Dual Src Kinase/Pretubulin Inhibitor KX-01, Sensitizes ERα-negative Breast Cancers to Tamoxifen through ERα Reexpression

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Abstract

Unlike breast cancer that is positive for estrogen receptor-α (ERα), there are no targeted therapies for triple-negative breast cancer (TNBC). ERα is silenced in TNBC through epigenetic changes including DNA methylation and histone acetylation. Restoring ERα expression in TNBC may sensitize patients to endocrine therapy. Expression of c-Src and ERs are inversely correlated in breast cancer suggesting that c-Src inhibition may lead to reexpression of ERα in TNBC. KX-01 is a peptide substrate-targeted Src/pretubulin inhibitor in clinical trials for solid tumors. KX-01 (1 mg/kg body weight-twice daily) inhibited growth of targeted Src/pretubulin inhibitor KX-01 xenografts in nude mice that was correlated with Src kinase inhibition. KX-01 also increased ERα mRNA and protein, as well as increased the ERα targets progesterone receptor (PR), pS2 (TFF1), cyclin D1 (CCND1), and c-myc (MYC) in MDA-MB-231 and MDA-MB-468, but not MDA-MB-157 xenografts. MDA-MB-231 and MDA-MB-468 tumors exhibited reduction in mesenchymal markers (vimentin, β-catenin) and increase in epithelial marker (E-cadherin) suggesting mesenchymal-to-epithelial transition (MET). KX-01 sensitized MDA-MB-231 and MDA-MB-468 tumors to tamoxifen growth inhibition and tamoxifen repression of the ERα targets pS2, cyclin D1, and c-myc. Chromatin immunoprecipitation (ChiP) of the ERα promoter in KX-01-treated tumors demonstrated enrichment of active transcription marks (acetyl-H3, acetyl-H3Lys9), dissociation of HDAC1, and recruitment of RNA polymerase II. Methylation-specific PCR and bisulfite sequencing demonstrated no alteration in ERα promoter methylation by KX-01. These data demonstrate that in addition to Src kinase inhibition, peptidomimetic KX-01 restores ERα expression in TNBC through changes in histone acetylation that sensitize tumors to tamoxifen.


Introduction

Breast cancer is the most common cancer in women and the cause of substantial morbidity and mortality. Estrogen receptor (ERα) expression in tumors is a marker for better prognosis and a predictor for response to endocrine therapy (1, 2). However, approximately one-third of breast cancers do not express ERα and these patients are generally associated with poor prognosis and worse clinical outcomes (3, 4). A subset of ERα-negative tumors termed triple-negative breast cancer (TNBC) lacks expression of ERα and progesterone receptor (PR), and does not overexpress the membrane receptor HER2. Patients with TNBC have several clinical characteristics that make them difficult to treat including rapid risk of recurrence at 1–3 years, increased mortality in the first 5 years, and rapid progression from distant recurrence to death (5, 6).

TNBC patients are not candidates for targeted therapies directed against ERα or HER2 and therapy is limited to cytotoxic chemotherapy and radiotherapy that is associated with significant toxicities. The opportunity to reexpress ERα in TNBC patients to sensitize tumors to less toxic endocrine therapy agents represents a promising therapeutic strategy, although there are currently no agents that achieve this result in the clinic. In experimental systems, certain histone deacetylase (HDAC) inhibitors and DNA methyltransferase (DNMT) inhibitors resulted in reexpression of ERα in ERα-negative breast cancer cells and sensitization of cells to endocrine therapy agents (7–9). These studies provided the basis for current clinical trials with HDAC inhibitors panobinostat, entinostat, and DNMT inhibitor 5-azacytidine (10) to reexpress ERα and sensitize tumors to ERα modulators such as tamoxifen.

ERα is silenced in TNBC through epigenetic changes including DNA methylation and altered histone acetylation (11, 12) and possibly additional signaling pathways that silence ERα expression.
c-Src is an oncogenic nonreceptor tyrosine kinase overexpressed in TNBC and identified as a therapeutic target for TNBC (13, 14). ERα and Src expression are inversely correlated in human primary breast cancers (15). Inhibition of Src may provide a mechanism for reexpression of ERα in TNBC.

Peptidomimetics represent a novel class of drugs that interact with the peptide substrate sites of proteins. KX-01 is the "first in class" peptidomimetic non-ATP kinase inhibitor that targets the substrate binding site of Src and inhibits its kinase activity and downstream targets (16, 17). In addition, a second mechanism of action for KX-01 at higher doses was identified as inhibition of tubulin polymerization (18, 19). KX-01 has completed phase 1 clinical testing for solid tumors (NCT00658970) and has completed a phase II trial for prostate cancer (NCT01074138; ref. 20). A phase Ib trial for acute myeloid leukemia is in progress (21) and a phase Ib/IIa clinical trial for KX-01 in mammary fat pads of nude mice and tumors were allowed to grow to approximately 100 mm3. Mice were randomly divided into 4 treatment groups (N = 5 mice, 7–10 tumors/group). Group 1 received pure distilled water by oral gavage twice daily, which served as vehicle control, group 2 was treated with KX-01 (1 mg/kg body weight, twice daily), group 3 mice were implanted with a tamoxifen pellet (5 mg, 60-day release) above the shoulder using a 10-gauge trochar, and group 4 mice were implanted with tamoxifen and treated with KX-01 (1 mg/kg body weight, twice daily). All mice were sacrificed on day 40 due to large tumor size exceeding 1,000 mm3 in the vehicle and tamoxifen alone groups. Tumor diameters were measured twice a week using digital calipers and tumor volume was calculated as 0.523 lM2 (where L is large diameter and M is small diameter). At sacrifice, tumors were removed from the mice and either immediately snap frozen with liquid nitrogen and stored at −80°C, or fixed with 10% formalin solution for IHC staining.

