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Src as a therapeutic target in biliary tract cancer

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Abstract
Src, a non-receptor tyrosine kinase, is involved in a number of cancer-related signaling pathways and aberrantly activated in biliary tract cancer (BTC). This study aimed to elucidate the potential role of Src as a therapeutic target in BTC. We tested bosutinib, an orally active c-Src/Abl kinase inhibitor, alone or in combination with cytotoxic agents using nine human BTC cell lines: SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, SNU-1196, HuCCT1, TFK-1, and EGI-1. Of these, SNU-308 and SNU-478 were relatively sensitive to bosutinib. Bosutinib abrogated phosphorylation of Src and its downstream molecules, significantly increased G1 cell-cycle arrest and apoptosis. Bosutinib significantly inhibited cell migration and invasion and decreased epithelial-mesenchymal transition markers. Bosutinib combined with gemcitabine or cisplatin showed synergistic anti-proliferative and anti-migratory effects. In addition, this combination further inhibited phosphorylation of Src and its downstream molecules and decreased epithelial-mesenchymal transition marker expression compared with bosutinib alone. We established a SNU-478 xenograft model for in vivo experiments, since SNU-478 was more tumorigenic than SNU-308. Bosutinib combined with gemcitabine or cisplatin showed significantly more potent anti-tumor effects than bosutinib alone. Bosutinib combined with gemcitabine further decreased Ki-67 expression and Src phosphorylation, and further increased TUNEL expression. Our data suggest that Src might be a potential therapeutic target in BTC. Bosutinib demonstrated promising anti-tumor activity alone or in combination with gemcitabine or cisplatin in BTC cells, which supports further clinical development in patients with advanced BTC.
Introduction

Src, a non-receptor tyrosine kinase, is involved in a number of cancer-related signaling pathways including FAK, PI3K, ERK, and STAT3, and promotes the proliferation, adhesion, migration, invasion, and metastasis of cancer cells (1-4). Its activity is increased in a variety of malignancies such as lung, skin, colorectal, breast, ovarian, and head and neck cancer (3-6). In addition, Src activation conferred therapeutic resistance to imatinib treatment for chronic myelogenous leukemia (7, 8), hormone treatment for breast and prostate cancer (9, 10), and trastuzumab treatment for breast cancer (11, 12). On the basis of these findings, Src-targeted agents have recently been investigated in preclinical and clinical studies in a variety of malignant diseases.

Biliary tract cancer (BTC) is an relatively uncommon malignancy with a poor prognosis (13). Most patients are diagnosed at an advanced stage and experience relapse despite radical surgery (14). Although the recent development of targeted therapeutics has significantly improved the clinical outcome of patients with advanced solid tumors, still there is no validated therapeutic target for advanced BTC and the prognosis of patients with advanced BTC remains still disappointing (15, 16). In recent years, only gemcitabine plus cisplatin has been established as a standard chemotherapy for BTC patients (17). Therefore, there is still an urgent unmet need for the development of novel therapeutic strategies for the treatment of advanced BTC, based on specific targets on cancer cells.

A recent study demonstrated that Src is also frequently overexpressed in BTC as in other malignant diseases, although its relationship with clinicopathologic parameters or histologic origin was not significant (18). In addition, blocking Src activity by novel Src inhibitors such as saracatinib (AZD-0530) and AZM555130 reduced the proliferative and invasive potential of human BTC cell lines (18, 19). However, these studies used only a limited number of BTC cell lines and only tested Src inhibitor monotherapy, without investigating combination
strategies with other chemotherapeutic agents that are already approved for BTC treatment. Therefore, further preclinical studies are necessary before designing clinical studies using Src inhibitors.

