Sensitizing estrogen receptor–negative breast cancer cells to tamoxifen with OSU-03012, a novel celecoxib-derived phosphoinositide-dependent protein kinase-1/Akt signaling inhibitor

Shu-Chuan Weng,[1] Yoko Kashida,[1] Samuel K. Kulp,[1] Dasheng Wang,[1] Robert W. Brueggemeier,[1] Charles L. Shapiro,[2] and Ching-Shih Chen[1]

[1]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and [2]Division of Hematology and Oncology, Department of Internal Medicine, College of Medicine, The Ohio State University, Columbus, Ohio

Abstract

Tamoxifen is a mainstay in the treatment of estrogen receptor (ER)–positive breast cancer patients. Although the efficacy of tamoxifen has been attributed to induction of tumor cell growth arrest and apoptosis by inhibition of ER signaling, recent evidence indicates that tamoxifen possesses ER-independent antitumor activities. Here, we use OSU-03012, a small-molecule inhibitor of phosphoinositide-dependent protein kinase-1 (PDK-1) to address the hypothesis that PDK-1/Akt signaling represents a therapeutically relevant target to sensitize ER-negative breast cancer to tamoxifen. OSU-03012 sensitized both ER-positive MCF-7 and ER-negative MDA-MB-231 cells to the antiproliferative effects of tamoxifen in an ER-independent manner. Flow cytometric analysis of phosphatidylserine externalization revealed that this augmented suppression of cell viability was attributable to a marked enhancement of tamoxifen-induced apoptosis by OSU-03012. Mechanistically, this OSU-03012-mediated sensitization was associated with suppression of a transient tamoxifen-induced elevation of Akt phosphorylation and enhanced modulation of the functional status of multiple Akt downstream effectors, including FOXO3a, GSK3α/β, and p27. The growth of established MDA-MB-231 tumor xenografts was suppressed by 50% after oral treatment with the combination of tamoxifen (60 mg/kg) and OSU-03012 (100 mg/kg), whereas OSU-03012 and tamoxifen alone suppressed growth by 30% and 0%, respectively. These findings indicate that the inhibition of PDK-1/Akt signaling to sensitize ER-negative breast cancer cells to the ER-independent antitumor activities of tamoxifen represents a feasible approach to extending the use of tamoxifen to a broader population of breast cancer patients. Considering the urgent need for novel therapeutic strategies for ER-negative breast cancer patients, this combinatorial approach is worthy of continued investigation. [Mol Cancer Ther 2008;7(4):800–8]

Introduction

Tamoxifen has been used extensively in the treatment of both advanced-stage and early-stage estrogen receptor (ER)–positive breast cancers and was recently approved as a chemopreventive agent for women at high risk for breast cancer (1, 2) The observed clinical efficacy of tamoxifen has been associated with its ability to induce growth arrest and apoptosis in breast cancer cells through the inhibition of estrogen binding to the ER. Nonetheless, tamoxifen at high concentrations ($\geq$10 μmol/L) also has been shown to mediate apoptosis in ER-negative cancer cells (3), which might be attributable to its ability to modulate the activation and/or expression status of a series of signaling targets in an ER-independent manner. Protein kinase C, transforming growth factor-β, calmodulin, the transcription factor c-Myc, and the mitogen-activated protein kinases (MAPK) p38 and c-Jun NH_2-terminal kinase are among the putative targets implicated in this ER-independent proapoptotic activity of tamoxifen (4). From a clinical perspective, these ER-independent antiproliferative effects of tamoxifen could possibly be exploited for the treatment of estrogen-unresponsive tumors, including ER-negative breast cancer, provided the concentrations of tamoxifen needed to modulate these apoptotic regulators could be attained therapeutically. This premise prompted our investigation of the combinatorial use of OSU-03012, a celecoxib-derived phosphoinositide-dependent protein kinase-1 (PDK-1)/Akt signaling inhibitor (5), with tamoxifen in ER-negative breast cancer cells. We hypothesized that PDK-1/Akt signaling represents a therapeutically relevant target to sensitize ER-negative breast cancer to tamoxifen by lowering the threshold for the ER-independent proapoptotic effects of tamoxifen. Extending the use of tamoxifen to the treatment of metastatic, hormone-insensitive breast cancer patients addresses an urgent need for the development of novel

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Requests for reprints: Ching-Shih Chen, Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, 336 L.M. Parks Hall, 500 West 12th Avenue, Columbus, OH 43210. Phone: 614-688-4008. E-mail: chen.844@osu.edu

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effective therapeutic approaches against ER-negative breast tumors.

Because PDK-1 is a proximal mediator of phosphatidylinositol-3-kinase signals, PDK-1 inhibitors influence a large portion of the phosphatidylinositol-3-kinase/Akt pathway. OSU-03012 has been shown to induce apoptosis at low micromolar concentrations in various types of solid tumor cells, including those of prostate (5), breast (6, 7), colon (8), lung (9), pancreas (10), and brain (11), chronic myelogenous leukemia cells (12), and chronic lymphocytic leukemia cells (13). It is noteworthy that, in a panel of breast cancer cells, OSU-03012 induced antiproliferative effects irrespective of differences in the functional and/or expression status of ER, HER2, and insulin-like growth factor-1 receptor 1 (6, 7). In this study, we show that OSU-03012 interacted with tamoxifen to enhance cell killing in both ER-positive ($ER\alpha^+$) MCF-7 and ER-negative ($ER\alpha^-$) MDA-MB-231 breast cancer cells.

Materials and Methods

Materials

Tamoxifen, 4-hydroxytamoxifen, 17β-estradiol, and gentamicin were purchased from Sigma-Aldrich. ICI-182780 was obtained from Tocris Bioscience. The PDK-1 inhibitor OSU-03012 was synthesized as described (5). For *in vitro* studies, stock solutions of test agents were prepared in DMSO and diluted in the indicated culture medium for treatment of cells (final concentration of DMSO, <0.1%). For *in vivo* studies, OSU-03012 and tamoxifen were prepared as suspensions in vehicle [0.5% (w/v) methylcellulose-0.1% (v/v) Tween 80 in sterile water] for oral administration to tumor xenograft-bearing immunocompromised mice. Antibodies against poly(ADP-ribose) polymerase, phospho-p27 (Thr^{157}), phospho-p38 (Thr^{180}/Tyr^{182}), GSK3β, phospho-GSKα/β ($Ser^{21/9}$), total-Akt, phospho-Akt (Ser^{473}), FOXO1, phospho-FOXO1 (Ser^{256}), FOXO3a, phospho-FOXO3a ($Ser^{318/321}$), FOXO4, and phospho-FOXO4 (Ser^{262}) were purchased from Cell Signaling Technology. Antibodies against ERα and β-actin were purchased from Santa Cruz Biotechnology and ICN Biomedicals, respectively.

