

Impact of the Src Inhibitor Saracatinib on the Metastatic Phenotype of a Fibrosarcoma (KHT) Tumor Model

MEIYU DONG*, LORI RICE*, SHARON LEPLER, CHRISTINE PAMPO and DIETMAR W. SIEMANN

Department of Radiation Oncology, University of Florida, Gainesville, FL 32610, U.S.A.

Abstract. *Background:* Src, a non-receptor tyrosine kinase frequently overexpressed and highly activated in malignancies, has been associated with a poor patient prognosis. The aim of the present studies was to examine the impact of an Src inhibitor (saracatinib) on a highly metastatic murine sarcoma cell line (KHT). *Materials and Methods:* Phosphorylation of Src and downstream effectors was determined using Western immunoblotting. Cell cycle was analyzed by flow cytometry using propidium iodide DNA staining, migration and invasion in modified Boyden chambers, activated MMP-9 by gel zymography, and visualization of pSrc and pFAK by confocal immunofluorescence. The number of KHT lung nodules in saracatinib-treated mice was compared to controls. *Results:* Saracatinib inhibited major pathways in the metastatic cascade *in vitro*, including Src and FAK activation. Functions required for metastasis, such as migration and invasion, were reduced when cells were exposed to 0.5 μ M and 1.0 μ M saracatinib, respectively ($p < 0.0001$). Pretreatment of KHT cells with either 1 μ M or 5 μ M saracatinib prior to tail vein injection decreased lung colonies in mice from 13.0 to 5.0 ($p < 0.05$) and less than 1.0 ($p < 0.01$), respectively. *Conclusion:* These findings suggest that Src inhibition by saracatinib may reduce the metastatic activity of tumor cells.

Approximately one in four deaths in the United States is caused by cancer (1), 90% of which have been attributed to distant metastases (2). New treatment strategies targeting metastases are clearly needed to improve patient survival. A number of recent studies have shown that Src signaling may play an important role in cancer progression. For example, overexpression of Src occurs in many solid tumors

including those in colon, breast, bladder, and pancreas (3-5). In addition, elevated Src expression or activity in tumors may be related to a poor prognosis (6). Published studies suggest that Src activation enhances the progression of cancer cells towards a metastatic phenotype by promoting survival, proliferation, migration, and invasion (4, 7-12).

Saracatinib (AZD-0530), *N*-(5-chloro-1, 3-benzodioxol-4-yl)-7-[2-(4-methylpiperazin-1-yl) ethoxy]-5-(tetrahydro-2H-pyran-4-yloxy)quinazolin-4-amine, is a small molecule, highly selective, orally available Src kinase inhibitor that specifically interferes with Src phosphorylation at tyrosine 419(human)/ 423(mouse) (13). In pre-clinical studies, saracatinib has been shown to suppress the motility and invasiveness of thyroid (14), lung (15), prostate (16), and head and neck squamous cell carcinoma (17) cell lines. Saracatinib was also found to inhibit the increased invasiveness of tamoxifen-resistant breast tumor cells *in vitro* (18), and to restore the sensitivity of a drug-resistant murine ovarian cell line to paclitaxel (19). Saracatinib is currently undergoing phase II evaluation in a number of clinical settings including breast, lung, head and neck, and ovarian cancer.

In the present investigation, the impact of saracatinib on tumor cell progression, invasion, and metastasis was evaluated in a highly aggressive and well-established murine sarcoma model.

Materials and Methods

Cell culture. KHT sarcoma cells (20) were grown in alpha minimal essential media (α -MEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine and maintained in a humidified incubator in the presence of 5% CO₂. Cell culture supplies were obtained from Invitrogen Corporation (Carlsbad, CA, USA).

Drug preparation. Saracatinib (AstraZeneca, Macclesfield, UK) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and subsequently diluted in phosphate-buffered saline (PBS) immediately before use, to concentrations from 0.5 to 5.0 μ M. In all cases, the final DMSO concentration was less than 0.5%.

Western immunoblotting. The abundances of phospho-Src (pSrc) and downstream effector proteins were assayed by Western immunoblotting. After treatment with 0.5-5.0 μ M saracatinib for 24 h, KHT cells were washed with cold PBS twice, lysed in RIPA

*These Authors contributed equally to this work.

