk between estrogen-bound ER and Src drives mitogenic pathways (16), p27 loss, and cell-cycle progression (15).

The lack of effective treatment for recurrent disease has stimulated development of targeted ovarian cancer therapies. Mitogenic pathways, including Src, Ras/Raf/MEK and PI3K/AKT, are frequently activated in ovarian cancer (17). Src is overexpressed or activated in most ovarian cancers and regulates proliferation and apoptosis (18–20). In ER-sensitive cancers, cross-talk between liganded ER and Src contributes to E2-mediated mitogenesis and ER-activated gene expression (16, 21–23). Saracatinib (AZD0530), a potent inhibitor of Abl and Src family kinases (24), inhibits invasion in vitro and xenograft growth in vivo (25) and was well tolerated in phase I trials (26). It is in phase II/III trials with chemotherapy for recurrent ovarian cancer (NCT00610714; NCT01196741), but has not been evaluated with antiestrogens in ovarian cancer.

Here, we investigated the hypothesis that constitutive Src expression contributes to resistance to ER-blockade in ovarian cancer. We generated an ER-blocker resistant ovarian cancer cell line and found that saracatinib reverses fulvestrant resistance in vitro and in vivo via transcriptional effects, cell-cycle arrest, autophagy, and apoptosis. These data provide novel evidence for cross-talk between ER and Src in ovarian cancer. Dual targeting of ER and Src more effectively inhibits ER-target gene expression and cell-cycle progression in vitro and ovarian cancer tumor growth in vivo. These data support further preclinical and clinical development of this therapeutic strategy to overcome cross-talk between ER and Src family pathways driving antiestrogen resistance.

### Materials and Methods

#### Drugs

Saracatinib ($10^{-6}$ mol/L) and fulvestrant (Fulv; $10^{-6}$ mol/L) were dissolved in dimethyl sulfoxide (DMSO) and ethanol, respectively (AstraZeneca). Saracatinib did not exceed $10^{-6}$ mol/L to avoid off target effects. The pure ERα agonist 4,4′,4″-[4-propyl-(1H)-pyrazole-1,3,5-triyl]trisphenol (PPT), the selective ERβ agonist 2,3-bis(4-hydroxyphenyl) propionitrile (DPN), the dual ERα agonist/ERβ antagonist (R,R)-5,11-Diethyl-5,6,12-tetrahydro-2,8-chrysenediol (THC), and the ERα antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidine-3-yl]-phenol (PHPTPP) were obtained from Tocris.

#### Cell culture

PEO1 ovarian cancer line and human ovarian surface epithelium (HOSE) cultures (X. Xu, University of Miami, Miami, FL), BG-1 (E. Denholm, NIEHS, NC) and a spontaneous antiestrogen-resistant variant, PEO1R, established after prolonged passage, were cultured in RPMI with 10% FBS. 293T cells were from American Type Culture Collection (ATCC). Lines were authenticated using ATCC guidelines. OCI-E1P (ovarian-carcinoma-Ince-endometrioid-primary-1) was cultured from a primary ER+ endometrioid ovarian cancer and used within 6 to 8 passages. Asynchronous cultures were treated with vehicle, $10^{-6}$ mol/L saracatinib, $10^{-6}$ mol/L fulvestrant, or both for 48 hours. PEO1R in media with 0.1% cFBS and BG-1 in 10% cFBS for 48 hours were then treated with ERα and ERβ agonists/antagonists.

#### Immunofluorescence microscopy

Cells ($3 \times 10^5$ cells) were serum/E2-deprived for 48 hours, then treated with $10^{-6}$ mol/L E2 for indicated times. Immunofluorescence (IF) used were anti-ERα (Abcam) or anti-pSrc (Cell Signalling) and secondary antibody Ab as described (27). Cells were fixed with 4%, 6-diamidino-2-phenylindole (DAPI) counterstained and analyzed by IF microscopy. Dual ER and pSrc staining used both antibodies together followed by secondary antibody linked to Texas Red for pSrc and GFP for ER.
Subcellular fractionation

Cells were serum/E2-deprived for 48 hours and then treated with E2 (10^{-8} mol/L) for 4 hours. Fractionation was done as described (28).

