Dasatinib inhibits the growth of prostate cancer in bone and provides additional protection from osteolysis

The need for therapies offering improved efficacy in the treatment of metastatic prostate cancer (CaP) has sparked an area of intense research. Although current docetaxel-based regimens offer increased survival over previous standard therapies for men with castration-resistant metastatic CaP, this only results in an improvement in median survival of 2–3 months, with a median overall survival of only 18–19 months (Petrylak et al., 2004; Tannock et al., 2004). Novel therapies with greater efficacy, either alone or in combination with docetaxel, are needed to further benefit afflicted men.

The Src family kinases (SFK) represent a nine-member group of non-receptor tyrosine kinases. Of this family, src is most extensively characterised. It is involved in cellular proliferation, survival, motility and angiogenesis (Yeatman, 2004). Src, as well as lyn, another SFK member, are overexpressed in CaP, and increased src expression correlates with disease progression (Goldenberg-Furmanov et al., 2004; Asim et al., 2008).

Src also regulates osteoclast function (Miyazaki et al., 2006). Src signalling is instrumental in formation of the sealing zone between the osteoclast and bone matrix, which is necessary for bone resorption (Destaing et al., 2008). Further support of src involvement in the regulation of osteoclast activity is demonstrated by the osteopetrotic phenotype observed in src knockout mice despite morphologically normal appearing osteoclasts (Soriano et al., 1991). Additionally, src inhibition was also reported to increase apoptosis of mature osteoclasts both in vitro and in vivo (Recchia et al., 2004).

The role of src in bone resorption is particularly relevant to CaP, which has a proclivity to metastasise to and invade bone (Roudier et al., 2003). CaP metastases are mainly osteoblastic in character; however, increased bone destruction is also associated with growth of CaP cells in the bone environment (Morrisey et al., 2007). Moreover, current treatment for advanced CaP patients includes androgen deprivation, which induces loss of bone mineral density (BMD; Galvão et al., 2008).

Dasatinib is an orally available inhibitor of Bcr-Abl kinase and SFK proteins and is FDA approved as a second line treatment for chronic myelogenous leukaemia and Philadelphia chromosome-positive acute lymphoblastic leukaemia. Recently, dasatinib has been shown to inhibit proliferation, cell adhesion, migration and invasion of CaP cells in vitro (Lombardo et al., 2004; Nam et al., 2005). Furthermore, dasatinib decreased the growth of PC-3 CaP cells injected orthotopically, reduced the number of metastases and inhibited growth at the metastatic site (Park et al., 2008; Yano et al., 2008).

CaP is a disease of significant heterogeneity and has historically been resistant to cytotoxic chemotherapies. Therefore, there is a substantial need to discover therapies that offer additive or synergistic properties (McCarty, 2004). Given the in vitro and preclinical activity of dasatinib, the frequency at which CaP metastasises to bone, and the role of src in the regulation of osteoclast function, we sought to determine the ability of dasatinib, alone and in combination with docetaxel, to inhibit the growth of CaP in bone and affect bone remodelling.
MATERIALS AND METHODS

Cell line

The C4-2B cell line (a gift from Dr Chung, Emory University) is a castration-resistant CaP cell line isolated from the bony metastasis of a mouse xenograft inoculated with C4-2 CaP cells, a subline of LNCaP CaP cells (Thalmann et al, 1994). C4-2B cells express androgen receptor, and when growing in the bone they elicit mixed osteolytic/osteoblastic reaction, which mimics the situation in human CaP bone metastases (Wu et al, 1998).

In vitro proliferation studies

C4-2B CaP cells were plated at a density of 200,000 cells per well in 2 ml of RPMI supplemented with 5% FBS. After 24 h, dasatinib (Bristol-Meyers-Squib Inc., Princeton, NJ, USA) was added to the cells at 20, 10, 5, 2.5, 1, and 0.5 μg/ml. PSA levels were measured directly from the media of treated and untreated cells (IMx Total PSA Assay; Abbott Laboratories, Abbott Park, IL, USA). Proliferation assays were performed in triplicate and repeated twice. PSA levels were measured directly from the media of treated and untreated cells (IMx Total PSA Assay; Abbott Laboratories, Abbott Park, IL, USA).

