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PRECLINICAL STUDY

Combined Src and ER blockade impairs human breast cancer proliferation in vitro and in vivo

Yi Chen · Edwin A. Alvarez · Diana Azzam · Seth A. Wander · Natalia Guggisberg · Mercè Jordà · Zhenlin Ju · Bryan T. Hennessy · Joyce M. Slingerland

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Abstract Antiestrogen therapies arrest susceptible estrogen receptor (ER)-positive breast cancers by increasing p27. Since Src phosphorylates p27 to promote p27 proteolysis, Src activation observed in up to 40% of ER-positive cancers may contribute to antiestrogen resistance. In this article, we show that treatment with the Src-inhibitor saracatinib (AZD0530) together with ER-blocking drugs increased breast cancer cell cycle arrest via p27. Saracatinib and fulvestrant together more effectively increased p27, reduced Ki67, and impaired MDA-MB-361 xenograft tumor growth in vivo than either of the drugs alone. In contrast, saracatinib monotherapy rapidly gave rise to drug resistance. Since combined ER and Src inhibition delays development of resistance in vivo, these data support further clinical investigation of saracatinib in combination with fulvestrant for women with ER-positive breast cancer. Proteomic analysis revealed striking bypass activation of the mTOR pathway in saracatinib-resistant tumors. mTORC1 activation also arose following long-term culture of ER-positive breast cancer lines in the presence of saracatinib. These data indicate the utility of proteomic analysis of drug-resistant tumors to identify potential means of drug resistance. The use of mTOR kinase inhibitors with saracatinib may subvert drug resistance and prove to be more effective than saracatinib alone.

Keywords Fulvestrant · Tamoxifen · Src inhibitor · Cell cycle · p27 · Breast cancer

Introduction

Estrogen regulates breast cancer development and growth. Hormonal therapies that inhibit estrogen effects on ER-positive human breast cancers include ER-blocking drugs,

Y. Chen · E. A. Alvarez · D. Azzam · S. A. Wander · N. Guggisberg · M. Jordà · J. M. Slingerland (✉)
Braman Family Breast Cancer Institute, University of Miami Sylvester Comprehensive Cancer Center, 1580 NW 10th Avenue, Miami, FL 33136, USA
e-mail: jslingerland@med.miami.edu

Y. Chen (✉)
Division of Antitumor Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai 201203, P. R. China
e-mail: ychen@jding.dhs.org

D. Azzam · J. M. Slingerland
Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, Miami, FL, USA

M. Jordà
Department of Pathology, University of Miami Miller School of Medicine, Miami, FL, USA

S. A. Wander
Cancer Biology Graduate Program, University of Miami Miller School of Medicine, Miami, FL, USA

Z. Ju · B. T. Hennessy
Department of Systems Biology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

B. T. Hennessy
Gynecological Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX, USA

J. M. Slingerland
Department of Medicine, University of Miami Miller School of Medicine, Miami, FL, USA

tamoxifen and fulvestrant (Faslodex or ICI 182780), and the aromatase inhibitors (AIs) which inhibit peripheral conversion of adrenal androgens to estrogens. The ER antagonist fulvestrant mediates receptor degradation and is of value in treatment of tamoxifen-resistant metastatic breast cancer. While metastatic breast cancers frequently respond to fulvestrant, these ultimately develop resistance. The median time to disease progression after fulvestrant as initial therapy of receptor-positive metastatic disease is only 8.2 months [1], and it is only 4 months when used after AI failure [2]. Thus, fulvestrant resistance is a challenging problem from the clinical perspective.

Antiestrogens mediate a G0/G1 cell cycle arrest in susceptible ER-positive breast cancer cells by increasing p27 [3–8], a key mediator of cell cycle arrest [4, 9]. Estrogens stimulate p27 proteolysis, cyclin E-Cdk2 activation, and cell cycle progression [4]. Antisense inhibition of p27 expression in estradiol-deprived, tamoxifen- or fulvestrant-arrested MCF-7 cells caused cell cycle re-entry [4]. Thus, p27 is a critical mediator of cell cycle arrest by hormonal therapies.

Ligand binding to ERα (hereafter, ER) rapidly activates signal transduction pathways. Ligand-activated ER rapidly recruits Src and Shc, activating MAPK [10–14] and phosphoinositide 3′-kinase signaling [12, 15] to promote quiescent cell re-entry into cycle and to shorten the G1 phase [16]. This non-genomic ER signaling is rapid, transient, and does not require ER-mediated gene expression, or genomic ER action. Given the two-way crosstalk between ER and Src, aberrant activation of either could lead to feed-forward loops to stimulate breast cancer growth.

Molecular targeted drugs may help patients overcome resistance to hormonal therapies. Oncogenic Src kinase activation via the ErbB family, and other cell surface receptors, [17] can mediate resistance to hormonal therapy in breast cancer [9, 18]. Src phosphorylates p27 which promotes p27 proteolysis [19, 20], and Src activation is significantly associated with low p27 protein levels in primary human breast cancers [19]. Thus, Src activation in breast cancer [19], through both estrogen-bound ER [10, 12, 21] and receptor tyrosine kinase action [17, 22], would lead to loss of p27 and impair the therapeutic efficacy of ER blockade.

