Dasatinib Is Preclinically Active against Src-Overexpressing Human Transitional Cell Carcinoma of the Urothelium with Activated Src Signaling

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Abstract

Dasatinib is an orally administered multitargeted kinase inhibitor that targets Src family tyrosine kinases, Abl, c-Kit, and PDGFR. A preclinical study was conducted to evaluate dasatinib alone or combined with cisplatin for human transitional cell carcinoma (TCC). Expression of Src in a human TCC tissue microarray was evaluated by immunohistochemistry. The activity of dasatinib and/or cisplatin was evaluated in six human TCC cell lines. Western blot was done to assess Src and phosphorylated-Src (p-Src) expression. The activity of dasatinib alone and in combination with cisplatin was determined in murine subcutaneous xenografts. Sixty-two percent to 75% of human TCC expressed Src. Dasatinib displayed significant antiproliferative activity at nanomolar concentrations against two human TCC cell lines (RT4 and Hu456) that exhibited high Src and p-Src expression and were cisplatin-resistant. RT4 cells were the most sensitive and displayed the highest level of Src pathway activation (p-Src/Src ratio). Dasatinib downregulated p-Src in either sensitive or resistant cells. TCC cells that were sensitive to cisplatin (5637 and TCC-SUP) were highly resistant to dasatinib and exhibited low Src expression. Dasatinib showed antitumor activity in RT4 murine xenografts, and the combination of dasatinib and cisplatin was significantly more active than placebo. Combination dasatinib plus cisplatin significantly inhibited proliferation and promoted apoptosis in vivo. In conclusion, dasatinib displayed significant preclinical antitumor activity against Src-overexpressing human TCC with active Src signaling and was highly active in combination with cisplatin in vivo. Further clinical development might be warranted in selected human subjects. Mol Cancer Ther; 9(5); OF1–8. ©2010 AACR.

Introduction

There will be ∼71,000 new cases of bladder cancer this year in the United States, of whom ∼25% will be muscle-invasive or metastatic (1). Front-line chemotherapy for metastatic transitional cell carcinoma (TCC) of the urothelium with cisplatin-based combinations including methotrexate, vinblastine, doxorubicin, cisplatin, or gemcitabine and cisplatin yields a median survival of ∼15 months (2). Although the response rate with these regimens is 40% to 70%, subsequent progression is universal and salvage chemotherapy is relatively ineffective (3). A significant proportion of patients are ineligible to receive optimal cisplatin-based combination chemotherapy due to renal dysfunction or poor performance status. The improvement in outcomes with neoadjuvant cisplatin-based combination chemotherapy for locally advanced muscle-invasive bladder cancer is modest (4). Therefore, efficacious and tolerable systemic agents need to be discovered for TCC.

The Src family kinases are non-receptor tyrosine kinases believed to play critical roles in tumor growth, angiogenesis, invasion and dissemination (5). The novel Src family and Abl kinase inhibitor, dasatinib, is a promising therapeutic agent with oral bioavailability. Dasatinib is approved by regulatory agencies for and has significant activity in Bcr/Abl-dependent chronic myeloid leukemia resistant or intolerant to imatinib (6). Dasatinib has been shown to have preclinical activity against human prostate, breast, pancreatic, lung and head and neck cancer, mesotheliomas and sarcomas dependent on Src kinase (7–14). Dasatinib inhibited cisplatin-induced Src phosphorylation in lung cancer cells (11). Cisplatin resistance induced by v-src transfection of gallbladder carcinoma cells was reversed by inhibitors of Src (15). Additionally, Src inhibition sensitizes ovarian cancer cells to both paclitaxel and platinum agents (16–18).

In vitro evaluation of human urothelial cancer cells showed that serum starvation promoted the activation...
of the Src tyrosine kinase (19). PP2, a Src family kinase inhibitor, elicited concentration-dependent growth inhibition in all bladder cancer cell lines (20). In another report, the tyrosine kinase activity of pp60c-Src was elevated in a subset of human bladder carcinomas compared with normal bladder mucosa (21). These data support a rationale to preclinically evaluate dasatinib alone and in combination with cisplatin chemotherapy for the therapy of TCC. Preclinical data may facilitate the selection of appropriate patients with TCC for the evaluation of dasatinib alone and in combination with cisplatin.

