INHIBITION OF PROTEIN KINASE c-Src REDUCES THE INCIDENCE OF BREAST CANCER METASTASES AND INCREASES SURVIVAL IN MICE.

IMPLICATIONS FOR THERAPY

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Discussion: 1028

Abbreviations:
Alkaline Phosphatase Activity (ALP); Dulbecco’s Modified Minimum Essential Medium (DMEM); Enhanced Chemiluminescence (ECL); Ethylene Diamino Tetra Acetic Acid (EDTA); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Granulocyte Macrophage Colony Stimulating Factor (GM-CSF); N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES); Horseradish Peroxidase (HRP); Macrophage Colony Stimulating Factor (M-CSF); Phosphate Buffered Saline (PBS); Receptor Activator of NF-
kB ligand (RANKL); Sodium Dodecyl Sulfate (SDS); Tartrate Resistant Acid Phosphatase (TRAcP); Transforming Growth Factor-β (TGF-β); Tumor Necrosis Factor-α (TNF-α).

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c-Src is a proto-oncogene, belonging to the non-receptor protein kinases family, which plays a prominent role in carcinogenesis. In this study we tested the hypothesis that c-Src could promote breast cancer metastasis acting on several cell types and that pharmacological disruption of its kinase activity could be beneficial for the treatment of metastases. Female BALB/c-\textit{nu/nu} mice were subjected to intracardiac injection of the human breast cancer cells MDA-MB-231 (MDA-231), which induced prominent bone and visceral metastases. These were pharmacologically reduced by treatment with the c-Src inhibitor (substituted 5,7-diphenyl-pyrrolo[2,3-d]-pyrimidine), CGP76030 (100 mg/kg/day p.o.), resulting in decreased morbidity and lethality. Metastases were more severe in mice injected with MDA-231 cells stably transfected with wild-type c-Src (MDA-231-Src\textsuperscript{WT}), while transfection in injected cells of a c-Src kinase-dead dominant-negative construct (MDA-231-Src\textsuperscript{DN}) resulted in reduced morbidity, lethality, and incidence of metastases similar to the mice treated with the inhibitor. An analogous beneficial effect of c-Src inhibition was observed in subcutaneous and intratibial implanted tumors. \textit{In vitro}, c-Src suppression reduced MDA-231 cell aggressiveness. It also impaired osteoclast bone resorption both directly and by reducing expression by osteoblasts of the osteoclastogenic cytokines interleukin-1\textbeta and interleukin-6, while PTHrP was not implicated. c-Src was also modestly but consistently involved in the enhancement of endothelial cell proliferation \textit{in vitro} and angiogenesis \textit{in vivo}. In conclusion, we propose that c-Src disruption affects the metastatic process and is thus a therapeutic target for the treatment of breast cancer.
INTRODUCTION

Breast cancer is a relatively common tumor, with an estimated incidence of 1.2 million new cases diagnosed worldwide every year (Henderson et al., 1993). The outcome of patients mainly depends on the development of distant metastases (Greenberg et al., 1996). Bone is the principal metastatic site in patients with mammary carcinomas (James et al., 2003), of whom approximately 20% survive for more than 5 years, while those with minor metastases in the bone can survive up to 10 years or more. In contrast, visceral metastases, although less common, are more likely to be fatal with a higher risk of early death.

Consistent evidence suggests the involvement of the proto-oncogene c-Src in the development and progression of many human cancers, including breast carcinomas (Otthenoff-Kalff et al., 1992; Verbeek et al., 1996; Ishizawar and Parsons, 2004; Dehm and Bonham, 2004). c-Src is a non-receptor tyrosine kinase whose deficiency in mice affects only bone cell function, with no effects in other organs (Soriano et al., 1991; Marzia et al., 2000). Our previous data demonstrated the ability of c-Src inhibitors belonging to the pyrrolopyrimidine class to reduce the malignant activities of prostate cancer cells in vitro (Recchia et al., 2003). c-Src kinase activity is significantly increased in human breast cancer tissues compared to benign breast tumors or adjacent normal breast tissues, and this elevated c-Src activity is correlated with poor metastasis-free survival (Hennipman et al., 1989; Verbeek et al., 1996). A role for c-Src in the development of breast cancer metastases has been elegantly demonstrated by Myoui (Myoui et al., 2003). However, the cellular mechanisms underlying its involvement in the metastatic disease are poorly understood. As a result, whether or not c-Src is an appropriate target for pharmacological therapy remains to be established.

In this study we tested the hypothesis that c-Src could promote breast cancer metastatic disease affecting various cell types and that, as a consequence,
pharmacological disruption of its kinase activity could be useful for the development of novel therapies for the treatment of both bone and visceral metastases arising from breast malignancy.
METHODS

Materials

DMEM, fetal bovine serum, penicillin, streptomycin, and trypsin were from GIBCO (Uxbridge, UK). Sterile plasticware was purchased from Falcon Becton-Dickinson (Cowley, Oxford, UK) or Costar (Cambridge, MA, USA). ECL kit, Hybond nitrocellulose. $[^3]H$-thymidine and $[^32]P$-$\gamma$-ATP were from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Anti-v-Src, anti-pY527 Src and anti-pY416 Src antibodies, c-Src wild-type (WT) and c-Src kinase-dead dominant-negative (DN) (c-Src$^{K296R/Y528F}$) constructs were obtained from Upstate Biotechnology (Lake Placid, NY). Ultra-Vision Detection System anti-Polyvalent HRP/DAB kit was from Lab Vision (Scaffold, UK). The Brilliant® SYBR® Green QPCR master mix was from Stratagene (La Jolla, CA). The anti-pan-phospho-tyrosines (pTyr), anti-actin, anti Ki-67 and anti-PTHrP antibodies were from SantaCruz Biotechnology, (Heidelberg, Germany). The monoclonal mouse anti-human Factor VIII-related antigen (FVIIIIRAg) was from Bømeda (Foster City, CA). Trizol, lipofectamine and PLUS reagent were purchased from Invitrogen (Carlsbad, CA). Rabbit muscle enolase and all chemicals, of the purest grade, were from Sigma Aldrich Chemical Co. (St. Louis, MO).

Cell lines

The human breast cancer cell line MDA-MB-231 (MDA-231) was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM L-glutamine.

