Inhibition of Src Tyrosine Kinase as Treatment for Human Pancreatic Cancer Growing Orthotopically in Nude Mice

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ABSTRACT

Purpose: The Src family comprises a family of nonreceptor intracellular tyrosine kinases that mediate a variety of cellular pathways. Src kinases are overexpressed in a variety of human tumors, including cancer of the colon, breast, and pancreas, and they are an integral part of tumor cell signaling pathways associated with migration, proliferation, adhesion, and angiogenesis.

Experimental Design: We investigated whether the blockade of Src kinase by daily oral administration of the novel Src tyrosine kinase inhibitor AZM475271 [kindly provided by AstraZeneca (Macclesfield, United Kingdom)], alone or in combination with intraperitoneal gemcitabine, can inhibit growth and metastasis of orthotopically implanted human pancreatic carcinoma cells in nude mice.

Results: Treatment with AZM475271 alone reduced the primary pancreatic tumor volume by approximately 40%, whereas AZM475271 plus gemcitabine reduced tumor volume by 90%. Furthermore, treatment with AZM475271 and gemcitabine significantly reduced metastasis: none of eight animals who received the combination treatment had lymph node or liver metastases, compared with five of five and three of five animals, respectively, in the control group (P = 0.001). Src inhibition by AZM475271 (alone or with gemcitabine) was associated with significantly reduced tumor cell proliferation, decreased tumor microvessel density, and increased apoptosis in vivo. Moreover, these effects were all significantly increased when gemcitabine was combined with AZM475271 compared with gemcitabine alone.

Conclusions: Src inhibition by AZM475271, either alone or in combination with gemcitabine, demonstrated significant antitumor and antimetastatic activity in an orthotopic nude mouse model for human pancreatic cancer. The combination of AZM475271 with gemcitabine sensitized tumor cells to the cytotoxic effect of gemcitabine.

INTRODUCTION

Pancreatic cancer remains an unsolved health problem. The estimated overall 5-year survival rate of only 1% to 4% is due to the inability to detect this disease at an early stage, the aggressiveness of the disease, and the lack of effective systemic therapies (1, 2). The standard chemotherapeutic agent for different stages of pancreatic cancer, gemcitabine, shows some efficacy, with response rates of about 12% to 27%; however, the median overall survival with treatment remains 4 to 6 months (3–5).

Recent advances in understanding the biology of pancreatic cancer may offer new therapeutic approaches. There is increasing interest in anticancer treatment strategies that combine standard chemotherapy with novel agents that are specifically targeted against pathological signaling pathways of cancer cells. Preclinical studies combining chemotherapy with drugs targeting the epidermal growth factor receptor (EGFR) or proangiogenic molecules such as vascular endothelial growth factor (VEGF) have shown potent efficacy in pancreatic cancer (6, 7).

Inhibition of the activity of intracellular signal transducers has been shown to inhibit proliferation, migration, metastasis, and invasion of cancer cells (8, 9). The signaling protein Src tyrosine kinase is a promising potential target for antitumor therapy and is currently under intensive investigation.

Src kinases are nonreceptor intracellular tyrosine kinases that mediate a variety of intracellular signaling pathways. They are cellular homologs of the products of the Rous sarcoma virus gene (v-src), which is the mutated and activated version of a normal cellular gene (c-src). There are eight members of this family: Src, Fyn, and Yes are ubiquitously expressed; Lck, Hck, Fgr, Lyn, and Blk have more tissue-restricted expression, mainly in hematopoietic cells (10). Src tyrosine kinases are known to be overexpressed in a variety of tumor types, such as human colon adenocarcinoma (11, 12), breast cancer (13, 14), and pancreatic carcinoma (15). Src family members are involved in numerous signaling pathways involved in proliferation, migration, tumor adhesion, and angiogenesis (16) and mediate signaling from many types of receptors including receptor tyrosine kinases (RTKs), integrins, and G-protein–coupled receptors (10). RTKs that signal through Src kinases include platelet-derived growth factor receptors, EGFRs, and fibroblast growth factor receptors (17). The Src family also appears to be required for growth factor–stimulated DNA syn-

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thesis, particularly for growth factors with RTKs such as platelet-derived growth factor receptor and EGFR (18, 19).

Thus, strategies that aim to inhibit Src kinase activity, either alone or in combination with standard chemotherapy, could be effective against primary tumor growth and metastasis of pancreatic carcinoma. Although Src overexpression and activation have been reported in human pancreatic carcinoma (15), the result of inhibiting this protein alone and in combination with chemotherapy has not been defined. The current study examined the in vivo effects of the novel Src tyrosine kinase inhibitor AZM475271 (20) in an orthotopic human pancreatic carcinoma model in nude mice.

MATERIALS AND METHODS

AZM475271. AZM475271 (Scheme 1) is a novel anilinoquinoxaline inhibitor of c-Src kindly provided by AstraZeneca (Macclesfield, United Kingdom).

