Chemical Therapeutics Discovery & Preclinical Development

Antitumor Activity of Saracatinib (AZD0530), a c-Src/Abl Kinase Inhibitor, Alone or in Combination with Chemotherapeutic Agents in Gastric Cancer

Hyun-Jin Nam1, Seock-Ah Im1,2, Do-Youn Oh1,2, Paul Elvin3, Hwang-Phill Kim1, Young-Kwang Yoon1, Ahruhm Min1, Sang-Hyun Song1, Sae-Won Han1,2, Tae-You Kim1,2, and Yung-Jue Bang1,2

Abstract

Src is a nonreceptor tyrosine kinase involved in the cross-talk and mediation of many signaling pathways that promote cell proliferation, adhesion, invasion, migration, and tumorigenesis. Increased Src activity has been reported in many types of human cancer, including gastric cancer. Therefore, this factor has been identified as a promising therapeutic target for cancer treatments, and targeting Src in gastric cancer is predicted to have potent effects. We evaluated the antitumor effect of a c-Src/Abl kinase inhibitor, saracatinib (AZD0530), alone or combined with chemotherapeutic agents in gastric cancer cell lines and a NCI-N87 xenograft model. Among 10 gastric cancer cell lines, saracatinib specifically inhibited the growth and migration/invasion of SNU216 and NCI-N87 cells. Saracatinib blocked the Src/FAK, HER family, and oncogenic signaling pathways, and it induced G1 arrest and apoptosis in SNU216 and NCI-N87 cells. Apoptosis required induction of the proapoptotic BCL2 family member Bim. Knockdown of Bim using siRNA decreased apoptosis induced by treatment with saracatinib, suggesting that Bim has an important role in saracatinib-induced apoptosis. Saracatinib enhanced the effects of lapatinib, an EGFR/HER2 dual inhibitor, in SNU216 and NCI-N87 cells. Furthermore, combined treatment with saracatinib and 5-fluorouracil (5-FU) or cisplatin exerted synergistic effects in both saracatinib-sensitive and saracatinib-resistant cells. Consistent with our in vitro findings, cotreatment with saracatinib and 5-FU resulted in enhanced antitumor activity in the NCI-N87 xenografts. These data indicate that the inhibition of Src kinase activity by saracatinib alone or in combination with other agents can be a strategy to target gastric cancer.

Mol Cancer Ther; 12(1); 16–26. ©2012 AACR.

Introduction

Src belongs to a family of nonreceptor protein tyrosine kinases (SFK). Activation of this factor can be regulated by interaction with focal adhesion kinase (FAK) and Crk-associated substrate (CAS). Src is involved in the cross-talk between many signaling pathways, including the integrin/FAK, EGF receptor (EGFR), Ras/Raf/MEK, PI3K/AKT, and JAK/STAT pathways, which promote cell proliferation, adhesion, invasion, migration, metastasis, and tumorigenesis (1, 2). A previous analysis found that 20% of human breast cancers overexpress both Src and EGFR, suggesting the involvement of Src in the EGFR signaling pathway (3). Another study found that Src activation is required for EGF-induced integrin-mediated migration and metastasis. This finding is indicative of cross-talk in the Src/EGFR/integrin pathway and shows that Src is a key regulator of signal transduction (4, 5). Elevation of Src expression and catalytic activity has been reported in a number of human cancers including lung, skin, colon, breast, ovarian, and head and neck malignancies (6). Src activity is also increased in gastric cancer (7, 8). The presence of activated Src in gastric cancer along with the role of this factor in cancer cell proliferation and metastasis raise the possibility that Src is a promising target for treating gastric cancer. However, the role of Src as a therapeutic target has not been widely studied.

Saracatinib (AZD0530) is a potent, orally administered small molecule that inhibits Src by blocking the ATP-binding site of Src kinases. The antiproliferative activity of saracatinib has been reported in several human cancer cell lines and xenograft models (9). Together with an estrogen receptor (ER) blockade, saracatinib was found to be effective in ER-positive breast cancer cell lines and a xenograft model (10). Saracatinib also exerted antitumor effects in a preclinical model of colorectal cancer,
suggesting that increased activation of the Src pathway is associated with sensitivity to saracatinib (11). However, it is not known which biomarkers can predict the sensitivity of solid tumors to Src inhibitors.

