SUPPLEMENTARY METHODS

MTT assay

Cell growth was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium- bromide (MTT) assay. Exponentially growing MDA-MB-231, MDA-MB-157, MDA-MB-468 cells (2 x10^4 cells/well) were seeded in 24-well plates in DMEM medium containing 5% FBS for 24 hours. Cells were incubated with vehicle and varying concentrations of KX-01 and dasatinib (10, 25, 50, 100, 250 nmol/L) for 48 hours. For combinational drug effect, KX-01 (25 nmol/L), paclitaxel (5 nmol/L), or combination (KX-01+ paclitaxel) were used. 100 µl MTT reagent (Invitrogen, Molecular Probes) was added to each well and plates were incubated for 2 hours at 37º C to allow MTT to form formazan crystals by reacting with metabolically active cells. Medium was replaced with 300 µl dimethylsulfoxide (DMSO) and plates were incubated for 15 minutes at room temperature with shaking. The optical density was measured at 595 nm. Cell growth was expressed as percent of vehicle.

Apoptosis ELISA assay

Apoptosis assays were performed using the nucleosome ELISA kit (Roche). This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) after apoptotic cell death. 8 x 10^3 MDA-MB-231 cells were incubated with KX-01, paclitaxel, or combinations in the concentrations described above for 24 hours in 96-well plates. The induction of apoptosis was evaluated by assessing the enrichment of nucleosomes in the cytoplasm as per manufacturer protocol and the data was normalized to cell number.

In vitro invasion assay

Invasion experiments were conducted in 24-well, two-compartment plates with Matrigel invasion chambers (Becton Dickinson). DMEM medium containing 10% FBS was added (500 µl/well) to the lower chambers to serve as the chemoattractant medium. Exponentially growing MDA-MB-231 cells were serum starved overnight, trypsinized and counted. The cells were suspended in serum-free medium containing various concentrations of KX-01 (10, 25 and 50 nmol/L). Cells in drug containing medium were added to the upper chambers (2.5×10^4 cells/well) and allowed to incubate for 24 hours. After incubation, the Matrigel inside the insert (along with the non-invaded cells) was removed with cotton swabs, and the outer side of the insert (where the invaded cells were located) was fixed and stained using Diff-Quik kit (Dade-Behring Diagnostics). Invaded cells on the underside of the insert were photomicrographed (original magnification, x 100). Six or more randomly selected fields were used for counting by Image J software (NIH, Bethesda, MD) particle count command. Each experiment was performed in triplicate and the experiment was repeated three times.

Correction for growth inhibition by KX-01

Since KX-01 inhibited MDA-MB-231 cell growth and induced apoptosis, it is conceivable that the fewer invading cells in the KX-01 treated group might be the consequence of the growth inhibition or cell death that occurred during the early part of the 24 hour incubation period. For this reason, the growth inhibitory effect of KX-01 on MDA-MB-231 cells was measured simultaneously under identical conditions in 24-well plates by viable cell count; the growth correction was applied to the invasion data. The percent viable cells after incubation with KX-01 10, 25 and 50 nmol/L was 95%, 86% and 70%, respectively and the vehicle group was

set as 100%. The number of cells invaded was counted and percent invasion was calculated by setting vehicle as 100%. The invasion was normalized to cell number as shown below,

percent invaded cells/percent viable cells × 100 = relative invasion (%).

3D-on top assay

Matrigel was thawed overnight at 4 °C and a thin layer of Matrigel was coated on pre-chilled 8-well chamber slides followed by incubation for 30 minutes at 37 °C to permit the Matrigel to gel. Exponentially growing MDA-MB-231 cells were trypsinized, centrifuged at 115 x g and 1.5 x 10^4 cells in 100 µl media were seeded onto the Matrigel coated surface of each well. Cells were allowed to settle and attach to the Matrigel for 30 minutes at 37 °C. Chilled media and Matrigel on ice were mixed at a 9:1 ratio and this Matrigel medium mixture was added to each well avoiding disturbance to the cells. KX-01 was added to the medium at varying concentrations (10, 25 and 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.