In vitro Cell Culture. L3.6pl human pancreatic cancer cells were established from COLO 357 fast-growing cells and cultured as described previously (6, 7).

In vitro Src Kinase Activity Assay. L3.6pl cells were plated into 10-mm dishes. After overnight incubation, the cells were treated for 4 hours with AZM475271 (1–10 μmol/L). After washing cells with ice-cold PBS, lysates were collected using lysis buffer (50 mmol/L HEPES [pH 7.2], 150 mmol/L NaCl, 1 mmol/L EGTA, 20 mmol/L NAF, 1% Triton X-100, 10% glycerol, 1 mmol/L β-glycerophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L Na3VO4) complemented with protease inhibitor mixture tablets (Roche Diagnostics, Mannheim, Germany). After centrifugation for 10 minutes at 9,000 × g, the supernatant was incubated at 4°C for 4 hours with 15 μg of v-src (Ab-1) monoclonal antibody (Oncogene Research Products, San Diego, CA) preadsorbed to protein A- and protein G-Sepharose (Sigma, Munich, Germany). The immune complex was washed three times with lysis buffer and twice with kinase buffer A [30 mmol/L HEPES (pH 7.5), 5 mmol/L MnCl2, 2 mmol/L dithiothreitol, and 0.1 mmol/L Na3VO4]. Beads were finally resuspended in 4 μL of 5% kinase buffer containing 5 μg of enolase (Sigma) and 10 μCi of [γ-33P]ATP (Perkin-Elmer, Wellesley, MA). After incubation at 30°C for 10 minutes, assays were terminated by the addition of 20 μL of 2× Laemmli sample buffer. The samples were heated at 95°C for 5 minutes and analyzed by SDS-12% PAGE. The gels were dried and subjected to autoradiography. Densitometry of the gels was performed by Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD).

In vivo Plasma Concentration. Healthy nude mice were treated with a single dose of 50 mg/kg AZM475271 by oral administration. The plasma concentration of AZM475271 was measured by mass spectrometry 2, 6, and 24 hours after oral feeding of the compound.

Orthotopic Implantation of Tumor Cells in the Pancreas of Athymic Nude Mice and Treatment of Established Human Pancreatic Cancer. To explore the in vivo antitumor effect of AZM475271 alone or with gemcitabine, we injected 1 × 106 viable L3.6pl cells into the pancreas of athymic nude mice (6). Seven days later, the mice were randomized into four treatment groups (n = 5–9). The first group received gemcitabine twice weekly (100 mg/kg intraperitoneally), the second group received AZM475271 daily (50 mg/kg orally), the third group received gemcitabine twice weekly (100 mg/kg intraperitoneally) and AZM475271 daily (50 mg/kg orally), and the control group received saline (PBS) twice weekly intraperitoneally. All mice were sacrificed on day 32, when the control animals became moribund. Detailed necropsy revealed that all of the mice had tumors in the pancreas.

Tumor volume (measured with a caliper through the skin), the weight of the pancreatic tumors, the incidence of regional (celiac and para-aortal) lymph node metastasis, and the number of liver metastases were recorded. Pancreatic carcinoma was confirmed by histopathology. For immunohistochemical staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin. Another part of the tumor was embedded in OCT compound (Miles, Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C.

Immunohistochemical Staining for Ki-67 (Proliferating Cells). Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was performed using a commercially available apoptosis detection kit (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI). CD31 staining for microvesSEL density. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining was performed using a commercially available apoptosis detection kit (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI). CD31 staining for microvesSEL density (MVD) was performed as described previously (7). For Ki-67 staining, tissue sections were deparaffinized and then overlaid with 10 mmol/L citrate buffer (pH 6.0) and heated for 15 minutes in a microwave at 750 W to remove the masking effect of formalin fixation. After cooling down, the slides were rinsed with PBS, and nonspecific binding sites were blocked with 5% bovine serum albumin (BSA) in PBS. After another washing step with PBS, the primary antibody (1:75; Zymed, San Diego, CA), a polyclonal rabbit anti-human antibody against Ki-67, was applied, and the slides were incubated for 2 hours at room temperature. The samples were then incubated with biotinylated goat antirabbit (Vector Laboratories, Burlingame, CA) secondary antibody (1:200) for 1 hour at room temperature, followed by incubation with an avidin-biotinylated horseradish peroxidase (HRP) complex from an ABC kit (Vector Laboratories). Seven to 10 high-power fields (0.159 mm2) per section of three to five tumors per treatment group were examined microscopically, and the average number of cells that stained positive for Ki-67 per treatment group was evaluated.