Saracatinib, (AZD0530) is a potent, orally available specific inhibitor of Src family kinases [23] in Phase II clinical trials for human cancers. It has antiproliferative activity with sub-micromolar IC50 values in several human cancer lines and xenografts [24]. Saracatinib and tamoxifen synergistically inhibit proliferation [18] and anchorage-independent growth [25] in cultured cells. Since growth inhibition by saracatinib is opposed by estrogen, Src inhibitor efficacy might be enhanced by ER blockade.

We recently found that saracatinib, when used together with the aromatase inhibitor anastrozole, reduced drug resistance and showed greater antitumor efficacy than either of the drugs alone in a xenograft model [9]. This study investigated the effects of ER blockade together with saracatinib on xenograft tumor growth and used proteomic analysis of resistant tumors to identify mechanisms underlying drug resistance.

Materials and methods

Chemicals

Saracatinib and fulvestrant were provided by Astra Zeneca, and tamoxifen was purchased from Sigma (St. Louis, MO, USA). Vehicle controls had no effect on analyses done.

Cell culture

ER-positive breast cancer cell lines, BT-474 and MDA-MB-361, were cultured as described [26].

Cell cycle effects of fulvestrant, tamoxifen, and saracatinib

BT-474 and MDA-MB-361 cells were treated with vehicle alone, fulvestrant (10 nM in BT-474, 100 nM in MDA-MB-361), 100 nM tamoxifen, 1 μM saracatinib, or both for 48 h. Cells were pulse labeled with 10 μmol/l bromodeoxyuridine, stained with anti-bromodeoxyuridine-conjugated FITC (Becton–Dickinson, Franklin Lakes, NJ) and propidium iodide, and cell cycle distribution assayed as described [4].

Growth inhibition of MDA-MB-361 tumor xenografts

Female oophorectomized, athymic BALB/c 4–6-week-old nude mice, purchased from NCI (Frederick, MD), were acclimatized, and implanted with a sustained release pellet containing 0.72 mg 17β estradiol. (Innovative Research of America, Sarasota, FL) 24 h before tumor inoculation. Subconfluent MDA-MB-361 cells were resuspended in Matrigel (10 mg/ml) (Becton–Dickinson) at 5×10^7 cells/ml. Each animal was injected with 0.1 ml of cell suspension into one mammary fat pad. Treatment was initiated at median tumor size 70 mm^3. Ten animals received no treatment, and ten each received daily fulvestrant (2.5 mg/mouse) subcutaneously, saracatinib (50 mg/kg) by oral gavage, or both drugs together. Saracatinib administered orally at 50 mg/kg inhibits Src kinase in xenograft tumors [24]. Tumor diameters were measured weekly and volumes calculated using $1/2 \times a \times b^2$ (where a and b are maximal tumor widths in two dimensions, $a > b$).

Immunoblotting and immunoprecipitation

Cells were lysed as described [4]. Western blot analysis used antibodies against MAPK, pMAPK, AKT, 4EBP1, p4EBP1, S6K, pS6K, mTOR, pmTOR, phospho-AKT, Src and phospho-Src (Cell Signaling), p27 from Transduction Laboratories (Lexington, KY), cyclin E1 monoclonal antibody E12 (Slingerland lab), and β-actin from Sigma. Other antibodies were from Santa Cruz. Protein levels on western blots were assayed for three different ECL exposures and quantitated by densitometry using Image Quant software from at least three different biological experiments.

The detection of cyclin E-bound proteins and cyclin E-Cdk2 kinase assays using Histone H1 substrate was as described [4]. Radioactivity in histone H1 was quantitated by phosphoimager from four different biological assays and plotted as % maximum activity.

Immunohistochemistry

Xenograft tumors were formalin fixed and paraffin embedded, and immunohistochemical analyses for $p27^{Kip1}$ (Becton–Dickinson antibody, 1:1000), Ki67 (DAKO, Carpinteria, CA, 1:100), and phospho-Src (SrcpY416, Stressgen, 1:100), respectively, were carried out as in [19, 27]. p27 and Ki67 were scored as percent of tumor nuclei positive [19, 27]. Src staining intensity was scored as described [19]. Each antigen was scored by two Pathologists (M.J. and N.G.) in at least 20 high power fields, and the mean score for each was plotted.

Reverse phase protein lysate array (RPPA)

The RPPA was carried out as described [28, 29] to quantify expression of over 100 proteins including MEK, ERK2, JNK, AKT, glycogen synthase kinase (GSK3), mTOR, S6K, p110 alpha, and phosphorylation of MEK at serines 217/221, MAPK at threonine 202 and tyrosine 204, JNK at threonine 183 and tyrosine 185, AKT at threonine 308 and serine 473, GSK3 at serine 21, mammalian target of rapamycin (mTOR) at serine 2448, S6K at threonine 389, and S6 at serines 235/236 using antibodies from Cell Signaling, Inc. (Danvers, MA), Epitomics, Inc. (Burlingame, CA-total MEK and p110 alpha antibodies), Lab Vision Corporation (Fremont, CA-total ER alpha antibody), and Santa Cruz (Santa Cruz, CA-total ERK2, JNK, and GSK3 antibodies).