Although a phase II clinical trial of saracatinib in unselected patients with advanced gastric cancer has been conducted, the responses of gastric cancer cells to Src inhibition have not been fully characterized (12). Therefore, we evaluated the antitumor activity of saracatinib in gastric cancers by examining the in vitro and in vivo effects of this compound. We evaluated the antitumor activity of saracatinib in gastric cancer cell lines by monitoring the activity of molecules involved in Src/FAK, HER family, and oncogenic signaling pathways, such as STAT3, AKT, and extracellular signal–regulated kinase (ERK), following treatment with saracatinib. Furthermore, we assessed the combined effects of saracatinib administered with chemotherapeutic agents, such as 5-fluorouracil (5-FU) and cisplatin, on gastric cancer cells and a xenograft model.

Materials and Methods

Reagents

Saracatinib (AZD0530), a potent Src inhibitor, was provided by AstraZeneca. Lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor, was supplied by Selleck Chemicals. 5-FU and cisplatin were obtained from Chongwoe Co., Ltd.. The chemical structures of saracatinib and 5-FU are shown in Supplementary Fig. S1.

Cell lines and culturing

Human gastric cancer cell lines (SNU1, 5, 16, 216, 601, 620, 638, 668, and 719) were supplied by the Korean Cell Line Bank; the identities of the cell lines were authenticated by DNA fingerprinting analysis (13). Human gastric cancer cells (NCI-N87) were purchased from the American Type Culture Collection (ATCC); authentication of the cell line was conducted by ATCC using a short tandem repeat analysis. NCI-N87 and SNU216 are cell lines in which HER2 is amplified. The copy number ratio of the HER2 gene determined by FISH analysis was found to be 4.34 for SNU216 cells and 8.4 for N87 cells (14, 15). Upon receipt, all cell lines were stored in liquid nitrogen, and passaged for less than 6 months before use for this study. The cells were maintained in RPMI-1640 media (HyClone Inc.) supplemented with 10% FBS in a humidified 5% CO₂ atmosphere at 37°C.

Cell growth inhibition assay

A tetrazolium dye (MTT, Sigma-Aldrich) assay was used to evaluate the inhibitory effect of saracatinib on cell growth. Human gastric cancer cell lines (SNU1, 5, 16, 216, 601, 620, 638, 668, 719, and NCI-N87) were seeded in 96-well plates, incubated for 24 hours and then treated with saracatinib (0.001, 0.01, 0.1, 1, and 10 μmol/L) for 72 hours at 37°C. After treatment with the drug, MTT solution was added to each well and the cells were incubated for 4 hours at 37°C before the medium was removed. Next, dimethyl sulfoxide (DMSO) was added to each well and the plates were shaken for 30 minutes at room temperature. Cell viability was determined by measuring the absorbance of all wells at 540 nm with a microplate reader (Versa Max; Molecular Devices). Six replicate wells were used for each analysis and at least 3 independent experiments were carried out. Data points shown represent the mean, whereas bars represent the SD.

To evaluate the effects of saracatinib administered in conjunction with other chemotherapeutic agents (5-FU or cisplatin), the cells were treated with serial dilutions of each drug alone or with a combination of saracatinib with either chemotherapeutic agent at a fixed ratio corresponding to the specific IC₅₀ of each drug. After 72 hours of drug exposure, cell proliferation was measured using an MTT assay as described earlier. Any synergistic effects resulting from cotreatment with the compounds were measured using the methods described by Chou and Talalay (16, 17). Analysis of the median effect was conducted using CalcuSyn software (Biosoft) to determine the combination index values (CI > 1: antagonistic effect, CI = 1: additive effect, and CI < 1: synergistic effect).

Migration and invasion assay

SNU216 cells were seeded in a 6-well plate (4 × 10⁵ cells/well). After 24 hours, the cell monolayers were scratched with a yellow tip. The plates were washed with PBS, and the cells were then incubated with medium alone or medium containing 1 μmol/L of saracatinib. After 24 hours, the plates were examined by light microscopy to monitor resealing of the cell monolayer.

The cell invasion was measured using a Cytoselect 24-well cell invasion assay kit (Cell Biolabs, Inc.). This 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. SNU216 cells were serum-starved for 24 hours. Next, a cell suspension containing 4 × 10⁵ cells/mL of serum-free media alone or serum-free media with 1 μmol/L of saracatinib were added to the inside of each insert. Each insert was then transferred to a lower well of the plate filled with media containing 10% FBS. After 24 hours of incubation, the invading cells were stained and quantified.

