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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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Abstract

Purpose—More effective, less toxic treatments for recurrent ovarian cancer (OVCA) are needed. Although >60% of OVCA 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. Since Src is activated in most OVCA, 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 OVCA. Dual ER and Src blockade effects on cell cycle, ER target gene expression, and survival were assayed in ERα+ OVCA lines, a primary human OVCA culture *in vitro*, and on xenograft growth.

Results—Most primary OVCA express ER. Src activity was greater in OVCA lines than normal epithelial lines. Estrogen activated Src, ER-Src binding and ER translocation from cytoplasm to nucleus. Estrogen mediated mitogenesis was via ERα, not ERβ. While each alone had little effect, combined saracatinib and fulvestrant increased p27, inhibited cyclin E-Cdk2 and cell cycle progression. Saracatinib also impaired induction of ER-target genes Myc and FOSL1; this was

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greatest with dual therapy. Combined therapy induced autophagy and more effectively inhibited OVCA 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 ER+ OVCA models. These data support further pre-clinical and clinical evaluation of combined fulvestrant and saracatinib in OVCA.

Keywords

Ovarian cancer; ER; fulvestrant; Src inhibitor; cell cycle; p27

Introduction

Ovarian cancer (OVCA) is the most lethal gynecological 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 OVCA. E2 drives proliferation via estrogen receptor α (ER α) and antiestrogens inhibit OVCA growth *in vitro* and *in vivo* (2-5). Data on ER β in OVCA is limited. OVCA lines and primary OVCAs express lower ER β than normal ovary and its loss correlates with malignant transformation (5, 6). ER α , hereafter ER, is expressed in OVCAs at levels similar to those in breast cancer (7). A meta-analysis (n=2500 patients) showed 67% ER protein expression in primary OVCAs (8). Despite high ER expression in OVCAs, numerous small trials of ER-blockers have been disappointing (9, 10). Tamoxifen, used as second-fourth line treatment for recurrent OVCA (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, heavily pre-treated recurrent OVCA (n=26) (10). To date, nine aromatase-inhibitor trials (total 229 patients), have yielded response rates from 0–35.7% and recurrent disease stabilization in 20–42% (11). OVCA ER expression was not routinely assayed in these trials.

In summary, therapies that block pro-tumorigenic estrogen effects in OVCA have not been studied in well-designed trials that consider tumor ER status. OVCAs manifest frequent *de novo* antiestrogen resistance and the 11-35% 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 (cdks) 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 OVCA (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 OVCA therapies. Mitogenic pathways, including Src, Ras/Raf/MEK and PI3K/AKT, are frequently activated in OVCA (17). Src is over-expressed or activated in most OVCAs and regulates proliferation and apoptosis (18-20). In E2 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 OVCA (NCT00610714; NCT01196741), but has not been evaluated with antiestrogens in OVCA.

Here, we investigated the hypothesis that constitutive Src expression contributes to resistance to ER-blockade in OVCA. We generated an ER-blocker resistant OVCA 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 OVCA. Dual targeting of ER and Src more effectively inhibits ER-target gene expression and cell cycle progression *in vitro*, and OVCA tumor growth *in vivo*. These data support further pre-clinical 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} M) and fulvestrant (Fulv) (10^{-6} M) were dissolved in DMSO and ethanol, respectively (Astra Zeneca). Saracatinib did not exceed 10^{-6} M to avoid off target effects. The pure ER α agonist 4,4',4''-[4-propyl-(1H)-pyrazole-1,3,5-triyl]tris-phenol (PPT), the selective ER β -agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), the dual ER α agonist/ER β antagonist (R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (THC) and the ER β -antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) were obtained from Tocris.

Cell culture

PEO1 OVCA line and human ovarian surface epithelium (HOSE) cultures (X. Xu, U of Miami, FL), BG-1 (E. Denholm, NIEHC, NC) and a spontaneous antiestrogen resistant variant, PEO1R established after prolonged passage, were cultured in RPMI with 10% FBS. 293T cells were from ATCC. Lines were authenticated using ATCC guidelines. OCI-E1P (ovarian-carcinoma-Ince-endometrioid-primary-1) was cultured from a primary ER+ endometrioid OVCA and used within 6-8 passages. Asynchronous cultures were treated with vehicle, 10^{-6} M saracatinib, 10^{-6} M 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 ER α and ER β agonists/antagonists.

