Preclinical Development

Peptidomimetic Src/Pretubulin Inhibitor KX-01 Alone and in Combination with Paclitaxel Suppresses Growth, Metastasis in Human ER/PR/HER2-Negative Tumor Xenografts

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Abstract

Src kinase is elevated in breast tumors that are ER/PR negative and do not overexpress HER2, but clinical trials with Src inhibitors have shown little activity. The present study evaluated preclinical efficacy of a novel peptidomimetic compound, KX-01 (KX2-391), that exhibits dual action as an Src and pretubulin inhibitor. KX-01 was evaluated as a single-agent and in combination with paclitaxel in MDA-MB-231, MDA-MB-157, and MDA-MB-468 human ER/PR/HER2-negative breast cancer cells. Treatments were evaluated by growth/apoptosis, isobologram analysis, migration/invasion assays, tumor xenograft volume, metastasis, and measurement of Src, focal adhesion kinase (FAK), microtubules, Ki67, and microvessel density. KX-01 inhibited cell growth \textit{in vitro} and in combination with paclitaxel resulted in synergistic growth inhibition. KX-01 resulted in a dose-dependent inhibition of MDA-MB-231 and MDA-MB-157 tumor xenografts (1 and 5 mg/kg, twice daily). KX-01 inhibited activity of Src and downstream mediator FAK in tumors that was coincident with reduced proliferation and angiogenesis and increased apoptosis. KX01 also resulted in microtubule disruption in tumors. Combination of KX-01 with paclitaxel resulted in significant regression of MDA-MB-231 tumors and reduced metastasis to mouse lung and liver. KX-01 is a potently active Src/pretubulin inhibitor that inhibits breast tumor growth and metastasis. As ER/PR/HER2-negative patients are candidates for paclitaxel therapy, combination with KX-01 may potentiate antitumor efficacy in management of this aggressive breast cancer subtype. \textit{Mol Cancer Ther}; 11(9); 1–12. ©2012 AACR.

Introduction

Patients with ER/PR/HER2-negative breast cancer subtype have been difficult to treat due to tumor heterogeneity and lack of definitive targets for targeted therapeutics (1). Chemotherapy remains the standard of care for patients, as hormonal therapies and HER2 targeting agents are not indicated (2). Paclitaxel is a microtubule-stabilizing agent widely used in breast cancer (3). Although ER/PR/HER2-negative breast cancer responds to these and other conventional agents, patients become resistant to treatment and relapse more frequently than patients with other breast cancer subtypes (4).

\textit{c-Src} is an oncogenic non–receptor tyrosine kinase that is upregulated in approximately half of all breast cancers (5). Src kinase has been associated with breast cancer proliferation, angiogenesis, cell motility, migration/invasion, and metastasis (6, 7). The role of Src in proliferation, migration, and invasion coupled with the elevated Src expression in breast cancer make Src a promising target for development of therapeutics. Moreover, Src inhibition has been identified as a therapeutic target for ER/PR/HER2-negative breast cancer (8). However as a single agent, the broad specificity Src/tyrosine kinase inhibitor dasatinib resulted in only a modest response rate of 5% in a phase II trial of 43 patients with advanced ER/PR/HER2-negative breast cancer (4, 9). Another Src inhibitor, sarcatinib failed as monotherapy in patients with hormone receptor–negative breast cancer (10). It is unknown why these compounds failed in these early trials or whether combinations with other drugs may have resulted in better response.

Peptidomimetics represent a novel class of drugs that interact with the peptide substrate sites of proteins. KX-01 (clinical-reference, KX2-391) was developed as a “first-in-class” peptidomimetic Src kinase inhibitor that binds to the peptide substrate site and inhibits Src kinase activity and downstream targets (11, 12). Orally bioavailable KX-01 completed phase I clinical testing (13) and is currently
in phase II trials for prostate cancer (14) as well as phase lb trial for acute myeloid leukemia (15). In addition to Src inhibition, KX-01 has a second mechanism of action (MOA) through binding to novel sites on α-β tubulin heterodimer that results in inhibition of microtubule polymerization (16). The dual activity of KX-01 to both inhibit Src and disrupt microtubules may provide KX-01 with additional antitumor activity for ER/PR/HER2-negative breast cancer in comparison to compounds that exhibit Src inhibition alone.

A previous study from this laboratory has shown that KX-01 combined with tamoxifen resulted in synergistic growth inhibition in ER-positive xenograft tumors, in part, through reduced phosphorylation and transcriptional activity of estrogen receptor-α (ERα; ref. 11). Given the elevated expression of Src in ER/PR/HER2-negative breast cancer (17), the present study evaluated the preclinical activity of peptidomimetic Src inhibitor KX-01 alone and in combination with chemotherapeutic drug paclitaxel. Because Src has been implicated in invasion and metastasis, this study also examined the anti-invasive and antimitastatic potential of KX-01 both in vitro and in vivo.

