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Osteosarcoma : searching for new treatment options

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Citation

Baranski Madrigal, Z. (2016, May 26). *Osteosarcoma : searching for new treatment options*. Retrieved from <https://hdl.handle.net/1887/39707>

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Title: Osteosarcoma : searching for new treatment options

Issue Date: 2016-05-26

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Dasatinib Src inhibitor selectively triggers
apoptosis and loss of invasive potential in human
osteosarcoma cells

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Manuscript in preparation

ABSTRACT

Conventional high-grade osteosarcoma is the most common primary bone malignancy with relatively high incidence in young people. About 40% of the patients develop metastases and have a very poor prognosis. New insights into osteosarcoma growth and progression that may lead to new therapeutic strategies are needed. Expression and activity of the Src cytoplasmic tyrosine kinase has been correlated with clinical stage and survival. Here, we studied the effect of pharmacological inhibitors of Src activity, including dasatinib, bosutinib and saracatinib in MOS and U2OS human osteosarcoma cell lines in 2D and 3D. All inhibitors decreased viability with an IC₅₀ in the micromolar range. Likewise, treatment with each of the inhibitors reduced the IC₅₀ of doxorubicin. However, only dasatinib treatment triggered caspase3/7 activation pointing to apoptosis. The selective activity of dasatinib correlated with its capacity to reduce Src activity. Next, the effects of the inhibitors were studied in MOS and U2OS cultures in 3D extracellular matrix (ECM) scaffolds. Under these conditions, all three inhibitors reduced viability but formation of branched networks in 3D ECM was selectively inhibited by dasatinib in presence of doxorubicin. The activity of focal adhesion kinase (FAK), a Src substrate that is important for cell migration, was exclusively sensitive to dasatinib. Indeed, in 3D ECM-embedded spheroid cultures dasatinib blocked cell migration capacity whereas the other inhibitors had no or partial effects. Together, these findings point to the use of dasatinib as a candidate drug to enhance apoptosis in response to chemotherapy and to reduce metastatic spread in patients with osteosarcoma.

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor that arises from mesenchymal stem cells that are capable of producing osteoid[1]. It has an overall incidence of 3 cases per million annually occurring predominantly in children and adolescents, with a second peak in people above 50 years of age[2]. At the moment of diagnosis, 10-20% of the patients present with metastasis, and about 30-40% of the patients with localized osteosarcoma will relapse mainly by presenting lung metastasis. Patients with relapsed disease have very poor prognosis with 23-33% 5-year overall survival[3].

Src is a nonreceptor tyrosine kinase that belongs to a family of 11 members, and it is widely expressed in a most tissues. Src acts as signal transducer from cell membrane receptors to downstream substrates. Src activity regulates cell morphology, adhesion, and migration, as well as survival and proliferation through activation of PI3K-Akt, Ras-Raf-MEK-ERK, and Jak-Stat and a cell-extracellular matrix (ECM) adhesion-signaling platform including the Src substrate focal adhesion kinase (FAK) [4,5]. Activation and expression of Src in colon cancer is associated with late tumor stage[6] and ability to metastasize[7]. Furthermore, Src activity and expression is also implicated in other malignancies such as breast cancer[8,9], ovarian cancer[10], lung cancer[11] and chondrosarcoma[12]. Notably, despite the fact that Src is overexpressed or constitutively active in many malignancies, mutations are rare in this gene. Therefore, in most cancers Src does not appear to drive tumor initiation or tumor formation, but may rather play a role in aspects of tumor progression[13,14].

As mentioned above, Src transduces signal from cell receptors among which is IGFR. This receptor was reported to be highly expressed in high grade conventional osteosarcoma[15], and its inhibition with antibodies proofed to increase event free survival duration[16]. Additionally,, in osteosarcoma Src expression and activity has been shown to correlate with clinical stage and patient survival, making Src a potential aiding marker to determine prognosis in osteosarcoma[17]. All together, these findings leads us to investigate the inhibition of Src as potential treatment for patients with osteosarcoma.

Dasatinib and bosutinib are two Src/Bcr-Abl inhibitors approved by the FDA for chronic myelogenous leukemia resistant to prior therapy[18-21]. Saracatinib, is a Src inhibitor that is currently in clinical trial for patients with recurrent osteosarcoma localized to the lung (NCT00752206), other cancers including melanoma (NCT00669019), prostate cancer (NCT01267266), and Alzheimer's disease (NCT01864655). The compounds have been tested as single agents in solid tumors with no evident clinical activity[22-26]. Here, we assessed the capacity of these inhibitors to attenuate human osteosarcoma cell survival and migration in 2D and 3D environments. The inhibitors were tested alone or in combination with the clinically relevant chemotherapeutic compound, doxorubicin.

MATERIALS AND METHODS

Reagents and antibodies. Doxorubicin was obtained from the Department of Clinical Pharmacology at LUMC, bosutinib, dasatinib and saracatinib were from SelleckChem (Huissen, Netherlands). Antibodies against ERK1/2(clone137F5), phospho-ERK(42/44) (#4695), AKT(9272), and phospho-AKT(Ser473) (#9271), were from Cell Signalling (Bioké, Leiden, The Netherlands). Antibodies against Src (clone GD11) and phospho-Src(Tyr418) (#44660G) were from Millipore (Amsterdam, The Netherlands) and Invitrogen (Bleiswijk, The Netherlands), respectively. Antibody against FAK (clone4.47) was from BioConnect (Huissen, The Netherlands). Antibodies against phospho-FAK(Tyr925) ([#MBS8507066](#)) and phospho-FAK(Tyr861) ([#MBS8507535](#)) were from Biosource (California, U.S.A.). Antibody against tubulin (T-9026) was from Sigma-Aldrich (Zwijndrecht, Netherlands).

Cell culture. Human osteosarcoma cell lines MOS, U2OS were previously described[27,28]. Cells were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and 25 U/mL penicillin and 25 µg/mL of penicillin-streptomycin. All cells were cultured in a humidified incubator at 37°C with 5% CO₂.

