Inhibition of Src Tyrosine Kinase Impairs Inherent and Acquired Gemcitabine Resistance in Human Pancreatic Adenocarcinoma Cells

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Abstract
Purpose: We tested the hypotheses that Src tyrosine kinase overactivity represents a chemoresistance mechanism and that Src inhibition may enhance gemcitabine cytotoxicity in pancreatic adenocarcinoma cells.

Experimental Design: Pancreatic adenocarcinoma cells PANC1, MiaPaCa2, Capan2, BxPC3, and PANC1\textsuperscript{GemRes}, a stably gemcitabine-resistant subline of PANC1, were exposed to combinations of gemcitabine and Src tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Src expression, phosphorylation (Tyr-416), and activity were analyzed by immunoblotting and \textit{in vitro} kinase assay. Expression of the M2 subunit of ribonucleotide reductase (RRM2), a putative chemoresistance enzyme, was quantified by Northern and Western blot. Cellular proliferation was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was characterized by YO-PRO-1/propidium iodide staining, fluorometric caspase profiling, and caspase inhibition (Z-Val-Ala-Asp-fluoromethyl ketone). The effects of constitutively active and dominant negative Src were determined. The therapeutic efficacy of PP2 in combination with gemcitabine was tested in nude mice orthotopically xenografted with PANC1\textsuperscript{GemRes}.

Results: Greater gemcitabine resistance was associated with higher Src phosphorylation and activity, both of which were higher in PANC1\textsuperscript{GemRes}, relative to PANC1; total Src levels were alike. PANC1\textsuperscript{GemRes} overexpressed RRM2. PP2 enhanced inherent gemcitabine chemosensitivity and attenuated gemcitabine resistance in PANC1\textsuperscript{GemRes}. Constitutively active Src increased gemcitabine chemoresistance; dominant negative Src impaired gemcitabine chemoresistance. PP2 augmented gemcitabine-induced caspase-mediated apoptosis, suppressed RRM2 expression, and decreased activity of the RRM2-regulating transcription factor E2F1 in PANC1\textsuperscript{GemRes}. PP2 and gemcitabine in combination substantially decreased tumor growth and inhibited metastasis \textit{in vivo}.

Conclusions: Increased Src tyrosine kinase activity represents a potential chemoresistance mechanism and a promising therapeutic target warranting further investigation in gemcitabine-resistant pancreatic adenocarcinoma.

Introduction
Pancreatic ductal adenocarcinoma is now the fourth most common cause of cancer-related death in the United States, accounting for approximately 30,000 deaths annually (1). Aggressive invasion and early metastasis are characteristic of the disease, such that 90% of patients have surgically unresectable disease at the time of diagnosis (2). Current chemotherapy regimens with the most effective agents such as the deoxycytidine analog gemcitabine (2’-2’-difluorodeoxycytidine) provide only marginal survival benefit (3).

Tyrosine kinases show promise as new therapeutic targets, and a number of tyrosine kinase inhibitors are currently undergoing clinical evaluation as cancer therapies (4, 5). Src is the \textit{c-Src} proto-oncogene, and it is the cellular homolog of the Rous sarcoma virus transforming protein v-Src (6). Accumulating evidence implicates Src as an important determinant of tumorigenesis, invasion, and metastasis (7, 8). Src is overexpressed in over 70% of pancreatic carcinoma cell lines, and Src kinase activity is often increased (9). Overexpression of activated c-Src has been reported to stimulate proliferation and migration and down-regulate E-cadherin expression in human pancreatic adenocarcinoma cell lines (10). However, relatively little is known regarding the effects of Src on chemoresistance. We therefore tested the hypotheses that increased Src activity represents a cytoprotective mechanism capable of promoting gemcitabine chemoresistance and that Src inhibition may augment gemcitabine-induced cytotoxicity. Our findings indicate that Src tyrosine kinase is a potential therapeutic target for the treatment of gemcitabine-resistant pancreatic adenocarcinoma.

Materials and Methods
Cell Lines and Cell Culture. Human pancreatic cancer cells were obtained from American Type Culture Collection (Manassas, VA). PANC1, MiaPaCa2, and Capan2 possess K-ras and p53 mutations. PANC1 and MiaPaCa2 are poorly differentiated; Capan2 is well differentiated. BxPC3 is moderately differentiated with wild-type K-ras (11, 12). PANC1, MiaPaCa2, and BxPC3 were maintained in DMEM containing 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD). Ca-
pan2 was cultured in McCoy’s 5A medium with 10% fetal bovine serum. All cells were incubated in a humidified (37°C, 5% CO₂) incubator, grown in 75-cm² culture flasks, and passaged on reaching 80% confluence.