Western blot analysis

Cells were incubated with saracatinib in 10 media supplemented with 10% FBS. Then, the cells were treated with lysis buffer. Equivalent amounts of protein (15 μg) from each cell lysate were separated by SDS-PAGE and then transferred to nitrocellulose membranes. After blocking with buffer, the membrane was incubated with primary antibodies at 4°C overnight. Antibodies against p-Src (pY416), Src, p-EGFR (pY1068), p-HER2 (pY1221/1222), p-HER3 (pY1289), p-STAT3 (pY-705), p-AKT (pS-473), p-ERK (pY42/p44), EGFR, HER2, HER3, STAT3, AKT,
ERK, caspase-3, -7, PARP, Bim, cyclin D, and p27kip1 were purchased from Cell Signaling Technology; FAK and p-FAK(Y925) antibodies were obtained from BD Biosciences; and anti-α-tubulin antibody was acquired from Sigma-Aldrich. P-Fan (Y961) antibody was purchased from Invitrogen, P-Fyn (Y530) antibody was from Novus, and Yes, Fyn, and p-Yes (Y537) antibodies were from Abcam. Integrin-αv and integrin-β-4, and -8 antibodies were obtained from BD Biosciences.

Cell-cycle analysis

After incubation with saracatinib at various concentrations (0.2, 1, and 5 μmol/L) for 48 hours, the cells were pelleted at 1,500 rpm for 5 minutes, fixed in 70% alcohol and stored at −20°C. The samples were then dissolved in 10 μL RNase (100 μg/mL) and subsequently incubated at 37°C for 10 minutes. Next, the samples were treated with propidium iodide. The DNA content of the cells (10,000 cells per experimental group) was determined using a FACS Calibur flow cytometer (BD Biosciences) equipped with a ModFit LT program (Verity Software House, Inc.), as previously described (18).

Annexin V-binding assay for apoptosis

After the cells were exposed to saracatinib or lapatinib, the degree of apoptosis was assessed using the Annexin V-binding assay according to the protocols of the manufacturer (BD PharMingen). The harvested cell suspension was then incubated with Annexin V for 15 minutes at room temperature in the dark and then analyzed by flow cytometry.

siRNA knockdown

siRNA specific for Bim, and nonspecific controls were purchased from Qiagen. Transfection procedures were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions at a final concentration of 40 nmol/L.

In vivo studies

Animal experiments were carried out in the animal facility of the Seoul National University (Seoul, Republic of Korea) in accordance with institutional guidelines. To measure the in vivo activity of saracatinib, 4- to 6-week-old female BALB/c athymic nude mice were purchased from Central Lab Animal, Inc. The mice were allowed to acclimatize to conditions in the animal facility for 1 week before being injected with cancer cells. Mice were injected subcutaneously with N87 cells in 100 μL of PBS (1 × 106 cells per 100 μL PBS). When the tumor reached a volume of 200 mm3, mice were randomly divided into treatment groups (5 mice/group) and received vehicle control, saracatinib suspended in a 1% solution of polysorbate 80 (Tween 80) in deionized water, 5-FU or a combination of saracatinib and 5-FU. Saracatinib was administered via oral gavage once daily at a concentration of 50 mg/kg for 3 weeks. 5-FU (50 mg/kg) was injected intraperitoneally once weekly for 3 weeks. The tumor was measured every other day using calipers and the volume was calculated with the following formula: \([\text{volume} = \text{length} \times \text{width}^2 / 2]\). At the end of the measurement period (day 21), the mice were sacrificed. The tumors were excised and fixed in neutral-buffered formalin for routine histologic examination and immunohistochemical staining. At the same time, total proteins were extracted from fresh tissue to detect protein expression and activity of Src and FAK.

Immunohistochemistry

Three core tissue biopsies (4 mm in diameter) were taken from each individual paraffin-embedded tissue sample (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (Superbiochips Laboratories). Each tissue array block contained samples from all mice. Sections 4 μm in thickness were cut from each triplicate tissue array block, deparaffinized, and dehydrated. Immunohistochemical detection of proliferating cells was conducted using an anti-Ki67 antibody. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was conducted for immunohistochemical detection of apoptosis using an Apoptag In situ Apoptosis Detection Kit (Chemicon International), in accordance with manufacturer’s recommendations.