The aim of our study was to investigate Src as a potential therapeutic target in BTC. We evaluated the therapeutic potential of bosutinib (4-anilino-3-quinolinecarbonitrile, also known as SKI-606) (20), an orally active small molecule c-Src/Abl kinase inhibitor, alone or in combination with cytotoxic agents using \textit{in vitro} and \textit{in vivo} models.
Materials and methods

Human BTC cell lines

A total of nine human BTC cell lines were used in this study. SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, and SNU-1196 cell lines were purchased from the Korean Cell Line Bank, Seoul, Korea on Mar. 2007 (21). HuCCT1 and TFK-1 cell lines were obtained from the RIKEN BioResource Center, Ibaraki, Japan on Mar. 2007. EGI-1 cell line was obtained from the Leibniz-Institut DSMZ (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany on Mar. 2007. The origin of each cell line was as follows: SNU-245, extrahepatic cholangiocarcinoma (common bile duct); SNU-308, gallbladder adenocarcinoma; SNU-478, ampulla of Vater adenocarcinoma; SNU-869, ampulla of Vater adenocarcinoma; SNU-1079, intrahepatic cholangiocarcinoma; SNU-1196, extrahepatic cholangiocarcinoma (hepatic duct bifurcation); HuCCT1, intrahepatic cholangiocarcinoma; TFK-1, extrahepatic cholangiocarcinoma; and EGI-1, extrahepatic cholangiocarcinoma (21-24). Most recent authentication of each cell lines was performed using ‘AmpFLSTR Identifiler PCR Amplification Kit (Catalog No. 4322288; Applied Biosystems, Foster, CA, USA)’ by the Korean Cell Line Bank on Mar. 8, 2016. The 3530xL DNA Analyzer (Applied Biosystems, Foster, CA) and the GeneMapper v5 (Applied Biosystems, Foster, CA) were used for DNA fingerprinting analysis. SNU-245, SNU-308, SNU-478, SNU-869, SNU-1079, SNU-1196, HuCCT1, and TFK-1 cell lines were maintained in RPMI-1640 media containing 10% fetal bovine serum (FBS; Welgene Inc., Gyeongsan, Korea) and 10 μg/mL gentamicin in a humidified atmosphere containing 5% CO₂ at 37°C. EGI-1 cell line was maintained in Dulbecco’s MEM supplemented with 10% FBS and 10 μg/mL gentamicin in the same condition.
**Tested agents**

Bosutinib was purchased from Selleck Chemicals LLC (Houston, TX, USA) for *in vitro* experiments and provided by Pfizer Inc. (New York, NY, USA) for *in vivo* experiments. The compound was dissolved in dimethyl sulfoxide (DMSO). Gemcitabine was purchased from Lilly Korea Co., Seoul, Korea. Cisplatin was purchased from JW Pharmaceutical Co., Seoul, Korea.

**Cell growth inhibition assay**

Cells were seeded in 96-well plates and exposed to increasing concentrations of targeted or cytotoxic agents for 72 h. After drug treatment, tetrazolium dye (MTT; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 4 h at 37°C. Then, the solution was removed carefully and DMSO was added. Cell viability was determined by measuring the absorbance at 540 nm with a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The half-maximal inhibitory concentration (IC$_{50}$) of chemotherapeutic agents was analyzed using SigmaPlot software (Systat Software, Inc., San Jose, CA, USA).

Matrigel (Trevigen, Gaithersburg, MD, USA) was used for three-dimensional cultures. Matrigel was thawed at 4°C and 200 μL was added per well to a 6-well plate. The plate was then incubated at 37°C for 30 min to promote gelling of the matrix. Next, harvested cells were mixed with 400 μL of 2% Matrigel, and then added into each well containing gelling matrix. After overnight incubation, each well was replaced by 2% Matrigel containing 0.1 or 0.5 μM bosutinib. Bosutinib-containing Matrigel was replaced every 4 days for 12 days.

**Western blot analysis**

Cells were treated with bosutinib for 48 h, and then lysed in RIPA buffer containing protease inhibitors on ice for 15 min. Next, protein obtained by centrifuge at 13,000 rpm for 20 min.
Equal amounts of proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed overnight at 4°C with primary antibodies. Primary antibodies against the following molecules were purchased from Cell Signaling Technology (Beverly, MA, USA): Src, phosphorylated Src (Tyr416), FAK, phosphorylated FAK (Tyr397 and Tyr925), AKT, phosphorylated AKT (Ser473), ERK, phosphorylated ERK (Thr202/Tyr204), STAT3, and phosphorylated STAT3 (Tyr705). Anti-Cyclin D, Cyclin E, Cyclin A, Cyclin B, p27, β-catenin, Lamin B, Vimentin, and Snail antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-β-actin antibody was purchased from Sigma-Aldrich. Antibody binding was detected using an enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ, USA). Anti-mouse and rabbit secondary antibodies were purchased from Thermo Scientific Inc. (Waltham, MA, USA). The data was normalized and quantified by ImageJ software (National Institute of Health, Bethesda, MD, USA).