In vitro 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Cell Proliferation Assay. A viability assay for L3.6pl cells treated with gemcitabine, AZM475271, or a combination of both agents was performed with a 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay kit (R&D Systems, Minneapolis, MN) with modifications. L3.6pl cells were removed from subconfluent cultures by trypsinization (0.25% trypsin and 0.02% EDTA), which was stopped with medium containing 10% fetal bovine serum. The cells were plated into 96-well plates (15,000 cells in 100 μL of 10% fetal calf serum (FCS) medium per 38-mm² well (the number of cells per well was determined by trypan blue method)). After 24 hours of attachment (37°C, 5% CO2), 200 μL of medium containing AZM475271 (1–25 μmol/L), gemcitabine (1–50 ng/mL), or both compounds were added, and the plates were incubated for another 48 hours. After washing with 200 μL of PBS, 10 μL of MTT reagent were added for another 2 hours at 37°C. When the purple precipitate was clearly visible under the microscope, 100 μL of detergent were added for another 2 hours in the dark. The absorbance per well was measured at 570 nm. The IC50 was defined as the dose that inhibited 50% of cell growth. All experiments were replicated three times.

In vitro c-src 3T3 Proliferation Assay. A mouse NIH3T3 fibroblast cell line transfected to overexpress active Src kinase (c-Src3T3) was made and provided by Sara Courneidne (Van Andel Research Institute, Grand Rapids, MI). This line has been shown to grow in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Paisley, United Kingdom) + 0.5% serum, conditions in which the parental NIH3T3 cells do not survive. In these low-serum conditions, the transfected cells are driven to proliferate through their expression of active Src kinase, and consequently, Src kinase inhibition should revert them to the parental phenotype. The c-Src3T3 cells are routinely cultured in DMEM + 5% FCS. For the assay, they were harvested with trypsin and plated to 96-well plates at 1.5 × 10⁴ cells per well. The following day, a dilution series was made from AZM475271 in neat dimethyl sulfoxide (DMSO). These were further diluted into DMEM + 5% FCS, and 100 μL of these dilutions were added per well to the cell plates. The final DMSO concentration per well was 0.5% in all wells. The plates were then incubated for an additional 24 hours. A colorimetric 5-bromo-2′-deoxyuridine (BrdUrd) Cell Proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics) was then used to assess proliferation according to the manufacturer’s instructions. Briefly, the cells were pulse-labeled with BrdUrd for 2 hours and fixed, and the cellular DNA was denatured with the provided solution and then incubated with Anti-BrdUrd-peroxidase for 90 minutes. The plates were then washed three times with PBS and patted dry, the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution was added, and the plates were incubated on a plate shaker for 10 to 30 minutes, until the positive control absorbance at 690 nm was circa 1.5. Positive control and blank wells were included on all plates containing compound diluent and MgCl₂ solution with and without ATP, respectively, to determine the dynamic range of the assay. The curves were plotted, and the IC₅₀ values for compound inhibition of cell proliferation were interpolated using Microcalc Origin following subtraction of the blank values.

In vitro Src Kinase Inhibition Test. A poly(Glu, Tyr) 4:1 random copolymer (Sigma-Aldrich, Poole, United Kingdom) was used as the tyrosine-containing substrate. This is stored as a 10 mg/mL stock solution in PBS at 20°C and diluted 1:200 with PBS to coat 96-well plates (100 μL/well). Substrate was plated the day before the assay, and the plates were covered with adhesive seals and stored overnight at 4°C. On the day of the assay, the substrate solution was discarded, and the plates were then incubated with 120 μL/well of 5% BSA in PBS for 10 minutes. The plates were then washed once with PBS containing 0.05% (v/v) Tween 20 (PBST) and then incubated with 50 mmol/L HEPES (pH 7.4) at 100 μL/well until the next stage. AZM475271 was dissolved in DMSO at 10 mmol/L. A dilution series was then made in double-distilled H₂O to give solutions at 4× the final required reaction concentrations. Solutions of 40 μmol/L ATP in 80 mmol/L MgCl₂ and 80 mmol/L MgCl₂ alone (for -ve controls) were prepared. Src kinase, expressed in sf9 insect cells by recombinant baculovirus containing the human c-src gene (Upstate Biotechnology, Lake Placid, NY), was diluted to 0.3 unit/mL in enzyme dilution buffer (100 mmol/L HEPES, 2 mmol/L dithiothreitol, 0.2 mmol/L sodium orthovanadate, and 0.02% BSA). The HEPES was discarded from the substrate plates, and the following additions were made in order: 25 μL/well compound dilution (water in the case of positive and negative controls); 25 μL/well ATP/MgCl₂ or MgCl₂ (negative controls) alone; and, finally, 50 μL/well Src kinase in dilution buffer to start the reaction. The final reaction concentrations were 0.15 unit/mL Src kinase, 20 mmol/L MgCl₂, and 10 μmol/L ATP (determined as the Km for ATP). The reaction time allowed was 15 minutes at room temperature on a plate shaker. The assay was stopped by washing the plates four times with PBST (150 μL/well). Detection of resultant tyrosine phosphorylation was facilitated by the addition of an anti–phospho-tyrosine monoclonal antibody conjugated to alkaline phosphatase (anti-pY/HRP, Santa Cruz Biotechnology, Santa Cruz, CA); this was diluted 1:5000 in PBST + 0.5% BSA + 0.1 mmol/L sodium orthovanadate, added at 100 μL/well, and incubated for 1 hour. The plates were then washed (six times). One tablet of the HRP substrate TMB (Sigma-Aldrich) was dissolved in 100 μL of DMSO and added per 10 mL of phosphate citrate buffer with sodium perborate (Sigma-Aldrich). TMB substrate solution (100 μL/well) was added. After 5 minutes of color development, the reaction was stopped by the addition of 50 μL/well 0.8 mol/L H₂SO₄, and the positive control wells now gave an A₄₅₀ nm of circa 1.2–1.5. Control and blank wells were included on all plates containing compound diluent and MgCl₂ solution with and without ATP, respectively, to determine the dynamic range of the assay. The curves were plotted, and the IC₅₀ values for compound enzyme inhibition were interpolated using KC3 Kinetica software (Bio-Tek Instruments, Winooski, VT) following subtraction of the blank values. The in vitro VEGF-R2 kinase inhibition assay determines the ability of AZM475271 to inhibit VEGF-R2 kinase activity and has been used as a selectivity screen. The method was as reported previously (14).

