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Src Signaling Interference Impairs the Dissemination of Blood-Borne Tumor Cells

Dietmar W. Siemann^{1,*}, Meiyu Dong¹, Chris Pampo¹, and Wenyin Shi²

¹Department of Radiation Oncology and Shands Cancer Center, University of Florida, Gainesville, FL, USA

²Department of Radiation Oncology, Thomas Jefferson University, Philadelphia, PA, USA

Abstract

Although solid tumors continuously shed cells, only a very small fraction of the neoplastic cells that enter the blood stream are capable of establishing metastases. In order to be successful these cells must attach, extravasate, proliferate, and induce angiogenesis. Preclinical studies have shown that small-molecule ATP-competitive Src kinase inhibitors can effectively impair metastasis associated tumor cell functions in vitro. However, the impact of these agents on the metastatic cascade in vivo is less well understood. The present studies examined the ability of saracatinib, a dual-specific, orally available inhibitor of Src and Abl protein tyrosine kinases, to interfere with the establishment of lung metastases in mice by tumor cells introduced into the blood stream. The results demonstrated that Src inhibition most effectively interfered with the establishment of secondary tumor deposits when treatments were administered while tumor cells were in the initial phases of dissemination.

Keywords

blood borne tumor cells; metastatic cascade; Src inhibition; saracatinib

Introduction

The ability of cancer cells to disseminate and form new foci of growth represents not only cancer's most malignant characteristic but also the most common reason for failure of conventional anticancer therapies. Indeed it is estimated that metastatic disease is the direct or indirect cause of nearly 90 percent of all deaths due to malignancy (Coghlin and Murray, 2010, Sleeman and Steeg, 2010, Wittekind and Neid, 2005). Still metastases are relatively rare events; in part because in order to establish progressive growth at a secondary site tumor cells must successfully navigate a complicated multi-step process which requires them to escape the primary tumor, survive in the blood stream, invade, proliferate, and induce angiogenesis. Thus, the very complexity of the metastatic cascade also offers the possibility for strategic interventions targeting key signal transduction pathways associated with the functional characteristics of the metastatic phenotype.

One such target may be the Src family of non-receptor protein tyrosine kinases (Martin, 2001, Summy and Gallick, 2003). Src plays a critical role in a variety of cellular signal transduction pathways associated with cell proliferation and survival (Basu and Cline, 1995, Frame, 2002, Taylor and Shalloway, 1996, Wei, et al., 2004). Its expression also promotes

*Correspondence to: Dietmar W. Siemann, PhD, Professor and Associate Chair, Department of Radiation Oncology, University of Florida, 2000 SW Archer Road, Gainesville, Florida 32610, 352-265-0287, siemadw@ufl.edu.

tumor cell detachment, migration and invasion through the regulation of focal adhesions (Parsons and Parsons, 1997, Timpson, et al., 2001) and interaction with integrins (Playford and Schaller, 2004) and proteolytic enzymes (Hauck, et al., 2002, Hiscox, et al., 2006, Pongchairerk, et al., 2005). In addition, Src expression has been associated with the angiogenic process, having been shown capable of modulating the expression of pro-angiogenic factors (Eliceiri, et al., 1999, Ellis, et al., 1998, Marx, et al., 2001), vascular permeability (Park, et al., 2007), and tube formation (Kilarski, et al., 2003, Kumar, et al., 2003).

In patients, Src is frequently over-expressed in malignancies (Egan, et al., 1999, Irby and Yeatman, 2000, Lutz, et al., 1998, Yeatman, 2004). Furthermore, elevated Src expression or Src pathway activation may be related to poor prognosis, tumor progression, and metastasis (Aligayer, et al., 2002, Cartwright, et al., 1994, Dehm and Bonham, 2004). Based on these observations, the inhibition of Src kinase activity has been identified as a novel anticancer treatment strategy (Green, et al., 2009, Hiscox and Nicholson, 2008) and lead agents dasatinib, saracatinib and bosutinib, are currently in clinical development in a number of solid tumor settings (Aleshin and Finn, 2010, Haura, et al., 2010, Koppikar, et al., 2008, Lara, et al., 2009, Lee and Gautschi, 2006, Saad and Lipton, 2010, Yu, et al., 2009).