**Selection of PANC1GemRes.** We chose the well-characterized pancreatic adenocarcinoma cell line PANC1 as the parental line from which to develop a gemcitabine-resistant cell line to study mechanisms of gemcitabine resistance. PANC1GemRes was developed as described previously (13): PANC1 cells were serially subcultured through incrementally increasing gemcitabine concentrations, starting at the IC₅₀, over 3 months. Persistence of stable resistance was confirmed by subculture in gemcitabine-free DMEM containing 10% fetal bovine serum; PANC1GemRes retained the capacity for proliferation when returned to medium containing 5 μM gemcitabine.

**Cell Treatment with Gemcitabine and 4-Amino-5-(4-chlorophenyl)-7-(2-butyl)pyrazolo[3,4-d]pyrimidine (PP2).** Cells (5 × 10⁵) were seeded onto 25-cm² culture flasks and allowed to attach overnight. After removal of media, the cells were rinsed once with PBS, fresh standard media (10% fetal bovine serum) containing gemcitabine (Eli Lilly) or PP2 (Calbiochem, San Diego, CA), a selective inhibitor of Src tyrosine kinase activity (14), were added, and the cells were exposed for 4 days. Previous studies have shown that PP2 at concentrations of 10–20 μM inhibits Src activity in cultured cells (15). A low PP2 concentration of 10 μM was chosen to minimize potential inhibition of other kinases, an effect reported to occur at higher concentrations (14). Medium was replaced with fresh medium containing the same concentration of these agents every 48 h. Control cells received standard media containing DMSO vehicle at a concentration of 0.2%.

**Proliferation Assay.** Cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Tryvigen Inc., Gaithersburg, MD), in accordance with the manufacturer’s instructions, and confirmed by cell counting. Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay have been shown to correlate well with [³H]thymidine incorporation in pancreatic cancer cell lines (16). Logarithmically growing cells were plated at 5 × 10³ cells/well in 96-well plates, allowed to adhere for 24 h, and cultured in the presence or absence of PP2 and gemcitabine. Cell proliferation was determined after 96 h. Plates were read using a V₅₀ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm corrected to 650 nm and normalized to controls. Each independent experiment was performed three times, with 10 determinations for each condition tested. The IC₅₀ of gemcitabine was calculated from these results. At identical time points, cells were trypsinized to form a single cell suspension. Intact cells, determined by trypan blue exclusion, were counted using a Neubauer hemocytometer (Hausser Scientific, Horsham, PA), and the number of cells per ml was calculated and compared with the control group to confirm the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide results. The same methodology was used to determine the IC₅₀ of 5-fluorouracil (5-FU).

**Apoptosis Staining.** After treatment, cells were washed, resuspended in 0.5 ml of PBS, and 1 μl/ml YO-PRO-1 and propidium iodide were added (Vybrant Apoptosis Assay Kit 4; Molecular Probes, Eugene, OR). Cells were incubated for 30 min on ice and then analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ), measuring fluorescence emission at 530 and 575 nm. Cells stained with the green fluorescent dye YO-PRO-1 were counted as apoptotic; necrotic cells stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10,000), giving the apoptotic index. Data were analyzed using CellQuest software (Becton Dickinson). All observations were reproduced at least three times in independent experiments.

**Caspase Profiling.** Whole cell lysates were assayed for caspase 2, 3, 8, and 9 activities using the BD ApoAlert Fluorometric Caspase Assay Plate (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer’s instructions. Plates were read (excitation, 360 nm; emission, 480 nm) using a CytoFluor 4000 multiwell fluorescence plate reader (Applied Biosystems, Foster City, CA). All measurements were performed in triplicate, each with four determinations for each condition and each caspase. The role of caspases in the apoptotic response was confirmed using the caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (Clontech).