Determination of Apoptotic Cells by Fluorescence-Activated Cell-Sorting Analysis. Quantification of apoptosis by fluorescence-activated cell-sorting (FACS) analysis was performed using annexin V-fluorescein isothiocyanate Apoptosis Detection Kit I (BD PharMingen, San Diego, CA). After incubation with AZM475271 (5 μmol/L), gemcitabine (50 ng/mL), or a combination of both drugs in vitro for 12 hours, cells were collected by gentle trypsinization, washed in PBS, and pelleted...
by centrifugation. Cells were resuspended in binding buffer, treated with 5 µL of annexin V (10 mg/mL) and 5 µL of propidium iodide (10 µg/mL), and incubated in the dark at room temperature for at least 20 minutes before processing for FACS analysis using the FACSCalibur machine. Ten thousand events were counted. The relative percentage of cells in the sub-G₀ phase was then quantitated and used as an estimate of the amount of cells undergoing apoptosis. Data were analyzed using WinMDI 2.8 software. Experiments were repeated four times, and the mean percentage of apoptotic cells was calculated.

**Boydoy Chamber Migration Assay.** Cell invasion was assessed using a modified Boyden chamber assay. Boyden chambers (8-µm pore size; Becton-Dickinson Labware, Franklin Lakes, NJ) were preincubated for 30 minutes with human fibronectin (13.4 ng/mL in PBS; Biotrend Chemikalien, Köln, Germany) and then dried and put into 12-well plates (Becton Dickinson Labware). Each upper chamber was filled with 1 x 10⁶ L3.6pl cells suspended in 0.5 mL of serum-free DMEM. The lower chambers contained 2 mL of the same medium containing 100 ng/mL human fibronectin. After 4 hours of incubation (37°C, 5% CO₂), the medium was removed, and the mean number of cells invading the lower chamber was assessed using a modified Boyden chamber assay. Boyden chambers (8-µm pore size; Becton-Dickinson Labware) were preincubated for 30 minutes with human fibronectin (13.4 ng/mL in PBS; Biotrend Chemikalien, Köln, Germany) and then dried and put into 12-well plates (Becton Dickinson Labware). Each upper chamber was filled with 1 x 10⁶ L3.6pl cells suspended in 0.5 mL of serum-free DMEM. The lower chambers contained 2 mL of the same medium containing 100 ng/mL human fibronectin. After 4 hours of incubation (37°C, 5% CO₂), the medium was removed, and the inner surfaces of the membranes were cleaned. Filters were stained with hematoxylin and eosin, and cells were counted in five random fields at ×100 magnification. The average of triplicate inserts from three representative experiments was obtained.

**Statistical Analysis.** Pancreatic tumor volume, body weight, and quantification of Ki-67, TUNEL, and CD31 were compared using one-way analysis of variance with a Student-Newman-Keuls multiple comparisons test (InStat 3.0 Statistical Software; Graphpad Software, San Diego, CA). The relative rates of liver and lymph node metastases within groups were compared by Fisher’s exact test. For all analyses, P < 0.05 was considered to be significant.

**RESULTS**

**In vitro Inhibition of Src Kinase Activity by AZM475271.** AZM475271 demonstrated strong dose-dependent inhibition of Src tyrosine kinase activity in the L3.6pl human pancreatic carcinoma cell line. Maximum reduction of Src kinase activity was observed after incubation for 4 hours with ≥5 µmol/L AZM475271 (4.76-fold reduction; Fig. 1).

**In vitro Evaluation of Plasma Concentration Levels.** After a single oral dose (gavage) of 50 mg/kg AZM475271 to healthy nude mice, the plasma concentration was measured 2, 6, and 24 hours after oral dosing with 50 mg/kg AZM475271.