Statistical analysis

Mean cyclin E-Cdk2 assays and IHC tumor scores for each treatment group were graphed as histograms, and error bars show standard error of the mean (SEM). Means were compared using the Student's t test.

Analysis of potential synergy between fulvestrant and saracatinib on tumor xenograft growth used the combination ratio as in [30]. 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 estradiol. The combination ratio compared the FTV that would be expected, if there were no synergy, with the observed FTV. The combination ratio was calculated as = (FTV of fulvestrant $\times$ FTV of saracatinib)/observed FTV of combination. Observed and expected FTV are described as

$$\text{Expected FTV} = (\text{mean FTV of fulvestrant}) \times (\text{mean FTV of saracatinib})$$

$$\text{Observed FTV} = \text{final tumor vol combined therapy} / \text{final tumor vol estradiol alone}$$

$$\text{Combination ratio} = \text{Expected FTV}/\text{Observed FTV}.$$

A combination ratio of greater than 1 indicates a synergistic effect; while a ratio of less than 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 [31]. Each protein and phosphoprotein measurement was corrected for loading using the average expression of the 86 proteins measured in each sample. NCSS software generated means' plots for the analyzed antibodies. The analysis of variance (ANOVA) was used to determine significant differences for the expression of individual proteins across the four treatment subgroups. ANOVA testing corrects each P value for all the comparisons performed across the four subgroups for each protein. In addition, the more rigorous Bonferroni correction was used to further address the testing of multiple (in this case 86) proteins using RPPA. In order to test n dependent or independent hypotheses on a data set, we tested each individual hypothesis at a statistically significant level of alpha/n to correct for the number of individual proteins tested. Thus, P values in the ANOVA test with values less than 0.05/86 or 0.0005814 retained statistical significance after the Bonferroni correction. Xcluster and Treeview softwares were used for the generation of dendrograms after log transformation and mean centering of the protein quantification data.

Results

Saracatinib and antiestrogens cause G1 arrest in ER-positive breast cancer cells

Fulvestrant and tamoxifen both arrest sensitive ER-positive breast cancer cells in G0/G1 by increasing p27 levels [4].

The effects of saracatinib together with these ER antagonists were assayed in two partially hormone-resistant ER-positive breast cancer lines with known ErbB2 and Src activation [17, 22]. In BT-474 cells treated for 48 h with either fulvestrant (Fas) or tamoxifen (Tam) treatment, the percent of cells in S phase (%S) decreased from 27 to 20% or 22%, respectively. Saracatinib (1 μM) alone reduced the %S of BT474 cells from 27 to 9%. Saracatinib combined with Fas or Tam caused a greater G1 arrest with the %S phase cells falling to 3% within 48 h (Fig. 1a).

MDA-MB-361 showed less cell cycle inhibition by 1 μM saracatinib alone than did BT-474. In MDA-MB-361, saracatinib alone reduced the %S from 40 to 29%. However, addition of saracatinib 1 μM together with either of the ER-blocking drugs for 48 h strongly inhibited cell cycle progression, reducing the %S phase cells to 2% (Fig. 1b).

Effects of ER blockade and saracatinib on signaling and cell cycle regulators

Saracatinib inhibited Src, and MAPK phosphorylation was reduced in both cell lines after 48 h of drug treatment.

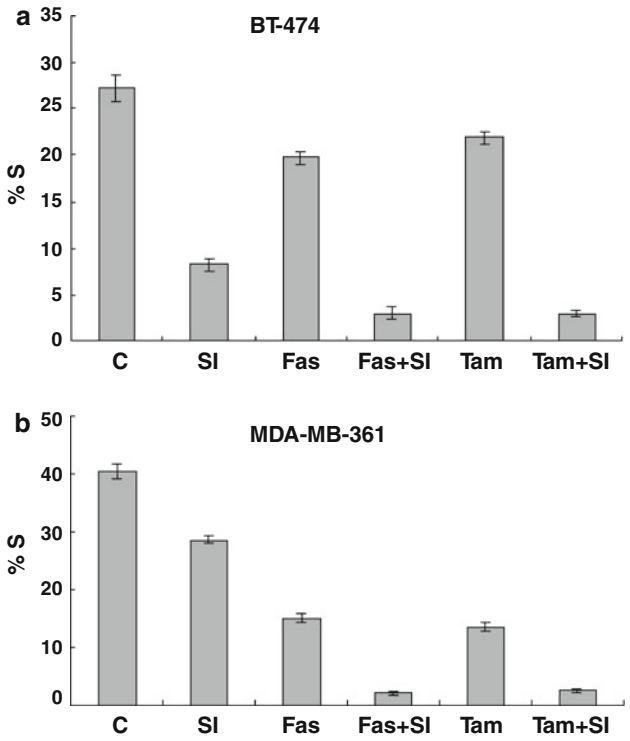


Fig. 1 Cell cycle effects of fulvestrant (*Fas*), tamoxifen (*Tam*), and saracatinib (*SI*). **a** Asynchronous (*Asyn*) BT-474, or **b** MDA-MB-361, cells were incubated for 48 h with saracatinib 1 μM (*SI*), Fas 10 nM (BT-474), or 100 nM (MDA-MB-361), Tam 100 nM or the indicated combination, and then recovered for cell cycle analysis by flow cytometry. *Bars* indicate standard error of the mean (SEM) from over >3 repeat assays

Neither Fas nor Tam alone affected Src or MAPK phosphorylation in either line, whereas combinations of either Fas or Tam with saracatinib inhibited these activities to a greater extent than did each ER-blocking drug alone (Fig. 2a) without affecting total kinase levels. pAKT was the most notably reduced by saracatinib together with either Tam or Fas in MDA-MB-361.