**Northern Blot Analysis.** Total RNA, extracted with Trizol reagent (Life Technologies, Frederick, MD), was electrophoresed through a denaturing gel (1.2% agarose, 2.2 M formaldehyde) and blotted onto positively charged nylon. After cross-linking with 120 mJ/cm² UV irradiation, the filter was prehybridized for 4 h at 42°C in 5× saline-sodium phosphate-EDTA, 5× Denhardt’s solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Oligonucleotide probes were identified as sequence specific by BLAST search, custom synthesized [M2 subunit of ribonucleotide reductase (RMR2), 5’-ACCATTGATTGTCGTAATGTCATTA-3′; β-actin, 5’-ATTTCGGCTCCGGCGCTTGGTGTAAGCTGTAGC-3′ (Sigma-Genosys, The Woodlands, TX)], and labeled with [α-³²P]ATP using the StripEZ kit (Ambion, Austin, TX). Hybridization was carried out with 50 ng of probe at 42°C for 16 h. The filter was washed four times in 5× saline-sodium phosphate-EDTA and 0.1% SDS for 10 min and exposed to XAR film (Eastman Kodak Co., Rochester, NY). After detection, the filter was stripped and reprobed for β-actin. Images were acquired using a UMAX PowerLook 3 scanner (Dallas, TX). Densitometric analysis was performed using Image-Pro Plus software version 4.0 (Media Cybernetics, Silver Spring, MD) and normalized to β-actin.

**Western Blotting.** Cells (2 × 10⁶) were harvested and rinsed twice with PBS. Cell extracts were prepared with lysis buffer [20 mM Tris (pH 7.5), 0.1% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin] and cleared by centrifugation at 12,000 × g at 4°C. Total protein concentration was measured using the BCA assay kit (Sigma, St. Louis, MO) with BSA as a standard, according to the manufacturer’s instructions. Cell extracts containing 30 μg of total protein were subjected to 10% SDS-PAGE, and the resolved proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). Equal protein loading was confirmed by Coomassie Blue (Bio-Rad, Hercules, CA) staining of the gel. After blocking with PBS containing 0.2% casein for 1 h at room temperature, membranes were incubated with 3–5 μg/ml antibody in PBS containing 0.1% Tween 20 overnight at 4°C. Anti-Src and anti-phospho-Src (Tyr-416) monoclonal antibodies...
were obtained from Upstate Biotechnology (Lake Placid, NY). Akt phosphorylation at Ser-473, a requirement for Akt activation (17, 18), was assessed using an anti-phospho-Akt (Ser-473) monoclonal antibody (Cell Signaling Technology Inc., Beverly, MA). Anti-Akt and anti-RRM2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Vectastain ABC kit (Vector Laboratory, Burlingame, CA) was used for 3,3′-diaminobenzidine chromogenic detection (Sigma), according to the manufacturer’s instructions. Densitometric analysis was performed as described above. Phospho-Src (Tyr-416) signal was normalized to total Src, and phospho-Akt signal was normalized to total Akt. The RRM2 signal was normalized to actin.

**Src Tyrosine Kinase Assay.** Src tyrosine kinase activity was determined in triplicate using a commercially available kinase assay kit (Sigma), according to the manufacturer’s instructions. Src immunoprecipitates (20 μg of total protein) were prepared using anti-Src monoclonal antibody immobilized onto protein G-Sepharose beads (Zymed Laboratories Inc., San Francisco, CA). Immunoprecipitates were washed and dissolved in tyrosine kinase buffer (final solution containing 0.3 mM ATP) and incubated for 30 min in 96-well plates coated with tyrosine kinase substrate solution (poly-Glu-Tyr). Phosphorylated substrate was quantified by chromogenic detection using horseradish peroxidase-conjugated anti-phosphotyrosine antibody. Optical densities were determined at 492 nm using a microplate Vmax spectrophotometer. Src kinase activity was compared with an epidermal growth factor receptor standard.

**Electrophoretic Mobility Shift Assay.** Oligonucleotide probes for E2F1 DNA binding (5′-ATT-TAA-GTT-TCG-CCT-TTC-TCA-A-3′; Santa Cruz Biotechnology) were labeled by combining 2 μl of oligonucleotide with 1 μl of T4 polynucleotide kinase (Promega, Madison, WI), 1 μl of [γ-32P]ATP (10 mCi/ml; Perkin-Elmer Life Sciences, Boston, MA), and 5 μl of nuclease-free water. This mixture was incubated at 37°C for 30 min. Unincorporated nucleotides were removed using G-25 MicroSpin columns (Amersham, Piscataway, NJ). DNA binding reactions contained 30,000 cpm of labeled probe in a final volume of 20 μl and 5 μg of nuclear protein extract. The reactions were incubated at room temperature for 30 min and run on 5% polyacrylamide gels in 0.25× Tris borate-EDTA buffer. Gels were dried and exposed to XAR film.