Cell Culture

ER-positive MCF-7 and ER-negative MDA-MB-231 cells were obtained from American Type Culture Collection and maintained in DMEM/Ham's F-12 (1:1) supplemented with 10% fetal bovine serum (FBS) and 10 μg/mL gentamicin (Sigma-Aldrich) at 37°C in a humidified incubator containing 5% CO_2.

Cell Viability Analysis

The viability of breast cancer cells was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium (MTS) assay (Promega) in five replicates. Cells were seeded at 7,000 per well in 96-well, flat-bottomed plates in 10% FBS-supplemented DMEM/Ham's F-12. After 24 h, the medium was replaced with that containing the indicated concentrations of individual agents or combinations of drugs and 5% FBS. Control cells were treated with DMSO vehicle at a concentration equal to that in drug-treated cells (final concentration, ≤0.1%). After 24- or 72-h treatment, 20 μL MTS reagent was added to each well and cells were incubated for up to 3 additional hours at 37°C. The absorbances were read on a plate reader at a single wavelength of 490 nm. The concentrations of agents that inhibited viability by 50% (IC_{50}) were calculated for single agents and combinations by the median-effect method of Chou and Talalay (14) using CalcuSyn software (Biosoft).

ER-Dependent Cell Proliferation Assay

MCF-7 cells were maintained in culture in DMEM/Ham's F-12 containing 10% charcoal-stripped FBS (Hyclone) for 5 days, after which cells were seeded at 4×10^4 per well into 24-well culture plates in the same medium. Twenty-four hours later, the medium was replaced with that containing the indicated concentrations of individual agents or combinations of drugs and 5% charcoal-stripped FBS. For the estradiol-treated groups, estradiol was added 30 min before the drug treatment. After treatments, cells were harvested and cell number in each well was counted using a Coulter counter (Beckman Coulter).

Immunoblotting

Treated cells were washed with PBS, collected by scraping into radioimmunoprecipitation lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and a mixture of protease inhibitors; Calbiochem], and then sonicated for 5 s. After brief centrifugation at 12,000 rpm, equivalent amounts of total protein from the soluble fractions of the cell lysates (20-50 μg) were resolved in SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with TBS containing 0.05% Tween 20 (TBST) and 5% nonfat milk for 1 h, the membrane was incubated with the appropriate primary antibody at 1:1,000 dilution in TBST-1% nonfat milk at 4°C overnight and then washed three times with TBST. The membrane was probed with horseradish peroxidase–conjugated secondary antibodies at 1:2,000 for 1 h at room temperature and washed with TBST three times. The immunoblots were visualized by enhanced chemiluminescence.

Transfection and Detection of FOXO3a-GFP

Cells were cultured on cover glasses in six-well culture plates and then transfected with the FOXO3a-GFP plasmid, which was kindly provided by Dr. Mickey C-T. Hu (M. D. Anderson Cancer Center; ref. 15), using the Fugene 6 transfection reagent (Roche). Twenty-four hours after transfection, cells were treated with OSU-03012 for 8 h and fixed in 4% formaldehyde. After washing with PBS, cells were mounted using Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (Vector), and green fluorescent protein fluorescence was visualized by microscopy. The number of nuclei with green fluorescent protein–positive signals was counted and expressed as a percentage of the total number of 4′,6-diamidino-2-phenylindole–positive nuclei.

Flow Cytometric Analysis for Apoptosis

For assessment of apoptosis, cells were treated for 24 h and then labeled with 5 μL Annexin V-FITC (Invitrogen) and 0.1 μg propidium iodide (Sigma-Aldrich) in 100 μL

binding buffer [10 mmol/L HEPES, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$ (pH 7.4)] containing 5×10^5 cells. Samples were mixed gently and incubated at room temperature in the dark for 15 min. Immediately before analysis by flow cytometry, 400 μL binding buffer was added to each sample. Two-color analysis of apoptosis was done using a BD FACSCalibur System (BD Biosciences). Fluorescence compensation on the flow cytometer was adjusted to minimize overlap of the FITC and propidium iodide signals. A total of 1.2×10^4 cells were acquired for each sample and a maximum of 1×10^4 cells within the gated region were analyzed.

In vivo Studies

Ovariectomized female NCr athymic nude mice (6-8 weeks of age) were obtained from the National Cancer Institute. The mice were group housed in plastic shoebox cages with autoclaved bedding and filtered air (4-5 mice per cage) with *ad libitum* access to sterilized food and water. Animal rooms were maintained at 22 ± 2°C with 12 h of fluorescent lighting per day. All experimental procedures using animals were done in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