Correspondence to: Lori Rice, Ph.D., Department of Radiation Oncology, 2033 Mowry Rd Rm 485E, Gainesville, FL 32610, U.S.A. Tel: +13522738251, Fax: +13522738252, e-mail: lrice@ufl.edu

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buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 1% Triton X-100; 0.1% SDS; 0.5% sodium deoxycholate; 1 μ M sodium orthovanadate; 5 μ M EDTA; 5 μ M sodium fluoride) containing a 1:20 dilution of mammalian protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), then collected and centrifuged at 14,000 rpm (4°C for 10 min). Total protein concentration was measured by Micro BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked in buffer containing 5% bovine serum albumin for 1 h at room temperature, and then incubated at 4°C overnight with primary antibodies for pSrc, [Y423-mouse/Y416-chicken/Y419-human], pPaxillin [Y118], pAkt [Y308], pERK1/2 [T202/Y204] (Cell Signaling Technology, Inc., Danvers, MA, USA), and pFAK [Y861, Y397] (Biosource International, Inc. Camarillo, CA, USA), followed by incubation for 1 h at room temperature with the appropriate secondary antibodies (species-specific horseradish peroxidase-conjugated). Immunoreactive bands were visualized by using enhanced chemiluminescence (ECL, Amersham Biosciences) and autoradiography. β -Actin were used as a loading control. Western immunoblotting was performed in triplicate experiments.

Cell proliferation and clonogenic cell survival. KHT cells (5×10^3) were seeded into 6-well plates and exposed to saracatinib (0.5-5.0 μ M) for 48 h. Cells in triplicate wells were counted daily for 4 days after plating and compared to controls. The effect of saracatinib (0.5-5.0 μ M) on the ability of KHT cells to produce colonies of at least 50 cells was determined by plating 100 cells plus drug in triplicate wells of 6-well plates. After 5 days, the media were removed, the plates stained with methyl blue, and the number of colonies in each well counted and compared to controls.

Cell cycle analysis. KHT cells were treated with 0.5-5.0 μ M saracatinib for 24 h. The cells were then harvested, fixed with ethanol, and treated with RNase. The nuclei were stained with propidium iodide and analyzed for DNA content by flow cytometry. The proportion of cells in each phase of the cell cycle was determined using Modfit LT software (Verity Software House, Topsham, ME, USA). This experiment was performed in duplicate.

Migration assay. KHT cells (1×10^5) were seeded into modified Boyden chambers (BD Biosciences, San Jose, CA, USA), in triplicate, and treated with 0.1-5.0 μ M saracatinib. After 24 h, cells were fixed and stained with crystal violet. Cells on top of the 8 μ M-pore membranes were removed using cotton swabs. Cells migrating through the membranes were counted at $\times 5$ magnification.

Invasion assay. KHT cells (5×10^5) suspended in medium containing 0.1% FBS were seeded into basement membrane matrix (Matrigel®)-coated invasion chambers (BD Biosciences), in triplicate, and treated with 0.5-5.0 μ M saracatinib. The lower chamber contained medium with 10% FBS. After 24 h, the cells and Matrigel in the upper chambers were removed using cotton swabs. Cells that had invaded through the Matrigel layer and migrated to the bottom side of the 8 μ M-pore membrane were fixed and stained with crystal violet. These cells were counted at $\times 5$ magnification.

Gelatin zymography. KHT cells (1.6×10^6) were seeded in 60 mm dishes and treated with 0.5-5.0 μ M saracatinib. After 24 h,

supernatants were collected and the proteins were separated on a 10% SDS-PAGE gel containing 1 mg/ml gelatin. After electrophoresis, the gels were washed for 15 min in 2.5% Triton X-100 and then incubated overnight in the same buffer at room temperature. After washing with deionized water, the gels were incubated for an additional 24 h at 37°C in a calcium-zinc buffer. The gel was stained with Coomassie brilliant blue R-250. Clear bands appearing at the expected locations for matrix metalloprotease (MMP)-9 and MMP-2 based on molecular weight markers were visualized using a transilluminating densitometer. The number of pixels per band was used to determine enzyme activity in each group. The zymography experiment was repeated three times with independent samples.

Localization and abundance of pSrc and pFAK by immunofluorescence confocal microscopy. KHT cells were cultured on chamber-slides and treated with 5 μ M saracatinib for 24 h. The cells were then fixed in 3.7% formaldehyde and permeabilized in 0.5% Triton X-100 in tris-buffered saline. Cells were hybridized with primary antibodies raised against Src, pSrc [Y423], FAK, and pFAK [Y861] and then stained with the appropriate secondary antibody conjugated to Alexa Fluor® 488 or Alexa Fluor® 594 (Invitrogen Corporation). Images of the stained cells were captured using a Leica scanning confocal microscope.

Formation of lung metastases. Six- to eight-week-old female C3H mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were maintained in a specific pathogen-free environment (University of Florida Health Science Center, Gainesville, FL, USA), with food and water provided *ad libitum*.