The EAHy926 endothelial cell line (Marr et al., 1997) was the kind gift of Dr. Cora-Jean S. Edgell, Department of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. Cells were cultured as above plus 1X HAT (hypoxanthine/aminopterin/thymidine). Immunocytochemical detection of von Willebrand
factor, αVβ3 integrin, and endothelial-specific antigen P1H12 (Solovey et al., 1997) confirmed the endothelial phenotype.

c-Src inhibitor

The c-Src inhibitor GP76030 was a substituted 5,7-diphenyl-pyrrolo[2,3d]pyrimidine synthesized in the Chemistry Research Laboratories of Novartis Pharma (Table 1) (Missbach et al., 1999; Missbach et al., 2000; Šuša et al., 2005). In vivo administration was performed by oral gavage (Missbach et al., 1999) (100 mg/kg/day) from the day after the intracardiac injection until the end of the experiment. For in vitro treatments, CGP76030 was dissolved in dimethyl sulfoxide at 10 mM and diluted in cell culture medium prior to use. Controls were carried out with a dimethyl sulfoxide concentration corresponding to the highest dose of test compound (0.2% v/v).

Cell transfection

The MDA-231 cell line was transfected with the pUSEamp expression vector containing wild type c-Src (MDA-231-SrcWT), or kinase-dead, dominant-negative c-Src (MDA-231-SrcDN) carrying a double mutation in the catalytic site and in the regulatory tyrosine-phosphorylation site 528 (K296R/Y528F). Transfected cells were selected for resistance to geneticin, with no clonal selection to avoid clonal variability. In preliminary experiments, empty vector-transfected cells were indistinguishable from non-transfected parental cells, therefore this latter variant was used as control cell line.

Animals

Four-week-old female immunocompromised BALB/c-\(\text{nu/nu}\) mice (Charles River, Milan, Italy) were maintained under sterile conditions and used for all in vivo experiments. Procedures involving animals and their care were conducted in conformity with national

**In vivo experimental metastases**

MDA-231, MDA-231-Src\textsuperscript{WT}, and MDA-231-Src\textsuperscript{DN} cells (1 \times 10^5/0.1 ml PBS) were injected into the left ventricle of BALB/c-\textit{nu/nu} mice anaesthetized with i.p. injection of pentobarbital (60 mg/kg) (8 mice per group) as described by Arguello et al. (1988) and Yoneda et al. (1997). A group of 8 mice injected with MDA-231 cells was treated with 100 mg/kg/day of c-Src inhibitor CGP76030 or with vehicle alone. A group of 8 mice was injected with PBS as control. Animals were daily monitored for body weight, behavior, and survival. Cachexia was evaluated as body weight decrease. Mice were also weekly subjected to deep anesthesia and X-ray analysis (36 KPV for 10 sec) using a Cabinet X-ray system (Faxitron model n.43855A; Faxitron X-Ray Corp., Buffalo Grove, IL) to follow the onset and progression of osteolytic lesions. At the end of the experiment (38 days) mice were sacrificed and subjected to final X-ray analysis and to anatomical dissection for evaluation of bone and visceral metastases, respectively.

**Subcutaneous xenograft implants**

BALB/c-\textit{nu/nu} mice were anaesthetized as described above, and cells (1 \times 10^5/0.1 ml PBS) were subcutaneously injected in the right flank using a tuberculin syringe with an 18G needle. Xenografts were monitored daily by measuring the average tumor diameter (two perpendicular axes) using a caliper. After 32 days, mice were sacrificed and tumor mass excised and weighed.
Xenograft histology

Subcutaneous tumors were fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2, and embedded in paraffin. Sections were cut using a Reichert-Jung 1150/Autocut microtome. Slide-mounted tissue sections (4 µm thick) were deparaffinized in xylene and hydrated serially in 100%, 95%, and 80% ethanol. Endogenous peroxidases were quenched in 3% H₂O₂ in PBS for 1 hour, then slides were incubated with the anti-ki67 or anti-FVIII:RAg primary antibodies for 1 hour at room temperature. Sections were washed three times in PBS, and antibody binding was revealed using the Ultra-Vision Detection System anti-Polyvalent HRP/DAB kit according to the manufacturer’s instructions.

Intratibial implants

BALB/c-<i>nu/nu</i> mice were anaesthetized, a syringe with a 27½G needle was inserted in the proximal end of the tibia, and 5 x 10⁴ tumor cells suspended in 25 µl PBS were injected into the intramedullary space. Radiographs were taken as described above at 20 and 32 days after injection.

Evaluation of osteolytic lesions

Radiographs were scanned using the Bio-Rad scanning densitometer (Hercules, CA), model GS800, and quantification of the area of interest was done using the Bio-Rad Quantity One® image analysis software. For histological examination, tibias were dissected, cleared of soft tissue, and fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2. Samples were then decalcified in EDTA and embedded in paraffin. Sections were cut and stained with trichrome stain (Masson) (Sigma-Aldrich kit No. HT15-1KT), or for the typical osteoclast marker, TRAcP (Sigma-Aldrich kit No. 85), according to the manufacturer’s instructions.
**Western blotting**

For protein extraction cells or tissues were lysed in RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Proteins were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with the primary antibody for 1 h at room temperature, washed and incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were revealed by ECL detection.

**In vitro c-Src kinase assay**

One mg of protein was extracted with RIPA buffer. Fifty microliters of protein G suspensions were incubated for 2 h at 4°C with 5 µg anti-Src antibody. The agarose beads were subsequently washed five times with ice-cold immunoprecipitation washing buffer (50 mM HEPES, pH 7.3, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM PMSF, 9 µg/ml leupeptin, 0.2 mM aprotinin) and incubated overnight at 4°C with cell lysates. Beads were then rewashed five times with washing buffer, then immunoprecipitates were washed and resuspended in kinase assay buffer (50 mM HEPES, pH7.5 and 0.1 M EDTA) and incubated for 20 min at 30°C in the same buffer plus 1:3 ATP mix (0.15 mM ATP, 30 mM MnCl₂ and 200 µCi/ml [³²P]-γATP, specific activity 3000Ci/mmöl, in kinase assay buffer) in the presence of rabbit muscle enolase. Reducing sample buffer was then added and samples were subjected to 10% SDS-PAGE. Electrophoretic gels were dried and exposed for two days to autoradiography films.

**Conventional and real Time RT-PCR**

Total RNA was extracted using the Trizol® procedure. One µg of RNA was reverse transcribed using M-MLV reverse transcriptase and the equivalent of 0.1 µg was used for
the PCR reactions. For real time PCR, the Brilliant® SYBR® Green QPCR master mix was used. PCR conditions and primer pairs used are listed in Tables 2 and 3.

**Conditioned media**

MDA-231, MDA-231-Src\textsuperscript{WT}, and MDA-231-Src\textsuperscript{DN} cells were allowed to grow in DMEM plus 10% FBS until 80% confluency. The medium was then replaced with serum-free medium, and after 48 hours supernatants were collected and stored at -20°C until use.