Materials and Methods

Cell lines, culture conditions, and reagents

The human breast cancer cell lines MDA-MB-231, MDA-MB-157, and MDA-MB-468 were obtained from the American Type Culture Collection. These cell lines lack expression of ERα and progesterone receptor (PR) and do not exhibit amplification/overexpression of HER2 (18). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin in a humidified incubator at 37 °C containing 5% CO2. No further authentication was conducted for cell lines. KX-01 was provided by Kinex Pharmaceuticals in powder form [KX-01:MSA (methanesulfonic acid) salt] that was water-soluble. Paclitaxel was purchased from Hospira Inc. and doxorubicin (methanesulfonic acid) salt from Bedford laboratories.

MTT and apoptosis assay

MTT and apoptosis assays (ELISA, Roche) were conducted as described (11). Cell growth was measured in MDA-MB-231, MDA-MB-157, MDA-MB-468 cells after incubation with vehicle and varying concentrations of KX-01, paclitaxel, doxorubicin, or dasatinib (5, 10, 25, 50, 100, 250 nmol/L) for 48 hours. Cell growth was expressed as percent of vehicle. Apoptosis was evaluated 24 hours after drug incubation.

Invasion assay

Invasion assays were conducted as described (19). Cells were incubated with KX-01 (10, 25, 50 nmol/L) for 24 hours. Invaded cells were counted (ImageJ software) and photomicrographed. The correction for growth inhibition by KX-01 is described in Supplementary Methods.

The number of cells invaded was counted and percentage invasion was calculated by setting vehicle as 100%. The invasion was normalized to cell number as follows:

$$\text{Relative invasion} \% = \frac{\text{Percent invaded cells}}{\text{Percent viable cells}} \times 100$$

Three-dimensional on-top assay

This assay was conducted as described (20) and is described in Supplementary Methods. KX-01 was added to the medium (10, 25, 50 nmol/L) at the time of cell plating. The Matrigel medium mixture and KX-01 was replaced every 2 days for the duration of the 4 days and photomicrographed.

Scratch assay

Scratch assay was conducted as described (19). Cells were incubated with vehicle or KX-01 (5, 10, and 25 nmol/L) for 24 hours after scratch and wound closure was photographed at ×40 magnification. Wound closure was evaluated by measuring wound width by ImageJ. Percentage of gap closure was calculated as:

$$\% \text{ gap closure} = \frac{\text{Gap area at time 0} - \text{Gap area at time 24 hours}}{\text{Gap area at time 0}} \times 100\%$$

Animals

Female athymic nude mice (BALB/c) aged between 4 and 5 weeks obtained from Charles River were housed in sterile cages and maintained in pathogen-free aseptic rooms with 12-hours light/dark schedule. Mice were fed with autoclaved food pellets and water ad libitum. All experiments were carried out in accordance with approved Institutional Animal Care and Use Committee protocol #2941R2 from Tulane University, New Orleans, LA.

Tumor xenograft studies

Xenograft procedures and KX-01 oral dosing were as described (11). Briefly, mammary fat pad tumors were established by injecting 5 × 10⁶ MDA-MB-231 cells in 150 μL of PBS-Matrigel mixture (1:2) orthotopically and bilaterally into the mammary fat pads of female nude mice (2 tumors/mouse). Treatments were started when tumors reached approximately 80 to 100 mm³. The first study used MDA-MB-231 xenografts and was conducted using vehicle (ultra-pure water) and 2 doses of KX-01 (1 and 5 mg/kg) administered twice daily by oral gavage (using metal 22-G feeding needle) for 28 days. A similar experiment was carried out with MDA-MB-157 xenografts (another ER/PR/HER2-negative model) to assess KX-01 response. A second study was conducted to test combination of KX-01 with paclitaxel on tumor growth. MDA-MD-231 tumor xenograft-bearing mice were treated with vehicle or KX-01 (5 mg/kg) twice daily, paclitaxel by intraperitoneal (i.p.) injection once a week, or combination of KX-01 + paclitaxel. Treatments were for 40 days
for all groups. A third study used MDA-MB-157 xenografts with the same combination treatment. A fourth study tested the effect of KX-01 or combination with paclitaxel for 24 days on larger MDA-MB-231 tumors (~300 mm³). Tumors were allowed to reach approximately 300 mm³ before beginning treatments. In this experiment, mice were treated with KX-01 at a higher dose of 15 mg/kg, and mice were treated once a day instead of twice a day. Paclitaxel was used at a dose of 20 mg/kg i.p. once a week. In all experiments, tumor caliper measurements were taken twice a week and tumor volume was calculated by the formula: \(0.523 \times L^2 M\) (where \(L\) is large diameter and \(M\) is small diameter). At the end of experiments, animals were sacrificed and tumors and mouse organs removed. Tissues were either stored in 10% neutral-buffered formalin for paraffin embedding, or snap-frozen for measurement of chromosome-17 by real-time PCR, and embedded for frozen sectioning for CD-31 staining. Immunohistochemistry (IHC) was conducted as described (11) on paraffin-embedded tumor tissues. The detailed immunohistochemical procedure is described in Supplementary Methods.