Cell-cycle analysis

Cells were bromodeoxyuridine (BrdUrd)-labeled, counterstained with propidium iodide, and cell-cycle distribution assayed as described (12).

Annexin-V staining

Drug-treated cells (48 hours) were analyzed by FITC Annexin-V Apoptosis Detection Kit I (BD Biosciences) as per manufacturer's instructions.

Detection of autophagic vesicles

Drug effects on autophagic vesicles were evaluated by Cyto-ID Autophagy Detection Kit (ENZO) as per manufacturer's instructions.

Plasmids and transfection

PEO1R were transduced with lentivirus carrying pLV411G effLuc-flag (IRES-hrGFP) as described (29). PEO1R were transduced with lentivirus encoding an Src-specific shRNA (5'-CAGATTGTCAACAACAG-3') or control shRNA (Open Biosystems, RHS4430-101028217 and RHS4346, respectively) and selected with puromycin.

Immunoblotting, immunoprecipitation, and kinase assay

Western blot analyses were conducted and quantitated by densitometry as described (12). Cyclin E antibody [HE-12, Harlow Lab (MGH)]; ERα, Cdk2, MAPK, pMAPK antibodies were from Santa Cruz; p27 from BD transduction; Akt, pAkt, Src and pSrc, LC3, and cleaved PARP antibodies from Cell Signaling.

Cyclin E/Cdk2 complexes were precipitated and immunoblotted for associated proteins or assayed for kinase activity with Histone H1 substrate as described (12). Substrate radioactivity was quantified by phosphoimager from 4 assays. ER-Src complexes were precipitated after 48 hours of serum/E2 deprivation and at intervals after E2 repletion and Src and ER detected by Western blot analysis as described (27).

RNA Extraction and real-time PCR

RNA was prepared using Trizol (Invitrogen). A total of 0.5 μg total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Biorad). Real-time PCR for c-Myc used (Forward-5'-GAGCTCTGGATACCTGCTG-3' and reverse 5'-AGGATAGTCCTTCCGGTGAGTGGAG-3'); FOS-L (forward-5'-CAGGGCGAGTACCTGCAAACTG-3' and reverse-5'-TCCTTCCGGGATTTGGCAAGAT-3'); and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as described (30).

Gene ARRAY and RPPA analysis

178 proteins and phosphoproteins were assayed using reverse phase protein arrays (RPPA) in 338 high-grade serous ovarian cancer from The Cancer Genome Atlas (TCGA) project as described (31). RPPA data processing used SuperCurve (K. Coombes and colleagues, SuperCurve: SuperCurve Package. R package version 1.4.1.2011) as described (31). Gene expression analysis of these samples used Affymetrix U133A GeneChips (12,042 genes) per Minimum Information about a Microarray Experiment (MIAME) guidelines of the Microarray Gene Expression Data Society as in ref. (32). The TCGA microarray data set used is available online at http://cancergenome.nih.gov/cancersselected.ovarian.

PEO1R xenografts

Luciferase-positive PEO1R^{Src-} cells (10^5 cells) suspended in collagen (33) were implanted under the renal capsule of female, BALB/C nude mice as described (34). Xenograft growth was compared in animals (10/group) +/- supplemental estradiol pellets at 0.72 mg/90 days (Innovative Research). In subsequent experiments, E2-supplemented animals (10/group) either received no treatment, fulvestrant 3.5 mg/week SQ, fulvestrant 5 mg/week SQ, saracatinib 25 mg/kg daily (via oral gavage), or both starting either 11 or 26 days after tumor implantation. This saracatinib dose inhibits Src kinase in xenografts (22, 23). Viable tumor burden (luciferase activity) was monitored weekly. Mice were imaged by Xenogen in vivo imaging system (IVIS). Bioluminescence plots of photon flux/time calculated for each mouse were normalized to day 0 signal values of 100 for all mice. Animals were weighed twice per week. Tumors were removed 75 days postimplantation or when morbidity required euthanasia as per University of Miami Animal care procedures.