**Osteoclast primary cultures**

Primary osteoclasts were differentiated from the bone marrow of 7-days-old CD1 mice. Bone marrow was flushed from the bone cavity of the long bones and minced in DMEM. Cells were recovered, plated in DMEM plus 10% FBS, and cultured up to 7 days in the presence of MDA-231, MDA-231-Src\textsuperscript{WT}, and MDA-231-Src\textsuperscript{DN} cell-conditioned media (dilution 1:4). Cultures were fixed in 3% paraformaldehyde in 0.1 M cacodylate buffer, and TRACP activity was detected histochemically as described above.

Osteoclasts were also differentiated onto bone slices and fixed in 3% paraformaldehyde in 0.1 M cacodylate buffer. Cells were then removed by ultrasonication in 1% sodium hypochlorite, and slices were stained with 0.1% toluidine blue. Pits were counted and the pit index computed according to Caselli et al. (1997).

**Osteoblast primary cultures**

Calvaria from 7-days-old CD1 mice were removed, cleaned free of soft tissues, and digested three times with 1 mg/ml \textit{Clostridium histolyticum} type IV collagenase and 0.25% trypsin for 20 min at 37°C, with gentle agitation. Cells from the second and third digestions were plated and grown in DMEM plus 10% FBS. At confluence, cells were trypsinized by standard procedures and plated according to the experimental protocol. These cells
expressed the osteoblast markers ALP, Runx-2, PTH/PTHR receptor, type I collagen, and osteocalcin (Marzia et al., 2000).

**Cell proliferation assay**

Cells were plated in 24-well multiplates (8,000 cells/well) and grown for 24 hours in DMEM plus 10% fetal bovine serum. They were then serum-starved for 24 hours in DMEM plus 0.2% BSA and incubated overnight with 2 μCi/ml [³H]-thymidine (specific activity 25 Ci/mmol). Cells to be treated with CGP76030 were serum-starved for 24 hours in DMEM plus 0.2% BSA, then incubated with the test compound or vehicle alone for further 24 hours in DMEM plus 0.2% BSA, during which 2 μCi/ml [³H]-thymidine was added after 12 hours from the beginning of treatment.

At the end of incubation, cells were dissolved in 0.1% SDS, precipitated with 100% trichloroacetic acid (TCA), and centrifuged at 3000 rpm for 15 min at 4°C. Pellets were dissolved in 0.1% SDS and the [³H]-thymidine incorporation measured in a β-counter.

**Cell adhesion**

MDA-231 cells were treated for 30 min in suspension with different concentrations of CGP76030 or with vehicle alone, then cells were plated on 96-well multiplates and allowed to adhere in the presence of the same treatments for two hours. At the end of incubation, cultures were washed three times with PBS to remove non-adherent cells, fixed with 80% methanol and stained with 0.5% crystal violet. Crystal violet was then dissolved in 0.1 N sodium citrate and absorbance evaluated at 595 nm in an ELISA plate reader.

**Migration and invasion assays**

Migration was performed by the modified Boyden-chamber method (Albini et al., 1987). Cells were added to 12 μm polycarbonate filters coated with 4.5 μg/cm² gelatin in
the upper compartment of the transwell chambers. After 6 hours (for MDA-231, MDA-231-Src\textsuperscript{WT}, and MDA-231-Src\textsuperscript{DN} cells) or 12 hours (for EAHy-926 cells), filters were stained with hematoxylin/eosin. Cell migration ability was evaluated by counting cells migrated to the lower side of the filters in five randomly chosen fields/filter at 200X. Invasion assays were performed in a similar manner except that i) the filters were coated with reconstituted Matrigel (35 µg/cm\textsuperscript{2}) and ii) the evaluation was performed after 12 hours (for MDA-231, MDA-231-Src\textsuperscript{WT}, and MDA-231-Src\textsuperscript{DN} cells) or 18 hours (for EAHy-926 cells). In both migration and invasion assays the chemoattractants employed were i) NIH3T3-conditioned media for MDA-231, MDA-231-Src\textsuperscript{WT}, and MDA-231-Src\textsuperscript{DN} cells, and ii) MDA-231-, MDA-231-Src\textsuperscript{WT}-, and MDA-231-Src\textsuperscript{DN}-conditioned media for EAHy-926 cells.

**Statistics**

All experiments were repeated at least three times. Data are expressed as the mean ± SEM. Statistical analysis was performed by one-way analysis of variance, followed by unpaired Student's t test. A p value <0.05 was conventionally considered statistically significant.
RESULTS

Pharmacological treatment with the c-Src inhibitor

Myoui et al (2003) have shown that reduced c-Src activity in breast cancer cells results in reduced tumor development both locally and in distant organs. c-Src is also known for its pivotal role in enhancing osteoclast activity (Miyazaki et al., 2004; Recchia et al., 2004) and maintaining the osteoblasts in a pre-differentiation status (Marzia et al., 2000). These latter cell types are involved in the osteolytic vicious circle in association with the breast cancer cells (Yoneda et al., 2005; Roodman, 2004) and the endothelial cells that supply angiogenesis and regulatory factors (Yoneda et al., 2005; Roodman, 2004; Guise et al. 2004). Therefore, given that all these cell types could be affected by disruption of c-Src tyrosine kinase activity, we tested whether the pharmacological treatment with an anti–c-Src compound could effectively counteract progression of experimental metastatic disease. We had previously demonstrated in an animal model of bone loss the therapeutic efficacy of the c-Src inhibitor, GCP76030 (Table 1), which prevents c-Src from binding to ATP (Missbach et al., 2000; Šuša et al., 2005). Therefore a similar regimen (100 mg/kg/day p.o.) was employed in mice intracardially injected with MDA-231 cells starting the day after cell inoculation. Indeed, in mice treated with CGP76030, we observed that cachexia (measured as body weight wasting, Fig. 1A) was delayed. Specifically, after 27 days from injection we found 85 % incidence of cachexia in control animals vs. 12.5% in the treated group, the latter reaching only 37.5% incidence at the end of experiment (Fig.1A). Accordingly, lethality (Fig.1B) was also delayed and its incidence reduced in treated littersmates relative to controls, since at day 31 from cell injection 62.5% of treated animals were still alive vs. 28.5% of control mice. Post-mortem examination of visceral organs in mice injected with the tumor cells evidenced detectable development of lymph node and lung metastases, with a significant reduction of lung metastasis and a trend towards decrease for lymph node lesions in treated mice relative to the control group (Fig.
1C). In control mice, osteolytic bone metastases appeared at day 26 post-inoculation and progressively increased to 57% (Fig. 1D). In contrast, in mice receiving the treatment with the c-Src inhibitor, no osteolytic lesions were detected until day 31, and the final incidence at sacrifice (day 38) was not higher than 25%. Western blot of tissue proteins extracted at sacrifice from tibias, showed a marked reduction of activating c-Src Y416 autophosphorylation, which is indispensable for full c-Src kinase activity, in mice treated with CGP76030 compared to controls (Figure 1E).