Target validation studies including Src transfection (Myoui, et al., 2003, Rucci, et al., 2006), antisense Src constructs (Wiener, et al., 1999), and Src mutation studies (Boyer, et al., 2002) support the role of Src as a key molecule in the metastatic cascade of cancer cells (Fizazi, 2007, Saad and Lipton, 2010). Furthermore, tissue culture studies have convincingly demonstrated that clinically advanced Src targeting agents not only significantly inhibit Src signaling in a variety of tumor cell lines but also severely impair metastasis-associated tumor cell functions (Dong, et al., 2010, Purnell, et al., 2009, Rice, et al., 2011, Schweppe, et al., 2009). However, the impact of these small molecule Src inhibitors on the metastatic cascade in vivo is far less well-documented. Preclinical studies of bladder (Green, Fennell, Whittaker, Curwen, Jacobs, Allen, Logie, Hargreaves, Dickinson, Wilkinson, Elvin, Boyer, Carragher, Ple, Bermingham, Holdgate, Ward, Hennequin, Davies and Costello, 2009) and head and neck (Ammer, et al., 2009) tumor models showed that saracatinib treatment impaired lymph node metastasis as was also the case for prostate cancer xenografts treated with dasatinib (Park, et al., 2008). In terms of hematogenous spread of cancer cells, bosutinib treatment of a murine breast cancer model reduced the number of liver, spleen, and lung metastases (Jallal, et al., 2007). Dasatinib inhibited metastasis of human pancreatic adenocarcinoma in a nude mouse model (Trevino, et al., 2006), but not the development of pulmonary metastases in a model of osteosarcoma (Hingorani, et al., 2009). Thus there exists not only a paucity of in vivo data, but a full understanding of how Src signaling inhibition by these agents perturbs the metastatic cascade in situ is lacking. The goal of the present study was to investigate which phase of the blood borne dissemination of tumor cells is impacted by Src inhibition.

Materials and Methods

Src kinase inhibitor

Stock solutions (10 mM) of saracatinib (AZD0530, AstraZeneca, Macclesfield, UK) were prepared by suspending the agent in 10% (v/v) Tween-80 and HEPES. Working dilutions were made by serial dilution of the stock solution in sterile saline. Saracatinib (10–25 mg/kg) was administered by oral gavage. For in vitro studies saracatinib was dissolved in DMSO (10 μ M) and diluted in PBS immediately prior to use such that the DMSO exposure in cell cultures was <0.1%. All chemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cell lines

Murine KHT sarcoma cells, received from Stanford University, Palo Alto, CA (Kallman, 1967), were grown in alpha minimal essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS) and 2 mmol/L L-glutamine (Invitrogen Corporation, Carlsbad, CA). Human microvascular endothelial cells of the lung (HMVEC-L, Lonza Biologics, Walkersville, MD) were grown in EBM-2-MV media (Lonza) supplemented with 5% FBS.

Tumor cell attachment

Control (0.1% DMSO) or saracatinib (0.5, 2.5 or 5 μ M) pretreated KHT sarcoma cells (1×10^6) were added to a monolayer of endothelial (HMVEC-L) cells. After 2 h, the number of tumor cells remaining unattached in the supernatant was counted.

Endothelial cell migration

HMVEC-L (5×10^2) were either pretreated with saracatinib (0–10 μ M) for 24 prior to seeding them into modified Boyden-chambers (BD Biosciences, Palo Alto, CA) or exposed to the agent during the 24 h assessment period. Cells remaining on top of the 8 μ -pore membrane then were removed using cotton swabs. Migrated cells on the other side of the membrane were fixed, stained with crystal violet, and counted at 5X magnification.

Tube formation

HMVEC-L (6×10^4) plated in triplicate were either pre-treated with 0–10 μ M saracatinib (24 or 48 h) or exposed to saracatinib after plating on Matrigel pre-coated 24-well plates (BD Biosciences, Palo Alto, CA) to assess tube formation. The cells were incubated for 24–72 h at 37°C and tube formation was observed and photographed.