**Immunofluorescence staining of \(\alpha\)-tubulin in MDA-MB-231 cells and tumors**

For *in vitro* experiments, MDA-MB-231 cells were incubated with 200 nmol/L KX-01 for 24 hours, fixed and immunostained with \(\alpha\)-tubulin antibody conjugated with AlexaFluor-488 (Invitrogen #32-2588). Fluorescence images were captured with a BD Pathway 855 Confocal fluorescence microscope at \(\times 60\) using Attovision software. Immunofluorescence for \(\alpha\)-tubulin was conducted in MDA-MB-231 tumors as described in Supplementary Methods. All fluorescent images were captured using an identical exposure time to permit comparison of staining intensity between treatment groups.

**Quantification of micrometastases**

Human DNA within the mouse organs was measured using quantitative real-time reverse transcriptase (RT)-PCR to detect human chromosome 17. The detection is based on a human-specific \(\alpha\)-satellite DNA sequence of the centromere region of human chromosome 17 (21). After necropsy (n = 5 animals per group), mouse organs were removed, homogenized, and DNA extracted and quantified using a NanoDrop Spectrophotometer (ThermoScientific). Quantitative real-time PCR procedure is described in Supplementary Methods. The \(C_T\) value obtained for human chromosome 17 was normalized to housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH; \(C_T\) value) that detected both mouse and human GAPDH as a measure of total DNA for the samples (GAPDH served as a loading control). Because the extracted DNA could have both mouse and human DNA, we used primers that detected both mouse and human GAPDH. \(\Delta C_T = C_T\) value of human chromosome 17 gene minus \(C_T\) value of mouse/human GAPDH. Standard curves for number of human cells within a mouse organ were generated by serial additions of MCF-7 human breast cancer cells (10⁸, 10⁷, 10⁶, 10⁵, 0 cells) to mouse whole lung, whole liver, or bone marrow (from one femur) just before homogenization of the tissues and isolation of DNA. Mouse organs were derived from untreated female nude mice at the same age as mice from the treatment groups. Human/mouse GAPDH was used as an internal control. Real-time RT-PCR for human/mouse GAPDH was carried out as described (22). Real-time PCR assays were conducted in triplicate. The standard curves for lung, liver, and femur were used to calculate the absolute number of human cells present in the lung, liver, and bone. Nonlinear regression analysis was used to interpolate unknowns from the standard curves (GraphPad Prism5). The detection limit for real-time PCR in this assay was 100 human cells per mouse lung, liver, or femur.

**Statistical analysis**

Data were expressed as mean ± SD. \(P < 0.05\) was considered significant. The mean and SD were calculated using Microsoft Excel or GraphPad Prism 5 software. Statistical significance was determined by 2-sample Student t tests (\(P < 0.05\), 2-tailed) and one-way ANOVA followed by Newman–Keuls multiple comparison test. Combination indices (CI) for combination drug treatments were conducted using CalcuSyn software (Biosoft). The extent of cooperation between KX-01 with paclitaxel was determined from the CI as follows: \(CI < 1.0\) indicated synergism; \(CI = 1.0\) indicated additivity; \(CI > 1.0\) indicated antagonism (23).

**Results**

Chemical structures of KX-01, dasatinib, and paclitaxel are shown in Fig. 1A.

**Effect of KX-01 and dasatinib on ER\(\alpha\)-negative breast cancer cells**

The ATP analogue Src inhibitor dasatinib was shown to inhibit growth of MDA-MB-231 breast cancer cells but not ER\(\alpha\)/PR/HER2-negative MDA-MB-468 cells (ref. 18; Fig. 1B). KX-01 at varying concentrations (10–250 nmol/L) inhibited *in vitro* growth of both MDA-MB-231 and MDA-MB-468 cells as well as another ER\(\alpha\)/PR/HER2-negative cell line, MDA-MB-157 (Fig. 1C). For dasatinib-resistant MDA-MB-468 cells, KX-01 inhibited growth by approximately 75.0% at the same concentration range where dasatinib inhibited growth by only approximately 20.0% (Fig. 1C). Moreover, KX-01 induced significant apoptosis in MDA-MB-468 cells at 25 nmol/L, whereas dasatinib did not induce apoptosis at a \(\times 10\) higher concentration (250 nmol/L; Supplementary Fig. S1).