Statistical analysis

All assays of cell-cycle distribution, cyclin E-Cdk2 kinase, IF and IP/Western blot analyses were done at least thrice. Xenograft studies were repeated twice. Data were summarized as mean ± SEM by treatment group and displayed in bar graphs. One- or two-way ANOVA was conducted to assess difference among treatment means. For 2 x 2 factorial experiments, interaction of the 2 factors was tested. A significant interaction is a statistical indication of synergism, meaning that the combined effect of 2 agents is manifested in a nonadditive manner. Following a significant ANOVA result (P ≤ 0.05) rejecting the null hypothesis that mean are the same across the treatment groups, the Tukey honestly significant difference (HSD) test was used for all pairwise mean comparisons. These multiple comparison procedures ensure actual family-wise error rates no greater than prespecified 5%. Analyses were conducted in SAS 9.3.

Results

Estrogen mediates Src activation, ER/Src binding, and ER translocation to the nucleus

PEO1R, a variant of the ovarian cancer ascites PEO1 line (3), developed antiestrogen resistance during prolonged culture. BG-1, a primary high-grade ovarian cancer line, is
antiestrogen sensitive (35). Both PEO1R and BG-1 express ER protein. In contrast, ER was not detected in 4 normal human ovarian surface epithelium (HOSE) lines (representative data, Fig. 1A). While prior work showed mitogenic kinase activation in ER-negative ovarian cancer lines (36, 37), this had not been reported in ER-positive lines. Both PEO1R and BG-1 showed activated/phosphorylated Src, MEK, and AKT compared with HOSE, whereas total levels were similar (Fig. 1A).

Estrogen–ER binding stimulates rapid, transient Src activation in breast cancer (16, 21–23) but such cross-talk has not been characterized in ovarian cancer. To investigate Src involvement in E2-stimulated ovarian cancer proliferation, PEO1R and BG-1 were E2- and serum-deprived in media with 0.1% cFBS for 48 hours and then treated with E2 (10^{-8} mol/L) for times indicated. Estrogen rapidly activated Src in both (Fig. 1B, top), and ER-bound activated Src was transiently detected in ER immunoprecipitates 15 minutes after E2 addition (Fig. 1B, bottom). ER and Src colocalize in the perinuclear cytoplasm by immunofluorescence microscopy at 15 minutes (Fig. 1C). Thus, E2 stimulates ER/Src binding and Src activation, which may contribute to its mitogenic effects.

Subcellular fractionation and IF showed that ER was strongly cytoplasmic in E2-deprived cells, but shifted into the nucleus 4 hours after E2 addition (Fig. 1D; and BG-1 data, Fig. 1E). Minimal membrane-bound ER was seen at either time. Thus, in contrast to breast cancer where ER is predominantly nuclear (38), both ovarian cancer lines express cytoplasmic ER in the absence of ligand, which translocates to the nucleus on E2 addition.

**E2 stimulates proliferation in ovarian cancer cells via ERα and stimulates xenograft growth**

To investigate antiestrogen resistance mechanisms, effects of estrogen deprivation with or without growth factor depletion were assayed. PEO1R did not arrest after 48 hours of E2 depletion alone (10% cFBS), but estrogen and serum-
depleted media (0.1% cFBS) for 48 hours reduced the percentage of S-phase (%S) from 50% to 15% (Fig. 2A). Notably, addition of E2 alone (10^{-8} or 10^{-9} mol/L) prevented 0.1% cFBS-induced growth arrest over 48 hours, with 50% of cells remaining in S-phase (E2, 10^{-8} mol/L). Thus, estrogen alone maintains proliferation in the absence of growth factors in PEO1R.

To assay further the ability of E2 to drive proliferation, cells were arrested in 0.1% cFBS for 48 hours. Addition of 10^{-8} mol/L estradiol alone, without serum, stimulated cell cycle re-entry with %S-phase peaking at 20 hours (Fig. 2B, see also Supplementary Fig. S1).

In contrast to PEO1R, BG-1 cells were more sensitive to estrogen deprivation (10% cFBS), showing a significant reduction in S-phase from 58% to 20% cells. Addition of E2 (10^{-8} or 10^{-9} mol/L) maintained proliferation at 48% S-phase (E2 10^{-8} mol/L; Fig. 2C). %S-phase pairwise comparisons in the presence or absence of estrogen (\* and \#) were significant at \( P < 0.0001 \) for both PEO1R and BG-1.