Western blotting. Cells were lysed with SDS protein buffer (125mM Tris/HCl pH 6.8, 20% glycerol, 4% SDS and 0.2% bromophenol blue). Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were blocked in 5% BSA-TBST (TRIS-0.05% Tween20), followed by overnight incubation with primary antibodies and 45 minutes incubation with HRP-conjugated secondary antibodies. Chemoluminescence was detected with a bioimager, LAS400 (GE Healthcare).

Measuring cell viability and apoptosis in 2D cultures. For cell viability, cells were processed using the ATPlite 1Step kit (Perkin Elmer) according to the manufacturer's instructions, followed by luminescence measurement. Apoptosis was measured by assessing caspase3/7 activity with CaspaseGlo 3/7 (Promega). The cells were exposed to the drug for 24 hours after which the reagent was added 1:1. Luminescence was measured in a Fluostar Optima plate reader.

3D collagen/matrigel culture assay. U2OS and MOS cells were cultured in 384-well plates (Greiner µclear) in a hydrogel containing Matrigel (Beckton Dickinson) and collagen I, supporting invasive growth of both cell lines. Cells in culture were trypsinized and directly added to the cooled gel solution. Using a robotic liquid handler (CyBio Selma 96/60), 14.5µL of gel-cell suspension was transferred to each well of a 384-well plate (2000 cells/well). After polymerization for 30 minutes at 37°C in an atmosphere of 5% CO₂, growth medium was added on top of the gel. After three days, when the cells had formed a network structure, compounds were diluted and added in quadruplicate wells for a period of 72 hours.

For measuring cell viability in 3D, a solution of 7g/L WST-1 (Serva Electrophoresis) and 8mg/L phenazinium methylsulfate (PMS; Sigma Aldrich) in 1x PBS were mixed in a 1:1 ratio and 5µL was added to each well. Plates were placed at 37°C for 5 hours, after which the absorbance at 450nm was measured using a FluoStar Optima late reader. Percentage viability was thereafter calculated by robust normalization (median) of the plates between positive control (no cells; 0% viability) and negative control (solvent; 100% viability) conditions.

For imaging, cells were fixed using 3.7% Formaldehyde (Sigma-Aldrich), permeabilized with 0.1% Triton-X100 and stained for F-actin using 50nM Rhodamine-Phalloidin (Sigma Aldrich) for 12 hours at 4°C. Subsequently, the plates were washed in PBS for at least 24 hours at 4°C. The plates were then imaged on a BD Pathway 855 inverted fluorescence microscope (BD Biosciences) using a 4x lens to capture Rhodamine-Phalloidin staining at focal planes spaced 50µm throughout the gel, capturing approximately 70% of a well. Subsequently, maximum intensity projections of the in-focus information of the Z-stacks was made using OcellO (OcellO B.V., Leiden, The Netherlands) image analysis tools.

3D collagen spheroid assay. Cell suspensions were injected into collagen scaffolds using automated injection as previously described[29,30]. 1 mg/ml rat tail collagen was prepared in complete growth medium supplemented with 1:5 dilution of 0.44M NaHCO₃ and 1:10 dilution of 1M Hepes pH 7.4. 60µL was added to each well of a 96-well µ-clear plate (Corning) and incubated for 1 hour at 37°C to allow polymerization. Cells were collected in medium containing 2% PVP, transferred to a needle and droplets of ~8nL were injected into the collagen gels resulting in spheroids of ~300µm diameter, using injection robotics from Life Science Methods, Leiden NL (<http://www.lifesciencemethods.com>). For DIC imaging of spheroids, a Nikon confocal microscope was used.

Statistical analysis. Dose response curve fitting and all statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). The unpaired two-tailed *t*-test

was used to compare between groups. Significant difference between groups in the 3D assay was calculated using 2way ANOVA with Bonferroni posttest.

RESULTS

Reduced human osteosarcoma cell viability in presence of bosutinib, dasatinib and saracatinib and selective Src kinase inactivation by dasatinib

We determined the effect of dasatinib, bosutinib and saracatinib on cell viability in MOS and U2OS human osteosarcoma cells. Responses to these inhibitors were highly similar for MOS and U2OS cells but differed considerably between the different inhibitors (Fig. 1A). Both cell lines showed no response to bosutinib concentrations $<1\ \mu\text{M}$ and a rapid decline in viability was observed as the bosutinib concentration increased from 1 to 5 μM . Instead, viability gradually decreased in response to 0.1-10 μM dasatinib and a similar trend, albeit less effective, was observed for saracatinib. IC₅₀ for bosutinib and dasatinib was $\sim 5\ \mu\text{M}$ and IC₅₀ was $>10\ \mu\text{M}$ for saracatinib (Fig 1B).

PI3K/AKT and Raf-MEK-ERK MAP kinase signaling pathways represent important drivers of survival and proliferation in many different cancer types. These two pathways are regulated by Src activity[31,32]. We tested if treatment of MOS and U2OS cells with the Src inhibitors affected these pathways. However, treatment with up to 2.5 μM bosutinib, dasatinib, or saracatinib did not affect phosphorylation of ERK (Fig 1C). In fact, treatment with saracatinib increased the levels of ERK phosphorylation particularly in U2OS. On the other hand, dasatinib and saracatinib suppressed AKT phosphorylation at 2.5 μM whereas bosutinib had no effect. Moreover, while 1 μM dasatinib effectively attenuated Src phosphorylation at Y418 in both cell lines, indicating attenuated Src kinase activity, bosutinib and saracatinib failed to do so even at 2.5 μM .