**Cell-cycle analysis**

Cells treated with bosutinib at various concentrations for 24 h were harvested, fixed with cold 70% ethanol, and stored at −20°C. The fixed cells were harvested by centrifugation, dissolved in 20 mg/mL RNase (Invitrogen, Carlsbad, CA, USA), and incubated at 37°C for 10 min. Next, the cells were stained with 20 μg/mL propidium iodide (PI) (Sigma-Aldrich). The DNA content of 10,000 cells per each experimental group was analyzed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Three independent experiments were performed for each condition.

**Apoptosis assays**
After the cells were treated with 1.0 μM bosutinib, the degree of apoptosis was measured using Annexin V-FITC and PI double-staining according to the protocols of the manufacturer (BD Biosciences). The cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences). Early apoptosis was defined as Annexin V-FITC-positive and PI-negative, while late apoptosis was Annexin V-FITC- and PI-positive. Results are expressed as the mean value of three independent experiments.

**Migration and invasion assays**

For the migration assay, cells were grown as monolayers in 6-well culture plates. After 24h, confluent monolayers were gently scratched with a sterile 200-μL pipette tip. The plates were washed with phosphate-buffered saline, and then DMSO or 0.1 μM or 1.0 μM bosutinib was added to the wells in medium. After 72 h for SNU-308 cells and 24h for SNU-478 cells, cell movement back into the area of the scratch was recorded by light microscopy. Cell migration was measured in 10 randomly selected microscopic fields for each experiment. Three independent experiments were performed for each condition.

The cell invasion assay was performed using a Cytoselect 24-well cell invasion assay kit (Cell Biolabs, Inc., San Diego, CA, USA). The kit included polycarbonate membrane inserts (8-μm pore size). The upper surface of the insert membrane was coated with a uniform layer of dried murine laminin I matrix. Cells were serum-starved for 24 h then a cell suspension containing 1 × 10^6 cells/mL in serum-free media alone or serum-free media with 1.0 μM of bosutinib was added to the inside of each insert. Each insert was then transferred to a lower well of the plate filled with media containing 20% fetal bovine serum. After incubation for 24 h, the invading cells were stained and extracted, and then quantified by measuring the absorbance at 560 nm with a VersaMax microplate reader (Molecular Devices). The data presented are representative of two independent experiments.
In vivo study

Animal experiments were performed at the Biomedical Center for Animal Resource Development of Seoul National University, Seoul, Korea, according to the institutional guidelines with prior approval from the institutional animal care and use committee. Of SNU-308 and SNU-478 cell lines that were sensitive to bosutinib in this study, we used the SNU-478 xenograft model for in vivo experiments, since the SNU-478 cell line was more tumorigenic in female Balb/c athymic nude mice than the SNU-308 cell line. A total of 30 female Balb/c athymic nude mice aged 4–6 weeks were supplied from Central Lab Animal, Inc., Seoul, Korea. The mice were adapted to local conditions for 1 week, and then injected subcutaneously in the right flank with $1 \times 10^7$ SNU-478 cells in 100 μL of PBS. After implantation of the tumor cells, the tumor volume was measured every week using calipers and calculated using the following formula: $(\text{width}^2 \times \text{height})/2$. When the tumor volume reached 200 mm$^3$, the mice were randomly divided into six treatment groups: control, bosutinib, gemcitabine, cisplatin, bosutinib plus gemcitabine, and bosutinib plus cisplatin. The control group was treated with 0.5% methanol and 0.4% Tween 80 in deionized water via oral gavage. Bosutinib at a dose of 150 mg/kg was administered via oral gavage once daily for 28 days. Gemcitabine (100 mg/kg) and cisplatin (4.5 mg/kg) were injected intraperitoneally twice a week for 28 days. When the tumor volume reached 1,500 mm$^3$, the mice were euthanized with CO$_2$. The tumors were excised and stored in liquid nitrogen for further western blot analysis or immunohistochemical stain.