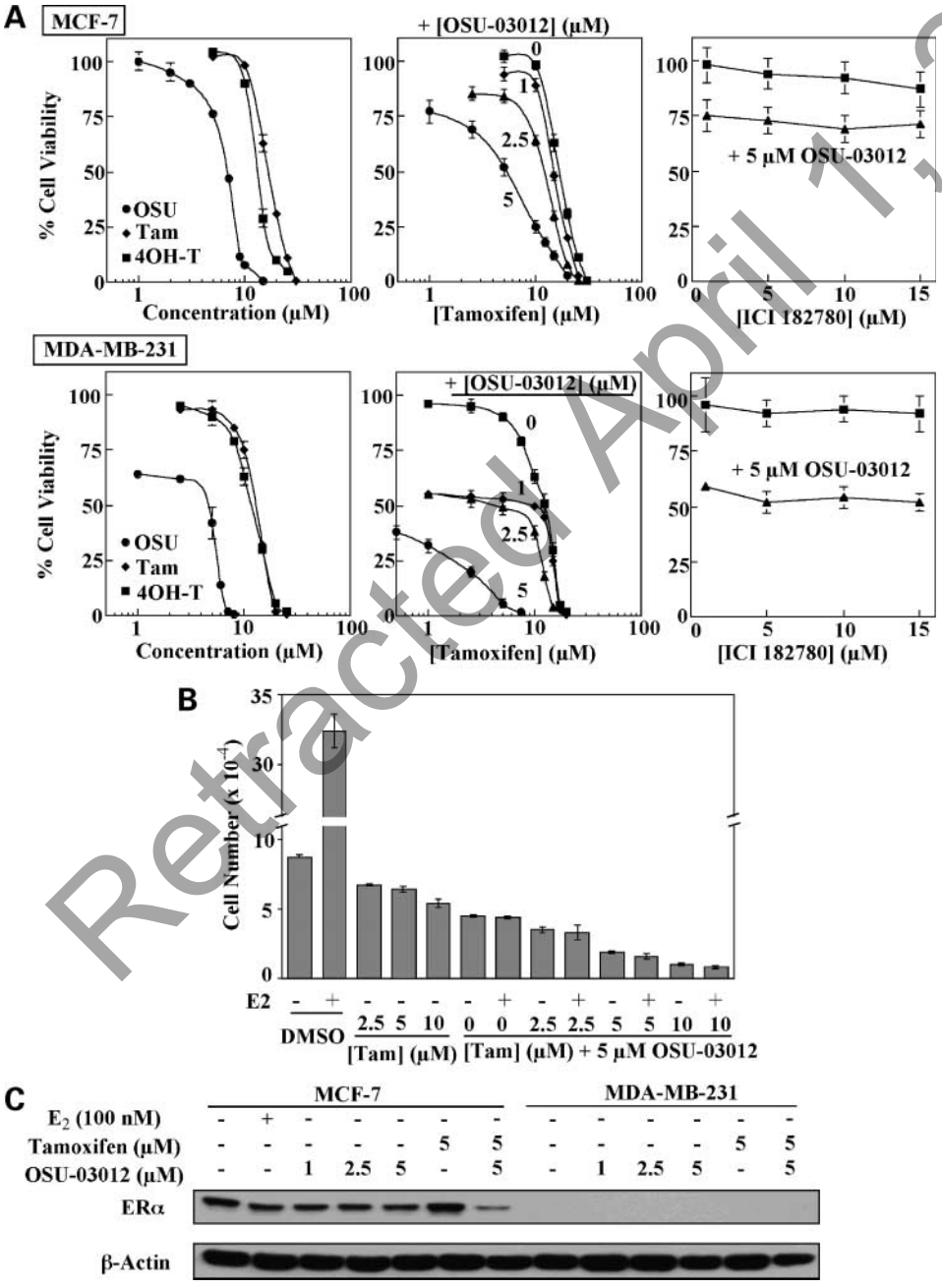

Figure 1. Sensitization of MCF-7 and MDA-MB-231 breast cancer cells to tamoxifen by OSU-03012 via an ER-independent mechanism. **A,** *top, left,* dose-dependent antiproliferative effects of OSU-03012 (*OSU*), tamoxifen (*Tam*), and 4-hydroxytamoxifen (*4OH-T*) on cell viability in MCF-7 cells; ●, OSU-03012; ♦, tamoxifen; ■, 4-hydroxytamoxifen. *Middle,* dose-dependent effect of OSU-03012 on the sensitivity of MCF-7 cells to the antiproliferative activity of tamoxifen. OSU-03012 doses: ■, 0 μmol/L; ♦, 1 μmol/L; ▲, 2.5 μmol/L; ●, 5 μmol/L. *Right,* effect of OSU-03012 on the response of MCF-7 cells to the pure ER antagonist ICI-182780. OSU-03012 doses: ■, 0 μmol/L; ▲, 5 μmol/L. *Bottom,* same sets of experiments were carried out in MDA-MB-231 cells in lieu of MCF-7 cells. Cell viability was determined by the MTS assay as described in Materials and Methods. *Points,* mean; *bars,* SD ($n = 5$). **B,** estradiol (*E2*) has no effect on OSU-03012-mediated sensitization of MCF-7 cells to the antiproliferative effect of tamoxifen. After 5 days of culture in the presence of 10% charcoal-stripped FBS, MCF-7 cells were seeded into 24-well culture plates (4×10^4 per well), treated as indicated in 5% charcoal-stripped FBS-containing medium for 72 h, and then harvested for counting of cell numbers as described in Materials and Methods. *Columns,* mean; *bars,* SD ($n = 3$). **C,** effect of OSU-03012, alone and in combination with tamoxifen, on ERα expression in MCF-7 cells. Cells were treated as indicated for 24 h. Immunoblotting was done as described in Materials and Methods.