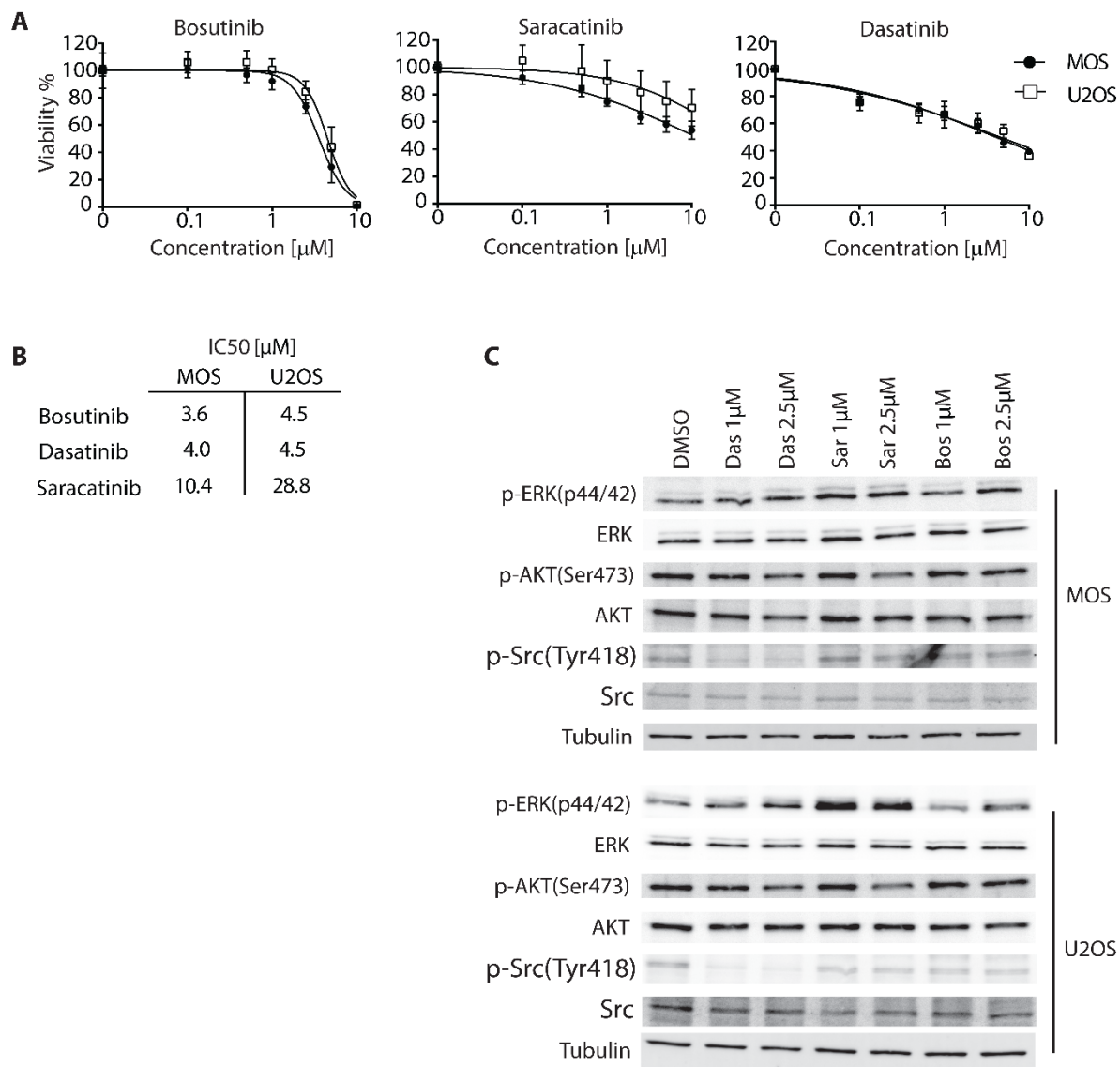


Figure 1. Effect of dasatinib, bosutinib and saracatinib in human osteosarcoma cells.

A) Dose response curves for dasatinib, bosutinib and saracatinib in two human osteosarcoma cell lines. Error bars represent the standard deviation of three experiments performed in triplicate. Cells were exposed for 72 hours. **B)** Table with IC50 values of bosutinib, dasatinib and saracatinib in MOAS and U2OS cells. **C)** Western blot analysis of phospho-ERK(p44/42), total ERK, phospho-AKT(Ser473), total AKT, phospho-Src(Tyr418), total Src, and tubulin loading control in MOS and U2OS cells under control (DMSO) conditions or after 48 hours treatment with 1 or 2.5μM of the indicated inhibitors.

Sensitization to doxorubicin in presence of bosutinib, dasatinib and saracatinib and selective induction of apoptosis by dasatinib

Src kinase activity may not be a bona fide cancer driver and mono therapy using either of these inhibitors may be ineffective. However, as Src stimulates pro-survival and proliferation