Immunohistochemistry

Four micrometer-thick sections from paraffin-embedded xenograft tumor tissues were deparaffinized and dehydrated. Immunohistochemistry detection of proliferating cells was
conducted using an anti-Ki-67 antibody (GeneTex, Inc., Irvine, CA, USA) at a dilution of 1:100. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were conducted for the immunohistochemistry detection of apoptosis using an ApopTag *In situ* Apoptosis Detection Kit (EMD Millipore, Billerica, MA, USA), in accordance with the manufacturer’s protocol. A phosphorylated Src antibody was used at a dilution of 1:200 and was purchased from Cell Signaling Technology.

**Statistical analysis**

Experimental data were expressed as the mean ± standard error (SE) and compared using the Student’s *t*-test. Data were analyzed and displayed using SigmaPlot software (Systat Software, Inc.). All statistical tests were two-sided, with significance defined as *P* < 0.05.
Results

_Bosutinib inhibits BTC cell proliferation by inhibition of Src phosphorylation and abrogation of its downstream signaling pathways_

A total of nine BTC cell lines were treated with bosutinib. Among them, SNU-308 and SNU-478 cells were sensitive to bosutinib with IC$_{50}$ values of 0.65 ± 0.06 μM and 0.63 ± 0.03 μM, respectively, compared with other cells (Fig. 1A and Supplementary Table 1). In contrast, TFK-1 cells were relatively resistant to bosutinib with an IC$_{50}$ value of 4.45 μM. In a three-dimensional culture system, bosutinib also showed an anti-proliferative effect that was dose dependent in both SNU-308 and SNU-478 cells (Fig. 1B).

Next, western blot analysis was performed to evaluate the effect of bosutinib on the downstream signaling pathways of Src (Fig. 1C). In SNU-308 and SNU-478 cells, bosutinib abrogated Src Tyr416 and FAK Tyr397 phosphorylation in a dose-dependent manner. In contrast, FAK Tyr925 phosphorylation was slightly inhibited by high-dose bosutinib in the SNU-308 cell line only, whereas it was upregulated in SNU-478 and TFK-1 cell lines. In addition, the phosphorylation of AKT and ERK in SNU-308 and SNU-478 cells was decreased with increasing concentrations of bosutinib. Bosutinib treatment decreased STAT3 phosphorylation in SNU-478 cells but it was increased in SNU-308 and TFK-1 cells. In TFK-1 cells, bosutinib did not significantly influence the phosphorylation of Src, AKT, and ERK when compared with SNU-308 and SNU-478 cells.

_Inhibition of Src by bosutinib induces G1 cell-cycle arrest in sensitive BTC cell lines_

The effects of bosutinib on the cell-cycle of SNU-308, SNU-478, and TFK-1 cells were evaluated using flow cytometry. Bosutinib significantly increased G1 cell-cycle arrest in a dose-dependent manner in both SNU-308 and SNU-478 cells (Fig. 2A). The sub-G1 fraction
was significantly increased by bosutinib treatment in SNU-478 cells (p = 0.008), and showed a tendency to be increased in SNU-308 cells with borderline significance (p = 0.062). In TFK-1 cells, G1 cell-cycle arrest was significantly increased after 1 μM bosutinib treatment but this was not observed at lower concentrations. The sub-G1 fraction in TFK-1 cells was not significantly increased by bosutinib treatment (p = 0.572). After 1 μM bosutinib treatment for 48h, the Annexin V-FITC/PI stain revealed that bosutinib significantly induced both early and late apoptosis in SNU-308 and SNU-478 cells (Fig. 2B). However, the tendency was not apparent in TFK-1 cells. Bosutinib decreased Cyclin D, Cyclin E, Cyclin A, and Cyclin B expression, and increased p27 expression in both SNU-308 and SNU-478 cells, but not in TFK-1 cells (Fig. 2C). Of note, the nuclear expression of p27 was significantly increased in both SNU-308 and SNU-478 cells, but not in TFK-1 cells (Fig. 2D).