Careful examination indicated that the animals' behavior was unremarkable, and no obvious detrimental effects were noted elsewhere in the body for the time-frame of the treatment, in line with the notion that c-Src disruption in mice affects only the skeleton (Soriano et al., 1991).

**MDA-MB-231 stable transfectants**

To investigate the cellular mechanisms affected by c-Src in the metastatic process of MDA-231 cells, we prepared stable transfectants carrying WT or DN c-Src constructs evaluating transfection efficiency by Western blot analysis. Fig. 2A (third row) shows that c-Src was abundantly expressed in MDA-231 parental cells and that both transfectants had higher levels of the protein. MDA-231-Src\textsuperscript{DN} cells, which carried the c-Src Y528F mutant, showed levels of phosphorylation of inactivating Y528 and activating Y416 comparable to those of the MDA-231 parental cell c-Src (Fig. 2A, second and first rows, respectively). In MDA-231-Src\textsuperscript{WT} cells, phosphorylation of both Y528 and Y416 was higher than in MDA-231 and MDA-231-Src\textsuperscript{DN} cells (Fig. 2A, second and first rows, respectively). Western blots with anti-pan-phosphotyrosines antibody of the same cell lysates showed a higher phosphorylation pattern in MDA-231-Src\textsuperscript{WT} cells and a proportionally lower degree of tyrosine phosphorylations in MDA-231-Src\textsuperscript{DN} cells compared to parental cells (Fig. 2B, upper panel). *In vitro* kinase assay evidenced a higher c-Src kinase activity in MDA-231-
SrcWT cells relative to MDA-231 (Fig. 2C, upper panel), while no activity was found in MDA-231-SrcDN.

**In vivo cellular mechanisms**

**Effect of c-Src on development of MDA-231 cell metastases in nude mice**

To test whether c-Src modulation directly affects the ability of MDA-231 cells to develop experimental metastases, mice were injected in the left ventricle with the parental cells or with cells carrying the c-Src WT and DN variants. In mice injected with MDA-231 and MDA-231-SrcWT cells, a similar progression and incidence of cachexia was noticed, while none of the mice injected with MDA-231-SrcDN cells showed weight wasting during the time frame of the observations (Fig. 2D). Death started slightly earlier, and its incidence was higher in mice injected with MDA-231-SrcWT cells relative to those injected with MDA-231 cells (70% vs. 57%, respectively). Noticeably, all animals injected with MDA-231-SrcDN cells survived for the whole length of the experiment (Fig. 2E).

Interestingly, a significant reduction of lung and lymph node metastases was noticed in MDA-231-SrcDN injected mice relative to the other two groups (Fig. 2F). In mice injected with MDA-231 or MDA-231-SrcWT cells, osteolytic bone metastases appeared at days 20 to 25 post-inoculation and progressively increased to 57% and 70% incidence, respectively (Fig. 2G). In contrast, in mice receiving MDA-231-SrcDN cells, no osteolytic lesions were detected until day 30, with a 25% incidence at sacrifice.

**Effect of c-Src on development of osteolytic lesions**

To investigate the role of MDA-231 cell c-Src in the osteolytic lesions induced by tumor cells, we performed intratibial inoculations of MDA-231, MDA-231-SrcWT, and MDA-231-SrcDN cells. Radiographs taken at 20 and 32 days from injection revealed the lowest frequency of tumor growth and osteolysis in tibias receiving MDA-231-SrcDN cells, whereas...
the frequency was similarly higher in tibias inoculated with MDA-231 and MDA-231-SrcWT cells (Table 4). Slightly smaller osteolytic areas were observed in those tibias inoculated with MDA-231-SrcDN cells that developed the lesion (50% of the mice) compared to the other two groups (Table 4, Fig. 3a). Histological examination of tibias injected with MDA–231 and MDA-231-SrcWT cells showed wide tumor burden and trabecular bone erosion in the osteolytic areas (Fig. 3b). Tibias endowed with MDA-231-SrcDN cells had smaller tumor mass confined within the medullary cavity (Fig. 3b). Histomorphometric analysis in sections histochemically stained for the osteoclast marker TRAcP evidenced a significant increase of osteoclast surface/bone surface and osteoclast number/bone surface in mice injected with MDA-231 cells compared to PBS-injected controls. This increase was even greater in mice receiving MDA-231-SrcWT cells, whereas it was smaller in tibias injected with MDA-231-SrcDN cells (Fig. 3c-d). Similar results were observed in the tibias of mice developing bone metastases by intracardiac injection of the three cell lines (not shown).

Effect of c-Src on sub-cutaneous growth of MDA-231 cells

c-Src is a tyrosine kinase known to stimulate cell proliferation. Therefore, we sought to test whether manipulation of c-Src in our transfectant cells changed their ability to grow in vivo when injected subcutaneously. Subcutaneous xenografts of MDA-231 cells grew at a rate appreciable by gross observations. The growth rate of MDA-231-SrcWT cells was significantly higher than that of parental cells, resulting in heavier and bigger tumors (Fig. 4Aa,b). In contrast, subcutaneous tumors formed by MDA-231-SrcDN cells were approximately 40% the weight and the size of those formed by MDA-231 cells (Fig. 4Aa,b), suggesting a lower proliferation rate. In agreement with this hypothesis, immunohistochemical analysis showed increased levels of the Ki67 proliferation marker in MDA-231-SrcWT tumors (Fig. 4B). Consistently, MDA-231-SrcDN tumors showed the lowest Ki67 expression among the three groups (Fig. 4B).
Tumor vascularization

One of the relevant steps for tumor growth and invasion is the capacity of cancer cells to interact with the endothelium and stimulate angiogenesis. Based on this characteristic, we tested the hypothesis that c-Src activity in MDA-231 cells could contribute to stimulation of tumor angiogenesis. We therefore evaluated blood vessel development in the subcutaneous tumor xenografts. Immunohistochemical detection of the endothelial marker Factor VIII showed well-developed capillaries in MDA-231 tumors (Fig. 4C). A trend towards an increase in microvascular density was observed in the MDA-231-SrcWT tumors (Fig. 4C). Basal stimulation was not c-Src dependent as no changes relative to MDA-231 cells were observed in MDA-231-SrcDN tumors.