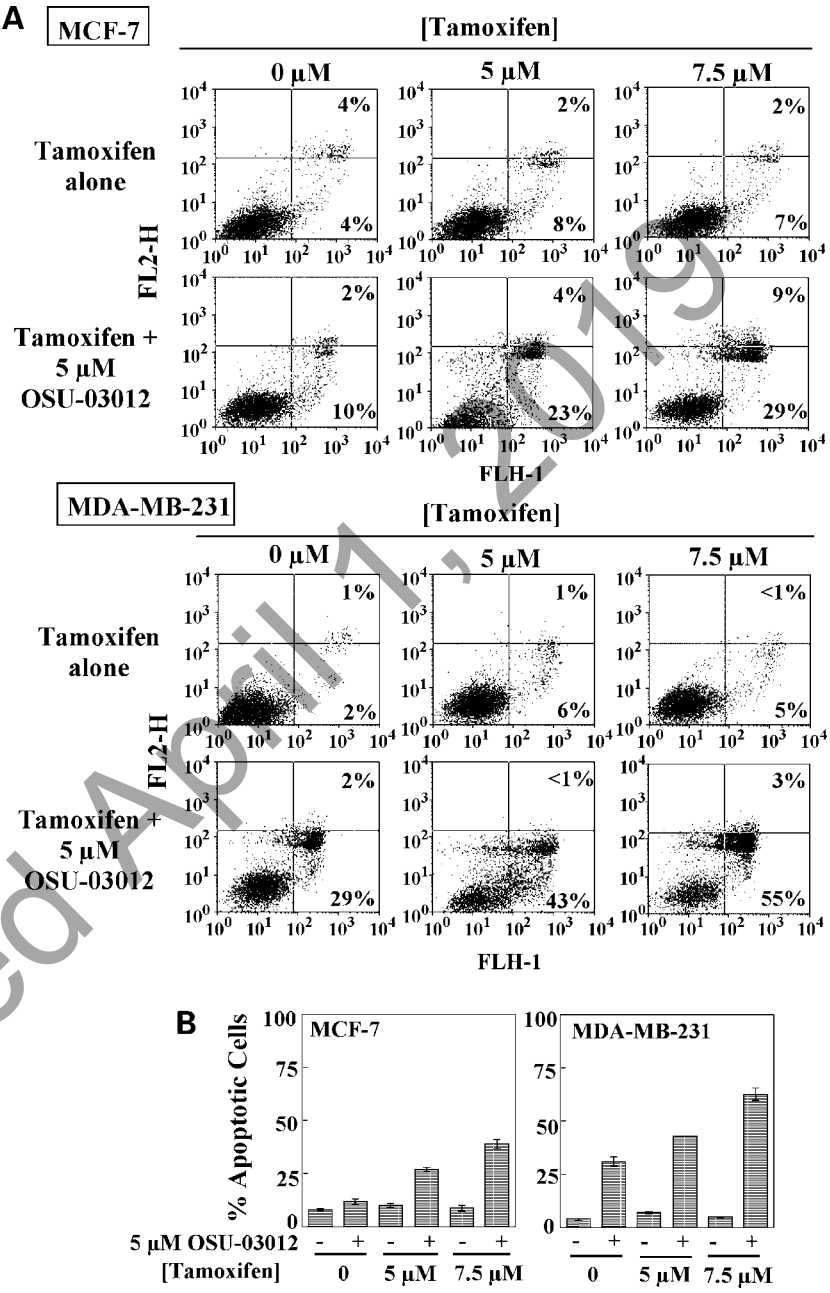

Figure 2. Annexin V flow cytometric analysis of apoptosis in MCF-7 and MDA-MB-231 cells receiving tamoxifen, OSU-03012, or the tamoxifen/OSU-03012 combination. **A,** MCF-7 and MDA-MB-231 cells were treated tamoxifen or OSU-03012 individually or in combination at the indicated concentrations in 5% FBS-containing DMEM/Ham's F-12 for 24 h followed by Annexin V/propidium iodide staining as described in Materials and Methods. Representative of two independent experiments. **B,** columns representing the flow cytometry data presented in **A** above. *Columns,* mean of two independent analyses; *bars,* SD. FL2-H, propidium iodide; FLH-1, Annexin V.

Each mouse was injected s.c. in the right flank with 5×10^5 MDA-MB-231 cells in a total volume of 0.1 mL serum-free medium containing 50% Matrigel (BD Biosciences). As tumors became established (mean starting volume, 59 ± 5 mm^3), mice were randomly assigned to treatment groups receiving (*a*) vehicle (0.5% methylcellulose-0.1% Tween 20 in water), (*b*) tamoxifen at 60 mg/kg, (*c*) OSU-03012 at 100 mg/kg, or (*d*) both tamoxifen and OSU-03012. All treatments were administered once per day by oral gavage (10 μL/g body weight) for the duration of the study. Tumors were measured weekly using calipers and their volumes calculated using a standard formula: $(\text{short axis})^2 \times \text{long axis} \times 0.52$. Body weights were measured weekly.

Immunohistochemistry

At terminal sacrifice, tumors harvested from mice were fixed in formalin and embedded in paraffin by routine procedures. Tumor specimens were submitted to The Ohio State University Veterinary Biosciences Histology/Immunohistochemistry Core for immunohistochemical staining of Ki-67 in representative 4 μm sections of tumor tissues. Proliferation indices were calculated as the number of immunopositive nuclei × 100% divided by the total number of cells per ×400 field.

Statistical Analysis

All experiments were carried out at least two times on different occasions. Values from *in vitro* experiments are presented as the mean ± SD. The medium-effect method was used to analyze dose-response data for single or multiple drugs. For *in vivo* data, values are expressed as mean ± SE. Comparison of variance and mean value were done using F test followed by t test ($P < 0.05$).

Results

OSU-03012 Enhances the Antiproliferative Effect of Tamoxifen and 4-Hydroxytamoxifen in MCF-7 and MDA-MB-231 Cells

The antitumor effects of OSU-03012, tamoxifen, and 4-hydroxytamoxifen, an active metabolite with at least 100-fold higher affinity for the ER, were assessed in ER-positive MCF-7 and ER-negative MDA-MB-231 cells by the MTS assay at 72 h. All three agents induced dose-dependent reductions in cell viability with IC_{50} values as follows: MCF-7: OSU-03012, 6.8 ± 0.2 μmol/L; tamoxifen, 16.2 ± 0.9 μmol/L; 4-hydroxytamoxifen, 13.1 ± 0.3 μmol/L; MDA-MB-231: OSU-03012, 4.0 ± 1.4 μmol/L; tamoxifen, 13.0 ± 0.2 μmol/L; 4-hydroxytamoxifen, 11.8 ± 0.2 μmol/L (Fig. 1A, *left*). It is noteworthy that OSU-03012 exhibited biphasic inhibition of viability in MDA-MB-231 cells, which was not noted in MCF-7 cells. This finding suggests that there existed distinct modes of mechanisms by which OSU-03012 mediated antiproliferative effects at concentrations below 5 μmol/L and above 1 μmol/L in MDA-MB-231 cells, which warrants investigation. On the other hand, both cell lines were comparably susceptible to the antiproliferative effects of tamoxifen and 4-hydroxytamoxifen irrespective of differences in their ER binding affinity and the cellular ER status.