Statistical analysis

All experiments were conducted in duplicate or triplicate and repeated at least twice. SE was used when calculating IC50 values using SigmaPlot version 9.0. All results that are presented in the graph were expressed as bars, ±SD. The statistical significance of the results was analyzed using an unpaired Student t test. P values less than 0.05 were considered to be statistically significant.

Results

SNU216 and NCI-N87 gastric cancer cells were sensitive to saracatinib

We examined the inhibitory effects of saracatinib in the growth of 10 gastric cancer cell lines (SNU1, 5, 16, 216, 601, 620, 638, 668, 719, and NCI-N87). The molecular alteration of these cells was previously reported (19). SNU216 and NCI-N87 cells were sensitive to saracatinib at IC50 values lower than 1 μmol/L (Table 1). As shown in Fig. 1A, saracatinib also exerted antimitorsomal (left) and anti-invasive effects (right; figure and graph) in SNU216 cells.

We next measured the basal expression levels of Src family kinases such as Src and FAK in gastric cancer cells. In a previous study, saracatinib-sensitive colorectal cancer cells and explants were found to have increased activation of Src and FAK (11). Similar to this report, we found that the protein expression of phosphorylated Src (Y416) and FAK (Y861, Y397, and Y925) were elevated in the SNU216 and NCI-N87 cells (Fig. 1B). We further compared the expression levels of various integrin family
members (integrin-α-2,-3,-5, and -V along with integrin-β-1,-4, and -8) in different gastric cancer cells (data not shown). Among these, mRNA and protein levels of integrin-β-8 were highest in the saracatinib-sensitive SNU216 and NCI-N87 cell lines (Supplementary Fig. S2). To determine whether saracatinib directly inhibits activity of Src family kinases (Src, Fyn, and Yes) and FAK, Western blotting was conducted to measure the total and phosphorylated levels of these proteins following treatment with saracatinib. The phosphorylation of Src, Fyn, Yes, and FAK in both SNU216 and NCI-N87 cells was decreased by saracatinib (Fig. 1C).

Because both sensitive cell lines (SNU216 and NCI-N87) were HER2-amplified cells, we tested whether HER2 amplification can be a marker for predicting sensitivity to Src inhibition. However, other breast cancer cell lines in which HER2 were amplified (SKBR3, MDA-MB-453, and BT474) were resistant to saracatinib. We also compared protein expression levels of different integrin family members (integrin-α-2,-3,-5,-V, and integrin-β-1,-4, and -8), FAK and Src. Among these, integrin-αv, integrin-β4,-8, and Src were highly expressed in the SNU216 and NCI-N87 cells as compared with HER2-amplified breast cancer cells (Fig. 1D).

**Src inhibition leads to blockage of the HER family and oncogenic signaling pathways**

We next examined the activity of HER family and oncogenic (STAT3, AKT, and ERK) signaling pathways after treatment with saracatinib. EGFR has been reported to activate Src, and combined inhibition of Src and EGFR abrogate the growth of head and neck squamous cell carcinoma (20, 21). Accordingly, decreased phosphorylation of HER family members and oncogenic signaling molecules such as AKT and ERK was detected in the SNU216 and NCI-N87 cells. In contrast, in SNU16 cells, p-HER2 was even increased at a dose-dependent manner, and no change occurred in the phosphorylation of other molecules with the treatment of saracatinib. In addition, saracatinib did not reduce STAT3 phosphorylation in the saracatinib-sensitive cell lines although STAT3 phosphorylation was increased in NCI-N87 cells following saracatinib treatment (Fig. 2A and B). Our findings were consistent with those of previous reports showing that STAT3 is reactivated after Src inhibition, and combined inhibition of Src and STAT3 results in an improved response (22).

**Saracatinib enhances antitumor effects of lapatinib in HER2-amplified gastric cancer cells**

According to a previous report, increased Src activation confers resistance to trastuzumab and targeting Src in combination with trastuzumab-sensitized trastuzumab-resistant cell lines (23). In addition, the integrin/FAK/Src pathway is highly activated in lapatinib-resistant cells, and inhibition of these pathways overcomes lapatinib resistance (24).

We therefore cotreated cells with saracatinib and lapatinib, an EGFR/HER2 tyrosine kinase inhibitor, to see if this combination enhances the effects on SNU216 and NCI-N87 cells. As shown in Fig. 2C and D, the number of apoptotic cells was significantly increased among the SNU216 and NCI-N87 cells treated with saracatinib plus lapatinib. Furthermore, additional decreases in the phosphorylation of EGFR, HER2, AKT, and ERK were observed in SNU216 and NCI-N87 cells treated with saracatinib plus lapatinib.