In vitro cellular mechanisms

Having demonstrated that c-Src tyrosine kinase pharmacological inhibition can delay the development of metastases in vivo, we sought to unravel the underlying cellular mechanisms in vitro.

MDA-231 cells

c-Src tyrosine kinase activity is involved in many critical cellular functions, and metastatic cells could be affected by c-Src manipulation at different levels. Therefore, we first investigated whether changes in c-Src activity could modulate MDA-231 cell proliferation. The in vitro [³H]-thymidine incorporation assay exhibited a significant decrease of MDA-231-SrcDN proliferation rate compared to MDA-231 cells, with a trend towards increased proliferation in MDA-231-SrcWT cell cultures (Fig. 5A).

Malignant cells exhibit the enhanced motility and invasive capacity indispensable for the metastatic process, and c-Src is known to modulate motility by its role in cytoskeletal
remodeling. Therefore, we tested whether c-Src manipulation could affect migration and invasion ability in MDA-231 cells. We observed that both were significantly increased in MDA-231-Src\textsuperscript{WT} cells, whereas they were reduced in MDA-231-Src\textsuperscript{DN} cells relative to MDA-231 cells (Fig. 5B,C).

Similar to downregulation of c-Src activity in MDA-231-Src\textsuperscript{DN} cells, \textit{in vitro} treatment of MDA-231 parental cells with the c-Src inhibitor CGP76030 caused a concentration-dependent decrease of cell proliferation, migration, and adhesion (Fig. 5D–F).

\textit{Paracrine stimulation of osteoclasts}

We and others had shown that paracrine factors released by breast cancer cells induce osteoclastogenesis and activate bone resorption (Yoneda et al., 2005; Roodman, 2004; Rucci et al., 2004). Because of the relevance of this paracrine activity for osteolysis at the bone metastatic site, we evaluated whether the manipulation of c-Src in MDA-231 cells could affect their ability to influence osteoclastogenesis. Indeed this was not the case, because similar osteoclast formation and bone resorption rates were observed in bone marrow cultures challenged with conditioned media from the three cell lines, independent of their wild-type or mutant c-Src expression (Fig. 6A,B).

In the context of bone metastasis, however, tumor cells could induce the expression of osteoclastogenic factors in osteoblasts (Yoneda et al., 2005; Roodman, 2004; Rucci et al., 2004). Therefore, we tested whether c-Src manipulation in MDA-231 cells could modulate their ability to influence osteoblast paracrine activities. Mouse calvarial osteoblast cultures treated with the conditioned media from our MDA-231 and MDA-231-Src\textsuperscript{WT} cultures showed an increase of \textit{IL-1\beta} and \textit{IL-6} mRNAs relative to untreated cells. This increase was not noticed in osteoblasts incubated with conditioned media from MDA-231-Src\textsuperscript{DN} cells (Fig. 6C, D). \textit{M-CSF}, \textit{GM-CSF}, \textit{PTHrP}, \textit{TNF-\alpha}, \textit{TGF-\beta}, \textit{IL-12}, \textit{IL-18} and \textit{Rankl/Opg} transcripts were unremarkable with no changes among the three groups of
cultures (data not shown). These results suggest an indirect role of MDA-231 cell c-Src in the paracrine stimulation of osteoclast formation via the osteoblast route, which could be selectively mediated by the osteoclastogenic cytokines interleukin-1β and interleukin-6 (Perez et al., 2001). Remarkably, none of the above cytokines were changed in the MDA-231-Src<sup>WT</sup> and MDA-231-Src<sup>DN</sup> cells relative to MDA-231 parental cells (Figure 7A). Starting from the results obtained by Myoui et al. (2003) who demonstrated a direct effect of c-Src on PTHrP production and activity, we evaluated PTHrP mRNA (Figure 7B, left panels) and protein (Figure 7B, right panels) expression in the MDA-231 cell lines, but failed to observe any modulation. Therefore, the MDA-231 paracrine factors that influence osteoblasts and are under c-Src control remain to be elucidated.

**Bone cells**

We had previously demonstrated (Recchia et al., 2004) and confirmed in this study (not shown) that the CGP76030 c-Src inhibitor reduced osteoclast formation and bone resorption and induced osteoclast apoptosis in vivo and in vitro. These results suggest that a direct inhibition of osteoclast activity could contribute to the reduced incidence of osteolytic lesions upon pharmacological inhibition of c-Src.

We had also demonstrated that c-Src inhibition stimulated osteoblast differentiation and bone formation (Marzia et al., 2000). Because of the role of osteoblasts in osteoclastogenesis, we asked whether c-Src inhibition by CGP76030 could directly affect the osteoclastogenic ability of osteoblasts. While reducing osteoblast proliferation and stimulating differentiation, treatment with CGP76030 had no effect on the expression of pro-osteoclastogenic cytokines, including *Rankl*, *IL-1β*, *IL-6*, *M-CSF*, *PTHrP*, *TGFβ*, *TNFα*, or anti-osteoclastogenic factors, such as *Opg*, *IL-12*, *IL-18*, and *GM-CSF* (not shown).
Endothelial cells

Due to the marginal effect shown in vivo on tumor vascularization, we next tested in vitro whether c-Src manipulation in tumor cells could affect endothelial activity by paracrine factors. EAHy926 endothelial cells were allowed to grow in the presence of conditioned media from the three tumor cell cultures. EAHy926 proliferation was slightly but consistently higher in the presence of MDA-231-SrcWT–conditioned medium relative to MDA-231 and MDA-231-SrcDN–conditioned media (Fig. 8A), mirroring the trend towards an increased microvascular density showed in vivo in the MDA-231-SrcWT xenografts. Again, basal stimulation by MDA-231 cell-conditioned medium was not dependent on c-Src because no changes were noticed in endothelial cultures incubated with MDA-231-SrcDN cell-conditioned medium.

EAHy926 cells were also allowed to migrate and invade a matrigel substrate in the presence of tumor cell-conditioned media. MDA-231 cell-conditioned medium showed a potent chemoattractant activity with a significant increase of EAHy926 cell migration and invasion in comparison to cells incubated with non-conditioned medium. However, c-Src modulation in tumor cells did not influence this activity (Fig. 8B,C), suggesting the release of selective c-Src–dependent paracrine factors slightly stimulating cell growth but not motility and invasion. MDA-231 cells expressed VEGF and βFGF, which could account for the potent paracrine effect on endothelial cells. However, these mRNAs were not modulated by over-expression of SrcWT or SrcDN (Fig. 8D). Therefore, the c-Src–dependent paracrine activity influencing endothelial proliferation remains to be elucidated.