**Bosutinib inhibits the migration and invasion of BTC cells**

Migration and invasion assays were performed to determine the effect of bosutinib on the migration and invasion activity of BTC cells. The migration assays indicated that bosutinib significantly inhibited the migratory activity of both SNU-308 and SNU-478 cells (Fig. 3A). Furthermore, bosutinib significantly inhibited the invasive activity of SNU-478 cells (Fig. 3B).

Next, we performed western blot analysis to investigate the effect of bosutinib on the expression levels of proteins related to epithelial-mesenchymal transition (EMT) in SNU-308 and SNU-478 cells. Bosutinib did not influence the expression levels of E-cadherin and β-catenin in both cell lines. However, Vimentin levels were slightly decreased and Snail expression was potently abrogated by bosutinib in a dose-dependent manner (Fig. 3C).
Src inhibition enhances the anti-proliferative and anti-migratory effects of cytotoxic agents in BTC

Because gemcitabine and cisplatin are standard chemotherapeutic agents in patients with advanced BTC, we investigated the combined effects of bosutinib with either gemcitabine or cisplatin in BTC cells. Bosutinib demonstrated synergistic anti-proliferative effects in combination with gemcitabine or cisplatin in SNU-308 and SNU-478 cells (Fig. 4A). The migratory effects of cytotoxic agents were potentiated by the addition of bosutinib (Fig. 4B). In addition, combination treatment of bosutinib with gemcitabine or cisplatin for 72 h significantly inhibited the migration of SNU-478 cells when compared with bosutinib monotherapy. In SNU-478 cells, combination treatment of bosutinib with gemcitabine or cisplatin further abrogated Src phosphorylation compared with bosutinib alone, and further downregulated Vimentin and Snail expression compared with bosutinib, gemcitabine, or cisplatin alone (Fig. 4C).

Src inhibition has potent antitumor effects in a xenograft model

A SNU-478 xenograft model using female Balb/c athymic nude mice demonstrated that bosutinib alone significantly delayed tumor growth compared with controls (Fig. 5A). Bosutinib combined with gemcitabine or cisplatin showed significantly more potent antitumor effects when compared with bosutinib, gemcitabine, or cisplatin monotherapy. Moreover, the combination treatment did not significantly influence the body weight of mice (data not shown).

Tumors treated with bosutinib alone exhibited an apparent decrease in cell proliferation by Ki-67 assays and an apparent increase in apoptosis by TUNEL assays (Fig. 5B). In addition, bosutinib treatment apparently decreased Src phosphorylation. Combination treatment of bosutinib and gemcitabine further decreased Ki-67 expression and Src phosphorylation, and
further increased TUNEL expression. In tumor lysates, the phosphorylation of Src and STAT3 was downregulated by bosutinib monotherapy. Moreover, when bosutinib was combined with gemcitabine, a greater decrease in Src and STAT3 phosphorylation was observed (Fig. 5C).
Discussion

Our data suggested that Src might be a potential therapeutic target in BTC. A previous study indicated that about 80% of BTC specimens expressed an activated Src protein similar to other malignancies (3-6, 18). In our study, a total of nine BTC cell lines were tested, and the IC_{50} values ranged from 0.63 to 4.45 μM in 3-day MTT assays. Our data support that a subset of BTC may be sensitive to the Src inhibitor, bosutinib.