Because E2 and fulvestrant both bind ER\( \alpha \) and ER\( \beta \), we tested which receptor mediates cell-cycle effects of estrogen. PEO1R cells were transferred to 0.1% cFBS, and BG-1 transferred to 10% cFBS, together with different steroid receptor ligands or antagonists for 48 hours before cell-cycle analysis (Fig. 2A, B, C).
cycle analysis (Fig. 2A, C). The pure ERα agonist, PPT (10^{-8} and 10^{-9} mol/L; refs. 5, 39), and the ERα agonist/ERβ antagonist THC (10^{-6} mol/L; ref. 40), both rescued E2 deprivation in these lines. Furthermore, the ERβ agonist, DPN, at 10^{-9} mol/L (5, 41) failed to induce proliferation and the pure ERβ antagonist PHPP (42) added with E2 did not abrogate E2 action (Fig. 2A and C). The mean %S phase did not differ significantly between asynchronous controls and cells maintained with added E2, PPT, THC, or PHPP (43), nor did they differ between DPN-treated and E2-deprived cells (+) in PEO1R or BG-1 (Fig. 2A and C). Thus, mitogenic effects of E2 in these ovarian cancer models are mediated by ERα and are not dependent on ERβ.

Because E2 alone could drive cell cycle reentry in vitro, E2 effects were further evaluated on PEO1R xenografts (Fig. 2D). PEO1R^{Luc+} was inserted under the renal capsule (34) of nude mice, with or without prior E2 pellet implantation. Estrogen-supplemented tumors showed sustained growth, with more than 100-fold higher photon flux/sec at 16 weeks than without E2 (Fig. 2D). Thus, while PEO1R grows in culture without E2 if sustained by serum, supplemental E2 is required for PEO1R xenograft growth.

**Src inhibition with saracatinib reverses resistance to fulvestrant**

Our prior work showed that estrogen rapidly activates Src, which phosphorylates p27 to promote p27 proteolysis (15). Because estrogen activated Src in these ovarian cancer lines (Fig. 1B), we postulated that combined Src and ER blockade would inhibit proliferation better than either alone. PEO1R, BG-1, and a primary ovarian tumor culture OCI-E1P were treated with the Src inhibitor, saracatinib, fulvestrant, or both (Fig. 3A–D). Asynchronous PEO1R showed no cell-cycle inhibition after 48 hours of fulvestrant (10^{-6} mol/L). Saracatinib (10^{-6} mol/L) alone modestly decreased the %S-phase from 50% to 29%. However, combined Src and ER-blockade significantly decreased the %S-phase to 10% compared with 29% with the Src inhibitor alone (44, P < 0.0001; Fig. 3A, left). In PEO1R, a synergistic effect on %S between the 2 drugs was indicated by a significant interaction (44, P = 0.008). The reduction in %S phase following dual therapy (Δ%S = 40) was greater than the sum of decreases in %S seen with either drug alone [21.97 = Fulv (0.6) and saracatinib (21.36; Fig. 3A; right)].

Saracatinib reduced p-Y416-phosphorylated–activated Src (pSrc), without changing total Src in PEO1R (Fig. 3B). Because Src mediates loss of p27 (15), drug effects on p27, cyclin E, and Cdk 2 were assayed. Fulvestrant alone had little effect on p27 or cell-cycle distribution; saracatinib increased p27 3-fold, whereas both together increased p27 by over 6-fold in PEO1R (Fig. 3B; left). After 48 hours treatment, cyclin E was immunoprecipitated and complexes were resolved and blotted for associated proteins (Fig. 3B, middle) or assayed for kinase activity (Fig. 3B; right). Cyclin E and Cdk2 levels were not affected by drug. Saracatinib alone and combined treatment increased p27 bound to cyclin E-Cdk2 by 4- and 6-fold, respectively, resulting in loss of cyclin E-Cdk2 activity (Fig. 3B; right). There was a significant synergistic interaction (P = 0.009) between fulvestrant and saracatinib for kinase inhibition.

The antiproliferative effects of ER and Src blockade were also enhanced by dual therapy in BG-1 (Fig. 3C). Fulvestrant increased p27 by 1.5-fold and reduced the %S phase from 40% to 18% (Fig. 3C; right). Saracatinib increased p27 2-fold and decreased S-phase from 40% to 27%. Both together increased p27 5-fold and S-phase fell significantly to 8%. The reductions in mean %S by fulvestrant or saracatinib alone compared with combination were both significant at P < 0.0001 (44). Drug interactions were additive but not synergistic (P = 0.102).