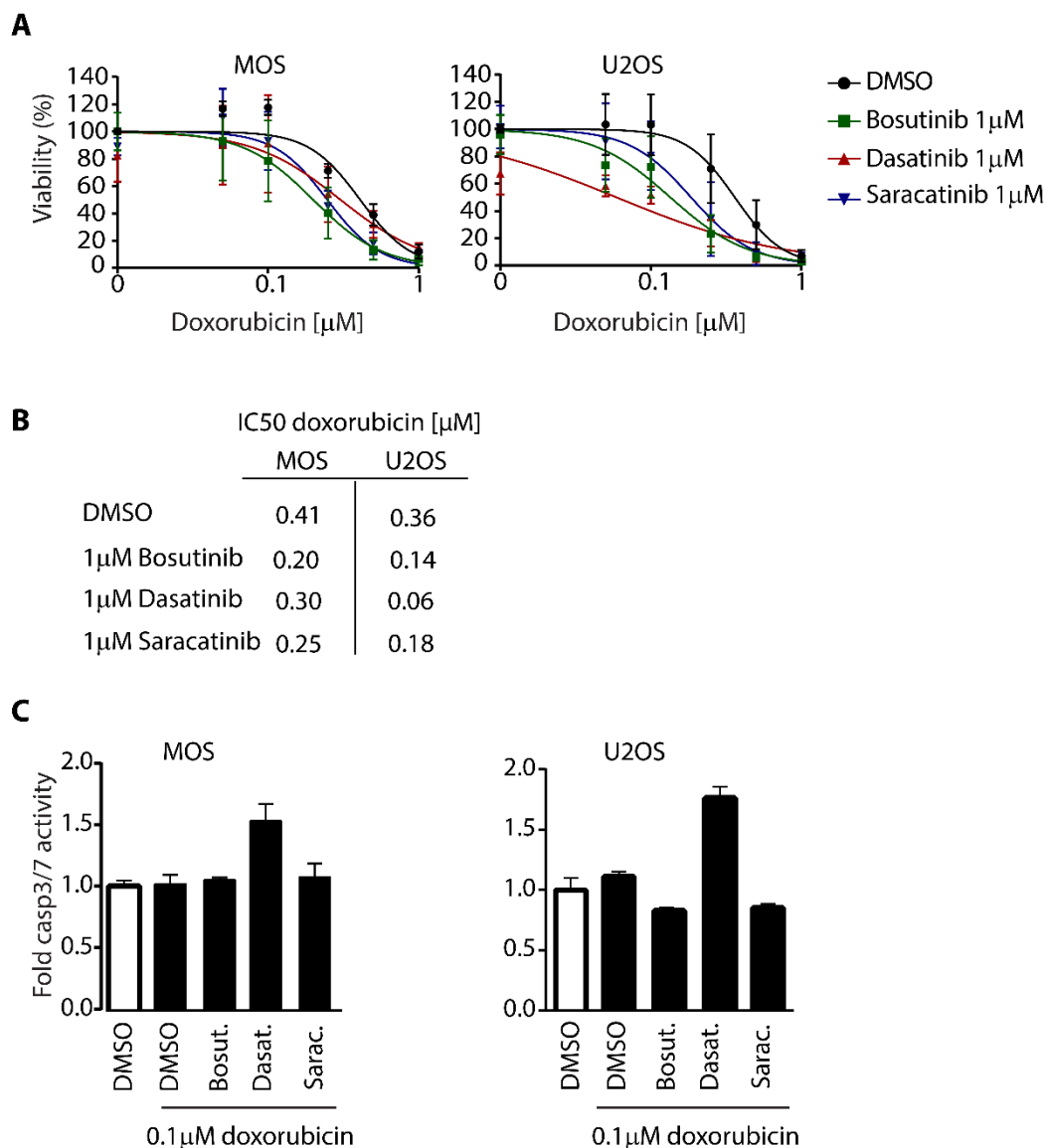


Figure 2. Effect of dasatinib, bosutinib and saracatinib in human osteosarcoma cells in the context of doxorubicin. A). Dose response curves for doxorubicin in two human osteosarcoma cell lines in absence (black) or presence of 1 μ M of dasatinib (red), bosutinib (green) or saracatinib (blue). Cells were exposed for 72 hours. Error bars represent mean $\pm$ SEM of three experiments **B).** Table with IC50 values for doxorubicin alone (DMSO) or in combination with 1 μ M bosutinib, dasatinib or saracatinib in MOS and U2OS cells. **C)** Caspase 3/7 activity in two human osteosarcoma cell lines under control conditions (white bars) or upon exposure for 24 hours to 0.1 μ M doxorubicin (black bars) in the presence of DMSO or 1 μ M dasatinib, bosutinib and saracatinib as indicated. Mean $\pm$ S.D is shown for one representative experiment of 3 performed in triplicate.

signaling pathways[13,14]. Its inhibition may render tumor cells more sensitive to chemotherapy. To investigate this, MOS and U2OS cells were exposed to 1 μ M of the inhibitor together with a dose range of doxorubicin for 72 hours. Indeed, both cell lines showed a reduction in viability already at lower doses of doxorubicin in presence of

dasatinib, bosutinib, or saracatinib, as compared to the response to doxorubicin alone (Fig. 2A). For MOS cells, the IC₅₀ for doxorubicin was reduced by 30-50%, and for U2OS cells a reduction of 50-80% was observed (Fig 2B). In order to assess whether decreased viability was related to apoptosis, we determined caspase3/7 activity. Interestingly, only treatment with dasatinib led to apoptosis either alone (not shown) or in combination with doxorubicin (Fig. 2C).

Reduced human osteosarcoma cell viability in 3D cultures in presence of bosutinib, dasatinib and saracatinib and selective morphological effects induced by dasatinib.

Next, we analyzed the effect of the panel of Src inhibitors in a 3D in vitro culture model. MOS and U2OS cells were suspended in a collagen-matrigel mixture and allowed to form a multicellular network for 72 hours. Subsequently cells were exposed to 1 μ M of dasatinib, bosutinib, or saracatinib alone or combined with a concentration range of doxorubicin. Inhibition of cell viability by the inhibitors alone as measured biochemically, was more pronounced compared to effects measured in 2D. All three inhibitors by themselves caused a reduction in viability of 40-50% (Fig 3A). Additional treatment with doxorubicin further decreased viability but no synergy was observed between doxorubicin and any of the inhibitors.

Next we used imaging and image analysis algorithms to measure “branch length” and “solidity” or roundness of the multicellular structures; parameters correlated cell migration [33]. Low concentrations of doxorubicin up to 0.1 μ M did not affect these parameters (Fig 3B). Exposure to 1 μ M of the Src inhibitors alone led to decreased branch length and increased solidity. However, in the presence of dasatinib MOS and U2OS cells were selectively responsive to low concentrations of doxorubicin; showing a decrease in branch length and a concomitant increase in solidity of the multicellular structures (Fig. 3B-D).

Selective inhibition of FAK activity and 3D osteosarcoma cell migration by dasatinib

To further investigate morphological effects caused by these inhibitors that may impact on osteosarcoma progression we made use of a 3D spheroid model. MOS and U2OS cells were injected as nL droplets into collagen gels as described before[29,30], and resulting spheroids