Supernatant levels of VEGF

KHT sarcoma cells (1.6×10^6) were seeded in 60 mm dishes and then treated with 0–10 μ M AZD0530 for a period of 24 h. Supernatants were collected and the concentrations of VEGF in the media were determined by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Intra-dermal angiogenesis assay

Six-to-eight-week-old female nude mice (Jackson Laboratories, Bar Harbor, ME) were injected intra-dermally with 1×10^5 KHT sarcoma cells in 4 ventral locations; 3 mice/group. Cells were either treated with saracatinib (0–0.5 μ M) prior to injection, or mice were treated with daily doses of saracatinib (0–10 mg/kg) after tumor cell inoculation. In either case the mice were euthanized 3 days later, the skin containing the 4 nodules was removed, and the number of blood vessels that intersected the tumor cell nodules was counted using a dissection microscope.

Artificial lung metastases

$2-5 \times 10^3$ KHT sarcoma cells were injected via the tail vein in a volume of 0.2 ml saline; 10 mice/group. Animals were euthanized 21 days later and lungs collected. The numbers of metastatic foci formed in the lungs were counted using a dissecting microscope. The sizes of individual lung nodules were measured using a scaled eyepiece accessory of the microscope.

Statistics

Statistical significance was determined using a Wilcoxon rank sum test. For all analysis, $p < 0.05$ was considered to be significant.

Animals

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). Mice were maintained in a specific pathogen-free environment (University of Florida Health Science Center, Gainesville, FL), with food and water provided ad libitum.

Results

Saracatinib is a highly selective, orally available small molecule that can inhibit Src kinase activity by interfering with Src phosphorylation at tyrosine 419-human/423-mouse (Hennequin, et al., 2006). In the highly metastatic KHT sarcoma model Src kinase inhibition by saracatinib results in significant impairment of metastasis associated tumor cell functions (Dong, Rice, Lepler, Pampo and Siemann, 2010). To determine whether saracatinib could impair the ability of KHT sarcoma cells to establish secondary tumor growth in situ, tumor cells were injected into the blood stream via the tail vein (on day 0), mice were treated with the Src inhibitor (at various times post tumor cell inoculation), and the number of metastatic foci formed in the lungs was determined 21 days later. In initial studies the mice were treated with daily 10 or 25 mg/kg doses of saracatinib beginning one day post tumor cell inoculation (Fig. 1). The results showed that such treatments led to significant reductions ($p < 0.05$, Wilcoxon rank sum test) in the number of lung colonies formed; both saracatinib doses being equally effective (Fig. 1a). For example, the 10 and 25 mg/kg doses resulted in 53/47% and 60/31% reductions in the number of lung colonies formed compared to those detected in mice receiving only the drug carrier. The median size of the lung foci detected did not differ significantly between saracatinib treated mice and drug carrier treated control animals (Fig. 1b).

Three additional experiments were conducted with the treatment protocol consisting of daily 10 mg/kg doses of saracatinib administered on days 1–20 after introducing the tumor cells into the blood stream (Fig. 2a). The results confirmed the ~2-fold reduction in lung colonies in saracatinib treated mice shown in Fig 1a. A second protocol in which the number of 10 mg/kg saracatinib doses was halved and the agent was administered only on days 3–7 and 10–14 also significantly reduced the number of neoplastic cell lung foci (Fig. 2b) but somewhat less effectively (35% versus 50% reduction in lung colony formation). The efficacy of the latter treatment protocol was primarily due to the initial five doses of saracatinib since (i) treating mice with this agent only on days 3–7 (Fig. 3a and 3b) was as effective at reducing lung colony formation (25 and 42% reduction) as was treating on days 3–7 plus 10–14, and (ii) treating only on days 10–14 failed to affect the number of lung colonies formed (Fig. 3a). Furthermore, five doses of saracatinib administered in the final days of lung colony growth (days 17–21) also had no effect on the resultant number of lung colonies (Fig. 3b).