In OCI-E1P, a culture established from a primary ovarian adenocarcinoma, both ER and pSrc were lower than in BG-1 and PEO1R (Fig. 3D, left). E1P showed a modest reduction in %S phase from 20% to 16% and 15% after fulvestrant or saracatinib alone, respectively. Both drugs together had a greater effect than either alone, reducing S-phase by 50%. Pairwise comparison between saracatinib and fulvestrant was significant (44, P < 0.002), with an additive but not synergistic effect (P = 0.395) between drugs (Fig. 3D, right). The efficacy of combined ER and Src inhibition in this primary ovarian cancer culture further supports its potential for therapeutic use in patients.

**Effects of Src knockdown together with fulvestrant**

Saracatinib inhibits Abl and multiple Src family members (24). To test the specific contribution of Src to saracatinib effects, PEO1R was stably transduced with shRNA to knockdown Src (shSrc; Fig. 3E, left) and effects of fulvestrant assayed. Although saracatinib modestly reduced proliferation (Fig. 3A), loss of Src alone had no cell-cycle effect compared with control; thus, inhibition of other Src family members seems to contribute to saracatinib effects (Fig. 3E, middle). Despite little effect of either Src knockdown or fulvestrant alone, treatment of the shSrc cells with fulvestrant reduced proliferation with a one-third decrease in S-phase from 27% to 20%. This was of borderline significance (P = 0.06) by multiple comparison analysis (ANOVA). However, comparison using 2-group t test was significant (P = 0.0001). Thus, loss of Src modestly augments effects of fulvestrant.

Quantitative PCR (Q-PCR) in parental versus shSrc-transduced PEO1R showed a compensatory increase of Src family members, Yes, Fyn, and Lyn expression after Src knockdown (Fig. 3E, right). Because saracatinib alone had a greater effect than Src downregulation, the antiproliferative effects of saracatinib are likely due in part to its inhibition of other Src family members.

**Analysis of ER protein and Src activation in primary human ovarian cancer**

To characterize ER expression in ovarian cancer and to identify ER-targets that might also be Src-regulated, 338 primary ovarian cancer were evaluated by RPPA and gene expression analysis (31). ERα protein was detected in 67% of primary ovarian cancers (228/338) on RPPA analysis. Comparison of TCGA gene expression data from these 338
primary ovarian cancer (31) with RPPA data showed ERα mRNA and protein levels were highly correlated \( P < 0.0001 \). To identify putative ER-targets that might be regulated by ER/Src cross-talk, we first evaluated Src activation. Within the ER-positive primary tumors, Src expression and pY416Src levels varied. The 25% of samples with the highest ERα expression quartile defined “low ERα” and the lowest expression quartile defined “low pSrc.” A total of 1,517 genes were upregulated in 25% of samples with the highest pY416Src (“high pSrc”) versus those in the lowest pY416Src level quartile. Among 632 genes preferentially expressed in “high ERα” ovarian cancer, 111 of those genes were also upregulated in “high pY416Src” tumors. In silico analysis revealed 60% of these have one or more estrogen response element (ERE) in their promoters, a marked excess compared with the expected frequency based on overall frequency of ERE (Supplementary Table S1).

**ER and Src inhibition both impair estrogen-induced ER target gene expression**

Few ER-target genes have been defined in ovarian cancer (5, 43). c-MYC, which bears a partial ERE promoter motif, was strongly expressed in both “high ERα” and “high pSrc” tumor groups and was chosen for further analysis. Cells

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Figure 3. Effects of combined ER and Src inhibition on signaling and cell-cycle regulators. A to D, PEO1R, BG-1, and OC-E1P were treated with 10\(^{-6}\) mol/L Fulv (F), 10\(^{-6}\) mol/L saracatinib (S), or both for 48 hours, followed by cell cycle and protein analysis. A, PEO1R cell-cycle distribution after 48 hours +/- drug. \( ^* \) \( P < 0.0001 \) for SI versus both; PEO1R showed synergism between the 2 drugs (\( P < 0.0001 \), right, mean \% S-phase). B, Western blot analysis for ER, total and activated Src, and p27 (left). Cyclin E was precipitated and associated proteins blotted (middle). Cyclin E was strongly expressed in both “high ERα” and “low pSrc” tumors.