Materials and Methods

Human TCC tissue microarray analysis. Formalin-fixed, paraffin-embedded specimens were available from 121 patients with T1 to T3 pathologic stage TCC who underwent radical cystectomy between 1995 and 2002 at Baylor College of Medicine-affiliated hospitals. Tumor tissue was cored (one tissue cylinder, 2 mm) and a microarray was constructed using the manual tissue arrayer (Beecher Instruments Microarray Technology). Immunohistochemistry for Src protein was done on the microarray to determine expression (0 to 3+) by using the Image-Pro Plus Software (Media Cybernetics, Inc.). The Src antibody used (Cell Signaling Technology) was derived from the rabbit and has specificity against Src, but could cross-react with other Src family proteins.

Cell culture and reagents. Six human TCC cell lines were evaluated: 5637, TCC-SUP, RT4, T24, RT112, and Hu456. 5637, TCC-SUP, RT4, and T24 cells were obtained from American Type Culture Collection. 5637 was cultured as a monolayer in RPMI 1640 (Invitrogen) supplemented with 2.5 g/L of glucose, 2 mmol/L of L-glutamine, 10 mmol/L of sodium pyruvate, 10 mmol/L of HEPES, 10% fetal bovine serum, and 1% penicillin-streptomycin. TCC-SUP was cultured as a monolayer in Eagle’s MEM (Invitrogen) with 10% fetal bovine serum, and 1% penicillin-streptomycin. RT4 and T24 cells were cultured as a monolayer in McCoy’s 5a medium (Invitrogen) and kept at 37°C with 5% CO2. Cell cycle and apoptosis assays. RT4 cells were passed at 80% confluence every 2 to 3 days for a total of three times to ensure exponential growth prior to use in the tumor experiments. The cells were trypsinized, washed in sterile PBS, and resuspended in RPMI 1640 at 106 cells/mL. Immediately prior to injection, cells were mixed (1:1) with Matrigel (BD Biosciences) and 5 × 106 cells were injected s.c. (100 μL/mouse) into one site of the flanks of 6- to 8-week-old female athymic nude mice (Charles River, Inc.). Mice were housed in barrier plastic cages and maintained on a 12-hour light/dark cycle with free access to food in a pathogen-free animal facility at Baylor College of Medicine.

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of Medicine. Mice developing palpable subcutaneous tumors were randomized to the treatment and control groups based on the tumor volume measurements. Tumor growth was followed by measuring the size of the tumors with a caliper, two to three times per week. Tumors were measured in two dimensions and tumor volume was estimated using the formula: $m_1^2 \times m_2 \times 0.5236$, where $m_1$ is the length of the short axis and $m_2$ is the length of the long axis. After 4 weeks, the experiment was terminated and tumors resected and prepared for histologic examination as described below.

**Administration of agents to mice.** All experiments involving animals were done under approved protocols granted by the Institutional Animal Care and Use Committee of Baylor College of Medicine. In a pilot study, two cohorts of five mice each bearing subcutaneous RT4 tumors were randomly assigned to groups with similar average tumor sizes. The two cohorts were treated as follows: no treatment (control) or dasatinib at 5 mg/kg/d once daily for 5 days a week for 4 weeks by oral gavage in a volume of 0.1 mL. In a second study, four cohorts of 12 to 13 mice per group were given either placebo p.o. once daily plus placebo intraperitoneally once weekly, dasatinib 5 mg/kg once daily plus placebo i.p. once weekly, placebo once daily p.o. plus cisplatin 4 mg/kg/wk i.p. once weekly or the combination of dasatinib 5 mg/kg once daily plus cisplatin 4 mg/kg i.p. once weekly. All treatments were administered for 4 weeks and oral therapy was administered for 5 days a week.

**Immunohistochemistry of murine tumors.** Xenograft tumors removed from mice were fixed in formaldehyde and embedded in paraffin. Expression of Ki-67, cleaved caspase-3, and CD31 in histologic sections were analyzed by immunohistochemistry using antibodies from Dako in five randomly selected murine xenografts from each group. Appropriate positive and negative controls were also stained. Four high-power fields (400×) were examined per tumor. Each image was interpreted for immunoreactivity using a 0 to 3+ scoring system for both the intensity of stain and percentage of positive cells by using the Image-Pro Plus Software (Media Cybernetics). Cells were considered positive for Ki-67 if they displayed 3+ expression, whereas cells with any expression of cleaved caspase-3 and CD31 were considered positive.

**Statistical analysis.** The significance of differences was determined by Student’s t test or one-way ANOVA measurement. Comparisons of inhibiting tumor growth between treatment groups or the control were done using one-way ANOVA and Tukey-Kramer HSD test done using JMP 5.1.2 statistical software package (SAS Institute). $P$ values were considered significant by Student’s t test, Tukey-Kramer HSD test, and if $P \leq 0.05$.