In BT-474, densitometric analysis of Western blots showed p27 increased by 1.6 ± 0.18-fold with Fas, by 1.5 ± 0.12-fold with Tam, and by 2.2 ± 0.02-fold with saracatinib alone, while Fas or Tam combined with saracatinib both increased p27 by 4.6 ± 0.09 and 4.6 ± 0.05-fold, respectively, over levels in asynchronous cells. In MDA-MB-361, p27 increased from 1.1- to 1.6-fold with either of the drugs alone, while Fas plus saracatinib increased p27 by 3.2 ± 0.29-fold. With Tam plus saracatinib, p27 increased 3.1 ± 0.31-fold. Cdk2 levels were modestly decreased by dual therapy, while cyclins D1 and E, and Cdk4 levels were not affected (Fig. 2b).

Fulvestrant, tamoxifen, and saracatinib inhibit Cyclin E-Cdk2 activity

After 48 h, cyclin E-Cdk2-bound p27 increased by 3.4-fold with saracatinib, 6.6-fold with Fas, and 6.8-fold with Tam alone in MDA-MB-361 (Fig. 2c). In MDA-MB-361, saracatinib/Fas increased cyclin E-Cdk2-bound p27 by 10-fold and saracatinib/Tam caused a 10-fold increase, and cyclin E-Cdk2 kinase inhibition was the greatest with dual therapy (Fig. 2d). Similar effects were observed in BT-474.

Saracatinib monotherapy increases ERα protein expression and transcriptional activity

Src has been shown to promote estrogen-driven ER proteolysis [26]. In both BT-474 and MDA-MB-361, 48 h of saracatinib increased ER protein levels (Fig. 3a), raising the possibility that escape from saracatinib monotherapy may arise through increased ER-mediated transcriptional activity. Q-PCR showed expression of cellular ER targets, *pS2* and *GREB1* increased after 48 h of saracatinib by 1.9- and 2.0-fold, respectively, compared with untreated in BT-474. Fulvestrant and tamoxifen alone and combination therapy all significantly inhibited *GREB1*, *pS2* mRNA levels in vitro (Fig. 3b, c). Similar results were found in MDA-MB-361 (Fig. 3b, c). The growth-promoting effects of the increase in ER levels and ER-transcriptional activity after saracatinib monotherapy could oppose its antitumor efficacy and promote drug resistance. These observations strengthen the rationale for use of saracatinib together with ER blockade in ER-positive cancers.