B, Cyclin E IP

Cyclin E–Cdk2 activity

BG-1

**Src Inhibition Reverses Antiestrogen Resistance in Ovarian Cancer**

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were deprived of serum and E2 (PEO1R) or E2 only (BG-1) for 48 hours and then treated with E2 alone with or without saracatinib, fulvestrant, or both for 6 hours. In PEO1R, E2 induction of c-MYC was partially inhibited by Fulv (P = 0.02 vs. both), and saracatinib (P = 0.01 vs. both) alone, but was reduced to below E2-deprived control by both together (P = 0.002 both vs. control, Fig. 4A). Drug effects were synergistic (P = 0.0019). Similarly, in BG-1, c-MYC expression was inhibited more by dual therapy than either drug alone (Fig. 4B). Dual therapy also decreased another ER-target gene, FOSL (5) more than either drug alone (Fig. 4C).

**Src inhibition in combination with ER blockade causes autophagy and apoptosis**

Anticancer drug efficacy can result from antiproliferative effects or cell death. Autophagy and apoptosis markers were assayed after 48 hours of saracatinib (10^{-8} M), fulvestrant (10^{-8} M), or both. Autophagy is a survival mechanism, but under prolonged stress, leads to cell death (44). Autophagic cells form intracellular vesicles where microtubule-associated protein light chain 3-1 (LC3-I) is cleaved to LC3-II (45). LC3-II levels in PEO1R were unaffected by fulvestrant, increased 2-fold with saracatinib, and more than a 4-fold with both, suggesting induction of autophagy (Fig. 5A).

To further evaluate autophagy, drug-treated PEO1R and BG-1 were labeled with Cyto-ID-Green autophagy dye, and autophagic lysosome fluorescence was assayed by flow cytometry analysis (46). Fulvestrant did not increase autophagic vesicle formation in either line. Saracatinib alone modestly increased autophagic vesicles. Autophagic vesicles increased significantly by 2-fold in PEO1R and 4-fold in BG-1 with both drugs compared with saracatinib alone (Fig. 5B and C, P < 0.0007 and P < 0.0006, respectively).

Drug effects on apoptosis were assayed by blotting poly (ADP-ribose) polymerase (PARP-1) cleavage products (47). PARP cleavage was not affected by fulvestrant but increased 3- to 4-fold with saracatinib (Fig. 5D), with no further increase with both drugs together. Thus, apoptosis contributes to saracatinib-mediated PEO1R growth inhibition but this is not augmented by fulvestrant. Evaluation of early and late effects on Annexin-V, another apoptosis marker, confirmed that saracatinib alone induced apoptosis (*, P = 0.005 SI vs. Ctrl) and the combination had little additional effect (**, P = 0.533 both vs. SI; Fig. 5E,F). Notably, no drugs caused caspase-3 and -9 cleavage in PEO1R (not shown).
Combined ER and Src blockade inhibits xenograft growth more effectively than monotherapy

We next examined if fulvestrant resistance could also be reversed by saracatinib in vivo. To approximate the peritoneal location of primary ovarian cancer, PEO1R cell pellets were implanted under the renal capsule of nude mice (34). Therapy was initiated 11 days after implantation with either fulvestrant 5 mg s.c./week, oral saracatinib 25 mg/kg daily or both, and tumor bioluminescence measured over 17 weeks (Fig. 6A). Analysis of photon flux growth curves using repeated measures modeling showed significant differences for combined treatment versus either fulvestrant (diff = −0.00018, P < 0.0001), saracatinib (diff = −0.00049, P < 0.0001), or control (diff = −0.00055, P < 0.0001). Tumor growth (photon flux) was minimally affected by saracatinib alone, slowed by fulvestrant, and declined dramatically with combination therapy between weeks 12 to 17 reflecting tumor regression. Although saracatinib alone showed
modest effects in vitro, drug resistance emerged rapidly with monotherapy in vivo. Animal weights increased gradually in all groups indicating good drug tolerance (see Supplementary Fig. S2).