Our initial results (Figs 1–3) implied that exposing mice to the Src inhibitor soon after the intravenous introduction of tumor cells had the greatest impact on the subsequent establishment of secondary site tumor growth. To further examine this possibility, mice injected with tumor cells were treated with one of three daily 10 mg/kg saracatinib protocols (Fig. 4). These were: (i) a single dose of saracatinib administered immediately (within 1 h) after tumor cell injection (day 0), (ii) 4 doses of the agent given on days 0–3, and (iii) 21 drug doses administered over the entire course of the experiment. Three independent studies were conducted and the results showed that the 3 treatment protocols were equally effective at significantly reducing the ability of KHT sarcoma cells to form lung colonies.

Previous in vitro investigations in our laboratory had shown that treating KHT sarcoma cells with saracatinib inhibited Src and FAK activation as well as tumor cell migration and invasion (Dong, Rice, Lepler, Pampo and Siemann, 2010). Another function of tumor cells that contributes to the in vivo metastatic process is attachment. To study the interaction between KHT tumor cells and endothelial cells, drug-treated and control tumor cells were added to a monolayer of endothelial cells. The results (Fig. 5) showed that saracatinib pretreatment significantly affected the ability of KHT tumor cells to attach to endothelial cells; i.e. the number of tumor cells that failed to attach to endothelial cells increased in a dose dependent manner. For example, during the 2 h assessment period, more than twice as many tumor cells in the 5 μ M saracatinib pretreated group remained unattached as compared to tumor cells in the untreated control group (Fig. 5).

Once established at a new site, progressive tumor growth requires the initiation of angiogenesis (Siemann and Horsman, 2009). Src has been shown to play a role in this process (Eliceiri, Paul, Schwartzberg, Hood, Leng and Cheresch, 1999, Ellis, Staley, Liu, Fleming, Parikh, Bucana and Gallick, 1998, Kilarski, Jura and Gerwins, 2003, Marx, Pavlakakis, McCowatt, Boyle, Levi, Bell, Cook, Biggs, Little and Wheeler, 2001, Park, Shah, Zhang and Gallick, 2007) and consequently a component of the antitumor effects associated with Src inhibitors may be attributed to their anti-angiogenic action (Fizazi, 2007, Saad and Lipton, 2010). To study this possibility the impact of saracatinib treatment on endothelial cell function was investigated in vitro. At the molecular level a 24 h exposure to saracatinib decreased pSrc in HMVEC-L cells in a dose dependent manner in the absence of changes in Src expression (Fig. 6a). However, these doses of saracatinib (1–10 μ M) did not affect HMVEC-L growth (data not shown) or migration (Fig. 6f). Furthermore, pretreating HMVEC-L with saracatinib for 24 or 48 h prior to assessing their ability to form tubes or exposing the cells to the agent during the tube formation period (24–48 h) had no effect on the ability of HMVEC-L to form tubes (Fig. 6b–e). Established endothelial tubes also were unaffected by saracatinib treatments of up to 72 h (data not shown). A 24 h saracatinib exposure did however significantly reduce KHT sarcoma cell VEGF secretion (Fig. 6g).

To study the effect of saracatinib treatment on KHT sarcoma cell-induced angiogenesis in vivo an intradermal assay was used (Shi and Siemann, 2002). Two treatment strategies were employed; tumor cells were either pretreated with non-cytotoxic doses of saracatinib (1 or 5 μ M (Dong, Rice, Lepler, Pampo and Siemann, 2010)) prior to injection, or the mice were treated with daily doses of saracatinib (10 or 25 mg/kg) beginning immediately after tumor cell injection. In either case, three days later, the mice were euthanized, the skin flap containing the inoculation site was excised, and the number of blood vessels induced was counted. The results (Fig. 7) showed that compared to controls, KHT tumor cells that were pretreated with saracatinib induced significantly fewer blood vessels (Fig. 7a). Similarly, skin flaps from mice treated with saracatinib also showed significant reductions in the number of blood vessels induced by KHT sarcoma cells (Fig. 7b).