Western blot analysis

C4-2B cells were treated with 100 nM dasatinib for 0.5, 1, 2, 4, 6, and 24 h. Whole cell lysates were prepared as we described previously (Brubaker et al, 2003). Protein concentrations were determined with a BCA assay (Bio-Rad Lab., Hercules, CA, USA). Total protein (20–50 μg) were resolved on 4–20% SDS–PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were blocked for 2 h with 5% BSA in for phospho-Src or with 5% non-fat milk. Rabbit polyclonal antibody to phospho-[Y 416]-Src Family Kinase (SFK; 1:1000; Cell Signaling Technology, Danvers, MA, USA), to PSA (2 μg/ml; DAKO, Carpenteria, CA, USA) and to GAPDH (1:2000; Cell Signaling Technology) were used. Goat anti-rabbit antibodies conjugated to HRP (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL (Genscript, Piscataway, NJ, USA) were used. Statistical analyses were performed using unpaired student t-tests and ANOVA (Prism Graphpad; Graphpad Software, San Diego, CA, USA). Significant results were determined as a P ≤ 0.05.

Immunohistochemistry

Five-micron sections were used for H&E and IHC. IHC was performed with rabbit polyclonal anti-phospho-SFK antibody (1:100; Cell Signaling). Negative control slides were performed with rabbit polyclonal IgG (1:100).

Statistical analyses

Statistical analyses were performed using unpaired student t-tests and ANOVA (Prism Graphpad; Graphpad Software, San Diego, CA, USA). Significant results were determined as a P ≤ 0.05.

RESULTS

In vitro effects of dasatinib on C4-2B cells

Previous studies have shown that dasatinib inhibits proliferation of LNCaP, PC-3 and DU-145 CaP cells in vitro (Lombardo et al, 2004; Nam et al, 2005). The effects of dasatinib on C4-2B have not been previously evaluated, and therefore, we first examined the effects of dasatinib on these cells in vitro. Similar to other CaP cell lines, dasatinib significantly decreased proliferation of C4-2B cells in a dose-dependent fashion (ANOVA, P < 0.0001) with an IC50 value of 3536 nM (Figure 1A). Concordant with the inhibition of proliferation, PSA levels in the media were also significantly decreased (ANOVA, P < 0.0001). We also calculated the ng/ml of PSA per 10^5 cells, and these data show that PSA production per 10^5 cells was only minimally affected by dasatinib up to 3000 nM (decreases of 1–4%). However, with the highest concentration of dasatinib tested, PSA per ng/ml per 10^5 cells was significantly lower than control group receiving placebo treatment (n = 8, PSA = 4.376 ± 1.13 ng/ml) (mean ± s.e.m.); (2) a group receiving docetaxel at 5 mg kg^-1 (Morgan et al, 2008; n = 7, PSA = 6.507 ± 1.38 ng ml^-1); (3) a group receiving dasatinib only at 50 mg kg^-1 (n = 8, PSA = 6.136 ± 0.99 ng ml^-1) and (4) a group receiving a combination of dasatinib 50 mg kg^-1 and docetaxel 5 mg kg^-1 (n = 8, PSA = 6.045 ± 1.098 ng ml^-1). There were no differences in enrolment PSA levels (ANOVA, P = 0.5834). Dasatinib was administered via gavage once a day for 5 days a week. Docetaxel was administered via intraperitoneal injection every 2 weeks. Animals were killed 7 weeks after enrolment or if compromised. Blood samples were drawn weekly for determination of serum PSA levels. Radiographs (Faxitron Specimen Radiography System, Model MX-20; Faxitron X-ray Corporation, Wheeling, IL, USA) and BMD levels were assessed just before killing (PIXImus Lunar densitometer; GE Healthcare, Waukesha, WI, USA). Calcium levels were determined in sacrifice serum (Quantrichm Calcium Assay Kit; BioAssay Systems, Hayward, CA, USA). For analyses, one animal from the control group and one animal from the docetaxel alone group were excluded because the tumour invaded into the surrounding soft tissue based on palpation and high serum PSA level (PSA > 100 ng/ml) 6 weeks after enrolment.