**Saracatinib leads to G1 arrest and apoptosis through induction of Bim in SNU216 and NCI-N87 cells**

To examine the effects of saracatinib on cell-cycle progression, flow cytometry was conducted. Saracatinib arrested SNU216 and NCI-N87 cells at the G1 phase, decreased cyclin D levels, and increased p27 expression (Fig. 3A and B). Furthermore, saracatinib markedly increased the sub-G1 phase fraction. PARP cleavage along with the activity of caspase-3 and -7 were also stimulated in response to saracatinib (Fig. 3A and C).

Bim (a proapoptotic BH3-only protein) has been reported to have an essential role in apoptosis. In an EGFR-mutant non–small cell lung carcinoma (NSCLC), knocking down Bim expression leads to an attenuation of apoptosis induced by gefitinib (25, 26). We therefore tested whether reduction of Bim expression affects on saracatinib-induced apoptosis in SNU216 and NCI-N87 cells. Both cell lines were transfected with Bim-specific or control siRNA. After 24 hours, the cells were treated with saracatinib and the number of apoptotic cells was determined after 48 hours with an Annexin V assay. Bim was induced by saracatinib in a dose-dependent manner in the SNU216 and NCI-N87 cells (Supplementary Fig. S3). The number of apoptotic cells was increased by Src inhibition; however, this number was significantly decreased by treatment with Bim-specific siRNA.
Figure 1. Saracatinib shows antimigratory and anti-invasive effects and affects Src and FAK signaling in saracatinib-sensitive cells. A, to examine the effects of saracatinib on cell migration, SNU216 cells were seeded in a 6-well plate. After 24 hours, cell monolayers were scratched and incubated in medium alone or with saracatinib. After another 24 hours, the cells were examined under light microscopy (left). A cell invasion assay was also carried out with SNU216 cells. The invading cells were stained and quantified as indicated in the graph at 560 nm (right). Bars, SD.

B, basal expression of Src and FAK pathway proteins in gastric cancer cell lines was analyzed by immunoblotting. C, activity of Src and FAK pathway proteins in SNU216, NCI-N87, and SNU16 cells was measured after treatment with saracatinib (0.2, 1, and 5 μmol/L) for 6 hours. D, growth inhibitory effects of saracatinib on the indicated cells were analyzed by an MTT assay (left), and protein expression levels of indicated antibodies were analyzed by immunoblotting (right).
These data indicate that Bim induction contributes to saracatinib-induced apoptosis. Saracatinib enhances the antitumor effects of chemotherapeutic agents in vitro and in vivo. We simultaneously treated 10 gastric cancer cell lines with saracatinib and 5-FU or cisplatin to determine whether saracatinib enhances the activity of these 2 chemotherapeutic agents, which are commonly used to treat patients with gastric cancer. Combined treatment with saracatinib and 5-FU or cisplatin produced synergistic effects in many of the gastric cancer cell lines even the cells were resistant to 5-FU or cisplatin alone (Table 2).

In NCI-N87 cells, 1 cell type in which the synergistic effects of saracatinib with 5-FU or cisplatin was observed, the number of apoptotic cells increased following the combined treatment of saracatinib and 5-FU or saracatinib and cisplatin (Fig. 4A).

We next confirmed the in vivo efficacy of saracatinib alone, 5-FU alone, or a combination of these 2 compounds in a N87 human gastric cancer xenograft model. Saracatinib alone showed significant antitumor activity, and the combination of saracatinib and 5-FU enhanced the individual antitumor activity of both drugs (Fig. 4B). Tumors treated with saracatinib alone exhibited a significant decrease in cell proliferation. When the tumors were treated with combination of
Saracatinib and 5-FU, more decreases of cell proliferation and more increases of apoptosis were observed by Ki67 and TUNEL assay (Fig. 4C). Furthermore, complete inhibition of Src/FAK activity was detected in the cells treated with a combination of these 2 drugs (Fig. 4D).
Discussion

Src is a mediator of numerous signal transduction pathways that are involved in many different steps of oncogenesis from proliferation to invasion and metastasis. Thus, targeting Src kinase for therapeutic purposes has been an important area of investigation. Blocking Src activity has anti-invasive and antimigratory effects, and inhibits the growth of various types of cancer (4, 20, 27, 28).