Wound healing/scratch assay

MDA-MB-231 cells were cultured to 80% confluency on 12-well plates. The medium was replaced with fresh medium containing vehicle or KX-01 (5, 10 and 25 nmol/L). A single strip of cells was scraped off the surface of the plate with a 200 µl disposable plastic pipette tip and the cells were cultured for an additional 24 hours at 37°C. Wound closure was viewed under a microscope and photographed (original magnification, x 40). The average extent of wound

closure was evaluated by measuring the width of the wound by Image J software. Percent gap closure was calculated as

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

Immunohistochemical staining

The paraffin-embedded tumor tissues were sectioned, mounted on microscope slides and preheated at 60ºC for 30 minutes. The sections were then deparaffinized and hydrated. The tumor sections were then antigen retrieved, quenched, blocked and incubated with primary antibodies against Src, FAK, phospho Y416Src (P-Y416 Src), phospho Y861FAK (P-Y861 FAK), or Ki67 at 4ºC overnight. The retrieval buffers used were Borg decloaker solution (Biocare Medical, Inc.) for Src, P-Y416 Src and P-Y861FAK , citrate buffer for Ki67, and 0.1 mol/L EDTA buffer for FAK staining. Dilutions of primary antibodies were: Src (1:200; Cell Signaling Technology, Inc.), P-Y416Src (1:100; Cell Signaling Technology, Inc.), FAK antibody (1:100; Cell Signaling Technology, Inc) and P-Y861FAK (1:100; Biosource Invitrogen Co.). After incubation with biotinylated secondary antibody (Vector labs) for 30 minutes, the slides were rinsed in PBS and incubated with ABC reagent (Vector labs). The stain was visualized by incubation in 3, 3-diaminobenzidine (DAB) for 1-2 minutes and counterstained with Harris hematoxylin for 25 seconds. Internal negative control samples were exposed to either rabbit IgG or 10% goat serum instead of the primary antibodies. Slides were dried and mounted with Permount (Fisher). Randomly selected bright field microscope images (original magnification, x 200) for each sample were obtained. All bright field images were visualized using a Nikon

OPTIPHOT microscope and photomicrographed by a Nikon Digital Sight High-Definition color camera (DS-Fi1) using NIS-Elements BR software.

Immunohistochemical staining for Ki67

To determine the percentage of proliferating cells, paraffin embedded tumor sections were stained with antibody to Ki67 (Themoscientific, Neo-markers, ready to use) as previously described. For quantification, three randomly selected bright field microscope images (original magnification, x 200) per sample were obtained as described above. The total cell number in each image was calculated by counting hematoxylin-positive cells using Image J software particle count command. DAB-positive cells were counted in the same way after using the color deconvolution command. Data were expressed as percent Ki67 positive cells.

Terminal deoxynucleotidyl transferase biotin dUTP nick-end labeling

To quantify the number of apoptotic tumor cells, paraffin embedded sections were analyzed with a commercially available terminal deoxynucleotidyl transferase biotin dUTP nick-end labeling (TUNEL) kit (DeadEnd Fluorometric TUNEL kit, [Roche]). The fluorescent TUNEL labeled cells were visualized using a fluorescent Nikon microscope and microphotographed using a Sensicam^{QE} High performance camera with IP Lab software (original magnification, x 100). Counting was performed by Image J; apoptotic index was expressed as apoptotic cells per field.