In a second experiment, drugs were started on day 21 after implantation when tumors were more established. To optimize detection of combined drug effects, the fulvestrant dose was lowered from 5 to 3.5 mg/week. Saracatinib dose was not changed. Tumors were harvested at 11 weeks. At the lower fulvestrant dose, only combination drug treatment significantly decreased tumor growth (ANOVA comparison of means, $P = 0.04$; Fig. 6B). Individual drug effects were not significant.

**Discussion**

Despite advances in understanding of signaling pathways driving ovarian carcinogenesis, the promise of targeted therapy is yet to be realized. Prior meta-analysis showed more than 60% of ovarian cancers express ER (8), and this was confirmed in a single large cohort of 338 primary ovarian cancer in the present study. Although ER expression in ovarian cancer is similar to breast cancer, efforts to target the ER therapeutically have shown limited efficacy, yielding short term benefit in 11% to 38% ovarian cancer (9, 10). Unfortunately, most trials have been small, not limited to ER+ disease, failed to correlate response with ER status and involved patients extensively treated with chemotherapy. In breast cancer, the benefit of ER blockade is limited to ER+ tumors (7). Thus, the potential efficacy of antiestrogens in ovarian cancer may be underestimated. Understanding mechanisms of antiestrogen resistance may identify targets whose inhibition could restore drug responses and provide new treatment options for ovarian cancer.

As observed herein, Src is activated in the majority of ovarian cancers (17). While they have many mitogenic effects, Src family kinases act ubiquitously to phosphorylate the Cdk inhibitor p27 to promote its degradation (14, 15). p27 is required for G1 arrest by antiestrogens (12, 22). Src inhibition by dasatinib also decreased proliferation by increasing p27 in ovarian cancer (48). Because p27 is required for growth inhibition by ER blockade (12), and since Src activates p27 proteolysis (15), we tested if the Src family kinase inhibitor saracatinib (24) could restore fulvestrant efficacy in resistant ER+ ovarian cancer. Here, we provide novel evidence for estrogen-stimulated ER cross-

![Figure 6. Effects of fulvestrant and saracatinib on ovarian cancer xenografts. PEO1RLUC+ was implanted under the renal capsule of estrogen-supplemented nude mice. A, drug initiated day 11 with Fulv (5 mg/week), saracatinib (25 mg/kg), or both. Tumor growth is graphed as mean normalized photon flux over time (± SEM). B, in a second in vivo experiment, treatments (Fulv 3.5 mg/week, saracatinib 25 mg/kg; both) were initiated at week 3; tumors were harvested week 11. Mean normalized photon flux at week 11 is graphed ±SEM for each; *, $P = 0.04$ versus control. Photographs show representative tumors.](image-url)
Src Inhibition Reverses Antiestrogen Resistance in Ovarian Cancer

talk with Src in ovarian cancer and that estrogen drives Src activation in the models tested. Moreover, we provide provocative in vitro and in vivo data indicating the potential for therapeutic efficacy of combined fulvestrant and saracatinib in ovarian cancer.

Our analysis of 338 primary ovarian cancer confirmed that most (67%) have detectable ER protein and show a range of pY416Src and total Src levels. Notably, approximately 60% of 111 genes preferentially expressed in primary ovarian cancer with the highest ER and pY416Src expression quartiles contained ERE motifs, supporting relevance of Src to ER action. Src activation in ER+PEO1R and BG-1 was greater than in benign ovarian epithelial lines.

Steroid hormones binding to ER, have been shown to activate Src in breast epithelial cells (16, 49). While ERα and ERβ are present in both ovarian cancer lines studied (5, 50), E2-stimulated proliferation was mediated by ERα alone. Antiestrogens have been shown to block ERα gene expression in ovarian cancer (5). Here, we show for the first time in ovarian cancer that estrogen rapidly and transiently activates Src, Src-ER binding and their colocalization in the perinuclear cytoplasm, followed by ER translocation to the nucleus. These rapid nongenomic ER-Src actions may be linked to ER-mediated transcription, as inhibition of estrogen-stimulated Src by saracatinib downregulated ER action at 2 ER-target genes: FOSL1 and c-MYC. Notably, this was greater with combined ER blockade and saracatinib than either alone. Thus, dual therapy may attenuate mitogenic ER target gene activation, contributing to the greater antiproliferative and antitumor efficacy observed with the combination over monotherapy.