Bosutinib monotherapy inhibited the phosphorylation of Src Tyr416 and FAK Tyr397, which is an autophosphorylation site for integrins (25-27). In contrast, bosutinib did not inhibit the phosphorylation of FAK Tyr925 residue, which is a Src-specific phosphorylation site. Previous preclinical studies of bosutinib showed conflicting results in terms of FAK phosphorylation. In colon cancer and breast cancer, bosutinib decreased FAK Tyr925 phosphorylation whereas phosphorylation of the Tyr397 residue was unchanged (28, 29). In contrast, FAK Tyr397 phosphorylation was significantly decreased by bosutinib in thyroid cancer (30). Another Src/Abl inhibitor dasatinib also inhibited FAK Tyr397 phosphorylation in colon cancer and pancreatic cancer (31, 32). The precise molecular mechanisms for these conflicting results need to be investigated further to determine how to abrogate the downstream signaling pathways of Src using Src inhibitors more effectively. In addition, bosutinib decreased AKT and ERK phosphorylation in a dose dependent manner in SNU-308 and SNU-478 cells, which were relatively sensitive to bosutinib. In contrast, conflicting results were shown for STAT3 phosphorylation. In SNU-478 cells, STAT3 phosphorylation was downregulated by bosutinib. However, in SNU-308 and TFK-1 cells, it was upregulated. This finding is in line with a previous study that demonstrated sustained Src inhibition induced altered JAK-STAT3 binding, leading to aberrant STAT3 activation (33). These results suggest that a STAT3 targeting strategy might be an option to increase the therapeutic efficacy or to overcome the resistance of Src inhibitors.
Cell-cycle analysis demonstrated that bosutinib induced G1 arrest and increased the sub-G1 fraction in a dose-dependent manner in SNU-308 and SNU-478 cells. Bosutinib decreased Cyclin D, Cyclin E, Cyclin A, and Cyclin B expression, and increased p27 expression in both sensitive BTC cell lines. In particular, the nuclear stabilization of p27 was also observed after bosutinib treatment, which has been suggested to be a mechanism of apoptosis induced by the Src inhibitor (34). Moreover, bosutinib monotherapy demonstrated potent anti-migratory and anti-invasive activity against BTC cell lines, and downregulated the expression of proteins related to EMT, which is associated with cancer progression and metastasis and often mediated by the MAPK and PI3K/AKT pathways (35, 36). These findings are also consistent with previous findings of bosutinib in other type of cancers (29, 30, 37, 38). The results of our preclinical studies support bosutinib as a single agent with promising anti-tumor activity in BTC, which warrants further clinical studies.

Our *in vitro* and *in vivo* models also demonstrated that bosutinib has synergistic anti-proliferative activity in combination with cytotoxic agents including gemcitabine and cisplatin, which are currently the standard treatment in patients with advanced BTC. Importantly, there was no significant effect on body weight in the *in vivo* models (15). The combination treatment further inhibited the migratory activity of BTC cells, decreased the phosphorylation of Src and its downstream molecules, decreased EMT marker expression levels such as Vimentin and Snail, and increased apoptosis in BTC cells. These results indicate that this novel combination strategy is worthy of further clinical studies in patients with advanced BTC, because there is currently no validated therapeutic target for advanced BTC despite the poor prognosis of these patients.

In spite of these promising results, alternative cancer survival and growth machineries are serious issues in anti-cancer drug discovery since it may eventually induce acquired resistance to Src inhibitors. Similarly, a previous study using a murine melanoma model
indicated that adoptive cell transfer therapy with cytotoxic T-cells unexpectedly induced treatment resistance through an inflammation-induced reversible loss of melanocytic antigens (39). More recently, Obenauf, et al. demonstrated that targeted therapy induces secretome changes in drug-sensitive cancer cells, paradoxically generating a tumor microenvironment that promotes the growth of drug-resistant clones (40). In case of Src inhibition, only few have been known about acquired resistance mechanisms. Aforementioned aberrant STAT3 activation by bosutinib could be a resistance mechanism of Src inhibitors (33). Recently, Lu, et al. demonstrated that IGFBP2/FAK pathway may be associated with dasatinib resistance in non-small cell lung cancer cells (41). Aberrant deregulation of FAK or its down-stream molecules by alternative pathway activation may contribute to Src inhibitor resistance. Therefore, further pre-clinical studies should focus on the identification and overcome of the alternative cancer survival and growth machineries of Src inhibitors in BTC.

A recent study identified that a bosutinib isomer (Bos-I) was more potent to inhibit Chk1 and Wee1 than ‘authentic’ bosutinib and synergized with gemcitabine in a pancreatic cancer cell line (42). However, the ability of Bos-I to inhibit Src and Abl was relatively less potent compared with ‘authentic’ bosutinib. Nevertheless, since Bos-I showed greater anti-tumor activity than ‘authentic’ bosutinib toward pancreatic cancer cells when combined with gemcitabine, further preclinical or clinical studies are needed to focus on the anti-proliferative activity of Bos-I combined with gemcitabine or cisplatin in BTC cells.