Immunofluorescence (IF) Microscopy

Cells (3×10^5) were serum/E2 deprived 48hrs, then treated with 10^{-8} M E2 for indicated times. IF used anti-ER α (Abcam) or anti-pSrc (Cell Signalling) and secondary antibody Ab as described (27). Cells were 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 hrs and then treated with E2 (10^{-8} M) for 4 hrs. Fractionation was as described (28)

Cell cycle analysis

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

AnnexinV staining

Drug treated cells (48 hrs) were analyzed by FITC AnnexinV Apoptosis Detection Kit I (BD Biosciences) per manufacturer.

Detection of autophagic vesicles

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

Plasmids and transfection

PEO1R were transduced with lentivirus carrying pLV411G effLuc-flag (IRES-hrGFP) as described (29). PEO1R were transduced with lentivirus encoding a Src specific shRNA (5'-CAGATTGTCAACAACACAG3-3') or control shRNA (Open Biosystems, RHS4430-101028217 and RHS4346, respectively) and selected with puromycin.

Immunoblotting, immunoprecipitation and kinase assay

Westerns were performed and quantitated by densitometry as described (12). Cyclin E antibody (HE-12, Harlow Lab (MGH)); ER α , Cdk2, MAPK, pMAPK antibodies were from Santa Cruz; p27 from BD transduction; Akt, pAkt, Src and pSrc, LC3, 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 phosphorimager from four assays.

ER-Src complexes were precipitated after 48hrs of serum/E2 deprivation and at intervals after E2 repletion and Src and ER detected by Western as described (27).

RNA Extraction and Real-time RT PCR

RNA was prepared using TRIzol® (Invitrogen). 0.5ug total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad, CA). Real-time PCR for c-Myc used (Forward-5'-GAGTCTGGATCACCTTCTGCTG-3' and reverse 5'-AGGATAGTCCTTCCGAGTGGAG-3'; FOS-L (forward-5'-CAGGCGGAGACTGACAACTG-3' and reverse-5'-TCCTTCCGGGATTTGCAGAT-3'); and 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 OVCA from The Cancer Genome Atlas (TCGA) project as described (31). RPPA data processing used SuperCurve (Coombes, K., et al., 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 MIAME guidelines of the Microarray Gene Expression Data Society as in (32). The TCGA microarray data set used is available on line at <http://cancergenome.nih.gov/cancersselected/ovarian>.

PEO1R xenografts

Luciferase-positive PEO1R^{LUC+} cells (10⁶) 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 0.72 mg/90 day (Innovative Research). In subsequent experiments, E2 supplemented animals (10/group) received either no treatment, fulvestrant 3.5mg/week, fulvestrant 5mg/week, saracatinib

25mg/kg daily or both via oral gavage 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/week. Tumors were removed 75 days post-implantation or when morbidity required euthanasia per UM Animal care procedures.

Statistical analysis

All assays of cell cycle distribution, cyclin E-Cdk2 kinase, IF and IP/Westerns were done at least thrice. Xenograft studies were repeated twice. Data were summarized as mean $\pm$ SEM by treatment group and displayed in bar graphs. One- or two-way analysis of variance was conducted to assess difference among treatment means. For 2 $\times$ 2 factorial experiments, interaction of the two factors was tested. A significant interaction is a statistical indication of synergism, meaning that the combined effect of two agents is manifested in a non-additive manner. Following a significant ANOVA result ($p < 0.05$) rejecting the null hypothesis that means are the same across the treatment groups, the Tukey's honestly significant difference (HSD) test was used for all pairwise mean comparisons. This multiple comparison procedure ensures actual family-wise error rates no greater than pre-specified 5%. Analyses were performed in SAS 9.3.