Inhibition of c-Src tyrosine kinase activity in the endothelial cell line by the c-Src inhibitor CGP76030 had no effect on proliferation, migration, and invasion, suggesting resistance of the endothelium to direct c-Src inhibition (Fig. 8E–G).
DISCUSSION

Development of metastases is responsible of poor outcome in patients with breast cancers and other carcinomas. Visceral metastases are fatal and known to cause remarkable morbidity and eventually mortality. Bone metastases are frequent in breast cancers and typically result in extensive painful osteolytic lesions and hypercalcemia (Cifuentes et al., 1979; Elte et al., 1986; Coleman and Rubens, 1987), which severely affect the patient’s quality of life. However, bone metastases can be dormant for many years, and patients carrying only this type of peripheral disease may survive significantly longer than those affected by visceral metastases (Greenberg et al., 1996). Many cell types are involved in the development of the osteolytic lesions, including cancer cells themselves, osteoclasts, osteoblasts, and endothelial cells, which altogether establish a “vicious circle” (Yoneda et al., 2005; Roodman, 2004). In this study, we have demonstrated that manipulation of c-Src tyrosine kinase activity by various means affects all these cell types, albeit to a different extent and with variable potency.

Significantly, blockade of c-Src tyrosine kinase activity by a specific pharmacologic inhibitor proved effective in reducing the incidence of metastases both in bone and in visceral organs, thus setting a background for the use of c-Src ATP binding antagonists to prevent or retard this severe complication of breast cancer. CGP76030 is a substituted 5,7-diphenyl-pyrrolo[2,3-d]pyrimidine that acts as a potent inhibitor of the c-Src tyrosine kinase activity (Susa et al., 2005). It shows selectivity vs. all other c-Src family members, except c-Yes, for which it has a fairly similar IC$_{50}$ in enzymatic assays. Lack of selectivity was also shown vs. the PDGF receptor and c-Kit, whereas the IC$_{50}$ was severalfold higher for members of the receptor and non-receptor tyrosine kinase families as well as for the serine/threonine protein kinases (Missbach et al. 1999; 2000; reviewed in Šuša et al., 2005, summarized in Table 1). It is therefore clear that not only inhibition of c-Src but also blockade of a few other tyrosine kinases may contribute to the improvement observed in
our experimental models. However, it is worth mentioning that systemic administration of the c-Src inhibitor shared a common trait with the action of c-Src kinase-dead dominant negative mutant transfected into the tumor cells, suggesting c-Src being central to the mechanism of action of CGP76030. This latter condition appeared to induce more potent effects than the treatment with the c-Src inhibitor. However, the two situations cannot be fully compared because the drug pharmacokinetics (Table 1) prevents to reproduce steady inhibition as that caused by the constitutive expression of a c-Src dominant negative transgene.

Remarkably, the pharmacological treatment used in this study promises further developments because it did not induce any obvious detrimental effect in our animals, in line with the notion that targeted disruption of the c-Src gene in mouse strongly affects only the skeleton, with no deleterious effects elsewhere in the body (Soriano et al., 1991). In a recent work, Yezhelyev et al. (2004) demonstrated that c-Src inhibition, either alone or in combination with conventional chemotherapy, shows antitumoral and antimetastatic activity in a orthotopic nude mouse model for human pancreatic cancer. A closely related inhibitor proved effective at inhibiting osteoclast-mediated bone resorption in healthy male volunteers, without significant adverse effects (Hannon et al., 2005), opening up a new avenue for the use in human diseases.

The development of visceral metastases and the incidence of morbidity and mortality were alike in mice inoculated with MDA-231 and with MDA-231-Src\textsuperscript{WT} cells. In contrast, the onset of bone metastases occurred earlier and the incidence was higher in mice receiving MDA-231-Src\textsuperscript{WT}. Our understanding of this difference points to the special role that the tyrosine kinase is likely to play for the homing tumor cells to the osteomedullary site, which is probably not maximized by the “physiological” expression of the gene in the tumor cells.
Likewise, while \textit{in vitro} migration and invasion of tumor cells were enhanced by over-expression of c-Src\textsuperscript{WT}, proliferation was not affected. This finding is in sharp contrast with the findings in subcutaneous implants of MDA-231-Src\textsuperscript{WT} cells, which were larger with a significantly higher expression of the proliferation marker, Ki67. This outcome suggests that \textit{in vivo} other determinants are likely to affect tumor growth. These may include environmental factors that could converge on the c-Src pathway, thus affecting cell proliferation in a synergistic fashion (Brown and Cooper, 1996).

Much more striking and interesting were the effects observed using MDA-231-Src\textsuperscript{DN} cells, because all of the parameters evaluated were negatively affected by over-expression of this inactive, kinase-dead mutant, albeit with slightly variable potency. Most importantly, our work demonstrated that the same negative effects could be reproduced both in \textit{in vivo} and \textit{in vitro} models receiving the c-Src inhibitor, CGP76030.

It is interesting to note that c-Src activity in MDA-231 cells also affected their ability to stimulate the other cells involved in the osteolytic “vicious circle”. Many important mediators are known to activate bone cells and endothelial cells, but none of those more commonly involved and investigated in this study were directly modulated in MDA-231 cells by changes in c-Src. Notably, in contrast with the data from Myoui et al (2003), who used similar MDA-231 transfectants and injection strategy, in our study PTHrP was not transcriptionally nor post-transcriptionally reduced upon c-Src inhibition. However, it is interesting to note that the lack of PTHrP changes did not prevent the beneficial effect of c-Src tyrosine kinase inhibition on the MDA-231 cell osteolytic lesions. It is clear however that as-yet-unknown factors released by MDA-231 cells, seemingly under the control of c-Src, can affect osteoclastogenesis (only via the osteoblasts) and endothelial proliferation (modestly but consistently). It will be a challenge in the future to gain knowledge on the specific MDA-231-secreted factor(s) influenced by c-Src, for example by the means of global gene expression profiling and/or proteomic analyses. Agents blocking these thus far
unrecognized factors could be used in combination with antagonists of the cytokines involved and with c-Src inhibitors, strengthening the emerging concept of personalized, multiple low-dose treatments for cancer-induced complications (Blumenschein et al., 1997; Ung et al., 1995).

In conclusion, we provide compelling evidence that in vivo pharmacological treatment with a c-Src inhibitor could be very promising for the treatment of breast cancer and its metastatic complications.
ACKNOWLEDGEMENTS

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REFERENCES


substrates and reduces bone resorption \textit{in vitro} and in rodent models \textit{in vivo}. Bone \textbf{24}:437-449.