**In vivo Inhibition of Pancreatic Cancer Growth and Metastasis by AZM475271 ± Gemcitabine.** Tumors appeared to be palpable at day 14 after tumor cell injection in all animals except mice treated with both AZM475271 and gemcitabine, in which the earliest possible palpation of the tumors was at day 17 after tumor cell injection. In all treated animals, the median tumor volume was significantly less than that in control mice (AZM475271-treated animals, 827 mm³; gemcitabine-treated animals, 393 mm³; AZM475271 + gemcitabine-treated animals, 124 mm³; control animals, 1359 mm³; Fig. 2; Table 1). Furthermore, after combination therapy, primary pancreatic tumor volume was significantly less than that seen with gemcitabine alone. The incidence of lymph node metastasis was also significantly reduced after combination therapy compared with controls. The incidence of liver metastasis was markedly reduced by treatment with AZM475271, either alone or in combination with gemcitabine, compared with controls (Table 1). Treatment with gemcitabine or AZM475271 alone did not significantly change animal weight; however, a reduction in body weight was observed in the combination therapy group at the end of the experiment, which was potentially related to cumulative toxicity (Table 1).

**Immunohistochemical Analyses.** We next analyzed the effect of AZM475271 alone and in combination with gemcitabine on tumor cell proliferation in vivo by assessing the levels of the nuclear antigen Ki-67, which is present in all phases of the cell cycle except G₀. The mean number of Ki-67-positive tumor cells in the pancreatic tumors of control mice was 480 ± 14. After therapy with gemcitabine or AZM475271 alone, the mean number of Ki-67-positive cells was 275 ± 33 and 178 ± 26, respectively (Fig. 3; Table 2). The lowest number of proliferating cells (155 ± 39) was found in tumors of mice treated with AZM475271 and gemcitabine. These results indicate an in vivo antiproliferative effect of AZM475271 alone and in combination with gemcitabine.

TUNEL staining was carried out to determine the ability of AZM475271 to induce apoptosis in pancreatic carcinoma cells.
Both gemcitabine and AZM475271 as single agents induced a significant enhancement of apoptotic cells in primary pancreatic tumors compared with control tumors. The mean number of TUNEL-positive cells was 27 ± 8 (gemcitabine) and 28 ± 14 (AZM475271) compared with 7 ± 3 (control; Fig. 3; Table 2). Maximum apoptotic effect was observed in the combination therapy group, which had a significantly higher number of TUNEL-positive cells (P < 0.005) compared with the groups treated with either gemcitabine (P = 0.005) or AZM475271 alone (P < 0.005).

MVD (measured with antibodies against CD31) was reduced by treatment with the Src kinase inhibitor but not by treatment with gemcitabine. We found a significant reduction in tumor MVD per field after treatment with AZM475271 (29 ± 16) or combination therapy (30 ± 7) compared with either control tumors (48 ± 12) or gemcitabine-treated tumors (49 ± 7; Fig. 3; Table 2). There was no significant difference in MVD in tumors treated with AZM475271 alone compared with tumors treated with AZM475271 + gemcitabine combination therapy.

**In vitro Antiproliferative Activity of AZM475271 ± Gemcitabine in L3.6pl Cells.** AZM475271 showed no antiproliferative effect on L3.6pl cells in vitro, although a rapid decrease in cell proliferation was detected at high concentrations (>20 μmol/L; Fig. 4A) that could be related to the ability of AZM475271 to induce cell death at these doses. Microscopic investigation revealed a significant decrease in the number of viable cells with 20 μmol/L AZM475271, indicating that the rapid inhibition of cell proliferation was caused by reduced cell numbers due to therapy-induced cell death (Fig. 4B).

To test whether AZM475271 would enhance the antiproliferative effect of gemcitabine, the MTT proliferation assay was performed using L3.6pl cells treated with gemcitabine (1–50 ng/mL) alone and in combination with 5 μmol/L AZM475271.

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**Table 1**  In vivo Efficacy of AZM475271 +i- Gemcitabine for Human Pancreatic Cancer in Nude Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pancreatic tumor</th>
<th>Metastases† (N)</th>
<th>Median (range) body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor incidence * (N)</td>
<td>Median (range) tumor volume (mm³)</td>
<td>Lymph node</td>
</tr>
<tr>
<td>Gemcitabine (twice weekly, 100 mg/kg)</td>
<td>5/5</td>
<td>393 (297–471); P &lt; 0.0004 ‡</td>
<td>2/5</td>
</tr>
<tr>
<td>AZM475271 (50 mg/kg/d)</td>
<td>9/9</td>
<td>827 (603–879); P &lt; 0.002 ‡</td>
<td>9/9</td>
</tr>
<tr>
<td>AZM475271 (50 mg/kg/d) + gemcitabine (twice weekly, 100 mg/kg)</td>
<td>8/8</td>
<td>124 (63–363); P &lt; 0.0001 ‡; P &lt; 0.002 §</td>
<td>0/8</td>
</tr>
</tbody>
</table>

* Data represent number of mice with tumors/number of mice receiving injections.
† Data represent number of mice with metastases/number of mice receiving injections.
‡ Compared with controls.
§ Compared with gemcitabine alone (unpaired Student’s t test).
There was no significant difference in IC$_{50}$ concentration of gemcitabine in combination with AZM475271 or as monotherapy (8.5 versus 10.5 ng/mL, respectively). In contrast, the IC$_{100}$ (no detectable proliferation) concentration of gemcitabine in combination with AZM475271 was significantly lower (25 ng/mL) than the IC$_{100}$ with gemcitabine monotherapy (50 ng/mL; Fig. 5).