Subcutaneous tumour models were also used for in vivo analysis. Approximately 2 x 10^6 C4-2B were injected subcutaneously into SCID mice. Once tumour volumes reached approximately 250 mm^3, animals were randomised to receive either placebo or dasatinib (50 mg kg^-1 gavage). Animals were killed 2 h after administration of drug and the tumours harvested. Half of each tumour was paraffin embedded and the other half flash frozen for molecular analysis. Five animals were examined from each group.

Immunohistochemistry

After killing, bothibia and subcutaneous tumours were fixed in 10% neutral buffered formalin for 24 h. The tumours were then decalcified for 48 h in 10% EDTA and then bothibia and subcutaneous tumours were processed for paraffin embedding. Five-micron sections where used for H&E and IHC. IHC was performed as we described previously (Kiefer et al, 2004) using the rabbit polyclonal anti-phospho-SFK antibody (1:100; Cell Signaling). Negative control slides were performed with rabbit polyclonal IgG (1:100).

In vivo studies

All animal procedures were performed in compliance with the University of Washington Institutional Animal Care and Use Committee and NIH guidelines. Intratibial injections were carried out as we described previously (Corey et al, 2002). Briefly, 6-week-old male SCID mice were injected with ~2 x 10^7 C4-2B cells into the proximal aspect of the right tibiae. A total of 48 animals were injected, and 4 weeks after the tumour cell injection animals that had serum PSA level > 0.6 ng ml^-1 (IMx Total PSA Assay) were randomised into four groups using the following design: (1) a
that of the control cells (44.2 ± 1.4%) but significant inhibition of proliferation was also noted at this concentration. These data suggest that dasatinib inhibits proliferation of C4-2B cells without largely affecting PSA production at concentrations between 12 and 3000 nM, although large decreases are seen with high concentrations of dasatinib (9000 nM).

We also show that dasatinib inhibited SFK activation in C4-2B cells. The inhibition of phospho-SFK formation was detected 30 min after treatment with 100 nM dasatinib and was sustained up to 24 h (Figure 1D). However, this western blot does not allow for determinations regarding the specific phosphorylation status of individual members of SFK.

It has been previously reported that lyn has a prominent role in the control of proliferation of PC-3 CaP cells in vitro whereas src played a more significant role as a mediator of migration (Park et al, 2008). Therefore in our subsequent experiments, we investigated the effects of dasatinib on phosphorylation of lyn and src, specifically, in C4-2B cells. We used a combination of immunoprecipitations (IP) with specific anti-lyn and src antibodies to pull out these proteins and a western blot with the anti-phospho-SFK Tyr416 antibody because there are no specific antibodies for phospho-lyn or phospho-src. Our IP results show that in C4-2B cells, dasatinib inhibited phosphorylation of src, although phosphorylation of lyn was unaffected (Figure 1C). We also show that treatment with dasatinib did not affect protein levels of src and lyn (Figure 1C).

**In vivo effects of dasatinib on tumour growth in bone and bone remodelling**

Administration of dasatinib significantly decreased serum PSA levels in tumour-bearing animals when compared to tumour-bearing control animals (Figure 2). Normalised values of serum PSA levels at killing to the enrolment serum PSA levels in animals treated with dasatinib were 2.3 ± 0.4 vs 9.2 ± 2.1 in the control animals (P = 0.004). Sacrifice serum PSA levels in animals treated with dasatinib in combination with docetaxel were 1.02 ± 0.2, which is significantly lower than PSA levels in dasatinib-alone-treated animals (P = 0.010) and control animals (P < 0.001). Administration of docetaxel decreased sacrifice serum PSA levels to 6.1 ± 0.7 but these differences did not reach significance (compared to control animals; ~30% decrease, P = 0.22). To further evaluate tumour burden, we also compared the weight of treated and untreated tumoured tibiae excised after killing. There were decreases in the weight of the treated tumoured tibiae vs control tumoured tibiae (expressed as percentage of control/docetaxel: 58.9 ± 18.4%, dasatinib: 65.0 ± 10.8% and dasatinib + docetaxel: 41.5 ± 3.6%). However, these results did not reach significance due to a large variation of the values in the control group. Representative radiographs taken before killing are shown in Figure 2B.