Discussion

Src kinases are a family of non-receptor tyrosine kinases that act as signal transduction modulators and are a critical component of many of the key signaling pathways pivotal in cancer progression (Dehm and Bonham, 2004, Frame, 2002, Lutz, Esser, Flossmann-Kast, Vogelmann, Luhrs, Friess, Buchler and Adler, 1998, Summy and Gallick, 2003). Src mediates signaling from several receptors and downstream effects of Src kinase signaling influence cell growth, proliferation, invasion and apoptosis (Frame, 2002). In particular, increased Src kinase signaling activity ultimately promotes an invasive tumor cell phenotype. In patients, elevated Src kinase activity has been documented in several tumors types (Aligayer, Boyd, Heiss, Abdalla, Curley and Gallick, 2002, Dehm and Bonham, 2004,

Lutz, Esser, Flossmann-Kast, Vogelmann, Luhrs, Friess, Buchler and Adler, 1998) associated with tumor progression, and linked to poor prognosis (Aligayer, Boyd, Heiss, Abdalla, Curley and Gallick, 2002, Cartwright, Coad and Egbert, 1994, Dehm and Bonham, 2004). As a consequence it has been suggested that Src kinase inhibition may hold significant therapeutic potential as an anti-metastatic therapy for solid tumors (Aleshin and Finn, 2010, Green, Fennell, Whittaker, Curwen, Jacobs, Allen, Logie, Hargreaves, Dickinson, Wilkinson, Elvin, Boyer, Carragher, Ple, Bermingham, Holdgate, Ward, Hennequin, Davies and Costello, 2009, Hiscox and Nicholson, 2008, Irby and Yeatman, 2000, Saad and Lipton, 2010, Summy and Gallick, 2003). However, in the discussion of anti-metastatic therapies it is essential to differentiate therapies that affect the progression of established secondary site neoplastic foci from interventions that impair the process of neoplastic cell dissemination.

In bone metastases, tumor cells dysregulate the normally balanced activities of osteoclasts and osteoblasts resulting in significant morbidity (Coleman, et al., 2001, Mundy, 2002). Src signaling is a key pathway involved in enhancing osteoclast activity and maintaining osteoblast pre-differentiation state (Miyazaki, et al., 2004, Yoneda and Hiraga, 2005). Src therefore represents a logical target for the treatment of advanced metastatic prostate or breast cancer (Fizazi, 2007, Saad and Lipton, 2010). In support, preclinical studies have shown that both dasatinib and saracatinib affect tumor growth in bone; a likely consequence not only of the agents' antineoplastic cell activities but also of their inhibitory effects on osteoclast-mediated bone resorption which plays a significant role in the growth and expansion of bone neoplastic lesions (de Vries, et al., 2009, Yang, et al., 2010). Furthermore, two recent clinical studies have shown that saracatinib inhibits bone resorption (Hannon, et al., 2010, Hannon, et al., 2012).

The focus of the present investigation was to assess the impact of saracatinib treatment on the ability of tumor cells to establish secondary lesions after entering the blood stream. We previously reported that KHT sarcoma cells pretreated with non-cytotoxic/non-cytostatic doses of saracatinib displayed a reduced ability to establish neoplastic lung lesions when injected intravenously (Dong, Rice, Lepler, Pampo and Siemann, 2010). We subsequently observed that this result was not limited to the KHT tumor model or the Src inhibitor saracatinib when we noted a similar outcome for 4T1 breast carcinoma cells pretreated with dasatinib (Saffran and Siemann, 2011); i.e. non-toxic doses of a Src inhibitor readily impaired the ability breast cancer cells to form pulmonary lesions.

The present studies indicate that in situ saracatinib treatment of mice bearing blood borne tumor cells exerts its primary effect on the development of pulmonary metastases rather than impairing the progression of established metastatic foci. Several lines of evidence support this conclusion. Initial studies in which mice were treated with daily doses of 10 or 25 mg/kg saracatinib beginning the day after iv tumor cell injection and lasting throughout the assessment period, showed that both treatments led to a significant decrease in the number of lung colonies formed (Fig 1a). However, the size of the neoplastic lesions formed in the treated and control mice were not significantly different (Fig. 1b) indicating that the primary therapeutic effect of the saracatinib treatment was a consequence of a reduction in the number of tumor cells capable of forming lung colonies. Moreover, the lack of a significant difference in efficacy between the two treatment groups (Fig. 1a) suggests that Src inhibition alone was not sufficient to completely block the development of lung metastases and implies that further increases in the dose of saracatinib would have had little additional therapeutic benefit.