**Results**

**Immunohistochemistry of human TCC tissue microarrays showed src expression.** Src expression of 2+ or 3+ determined by immunohistochemistry was found in the vast majority of TCC tumors from 121 patients with pathologic stages T2 to T4 disease (Fig. 1). The adjacent non-tumor bladder tissue commonly did not express detectable levels of Src. The level of expression varied from ~62% of T2 stage cases to 75% of T4 cases in each stage group. No statistical differences or correlations were found between pathologic stage and level of Src expression.

**Bladder TCC cell lines that show upregulated Src signaling are inhibited by dasatinib.** Both total Src and the corresponding activated phosphorylated-Src (p-Src) was determined by Western analysis. Src and p-Src protein expression seemed to be expressed at the highest levels in Hu456, RT112, and RT4 cells, whereas 5637, T24, and TCC-SUP cells showed lower levels of expression (Fig. 2A). Among the higher Src expressing TCC cell lines, the p-Src to total Src ratio was highest in RT4 cells, indicating a highly activated Src signaling...
pathway (Fig. 2B). Therefore, the RT4 cell line was selected to evaluate the modulation of Src and p-Src after exposure to dasatinib. Additionally, RT112 cells were also chosen to evaluate modulations by dasatinib, given the high Src expression coupled, in contrast to RT4 cells, with a lower level of Src activation and relative resistance of cell viability to dasatinib (see next section). Src activation (i.e., the level of p-Src), but not total Src expression, was correspondingly inhibited by low nanomolar concentrations of dasatinib in both RT4 and RT112 cell lines (Fig. 2C).

**Dasatinib inhibits viability of high Src-expressing cells in vitro and induces a G<sub>0</sub>-G<sub>1</sub> arrest.** Dasatinib reduced cell viability (MTT assay) at a relatively low IC<sub>50</sub> concentration of ~100 nmol/L in both RT4 and Hu456 cell lines, whereas the other cell lines were less sensitive to dasatinib with IC<sub>50</sub> >1,000 nmol/L (Fig. 3A). Given the high sensitivity of RT4 cells to concentrations of dasatinib attainable in vivo and the earlier demonstration of the highest level of activation of Src signaling (i.e., high p-Src/total Src ratio) in RT4 cells, we chose the RT4 cell line for further evaluation of the activity of dasatinib with or without cisplatin. Both RT4 and Hu456 cells were highly resistant to cisplatin (IC<sub>50</sub> >10 μg/mL), whereas 5637 and T24 cells were sensitive (Fig. 3B). Dasatinib combined with cisplatin showed additive inhibition of RT4 cells in vitro (Fig. 3C). Dasatinib (100 nmol/L) induced a G<sub>0</sub>-G<sub>1</sub> cell cycle arrest in RT4 cells after 24 and 48 hours of exposure (Fig. 3D). Significant increases of apoptosis of RT4 cells were not detected by 100 or 500 nmol/L of dasatinib (at 48 or 72 hours) compared with untreated controls (data not shown). Given the absence of activity against cell viability of cisplatin and the lack of synergistic activity of combination cisplatin-dasatinib against viability (Fig. 3C), evaluation of the antiapoptotic activity of the combination was not considered warranted.

**Combined dasatinib and cisplatin chemotherapy shows significant in vivo antitumor efficacy against RT4 xenografts.** In an initial pilot experiment using five mice per group bearing established RT4 tumors, mice treated with 5 mg/kg of dasatinib orally once daily displayed a trend for improved antitumor activity compared with controls treated with solvent only (data not shown). We repeated this experiment with 12 to 13 mice per group and compared the effect of monotherapies (dasatinib alone at 5 mg/kg/d or cisplatin alone at 4 mg/kg/wk) or combined therapy (dasatinib 5 mg/kg/d and cisplatin...
4 mg/kg/wk) with mock-treated controls (vehicle only; Fig. 4). Tumor growth was analyzed by one-way ANOVA at day 28 after the beginning of treatment. There was a significant difference between treatment groups \((F = 3.84, df = 3; P < 0.02)\) that showed the combination drug therapy to be significantly better at reducing tumor growth than the no treatment control. No significant differences were seen between control and the monotherapies or between the monotherapies themselves (Fig. 4).