Materials and Methods

Cell culture and reagents

ERα/PR/Her2-negative MDA-MB-231, MDA-MB-468 breast cancer cell lines and the ERα/PR-positive MCF-7 breast cancer cell line were obtained from ATCC. Cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin (Invitrogen). To generate an MDA-MB-468 luc− cell line, MDA-MB-468 cells were transduced with lentiviral particles expressing the luciferase gene and RFP. Transduced cells were then selected for antibiotic resistance (G418; Invitrogen) and surviving colonies were screened for bioluminescence in complete media supplemented with 150 μg/mL n-luciferin (Gold Biotechnology) by in vitro imaging using the IVIS XRMS small-animal imaging system (PerkinElmer). Bioluminescent and RFP-positive cells were grown in culture and characterized for stable luminescence in vivo and tumorigenic potential in vivo. Cells were maintained in a humidified environment of 5% CO2 at 37°C. KX-01 was provided by Athenex Pharmaceuticals. Tamoxifen pellets were purchased from Athenex Pharmaceuticals.

Tumor xenograft study

Female nude mice (4–6 weeks old; BALB/c nude) were purchased from Charles River Laboratories and maintained in pathogen-free conditions. The use and care of animals in this study is approved by the Institutional Animal Care and Use Committee protocol #2941R2 from Tulane University (New Orleans, LA). Xenograft procedures and KX-01 oral dosing was done as described in our previous studies (17, 18). Briefly, we used the MDA-MB-231 xenograft model and tested two doses of KX-01 (1 and 5 mg/kg body weight, twice daily by oral gavage) for 30 days. KX-01 (5 mg/kg) resulted in significant tumor growth inhibition associated with increased apoptosis and microtubule disruption. KX01 (1 mg/kg) exhibited a more modest tumor growth inhibition but no significant apoptosis or microtubule disruption was detected in the tumors (18). This study used KX-01 at 1 mg/kg body weight, a dose that inhibits Src kinase activity. MDA-MB-231 cells (5 × 106) were injected bilaterally into the mammary fat pads of nude mice and tumors were allowed to grow to approximately 100 mm3. Mice were randomly divided into 4 treatment groups (N = 5 mice, 7–10 tumors/group). Group 1 received pure distilled water by oral gavage twice daily, which served as vehicle control, group 2 was treated with KX-01 (1 mg/kg body weight, twice daily), group 3 mice were implanted with a tamoxifen pellet (5 mg, 60-day release) above the shoulder using a 10-gauge trochar, and group 4 mice were implanted with tamoxifen and treated with KX-01 (1 mg/kg body weight, twice daily). All mice were sacrificed on day 40 due to large tumor size exceeding 1,000 mm3 in the vehicle and tamoxifen alone groups. Tumor diameters were measured twice a week using digital calipers and tumor volume was calculated as 0.523 × L × M2 (where L is large diameter and M is small diameter). At sacrifice, tumors were removed from the mice and either immediately snap frozen with liquid nitrogen and stored at −80°C, or fixed with 10% formalin solution for IHC staining.

Bioluminescent imaging

A similar experiment as described for MDA-MB-231 xenografts was carried out with MDA-MB-468 xenografts using MDA-MB-468 luc+ cells. Approximately 5 × 106 MDA-MB-468 luc+ cells were injected into the mammary fat pads of nude mice to form primary tumors. Bioluminescent imaging was performed with a highly sensitive, cooled CCD camera mounted in a light-tight specimen box (IVIS XRMS, PerkinElmer). Imaging and quantification of signals were controlled by the acquisition and analysis software Living Image (PerkinElmer). For in vivo imaging, animals were given the substrate n-luciferin by intraperitoneal injection at 150 mg/kg in Dulbecco PBS (DPBS; Invitrogen) and anesthetized (1%–3% isoflurane). Mice were then placed into the IVIS box containing a light-tight camera with continuous exposure to 1%–2% isoflurane. Imaging times ranged from 1 second to 5 minutes depending on the tumor and the time point. Generally, one animal was imaged at a time. The low levels of light emitted from the bioluminescent tumors or cells were detected by the IVIS camera system, integrated, digitized, and displayed. Regions of interest from displayed images were identified around the tumor sites and were quantified as total photon counts or photons/sec using Living Image software (PerkinElmer). Background bioluminescence in vivo was the region of 1 × 108 photon counts or 1–2 × 107 photons/s. Tissues were subsequently fixed in 10% formalin (Sigma) and prepared for IHC evaluation.