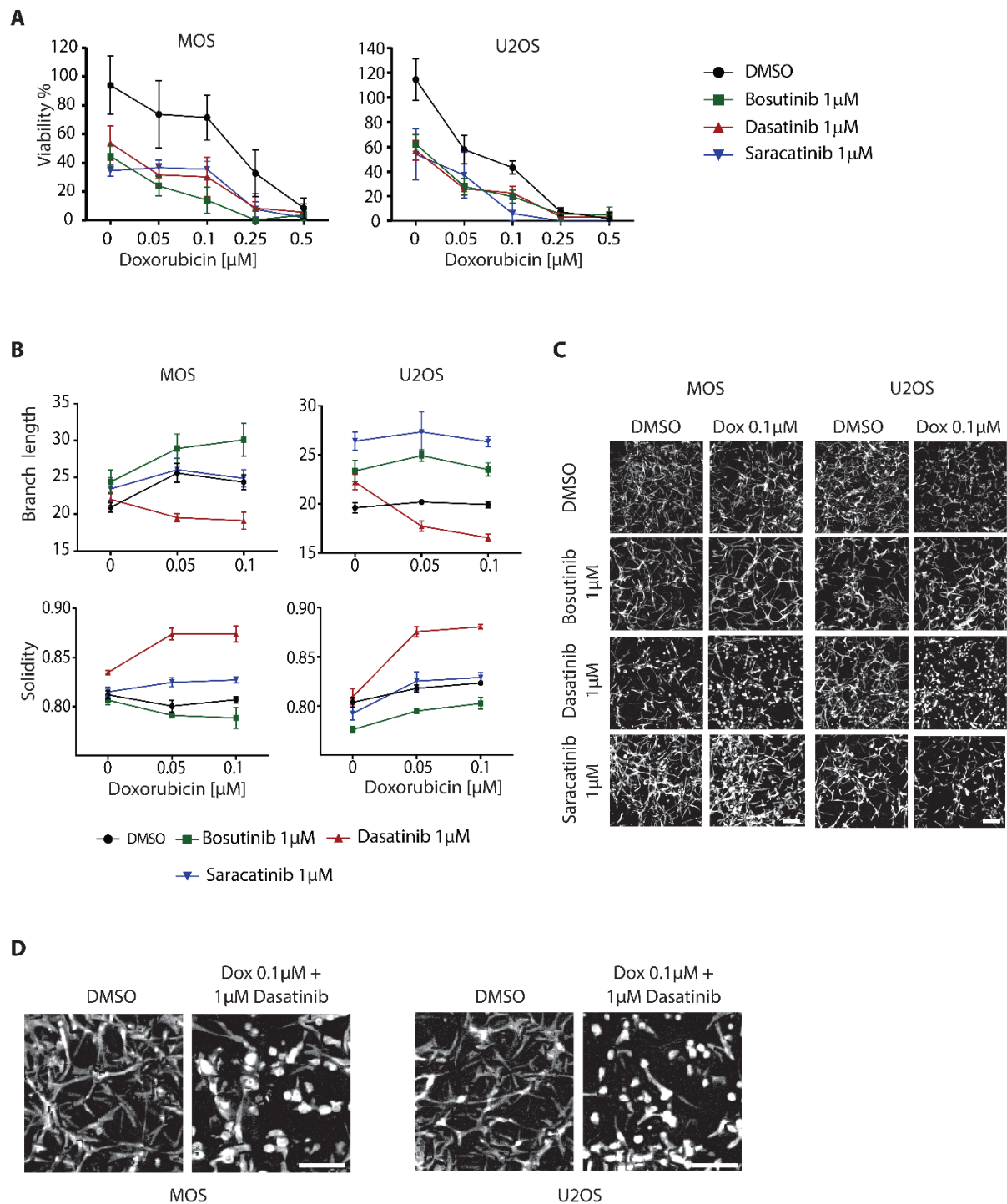


Figure 3. Effect of dasatinib, bosutinib and saracatinib in human osteosarcoma cells in the context of doxorubicin in 3D cultures. A,B) Doxorubicin dose response curve for human osteosarcoma cells grown in collagen/matrige[®] mixture under control conditions (DMSO; black line) or in presence of 1µM dasatinib (red), bosutinib (green), or saracatinib (blue). Cells were exposed for 72 hours. **A)** Viability was assessed using WST/PMS absorbance. Error bars represent mean $\pm$ SEM of three experiments. Values were normalized to median of DMSO. **B)** Image analysis was used to assess average branch length (top graphs) and solidity (bottom graphs). Error bars represent mean $\pm$ s.d of one representative experiment done in quadruplicate. **C)** Representative images such as those used

for graphs in B. Each image is a compression of 11stacks of the 3D culture. **D)** Zoom-in of selected areas from images in C, showing marked effect of doxorubicin/dasatinib combination.