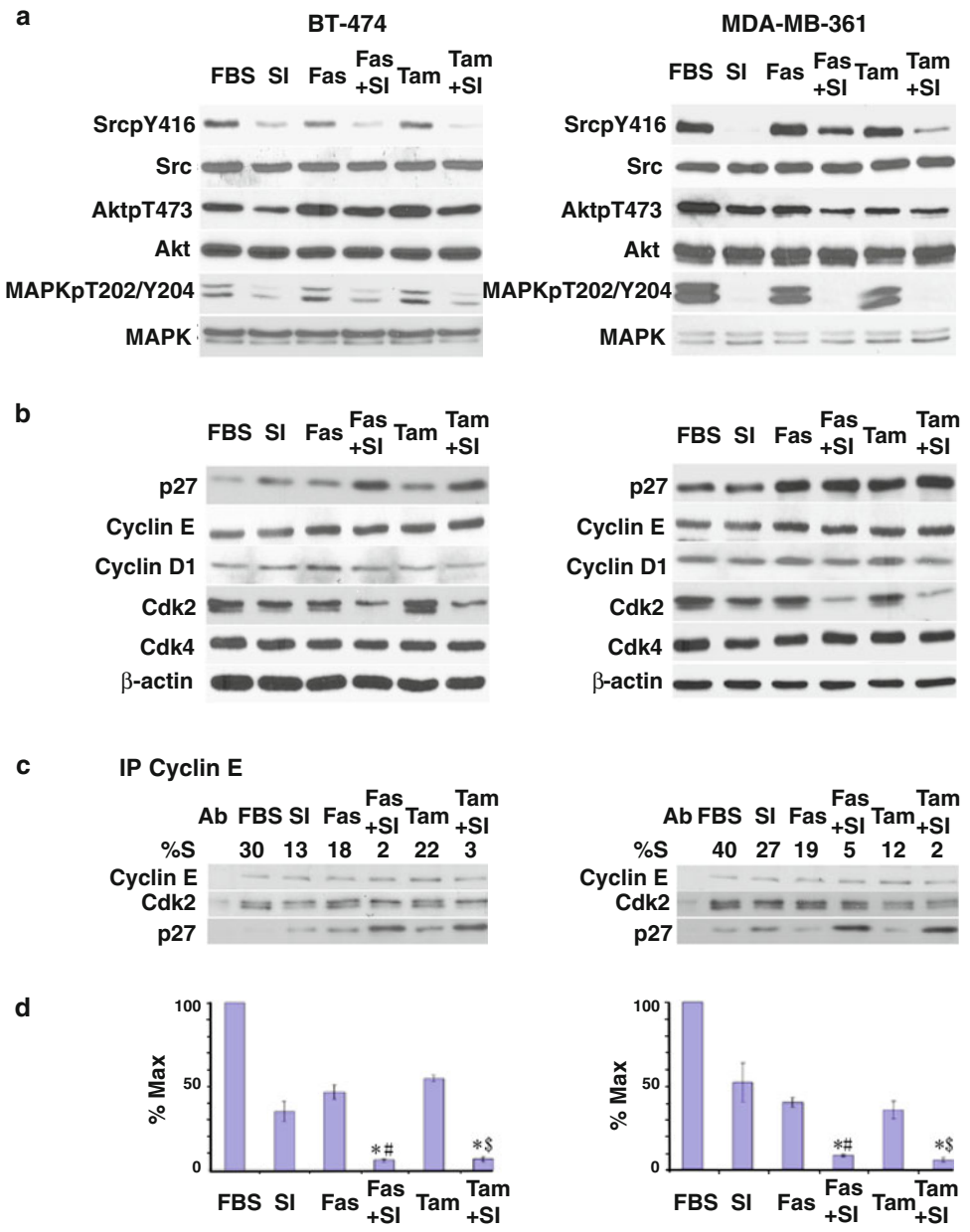


Fig. 2 Effects of fulvestrant (*Fas*), tamoxifen (*Tam*), and saracatinib (*SI*) on signaling and cell cycle regulators. Cells were treated with saracatinib (*SI*), fulvestrant (*Fas*), tamoxifen (*Tam*), both ER blockade and SI (*Fas* + *SI* or *Tam* + *SI*), or no drug (*asyn*) for 48 h, and then collected for western analysis of **a**, the indicated signaling kinases and **b**, cell cycle regulators. Cyclin E immunoprecipitates were **c**, resolved and immunoblotted for associated proteins and **d**, were assayed for associated kinase activity as described in Methods. Ab indicates antibody only controls. The percentage of cells in S phase (%S) after 48 h is indicated. Radioactivity in histone H1 substrate was quantitated, and mean values from four repeat assays were graphed as a percent of maximum kinase activity ±SEM. Student's *t* test indicates * $P < 0.05$ for comparison versus SI, # $P < 0.05$ for comparison versus Fas, and $ $P < 0.05$ for comparison versus Tam

Combined therapy with fulvestrant and saracatinib decreases xenograft proliferation and emergence of drug resistance

The antitumor efficacy of combined ER blockade and Src inhibition was assayed in MDA-MB-361 xenografts in vivo in nude mice. Saracatinib alone (50 mg/kg) initially inhibited tumor growth, but resistance developed within 21 days, and treated tumor volumes were similar to untreated controls thereafter (Fig. 4a). Fulvestrant alone suppressed tumor growth significantly, compared with control, yielding an inhibition rate of 31.4% (Fig. 4a, b). Combined fulvestrant and saracatinib therapy caused more potent inhibition of tumor growth than either drug alone, yielding an inhibition rate of 65.5% by 42 days (Fig. 4a, b).

Combination therapy was evaluated using the combination ratio of the expected over the observed fractional tumor volume (FTV). A combination ratio of greater than 1 indicates a synergistic effect. Although saracatinib monotherapy had little effect, use of saracatinib combined with fulvestrant delayed the emergence of drug resistance, yielding a greater antitumor effect than with either drug alone. Within 1 week of therapy, the combination ratio was 1.29 (see table in Fig. 4b), indicative of synergy between fulvestrant and saracatinib. At all the subsequent time points, the combination ratios