IHC

IHC staining was performed on 10% neutral buffered formalin-fixed paraffin-embedded tumor samples as described previously (17, 18). Briefly, sections mounted on slides were deparaffinized in xylene, dehydrated in ethanol, rinsed in water, and antigen retrieval was carried out with 0.01 mol/L citrate buffer (pH 6.0) for 20 minutes in a steamer and then incubated with 3% hydrogen peroxide for 5 minutes. After washing with PBS, sections were...
blocked by incubation in 10% normal goat serum for 30 minutes, followed by overnight incubation with primary antibody. The source of the primary antibody and the dilutions used for IHC were as follows: ERα (1:100), Ki67 (prediluted; NovoMarkets), PR (1:100; Thermo Scientific), vimentin (1:100; Vector Laboratories), E-cadherin (1:400), β-catenin (1:800), total Src (1:200), and phospho-Y416 Src (1:100) from Cell Signaling Technology Inc. After overnight incubation with primary antibody, slides were washed with PBS followed by 30 minutes incubation with biotinylated secondary antibody (Vector Laboratories), rinsed in PBS, and incubated with ABC reagent (Vector Laboratories) for 30 minutes. The stain was visualized by incubation in 3, 3-diaminobenzidine (DAB) and counterstained with Harris hematoxylin.

Internal negative control samples incubated with either nonspecific rabbit IgG or 10% goat serum instead of the primary antibody showed no specific staining. Slides were dehydrated and mounted with Permount (Fisher Scientific). Slides were visualized using a Nikon OPTIPHOT microscope and randomly selected bright-field microscope images (magnification, ×200) were captured by Nikon Digital Sight High-Definition color camera (DS-Fi1) using NIS-Elements BR software.

**Quantitative real-time RT-PCR**

Total RNA was extracted from MDA-MB-231, MDA-MB-468, and MCF-7 (positive control) tumors using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Five micrograms of total RNA was reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). In the real-time PCR step, PCR reactions were performed in triplicates with 1 μL cDNA per reaction and primers specific for ERα (Hs01046818_m1) and GAPDH (Hs99999905_m1) provided by InVentoried Gene Assay Products (Applied Biosystems) using the Fast Start TaqMan probe master (Roche Diagnostics) in a q5 Bio-Rad thermocycler. Thermal cycling was initiated at 94°C for 4 minutes followed by 40 cycles of PCR (94°C, 15 seconds; 60°C, 30 seconds). GAPDH was used as an endogenous control and vehicle control was used as a calibrator. The relative changes of gene expression were calculated as:

$$\frac{2^{-\Delta\Delta C_{T}}}{\Delta C_{T} (treated \ samples)} - \Delta C_{T} (untreated \ control \ samples),$$

where $\Delta C_{T} = C_{(treated)} - C_{(GAPDH)}$ and $C_{(t)}$ represents threshold cycle number. The real-time RT-PCR was performed in triplicates and repeated at least two times.

**Chromatin immunoprecipitation assay for the ERα promoter**

Tumor samples that were snap frozen in liquid nitrogen and stored at −80°C were used for chromatin immunoprecipitation (ChIP) assays. MDA-MB-231 tumors from the treatment groups (i) vehicle control (VC), (ii) KX-01, (iii) tamoxifen, and (iv) TAM + KX-01 were used. MCF-7 tumors were used as a positive control for ERα expression. The ChIP assay was performed using the Magna ChIP G tissue kit according to the manufacturer’s protocol (Millipore) and our previous studies (23). Briefly, a 5-mm tumor tissue piece was obtained using a microdissection punch and the sample was dispersed in 1 mL Magna ChIP G tissue stabilization solution with protease inhibitors and then cross-linked using 1% formaldehyde treatment [prepared fresh; 270 μL of 37% formaldehyde (Sigma) to 10 mL of PBS]. Glycine (125 mM/L) was used to quench the formaldehyde and block further cross linking. After centrifugation at 800 × g at 4°C for 5 minutes, the pellet was rinsed in PBS, suspended in 500 μL Magna ChIP G tissue lysis buffer, vortexed well, and incubated on ice for 15 minutes. Cells were then centrifuged at 800 × g at 4°C for 5 minutes and the supernatant was removed. The cell pellet was resuspended in 125 μL Magna ChIP dilution buffer in a 1.5-mL tube, and the samples were sonicated using the Bioruptor automatic sonicator (Diagenode) at 4°C for 12 cycles of 30 seconds “ON”/30 seconds “OFF” to shear chromatin and generate DNA fragments of 200–1,000 base pairs. Five microliters (1%) of the content was removed and saved in 4°C as input. The sheared cross-linked chromatin was immunoprecipitated (IP) using ChIP-validated antibodies to acetyl-histone H3, acetyl-histone H3-lys9 (H3K9), trimethyl-histone H3-lys9 (Upstate Biotechnology), HDAC1, and RNA Pol II (Santa Cruz Biotechnology). Each IP reaction consisted of 125 μL of chromatin + 375 μL of dilution buffer with protease inhibitors + 20 μL of protein G magnetic beads + 5 μg of primary antibody. The IP reactions were incubated at 4°C overnight with rotation. IgG from the same species as the primary antibodies served as negative controls. Magnetic beads were separated using a magnetic separator (Biolabs) and the supernatant was discarded. The Protein G magnetic beads–antibody–chromatin complex was captured with a series of wash buffers provided in the Magna ChIP G tissue Kit: one time each for 5-minute each wash on a rotating platform followed by magnetic clearance and careful removal of the supernatant fractions: 500 μL low salt immune complex wash buffer, 500 μL high salt immune complex wash buffer, 500 μL LiCl immune complex wash buffer, 500 μL TE buffer. Following immunoprecipitation, protein–DNA cross-links were reversed by adding 100 μL Magna Chip elution buffer with protease K and incubated at 62°C for 2 hours with shaking followed by incubation at 95°C for 10 minutes. Samples were allowed to cool to room temperature and the magnetic beads were separated and supernatant was transferred to a new tube and DNA was purified using spin columns according to the manufacturer’s protocol. ChIP-purified DNA was amplified by standard PCR using primers for the ERα promoter (sense, 5’-CAAGCATCCTGAGGGACGTCTAG-3’; antisense, 5’-GATCCACGTCCGTGCTGCGGCTG-3’) yielding a 150-bp fragment using the following reaction conditions: 2 μL of ChIP-purified DNA or 1% total input DNA, 200 μmol/L of each primer, 1.5 μmol/L MgCl2, 200 μmol/L dNTP, 10 μL PCR gold buffer (Applied Biosystems), and 2 units of Hot start AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total volume of 20 μL. The reaction was initiated at 94°C for 4 minutes followed by 30 cycles of PCR (94°C, 30 seconds; 56°C, 30 seconds; 72°C, 1 minute), and extended at 72°C for 5 minute. After amplification, PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining using a Gel Doc 2000 instrument (Bio-Rad). All ChIP assays were performed three times yielding similar results.