Combined Src and ER blockade also caused a more significant growth arrest, with greater increases in p27 and cyclin E-Cdk2-bound p27, than observed with monotherapy in 2 ER+ ovarian cancer lines. Greater growth inhibition by combined Src and ER blockade was confirmed in a primary ovarian cancer tumor culture, E1P. In PEO1R, saracatinib and fulvestrant synergistically inhibited cell cycle and cyclin E-Cdk2, yet in BG-1 and E1P lines, drug effects were additive. This may be due to higher Src levels/activity observed in PEO1R compared with BG-1 and E1P. These findings are consistent with recent reports showing Src inhibition augments antiestrogen effects in breast cancer models (23, 51). Our ability to grow tumor cultures from primary ovarian cancers may allow future prediction of treatment responsiveness to individualize use of targeted therapies.

Saracatinib inhibits multiple Src family members (24, 25). Unlike saracatinib, Src knockdown on its own failed to inhibit proliferation, implicating other Src family members in the observed drug effects. However, while neither fulvestrant alone nor shSrc inhibited cell cycle, loss of Src did cooperate with ER blockade to impair cell-cycle progression, suggesting that Src and ER signaling both drive proliferation in this ER+ ovarian cancer model.

Combined Src and ER blockade may impair tumor growth not only through antiproliferative effects, but also via autophagy. Autophagy is induced by adverse conditions, and when sustained, may lead to type II programmed cell death (44, 52). Fulvestrant alone did not induce autophagy, consistent with its known cytostatic effect. Interestingly, while LC3-II and autophagic vesicle fluorescence rose with saracatinib alone, combination therapy had a significantly greater effect. Thus, autophagic cell death via type II apoptosis is activated by Src inhibition and augmented by dual ER and Src blockade. In contrast, PARP cleavage and Annexin-V staining, observed with saracatinib, were not significantly increased by dual therapy suggesting combined therapy does not increase apoptosis via these pathways. Notably, the increase in Annexin-V staining by saracatinib was not accompanied by caspase-3 or -9 cleavage. Annexin-V staining reflects membrane phosphatidylserine (PS) exposure. While this is commonly activated by caspase-9 and -3, it can occur via alternate mechanisms (53). Src inhibitor–induced autophagy and apoptosis may both contribute to saracatinib-mediated antitumor efficacy (54).

We have characterized a new model for the study of antiestrogen resistance in ovarian cancer. The PEO1R model is antiestrogen resistant but retains estrogen sensitivity in vitro and in vivo. PEO1R xenografts required estrogen for growth in vivo. Ovarian cancer spreads by peritoneal dissemination. Implantation of luciferase tagged PEO1R under the renal capsule allows highly sensitive and quantitative monitoring of ovarian xenograft growth in its natural environment adjacent to the ovary (54). While short-term saracatinib was effective in vitro, resistance rapidly emerged in vivo. Estrogen-driven Src activation, recruitment of other mitogenic pathways, and upregulation of other Src family kinases may all contribute to failure of saracatinib alone. It is noteworthy that bypass activation of MEK and PI3K/mTOR pathways was observed with prolonged saracatinib monotherapy in breast cancer culture and xenografts (22, 23).

Notably, when combined with fulvestrant, saracatinib resistance did not emerge in ovarian cancer xenografts. While most targeted therapies reduce the rate of tumor growth, combined saracatinib and fulvestrant caused tumor regression. The late regression may reflect induction of autophagy leading to late loss of tumor viability. The greater antitumor efficacy of dual therapy may result from more effective inhibition of estrogen-activated gene expression, more profound cell-cycle inhibition, and greater cell death.

Although clinical data suggest ovarian cancer is largely unresponsive to hormonal therapies, our data raise the provocative possibility that dual ER and Src blockade may prevent or reverse antiestrogen resistance in this disease. These data support further preclinical evaluation with the ultimate goal of clinical investigation of this drug combination.

Disclosure of Potential Conflicts of Interest

G.B. Mills has a commercial research grant from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Src Inhibition with Saracatinib Reverses Fulvestrant Resistance in ER-Positive Ovarian Cancer Models *In Vitro* and *In Vivo*

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