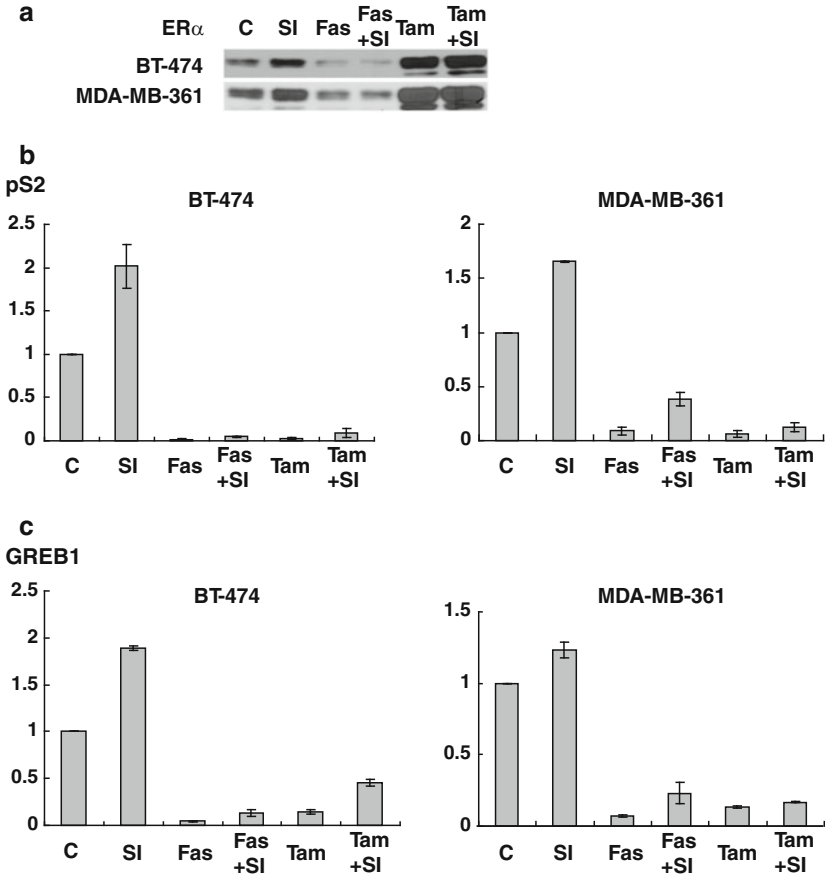
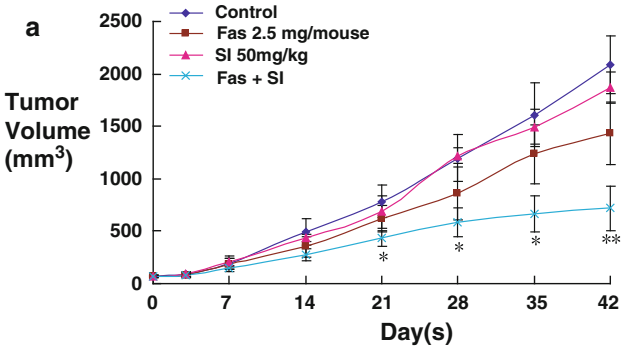


Fig. 3 Saracatinib increases ER protein levels and transcription activity. Cells were treated with no additional drug, saracatinib (*SI*), fulvestrant (*Fas*), tamoxifen (*Tam*), or both saracatinib and ER blocker together (*Fas* + *SI* or *Tam* + *SI*) for 48 h, and then collected for **a** Western blot for ER, **b** QPCR for *pS2*, and **c** Q-PCR for *GREB1*



b

	Day(s)	3	7	14	21	28	35	42
	FTV_{Fas}	0.90	0.94	0.71	0.79	0.72	0.77	0.69
	FTV_{AZD}	1.01	1.00	0.88	0.87	1.02	0.93	0.90
$FTV_{Fas+AZD}$	Expected	0.90	0.94	0.63	0.69	0.73	0.71	0.61
	Observed	0.95	0.73	0.56	0.55	0.54	0.43	0.38
	Ratio	0.96	1.29	1.12	1.25	1.34	1.66	1.60

Fig. 4 Fulvestrant and saracatinib effects on MDA-MB-361 breast cancer growth in vivo. **a** Tumor growth curve graphs relative tumor volume over time in each treatment group. Data are expressed as the mean ±SEM. *Non-overlapping error bars* indicate statistically significant treatment responses. Student's *t* test indicates * $P < 0.05$ or ** $P < 0.01$ for comparison of dual therapy versus control. **b** Fractional tumor volume (FTV) in fulvestrant and SI-treated groups relative to untreated control is tabulated

exceeded 1, reaching a maximum of 1.66 at 35 days of treatment.

In order to define molecular endpoints that predict antitumor response, p27, pY416-phosphorylated activated Src (Src-pY416), and Ki67 were assayed by immunohistochemistry in treated and untreated xenograft tumors recovered at the end of the experiment. Nuclear p27 levels were significantly higher, and Src activation and Ki67 levels were lower after combination treatment than after monotherapy or in untreated controls (Fig. 5a).

Proteomic analysis reveals mTOR activation in saracatinib-resistant xenografts

Proteomic analysis of xenograft tumors showed no activation of apoptosis in any treatment group. Xenograft tumors persisting after drug treatment showed significant mTOR activation, with increased mTORpS2448 ($P = 8e5$), 4EBP1 pT37pT46 ($P = 0.00019$), and S6KpT389 ($P = 0.01$) in the absence of PI3K, PDK1, AKT, or SGK activation. EGFR ($P < 1 \times 10^{-8}$) was also significantly elevated after drug treatment. Activation of SAPK/Erk6 (p38pT180Y182) ($P = 0.0086$), a downstream effector of EGFR, Src, and