**Methylation-specific PCR analysis**

Genomic DNA was isolated from MDA-MB-231 tumors treated with VC, TAM, KX-01, and TAM + KX-01, and from MCF-7 tumors using the QIAamp DNA Mini Kit (QIagen) according to the manufacturer’s instructions. Five-hundred nanograms of genomic DNA was bisulfite treated using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer’s instructions. The bisulfite treatment converts unmethylated cytosine residues, but not methylated cytosines, to uracil (detected as thymine following PCR). One-hundred nanograms of bisulfite-converted DNA was used as a template for methyl-specific PCR. ERα-positive MCF-7 tumor was used as an unmethylated (U) control for the ERα promoter,
whereas vehicle-treated ERα-negative MDA-MB-231 tumors were considered as a methylated control for the ERα promoter. The methylation status of bisulphite-modified DNA at the critical region of the ERα promoter CpG islands was characterized by methyl-specific PCR using the following primers (8, 11, 24):

ER unmethylated (U) forward primer: 5’GGTGTATTGCTAGTAGTAAAGTTGT 3’; reverse primer: 5’CCCATTAACAAAAACCATCTAACACC 3’;

ER methylated (M) forward primer: 5’GTTGATTTGGATAGTAGTAAAGTTGT 3’; reverse primer: 5’CGTAAAAAACCGATCTAAACCG 3’.

The PCR mixture contained 100 ng DNA, 200 nmol/L of each primer, 1.5 mmol/L MgCl₂, 200 μmol/L dNTPs, 10× PCR gold buffer (Applied Biosystems), and 2 units of Hot start AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total volume of 20 μL. The reaction was initiated at 95°C for 5 minutes followed by 31 cycles of PCR (95°C, 30 seconds; 55°C, 30 seconds; 72°C, 30 seconds), and extended at 72°C for 5 minutes. PCR products were subjected to electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Pictures were taken using a Gel Doc 2000 instrument. Assays were performed three times yielding similar results.

**Bisulfite sequencing analysis**

Bisulfite modification was carried out using Zymo Research EZ Methylation kit (D5004). Sample DNA (200–500 ng) was used for bisulfite modification following by the PCR amplification. –533 to +120 from ATG site (−172 to +481 from transcriptional start site TSS) methylation sequencing was performed by EpigenDx to determine the site-specific methylation changes in the ERα promoter region.

**Statistical analysis**

Statistical significance was evaluated using the Student t tests (P < 0.05; two-tailed) and one-way ANOVA following by Tukey multiple comparison test. Data were expressed as mean ± SD. P < 0.05 was considered statistically significant. The mean and SD values were calculated using Microsoft Excel or Graphpad Prism.

**Results**

**Oral administration of KX-01 sensitized ERα-negative MDA-MB-231 and MDA-MB-468 breast tumor xenografts to tamoxifen**

When MDA-MB-231 tumor volumes reached approximately 80–100 mm³, mice were treated with vehicle (ultrapure water), KX-01 at 1 mg/kg twice daily, tamoxifen (5 mg pellet; 60-day release), or tamoxifen + KX-01 continuously for up to 40 days. KX-01 used at 1 mg/kg body weight resulted in some tumor growth inhibition beginning at day 18 (Fig. 1A), but the drug efficacy at 1 mg/L was less compared with our previous study that used 5 mg/kg KX-01 (18). Tumor growth inhibition by 1 mg/kg body weight KX-01 alone was correlated with inhibition of Src kinase (Supplementary Fig. S1), indicating that Src kinase inhibition likely contributed to KX-01 efficacy in MDA-MB-231 tumors. Mice implanted with tamoxifen pellet alone did not exhibit tumor growth inhibition compared with the vehicle control (Fig. 1A). On day 40, mice in the control and tamoxifen treatment groups had to be sacrificed due to high tumor burden. At day 40, KX-01 alone and tamoxifen + KX-01 reduced tumor volume by 59% and 70%, respectively, compared with vehicle. Tumor volume for the tamoxifen + KX-01 group was significantly reduced compared with the KX-01 alone group (Fig. 1A and B), and the difference in tumor volume between these treatments increased at day 48 (P < 0.01) and at day 60 (P < 0.001) (data not shown). The final tumor weights (day 60) for the tamoxifen + KX-01 group was 32% lower compared with the KX-01 alone group (Fig. 1F).