Untreated KHT cells, or cells treated with 1 or 5 μ M saracatinib for 24 h, were injected (1×10^5) into C3H mice *via* the tail vein (20 mice/group). Three weeks later, the mice were euthanized and the lungs were removed and fixed in Bouin's solution for 24 h. The number of lung nodules was counted and the sizes were determined using an eyepiece with a vernier scale under a light microscope.

All research was governed by the principles of the Guide for the Care and Use of Laboratory Animals (USPHS), and approved by the University of Florida Institutional Animal Care and Use Committee (Gainesville, FL, USA).

Statistical analyses. The statistical significance of differences of *in vitro* data was determined by Student's *t*-test (two-tailed); *in vivo* data were analyzed by Wilcoxon rank sum test.

Results

Src signaling. The effects of saracatinib on phosphorylation of Src in KHT cells are shown in Figure 1. In KHT cells exposed to saracatinib for 24 h, the level of Src protein expression was unchanged, but the autophosphorylation of Src at tyrosine 423 was reduced in a dose-dependent manner. Similarly, the abundance of FAK protein remained constant, but phosphorylation of FAK at tyrosine (Y) 861, an Src-dependent phosphorylation site (21), was reduced (Figure 1). However, as expected, there was no change in phosphorylation at Y397, an Src-independent site. The abundances of pPaxillin, pERK1/2, and pAkt also were unchanged.

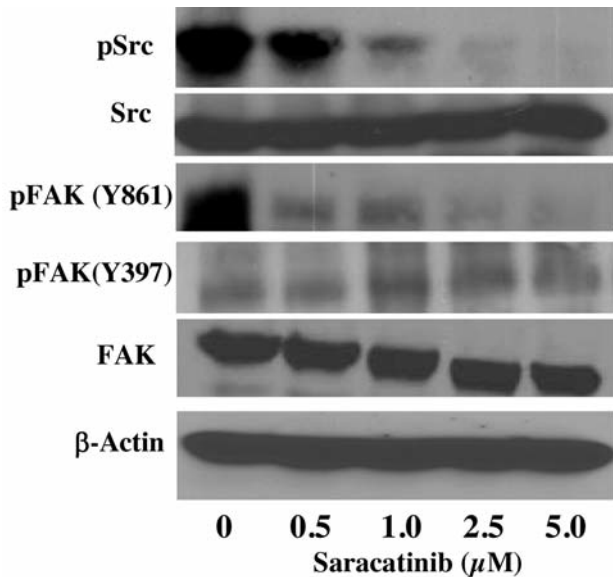


Figure 1. Effects on total and phosphorylated Src and FAK protein expression in KHT cells after 24 h exposure to saracatinib (0.5 to 5.0 μM) as determined by Western immunoblotting. Actin was used as a loading control.

Cell proliferation and clonogenic cell survival. Exposure to saracatinib (0.5–5.0 μM) for four days resulted in a decrease in proliferation of KHT cells (Figure 2A). However, clonogenic cell survival over the same dose range was not different from controls (Figure 2B) indicating that saracatinib produces a cytostatic effect rather than cell death.

Cell cycle. Cell cycle analysis (Figure 3) indicated that compared to controls, KHT cells treated with 2.5 μM or 5 μM saracatinib accumulated in the G_1 phase of the cell cycle (51 vs. 82%), with a concomitant decrease in G_2/M (13 vs. 7%) and S phases (35 vs. 11%) after 24 h drug exposure.

Cell migration and invasion. A modified Boyden chamber assay was used to investigate whether saracatinib treatment inhibited KHT cell motility and invasiveness. A concentration of 0.5 μM saracatinib was sufficient to significantly reduce KHT cell migration through an 8 μm pore membrane, with 1.5 μM saracatinib resulting in almost complete inhibition (Figure 4A). Similarly, when cells invaded a thin layer of Matrigel to reach medium with a chemoattractant (10% FBS), exposure to 0.5 μM saracatinib resulted in a decrease in cells found on the opposite side of the membrane, with significant reductions in cell numbers from 1.0 μM saracatinib (Figure 4B).

Activation of MMP-9 and MMP-2. Gelatin zymography analysis showed that the active MMP-9 significantly