**Combination of KX-01 with paclitaxel resulted in synergistic growth inhibition of breast cancer cells *in vitro***

A previous study from this laboratory showed that KX-01 inhibited growth of a panel of breast cancer cell lines...
Figure 1. KX-01 induced growth inhibition and apoptosis in ER/PR/HER2-negative breast cancer cells and synergized with paclitaxel in vitro. A, chemical structures of KX-01, dasatinib, and paclitaxel. B and C, growth-inhibitory effects of the broad specificity Src inhibitor dasatinib (B) and KX-01 (C) at varying concentrations (10, 25, 50, 100, 250 nmol/L) on ER/PR/HER2-negative MDA-MB-231, MDA-MB-157, and MDA-MB-468 cells. Cells were incubated with drugs for 48 hours and growth inhibition was assessed by MTT assay. Results presented as % vehicle/C6±SD (n=3). D, KX-01 induced synergistic growth inhibition in MDA-MB-231 cells when combined with paclitaxel (PAX). Cell growth was measured by MTT assay. Cells were incubated with KX-01 (25 nmol/L), PAX (5 nmol/L), or combination for 24, 48, and 72 hours. Results presented as % vehicle ± SD (n=3). E, KX-01 in combination with PAX resulted in enhanced apoptosis in MDA-MB-231 cells. Apoptosis assay was conducted following 24-hour incubation with vehicle KX-01 (25 nmol/L), PAX (5 nmol/L), or KX-01 + PAX. * P < 0.05 (Student t test), significantly different compared with vehicle (VC), # P < 0.05 (Student t test), statistical evidence of enhanced apoptosis in combination treatment compared with either drug alone. Data are representative of 3 independent experiments.
with different ERα status (ER-positive MCF-7 cells and ER-negative MDA-MB-231, MDA-MB-157, MDA-MB-468, and BT-549 cells) but did not affect growth of nontumorigenic breast epithelial MCF-10A cells (11). After 24-hour incubation, KX-01 (25 nmol/L) alone or paclitaxel (5 nmol/L) alone at suboptimal growth inhibitory concentrations produced modest growth inhibition of 11.2% and 14.1%, respectively (Fig. 1D). Co-incubation of KX-01 with paclitaxel resulted in synergistic 43.2% growth inhibition of MDA-MB-231 cells compared with vehicle-treated cells (Fig. 1D). A dose response for growth inhibition was determined over concentration ranges of 5 to 100 nmol/L for KX-01 alone and 1 to 10 nmol/L for paclitaxel (data not shown). From these data, CI values were calculated (as described in ref. 23) for KX-01 + paclitaxel over a concentration range (Supplementary Table S1A). Similar patterns of synergistic growth inhibition of KX-01 with paclitaxel at various concentrations were observed in MDA-MB-157 and MDA-MB-468 cells (Supplementary Table S1A).

**Combination of KX-01 with paclitaxel induced apoptosis in MDA-MB-231 breast cancer cells**

To determine whether synergistic growth inhibition by combination of paclitaxel + KX-01 resulted from increased apoptosis, an ELISA-based DNA fragmentation assay was used. KX-01 or paclitaxel at the concentrations used in Fig. 1D resulted in modest apoptosis in MDA-MB-231 cells (Fig. IE). Combination of KX-01 with paclitaxel enhanced apoptosis 2-fold compared with single drug treatments (Fig. IE).

**Oral administration of KX-01 reduced growth of ER/PR/HER2-negative breast tumor xenografts in mice**

MDA-MB-231 and MDA-MB-157 xenograft tumors (80–100 mm³) in nude mice were established to determine the effect of KX-01 on tumor growth in vivo. Mice were treated with vehicle (ultra-pure water), KX-01 at 1 mg/kg and KX-01 at 5 mg/kg for 28 days, twice daily. KX-01 resulted in marked tumor growth inhibition in both tumor models (Fig. 2A and Supplementary Fig. S2A). Mice receiving KX-01 at 1 and 5 mg/kg showed reduced MDA-MB-231 tumor volume compared with vehicle from days 18 and 15, respectively (P < 0.05; Fig. 2A). KX-01 at 1 and 5 mg/kg reduced MDA-MB-231 tumor volume 35.3% and 79.0%, respectively (Fig. 2A), and reduced MDA-MB-157 tumor volume 23.5% and 42.0%, respectively (Supplementary Fig. S2A).

**KX-01 decreased activity of Src and downstream target focal adhesion kinase in MDA-MB-231 tumor xenografts**

To determine whether KX-01 effectively inhibited activation of Src in primary tumors, tumors were prepared for IHC using antibodies to the active forms of Src kinase (p-Y416-Src) and its downstream target focal adhesion kinase (FAK; p-Y661-FAK). KX-01 significantly inhibited Src phosphorylation (Supplementary Fig. S2B) and FAK phosphorylation (Fig. 2C) with no significant effect on expression of total Src or FAK.

**KX-01 reduced proliferation and angiogenesis and increased apoptosis in MDA-MB-231 tumor xenografts**

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was used to measure apoptotic cells. KX-01 significantly enhanced the number of apoptotic cells 3- to 4-fold compared with vehicle-treated tumors (P < 0.001; Fig. 2D and G). KX-01 reduced the percentage of tumor cells positive for proliferation marker Ki67-positive tumor cells to 37.3% compared with vehicle (58.7%; P < 0.001; Fig. 2E and H). To evaluate the effect of KX-01 on angiogenesis, microvessel density (MVD) was assessed by counting CD31-positive microvessels. KX-01 significantly reduced MVD (P < 0.05) compared with vehicle (Fig. 2F and I). In summary, these data show that KX-01 significantly reduced growth of MDA-MB-231 tumors that was attributed, in part, to reduced tumor cell proliferation, increased apoptosis, and decreased blood vessel density (modestly) that was coincident with reduced Src kinase activity in the tumors.