In summary, our data suggest that Src might be a potential therapeutic target in BTC. Bosutinib, an orally active small molecule c-Src/Abl kinase inhibitor, demonstrated promising anti-tumor activity alone or in combination with gemcitabine or cisplatin by significantly inhibiting the phosphorylation of Src and its downstream molecules, inducing G1 cell-cycle arrest via the nuclear stabilization of p27 in part, decreasing migration, invasion, and EMT, and increasing apoptosis in BTC in vitro and in vivo models. The results of our
preclinical study support the further clinical development of bosutinib monotherapy or combination therapy with other cytotoxic agents in patients with advanced BTC.
References


Figure legends

Figure 1. Bosutinib inhibits the proliferation of BTC cells via the inhibition of Src phosphorylation.

(A) The effects of bosutinib on BTC cell proliferation were evaluated by MTT assays. Cells were treated with increasing doses of bosutinib for 72 h. (B) A Matrigel-embedded three-dimensional culture system was used to investigate the anti-proliferative activity of bosutinib in SNU-308 and SNU-478 cells. Cells were treated with 0 (DMSO), 0.1, and 0.5 μM of bosutinib every 3 days for 12 days, and then examined under light microscopy. (C) Western blot analysis was performed to evaluate the effect of bosutinib on Src signaling pathways in BTC cells. SNU-308, SNU-478, and TFK-1 cells were treated with increasing concentrations of bosutinib (0 [DMSO], 0.1, 0.5, and 1.0 μM) for 48 h, after which protein extracts were immunoblotted with the indicated antibodies. The molecular weight of each protein is as follows: Src, 60 kDa; FAK, 125 kDa; AKT, 60 kDa; ERK, 42/44 kDa; STAT3, 79/86 kDa; and β-actin, 42 kDa.

Figure 2. Bosutinib induces the G1 cell-cycle arrest of sensitive BTC cells.

(A) Cell-cycle analysis of SNU-308, SNU-478, and TFK-1 cells was performed using flowcytometry after increasing concentrations of bosutinib treatment (0 [DMSO], 0.1, 0.5, and 1.0 μM) for 24 h. The percentage of cells in the sub-G1, G1, S, and G2/M phases are shown in the bar graph. The columns represent the mean of three independent experiments and error bars are shown (± SE). *p < 0.01. (B) After the cells were treated with 1.0 μM bosutinib, apoptosis was quantified using Annexin V-FITC and PI staining and flow cytometry. Early apoptosis was defined as Annexin V-FITC-positive and PI-negative, while late apoptosis was Annexin V-FITC- and PI-positive. Results are expressed as the mean value...
of three independent experiments. *p < 0.05. (C) Western blot analysis demonstrated the effect of bosutinib on the expression levels of cell-cycle checkpoint molecules including Cyclin D, Cyclin E, Cyclin A, Cyclin B, and p27 in SNU-308, SNU-478, and TFK-1 cells. The cells were treated with increasing doses of bosutinib (0 [DMSO], 0.1, 0.5, and 1.0 μM) for 48 h. (D) Nuclear or cytosolic expression levels of p27 were measured after bosutinib treatment. Western blot data were normalized to Lamin B levels using ImageJ software (National Institute of Health). *p < 0.01.

**Figure 3. Bosutinib inhibits the migration and invasion of BTC cells.**

(A) Migration assays were performed to visualize the effects of bosutinib on migration activity in BTC cells. SNU-308 and SNU-478 cells were seeded in a 6-well plate. After 24 h, cell monolayers were scratched with a sterile 200-μL pipette tip and incubated in culture medium alone or with bosutinib. After 72 h for SNU-308 cells and 24h for SNU-478 cells, the cell migration activity was observed under light microscopy. **p < 0.005. (B) Invasion activity was examined using a chamber system coated with laminin I matrix. The invaded cells were stained and quantified at 560 nm. **p < 0.005. (C) Western blot analysis of proteins associated with EMT was performed after treatment with increasing concentrations of bosutinib (0 [DMSO], 0.1, 0.5, and 1.0 μM).