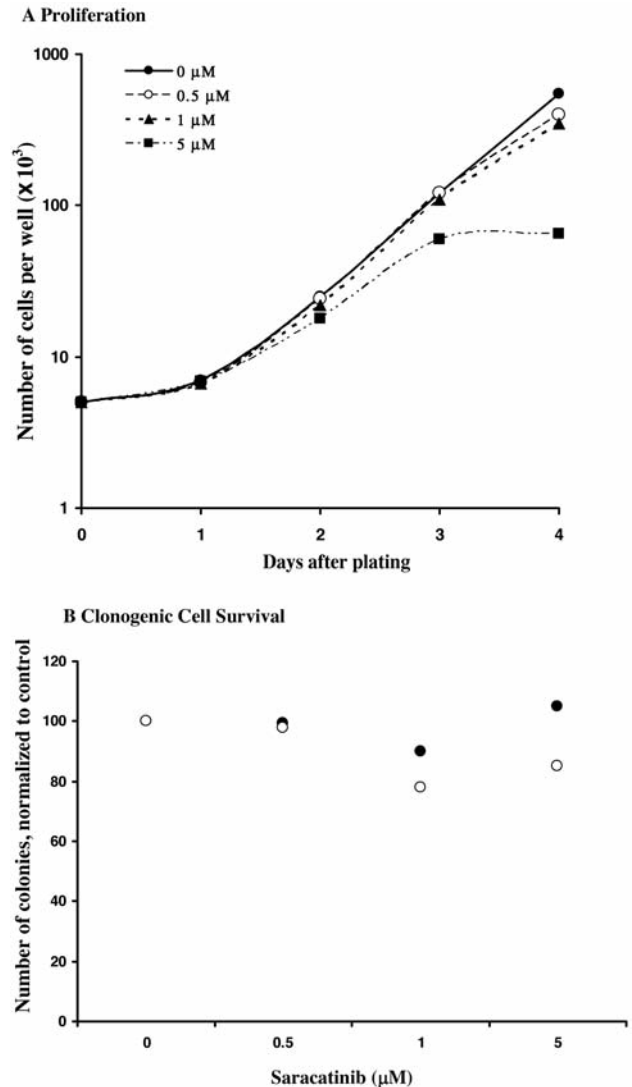


Figure 2. A: Cell proliferation. KHT cells (5×10^3) were plated in 6-well plates and exposed to saracatinib (0.5 to 5.0 μM) for four days. The log of the average number of cells from triplicate wells in each treatment group is presented for each day after plating. B: Clonogenic cell survival. KHT cells (100) were plated in 6-well plates and exposed to saracatinib (0.5 μM) for five days. The average number of KHT colonies (>50 cells) counted in triplicate wells for each treatment group is presented from two independent experiments.

decreased in a dose-dependent manner after 24 h saracatinib exposure, whereas the amount of pro-MMP-9 remained unchanged (Figure 5 A and B). However, saracatinib did not affect the amount of pro-MMP-2 nor of its active form.

Localization and abundance of pSrc and pFAK. Immunofluorescent confocal microscopy images showed that exposure to 5.0 μM saracatinib for 24 h caused KHT cells to assume a rounded appearance with less abundant staining for