**Combination of KX-01 with paclitaxel resulted in regression of ER/PR/HER2-negative tumor xenografts**

The in vitro observations of synergistic growth inhibition of breast cancer cell lines co-incubated with KX-01 and paclitaxel were evaluated in vivo using MDA-MB-231 and MDA-MB-157 tumor xenografts. KX-01 administered alone (5 mg/kg, twice daily) decreased MDA-MB-231 tumor volume by 61.1%, and treatment with paclitaxel alone (3 mg/kg, once a week) decreased tumor volume by 33.4% compared with vehicle (P < 0.01, Fig. 3A). Combination of KX-01 with paclitaxel reduced tumor volume by 93.0% (Fig. 3A). Most significantly, combination of KX-01 with paclitaxel resulted in complete regression of tumors in 2 of 5 mice. In MDA-MB-157 xenografts, the same single-agent doses of KX-01 and paclitaxel reduced tumor volumes by 42.0% and 16.3%, respectively (Fig. 3B). Combination of KX-01 + paclitaxel reduced tumor volumes by 71.1% compared with vehicle (P < 0.01; Fig. 3B). No noticeable toxicity or significant body weights reduction was observed in either drug alone or combinational treatment groups (Supplementary Fig. S3A). Hematoxylin and eosin (H&E) staining of MDA-MB-231 tumor sections revealed more acellular, necrotic regions in tumors from the combination treatment groups than form single drug treatments (Supplementary Fig. S3B, arrows).

**Combination of KX-01 with paclitaxel resulted in regression of larger, established MDA-MB-231 tumors**

Xenograft experiments described in Figs. 2 and 3 and Supplementary Fig. S2 began drug treatments when
tumors reached a palpable size of approximately 80 to 100 mm$^3$. To determine whether combination of KX-01 with paclitaxel would cause regression of larger and more established tumors, MDA-MB-231 xenografts were allowed to reach approximately 300 mm$^3$ before treatments began. Animals were treated with KX-01 at a higher dose of 15 mg/kg only once a day, which when compared with 5 mg/kg KX-01 twice a day showed similar tumor growth inhibition in MDA-MB-231 xenografts (data not shown). Previous studies showed that a higher dose of paclitaxel was needed for efficacy against larger, established MDA-MB-231 tumors as compared with smaller tumors (24, 25). For this reason, paclitaxel was used at a dose of 20 mg/kg, once a week. KX-01 alone resulted in significant tumor growth inhibition but without tumor regression; tumor volume was decreased by 48.2% ($P < 0.001$ vs. vehicle; Fig. 4A). Paclitaxel alone resulted in significant tumor growth inhibition but without tumor regression; tumor volume was decreased by 35.4% ($P < 0.001$; Fig. 4A) with no significant reduction in mouse body weight. B and C, KX-01 inhibited phosphorylation of Src and downstream mediator FAK in MDA-MB-231 xenografts. IHC was conducted as described in Supplementary Methods for total Src, phospho-Src-Y416 (p-Y416-Src), FAK, and phospho-FAK-Y861 (p-Y861-FAK). Representative photomicrographs are presented ($>$200). D–F, KX-01 induced apoptosis and reduced proliferation and angiogenesis in MDA-MB-231 xenografts. Paraffin-embedded tumor sections from vehicle and KX-01 (5 mg/kg) groups were stained for TUNEL (D) and Ki67 (E). Fluorescent (TUNEL) and bright-field (Ki67) photomicrographs were taken and representative images are presented ($>$200). F, frozen tumor sections were stained for mouse CD31. Bright-field microscope images ($>$40) were taken, and representative photomicrographs are presented. G–I, quantitative representation of TUNEL, Ki67, and CD-31 staining. VC, vehicle control.
weight (Supplementary Fig. S3C). Combination of KX-01 (15 mg/kg) with paclitaxel (20 mg/kg) resulted in 90.6% tumor regression to a volume that was markedly below the starting tumor volume ($P < 0.001$; Fig. 4A) with approximately 10% mouse body weight reduction but without noticeable toxicity (initial mean body weight = 19.4 g; final mean body weight = 17.6 g; Supplementary Fig. S3C). By day 12, mice receiving KX-01 + paclitaxel showed significant tumor regression compared with single treatments ($P < 0.001$, Fig. 4A). H&E staining of MDA-MB-231 tumor sections showed more acellular, necrotic regions in tumors from combination treatments than from single treatments (Supplementary Fig. S3D, arrows).