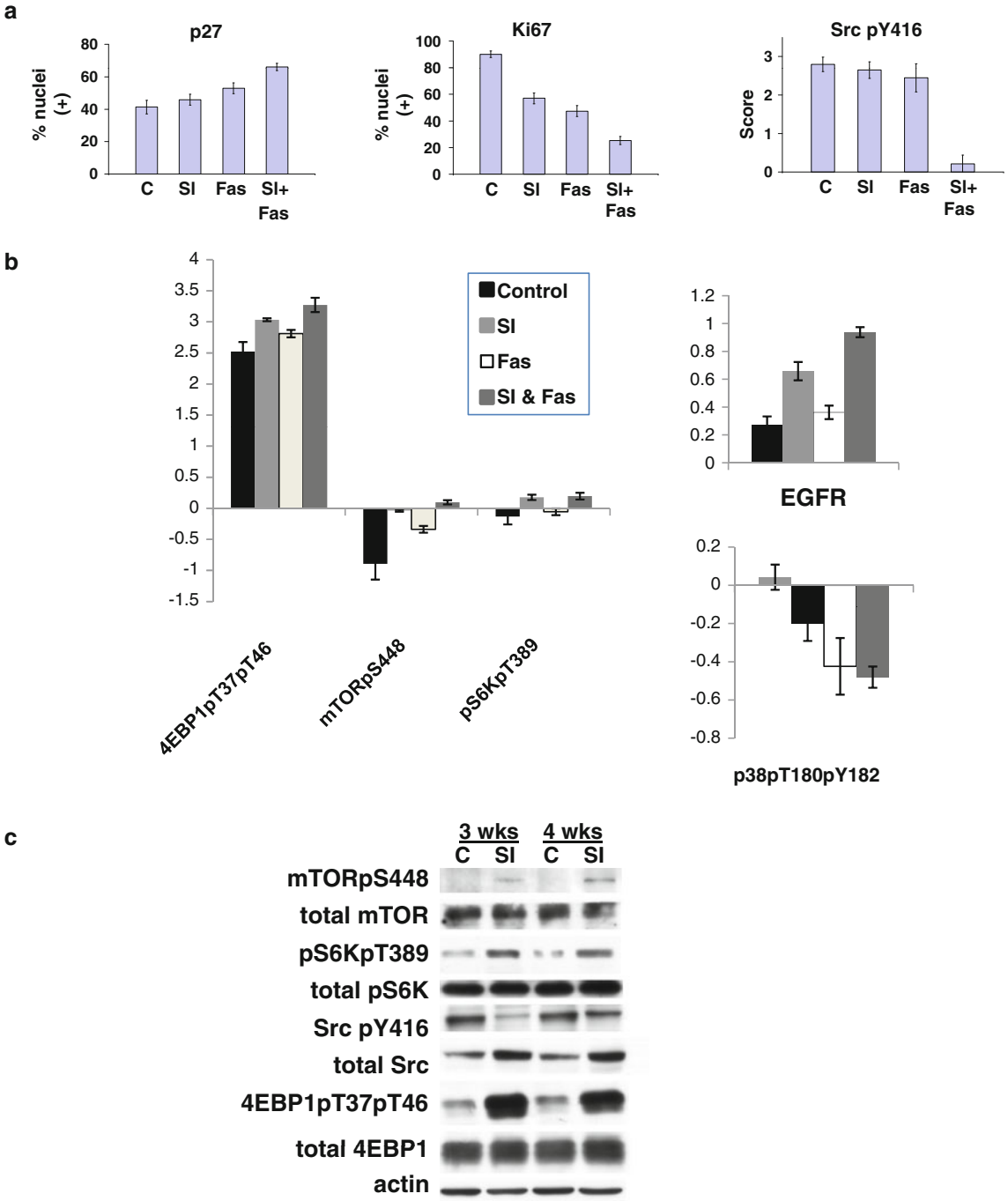


Fig. 5 Activation of bypass pathways after saracatinib treatment. Xenograft tumors were recovered after treatment with the indicated drugs and **a** stained for p27, Ki67, and Y416-phosphorylated active Src (Src-pY416) by immunohistochemistry or **b** analyzed by RPPA. Mean IHC scores from >20 high-power fields are plotted (±SEM) in **a**. Log values are plotted in **b** from RPPA for proteins whose expression was significantly altered by drug treatment. **c** MDA-MB-361 cells were cultured in the continuous presence of either vehicle control (*C*) or saracatinib 1 μM (*SI*) and lysed at the indicated times, and total or phospho-activated proteins assayed by Western. Data from untreated MDA-MB-361 cells and long-term vehicle-treated controls were identical (not shown)

mTOR, was also notable in resistant tumors. These data are presented in Fig. 5b. When the more rigorous Bonferroni correction was applied to account for multiple comparisons, EGFR, mTORpS2448, and 4EBP1pT37pT46 all retained significance. In contrast to the previous observations in saracatinib and saracatinib/anastrozole treated aromatase-transfected MCF-AROM5 xenografts [9], the MEK/MAPK pathway was not activated by saracatinib compared to other treatment groups. The compensatory activation of EGFR and mTOR pathways after long-term

therapy may contribute to resistance to the antitumor effects of saracatinib.

Derivation of saracatinib-resistant MDA-MB-361 in long-term cell culture

While Src was strongly inhibited by 48 h saracatinib in cultured MDA-MB-361 (Fig. 2a), resistant xenograft tumors recovered after 6 weeks of saracatinib monotherapy showed little Src inhibition by IHC compared to untreated controls. RPPA analysis showed mTOR activation in saracatinib-resistant MDA-MB-361 xenografts in this study, as was also observed in saracatinib-resistant MCF-AROM5 xenografts [9]. In order to further evaluate saracatinib effects on mTOR, MDA-MB-361 cells were cultured in the continued presence of 1 μM saracatinib. Drug-resistant cells recovered at 3 and 4 weeks of treatment also showed activation of mTOR complex 1 (mTORC1), with increased phospho-mTORpS448 and phosphorylation of its substrates, S6KpT389 and 4EBP1pT37pT46, compared to cells cultured with vehicle alone (Fig. 5c). A similar activation of mTORC1 was also observed within 4 weeks of culture of MCF-7 in the presence of saracatinib (not shown). Src inhibition, as detected by loss of reactivity to SrcpY416, was attenuated with long-term culture and the total Src levels increased.