Results

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

PEO1R, a variant of the OVCA ascites PEO1 line (3), developed antiestrogen resistance during prolonged culture. BG-1, a primary high grade OVCA line, is antiestrogen sensitive (35). Both PEO1R and BG-1 express ER protein. In contrast, ER was not detected in four normal human ovarian surface epithelium (HOSE) lines (representative data, Fig. 1A). While prior work showed mitogenic kinase activation in ER negative OVCA 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 to HOSE, while 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 OVCA. To investigate Src involvement in E2-stimulated OVCA proliferation, PEO1R and BG-1 were E2 and serum deprived in media with 0.1% cFBS for 48 hrs and then E2 (10^{-8} M) treated for times indicated. Estrogen rapidly activated Src in both (Fig. 1B upper panel), and ER-bound activated Src was transiently detected in ER immunoprecipitates 15 minutes after E2 addition (Fig 1B lower panel). ER and Src co-localize in the perinuclear cytoplasm by immunofluorescence microscopy at 15 min. (Fig1C). Thus, E2 stimulates ER/Src binding and Src activation, which may contribute to its mitogenic effects.

Subcellular fractionation and immunofluorescence showed ER was strongly cytoplasmic in E2 deprived cells, but shifted into the nucleus 4 hrs 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 OVCA lines express cytoplasmic ER in the absence of ligand, which translocates to the nucleus on E2 addition.

E2 stimulates proliferation in OVCA cells via ER α 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 hrs of E2 depletion alone (10% cFBS), but estrogen and serum depleted media (0.1% cFBS) for 48hrs reduced the %S phase from 50% to 15% (Fig 2A). Notably, addition of E2 alone (10^{-8} or 10^{-9} M) prevented 0.1% cFBS-induced growth arrest over 48 hrs, with 50% of cells remaining in S phase (E2 10^{-8} M shown). Thus, estrogen alone maintains proliferation in the absence of growth factors in PEO1R.

To assay further the ability of E2 to drive PEO1R proliferation, cells were arrested in 0.1% cFBS for 48 hrs. Addition of 10^{-8} M estradiol alone, without serum, stimulated cell cycle re-entry with % S phase peaking at 20hrs (Fig. 2B, see also Supplemental Fig 1).

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} M) maintained proliferation at 48% S phase (E2 10^{-8} M shown) (Fig 2C). %S phase pairwise comparisons in the presence or absence of estrogen (*and ‡ were significant at $p < .0001$ for both PEO1R and BG-1.

Since E2 and fulvestrant both bind ER α and ER β , 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 hrs prior to cell cycle analysis (Fig 2A,C). The pure ER α agonist, PPT (10^{-8} and 10^{-9} M) (5, 39), and the ER α agonist/ER β antagonist THC (10^{-6} M) (40), both rescued E2 deprivation in these lines. Furthermore, the ER β agonist, DPN at 10^{-9} M (5, 41) failed to induce proliferation and the pure ER β antagonist PHTPP (42) added with E2 did not abrogate E2 action (Figs 2A,C). The mean %S phase did not differ significantly between asynchronous controls and cells maintained with added E2, PPT, THC or PHTPP (^), nor did they differ between DPN treated and E2 deprived cells (+) in PEO1R or BG-1 (Fig2A,C). Thus, mitogenic effects of E2 in these OVCA models are mediated by ER α and not dependent on ER β .

Since E2 alone could drive cell cycle re-entry *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 >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 estrogen rapidly activates Src, which phosphorylates p27 to promote p27 proteolysis (15). Since estrogen activated Src in these OVCA 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 hrs of fulvestrant (10^{-6} M). Saracatinib (10^{-6} M) alone modestly decreased the %S phase from 50% to 29%. However, combined Src and ER-blockade significantly decreased the %S phase to 10% compared to 30% with the Src inhibitor alone (* $p < 0.0001$) (Fig 3A, left). In PEO1R, a synergistic effect on %S between the two drugs was indicated by a significant interaction ($p = .008$). 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 [$21.97 = \text{Fulv} (0.6) + \text{saracatinib} (21.36)$] (Fig 3A right panel).

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 three-fold; while both together increased p27 by over 6-fold in PEO1R (Fig3B-left panel). After 48 hrs 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 panel). 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 panel). 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 panel). Saracatinib increased p27 two-fold and decreased S phase from 40 to 27%. Both together increased p27 five-fold and S phase fell significantly to 8%. The reductions in mean %S by fulvestrant or saracatinib alone compared to combination were both significant at $p<0.0001$ (*). Drug interactions were additive 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 combination was significant ($*p<0.002$), with an additive 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 utility 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 to control, thus inhibition of other Src family members appears 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 two-group t-test was significant ($p=.0001$). Thus, loss of Src modestly augments effects of fulvestrant.