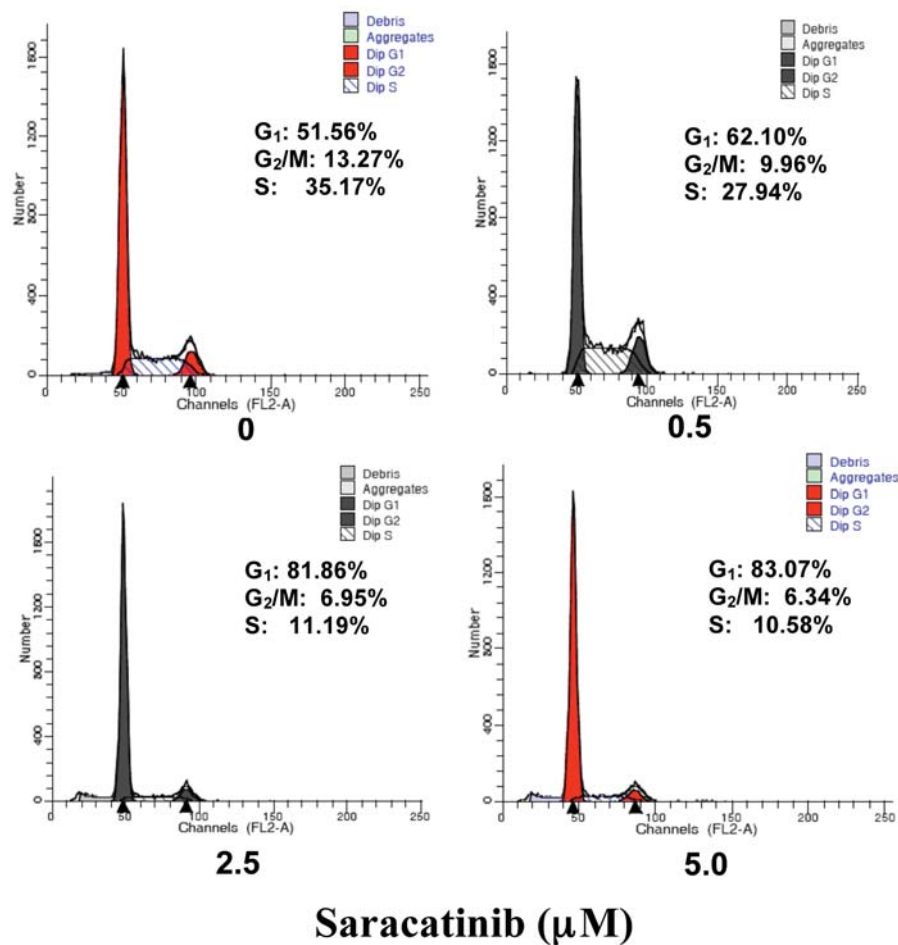


Figure 3. KHT cell cycle distribution assessed by flow cytometry 24 h after exposure to 0.5-5.0 μ M saracatinib. The results shown are representative of two independent experiments.

pSrc and pFAK and a loss of the characteristic dagger-like appearance of focal adhesions (Figure 6).

Formation of lung metastases. KHT cells pre-treated with saracatinib for 24 h were injected *via* tail vein into C3H mice to evaluate the effect of this agent on *in vivo* metastatic activity. When lung nodules were counted 3 weeks post-injection, the data showed a mean reduction in nodule number from 13 to 5 and to less than 1 for 1 μ M ($p < 0.05$) and 5 μ M ($p < 0.01$), respectively, compared to controls (Figure 7A and B). However, nodule size distributions in the treated groups were not different from controls (Figure 7C).

Discussion

Accumulating evidence suggests that Src tyrosine kinases may play critical roles in regulating cancer progression. Increases in Src expression and phosphorylation have been

detected in a variety of human tumors (3-5) and are associated with late-stage disease (22). Consequently, several Src inhibitors including saracatinib, dasatinib, and SKI-606 are currently under investigation as novel therapeutic agents for the treatment of various types of cancer (23).

In the present study, the activity of one of these inhibitors (saracatinib) was assessed in a highly metastatic murine tumor cell line (KHT sarcoma). Initial experiments demonstrated that exposure to saracatinib at concentrations up to 5 μ M resulted in a decrease in cell proliferation (Figure 2A) that may be explained by an accumulation of cells in the G₁ phase of the cell cycle (Figure 3). However, there was no increase in cell death, as determined by clonogenic cell survival assays (Figure 2B). Such treatment did, however, lead to a significant dose-dependent impairment of KHT sarcoma cell migration and invasion (Figure 4 A and B). These findings are similar to those reported previously for thyroid and lung cancer cells exposed to saracatinib (14-15).