The ability of OSU-03012 to sensitize breast cancer cells to tamoxifen was shown by the shift of the dose-response curve for tamoxifen to the left in response to increasing levels of OSU-03012 in MCF-7 cells and, more prominently, in MDA-MB-231 cells (Fig. 1A, *middle*). Three lines of evidence suggest that this OSU-03012-induced sensitization was mediated through an ER-independent mechanism. First, as just described, prominent sensitization was observed in the ER-negative MDA-MB-231 cells. Second, this sensitization was specific to tamoxifen as the responses of MCF-7 and MDA-MB-231 cells to the combination of OSU-03012 (5 μmol/L) with the pure antiestrogen, ICI-182780, were nearly identical to their responses to OSU-03012 alone (Fig. 1A, *right*). Thus, this finding indicates that the ~25% reduction in viability of cells treated with the combination was attributable to the activity of OSU-03012, suggesting that the response to ICI-182780 was unaltered

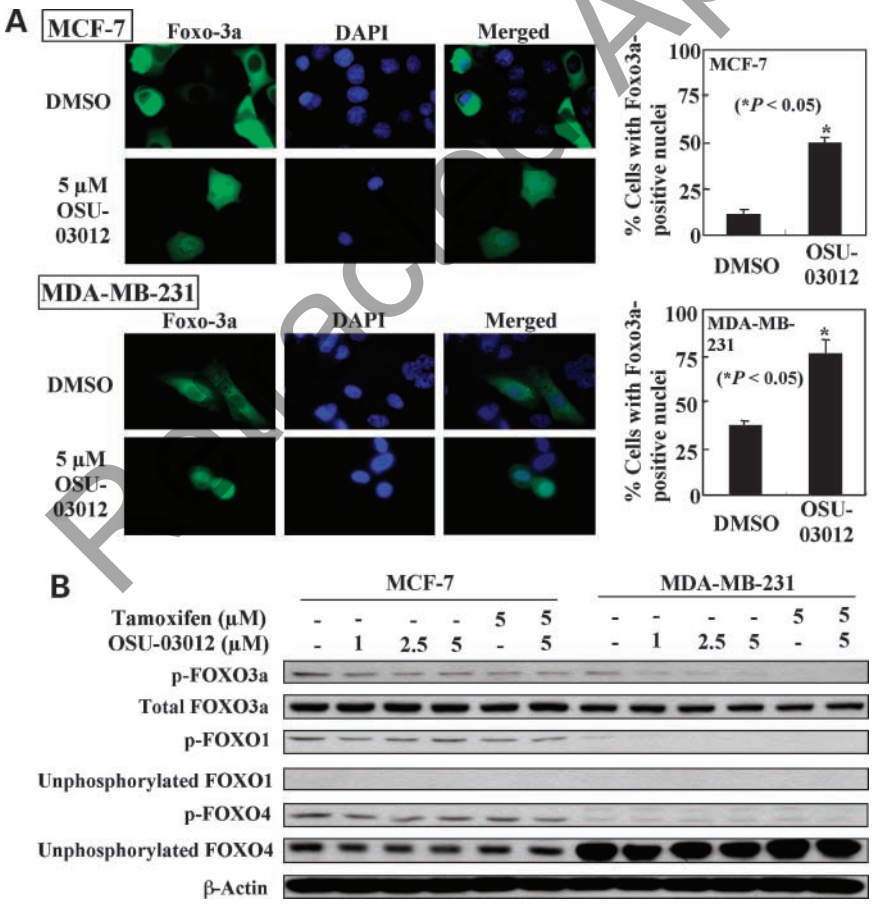

Figure 3. Effect of OSU-03012 on the intracellular localization of FOXO3a and expression levels of other FOXO proteins in MCF-7 and MDA-MB-231 cells. **A,** immunocytochemical analysis of the effect of OSU-03012 on the intracellular localization of green fluorescent protein–tagged FOXO3a in MCF-7 and MDA-MB-231 cells. Cells were treated with 5 μmol/L OSU-03012 in 5% FBS-containing DMEM/F-12 medium for 8 h. The percentage of cells with green fluorescent protein–positive nuclei was determined by fluorescence microscopy. *Columns,* mean; *bars,* SD ($n = 3$). **B,** effect of OSU-03012, alone and in combination with tamoxifen, on the expression and phosphorylation status of FOXO protein family members in MCF-7 and MDA-MB-231 cells. Cells were treated as indicated for 24 h. Immunoblotting was done as described in Materials and Methods.