In the present study, we found that inhibiting Src with saracatinib had growth inhibitory effects on gastric cancer cells, such as SNU216 and N87 cells (Table 1). Furthermore, this compound exerted antimigratory and anti-invasive effects on the SNU216 cells (Fig. 1A).

There have been many efforts to identify predictive biomarkers for Src inhibitor. Potential markers predicting sensitivity to dasatinib, a multitargeting inhibitor of tyrosine kinases that reduces the activities of c-Src, Bcr-Abl, and other kinases, were evaluated by gene expression profiling in a panel of 23 breast cancer cell lines. The results of this study have identified 6 genes that are highly correlated with dasatinib sensitivity (EPHA2, CAV1, CAV2, ANXA1, PTRF, and IGFBP2; ref. 29). In addition, elevated expression of moesin, caveolin-1, and yes-associated protein-1 was found to be a sensitive marker for dasatinib (30).

It has also been suggested that LRRC19 and IGFBP2 identified by the K-TSP classifier are potential predictive biomarkers for saracatinib sensitivity in pancreatic cancer (31). Furthermore, Src overexpression was suggested as genotype-associated indicator of sensitivity to Src inhibitor such as saracatinib and dasatinib in ovarian, breast, NSCLC, or prostate cancers (32). A second K-TSP classifier (TOX, GLI3R, TSPAN7, BCA54, and PARDEG-C-NXN) and increased activation of the Src pathway were also suggested to be predictive markers for saracatinib sensitivity in colorectal cancer (11). In the present study, we found that the levels of both Src (Y416) and FAK (Y861, Y397, and Y925) activity were elevated in SNU216 and NCI-N87 cells that are HER2-amplified, saracatinib-sensitive cells (Table 1 and Fig. 1B).

We next tested whether HER2 expression levels affect sensitivity to saracatinib. First, we compared the activity of saracatinib on HER2-amplified SNU216, and NCI-N87 cells with HER2-amplified SKBR3, MDA-MB-453, and BT474 cells. In contrast to gastric cancer cell lines with HER2 amplification (SNU216 and NCI-N87), breast cancer cell lines with HER2 amplification (SKBR3, MDA-MB-453, and BT474) were resistant to saracatinib. We then compared the basal expression of Src, FAK, and integrin family members among the HER2-amplified cell lines. Cross-talk between EGFR and Src has been previously reported (20, 21), and integrins also cooperate with growth factor receptors, such as EGFR as well as the FAK/Src signaling pathway (33, 34). The integrin/FAK/Src pathway was highly activated in lapatinib-resistant cells, and inhibition of this pathway overcame lapatinib resistance (24).

In our study, we found that integrin-αv, integrin-β4, -8, and p-Src were highly expressed in the saracatinib-sensitive SNU216 and NCI-N87 cells as compared with SKBR3, MDA-MB-453, and BT474 cells (Fig. 1D). Next, we verified the effects of saracatinib on oncogenic signaling factors such as AKT and ERK in LS174T (HER2-nonamplified, saracatinib-sensitive cell line) and SKBR3 (HER2-amplified, saracatinib-resistant cell line). Sensitivity of LS174T cells to saracatinib was reported in a previous study (11), whereas sensitivity of SKBR3 cells to saracatinib was observed in our study (Fig. 1D). With the treatment of saracatinib, p-AKT and p-ERK were downregulated in LS174T cells, however, there was no significant change in SKBR3 cells (Supplementary Fig. S4). These results indicate that both EGFR and the integrin/FAK/Src signaling pathways have important roles in the antitumor effects of saracatinib in gastric cancer.