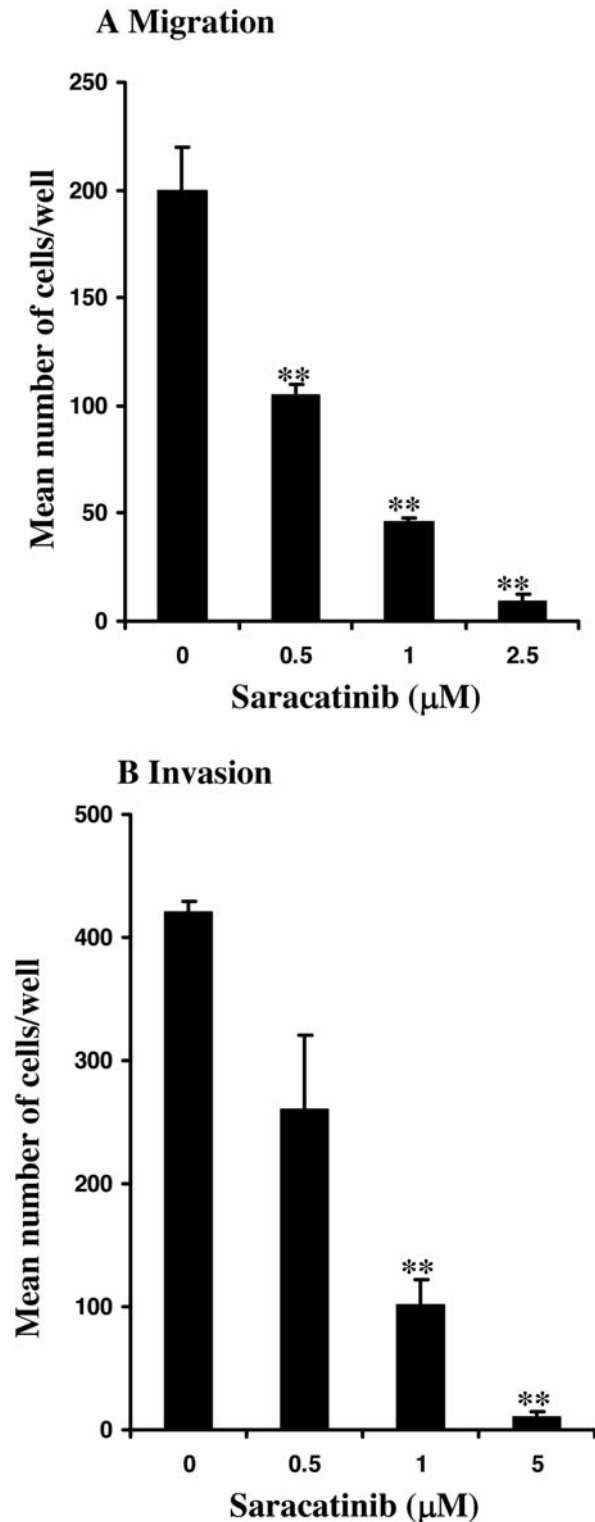


Figure 4. Effect of 24 h saracatinib exposure on KHT cell motility in a modified Boyden chamber assay. A: Migration of cells through an 8 μ pore-size membrane. B: Invasion of cells through a Matrigel-coated 8 μ pore-size membrane. Graphs show the number of cells found on the bottom surface of the membranes after 24 h (mean number of cells per well \pm SE for 3 experiments). ** $p < 0.0001$ vs. control.

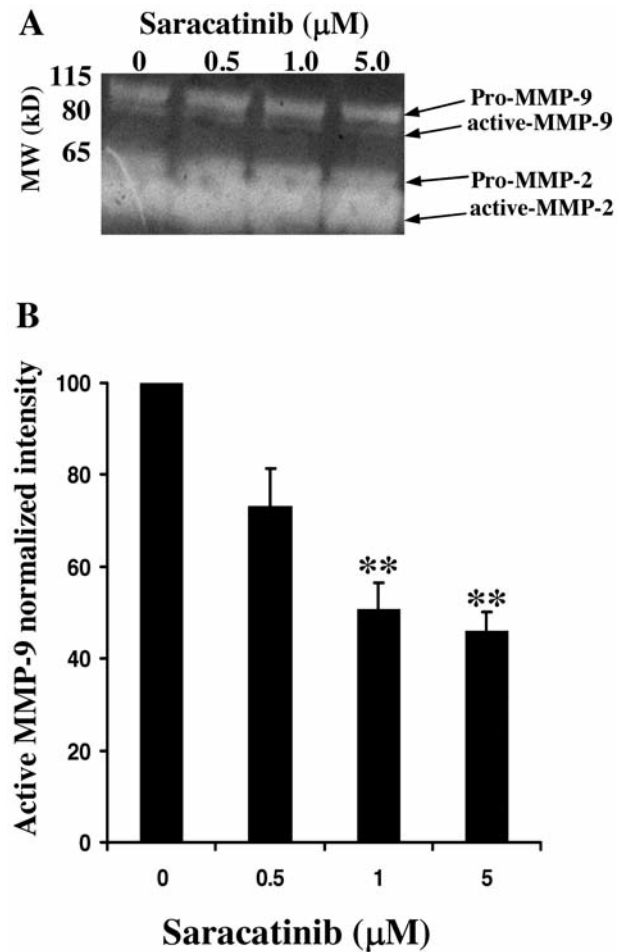


Figure 5. Effect of 24 h saracatinib exposure (0.5-5 μ M) on MMP-9 and MMP-2 activity in KHT cells analyzed by gelatin zymography. A: Image of representative zymograph gel showing reduction in intensity of bands corresponding to active MMP-9. No change in intensity of bands corresponding to pro-MMP-9 or pro- or active MMP-2 was detected. B: Graph showing pixel intensity of active MMP-9 bands as determined by densitometry. Results shown are normalized means \pm SE for 3 experiments. ** $p < 0.01$ vs. control.

Phosphorylation of molecules downstream of Src, such as paxillin, Akt and ERK, was unchanged, indicating they probably do not play a major role in decreased cell invasion after saracatinib exposure.