In MDA-MB-468 tumors, tamoxifen alone (10 mg pellet; 60-day release) and KX-01 alone (1 mg/kg body weight, twice daily) had no effect on tumor volume compared with vehicle (Fig. 1C–E) demonstrating resistance of MDA-MB-468 tumors to both drugs. However, cotreatment with tamoxifen + KX-01 reduced tumor volume 67% compared with vehicle (Fig. 1C–E). The final tumor weights for the tamoxifen + KX-01 group was 43% lower compared with the KX-01 alone group (Fig. 1G).

**KX-01 induced expression of ERα in MDA-MB-231 and MDA-MB-468 tumor xenografts**

MDA-MB-231 and MDA-MB-468 tumor sections were examined for the effect of KX-01 on protein levels of ERα and the ERα target PR, which is a marker for a functional ERα signaling pathway. IHC analysis revealed that ERα and PR expression was absent in tumors from the vehicle control and tamoxifen-treated group in both MDA-MB-231 and MDA-MB-468 tumors, but KX-01 alone and KX-01 + TAM significantly increased ERα and PR expression (Fig. 2A–D). These results demonstrate that treatment with KX-01 in two TNBC xenograft tumors (MDA-MB-231 and MDA-MB-468) resulted in reexpression of ERα, a requirement for tumor sensitivity to tamoxifen. Proliferation marker Ki67 was significantly reduced in tumors treated with TAM + KX-01 compared with KX-01 alone or control treatment (Fig. 2A–D).

To further evaluate the KX-01 effect on reexpression of ERα, we assessed two TNBC patient-derived xenograft (PDX) tumors. PDX tumors propagated in mice were excised and cultured in medium ex vivo with vehicle or KX-01 (25, 50 nmol/L) for 72 hours. KX-01 (25 and 50 nmol/L) increased ERα mRNA 2.7- and 3.4-fold, respectively (Supplementary Fig. S2).

To further assess the restoration of ERα signaling by KX-01, MDA-MB-231 tumors were assessed for expression of additional ERα target proteins, c-myc, cyclin D1, and pS2. KX-01 induced expression of cyclin D1 and pS2 protein. Cotreatment of KX-01 + tamoxifen resulted in suppression of c-myc, cyclin D1, and pS2 protein levels (Supplementary Fig. S3). These results indicate that KX-01 could restore ERα target proteins in MDA-MB-231 tumors and that cotreatment with tamoxifen could suppress the KX-01–induced expression.

It is possible that KX-01 sensitized tumor cells to off-target effects of tamoxifen to induce apoptosis (25). To address this possibility, the level of apoptosis was measured in tumors from all treatment groups in MDA-MB-231 tumors (Supplementary Fig. S4). Tamoxifen alone did not induce apoptosis. KX01 induced a very modest level the apoptosis, and there was no additional apoptosis in the KX01 + tamoxifen treatment group. To further address off-target effects of tamoxifen, and a requirement for ERα reexpression to sensitize tumors to tamoxifen, we used another TNBC xenograft model, MDA-MB-157, that does not express significant ERα protein in response to KX-01 treatment (Supplementary Fig. S5A). Tamoxifen alone did not induce apoptosis, KX-01 alone induced a modest level of apoptosis, and KX-01 + tamoxifen induced the same level of apoptosis as KX-01 alone. 

Published OnlineFirst July 27, 2017; DOI: 10.1158/1541-7786.MCR-16-0297-T

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in MDA-MB-157 tumors (Supplementary Fig. S6). Tamoxifen did not result in tumor growth inhibition in the presence and absence of KX-01 (Supplementary Fig. S5B). These data indicate that when KX-01 does not result in significant ERα protein expression in these TNBC tumors, the tumors were not sensitized to tamoxifen growth inhibition. The data with MDA-MB-157 tumors further indicate that KX-01 targets mechanisms other than ERα reexpression that contribute to the antitumor efficacy.

**ERα reexpression and sensitivity to tamoxifen was reversible upon KX-01 withdrawal**

To determine the reversibility of ERα reexpression and tamoxifen sensitivity by KX-01 in MDA-MB-231 tumors, tumor-bearing animals were treated with KX-01 for 14 days, KX-01 treatment was withdrawn, and then the animals were then divided and treated with either tamoxifen or vehicle for an additional 16 days. There was no significant difference in tumor volume between the tamoxifen and vehicle treatment groups at day 30 (Supplementary Fig. S7A). The tumors in both treatment groups did not exhibit significant ERα expression (Supplementary Fig. S7B) as compared with continuous KX-01 treatment (Fig. 2A and C). MCF-7 tumor sections were used as a positive control that demonstrated a robust ERα expression (Supplementary Fig. S7C).