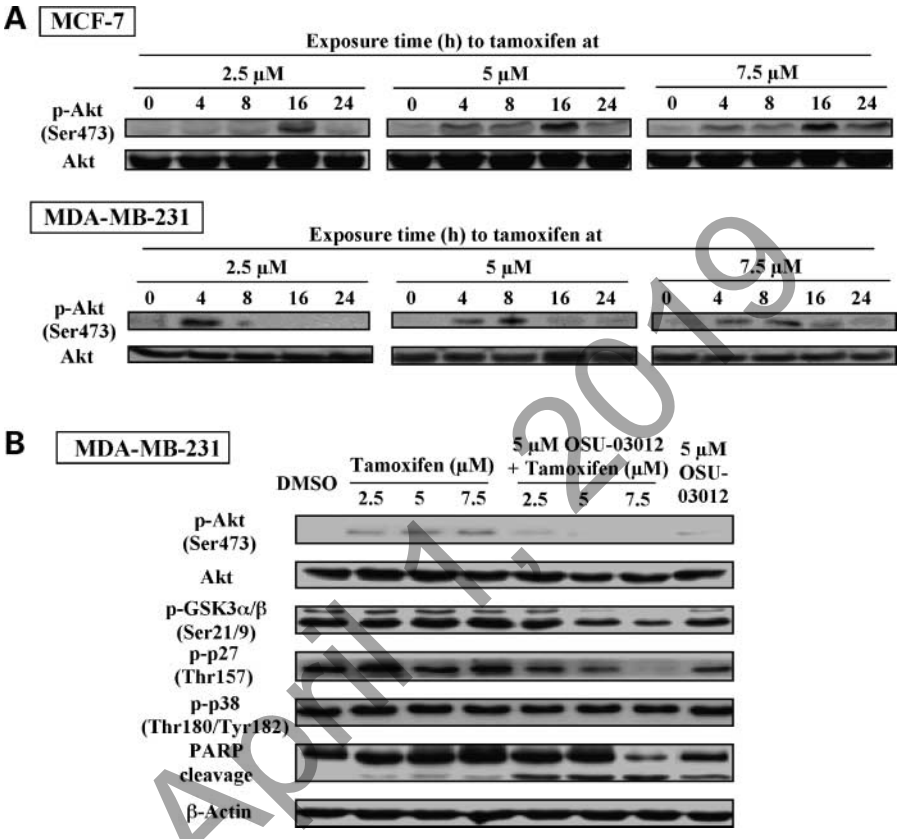

Figure 4. Effect of tamoxifen, OSU-03012, and the tamoxifen/OSU-03012 combination on the phosphorylation status of Akt and its downstream effectors GSK3α/β and p27 in MCF-7 and MDA-MB-231 cells. **A,** transient up-regulation of Akt phosphorylation in MCF-7 and MDA-MB-231 cells treated with tamoxifen. Cells were exposed to tamoxifen at 2.5, 5, or 7.5 μmol/L for the indicated times. Immunoblotting for p-Ser^{473}-Akt and total Akt was done as described in Materials and Methods. **B,** dose-dependent effect of tamoxifen alone or in combination with 5 μmol/L OSU-03012 on the phosphorylation status of Akt and its substrates GSK3α/β and p27 and on poly(ADP-ribose) polymerase cleavage in MDA-MB-231 cells. The MAPK p38 was used as a negative control to show the specificity of Akt inhibition. MDA-MB-231 cells were exposed to individual treatments for 8 h. Immunoblotting was done as described in Materials and Methods.

by the presence of OSU-03012. Lastly, MCF-7 cells (4×10^4 per well) were exposed to various treatments with or without 1 nmol/L estradiol in medium containing charcoal-stripped serum, and cell numbers were counted after 6 days. As shown, although estradiol significantly increased the number of vehicle-treated MCF-7 cells, it could not diminish the suppressive effect of the combination therapy on cell proliferation (Fig. 1B).

To gain some insight into the effects of OSU-03012, both alone and in combination with tamoxifen, on ERα signaling, expression levels of ERα were determined by immunoblotting in treated MDA-MB-231 and MCF-7 cells (Fig. 1C). In the ER-negative MDA-MB-231 cells, none of the treatments resulted in an observable reexpression of ERα that could have potentially restored tamoxifen sensitivity, thereby providing further support for an ER-independent mechanism of OSU-03012-induced tamoxifen sensitization in ER-negative cells. In contrast, OSU-03012 noticeably reduced ERα expression in MCF-7 cells at 1, 2.5, and 5 μmol/L to a level comparable with that observed after estradiol treatment. Moreover, in combination with 5 μmol/L tamoxifen, 5 μmol/L OSU-03012 caused a substantially greater reduction in ERα levels in treated MCF-7 cells. This finding suggests that a role for suppressed ER signaling in OSU-03012-induced sensitization to tamoxifen cannot be entirely discounted in ER-positive MCF-7 cells.

OSU-03012 Sensitizes MCF-7 and MDA-MB-231 Cells to the Apoptotic Effects of Tamoxifen

As indicated by Annexin V analysis of phosphatidylserine externalization, OSU-03012-mediated sensitization was, at least in part, attributable to the enhancement of tamoxifen-induced apoptosis (Fig. 2A). Relative to MCF-7 cells, MDA-MB-231 cells exhibit substantially higher susceptibility to the apoptotic and chemosensitizing effects of OSU-03012. As shown in Fig. 2B, normalization to the DMSO-treated controls revealed that OSU-03012 alone at 5 μmol/L induced 4% and 28% apoptotic death in MCF-7 and MDA-MB-231 cells, respectively, whereas tamoxifen alone at 5 and 7.5 μmol/L lacked apoptotic activity in either cell line. However, in combination with 5 μmol/L OSU-03012, tamoxifen at 5 and 7.5 μmol/L caused 19% and 30% apoptotic death, respectively, in MCF-7 cells vis-à-vis 41% and 55%, respectively, in MDA-MB-231 cells. Moreover, our data indicate that the sensitizing effect of OSU-03012 on tamoxifen-induced apoptosis could not be diminished by the presence of estradiol (data not shown), providing further support for the dissociation of this chemosensitization from ER signaling.

Functional Role of Akt Inhibition in OSU-03012-Mediated Sensitization of Breast Cancer Cells to Tamoxifen

Although MCF-7 and MDA-MB-231 cells exhibit low levels of Akt phosphorylation (6, 16, 17), we hypothesized

that Akt signaling still represented a therapeutically relevant target for OSU-03012 to sensitize these cells to tamoxifen via two potential mechanisms. First, OSU-03012-mediated Akt inhibition would lead to the activation of a series of apoptosis regulators, which might interact synergistically with the non-ER targets of tamoxifen to facilitate apoptosis signaling. Second, OSU-03012 could antagonize tamoxifen-induced Akt activation, thereby overcoming therapeutic resistance.

To test our hypothesis, we first examined the effect of OSU-03012 on the functional status of several Akt downstream effectors, the FOXO family of forkhead transcription factors (reviewed in ref. 18), GSK3α/β (19, 20), and p27 (21, 22), in MCF-7 and MDA-MB-231 cells. It is well understood that Akt plays an integral role in regulating the activity of FOXO proteins (reviewed in ref. 18) by modulating their intracellular location through phosphorylation. Immunofluorescent labeling of FOXO3a in MCF-7 and MDA-MB-231 cells indicated that OSU-03012 treatment resulted in a multifold increase in nucleus-associated FOXO3a in comparison with its apparent cytoplasmic sequestration in DMSO vehicle-treated cells, suggesting nuclear translocation of FOXO3a in response to Akt inhibition (Fig. 3A). This alteration in FOXO3a intracellular localization was associated with OSU-03012-induced reductions in the phosphorylation status of FOXO3a as well as the Akt substrates, GSK3α/β and p27, at the Akt-specific phosphorylation sites, p-Ser^{318}/Ser^{321}-FOXO3a (Fig. 3B) and p-Ser^{21}/Ser^{9}-GSK3α/β and p-Thr^{157}-p27, respectively (Fig. 4B). In addition, the effect of tamoxifen and OSU-03012 on the Akt-sensitive phosphorylation status of other FOXO proteins, specifically FOXO1 and FOXO4, was examined. As shown in Fig. 3B, the low endogenous level of p-Ser^{256}-FOXO1 in MDA-MB-231 cells was diminished by tamoxifen and OSU-03012 but without a detectable corresponding change in the expression of the unphosphorylated protein. Changes in the phosphorylation of Ser^{262}-FOXO4 could not be detected in either cell line after treatment. These findings suggest that, among the FOXO proteins, FOXO3a may play the more prominent role in the effects of OSU-03012 on the sensitivity of ER-negative breast cancer cells to tamoxifen.