**In vitro c-src 3T3 Proliferation.** To demonstrate the potency of AZM475271, the IC$_{50}$ of c-src–transfected 3T3 fibroblasts was evaluated using a colorimetric BrdUrd Cell Proliferation ELISA kit (Roche Diagnostics) after incubation with different doses of AZM475271. The antiproliferative IC$_{50}$ was 0.5 μmol/L ($n=6$; 0.5, 0.66, 0.56, 0.56, 0.45, and 0.42 μmol/L) in comparison with other tumor cell lines such as PC3 (human prostate cancer), DU145 (human prostate cancer), and A549 (human lung cancer) with an antiproliferative IC$_{50}$ of 32, 16, and 17 μmol/L, respectively.

**In vitro Src Kinase and KDR Inhibition.** To demonstrate the selectivity of AZM475271, an in vitro src and KDR inhibition ELISA was performed measuring the IC$_{50}$ concentration of AZM475271 to inhibit the phosphorylation of c-src, lck, and c-yes was 0.01, 0.03, and 0.08 μmol/L, respectively, in comparison with an IC$_{50}$ of 0.7 μmol/L AZM475271 to inhibit KDR.

**In vitro Induction of Cell Death by AZM475271 ± Gemcitabine in L3.6pl Cells.** FACS analysis was used to determine whether the antitumor effect of AZM475271 alone or in combination with gemcitabine was due to cell death. L3.6pl cells were treated with AZM475271 (5 μmol/L), gemcitabine (50 ng/mL), or a combination of both drugs, resulting in apoptosis in 12.6 ± 3.6%, 11.0 ± 2.1%, and 52.2 ± 16.1% of the tumor cells, respectively (Fig. 6).

**Table 2 Immunohistochemical analysis of human pancreatic carcinoma in the pancreas of control and treated mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor cells</th>
<th>Endothelial cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ki-67</td>
<td>TUNEL</td>
</tr>
<tr>
<td>Saline (control)</td>
<td>480 ± 14</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>Gemcitabine (twice weekly, 100 mg/kg)</td>
<td>275 ± 33; $P &lt; 0.001$†</td>
<td>27 ± 8; $P &lt; 0.001$†</td>
</tr>
<tr>
<td>AZM475271 (50 mg/kg/d)</td>
<td>178 ± 26; $P &lt; 0.0005$‡</td>
<td>28 ± 14; $P &lt; 0.001$†</td>
</tr>
<tr>
<td>AZM475271 (50 mg/kg/d) + gemcitabine (twice weekly, 100 mg/kg)</td>
<td>155 ± 39; $P &lt; 0.0001$‡</td>
<td>54 ± 18; $P &lt; 0.0001$‡</td>
</tr>
</tbody>
</table>

* Number of positive cells per field from 10 random 0.159-mm$^2$ fields at ×100 magnification.
† Compared with controls.
‡ Compared with gemcitabine alone;
§ Compared with gemcitabine or AZM475271 alone (unpaired Student’s t test).
**In vitro Inhibition of Migration by AZM475271 of L3.6pl Cells.** To investigate the antitumor effect of Src inhibition demonstrated in vivo, we assessed whether AZM475271 treatment of L3.6pl cells prevented cell migration in a modified Boyden chamber assay. AZM475271 treatment produced significant inhibition of L3.6pl tumor cell migration at concentrations of 1 and 5 μmol/L (Fig. 7).

**DISCUSSION**

Src tyrosine kinases are involved in the transduction of downstream signals from tumor cell receptors such as RTKs and integrins. The family of Src kinases regulates a number of tumor cell-specific functions such as migration, adhesion, cell growth and differentiation, and survival. Therefore, there is increasing interest in elucidating Src function in tumor cells and the effects of Src inhibition. Src overexpression and increased activity have been reported in several pancreatic cancer cell lines (15); therefore, inhibition of Src kinase activity has potential as a strategy for the treatment of pancreatic carcinoma.