We have previously published that despite the stimulation of bone formation by the growth of C4-2 cells in the tibia, tumoured tibiae showed decreases in BMD and trabecular bone volume (Pfitzenmaier et al, 2003; Morrissey et al, 2007). Knowing the significant role src plays in the regulation of osteoclast activity, we, therefore, examined alterations in BMD in tibiae of animals treated with dasatinib. The growth of C4-2B cells in the tibiae of untreated control animals resulted in ~20% decrease in BMD vs contralateral non-tumoured tibiae (Figure 3B). Our results show that treatment with dasatinib resulted in 25 ± 1.4% greater BMD of tumoured tibiae over control tumoured tibiae (P < 0.001; Figure 3A). In this experiment, treatment with docetaxel did not significantly affect BMD of tumoured tibiae vs control tumoured tibiae (P = 0.96). We also compared loss of BMD between tumoured and contralateral non-tumoured tibiae in the untreated...
Dasatinib alone and in combination with docetaxel significantly inhibited this loss of BMD (P<0.001; Figure 3B). Tumoured tibiae treated with dasatinib showed only an 8% loss in BMD compared to contralateral non-tumoured tibiae. Again, docetaxel alone did not affect the loss in BMD between contralateral tibiae (22% loss in BMD). Offering further proof of inhibition of osteolysis, on H&E stains, we show increased bone formation in tumoured tibiae receiving treatment with dasatinib alone and in combination (Figure 3D).

The inhibition of osteolysis by dasatinib was not limited to the tumoured tibiae. When we compared the BMD of non-tumoured tibiae of those animals treated with dasatinib alone and in combination vs non-tumoured tibiae of the docetaxel-treated and control animals, our results showed that dasatinib caused increased BMD of the normal tibiae of treated animals (10±1.9%, P=0.0007). Examination of BMD of the entire animal revealed an increase of 6±1.2% (P=0.001) in the dasatinib and combination therapy-treated animals over the docetaxel-treated and control animals. The systemic inhibition of osteolysis by dasatinib was further confirmed by the significant decreases in calcium levels in sacrifice serum from those animals receiving dasatinib and combination therapy vs control and docetaxel-alone-treated animals (dasatinib alone and in combination: 1.34±0.09 mmol l⁻¹; docetaxel alone and control animals: 1.61±0.06 mmol l⁻¹; P=0.027).

Immunohistochemistry of C4-2B subcutaneous tumours

To demonstrate that dasatinib inhibits SFK phosphorylation in vivo, we first stained intratibial tumours; however, the process of decalcification negatively affects the stability of phosphorylated proteins and we obtained negative results. Therefore, as an alternative, we have chosen to perform IHC on subcutaneous tumours treated with the same dose of dasatinib. Our results show strong immunoreactivity of phospho-SFK in the control C4-2B subcutaneous tumours, and minimal staining in the dasatinib-treated tumours (Figure 2C). Appropriate negative controls exhibited no immunoreactivity (data not shown). ×10 magnification.

DISCUSSION

Therapy for patients with metastatic CaP typically involves androgen deprivation. Although this therapy is effective initially, most patients will ultimately develop castration-resistant CaP. At that point, the current standard of care is chemotherapy with docetaxel, which offers an incremental survival advantage for these patients. This underscores the need for additional therapies in treating patients with metastatic CaP. A potential avenue for improving outcomes for patients with metastatic CaP involves the use of small molecule inhibitors of signalling pathways either alone or in combination (McCarty, 2004). SFK inhibitors fall into this
category and there are several of these being evaluated in both the preclinical and clinical setting. As src levels are increased in advanced CaP and possibly even more so in castration-resistant disease, we sought to investigate the efficacy of a src inhibitor, dasatinib, in treating CaP growth in bone.

We have demonstrated that dasatinib inhibits proliferation of C4-2B CaP cells in vitro. Most importantly, we showed that PSA production is not affected in the absence of growth inhibition. This point is critical to our in vitro studies as it allows for PSA levels to be used as a surrogate marker for tumour growth in bone in response to this therapy. Also of interest, despite recent literature demonstrating the importance of the SFR member, lyn, in regulating proliferation of PC-3 CaP cells (Park et al, 2008), we show that lyn phosphorylation is unaffected in C4-2B cells on treatment with dasatinib. Thus implying that lyn inactivation is not involved in the inhibition of proliferation by dasatinib in these cells.