Discussion

The pivotal role of estrogen in breast cancer development and progression is underscored by the success of drugs that target estrogen synthesis (via aromatase inhibition) and action (via ER blockade) in the treatment and chemoprevention of breast cancer [32]. Unfortunately, after an initial response, most tumors acquire resistance to hormonal therapy [33, 34]. Acquired resistance to endocrine therapy in preclinical models has been attributed to agonistic effects of tamoxifen, to increased genomic ER-signaling and to ligand-independent mitogenic effects of increased peptide growth factor signaling resulting in crossalk ER activation [35–37]. Various signal transduction inhibitors can target these latter pathways to inhibit hormone-resistant growth. In experimental models of hormone-sensitive breast cancer, combinations of endocrine agents with signal transduction inhibitors provide significantly greater growth inhibition than either alone, delaying the emergence of resistance [35–37].

Estrogen stimulation of estrogen-deprived cells decreases the cell cycle inhibitor p27, leading to cyclin E-Cdk2 activation, and cell cycle entry [4, 38–40]. Tyrosine phosphorylation of p27 by Abl and Src family kinases impairs the Cdk2 inhibitory action of p27 and promotes p27 proteolysis [19, 20]. Since p27 is required for the therapeutic growth inhibitory effects of estrogen deprivation or ER blockade [4], and since Src activates p27 proteolysis [19], we reasoned that Src inhibitors could potentially cooperate with ER blockade to augment antitumor efficacy toward ER-positive breast cancer.

The preclinical data presented in this article suggest the potential for therapeutic synergy between ER blockade and the novel Src inhibitor, saracatinib, in vitro and in vivo. While Fas and Tam both partially arrested BT-474 and MDA-MB-361 cells in G1, this occurred without appreciable Src or MAPK inhibition. Tamoxifen and fulvestrant appear to inhibit cell cycle by action on p27 through additional mechanisms that are either independent of and/or downstream of Src or MAPK. Saracatinib together with ER blockade more effectively increased p27 to mediate cell cycle arrest. Moreover, combined fulvestrant/saracatinib exhibited greater antitumor efficacy in vivo with greater Src inhibition and a greater increase in p27, and reduction of Ki67 observed in dual-treated xenografts than that of was observed with either of the drugs alone, supporting further investigation of this combination therapy in clinical trials. These data also support further immunohistochemical evaluation of p27, Src-pY416, and Ki67 as putative indicators of treatment response in clinical trials.

Despite the activation of Src in the MDA-MB-361 line, the antitumor efficacy of saracatinib monotherapy was limited and resistance rapidly emerged. A similar emergence of resistance to saracatinib monotherapy was also observed in xenografts of an aromatase-transfected MCF-7 variant, MCF-AROM5 [9]. In this study, EGFR, mTORpS448, and 4EBP1pT73pT46 all increased with saracatinib treatment of xenograft tumors, yielding P values < 0.0005814. mTOR complex 1 activation was observed not only in xenografts persisting after 6 weeks of drug, but also after long-term drug exposure in tissue culture. Thus, saracatinib resistance appears to emerge in part due to the marked increase in EGFR levels and/or activation of the mTOR complex 1 pathways, which would drive growth and ultimately overcome drug effects.

The potential for RPPA analysis in xenograft models to predict mechanisms of resistance to targeted therapies and to suggest pathways whose inhibition may complement the therapeutic efficacy of Src inhibitors warrants further investigation in preclinical models and in subsequent clinical trials. The data presented here in support further study of the antitumor efficacy of saracatinib together with either rapalogs or with the newer mTOR kinase inhibitors. Moreover, while Phase I trials of saracatinib monotherapy have shown good tolerability, the preclinical data of this study suggest that this drug may prove to be more clinically effective in combination with antiestrogens in ER-positive breast cancers.

Src inhibition by saracatinib led to an increase in ER protein levels. This is consistent with the finding that the crosstalk between ligand-activated ER and cSrc promotes proteasomal ER degradation [26]. Src inhibition has been shown to impair ligand-activated ER ubiquitylation and ER proteolysis, while Src induction shortened the ERt1/2 [26]. Saracatinib alone not only increased ER protein levels in MDA-MB-361 and BT-474 in vitro, but also led to an increased expression of ER target genes *PS*2 and *GREB1*. Saracatinib-mediated upregulation of ER levels and promitogenic ER-transcriptional activity could present an additional compensatory mechanism contributing to saracatinib drug resistance. These data also support further development of combination saracatinib and fulvestrant therapy, since the latter not only blocks ER action, but also strongly activates ER proteolysis.

In summary, this study demonstrates that saracatinib combined with fulvestrant more effectively inhibits proliferation of ER-positive breast cancer cells in vitro and in vivo than either drug alone. These data support further clinical investigation of the therapeutic potential of saracatinib in combination with fulvestrant for women with ER-positive breast cancer.

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Conflicts of interest statement The authors declared no conflict of interest.

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