In this report, we have demonstrated the antitumor efficacy of the orally bioavailable Src kinase inhibitor AZM475271 (AstraZeneca), alone or combined with gemcitabine, in an orthotopic nude mouse model of pancreatic cancer. In vitro, we observed a strong dose-dependent inhibition of Src kinase in the highly metastatic human pancreatic cancer cell line L3.6pl after treatment with AZM475271. At 5 μmol/L AZM475271, Src kinase activity was reduced by 4.76-fold, confirming that AZM475271 inhibits Src kinase in L3.6pl human pancreatic cancer cells. To demonstrate the selectivity of the compound, an in vitro src and KDR inhibition ELISA was performed measuring the IC50 of AZM475271 necessary to inhibit the phosphorylation of src kinase and KDR of an immobilized substrate. The IC50 of AZM475271 to inhibit the phosphorylation of c-src, lck, and c-yes was 0.01, 0.03, and 0.08 μmol/L, respectively, in comparison with an IC50 of 0.7 μmol/L AZM475271 to inhibit KDR phosphorylation.

We observed antiproliferative activity against active Src engineered NIH3T3 fibroblasts. The antiproliferative IC50 was 0.5 μmol/L (n = 6; 0.5, 0.66, 0.56, 0.56, 0.45, and 0.42 μmol/L) in comparison with other tumor cell lines such as PC3 (human prostate cancer), DU1145 (human prostate cancer), and A549 (human lung cancer) with an antiproliferative IC50 of 32, 16, and 17 μmol/L, respectively.

In vivo, treatment with AZM475271 alone resulted in 40% inhibition of primary pancreatic tumor growth. However, the combination of AZM475271 and gemcitabine further inhibited pancreatic tumor growth up to 90%. Combination therapy (AZM475271 + gemcitabine) was also associated with a growth delay of primary pancreatic tumors after orthotopic tumor cell injection. Our results indicate that the inhibition of Src kinase by AZM475271 in human pancreatic cancer has antitumor efficacy that is markedly enhanced by combination with gemcitabine. Based on the in vivo evaluation of the plasma concentration levels of AZM475271 after single oral application of 50 mg/kg, we can assume that in relation to the in vitro experiments, biological effective plasma concentration levels are obtained as well in vivo and the antitumorogenic and antimetastatic effect in the orthotopic pancreatic cancer model can be related to AZM475271.

We considered two theories to explain the potential antitumor effects of AZM475271: first, the theory that AZM475271 directly inhibits the proliferation of tumor cells. To verify this theory, we performed immunohistochemical analysis for proliferating cells (Ki-67) in the tumor samples treated with AZM475271. Quantification of the Ki-67–positive cells showed that inhibition of the Src kinase activity correlated with a decreased number of dividing tumor cells both after monotherapy and after combination therapy with gemcitabine. However, the in vitro proliferation assay for L3.6pl cells treated with AZM475271 revealed no antiproliferative effect as a result of Src kinase inhibition. There was no significant difference in the percentage of inhibition of cell proliferation after incubation with AZM475271 (1–15 μmol/L). It was microscopically confirmed that the inhibition of cell proliferation observed after treatment with ≥20 μmol/L was a result of reduced cell numbers due to therapy-induced cell death.

These contrasting in vitro and in vivo results led us to formulate our second theory, that the antiproliferative effect of
AZM475271 observed in vivo was related to an indirect regulation of tumor cell proliferation via inhibition of tumor-induced angiogenesis. Indeed, we observed a reduced amount of microvessels in pancreatic tumors treated with AZM475271 as monotherapy or in combination with gemcitabine, suggesting that inhibition of the Src kinase in pancreatic tumors in vivo resulted in an antiangiogenic effect. Moreover, the antiangiogenic activity of the Src kinase inhibitor was also evident in vitro, where proliferation, invasion, and migration of the endothelial cells were inhibited by AZM475271 using a rat aortic rings assay (data not shown).

Chou et al. (21) recently reported that Src kinase promotes tumor angiogenesis through regulation of VEGF-induced signaling. Together with the evidence that regulation of the VEGF expression by tumor cells (and consequently tumor angiogenesis) correlates with Src kinase activity (22), our results indicate that the antiangiogenic effect of Src kinase inhibition in human pancreatic carcinoma caused an additional inhibition of tumor cell proliferation. The same effect was shown previously in a colon cancer model (23).

Although the number of microvessels in the animals treated with AZM475271 in combination with gemcitabine was found to be similar to that observed after AZM475271 monotherapy, combination therapy resulted in 90% inhibition of primary pancreatic tumor growth, whereas AZM475271 monotherapy was able to reduce primary pancreatic tumor growth by ~40% compared with control tumors. Such strong tumor growth inhibition after combination treatment with AZM475271 + gemcitabine cannot be completely explained by the ability of AZM475271 to reduce the amount of microvessels.