**Combination therapy with dasatinib and cisplatin increases apoptosis and reduces cell proliferation in RT4 xenografts.** Treatment with the combination of dasatinib plus cisplatin resulted in a significant increase in apoptosis (measured as cleaved caspase-3 expression) when compared with mock-treated controls (one-way ANOVA: four high-powered fields from each of three to four tumors, \(n = 15\) total for each therapy; \(F = 9.67, df = 3; P \leq 0.0001\)), whereas monotherapy with cisplatin or dasatinib showed a trend towards increased apoptosis but were not significantly different from the mock-treated control tumors (Fig. 5). Correspondingly, cellular proliferation (measured by Ki-67 expression) was significantly reduced when the combination of dasatinib plus cisplatin was compared with cisplatin alone or the untreated control (one-way ANOVA: four high-powered fields from
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Figure 5. Immunohistochemical quantification of apoptosis, proliferation, and angiogenesis from resected RT4 tumor xenografts at 4 weeks. RT4 xenograft tumors removed from mice, treated as described in Fig. 4, were analyzed for expression of Ki-67, cleaved caspase-3 and CD31 by immunohistochemistry. Four high-power fields (400×) were examined per tumor. Each image was interpreted for immunoreactivity using a 0 to 3+ scoring system for both the intensity of stain and percentage of positive cells by using the Image-Pro Plus Software (Media Cybernetics). Cells were considered positive for Ki-67 if they displayed 3+ expression. Cells with any level of caspase-3 or CD31 expression were considered positive. Data were expressed as a percentage of control staining. Error bars represent the SEM of independent high-powered fields within each treatment group.

Discussion

This study shows the significant preclinical antitumor activity (both in vitro and in vivo) of dasatinib in human TCC cell lines in which Src is active at constitutively high levels. Among the high-Src expressing cells (RT4, Hu456, and RT112), higher ratios of p-Src/total Src reflecting Src activation (RT4 cells) seemed to be predictive for sensitivity to dasatinib. In contrast, cells with high Src but relatively low p-Src (RT112) were relatively resistant to dasatinib, despite downregulation of p-Src by exposure to dasatinib. This suggests that the baseline level of expression and activation of the Src pathway might be more predictive for benefit from dasatinib than dasatinib-induced downregulation of the pathway. Preclinical studies in other malignancies have also shown p-Src inhibition to be predictive for the activity of dasatinib (25, 26). Although dasatinib significantly reduced overall viability and proliferation of RT4 cells, apoptosis was not increased. This suggests that dasatinib is probably best used as an adjunct in combination with an apoptosis-inducing agent. These findings also imply that Src signaling might be an important factor driving the growth of a subset of TCC tumors. Additionally, this study showed that a dose of dasatinib that attains low nanomolar concentrations in vivo could be effective at inhibiting the growth of Src-expressing TCC when combined with one of the current standard chemotherapeutic agents, cisplatin.

In a tissue microarray of TCC tumors from 121 patients with pathologic T2 to T4 disease, the vast majority (62–75%) expressed Src (2+ or 3+), whereas adjacent nontumor bladder tissue showed little or no expression. We have reported only 2+/3+ intensity because we believe these to be more relevant than reporting all levels of expression. When examining by 1+ to 3+ expression, tumors from all stages expressed Src and discrimination between different stages was not possible. Intriguingly, higher stages seemed to numerically express Src less commonly than lower stages, but statistically significant differences were not found, probably due to the small numbers of tumors per stage. Src expression decreased with higher TCC tumor stage in another recent study, which suggests that its inhibition may be more relevant in patients with lower tumor stages (27). Although the level of activated Src signaling (i.e., p-Src) in human tumor specimens is of great interest, we did not perform these studies due to the lack of fresh or frozen tumor tissue. The immunohistochemical studies were done in a tissue microarray of formalin-fixed, paraffin-embedded tumors and the estimation of phosphorylated proteins in such tissue is probably unreliable. We have also been unable to correlate the level of Src expression with clinical outcomes because clinical outcome data are insufficient at this time (but future studies of this important issue are planned). It is noteworthy that a biomarker could be predictive of benefit from an agent, without being prognostic.