**KX-01 treatment increased epithelial markers and reduced mesenchymal markers in MDA-MB-231 and MDA-MB-468 tumors**

ERα expression is a marker for a well-differentiated breast tumor with epithelial-like phenotype. As MDA-MB-231 tumors...
exhibit a mesenchymal phenotype, the reexpression of ERα by KX-01 suggested that the tumors may have undergone a mesenchymal to epithelial transition (MET). Both MDA-MB-231 and MDA-MB-468 tumors express the mesenchymal marker vimentin and exhibit β-catenin staining in the cytoplasm and nucleus. These tumors are negative for the epithelial marker E-cadherin, a cell–cell adhesion protein that is increased by Src inhibition (26). KX-01 treatment increased E-cadherin expression in tumor cell membranes and markedly reduced vimentin expression (Fig. 3A and B). Nuclear β-catenin contributes to breast tumorigenesis by regulating genes that are involved in proliferation, invasion, and EMT (27). When β-catenin is expressed in the cell membrane with E-cadherin, signaling-competent nuclear β-catenin levels diminished and cell proliferation and invasion were suppressed (28). β-Catenin is located predominantly in the nucleus of untreated MDA-MB-231 and MDA-MB-468 tumors. KX-01 treatment resulted in marked redistribution to the cell membrane (Fig. 3A–D). Reexpression of ERα protein and the epithelial marker E-cadherin by KX-01 in MDA-MB-231 was further demonstrated by Western blot analysis (Supplementary Fig. S8). Taken together, these data demonstrate that KX-01 induced epithelial markers and suppressed mesenchymal markers in two TNBC xenograft tumors.
KX-01 induced histone modifications in the ERα promoter region of MDA-MB-231 tumors

We sought to investigate the mechanisms involved in ERα reexpression mediated by treatment with KX-01. KX-01 resulted in 3- to 4-fold increase in ERα mRNA in MDA-MB-231 tumors (Fig. 4A). Previous studies have reported that histone acetylation and methylation in the ERα promoter regulate expression in MDA-MB-231 cells in vitro (24, 29–31). Histone modification patterns in MDA-MB-231 tumors were analyzed by ChIP assays using antibodies to both transcriptionally active (acetyl-H3, acetyl-H3 Lys9) and inactive (trimethyl-H3 Lys9) markers of chromatin (24). KX-01 and KX-01 + tamoxifen resulted in enrichment of the active histone acetylation chromatin markers, acetyl-H3 and acetyl-H3 Lys9 (Fig. 4B). The inactive, trimethyl-H3 Lys9 mark was not changed in any of the treatment groups compared with vehicle (Fig. 4B). Remarkably, KX-01 treatment resulted in HDAC1 dissociation from the ERα promoter, and a concomitant recruitment of RNA polymerase II (Fig. 4B). KX-01 did not alter histone deacetylase 1 (HDAC1) levels or activity in tumors (Supplementary Fig. S9). Collectively, these data demonstrate that KX-01 induced alterations in histone acetylation that were consistent with a recruitment of RNA polymerase II to the ERα promoter and transcriptional increase in ERα mRNA (Fig. 4A).

KX-01 did not alter DNA methylation status of the ERα promoter in MDA-MB-231 tumors

The ERα promoter in human ERα-negative breast cancer cell lines is highly methylated at CpG islands (29). More than 25% of ERα-negative breast cancer cells exhibit aberrant methylation in the ERα promoter suggesting that DNA methylation plays a critical role in regulating ERα expression (11, 32). The methylation status of the ERα promoter region covering +375 to +495...
CpG islands was examined in MDA-MB-231 tumors using methylation-specific PCR (MSP) analysis. As a control, the ERx promoter region in ERx-positive MCF-7 breast cancer cells was predominantly unmethylated (Fig. 5B, lanes 9–10). In contrast, the ERx promoter in vehicle-treated MDA-MB-231 tumors was partially hypermethylated (Fig. 5B, lanes 1–2). Treatment with KX-01 or tamoxifen alone or in combination did not significantly alter the methylation status of the ERx promoter in MDA-MB-231 tumors (Fig. 5B, lanes 3–7). These data indicated that reexpression of ERx in MDA-MB-231 tumors by KX-01 was not the result of alteration in the methylation status of the ERx CpG islands. To elucidate the effects of methylation on the ERx promoter region, we examined the methylation status of the ERx promoter region from −66 to −356 covering most of the CpG dinucleotides. Bisulfite-sequencing was used to examine ERx methylation patterns MDA-MB-231 tumors. ERx-positive MCF-7 breast cancer cells served as control. The ERx promoter region of MCF-7 cells maintained an unmethylated status, whereas the ERx promoter of MDA-MB-231 tumors was hypermethylated on CpG islands (~80%; Fig. 5C). There was no significant change in the methylation status of the ERx promoter in MDA-MB-231 tumors from animals treated with vehicle (84.8% ± 14.7%), tamoxifen (78% ± 10%), KX-01 (75% ± 12%), and KX-01 + tamoxifen (81.4% ± 15.1% Fig. 5C), indicating that alterations in DNA methylation does not contribute to ERx reexpression by KX-01. These results indicated that KX-01–induced changes in histone modifications of the ERx promoter was of greater importance for ERx reexpression that were changes in DNA methylation in TNBC.