To gain additional support for the notion that targeting Src signaling would predominantly affect the early stages of tumor cell dissemination, a series of experiments utilizing a variety

of in situ treatment schedules were undertaken (Figs 2–4). The results showed that the timing of the administration of the Src inhibitor was far more critical to its ability to impair the metastatic cascade than the total dose. For example, reducing the number of daily 10 mg/kg saracatinib doses from 20 to 10 had little impact on its efficacy (Fig. 2). Furthermore, when comparing the results illustrated in Figs 2 and 3 it is readily apparent that saracatinib treatments administered during the first week after intravenous injection of tumor cells were responsible for the observed reduction in the number of pulmonary tumor nodules with treatments commencing on day 10 or 14 failing to have a significant effect. Perhaps most intriguing a single dose of the Src inhibitor administered shortly after the tumor cells were introduced into the circulation proved as effective at inhibiting the establishment of tumors in the lungs as any of the other treatment schedules investigated.

These findings suggest that those tumor cells undergoing extravasation (attachment, invasion, migration) were particularly vulnerable to saracatinib therapy. In support, in vitro studies have shown that saracatinib doses that are non-toxic and non-cytostatic significantly inhibit KHT sarcoma cell migration and invasion (Dong, Rice, Lepler, Pampo and Siemann, 2010) as well as attachment to endothelial cells (Fig. 5). The lack of a measurable response to saracatinib treatment in established lung tumor nodules (> 10 days after iv injection) is consistent with our finding that treatment of mice bearing 200 mg primary KHT tumors with 10 or 25 mg/kg saracatinib had no effect on the growth rate of these tumors (data not shown). The latter result is in keeping with the reports of others of modest antitumor effects with Src inhibitors. In the case of saracatinib, neither bladder cancer (Green, Fennell, Whittaker, Curwen, Jacobs, Allen, Logie, Hargreaves, Hickinson, Wilkinson, Elvin, Boyer, Carragher, Ple, Birmingham, Holdgate, Ward, Hennequin, Davies and Costello, 2009) nor head and neck squamous cell carcinoma (HNSCC) (Ammer, Kelley, Hayes, Evans, Lopez-Skinner, Martin, Frederick, Rothschild, Raben, Elvin, Green and Weed, 2009) xenograft growth was significantly inhibited when treated with saracatinib while only a subset of human pancreatic xenografts responded to this agent (Rajeshkumar, et al., 2009). Similarly, dasatinib also has shown only limited efficacy against solid tumors in preclinical investigations (Hingorani, Zhang, Gorlick and Kolb, 2009).

Interestingly the lack of tumor growth inhibition occurs despite evidence that small-molecule ATP-competitive Src kinase inhibitors can suppress angiogenesis (Eliceiri, Paul, Schwartzberg, Hood, Leng and Cheresch, 1999, Ellis, Staley, Liu, Fleming, Parikh, Bucana and Gallick, 1998, Kilarski, Jura and Gerwins, 2003, Park, Shah, Zhang and Gallick, 2007). In the present investigation saracatinib treatment (1–10 μ M) suppressed Src phosphorylation in endothelial cells (Fig. 6a) but did not affect endothelial cell tube formation (Fig. 6b–e) or migration (Fig. 6f). However, secretion of VEGF by KHT sarcoma cells was significantly reduced (Fig. 6g). Furthermore both saracatinib pretreated KHT cells and KHT sarcoma cell bearing mice treated with saracatinib demonstrated clear evidence of an in vivo antiangiogenic effect of saracatinib (Fig. 7). Thus it is conceivable that antiangiogenic effects of saracatinib may contribute to early phases of lung nodule development but these clearly are not sufficient to impact the growth of established lung colonies.