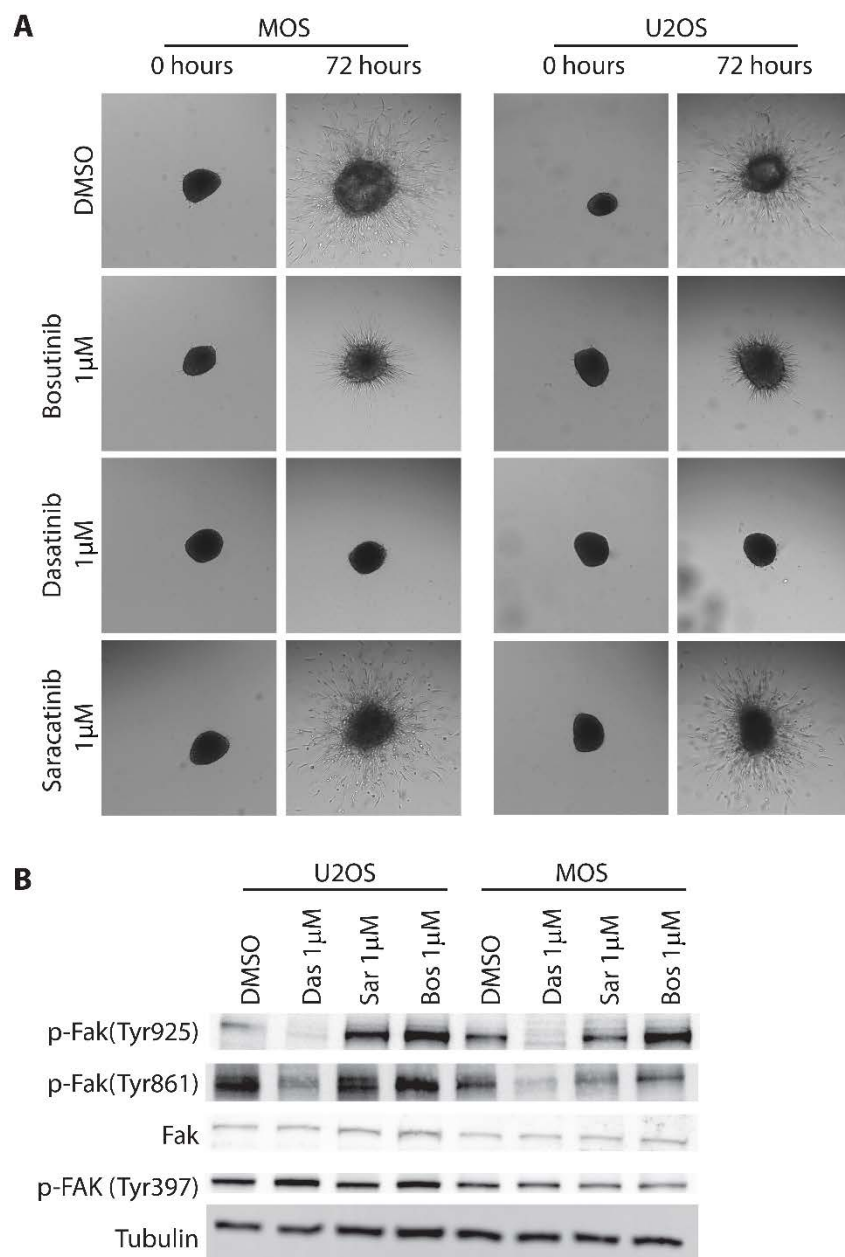


Figure 4. Dasatinib inhibits Fak activation and stops collagen invasion in osteosarcoma.

A) U2OS and MOS cell-derived collagen-embedded spheroids directly after cell-injection (0 hours) and after 72 hours incubation under control conditions (DMSO) or in presence of 1μM of the indicated inhibitors. Images were obtained using a Nikon confocal microscope. **B)** Western blot analysis of total FAK and phospho-FAK(Tyr397), (Tyr861), (Tyr925), and tubulin loading control for U2OS and MOS cells maintained for 48 hours under control conditions (DMSO) or in presence of 1μM of the indicated inhibitors.

were exposed to DMSO or 1μM bosutinib, dasatinib and saracatinib for 72hours. Saracatinib did not affect spheroid outgrowth or 3D cell migration and bosutinib had an intermediate effect while dasatinib treatment completely blocked outgrowth and collagen invasion of MOS and U2OS cells in this model (Fig 4A).

Src promotes invasion and metastasis and plays a key role as a regulator of cell-ECM adhesions containing the Src substrate FAK. The Src/FAK complex integrates signals from the extracellular environment and controls and coordinates adhesion dynamics and cell migration[34,35]. FAK is autophosphorylated at Tyr397 upon integrin-mediated adhesion creating a binding site for Src, which subsequently phosphorylates FAK at Tyr407, 576, 577, 861 and 925[34,36]. We analyzed FAK phosphorylation after 48hour treatment with 1 μ M dasatinib, bosutinib or saracatinib. The FAK autophosphorylation site was not affected by any of the inhibitors. However, in agreement with its selective inhibition of cell migration through 3D ECM scaffolds, phosphorylation of FAK at Src substrates Tyr861 and Tyr925 was selectively inhibited by dasatinib. Whereas Saracatinib and bosutinib had no apparent effect (Fig 4B).

DISCUSSION

In this study we investigated the effect of Src inhibitors dasatinib, bosutinib, and saracatinib in two human osteosarcoma cell lines. Impacts on cell viability and migration were tested as single agent as well as in combination with the chemotherapeutic compound doxorubicin, which is used in the clinic for treatment of osteosarcoma. Src activity regulates the PI3K-Akt, Ras-Raf-ERK, Jak-Stat and FAK-Paxillin pathways. In osteosarcoma, none of the inhibitors interfered with ERK and AKT phosphorylation, and only dasatinib inhibited Src and Fak activation in MOS and U2OS cell lines. Notably, the inhibitors have other targets such as other members of the Src family, Bcr-Abl, MAPK kinases, Eph receptors, cKit, STK6, PDGFR and TEC family kinases[37,38]. An interesting study that mapped the target profile of bosutinib in chronic myeloid leukemia cells identified new targets and to what extent targets were inhibited[39]. The MAPK family was found to be a major target, but MEK1 and MEK2 were not significantly inhibited[39,40]. These results may explain why ERK activation was not inhibited by any of the inhibitors in our study. Furthermore, the fact these inhibitors do not completely inhibit the activity of a kinase, can explain why saracatinib and bosutinib did not show appreciable inhibition of Fak phosphorylation, and failed to affect cell migration. The autophosphorylation site (Tyr397) of Fak causes a conformational change allowing Src binding and further Fak phosphorylation in Tyr576/577, Tyr861 and Tyr925. The phosphorylation of these sites is important for the interaction with integrins and E-cadherin[34]. While bosutinib has been reported to inhibit Fak-(Y925) phosphorylation in breast cancer cells, in the two osteosarcoma cell lines used only dasatinib inhibited Src-mediated phosphorylation of Fak Tyr861 and Tyr925[41].

To study the effect of dasatinib, bosutinib or saracatinib on the migratory behavior of osteosarcoma cells, we used 3D cell culture systems. 3D cultures may better reflect the tumor microenvironment as compared to 2D cultures and cell matrix adhesions and migratory behavior are closer to the *in vivo* situation [42-45]. In the two 3D systems we used, including mixture of cells in collagen/matrigel and microinjection of cells to examine migration from spheroids in collagen gels, collagen type I is the major ECM component and this is also the main component (90%) of the ECM of bones[46]. Our finding that dasatinib selectively blocks osteosarcoma cell migration in this environment correlates its selective inhibition of Src-mediated Fak phosphorylation. Thus, dasatinib treatment likely interferes with the Src/Fak signaling platform to prevent cell migration and may thus interfere with metastatic capacity.