**KX-01 disrupted microtubules in MDA-MB-231 cells and tumors**

In addition to Src inhibition, KX-01 was shown to inhibit microtubule polymerization *in vitro* indicating a second MOA (16, 26). KX-01 (200 nmol/L, 24 hours) markedly inhibited microtubule formation in MDA-MB-231 cells *in vitro* (Supplementary Fig. S4A). To assess the effect of KX-01 on microtubule organization in tumor tissues, paraffin-embedded MDA-MB-231 tumor sections were incubated with an anti-tubulin fluorescent antibody. In vehicle-treated animals, tumor cells exhibited staining of distinct microtubule fibers demonstrative of an intact microtubule network (Fig. 4B). In KX-01–treated mice, tumor cells exhibited a more diffuse staining pattern with markedly reduced fluorescence intensity indicative of a disrupted and reduced microtubule network in the cells. In contrast, paclitaxel resulted in more intensely stained microtubule arrays, consistent with the microtubule-stabilizing activity of paclitaxel (Fig. 4B). Interestingly, the tumors from mice treated with KX-01 + paclitaxel showed a microtubule-staining pattern that was markedly different from either single treatment. KX-01 + paclitaxel resulted in a diffuse tubulin-staining pattern indicating loss of the microtubule network, as well as evidence of fragmented microtubules (arrows). Taken together, these data provide direct evidence that KX01 alone, and in combination with paclitaxel, resulted in disruption of the microtubule network in tumors.

**KX-01 inhibited breast cancer cell outgrowth, invasion, and migration *in vitro***

Cell invasiveness was assessed using a 3D invasion assay in which MDA-MB-231 cells were cultured in reconstituted extracellular matrix to mimic an *in vivo* microenvironment (20). Treatment of MDA-MB-231 cells with 25 and 50 nmol/L KX-01 reduced invasive stellate structures that are a hallmark of invasive cells (Fig. 5A). There was almost complete inhibition of stellate structures at 50 nmol/L KX-01. The effect of KX-01 on MDA-MB-231 invasion *in vitro* was assessed using the Boyden chamber Matrigel invasion assay. A significant decrease in MDA-MB-231 cell invasion by 52.9% occurred with 50 nmol/L KX-01 ($P < 0.05$; Supplementary Fig. S4B). The anti-invasive effect was independent of the growth-inhibitory effect of KX-01, as invasion data were normalized to cell number (see Materials and Methods). Cancer cell migration was assessed by a wound-healing/scratch assay. Concentrations of 5, 10, and 25 nmol/L KX-01 significantly decreased MDA-MB-231 cell migration by 11.7%, 15.6%, and 39.9%, respectively, compared with vehicle (Fig. 5C; $P < 0.05$; Supplementary Fig. S4C).

**Effect of KX-01 on MDA-MB-231 micrometastasis *in vivo***

Although the relatively short treatment time (40 days) for the MDA-MB-231 xenograft tumors was not sufficient for tumors to exhibit visual macrometastases to mouse organs, 40 days were sufficient to measure...
treatments began. Mice (n = 5 per group) were treated with vehicle, KX-01 (15 mg/kg) by oral gavage once daily, paclitaxel (PAX; 20 mg/kg) once a week i.p. (arrows), or KX-01 + PAX. Tumor volumes were recorded as mm³ ± SD. *P < 0.05 (one-way ANOVA followed by Newman–Keuls multiple comparison test). B, paraffin tumor sections from treatment groups were analyzed by immunofluorescence with anti-tubulin antibody conjugated with Alexafluor-488 (green) and imaged with a confocal microscope (×60). White arrows indicate fragmented microtubules in the KX-01 + PAX group.

Figure 4. Combination of high-dose KX-01 with paclitaxel induced regression of established, large MDA-MB-231 tumors. A, MDA-MB-231 tumors were established in nude mice (tumor volume = 300 mm³) before treatments began. Mice (n = 5 per group) were treated with vehicle, KX-01 (15 mg/kg) by oral gavage once daily, paclitaxel (PAX; 20 mg/kg) once a week i.p. (arrows), or KX-01 + PAX. Tumor volumes were recorded as mm³ ± SD. *P < 0.05 (one-way ANOVA followed by Newman–Keuls multiple comparison test). B, paraffin tumor sections from treatment groups were analyzed by immunofluorescence with anti-tubulin antibody conjugated with Alexafluor-488 (green) and imaged with a confocal microscope (×60). White arrows indicate fragmented microtubules in the KX-01 + PAX group.