Our in vivo studies have indicated that dasatinib decreased tumour growth in the bone environment. First, we used serum PSA levels to evaluate effects on tumour growth, and our data showed that dasatinib caused decreases in serum PSA. This effect is important, as PSA levels are the major surrogate marker of anti-tumour response in tumours growing intratibially while under treatment. Although it is generally accepted that in primary disease PSA correlates with tumour volume, it is also noted from preclinical and clinical data that in advanced castration-resistant CaP, changes in serum PSA levels do not necessarily reflect changes in tumour volume. We believe, however, that our in vitro results allow us to extrapolate that the decreases in serum PSA levels seen in mice bearing intratibial tumours are synonymous with decreases in tumour volume. To further confirm dasatinib inhibition of tumour growth by means other than serum PSA levels, we examined the weight of the tibiae with tumour. The decreases in weight of the tibiae observed in those groups receiving either docetaxel, dasatinib or a combination compared to the control group help to substantiate the claim that treatment resulted in decreased tumour burden. This may be especially accurate for dasatinib (alone or in combination)-treated tibiae where decreases in tibiae weight were seen despite increases in BMD, which implies that the weight loss is related to decreases in tumour volume and not bone destruction. The increased BMD of the groups receiving dasatinib alone or in combination may also be a result of decreased tumour growth and not solely related to the effect that dasatinib has on regulating osteoclast activity. Altogether, these data indicate that dasatinib has a significant cytotoxic effect on CaP cells in the bone environment.

An issue to mention is the lack of significant effects seen in the docetaxel monotherapy group. Our previous work with docetaxel had shown that the dose of 5 mg kg⁻¹ to be an efficacious dose, albeit those studies were performed on a different cell line. In this study, the docetaxel when used alone did slow tumour growth by ~30%, but these results did not reach significance. However, when docetaxel was used in combination with dasatinib, the dose resulted in significant decreases in PSA levels beyond those seen with the administration of dasatinib alone. We have previously seen this increased efficacy with the use of other pharmaceuticals in combination with docetaxel over either agent alone (Brubaker et al, 2006; Morgan et al, 2008). In addition, src inhibition has been reported to increase sensitivity of other advanced cancers to several already used chemotherapies and specifically to docetaxel resistance (Yezhelyev et al, 2004; Han et al, 2006). This phenomenon may have important implications for patient care as of the efficacy of docetaxel therapy may be enhanced with the addition of dasatinib. Nonetheless, final conclusions regarding effects of dasatinib in this regard will require additional clinical investigations.

Src is an important player in forming the cell–cell junctions necessary for bone resorption to take place, and it has been clearly shown that inhibition of src activity decreases bone osteolysis. However, our results are the first to show that dasatinib treatment results in increases in BMD of normal bone as well as increases in BMD of bone harbouring an osteolytic tumour. The ability of dasatinib to increase BMD may prove to be beneficial to patients with CaP where skeletal related events (SRE; ie, pathologic fractures, spinal cord compression) and bone pain are a significant source of morbidity. Additionally, even patients without bone metastases suffer an increased risk of osteoporotic fractures from the effects of androgen deprivation. Agents that can cause increases in BMD have been investigated in metastatic prostate cancer. The bisphosphonate, zoledronic acid, has been shown to increase time to first SRE as well as reduced the number of SREs in patients with castration-resistant metastatic prostate cancer (Saad et al, 2004). Inhibition of RANKL has also been shown to increase BMD in experimental prostate cancer bone metastasis models (Ignatowski et al, 2008) and inhibitors (AMG162, denosumab) are currently in clinical trials. These agents have been shown to affect tumour growth mainly via indirect mechanisms by inhibiting bone lysis. In contrast, decreasing activity of src has been shown not only to inhibit osteolysis but also directly inhibit tumour growth. Therefore, the use of compounds such as dasatinib has the potential to provide pronounced benefit for metastatic prostate cancer patients.
In summary, our results indicate that dasatinib inhibits the growth of CaP in bone, and these inhibitory effects are increased in combination with docetaxel. Additionally, our study demonstrates decrease in osteolysis in dasatinib-treated animals, both in the tumour as well as normal bone. Our results suggest that dasatinib alone or in combination should be further investigated in a clinical setting for patients with metastatic CaP, and these studies are currently ongoing.

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