In addition, dasatinib selectively triggers apoptosis and causes morphological alterations in 3D cultures in the presence of doxorubicin. It was previously reported that dasatinib has the capacity to sensitize chondrosarcoma cells to doxorubicin (jolieke refe). Furthermore, a new Src inhibitor, A-770041, was shown to increase sensitivity to doxorubicin in osteosarcoma cells (refDuan et al. BMC Cancer 2014, 14:681). Notably, a decrease in the IC50 of doxorubicin is observed when combined with each the inhibitors indicating that dasatinib selectively affects some, but not all aspects of these inhibitors. Several studies have shown that these three inhibitors do not have an effect as single agents in solid tumors. For example, dasatinib inhibits activation of Src and Fak *in vitro* and *in vivo*, but it does not induce apoptosis or prevent tumor metastasis to the lungs in a xenograft osteosarcoma mice[47]. However, others showed that for biliary tract carcinomas saracatinib was effective in a preclinical model, and both dasatinib and saracatinib are effective in leukemia[48-51] indicating that the therapeutic effect of these inhibitors is cancer type-dependent. Despite the lack of activity as a single agent, the combination of dasatinib, bosutinib or saracatinib with doxorubicin in breast cancer or pancreatic cancer cells did lead to a synergistic effect *in vitro* and *in vivo*[52-54].

Altogether, we find that dasatinib selectively inhibits activity of the Src/Fak signaling complex in osteosarcoma cells and, most likely as a consequence of this, migration in collagen scaffolds. Furthermore, while all three inhibitors decreased the IC50 of doxorubicin, dasatinib selectively triggers apoptosis and morphological changes in the context of doxorubicin. Our findings point to the combination of dasatinib and doxorubicin as a potential therapy for osteosarcoma to prevent or minimize metastasis.

CONFLICT OF INTEREST

L.S. Price is founder and co-owner of Ocello B.V. a contract research company that offers screening services using 3D tissues. This role has had no bearing on the content of the manuscript.

REFERENCES

1. Mohseny AB, Szuhai K, Romeo S, *et al.* Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. *J Pathol* 2009; **219**: 294-305.
2. Rosenberg AE, Cleton-Jansen A-M, Pinieux Gd, *et al.* Conventional osteosarcoma. In: WHO Classification of Tumours of Soft Tissue and Bone. (ed)[^](eds). IARC: Lyon, 2013; 282-288.
3. Buddingh EP, Anninga JK, Versteegh MI, *et al.* Prognostic factors in pulmonary metastasized high-grade osteosarcoma. *Pediatric blood & cancer* 2010; **54**: 216-221.
4. Amundson SA, Myers TG, Scudiero D, *et al.* An informatics approach identifying markers of chemosensitivity in human cancer cell lines. *Cancer research* 2000; **60**: 6101-6110.
5. Yeatman TJ. A renaissance for SRC. *Nature reviews Cancer* 2004; **4**: 470-480.
6. Talamonti MS, Roh MS, Curley SA, *et al.* Increase in activity and level of pp60c-src in progressive stages of human colorectal cancer. *The Journal of clinical investigation* 1993; **91**: 53-60.
7. Jones RJ, Avizienyte E, Wyke AW, *et al.* Elevated c-Src is linked to altered cell-matrix adhesion rather than proliferation in KM12C human colorectal cancer cells. *British journal of cancer* 2002; **87**: 1128-1135.
8. Biscardi JS, Ishizawar RC, Silva CM, *et al.* Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast cancer research : BCR* 2000; **2**: 203-210.
9. Hynes NE. Tyrosine kinase signalling in breast cancer. *Breast cancer research : BCR* 2000; **2**: 154-157.
10. Wiener JR, Windham TC, Estrella VC, *et al.* Activated SRC protein tyrosine kinase is overexpressed in late-stage human ovarian cancers. *Gynecologic oncology* 2003; **88**: 73-79.
11. Leung EL, Tam IY, Tin VP, *et al.* SRC promotes survival and invasion of lung cancers with epidermal growth factor receptor abnormalities and is a potential candidate for molecular-targeted therapy. *Molecular cancer research : MCR* 2009; **7**: 923-932.
12. van Oosterwijk JG, van Ruler MA, Briaire-de Bruijn IH, *et al.* Src kinases in chondrosarcoma chemoresistance and migration: dasatinib sensitises to doxorubicin in TP53 mutant cells. *British journal of cancer* 2013; **109**: 1214-1222.
13. Frame MC. Src in cancer: deregulation and consequences for cell behaviour. *Biochimica et biophysica acta* 2002; **1602**: 114-130.
14. Summy JM, Gallick GE. Src family kinases in tumor progression and metastasis. *Cancer metastasis reviews* 2003; **22**: 337-358.
15. Hassan SE, Bekarev M, Kim MY, *et al.* Cell surface receptor expression patterns in osteosarcoma. *Cancer* 2012; **118**: 740-749.
16. Kim SY, Toretsky JA, Scher D, *et al.* The role of IGF-1R in pediatric malignancies. *Oncologist* 2009; **14**: 83-91.
17. Hu C, Deng Z, Zhang Y, *et al.* The prognostic significance of Src and p-Src expression in patients with osteosarcoma. *Medical science monitor : international medical journal of experimental and clinical research* 2015; **21**: 638-645.
18. Kantarjian H, Jabbour E, Grimley J, *et al.* Dasatinib. *Nature reviews Drug discovery* 2006; **5**: 717-718.