The ability of cells to migrate and invade is also dependent on their integrin-mediated interaction with the extracellular matrix (ECM), which in turn, is regulated by focal adhesion molecules. FAK is a non-receptor tyrosine kinase that can interact with Src to modulate the dynamics of focal adhesions (24). Upon integrin engagement, FAK is phosphorylated at tyrosine 397. This allows pFAK to recruit pSrc, which subsequently promotes phosphorylation of FAK at several additional tyrosine sites including Y861. The present results

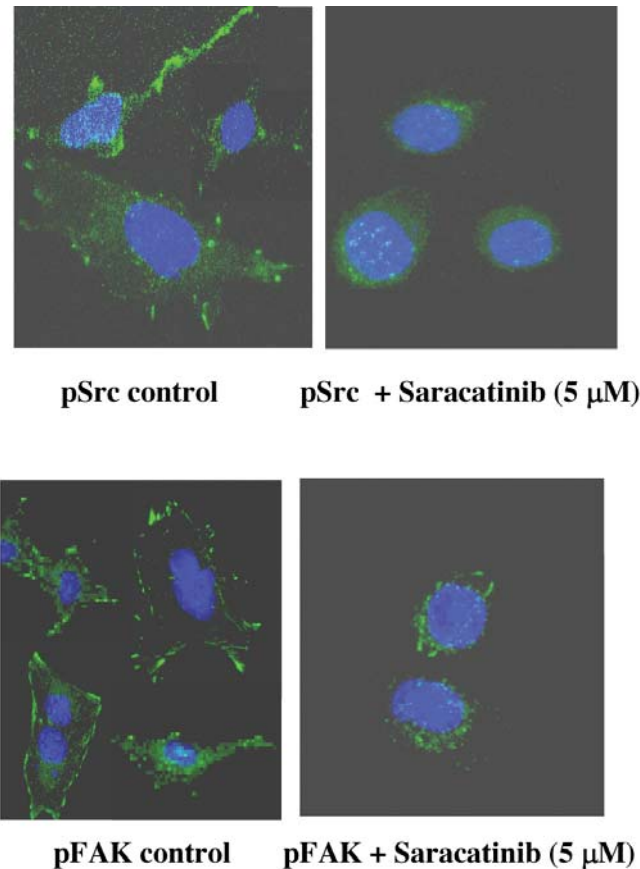


Figure 6. Distribution of pSrc (Y423) and pFAK (Y861) after 24 h exposure to 5 μ M saracatinib, as determined by immunofluorescent confocal microscopy. Green: Alexa Fluor[®] 488, pSrc, pFAK; Blue: DAPI, nuclei.

showed that saracatinib reduced phosphorylation of Src and FAK (Y861), as determined by Western immunoblotting (Figure 1). In addition, when examined by immunofluorescent staining, saracatinib treatment led to changes in cell morphology and reduced staining intensity of both pFAK and pSrc (Figure 6). These observations strongly suggest that the focal adhesions of tumor cells are greatly disrupted by saracatinib-induced inhibition of Src activation.

Since tumor cell invasion involves the proteolytic degradation of ECM components by MMPs, the effect of saracatinib treatment on these proteases in KHT sarcoma cells was also examined. MMP-9 and MMP-2 have previously been reported to be regulated by Src in other tumor cell lines including RK3E (25), astrocytes (26), and MCF-7 cells (27). In the present studies, MMP-9 and MMP-2 secretion levels were unchanged after saracatinib treatment but the amount of active MMP-9 decreased (Figure 5A and B). This observation suggests that a reduction in enzyme activity following saracatinib treatment may contribute to the observed reduction in KHT cell invasiveness.

KHT cells were derived from a highly aggressive sarcoma which arose spontaneously in a C3H/Km mouse in 1967 (20). It metastasizes early and rapidly such that even when the primary tumor is controlled by irradiation, the animal will die of extensive lung metastases (28). Attempts to control the lung metastases by irradiation have reportedly resulted in treatment failures due to ovarian and renal metastases confirmed to have arisen from the lung nodules (29). This model thus may mimic highly malignant and metastatic diseases that present clinically, in which controlling the spread of tumors to distant sites is necessary to improving treatment outcomes.

To examine whether the *in vitro* effects of saracatinib treatment on tumor cell function would affect the ability of KHT sarcoma cells to establish secondary tumor deposits in the lungs of mice, tumor cells pretreated with non-cytotoxic doses of saracatinib and untreated control tumor cells were injected into mice *via* the tail vein. The results demonstrated that when equal numbers of tumor cells were injected, fewer saracatinib-pretreated KHT cells were able to form tumor