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). Since 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 OVCA

To characterize ER expression in OVCA and to identify ER-targets that might also be Src-regulated, 338 primary OVCA were evaluated by RPPA and gene expression analysis (31). ER α protein was detected in 67% of primary OVCA (228/338) on RPPA analysis. Comparison of TCGA gene expression data from these 338 primary OVCA (31) with RPPA data showed ER α 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 ER α mRNA were defined as “High ER α ” and the lowest expression quartile defined “Low ER α ”. 1517 genes were upregulated in the 25% of samples with the highest pY416Src (“High pSrc”) versus those in the lowest pY416 Src level quartile. Among 632 genes preferentially expressed in “High ER α ” OVCA, 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 to the expected frequency based on overall frequency of ERE (Supplementary Table 1).

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

Few ER-target genes have been defined in OVCA (5, 43). *c-MYC*, which bears a partial ERE promoter motif, was strongly expressed in both “High ER α ” and “High pSrc” tumor groups and was chosen for further analysis. Cells were deprived of serum and E2 (PEO1R) or E2 only (BG-1) for 48 hrs and then treated with E2 alone with or without saracatinib, fulvestrant or both for six 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). The estrogen-stimulated cell cycle re-entry of E2 starved cells was also partly inhibited by each drug, but most profoundly reduced by both together ($p<0.0001$ for SI+PPT or Fulv+PPT vs both+PPT) (shown for PEO1R, Fig 4D).

Src inhibition in combination with ER blockade causes autophagy and apoptosis

Anti-cancer drug efficacy can result from anti-proliferative effects or cell death. Autophagy and apoptosis markers were assayed after 48 hrs of saracatinib (10^{-6} M), fulvestrant (10^{-6} 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-I (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 (46). Fulvestrant did not increase autophagic vesicle formation in either line. Autophagic vesicles increased significantly by 2-fold in PEO1R and 4-fold in BG-1 with both drugs compared to saracatinib alone (Figure 5B, C, $p=0.0007$ and $p<0.006$, 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 3-4-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 AnnexinV, another apoptosis marker, confirmed that saracatinib alone induced apoptosis ($*p=0.005$ SI vs Ctrl) and the combination had little additional effect ($^{\wedge}p=0.533$ both vs SI) (Fig E,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 OVCA, PEO1R^{luc+} cell pellets were implanted under the renal capsule of nude mice. Therapy was initiated eleven days after implantation with either fulvestrant 5mg 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<.0001), saracatinib (diff=-0.00049, p<.0001), or control (diff=-0.00055, p<.00011). Tumor growth (photon flux) was minimally affected by saracatinib alone, slowed by fulvestrant and declined dramatically with combination therapy between weeks 12-17 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 Figure 2).

In a second experiment, drugs were started on day 21 post 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 >60% of OVCA express ER (8), and this was confirmed in a single large cohort of primary OVCA in the present study. Although ER expression in OVCA is similar to breast cancer, efforts to target the ER therapeutically have shown limited efficacy, yielding short term benefit in 11-38% OVCA (9, 10). Unfortunately, most trials have been small, not limited to ER+ disease, failed to correlate response with ER status and were in 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 OVCA may be underestimated. Understanding mechanisms of antiestrogen resistance may identify targets whose inhibition could restore drug responses and provide new treatment options for OVCA.

As observed herein, Src is activated in the majority of OVCA (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 OVCA (48). Since 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+ OVCA. Here, we provide novel evidence for estrogen-stimulated ER cross-talk with Src in OVCA 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 OVCA.

Our analysis of 338 primary OVCA 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 OVCA 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 ER α and ER β are present in both OVCA lines studied (5, 50), E2-stimulated proliferation was mediated by ER α alone. Antiestrogens have been shown to block ER α gene expression in OVCA (5). Here we show for the first time in OVCA that estrogen rapidly and transiently activates Src, Src-ER binding and their co-localization in the perinuclear cytoplasm, followed by ER translocation to the nucleus. These rapid non-genomic ER-Src actions may be linked to ER-mediated transcription, since inhibition of estrogen-stimulated Src by saracatinib downregulated ER-action at two ER-target genes: *FOSL* 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 anti-tumor 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 two ER + OVCA lines. Greater growth inhibition by combined Src and ER blockade was confirmed in a primary OVCA tumor culture. 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 to 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 OVCA 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+ OVCA model.