19. Stansfield L, Hughes TE, Walsh-Chocolaad TL. Bosutinib: a second-generation tyrosine kinase inhibitor for chronic myelogenous leukemia. *Ann Pharmacother* 2013; **47**: 1703-1711.
20. Shah NP, Tran C, Lee FY, *et al.* Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science* 2004; **305**: 399-401.
21. Boschelli F, Arndt K, Gambacorti-Passerini C. Bosutinib: a review of preclinical studies in chronic myelogenous leukaemia. *European journal of cancer* 2010; **46**: 1781-1789.
22. Reddy SM, Kopetz S, Morris J, *et al.* Phase II study of saracatinib (AZD0530) in patients with previously treated metastatic colorectal cancer. *Invest New Drugs* 2015; **33**: 977-984.
23. Mackay HJ, Au HJ, McWhirter E, *et al.* A phase II trial of the Src kinase inhibitor saracatinib (AZD0530) in patients with metastatic or locally advanced gastric or gastro esophageal junction (GEJ) adenocarcinoma: a trial of the PMH phase II consortium. *Invest New Drugs* 2012; **30**: 1158-1163.
24. Gucalp A, Sparano JA, Caravelli J, *et al.* Phase II trial of saracatinib (AZD0530), an oral SRC-inhibitor for the treatment of patients with hormone receptor-negative metastatic breast cancer. *Clin Breast Cancer* 2011; **11**: 306-311.
25. Schilder RJ, Brady WE, Lankes HA, *et al.* Phase II evaluation of dasatinib in the treatment of recurrent or persistent epithelial ovarian or primary peritoneal carcinoma: a Gynecologic Oncology Group study. *Gynecologic oncology* 2012; **127**: 70-74.
26. Taylor JW, Dietrich J, Gerstner ER, *et al.* Phase 2 study of bosutinib, a Src inhibitor, in adults with recurrent glioblastoma. *J Neurooncol* 2015; **121**: 557-563.
27. Mohseny AB, Machado I, Cai Y, *et al.* Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. *Lab Invest* 2011; **91**: 1195-1205.
28. Ottaviano L, Schaefer KL, Gajewski M, *et al.* Molecular characterization of commonly used cell lines for bone tumor research: a trans-European EuroBoNet effort. *Genes Chromosomes Cancer* 2010; **49**: 40-51.
29. Truong HH, de Sonnevile J, Ghotra VP, *et al.* Automated microinjection of cell-polymer suspensions in 3D ECM scaffolds for high-throughput quantitative cancer invasion screens. *Biomaterials* 2012; **33**: 181-188.
30. Truong HH, Xiong J, Ghotra VP, *et al.* beta1 integrin inhibition elicits a prometastatic switch through the TGFbeta-miR-200-ZEB network in E-cadherin-positive triple-negative breast cancer. *Science signaling* 2014; **7**: ra15.
31. Dhillon AS, Hagan S, Rath O, *et al.* MAP kinase signalling pathways in cancer. *Oncogene* 2007; **26**: 3279-3290.
32. Martini M, De Santis MC, Braccini L, *et al.* PI3K/AKT signaling pathway and cancer: an updated review. *Annals of medicine* 2014; **46**: 372-383.
33. Di Z, Klop MJ, Rogkoti VM, *et al.* Ultra high content image analysis and phenotype profiling of 3D cultured micro-tissues. *PLoS One* 2014; **9**: e109688.
34. McLean GW, Carragher NO, Avizienyte E, *et al.* The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. *Nature reviews Cancer* 2005; **5**: 505-515.
35. Mitra SK, Schlaepfer DD. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Current opinion in cell biology* 2006; **18**: 516-523.
36. Westhoff MA, Serrels B, Fincham VJ, *et al.* SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Molecular and cellular biology* 2004; **24**: 8113-8133.
37. Zarbock A. The shady side of dasatinib. *Blood* 2012; **119**: 4817-4818.

38. Shi H, Zhang CJ, Chen GY, *et al.* Cell-based proteome profiling of potential dasatinib targets by use of affinity-based probes. *J Am Chem Soc* 2012; **134**: 3001-3014.
39. Remsing Rix LL, Rix U, Colinge J, *et al.* Global target profile of the kinase inhibitor bosutinib in primary chronic myeloid leukemia cells. *Leukemia* 2009; **23**: 477-485.
40. Winter GE, Rix U, Carlson SM, *et al.* Systems-pharmacology dissection of a drug synergy in imatinib-resistant CML. *Nat Chem Biol* 2012; **8**: 905-912.
41. Vultur A, Buettner R, Kowolik C, *et al.* SKI-606 (bosutinib), a novel Src kinase inhibitor, suppresses migration and invasion of human breast cancer cells. *Molecular cancer therapeutics* 2008; **7**: 1185-1194.
42. Ravi M, Paramesh V, Kaviya SR, *et al.* 3D cell culture systems: advantages and applications. *J Cell Physiol* 2015; **230**: 16-26.
43. Thoma CR, Zimmermann M, Agarkova I, *et al.* 3D cell culture systems modeling tumor growth determinants in cancer target discovery. *Advanced drug delivery reviews* 2014; **69-70**: 29-41.
44. Cukierman E, Pankov R, Stevens DR, *et al.* Taking cell-matrix adhesions to the third dimension. *Science* 2001; **294**: 1708-1712.
45. Baker EL, Srivastava J, Yu D, *et al.* Cancer cell migration: integrated roles of matrix mechanics and transforming potential. *PLoS One* 2011; **6**: e20355.
46. Boskey AL. Bone composition: relationship to bone fragility and antiosteoporotic drug effects. *Bonekey Rep* 2013; **2**: 447.
47. Hingorani P, Zhang W, Gorlick R, *et al.* Inhibition of Src phosphorylation alters metastatic potential of osteosarcoma in vitro but not in vivo. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2009; **15**: 3416-3422.
48. Cavalloni G, Peraldo-Neia C, Sarotto I, *et al.* Antitumor activity of Src inhibitor saracatinib (AZD-0530) in preclinical models of biliary tract carcinomas. *Molecular cancer therapeutics* 2012; **11**: 1528-1538.
49. Cortes JE, Kantarjian HM, Brummendorf TH, *et al.* Safety and efficacy of bosutinib (SKI-606) in chronic phase Philadelphia chromosome-positive chronic myeloid leukemia patients with resistance or intolerance to imatinib. *Blood* 2011; **118**: 4567-4576.
50. Apperley JF, Cortes JE, Kim DW, *et al.* Dasatinib in the treatment of chronic myeloid leukemia in accelerated phase after imatinib failure: the START a trial. *J Clin Oncol* 2009; **27**: 3472-3479.
51. Yu EY, Wilding G, Posadas E, *et al.* Phase II study of dasatinib in patients with metastatic castration-resistant prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2009; **15**: 7421-7428.
52. Pichot CS, Hartig SM, Xia L, *et al.* Dasatinib synergizes with doxorubicin to block growth, migration, and invasion of breast cancer cells. *British journal of cancer* 2009; **101**: 38-47.
53. Beeharry N, Banina E, Hittle J, *et al.* Re-purposing clinical kinase inhibitors to enhance chemosensitivity by overriding checkpoints. *Cell Cycle* 2014; **13**: 2172-2191.
54. Liu KJ, He JH, Su XD, *et al.* Saracatinib (AZD0530) is a potent modulator of ABCB1-mediated multidrug resistance in vitro and in vivo. *Int J Cancer* 2013; **132**: 224-235.