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FIGURE LEGENDS

Figure 1. In vivo treatment with c-Src inhibitor. Four-week-old female BALB/c-nu/nu mice were intracardiacally injected with a suspension (100,000 cells/100 µl PBS) of MDA-MB-231 cells. From the day after inoculation they were treated daily with the c-Src inhibitor, CGP76030 (100 mg/kg/day) or with vehicle alone (control). Mice were monitored daily for cachexia (decrease of body weight) (A) and survival (B), and weekly subjected to deep anesthesia and X-ray analysis to check the onset of bone metastases (D). Animals were then sacrificed and subjected to anatomical dissection and X-ray analysis to evaluate visceral (C) and bone metastases (D), respectively. Similar results were observed in three independent experiments (N mice/group = 8). Insert in (D): X-ray of hindlimbs of PBS- and MDA-231-injected mice showing in the latter osteolytic lesions (arrows). In (C) data are the mean ± SEM. *p<0.05. (E) Fifty micrograms of proteins extracted from tibias of intracardiac injected mice treated with vehicle or CGP76030 were subjected to 12% SDS-PAGE. The filter was immunoblotted with anti-phosphoSrc-(Tyr416) antibody, stripped and reprobed with an anti-v-src antibody and with anti-actin antibody as loading control.

Figure 2. Evaluation of MDA-MB-231 stable transfectants. MDA-MB-231 cells (MDA-231) and the cell lines stably transfected with Src wild type (MDA-231-SrcWT) and Src kinase-dead/dominant-negative constructs (MDA-231-SrcDN) were lysed in RIPA buffer and 50 µg of proteins were subjected to 12% SDS-PAGE. Filters were immunoblotted with anti-phosphoSrc-(Tyr 416) and –phosphoSrc-(Tyr 528) antibodies, stripped, and reprobed with anti-v-src antibody and with anti actin antibody, as loading control (A), or immunoblotted with anti-pan-phosphoTyr (pTyr) antibody (B upper panel) and reprobed with anti-actin antibody as loading control (B lower panel). c-Src immunoprecipitates from the indicated cells were...
incubated with rabbit muscle enolase as a substrate in the presence of \(^{32}\text{P}\)-γATP and the reaction products were subjected to SDS-PAGE and autoradiography (C upper panel). Filters were then Western blotted for v-Src and for actin as loading controls (C, middle and lower panels). **Intracardiac injection of MDA-231-stable transfectants.** Four-week-old female BALB/c-\textit{nu/nu} mice were inoculated in the left ventricle with a suspension (100,000 cells/100 μl PBS) of MDA-231, MDA-231-Src\textsuperscript{WT}, or MDA-231-Src\textsuperscript{DN} cell lines. Mice were monitored daily for cachexia (decrease of body weight) (D) and survival (E) and monitored weekly under deep anesthesia for bone metastases by X-ray analysis (G). Animals were then sacrificed and subjected to anatomical dissection and X-ray analysis to evaluate visceral (F) and bone metastases (G), respectively. Similar results were observed in three independent experiments (N mice/group = 8). Data in (F) are the mean ± SEM. *\(p<0.05\).

**Figure 3. Intratibial injection.** X-ray analysis (arrows, osteolytic areas) (a) and histological analysis (b, c) of the hindlimbs of female BALB/c-\textit{nu/nu} mice injected in the proximal end of the tibia with MDA-231, MDA-231-Src\textsuperscript{WT} or MDA-231-Src\textsuperscript{DN} cells (5 x 10\(^4\) cells/25 μl PBS). (b) Trichromic staining; (*, tumor mass). (c) Histochemical purple staining (arrows) for TRAcP activity as a marker of osteoclasts. (d) Histomorphometric analysis of tibias injected with vehicle (PBS), or MDA-231, MDA-231-Src\textsuperscript{WT}, or MDA-231-Src\textsuperscript{DN} cells (Oc.S/BS, Osteoclast Surface/Bone Surface; Oc.N/BS, Osteoclast Number/B.S. N. mice/group = 4).

**Figure 4. In vivo tumor growth.** Female BALB/c-\textit{nu/nu} mice were subcutaneously injected with MDA-231, MDA-231-Src\textsuperscript{WT}, and MDA-231-Src\textsuperscript{DN} cell lines (1 x 10\(^6\) cells/100 μl PBS). After 38 days, animals were sacrificed, the tumors excised (A,a) and their weight (A,b) determined (N mice/group = 4; *\(p=0.05\) vs. MDA-
Figure 5. In vitro characterization of MDA-231 cells. (A) MDA-231, MDA-231-Src\(^{WT}\), and MDA-231-Src\(^{DN}\) cells were serum-starved and incubated overnight with 2 \(\mu\text{Ci/ml }[^{3}\text{H}]\text{-thymidine}\) as described in the Methods to assess cell proliferation (\(^{\#}p<0.05\) vs. MDA-231-Src\(^{WT}\) and MDA-231 cells). (B) Cells were plated in transwells, onto membranes coated with 4.5 \(\mu\text{g/cm}^2\) gelatin. After 6 hours the number of cells that migrated through the membrane was estimated (\(^{*}p<0.002\) vs. MDA-231; \(^{#}p=0.05\) vs. MDA-231). (C) Cells were plated in transwells, onto membranes coated with 12.5 \(\mu\text{g/ml matrigel}\). After 12 hours, the number of cells invading the matrigel substrate was estimated (\(^{*}p=0.002\) vs. MDA-231; \(^{#}p=0.02\) vs. MDA-231). (D) MDA-231 cells were serum-starved for 24 hours in DMEM plus 0.2% BSA and incubated with the c-Src inhibitor at the concentrations indicated, or with vehicle (dimethyl sulfoxide) alone for 24 hours in DMEM plus 0.2% BSA, and with 2 \(\mu\text{Ci/ml }[^{3}\text{H}]\text{-thymidine}\) added after 12 hours from the beginning of treatment. (C, control; \(^{*}p<0.02;^{**}p<0.002\) vs. control). (E) MDA-231 cells were cultured in transwells, onto membranes coated with gelatin, and treated with CGP76030 at the concentrations indicated on abscissa. After 6 hours the number of cells that migrated through the membrane was estimated (C, control; \(^{*}p<0.05;^{**}p<0.005\) vs. control). (F) Cells were trypsinized, treated with dimethyl sulfoxide (control) or CGP76030 for 30 min at the concentrations indicated on abscissa, and allowed to adhere in the presence of the same
treatments for two hours (*p<0.05; **p<0.005 vs. control). Results are the mean ± SEM of three independent experiments.