micrometastasis by quantitating human DNA in mouse tissues. At the termination of experiments described in Fig. 3A, lung, liver, and the bone marrow from femurs were removed (n = 5 mice), and the amount of human DNA in these mouse tissues was measured by quantitative real-time RT-PCR directed toward an α-satellite sequence specific for human chromosome 17. Standard curves for the number of human cells within control mouse organs were generated by serial additions of human breast cancer cells to whole lung, whole liver, and bone marrow (one femur) just before homogenization of the tissues and DNA isolation (Supplementary Fig. S5A). The incidence of metastasis to the lung in the vehicle group was 4 of 5 mice (80%). The mean number of MDA-MB-231 cells in the mouse lung for the vehicle group was 6.0 × 10⁶ cells (number extrapolated from the standard curve, Supplementary Fig. S5A). KX-01 alone (5 mg/kg) or KX-01 + paclitaxel (3 mg/kg) significantly reduced metastasis to the lungs (P < 0.05, Fig. 6A). No human cells were detected in the lungs of mice treated with either KX-01 alone or KX-01 + paclitaxel. The incidence of metastasis to the bone marrow was 3 of 5 mice (60%), and the mean number of MDA-MB-231 cells in the bone marrow for the vehicle group was 7.1 × 10⁵ cells. Although there were no detectable human cells in the bone marrow from mice treated with KX-01 alone or paclitaxel + KX-01, the differences between these treatment groups and vehicle were not statistically significant (Fig. 6B). In addition, there was no statistical difference between vehicle and paclitaxel alone for metastatic cells in the bone marrow (Fig. 6B). The incidence of micrometastasis to liver in the vehicle group was 4 of 5 mice (80%) and the mean number of MDA-MB-231 cells in the liver for the vehicle group was 3.0 × 10⁶ cells. As with the lungs and bone marrow, no detectable human cells were found in the livers of mice treated with KX-01 alone or KX-01 + paclitaxel (Fig. 6C). There was a statistically significant reduction in liver metastasis in the KX-01 alone group and the KX-01 + paclitaxel group compared with the vehicle group (P < 0.05; Fig. 6C). Paclitaxel alone did not significantly alter liver metastasis compared with vehicle. Taken together, these data show that KX-01 alone and in combination with paclitaxel significantly reduced metastasis of MDA-MB-231 cells to the lung and liver.

Discussion

Activation of Src kinase has been shown to decrease apoptosis of cancer cells and to promote cell mitosis, cell motility/invasion, and neo-angiogenesis to tumors (6, 7). In the present study, the clinical Src/pretubulin inhibitor KX-01 as a single-agent inhibited growth, migration, and invasive potential and increased apoptosis of ER/PR/HER2-negative breast cancer cells in vitro. Treatment of mice bearing ER/PR/HER2-negative tumors with KX-01 resulted in a dose-dependent decrease in tumor volume with concomitant reduction in proliferation, MVD, and increased apoptosis. When KX-01 was combined with paclitaxel, there was significantly reduced tumor volume and tumor regression compared with either drug alone without noticeable toxicities. KX-01 also exhibited an antimetastatic effect against MDA-MB-231 tumors. The antitumor and antimetastatic effects of KX-01 were coincident with marked inhibition of Src activity and disruption of the microtubule network in the primary tumors. ER/PR/HER2-negative breast cancers are intrinsically more chemoresistant than other breast cancer subtypes (27). Given the lack of targeted agents and the rapid onset of chemoresistance in metastatic ER/PR/HER2-negative breast cancers, there is urgent need for novel therapeutic approaches to target these tumors. Combinations of targeted agents with conventional chemotherapeutic agents
are currently being investigated and have proven clinically successful in several cancers including breast cancer (28, 29). The present study presents KX-01 as a first-in-class clinical Src/pretubulin inhibitor that results in significant tumor regression when combined with standard-of-care chemotherapy agent paclitaxel showing that this combination could be considered in the clinical setting.

KX-01 efficacy in preclinical tumor models was correlated with strong inhibition of Src and FAK activity, suggesting that Src inhibition contributed to the antitumor effect. In addition to Src inhibition, KX-01 also inhibits microtubule polymerization as a second MOA (16, 26). This second MOA likely contributes to the robust apoptosis and antitumor activity in comparison with drugs that exhibit only Src inhibition. In this regard, KX-01 as a single compound may exert similar effects as combination of clinical Src inhibitors with microtubule-targeting chemotherapy (30) and may also be effective in Src inhibitor-resistant tumors.

Paclitaxel stabilizes microtubules resulting in mitotic blockade and subsequent cell-cycle arrest or apoptosis. Interestingly, KX-01 and paclitaxel disrupt microtubules by different means; inhibition of microtubule assembly and stabilization of microtubules, respectively, as was observed by the markedly different microtubule-staining patterns in tumors exposed to these 2 agents (Fig. 4B). The combination of KX-01 with paclitaxel resulted in a reduced microtubule network along with fragmentation of many of the remaining microtubules. It was recently shown that microtubule-inhibiting drugs can cause microtubule minus-end detachment from the microtubule-organizing centers (ref. 31). It is possible that KX-01 through inhibition of tubulin polymerization has a similar effect in causing microtubule detachment from microtubule-organizing centers. However in the KX-01 + paclitaxel group, the resulting detached microtubules are protected by paclitaxel from further KX-01–induced depolymerization, thus giving rise to fragmented microtubules. It is likely that these 2 different mechanisms for microtubule disruption contribute to the antitumor synergy observed when KX-01 and paclitaxel were combined.