### Table 2. Enhanced efficacy of saracatinib with 5-FU or cisplatin in gastric cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>5-FU</th>
<th>Cisplatin</th>
<th>5-FU</th>
<th>Cisplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNU216</td>
<td>3.28</td>
<td>4.58</td>
<td>3.844</td>
<td>0.838</td>
</tr>
<tr>
<td>NCI-N87</td>
<td>2.64</td>
<td>1.18</td>
<td>0.142</td>
<td>0.197</td>
</tr>
<tr>
<td>SNU1</td>
<td>&gt;10</td>
<td>5.74</td>
<td>1.348</td>
<td>1.458</td>
</tr>
<tr>
<td>SNU5</td>
<td>&gt;10</td>
<td>2.96</td>
<td>0.119</td>
<td>0.914</td>
</tr>
<tr>
<td>SNU16</td>
<td>5.86</td>
<td>8.86</td>
<td>0.193</td>
<td>1.562</td>
</tr>
<tr>
<td>SNU601</td>
<td>4.62</td>
<td>1.54</td>
<td>0.522</td>
<td>2.586</td>
</tr>
<tr>
<td>SNU620</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.784</td>
<td>0.597</td>
</tr>
<tr>
<td>SNU638</td>
<td>&gt;10</td>
<td>4.99</td>
<td>1.745</td>
<td>4.609</td>
</tr>
<tr>
<td>SNU668</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>2.9</td>
<td>1.048</td>
</tr>
<tr>
<td>SNU719</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.035</td>
<td>0.007</td>
</tr>
</tbody>
</table>

NOTE: The IC_{50} values of 5-FU and cisplatin determined using an MTT assay are shown along with the CI for the combinations of saracatinib with 5-FU or cisplatin in gastric cancer cell lines (CI > 1, antagonistic effect; CI = 1, additive effect; CI < 1, synergistic effect).
FAK/Src signaling pathway may cooperatively modulate the sensitivity to Src inhibition. According to previously reported data for IC_{50} profiles of kinases, saracatinib shows inhibitory activity in Src family kinases such as Src (IC_{50} 2.7 nmol/L), Yes (IC_{50} 4 nmol/L), Fyn (IC_{50} 10 nmol/L), and EGFR (IC_{50} 66 nmol/L; ref. 35). In our study, saracatinib inhibited the phosphorylation of Src, Yes, Fyn, and FAK (Fig. 1C). In

Figure 4. Saracatinib administered in combination with 5-FU or cisplatin exerts synergistic effects against gastric cancer cells and enhances the antitumor activity of 5-FU in an NCI-N87 xenograft model. A, NCI-N87 cells were treated with the indicated concentrations of saracatinib, 5-FU (left), and cisplatin (right) alone, or combinations (saracatinib plus 5-FU or saracatinib plus cisplatin). The cells were then harvested for an Annexin V-binding assay 48 hours after treatment. Columns, mean of 3 independent experiments; bars, SD. *, P < 0.01. B, mice were treated with vehicle alone, saracatinib (50 mg/kg), 5-FU (50 mg/kg), or saracatinib (50 mg/kg) plus 5-FU (50 mg/kg). *, P < 0.001 compared with the control group on day 21; **, P < 0.05 compared with the saracatinib-treated group on day 21. C, immunohistochemistry was conducted to monitor Ki67 and TUNEL expression in the groups treated with saracatinib, 5-FU, or saracatinib plus 5-FU. Representative images of the immunohistochemistry assay are shown. C, on the final day of treatment (day 21), total cell proteins were extracted from tissues for immunoblotting with the indicated antibodies.
addition, saracatinib downregulated the activity of HER family members and downstream pathways (Fig. 2A and B). The enhanced effects of combined activation of targeting Src and EGFR signaling pathways are previously reported (6, 20, 36–38). Furthermore, targeting Src in combination with trastuzumab-sensitized trastuzumab-resistant cell lines (23), and inhibition of integrin/FAK/Src pathway overcame lapatinib resistance (24). Thus, we treated the SNU216 and NCI-N87 cells with combination of saracatinib and lapatinib. Apoptosis and inhibition of phosphorylation of EGFR, HER2, AKT, and ERK were enhanced with this combination treatment (Fig. 2C and D). Given that saracatinib inhibited both Src/FAK and EGFR signaling pathways and lapatinib enhanced antitumor effects of saracatinib, we concluded that cross-talk may exists between Src and EGFR signaling.

Bim has been emerging as an essential component of the apoptotic process triggered by EGFR tyrosine kinase inhibition by gefitinib or lapatinib (25, 28). In our study, apoptosis observed with saracatinib accompanied by induction of Bim. Bim knockdown decreased the number of apoptotic cells following treatment with saracatinib. These findings suggest an important role of Bim induction in saracatinib-mediated apoptosis (Fig. 3D and Supplementary Fig. S3).