Combined Src and ER blockade may impair tumor growth not only through anti-proliferative 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 AnnexinV 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 AnnexinV staining by saracatinib was not accompanied by Caspase 3 or 9 cleavage. AnnexinV 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 anti-tumor efficacy (54).

We have characterized a new model for the study of antiestrogen resistance in OVCA. The PEO1R model is antiestrogen resistant but retains estrogen sensitivity *in vitro* and *in vivo*. PEO1R xenografts required estrogen for growth *in vivo*. OVCA 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 (34). 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 OVCA 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 anti-tumor 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 OVCA 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 pre-clinical evaluation with the ultimate goal of clinical investigation of this drug combination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Most ovarian cancer patients suffer disease recurrence and available chemotherapies are limited and toxic. Antiestrogen therapies in recurrent ovarian cancer, including fulvestrant, have been well tolerated with minimal toxicity, but show <30% clinical benefit and this lasts for only a few months. Efficacy is limited by de novo resistance and by rapid development of resistance to antiestrogens over time. Our data demonstrate that combined targeting of estrogen-ER-driven Src kinase signaling pathways, in addition to ER blockade, has potential to reverse or prevent antiestrogen resistance *in vitro* and *in vivo*. The novel combination of the Src inhibitor, saracatinib and ER blocker, fulvestrant, may offer a new therapeutic option for patients with recurrent ovarian cancer. Present findings will stimulate further preclinical studies that may lead ultimately to clinical validation in trials with potential to impact clinical treatment and prevention of antiestrogen resistant ovarian cancer.

Figure 1.

Estrogen activates Src, ER/Src binding and ER translocation to the nucleus. A, Western shows ERα, total and activated Src, MEK and AKT in PEO1R, BG-1 and HOSE. (B-E) E2(10⁻⁸M) was added to E2 deprived PEO1R or BG-1 at t=0 and cells were recovered at indicated times. B, Westerns show phosphorylated or total Src (top). ERα immunoprecipitates were resolved and immunoblotted for pSrc or ERα (bottom). C, IF shows perinuclear ERα and pSrc co-localization 15 min after E2 addition. D, Sub-cellular fractions were blotted for ER and actin. E, ERα localization in BG-1 at t=0 and after 4 hrs E2.

Figure 2.

E2 stimulates proliferation via ER α and is required for PEO1R xenograft growth. A, PEO1R in 10% FBS were transferred to media with 10% cFBS, 0.1% cFBS alone or 0.1% cFBS together with E2 (10^{-9} M), PPT (10^{-9} M), THC (10^{-6} M), DPN (10^{-9} M), or [E2(10^{-9} M)+PHTPP(10^{-6} M)] for 48hrs and cell cycle distribution analyzed. * $p < .0001$ for 10%FBS vs 0.1% cFBS, ‡ $p < .0001$ for 0.1% cFBS vs E2; ^ $p = ns$ for E2, PPT and THC; + $p = ns$ for DPN vs 0.1% cFBS. B, PEO1R were E2 deprived in 0.1% cFBS for 48hrs (T=0 hrs), then E2 (10^{-8} M) was added and cells recovered for cell cycle analysis at indicated times (see also Supplemental Figure 1). C, BG-1 in 10%FBS were transferred to media with 10% cFBS +/- steroids as above for 48 hrs; * indicates $p < .0001$ for 10%FBS vs 10% cFBS, ‡ $p < .0001$ for 10% cFBS vs E2; ^ $p = ns$ for E2, PPT and THC; + $p = ns$ for DPN vs 10% cFBS. D, PEO1R^{luc+} xenograft growth/time +/- E2 supplementation assayed by IVIS (normalized photon flux). (All graphs shown mean +/- SEM).

Figure 3.