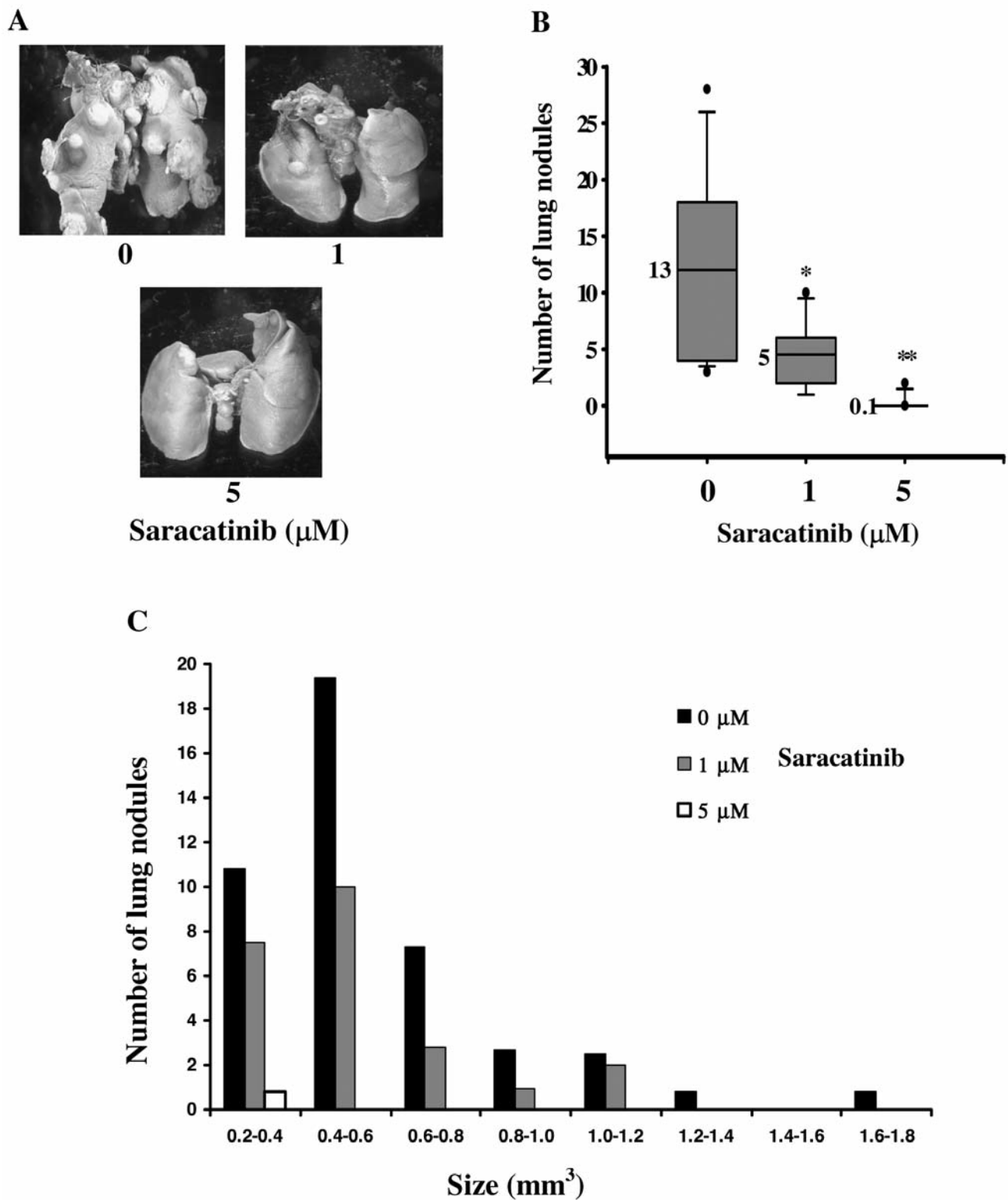


Figure 7. Effect of 24 h pre-treatment of KHT cells with saracatinib (1 or 5 μ M) on the formation of lung colonies after tail vein injection. A: Images of representative lungs taken from mice three weeks after injection. B: Median numbers of lung nodules in each treatment group (horizontal line) and the 25th and 75th percentiles (box boundaries), respectively. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively. * p <0.05, ** p <0.01 vs. control. C: Size distribution of lung nodules.

nodules in the lungs compared to controls (Figure 7A and B). This reduction in the number of lung nodules occurred in the absence of a significant difference in the size distributions of colonies arising from control and drug-pretreated tumor cells (Figure 7C). Taken together, these findings imply that the effects of saracatinib exposure on the ability of KHT tumor cells to form lung nodules was a consequence of the drug impeding the ability of tumor cells to establish secondary tumor deposits and not due to a lasting drug effect on subsequent tumor cell growth. Mechanistically, saracatinib exposure impaired KHT cell cycle progression (Figure 3), migration (Figure 4A) and invasion (Figure 4B), key steps required by tumor cells to escape the blood stream and to establish new lesions. However, further studies will be needed to resolve the relative contribution each of these processes may make to the observed final result of a reduction in the number of lung nodules.

In conclusion, this study shows that saracatinib, a specific Src inhibitor, has significant effects on key cellular processes associated with the metastatic cascade. Therefore, our results suggest that molecular targeting agents that interfere with Src pathways should be considered when developing novel therapeutic treatment strategies for patients at risk of metastatic disease.

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