Discussion

Surgery, chemotherapy, and radiation are mainstays for therapeutic management of TNBC. Targeted therapy is limited in TNBC due to the paucity of druggable targets such as ERx and HER2/neu. ERx is silenced in TNBC through epigenetic changes including DNA methylation and altered histone acetylation and hyperactivation of kinases (24, 29, 33). In this study, clinical Src/pretubulin inhibitor KX-01 resulted in a robust reexpression of ERx in TNBC tumor models that coincided with activating epigenetic marks in the ERx promoter. Tumors treated with low-dose KX-01 became sensitized to the endocrine therapy agent tamoxifen and also exhibited a decrease in mesenchymal markers and an increase in epithelial markers. This study describes a novel application of a clinical peptidomimetic Src kinase inhibitor, KX-01, for TNBC resulting in ERx reexpression that occurs through epigenetic changes in the tumor. ERx reexpression and mesenchymal to epithelial reprogramming of TNBC tumors by KX-01, may sensitize tumors to tamoxifen or other endocrine therapy agents and limit metastatic spread of TNBC.

Restoration of ERx expression in TNBC patients is an appealing treatment strategy that could sensitize tumors to endocrine therapy and avoid or reduce the levels of cytotoxic chemotherapy needed for disease management. In contrast to irreversible genetic mutations, epigenetic changes, such as occurring in the ERx gene, are potentially reversible (34), making these changes amenable to pharmacological interventions (35). Currently, there are no agents that achieve reexpression of ERx in the clinic although certain HDAC inhibitors, demethylating agents, epigallocatechin-3-gallate (a major polyphenol in green tea), and arsenic trioxide have been shown to reexpress ERx in experimental models (36, 37). Src expression and activity is inversely correlated with ERx levels in human primary breast cancers (15, 38, 39) suggesting that Src kinase inhibition may be a strategy for reexpression of ERx and restoration of sensitivity to endocrine therapies.

This study identified a previously unknown preclinical application of KX-01 at low doses that results in the reexpression of ERx in TNBC tumors. KX-01 is a novel peptidomimetic compound with two identified MOA’s (18, 19): (i) inhibition of Src kinase that was evident at both low dose (1 mg/kg twice daily; Supplementary Fig. S1) and high dose (5 mg/kg twice daily) KX-01 in MDA-MB-231 tumors (18), and (ii) microtubule disruption evident only at higher doses [≥5 mg/kg twice daily (ref. 18; Supplementary Fig. S10). At these higher doses of ≥5 mg/kg twice daily, KX-01 did not result in reexpression of ERx in MDA-MB-231 tumors (data not shown). The reexpression of ERx only at the lower KX-01 dose suggests that the Src inhibition MOA of KX-01, but not microtubule disruption, was contributing to the ERx reexpression. These dose-dependent changes in the MOA and action of KX-01 are reminiscent of other drugs such as cyclophosphamide that exhibits an...
immunosuppressive and tumoricidal effect at high dose, but an immunostimulatory effect at low dose (40).

Histone acetylation/deacetylation is the most prominent post-translational modification of histones and a crucial determinant of gene expression (41). Histone deacetylation of core histones generates an overall positive charge on lysine residues that when reacted with negatively charged DNA, results in a more compact nucleosome that limits transcription due to the physical inability of RNA polymerase to access the DNA (42). Histone acetylation reactions with positively charged lysine residues, results in a more compact nucleosome that limits transcription due to the physical inability of RNA polymerase to access the DNA (42).

Histone acetylation changes are consistent with the findings that dynamic and reversible chromatin modifications regulate gene expression. Previous studies demonstrated that silenced ERα could be reexpressed solely by changes in histone acetylation without alteration of the DNA methylation pattern. Treatment with the HDAC inhibitor LBH589 or epigallocatechin-3-gallate restored ERα mRNA and protein expression in MDA-MB-231 cells without demethylation of the CpG islands within the ERα promoter (8, 43). Taken together, these studies support the hypothesis that changes in histone acetylation alone can restore the expression of the silenced ERα gene without altering the DNA methylation state at the ERα promoter, and provide a mechanistic explanation for the transcriptional activation of the silenced ERα gene by KX-01.

There are several potential mechanisms by which inhibition of Src by KX-01 may alter histone modifications and impact chromatin structure in the ERα promoter. Yu and colleagues reported that Src phosphorylated the HDAC Inhibitor of Growth 1 (ING1), resulting in nuclear to cytoplasmic localization and decrease in protein stability (44). C-terminal Src kinase (Csk)-binding protein (Cbp)/PAG1 expression was repressed by Src-mediated alterations in histone H4 acetylation and trimethylation of the ERα promoter (45). The DNA methylation status of the ERα promoter in tumors from VC, TAM, KX-01, TAM + KX-01, ERα-positive MCF-7 tumors were used as a control for the unmethylated ERα promoter and H2O used as negative no template control. A representative gel photograph is presented and the experiment was repeated three times. The DNA methylation status of the ERα promoter in tumors from VC, TAM, KX-01, TAM + KX-01 treatment mice were detected by sodium bisulfite methylation sequencing. MCF-7 and no template were used as controls. U, Unmethylated; M, methylated; VC, vehicle control; TAM, tamoxifen.