**Figure 6. Effect of tumor-cell–conditioned media on osteoclastogenesis and bone resorption.** (A) Bone marrow from long bones of 7-day-old mice was cultured onto 24-well multiplates in the presence of DMEM (control medium) or conditioned media (CM) from MDA-231, MDA-231-SrcWT, and MDA-231-SrcDN. After 8 days, cultures were fixed and differentiated osteoclasts were stained for the TRAcP enzyme and counted. (B) Bone marrow was cultured as described in A onto bone slices. After 8 days, slices were fixed and stained with toluidine blue and the pit index was calculated. Data are expressed as mean ± SEM of three independent experiments (*p<0.05 vs. control medium). **Osteoblast transcriptional expression of osteoclast-regulating cytokines.** Confluent osteoblasts were starved overnight in serum-free DMEM. Medium was then replaced with DMEM (control medium) or with tumor cells CM, and cells were incubated for further 24 hours. The RNA was extracted. One µg RNA was reverse-transcribed and the equivalent of 0.1 µg was subjected to Real Time PCR using the Brilliant® SYBR® Green QPCR master mix and primer pairs and conditions specific for *IL-1beta* (C) and *IL-6* (D) (Table 2). Expression levels were normalized vs. *GAPDH* expression. Values are presented as relative expression. Similar results were observed in three independent experiments (*p<0.05 vs. control medium and vs. MDA-231-SrcDN- CM).

**Figure 7. Transcriptional expression of osteoclast-regulating cytokines.** (A, B left panels) RNA from MDA-231, MDA-231- SrcDN and MDA-231-SrcWT was extracted and reverse transcribed. The equivalent of 0.1 µg was subjected to PCR for the indicated genes using the primer pairs and conditions described in Table 3. As positive control for *RankL* and *TGFβ* amplification, human peripheral
blood mononuclear cells (hPBMC) mRNA was used. (B, right panel) MDA-231, MDA-231-SrcWT and MDA-231-SrcDN cells were lysed in RIPA buffer and 50 µg of proteins were subjected to 12% SDS-PAGE. Filters were immunoblotted with anti-PTHrP antibody, stripped, and reprobed with an anti-actin antibody, as loading control. Similar results were obtained in three independent experiments.

**Figure 8. Effect of c-Src modulation on endothelial cells.** (A) EAHy926 endothelial cells were subjected to the [³H]-thymidine incorporation test under treatment with tumor-cell–conditioned media to evaluate cell proliferation (*p<0.02 vs. control; §p<0.05 vs. MDA-231). (B) EAHy926 cells were cultured in transwells onto membranes coated with gelatin and allowed to migrate using tumor-cell–conditioned media as chemoattractants. After 12 hours, the number of cells migrating through the membrane was estimated (*p<0.02 vs. control). (C) EAHy926 cells were cultured in transwells as in (B), but onto membranes coated with matrigel for testing invasion ability. After 18 hours, the number of cells migrating through the membrane was estimated (*p<0.05 vs. control). Results are the mean ± SEM of three independent experiments. (D) mRNA from MDA-231, MDA-231-SrcWT, and MDA-231-SrcDN cells were extracted and reverse-transcribed, and the cDNAs were subjected to RT-PCR for VEGF, bFGF, and GAPDH amplification. Results are representative of three evaluations. EAHy926 cells were treated with the c-Src inhibitor CGP76030 as described in Fig. 4D-F and subjected to the proliferation test by [³H]-thymidine incorporation (*p<0.02 vs. control) (E), migration test through gelatin (F), and invasion test through matrigel (G). Data are the mean ± SEM of three independent experiments.
Table 1 – Characteristics of the CGP76030 c-Src inhibitor

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The data are derived from Šuša et al., 2005
Table 2 – Mouse primer pairs and PCR conditions

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<td>GAPDH</td>
<td>Fw 5'-CACCATGGAGAAGGCGGGG-3' Rv 5'-GACGGACACATTGGGAGTAG-3'</td>
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<td>Fw 5'-AAAGCACCCTGTAGAAACA-3' Rv 5'-CCGTTTTATCCTCTCTACACTC-3'</td>
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Table 3 – Human primer pairs and PCR conditions

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<tr>
<td>IL-18</td>
<td>Fw: 5'-ACATAGGTCATGATCTGACGG-3' Rv: 5'-GAAGATTCAATTGCGATCATT-3'</td>
<td>32 cycles: 94°C 1 min, 58°C 1 min, 72°C 1 min</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Fw: 5'-CCTGCCACAGATCCCTATT-3' Rv: 5'-GTGACCTCTTGGCAGTAGT-3'</td>
<td>35 cycles: 94°C 1 min, 61°C 1 min, 72°C 1 min</td>
</tr>
<tr>
<td>FGF</td>
<td>Fw 5'-AGCAGCAGCTAGGAGCTTCT-3' Rv 5'-CTGAGATTTCCCGAAGCGCA-3'</td>
<td>40 cycles: 94°C 45 sec, 60°C 45 sec, 72°C 45 sec</td>
</tr>
<tr>
<td>VEGF</td>
<td>Fw 5'-CTACCTCCACCACTGGAG-3' Rv 5'-TGGTGATGCGACTTCA-3'</td>
<td>40 cycles: 94°C 45 sec, 60°C 45 sec, 72°C 45 sec</td>
</tr>
</tbody>
</table>
Table 4: - Evaluation of the incidence of osteolytic lesions (X-ray analysis), osteolytic area (X-ray analysis) and tumor diameter (histological examination) at 20 and 32 days after intratibial injection of MDA, MDA-Src\textsuperscript{WT} or MDA-Src\textsuperscript{DN} cells in BALB/c-\textit{nu}/\textit{nu} mice.

<table>
<thead>
<tr>
<th>Days after injection</th>
<th>Incidence of osteolytic lesions (%)</th>
<th>Osteolytic area (mm\textsuperscript{2})</th>
<th>Tumor diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>MDA</td>
<td>50%</td>
<td>67%</td>
<td>4.5 ± 1.1</td>
</tr>
<tr>
<td>MDA-Src\textsuperscript{WT}</td>
<td>50%</td>
<td>75%</td>
<td>4.2 ± 1.26</td>
</tr>
<tr>
<td>MDA-Src\textsuperscript{DN}</td>
<td>17%</td>
<td>50%</td>
<td>3.7±0.57</td>
</tr>
</tbody>
</table>

*\(P = 0.05\) vs MDA
§\(P < 0.05\) vs MDA-Src\textsuperscript{WT}

N. mice/group = 4.
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8