Invading cells produce various membrane protrusions that promote migration and proteolysis of extracellular matrix (ECM; 32). Src has been shown to regulate microtentacles and invadopodia (32). Src is both necessary and sufficient for the formation of invadopodia at sites of integrin-mediated ECM adhesion (33). Cells coordinate focal adhesions and invadopodia to provide traction (34). MDA-MB-231 cells are highly invasive and migratory and are shown to produce microtentacle protrusions and invadopodia (32). Src inhibition by KX-01 significantly reduced formation of invasive stellate structures in MDA-MB-231 cells and inhibited cell migration and invasion. These findings correlated with KX-01 inhibition of Src and FAK in tumors.
Metastasis is the final step in the progression of many solid tumors with the lungs, liver, and bone as frequent sites for breast cancer metastasis (35, 36). The brief treatment period (40 days) of MDA-MB-231 xenografts permitted an evaluation of micrometastasis to mouse organs from the primary tumor. KX-01 alone or in combination with paclitaxel resulted in complete absence of detectable micrometastasis to the lung and liver. It should be noted that the reduction in micrometastasis could be attributed, in part, to the reduced primary tumor burden in the treated animals. Because the tumor xenografts had to be terminated within 30 to 40 days due to the large size of the primary tumors, micrometastases to mouse organs were limited, and sectioning/H&E staining of mouse organs to quantitate micrometastases was not possible. As an alternate approach to measure very early micrometastases, we quantitated levels of human chromosome 17 microsatellite regions by PCR using an established procedure (21, 37) that we have previously used (38). Although precaution was taken to rinse mouse organs thoroughly with PBS to remove blood and blood clots before DNA isolation, a caveat to this procedure is the presence of any circulating tumor cells in the vasculature of the organs that could contribute to the DNA quantitation. Future studies will evaluate KX-01 in impacting multiple steps of the metastatic process using appropriate metastatic models and histopathologic methods to quantitate metastatic burden.

To determine whether KX-01 could synergize with cytotoxic drugs other than paclitaxel, doxorubicin (a topoisomerase II inhibitor widely used for the treatment of breast cancer) was used in combinational treatments. In ER/PR/HER2/neu-negative cell lines, lower concentrations of KX-01 (10 and 25 nmol/L) synergized with
doxorubicin for cell growth inhibition (Supplementary Table S1B). However, at higher KK-01 concentrations (50 nmol/L), combination with doxorubicin resulted in antagonism with CI values above 1.0 (Supplementary Table S1B). In MDA-MB-231 and MDA-MB-157 tumor xenograft experiments, combination of KK-01 + doxorubicin resulted in significant tumor growth inhibition that was greater than either drug alone (Supplementary Fig. S2B and S2C). However unlike combination with paclitaxel, the antimetastatic effect of KK-01 in vivo was antagonized by combination with doxorubicin (Supplementary Fig. S5B). Whereas KK-01 alone reduced lung metastasis, when combined with doxorubicin, there was no significant difference in lung metastasis compared with vehicle. It is unknown why combination of KK-01 with doxorubicin did not result in complete synergy for cell growth inhibition and reduced metastasis across a broad concentration range of KK-01 as was shown for combination with paclitaxel. The 2 cytotoxic agents have different MOAs that likely underlie the efficacy when combined with KK-01. As noted above, the synergy between KK-01 and paclitaxel may be due to targeting 2 different mechanisms for microtubule inhibition. Doxorubicin as a DNA-damaging agent may not complement KK-01 as a DNA-damaging agent may not complement KK-01 as effectively. It will be important to identify concentrations of doxorubicin + KK-01 that will exhibit desired clinical outcomes. Additional studies are needed to identify the mechanisms underlying the mixed efficacy of doxorubicin combined with KK-01.

ER/PR/HER2-negative breast cancer represents 15% of breast cancers, but this tumor subtype expresses elevated Src kinase compared with ER-positive breast cancer (39, 40). ER/PR/HER2-negative breast cancer presents more frequently in younger women and in African-American women (41). In urban hospitals that treat a larger proportion of African-American women, the incidence of ER/PR/HER2-negative breast cancer is higher than 15%. Patients who present with this tumor subtype represent an unmet medical need for clinical care of breast cancer. Although it is unlikely that inhibition of Src alone would significantly impact patient care, the present study showed that targeting 3 MOAs (Src inhibition, tubulin disruption, and tubulin stabilization) can achieve greater efficacy in tumor growth inhibition than treatment with compounds that would target only a single MOA. Furthermore, paclitaxel dose is limited in the clinic due to neurotoxicity. The inclusion of KK-01 with paclitaxel therapy may permit lower doses of paclitaxel to be used while maintaining or improving efficacy.

Disclosure of Potential Conflicts of Interest
D. Hangauer is Chief Scientific Officer for Kinex Pharmaceuticals LLC that supplied KK-01 and dasatinib for this study. B.G. Rowan received a $25,000 research grant from Kinex Pharmaceuticals to support the research presented in this study. No potential conflicts of interest were disclosed by the other authors.

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References
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