Subsequently, we examined the effect of OSU-03012 on the phosphorylation status of Akt in tamoxifen-treated MCF-7 and MDA-MB-231 cells by Western blotting. As shown in Fig. 4A, tamoxifen treatment caused a transient increase in Akt phosphorylation in a time- and dose-dependent manner in MCF-7 cells and, to a substantially lesser extent, MDA-MB-231 cells. Tamoxifen-induced Akt phosphorylation peaked at 16 h after treatment in MCF-7 cells and between 4 and 8 h in MDA-MB-231 cells. The ability of OSU-03012 to antagonize this tamoxifen-induced up-regulation of phosphorylated Akt was clearly evident in MDA-MB-231 cells (Fig. 4B). Moreover, in addition to decreasing the level of p-Ser^{473}-Akt in tamoxifen-treated cells, OSU-03012 also interacted with tamoxifen to reduce in a dose-dependent manner the phosphorylation levels of the two Akt substrates, Ser^{21}/Ser^{9}-GSK3α/β and

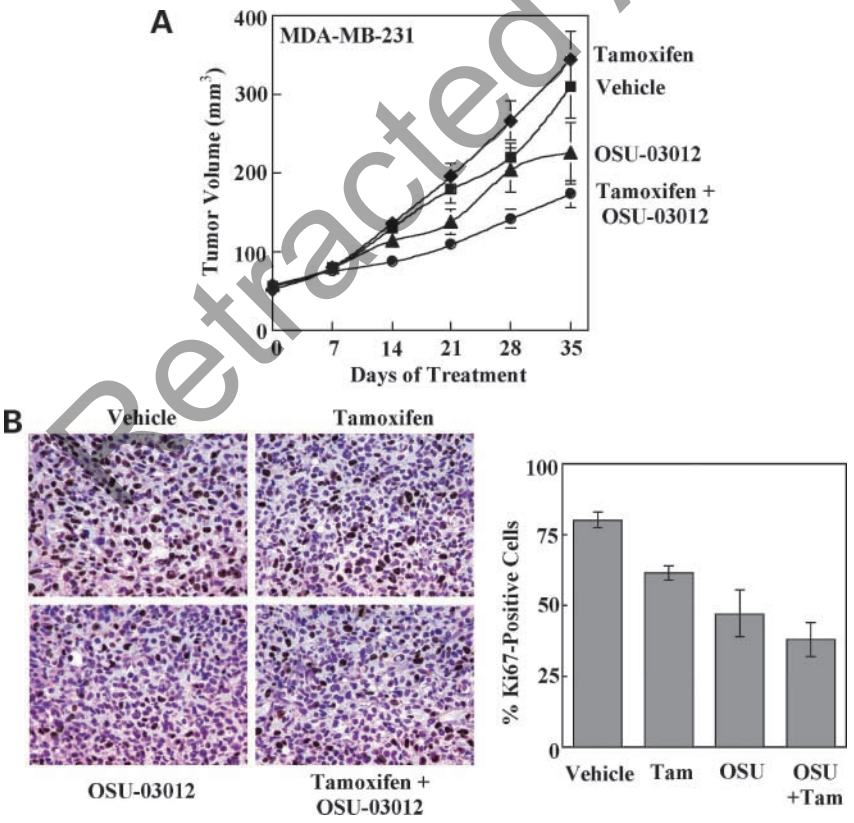

Figure 5. Effects of daily oral treatment with tamoxifen (60 mg/kg), OSU-03012 (100 mg/kg), and the tamoxifen/OSU-03012 combination (60 and 100 mg/kg, respectively) on the growth of established s.c. MDA-MB-231 tumors in ovariectomized female athymic nude mice. MDA-MB-231 tumors were established in each mouse by s.c. injection of 5×10^5 MDA-MB-231 cells in a total volume of 0.1 mL of cold serum-free medium containing 50% Matrigel. Mice with established tumors (starting mean tumor volume, 59 ± 5 mm^3) were randomly assigned to four groups (n = 10-12) that received the indicated treatments once daily by oral gavage for the duration of the study as described in Materials and Methods. **A,** mean tumor volumes for each treatment group as a function of day of treatment. *Points,* mean tumor volume; *bars,* SE (n = 10-12). **B,** immunohistochemical evaluation of intratumoral proliferation in MDA-MB-231 xenograft tumors. Immunostaining for Ki-67 in formalin-fixed, paraffin-embedded tumor tissues was done, and proliferation indices were calculated as described in Materials and Methods. *Left,* immunohistochemistry showing Ki-67 expression (brown intranuclear staining) in MDA-MB-231 tumors from each treatment group. Tissues were counterstained with hematoxylin. *Right,* proliferation indices in MDA-MB-231 tumors from each treatment group. *Columns,* mean of five ×400 fields; *bars,* SD.

Thr^{157}-p27. Similar results were also obtained in MCF-7 cells (data not shown). In contrast, the level of phosphorylated Thr^{180}/Tyr^{182}-p38 MAPK remained unaltered in drug-treated cells, suggesting that this dephosphorylation effect of the drug combination was specific for components of the Akt pathway. Consistent with the flow cytometry data described previously (Fig. 2), these alterations in Akt signaling were associated with a dose-dependent increase in apoptosis as indicated by poly(ADP-ribose) polymerase cleavage (Fig. 4B). Together, these *in vitro* findings support the existence of mechanistic interactions between OSU-03012-mediated Akt inhibition and the ER-independent actions of tamoxifen in facilitating apoptosis signaling in breast cancer cells.