**Figure 4. Bosutinib enhances the anti-proliferative and anti-migratory effects of cytotoxic agents.**

(A) Anti-proliferative activity of 0.5 μM bosutinib in combination with 0.01 μM gemcitabine or 1.0 μM cisplatin was evaluated by MTT assays. *p < 0.01 and **p < 0.005. (B) For migration assays, SNU-478 cell monolayers were scratched with a sterile 200-μL pipette tip, and then treated with 0.1 μM bosutinib, 0.1 μM gemcitabine, or 2.0 μM cisplatin alone or in...
combination. The distance between the cells was measured. **p < 0.005. (C) In SNU-478 cells, the western blot analysis of molecules associated with Src signaling pathways and EMT was performed after treatment with 1.0 μM bosutinib, 0.1 μM gemcitabine, or 2.0 μM cisplatin alone or in combination for 48 h.

**Figure 5. Bosutinib has potent anti-tumor effects in combination with gemcitabine or cisplatin in a SNU-478 xenograft model.**

(A) Mice were treated with vehicle alone, bosutinib (150 mg/kg), gemcitabine (100 mg/kg), cisplatin (4.5 mg/kg), bosutinib plus gemcitabine, or bosutinib plus cisplatin for 28 days. *p < 0.01. (B) The tumors were harvested and analyzed by immunohistochemistry. Ki-67 and TUNEL expression and Src phosphorylation were evaluated in a SNU-478 xenograft model. (C) Proteins were extracted from excised tumors for immunoblotting with the indicated antibodies. Results of representative samples were shown.
Figure 1.

(A) Percentage of viable cells (%) with varying concentrations of Bosutinib. Different cell lines are represented by different lines.

(B) Microscope images of SNU-308 and SNU-478 cells treated with 0 μM, 0.1 μM, and 0.5 μM Bosutinib.

(C) Western blot analysis of various proteins in SNU-308, SNU-478, and TFK-1 cells treated with different concentrations of Bosutinib. The proteins analyzed include p-Src (Tyr416), Src, p-FAK (Tyr925) and (Tyr397), FAK, p-AKT, AKT, p-ERK, ERK, p-STAT3, STAT3, and β-actin.
Figure 2.

(A) 

![Cell cycle phases for SNU-308, SNU-478, and TFK-1](image1)

(B) 

![Cell cycle population for early and late apoptosis for SNU-308, SNU-478, and TFK-1](image2)

(C) 

![Western blots for SNU-308, SNU-478, and TFK-1](image3)
Figure 2.

(D) 

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<th></th>
<th>SNU-308</th>
<th>SNU-478</th>
<th>TFK-1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td><img src="image1" alt="" /></td>
<td><img src="image2" alt="" /></td>
<td><img src="image3" alt="" /></td>
</tr>
<tr>
<td><strong>1 μM</strong></td>
<td><img src="image4" alt="" /></td>
<td><img src="image5" alt="" /></td>
<td><img src="image6" alt="" /></td>
</tr>
</tbody>
</table>

Relative band intensity (%)

Cytosol  | Nucleus  | Cytosol  | Nucleus  | Cytosol  | Nucleus  |
0  |       | 200 | 100 |     | 200 |     |
100 |      |   |   | 100 |     | 100 |     |
200 |      |   |   |   |     |   |     |

* indicates statistical significance.
Figure 3.

(A) SNU-308

Control

0.1 μM

1.0 μM

0 h

72 h

SNU-478

Control

0.1 μM

1.0 μM

0 h

24 h

(B) SNU-478

Control

1.0 μM

(C) SNU-308

SNU-478

E-cadherin

β-catenin

Vimentin

Snail

β-actin

**
Figure 4.

(A) SNU-308 SNU-478 TFK-1

(B) SNU-478

(C) p-Src p-STAT3 p-ERK E-cadherin Vimentin Snail

β-actin

β-actin

Con Bos Gem Cis B+G B+C
Figure 5.

(A) SNU-478 Xenograft

(B) Comparison of treatment effects on tumor volume.

(C) Immunohistochemical analysis showing expression levels of p-Src, Src, p-STAT3, p-ERK, and β-actin across different treatment groups.
Molecular Cancer Therapeutics

Src as a therapeutic target in biliary tract cancer
Ah-Rong Nam, Ji-Won Kim, Ji Eun Park, et al.

Mol Cancer Ther Published OnlineFirst April 22, 2016.

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