Effects of combined ER and Src inhibition on signaling and cell cycle regulators. (A-C). PEO1R, BG-1, and OC-E1P were treated with 10⁻⁶M Fulv (F), 10⁻⁶M saracatinib (SI) or both for 48 hrs, followed by cell cycle and protein analysis. A, PEO1R cell cycle distribution after 48 hrs +/-drug. * p<0.0001 for SI vs both, PEO1R showed synergism between the two drugs (p<0.0001) (right panel mean %S phase). B, Western for ER, total and activated Src and p27 (left panel). Cyclin E was precipitated and associated proteins blotted (middle panel). Cyclin E precipitates were assayed for kinase activity and radioactivity in histone H1 substrate shown (right panel, top) and graphed as %max activity (right panel, bottom); * p<0.0003 SI vs both. C, BG-1 cell cycle distribution at 48 hrs +/-drug (left, *p<0.0001 Fulv vs both); Western in BG-1 (right). D, Western in asynchronous BG-1, PEO1R, and E1P (left). Cell cycle analysis of E1P +/- 48 hrs drug; * p=.005 SI vs both (right). E, PEO1R cells were stably transduced with shRNA-Src or shRNA-control and treated +/-Fulv (10⁻⁶M) 48 hrs. Western shows Src knockdown (left). Cell cycle distribution graphed, * indicates p=0.06 shSrc vs shSrc+Fulv (middle); QT-PCR for Src, Yes, Fyn and Lyn in PEO1R +/- Src knockdown. All data are graphed as mean +/-SEM (right).

Figure 4.

Saracatinib and fulvestrant impair estrogen induced ER α target gene activation and cell cycle re-entry. (A-C) After 48hrs E2 deprivation in 0.1% cFBS for PEO1R and 10% cFBS for BG-1, cells were treated with E2 (10^{-8} M) +/- drug for 6hrs. *c-MYC* expression A, - in PEO1R, B, in BG-1 and C, *FOSL* in BG-1 were assayed by Q-PCR and graphed as mean fold-change vs control +/- SEM. For *c-MYC* in PEO1R, * $p=0.01$ for E+SI vs both+E and ^ $p=0.02$ for E+Fulv vs E+Both. For *c-MYC* in BG-1, * $p<0.001$ for E+SI vs E + Both and ^ $p=0.08$ for E+Fulv vs E+Both. For *FOSL* in BG-1, * $p=0.03$ for E+SI vs E+Both and ^ $p=0.001$ for E+Fulv vs Both+E. D, Cell cycle distribution after 48 hrs in media with 0.1% cFBS, PEO1R followed by addition of E2 (10^{-9} M), or PPT (10^{-9} M) alone or with indicated drugs for 20 hrs; * $p<0.0001$ for SI+PPT vs PPT+Both and ^ $p<0.0001$ for Fulv+PPT vs PPT+Both.

Figure 5.

Effects of ER and Src inhibition on apoptosis and autophagy. PEO1R and BG-1 were treated with 10^{-6} M Fulv (F), 10^{-6} M saracatinib (SI) or both drugs for 48 hrs. A, Western of LC3-II +/- drug. Lane 1 shows paclitaxel-treated 293T as positive control, lanes 2-5 PEO1R +/- indicated drugs. B, Profiling of Cyto-ID autophagy as histogram-overlays. C, Bar graph of % shift in Cyto-ID fluorescence (+/-SEM) from (B), indicates increase in autophagic vesicles *indicates $p < 0.0007$ (PEO1R) for SI and both vs control and $\ddagger p < 0.006$ (BG-1) for SI and both vs control. D, Western of PARP and cleaved PARP +/- drug; lane 1 shows 293T/taxol control, lanes 2-5 PEO1R +/- indicated drugs. E, AnnexinV labeling of PEO1R for early/late apoptosis by flow cytometry +/-drug (% AnnexinV+ cells shown). F. Percent cells with early/late AnnexinV staining +/-SEM from (E); * $p=0.005$ Ctrl vs SI; ^ $p=0.533$ SI vs both for early apoptosis; late apoptosis all treatments $p>0.05$.

Figure 6.

Effects of fulvestrant and saracatinib on OVCA xenografts. PEO1R^{LUC+} was implanted under the renal capsule of estrogen supplemented nude mice. A, Drug initiated day 11 with Fulv (5mg/week), saracatinib (25mg/kg) or both. Tumor growth is graphed as mean normalized photon flux over time ($\pm$ SEM). B, In a second *in vivo* experiment, treatments (Fulv 3.5 mg/week, saracatinib 25mg/kg; both) were initiated at week 3; tumors were harvested week 11. Mean normalized photon flux at week 11 is graphed $\pm$ SEM for each; * indicates $p=0.04$ vs control: photographs show representative tumors.