The inverse correlation of in vitro sensitivity of TCC cells to cisplatin and dasatinib is intriguing and suggests that these agents target different subsets of cells. Given that low Src-expressing TCC cells (TCC-SUP and 5637) were more sensitive to cisplatin, it may be postulated that inhibition of Src signaling may render cells more sensitive to cisplatin. Indeed, another preclinical study has shown that Src induced cisplatin resistance in gallbladder carcinoma cells, which was reversed by herbimycin A or radicicol (Src family inhibitors; ref. 15). In this preclinical study, Src seemed to induce repair of cisplatin-DNA interstrand cross-links (15). However, the combination of dasatinib and cisplatin in vitro was additive and not synergistic against the high-Src expressing RT4 cell line. Therefore, Src-overexpressing TCC cells may be resistant to cisplatin independent of Src signaling. Although cell lines in vitro are relatively biologically homogeneous, in vivo tumors are more heterogeneous. This might explain the significant enhancement of outcomes with the combination of cisplatin and dasatinib in vivo against a Src-overexpressing cell line derived tumor, whereas monotherapy did not attain statistical significance for improved outcomes compared with controls. Cisplatin also has antistromal effects in vivo that confer antitumor activity and this may have contributed to significant efficacy for the combination in vivo by targeting different components of the tumor. Dasatinib has previously shown increased preclinical activity in combination with...
multiple different chemotherapeutic agents in different malignancies (11, 28, 29). Immunohistochemistry of a subset of randomly selected RT4 xenografts showed significant antiproliferative and proapoptotic activity for the combination of dasatinib and cisplatin compared with placebo-treated controls. Antiangiogenic activity was numerically higher with the combination compared with placebo-treated controls, but the difference was not statistically significant, probably due to the small sample size of five mice per group examined by immunohistochemistry.

Although we have shown significant preclinical antitumor activity for dasatinib in Src-overexpressing TCC cells with activated Src signaling, the inhibition of Src signaling and the presence of the target in a significant proportion of human TCC tumors, we raise some caveats and limitations of our experiments. Activity in a larger panel of cell lines, more animal models, and autotrophic or orthotopic models might better predict for clinical activity in human subjects with TCC. We only evaluated concurrent therapy with dasatinib and cisplatin and a determination of the sequence dependence of antitumor activity was beyond the scope of our project. Given that dasatinib induced G0-G1 arrest, it is possible that dasatinib may take some cells out of their cycle and render them unavailable for cisplatin killing. Therefore, a sequence of dasatinib followed by cisplatin after an interval could potentially be more efficacious. Additionally, dasatinib has been shown in other studies to inhibit cell migration and invasion at an earlier time point before inhibiting cell proliferation, and the investigation of these properties was also beyond the scope of our project (10, 12). It is unclear that inhibition of Src signaling could alter the natural history of human subjects with TCC. Although our preclinical data suggests that high Src expression coupled with activated Src-signaling predicts for the activity of dasatinib, inhibition of other molecular targets dependent on Src signaling may also explain this association. Additionally, inhibition of other unevaluated targets not associated with Src may also partly account for activity, e.g., the Btk tyrosine kinase, which has been found to be another major target of dasatinib (30). Ovarian cancer cell lines with high expression of Yes, Lyn, EphA2a but not Src, FAK, Kit, or PDGFR-β were sensitive to dasatinib, which suggests a potential role for other molecular targets (18). However, there is no evidence supporting the expression of other key targets of dasatinib, Kit and Abl, in TCC. Intriguingly, a recent preclinical study suggests the beneficial antimitotic effect of Src in TCC with the finding that phosphorylation by Src enhanced metastasis suppression by RhoGDI2 (27). Therefore, the further validation of Src as a molecular target at least in a subset of patients with TCC is warranted.

Clinical trials are evaluating the antitumor activity of dasatinib in a broad array of solid malignancies. Additionally, osteolytic activity of osteoclasts is inhibited by dasatinib, although it is unclear if this activity is dependent on the targeting of Src (31). Recent clinical studies corroborate these data and reveal biological activity in the setting of chemo-naïve patients with metastatic castration-resistant prostate cancer with frequent declines in markers of osteolysis (32). These data may be relevant in human subjects with metastatic TCC because this malignancy metastasizes to the bone quite frequently. Further thoughtful development of dasatinib for Src-overexpressing TCC, with a special focus on those with activated Src-signaling, might be warranted. A concurrent focus on elucidating the role of Src signaling in driving the growth of TCC and affecting clinical outcomes as well as sensitivity to dasatinib is necessary. Given the need for tolerable and convenient options, a rationale can be made to study dasatinib alone and in combination with cisplatin chemotherapy in appropriate patients with advanced TCC. Indeed, an ongoing trial led by the Baylor College of Medicine and the Hoosier Oncology Group evaluates 3 to 5 weeks of neoadjuvant dasatinib alone in patients with muscle-invasive TCC of the bladder followed by radical cystectomy. Analysis of baseline biopsy and post-therapy cystectomy tumor tissue for modulation of the Src pathway and downstream proliferation, apoptosis, and angiogenesis will be conducted to obtain signal activity. Additionally, this trial will permit the correlation of biological activity with the level of baseline expression of Src and Src signaling to determine their predictive ability. If signal activity is detected, further rational development of dasatinib in the metastatic setting may be warranted in appropriately selected patients with TCC.

Disclosure of Potential Conflicts of Interest

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