Immunohistochemical staining of CD31/PECAM and quantification of microvessel density

To determine tumor microvessel density, the frozen tumor xenograft samples were sectioned, fixed in acetone and air dried. Sections were then incubated in 3% H_2O_2 in PBS for 5

minutes, blocked with 10% rabbit serum for 30 minutes, and subsequently incubated with anti-mouse CD31/PECAM rat antibody (BD Pharmigen BD Bioscience; 1:50 diluted in 10% rabbit serum) overnight at 4ºC. Secondary antibody reaction was performed with peroxidase-conjugated rabbit anti-rat secondary antibody (Vector Labs). The stain was visualized by incubation with DAB (Vector labs) followed by counterstaining with Harris hematoxylin. Five randomly selected bright field microscope images (original magnification, x 40) per sample were obtained as described above, and positively stained microvessels were counted using Image J.

Immunofluorescence staining of alpha tubulin in MDA-MB-231 cells and tumors

For *in vitro* experiments, MDA-MB-231 cells were plated in 8 well chamber slides with DMEM medium supplemented with 10% FBS. Cells were incubated with 200 nmol/L KX-01 for 24 hours. Cells were fixed in 3.7% paraformaldehyde in PBS, and then permeabilized in 0.3% Triton X-100 in PBS. After 1% BSA block, cells were immunostained for 2 hours at room temperature with alpha-tubulin antibody conjugated with Alexa fluor 488 (Invitrogen #32-2588) and nuclei were stained with DAPI. Fluorescence was visualized with a BD Pathway 855 Confocal fluorescence microscope at 60x using Attovision software. For MDA-MB-231 tumors, paraffin embedded tumor sections were deparaffinized, antigen retrieved using citrate buffer, blocked with 10% goat serum and incubated with Alexa Fluor 488 conjugated anti-tubulin antibody for 2 hours at room temperature. After rinsing the slides in PBS, the fluorescent slides were mounted using Vectashield mounting media (Vector Labs) and sealed. Sections were then examined on a BD Pathway 855 Confocal fluorescence microscope. Z-stack images (using Olympus PLAPO 60X oil-immersion objective; NA 1.4; 0.15 mm WD) were captured with a Hamamatsu ORCA-AG camera system using BD AttoVision Software. All fluorescent images

were captured using an identical exposure time to permit comparison of staining intensity between treatment groups.

Quantitative real-time PCR

Human DNA within the mouse organs was measured using quantitative real time RT-PCR to detect human chromosome-17. Quantitative real-time PCR was performed in a volume of 25 µl that contained 12.5 µl FastStart Taqman Probe Master for probes (Roche), 200 nmol/L each of the forward and reverse primers, 100 nmol/L TaqMan probe, and 250 ng target DNA template (see below for primers and probes). Reactions were incubated at 50 ºC for 2 minutes and at 95 ºC for 10 minutes, followed by 45 cycles at 95 ºC for 15 seconds, and 60 ºC for 1 minute using a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Human/mouse GAPDH was used as an internal control**.** Real time RT-PCR for human/mouse GAPDH were performed in a volume of 25 µl that contained 12.5 µl iQ SYBR Green Supermix (Bio-Rad), 900 nmol/L each of the forward and reverse primers (see below for primers), and 250 ng target DNA template. All real-time PCR assays were performed in triplicate. The SC obtained for lung, femur and liver was used to calculate the absolute number of human cells present in the lung, liver and bone marrow of mouse tissue samples, respectively. Non-linear regression analysis was used to interpolate unknown from the SC (GraphPad Prism 5). The detection limit for real-time PCR in this assay was 100 human cells per mouse lung, femur or liver.

Human Cr17_1a forward primer: 5'-GGG ATA ATT TCA GCT GAC TAA ACAG-3'

Human Cr17_4b reverse primer: 5'-AAA CGT CCA CTT GCA GAT TCT AG-3'

TMsat_probe : 6FAM-CAC GTT TGA AAC ACT CTT

XT TTG CAG GATC p (X=Tamra)

Mouse/human GAPDH forward primer: 5’- CAG CGA CAC CCA CTC CTC CAC CTT -3’

Mouse/human GAPDH reverse primer: 5’- CAT GAG GTC CAC CAC CCT GTT GCT -3’