Because aberrant Src signaling has been linked to cancer cell functions associated with an enhanced metastatic phenotype, Src inhibitors are being actively pursued for their potential as anti-metastatic anticancer agents. As such Src signaling interventions could prove efficacious against secondary disease or impact the spread of neoplastic cells from a primary tumor; a therapeutic distinction often blurred in experimental investigations of such agents. Yet a clearer understanding and a better distinction of how Src inhibitors impact the metastatic cascade and/or established metastases could have significant impact on the clinical settings in which to apply such agents. The present studies were undertaken to investigate whether molecular intervention in Src-associated signaling by saracatinib could

reduce the hematogenous spread of KHT sarcoma cells. In particular, these studies sought to determine which stage in the process of establishing viable progressive secondary tumor growth was most susceptible to Src inhibitor therapy. The results demonstrated that exposing mice to saracatinib while tumor cells were in the “extravasation and establishment of new growth” phases of the metastatic process was therapeutically be most beneficial.

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Fig. 1. Median number (a) and sizes (b) of neoplastic cell foci in the lungs of mice determined 21 days after iv injection of KHT sarcoma cells. Beginning the day after cell injection, the mice were treated daily with either the drug carrier or saracatinib (10 or 25 mg/kg). Bars and hatched areas = 90% and 75% confidence intervals, respectively; stars = statistical significance of $p < 0.05$ (Wilcoxon's rank-sum test) compared with controls.

Fig. 2. Median number of neoplastic cell foci in the lungs of mice determined 21 days after iv injection of KHT sarcoma cells. In (a) 5×10^3 (squares, triangles) and 2×10^3 (circles) KHT cells were injected. In (b) 2×10^3 KHT cells were used. Treatment in (a) was daily 10 mg/kg doses of saracatinib commencing on day 1; treatment in (b) was 10 mg/kg administered daily on days 3–7 and 10–14. Bars and hatched areas = 90% and 75% confidence intervals, respectively; stars = statistical significance of $p < 0.05$ (Wilcoxon's rank-sum test) compared with controls.

Fig. 3.

Median number of lung colonies assessed 21 days post tumor cell injection in mice treated with daily doses of 10 mg/kg saracatinib either on days 3–7 or 10–14 (**a**) or days 3–7 or 17–21 (**b**). Bars and hatched areas = 90% and 75% confidence intervals, respectively; stars = statistical significance of $p < 0.05$ (Wilcoxon's rank-sum test) compared with controls.

Fig. 4. Median number of KHT sarcoma lung colonies determined 21 days after injecting 5×10^3 (circles) or 2×10^3 (squares, triangles) tumor cells. Saracatinib treatment(s) consisted of a single 10 mg/kg dose administered on day 0, four doses of 10 mg/kg given on days 0–3, or daily doses of 10 mg/kg administered through the course of the experiments. Bars and hatched areas = 90% and 75% confidence intervals, respectively; stars = statistical significance of $p < 0.05$ (Wilcoxon's rank-sum test) compared with controls.

Fig. 5.

Number of non-adherent KHT sarcoma cells remaining in the supernatant 2 h after 1×10^6 tumor cells were added to a monolayer of endothelial cells. Tumor cells were pretreated with 0.1% DMSO (control) or saracatinib (0.5, 2.5 or 5 μM) for 24 h prior to their addition to the endothelial cells. After 2 h, the number of tumor cells remaining unattached in the supernatant was counted. Data are the mean number of cells in 6-well plates ($\pm$ SE for 3 experiments); stars = $P < 0.05$ versus control.

Fig. 6.

The effect of a 24 h exposure of saracatinib (1–10 μM) on HMVEC-L Src and Src phosphorylation (a), tube formation (b–e), and motility (f) are illustrated. Bars in b–e equal 100 μ. The level of VEGF determined in the media of KHT sarcoma cells after a 24 h saracatinib treatment is shown in (g). Data are the results from three independent experiments ± SE; stars = $P < 0.05$ versus control.

Fig. 7.

The effect of saracatinib on the ability of KHT sarcoma cells to induce angiogenesis was determined by either pretreating the tumor cells for 24 h with saracatinib (0–5 μM) prior to injection (**a**) or treating the mice with daily 10 mg/kg doses of saracatinib post tumor cell inoculation (**b**). Bars and hatched areas = 90% and 75% confidence intervals, respectively; stars = statistical significance of $p < 0.05$ (Wilcoxon's rank-sum test) compared with controls.