We also investigated the effects of combination treatment of saracatinib with 5-FU or cisplatin. Saracatinib exerted synergistic effects in 6 of 10 gastric cancer cell lines in combination with 5-FU (Table 2). A combination of saracatinib and 5-FU increased apoptosis in NCI-N87 cells (Fig. 4A) and enhanced the antitumor activity of saracatinib in the NCI-N87 xenografts (Fig. 4B–D). Previously, it was reported that Src inhibition reverts chemoresistance to 5-FU through thymidylate synthase regulation, and the effect of Src kinase inhibition on 5-FU chemosensitivity might be accomplished by blocking 5-FU-induced EGFR-AKT activation (39). Therefore, we tested to see if the synergy between saracatinib and 5-FU observed in our study might have resulted from thymidylate synthase downregulation. Saracatinib significantly decreased thymidylate synthase levels in NCI-N87 (Supplementary Fig. S5). Interestingly enough, SNU5 cells, which are resistant to 5-FU, also showed the synergism between saracatinib with 5-FU by downregulating thymidylate synthase (Supplementary Fig. S5).

Saracatinib also enhanced the efficacy of cisplatin in gastric cancer cell lines. One study found that Src activity mediates oxaliplatin (platinum-based chemotherapeutic agent) sensitivity and resistance, and reduced expression of Src leads to increased cytotoxicity in response to oxaliplatin (40). Therefore, sensitivity to cisplatin and other platinum-based chemotherapeutic agents might also be affected by altered Src expression or activity in a similar manner. Furthermore, since Src is a downstream target of multiple pathways including insulin-like growth factor (IGF)-1R, EGFR, hepatocyte growth factor receptor, and platelet-derived growth factor receptor (PDGFR), it is necessary to further investigate the effects of saracatinib on these various signaling pathways.

In summary, our findings suggest that saracatinib shows antitumor effects in SNU216 and NCI-N87 cells through blockade of Src/FAK, HER family, and oncogenic signaling pathways. Saracatinib leads to G1 arrest as well as apoptosis in association with induction of Bim. Intriguingly, saracatinib enhanced the effects of lapatinib. Furthermore, saracatinib enhanced the antitumor effects of cytotoxic agents such as 5-FU or cisplatin not only in saracatinib-sensitive cells, but also in cells resistant to saracatinib. These preclinical data indicate that inhibition of Src kinase activity by saracatinib alone or in combination with other agents can be one of strategy to target gastric cancer.

Disclosure of Potential Conflicts of Interest
S.-A. Im was employed as Research Scientist in AstraZeneca (2007–2010) and is a consultant/advisory board member of AstraZeneca. D.-Y. Oh has other commercial research support and honoraria from speakers bureau of Hannmi Pharm. Y.-J. Bang has commercial research grant, honoraria from speakers bureau, and is a consultant/advisory board member of AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: H.-J. Nam, S.-A. Im, D.-Y. Oh, Y.-J. Bang
Development of methodology: H.-J. Nam, S.-A. Im, D.-Y. Oh, Y.-K. Yoon, Y.-J. Bang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-J. Nam, S.-A. Im, D.-Y. Oh, Y.-K. Yoon, A.R. Min
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-J. Nam, S.-A. Im, D.-Y. Oh, T.-Y. Kim, Y.-J. Bang
Writing, review, and/or revision of the manuscript: H.-J. Nam, S.-A. Im, D.-Y. Oh, P. Elvin, Y.-J. Bang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-J. Nam, S.-A. Im, D.-Y. Oh, H.-P. Kim, S.-H. Song
Study supervision: H.-J. Nam, S.-A. Im, D.-Y. Oh, S.-W. Han

Grant Support
Y.-J. Bang was supported by research grants from the Oncology Research collaboration of AstraZeneca Inc. (06-2007-301-0), and S.-A. Im was supported by fellowship grant from AstraZeneca Inc. T.-Y. Kim was supported from the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093820). D.-Y. Oh was supported from Seoul National University Hospital (30-2010-017-0).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 3, 2012; revised September 25, 2012; accepted November 2, 2012; published OnlineFirst November 9, 2012.

References


Molecular Cancer Therapeutics

Antitumor Activity of Saracatinib (AZD0530), a c-Src/Abl Kinase Inhibitor, Alone or in Combination with Chemotherapeutic Agents in Gastric Cancer

Hyun-Jin Nam, Seock-Ah Im, Do-Youn Oh, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0109

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/11/09/1535-7163.MCT-12-0109.DC1

Cited articles
This article cites 40 articles, 19 of which you can access for free at:
http://mct.aacrjournals.org/content/12/1/16.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/12/1/16.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.