In vivo Efficacy of the Combination of Tamoxifen and OSU-03012 in a MDA-MB-231 Tumor Xenograft Model

To further evaluate the antitumor potential of the OSU-03012/tamoxifen combination regimen in ER-negative breast cancer, ovariectomized female athymic nude mice bearing established s.c. MDA-MB-231 tumor xenografts (starting mean tumor volume, 59 $\pm$ 5 mm^3) were treated daily for 35 days by gavage with tamoxifen at 60 mg/kg, OSU-03012 at 100 mg/kg, the combination of both drugs at the same respective dose levels, or vehicle. All treatments were well tolerated without overt signs of toxicity and without significant change in body weights compared with the vehicle-treated group throughout the course of this study. As shown in Fig. 5A, treatment with tamoxifen alone had no appreciable effect on MDA-MB-231 tumor growth, but both OSU-03012 alone and the combination regimen significantly inhibited MDA-MB-231 tumor growth by 30% and 50% ($P < 0.05$), respectively, after 5 weeks of treatment. Immunohistochemical evaluation of Ki-67 expression revealed diminished proliferation within tumors from all treatment groups with a significantly greater reduction in mice treated with the tamoxifen/OSU-03012 combination than in those treated with either agent alone ($P < 0.05$; Fig. 5B). These *in vivo* data are consistent with our *in vitro* findings regarding the effect of OSU-03012 on sensitizing MDA-MB-231 cells to tamoxifen via an ER-independent mechanism.

Discussion

Tamoxifen, a selective ER modulator, mediates antiproliferative effects in ER-positive breast cancer cells with nanomolar potency through the disruption of estrogen binding to the ER. Recent studies have indicated that tamoxifen is also effective against ER-negative tumor cells, including those of liver, ovary, pancreas, and breast (4), although at therapeutically unattainable concentrations. Although the ER-independent mechanism by which tamoxifen facilitates apoptosis remains elusive, putative molecular targets include protein kinase C, transforming growth factor-β, calmodulin, c-myc, ceramide, and MAPKs. From a mechanistic perspective, these ER-independent proapoptotic mechanisms could be pharmacologically exploited by targeting PDK-1/Akt signaling to lower the apoptosis threshold in ER-negative breast cancer cells. This hypothesis is of clinical relevance in light of recent evidence that PDK-1/Akt signaling is frequently upregulated in breast cancers (6, 23, 24). The findings presented here provide the proof-of-principle of this hypothesis by showing the ability of the PDK-1/Akt signaling inhibitor OSU-03012 to sensitize MDA-MB-231 cells to the antiproliferative effects of tamoxifen.

The molecular basis for this OSU-03012-mediated sensitization may be 3-fold. First, as a possible compensatory mechanism, tamoxifen treatment, in the range of 2.5 to 7.5 μmol/L, led to a transient increase in Akt phosphorylation in MCF-7 cells and, to a much lesser extent, in MDA-MD-231 cells. This finding is reminiscent of that in a recent report describing a transient increase in p-Akt in MCF-7 cells, but not in MDA-MB-231 cells, after treatment with tamoxifen at 40 to 80 nmol/L (17). The discrepancy between these reported data and our findings in MDA-MB-231 cells might be attributed to differences in the dose of tamoxifen used between these two studies. Nevertheless, this transient tamoxifen-induced elevation of Akt phosphorylation may serve a protective function, which is counteracted by OSU-03012 leading to increased cellular sensitivity to tamoxifen. Second, our data suggest that OSU-03012-mediated Akt inhibition interacted cooperatively with the ER-independent actions of tamoxifen in modulating the functional status of multiple Akt downstream effectors, including FOXO3a, GSK3α/β, and p27. Third, OSU-03012-induced apoptosis in cancer cells has been associated with effects on pathways other than PDK-1/Akt signaling, including the disruption of mitochondrial membrane potential and activation of caspase-9 (5, 9), induction of endoplasmic reticulum stress responses (11), inhibition of p21-activated kinase 1 activity (25), inhibition of Janus-activated kinase 2/signaling transducer activator of transcription 3 and MAPK pathways, and downregulation of cyclins A and B and the inhibitor of apoptosis protein (22) members, X-linked inhibitor of apoptosis, and survivin (26). Thus, these and perhaps other OSU-03012-induced apoptotic pathways may merge with those induced by tamoxifen to culminate in enhanced breast cancer cell death. Which of the putative ER-independent targets of tamoxifen interact with these PDK-1-dependent or PDK-1-independent OSU-03012-induced pathways in cotreated cells remains undefined. Our finding that the phosphorylation status of p38 was not altered by OSU-03012 or tamoxifen suggests that its upstream regulators, such as the putative tamoxifen targets, protein kinase C, calmodulin, c-myc, ceramide, and MAPK, are not involved.

The therapeutic potential of this combination regimen was shown in its superior activity in suppressing established MDA-MB-231 xenograft tumor growth in comparison with that of individual agents (Fig. 5). This *in vivo* finding provides a proof-of-principle that Akt signaling represents a clinically relevant target to sensitize ER-negative breast cancer cells to the ER-independent proapoptotic actions of tamoxifen. This strategy is distinct from that underlying the use of demethylating agents and histone deacetylase inhibitors to restore tamoxifen

sensitivity in MDA-MB-231 cells by reactivating the expression of ER mRNA and functional protein (27). Other approaches reported to enhance tamoxifen sensitivity in ER-negative breast cancer cells include those aimed at triggering apoptotic pathways, such as the induction of ceramide synthesis with persin, a plant toxin (28), and cotreatment with tumor necrosis factor–related apoptosis-inducing ligand (29), both of which modulate tamoxifen responsiveness independent of ER status. Strategies that suppress survival pathways associated with tamoxifen resistance have also been reported, such as inhibition of the cytoprotective protein, clusterin, by immunoneutralization or antisense therapy to counteract its induction in response to tamoxifen treatment in ER-positive cells (30), and inhibition of phosphatidylinositol-3-kinase in chronically estrogen-deprived, aromatase-transfected, ER-positive breast tumor xenografts (31). In addition, the small-molecule zinc finger inhibitor disulfide benzamide effectively restored tamoxifen sensitivity in resistant ER-positive breast cancer cell lines through targeted disruption of ER DNA-binding domain, subsequent modulation of cofactor recruitment, and inhibition of ERE transactivation (32). These and other research efforts addressing tamoxifen sensitivity underscore the major challenge that acquired and *de novo* resistance to antiestrogens poses to the clinical management of breast cancer. Considering the urgent need for novel strategies for the treatment of ER-negative breast cancers, the pharmacologic exploitation of both ER-dependent and ER-independent antitumor activities of tamoxifen with combinatorial approaches represents a paradigm shift in endocrine therapy for breast cancer that is worthy of further investigation.

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