Other preclinical studies in pancreatic cancer have demonstrated that the antitumor efficacy of gemcitabine can be enhanced by additional treatment with inhibitors of the tyrosine kinase receptors (24, 25), thus it is possible that inhibition of Src kinase function can enhance the antitumor effect of gemcitabine in pancreatic cancer. We tested whether the combination of AZM475271 and gemcitabine affects tumor cell proliferation in vitro and found that the IC_{50} dose for gemcitabine used in combination with 5 μmol/L AZM475271 was not significantly different from the IC_{50} dose of gemcitabine used as monotherapy (8.5 versus 10.5 ng/mL, respectively). In contrast, the concentration of gemcitabine that produced no detectable cell growth was markedly reduced by combination with 5 μmol/L AZM475271 (IC_{100} 25 ng/mL) compared with gemcitabine monotherapy (IC_{100} 50 ng/mL). These data suggest that Src kinase inhibition by AZM475271 sensitizes tumor cells to the cytotoxic effect of gemcitabine. Interestingly, it has also been demonstrated that active Src kinase promotes survival of ovarian cancer cell lines and that inhibition of Src kinase sensitizes ovarian cancer cells toward other chemotherapeutic agents (paclitaxel and cisplatinum; ref. 26).

We next investigated whether AZM475271 treatment alone could induce apoptosis in human pancreatic tumors in vivo and whether the inhibition of Src kinase could enhance the apoptotic effects of gemcitabine. The mean number of apoptotic cells in the tumors treated with AZM475271 alone was significantly elevated compared with that in control tumors (28 ± 14 versus 7 ± 3, respectively), indicating that inhibition of Src kinase induces apoptosis of human pancreatic tumor cells. Moreover, the amount of apoptotic cells was markedly higher after combination therapy (54 ± 18) compared with AZM475271 monotherapy, demonstrating that Src inhibition enhances the apoptotic effects of gemcitabine.

A key signaling pathway downstream of Src associated with oncopogenesis is signal transducers and activators of transcription (STAT), which has been shown to prevent apoptosis of tumor cells and is also known to be activated in pancreatic cancer cells (27). Constitutive activation of STATs is linked to persistent activity of tyrosine kinases, including Src, EGFR, Janus kinases, Bcr-Abl, and many others. Persistent signaling of specific STATs, in particular, STAT3 and STAT5, has been demonstrated to participate in oncopogenesis through up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators such as Bcl-xL, Mcl-1, cyclins D1 and D2, and c-Myc. Inhibition of constitutively active STAT signaling pathways has been shown repeatedly to inhibit tumor cell growth in vitro and in vivo (27). Furthermore, previous studies have shown that the transcription factor STAT3 mediates VEGF gene transcription and its activation. In addition, it has been shown that VEGF can differentially induce STAT3 activation and that this effect is linked to VEGFR2/STAT3 complex formation, which correlates with VEGF autocrine ability to stimulate its own gene expression (28).

Therefore, it is very possible that at least some of the antitumor effects as well as the sensitization to gemcitabine can be attributed to inhibition of STAT3 signaling downstream of Src.

In vitro FACS analysis detected a significantly elevated number of apoptotic cells after treatment with AZM475271 and gemcitabine compared with gemcitabine monotherapy and controls, indicating that inhibition of Src kinase in human pancreatic cancer cells promotes apoptosis and markedly enhances gemcitabine-induced cell death.

Additionally, we demonstrated an in vivo antimetastatic effect after Src kinase inhibition in human pancreatic cancer growing orthotopically in nude mice. A decreased incidence of lymph node metastases and a complete inhibition of the liver metastases were associated with administration of AZM475271 monotherapy as well as combination therapy. The antimetastatic effect was confirmed in vitro, where analysis of tumor cell migration using a modified Boyden chamber assay revealed a strong dose-dependent inhibition of L3.6pl cell migration. AZM475271 is a potent inhibitor of L3.6pl migration in vitro at a concentration of 1 μmol/L. Similar results were obtained in Src-containing migrating human lung cancer cells (A549), in which AZM475271 showed antimigratory activity even at submicromolar concentrations (20). Recent evidence has demonstrated a major role for Src activity in the control of cell adhesion and cytoskeletal changes, which in turn regulate cell invasion and migration (29). Therefore, therapeutic inhibition of Src might primarily be useful in preventing metastasis, rather than inhibiting tumor growth. This has important implications for the preclinical and clinical assessment of such agents, and the design of relevant endpoints is necessary for the investigation of anti-invasive compounds such as Src inhibitors. Our results are consistent with such a role, with the demonstration of in vivo antimetastatic and in vitro antimigration effects of AZM475271.
In summary, we have demonstrated that AZM475271 inhibition of Src kinase, either alone or in combination with gemcitabine, has significant antitumor activity against primary tumor growth and metastasis in an orthotopic nude mouse model for human pancreatic cancer. The antitumor efficacy of AZM475271 is mediated in part by direct induction of tumor cell death and in part by an indirect antiproliferative effect based on reduced MVD, and the anti-invasive properties of AZM475271 may account for the observed inhibition of metastasis. Moreover, we have shown that the inhibition of Src kinase markedly increased the antitumor activity of gemcitabine based on the ability of AZM475271 to sensitize tumor cells against the cytotoxic effect of gemcitabine.

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Inhibition of Src Tyrosine Kinase as Treatment for Human Pancreatic Cancer Growing Orthotopically in Nude Mice

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