![Effect of KX-01 on ERα promoter methylation status in MDA-MB-231 tumors.](image)

**Figure 5.**

Effect of KX-01 on ERα promoter methylation status in MDA-MB-231 tumors. **A,** A schematic overview of the Csp island (denoted by the dark shaded area) of the ERα promoter region is shown. ATG, start codon; TSS transcription start site. **B,** The methylation pattern of the ERα promoter was analyzed using a MSP (methylation-specific PCR) with previously reported primer set ERS (4) in all four treatment groups (VC, TAM, KX-01, TAM + KX-01). ERα-positive MCF-7 tumors were used as a control for the unmethylated ERα promoter and H2O used as negative no template control. A representative gel photograph is presented and the experiment was repeated three times. **C,** The DNA methylation status of the ERα promoter in tumors from VC, TAM, KX-01, TAM + KX-01 treatment mice were detected by sodium bisulfite methylation sequencing. MCF-7 and no template were used as controls. U, Unmethylated; M, methylated; VC, vehicle control; TAM, tamoxifen.

Tamoxifen sensitivity in the MDA-MB-231 and MDA-MB-468 tumors occurred only after KX-01 treatment suggesting that ERα reexpression is needed to restore tamoxifen sensitivity to TNBC tumors. As PR is a marker for a functional ERα signaling pathway, the reexpression of PR is also a candidate biomarker for a tumor that would be responsive to endocrine therapy. MDA-MB-231 and
MDA-MB-468 tumors are histologically different. MDA-MB-231 cells were derived from a adenocarcinoma; the cells are highly invasive expressing mesenchymal markers and are representative of late-stage breast cancer (50). MDA-MB-468 cells were derived from a patient with a histologically different tumor (ductal carcinoma) and exhibit less mesenchymal features than MDA-MB-231 cells (51). The sensitization to endocrine therapy by KX-01 of these two TNBC models that have different features may serve as a paradigm for future studies of tumor response to KX-01 for endocrine therapy sensitivity.

Inhibition of Src kinase has been shown to prevent ERα protein degradation in breast cancer (15). In addition to increasing ERα mRNA in triple-negative tumors, it is possible that KX01 may also inhibit ongoing degradation of ERα protein. We measured ERα mRNA in three TNBC cell lines with and without KX01 treatment. Compared with MCF-7 cells that express robust levels of ERα mRNA, all TNBC cell lines had very low (MDA-MB-468, MDA-MB-157) to barely detectable (MDA-MB-231) ERα mRNA that was markedly increased by KX01 treatment (Supplementary Fig. S11). In MDA-MB-157 tumors, KX01 markedly increased ERα mRNA without a significant increase in ERα protein (Supplementary Fig. S5A), suggesting that KX-01 treatment has a greater effect on inducing ERα mRNA than in inhibiting ERα protein turnover in these tumors. Although the major contribution of KX01 to ERα reexpression in MDA-MB-231 and MDA-MB-468 is likely increased ERα mRNA, we cannot exclude that some of the ERα protein reexpression observed may be due to KX-01 inhibiting ERα protein turnover.

In addition to reexpression of ERα/PR by KX-01, MDA-MB-231 and MDA-MB-468 tumors also exhibited an increase in the epithelial marker E-cadherin expression, and a concomitant decrease in mesenchymal markers nuclear β-catenin and vimentin. Epithelial-to-mesenchymal transition (EMT) has been recognized as a critical feature of embryogenesis, organogenesis, and has been shown to play a critical role in cancer progression and metastasis (52). Human breast cancers exhibit a strong direct correlation between ERα and E-cadherin expression and studies have shown that ERα signaling can regulate E-cadherin expression and EMT (53, 54). In addition, Src inhibition has been shown to inhibit EMT and reduce metastasis in many cancers including breast cancer (26, 55). Although the effect on primary TNBC growth inhibition by KX-01 + tamoxifen was modest, the paradigm of ERα re-expression by a clinical agent, KX-01, provides opportunity to test additional endocrine therapy agents and/or combination with other none-endocrine therapies that are effective in ER-positive breast cancer. As TNBC is frequently metastatic, another potential clinical benefit of KX-01 is the ability to induce MET that could limit metastatic spread. In this regard, a number of differentiation therapies induce MET and limit breast cancer metastasis (36–60). It is possible that patient tumors exhibiting silenced ERα that is due predominantly to chromatin remodeling (deacetylated histones) and that also exhibit elevated Src kinase may be candidates for KX-01 therapy to reexpress ERα and induce MET in the tumors.

Disclosure of Potential Conflicts of Interest

D. Hangauer has ownership interest (including patents) in Athenex Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Mr. Tripp Frasch for assistance with preparing figures.

Grant Support

This study was supported, in part by DK068432 (to B.G. Rowan), LA CATS Center grant 1 U54 GM104940 (to B.M. Collins-Burow), and CA125806 (to M.E. Burow). This work has been supported in part by the Bio-specimen Core Laboratory of the Louisiana Cancer Research Consortium.

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Received September 2, 2016; revised February 22, 2017; accepted July 19, 2017; published OnlineFirst July 27, 2017.

References


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Molecular Cancer Research

Dual Src Kinase/Pretubulin Inhibitor KX-01, Sensitizes ERα -negative Breast Cancers to Tamoxifen through ERα Reexpression

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doi:10.1158/1541-7786.MCR-16-0297-T

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