**Expression Constructs.** c-Src (Y529F) constitutively active mutant or the c-Src (K295R) dominant negative kinase inactive mutant cDNAs (Upstate Biotechnology) were cloned into the pUSEamp vector (Upstate Biotechnology) as described elsewhere (19), in accordance with the manufacturer’s instructions. PANC1 and PANC1GemRes cells at 80% confluence in 60-mm dishes were washed with Opti-MEM according to vendor’s instruction. Five μg of pUSEamp(-), pUSEamp-Src(Y529F), or pUSEamp-Src(K295R) were preincubated with LipofectAMINE at 25°C for 20 min and placed onto the cells in a volume of 500 μl. After incubation at 37°C for 3 h (with intermittent rocking every half hour), standard medium was added. After 48 h of incubation, cells were analyzed for Src kinase activity by in vitro kinase assay, and the gemcitabine IC50 was determined as described.

### Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gemcitabine</th>
<th>Gemcitabine + PP2</th>
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<tbody>
<tr>
<td>PANC1</td>
<td>50 ± 5 nM</td>
<td>4 ± 1 nM</td>
</tr>
<tr>
<td>PANC1GemRes</td>
<td>10 ± 2.5 nM</td>
<td>6 ± 1 nM</td>
</tr>
<tr>
<td>MiaPaCa2</td>
<td>40 ± 2.5 nM</td>
<td>4 ± 1 nM</td>
</tr>
<tr>
<td>BxPC3</td>
<td>18 ± 2.5 nM</td>
<td>2 ± 1 nM</td>
</tr>
<tr>
<td>Capan2</td>
<td>12 ± 2.5 nM</td>
<td>1 ± 0.5 nM</td>
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*PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.*

*P < 0.05 versus gemcitabine alone.

**Nude Mouse Orthotopic Xenograft Model.** Male athymic nu/nu mice (age, 5 weeks; weight, 20–22 g; specific pathogen free) were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed in microisolator cages with autoclaved bedding in a specific pathogen-free facility with 12-h light/dark cycles. Animals received water and food ad libitum and were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals. Mice were anesthetized with i.p. ketamine (200 mg/kg) and xylazine (10 mg/kg) and inoculated with 10^6 gemcitabine-resistant PANC1GemRes cells in 20 μl of PBS by surgical orthotopic implantation into the pancreas as described previously (20). After inoculation, mice were randomized to three treatment groups: (a) treatment group 1 (n = 8) received 2 mg/kg PP2 in 1% DMSO by i.p. injection three times a week; (b) treatment group 2 (n = 8) received gemcitabine (100 mg/kg) in the same volume of 1% DMSO vehicle as received by group 1, three times a week; and (c) treatment group 3 (n = 8) received 2 mg/kg PP2 and 100 mg/kg gemcitabine in the same volume of DMSO as groups 1 and 2, three times a week. The control group received the same volume of 1% DMSO vehicle as the other groups, three times a week. Treatment was commenced 1 day after implantation, and necropsy was performed 4 weeks after implantation. Primary tumors were identified, weighed, and normalized to total body mass. Tumor growth inhibition rate was calculated using the following formula: tumor growth inhibition rate (%) = (1 – M × /Mc) × 100, where Ma and Mc are the mean normalized tumor masses of treatment and control groups, respectively (21). Liver metastases were counted and confirmed histologically.

**Statistical Analysis.** Data are expressed as means ± SD. Analysis was performed using ANOVA, unpaired t test, and Mann-Whitney U test, as appropriate, using Statistica 5.5 (Stat Soft, Tulsa, OK). P < 0.05 was considered statistically significant.

**Results**

**Gemcitabine Chemoresistance Is Associated with Increased Src Kinase Activity.** PANC1 and MiaPaCa2 cells exhibited the greatest inherent resistance to gemcitabine (IC50 = 50 and 40 nM, respectively). BxPC3 and Capan2 were more susceptible (IC50 = 18 and 12 nM, respectively; Table 1). PANC1GemRes demonstrated stable gemcitabine
Src Inhibition Impairs Gemcitabine Resistance

PP2 Impairment of Gemcitabine Chemosensitivity Is Associated with Increased Caspase-Mediated Apoptosis.

We compared the responses of PANC1\textsuperscript{GemRes} and parental PANC1 to characterize mechanisms of acquired gemcitabine resistance. Treatment with 1 \( \mu \text{M} \) gemcitabine induced a greater increase in the apoptotic fraction of PANC1 than in that of PANC1\textsuperscript{GemRes}. Although treatment with 10 \( \mu \text{M} \) PP2 alone caused only minor increases in the apoptotic fractions of PANC1 and PANC1\textsuperscript{GemRes}, PP2 substantially augmented the gemcitabine-induced apoptotic fraction in both cell lines (Fig. 4). The magnitude of this effect was especially marked in PANC1\textsuperscript{GemRes}. This increase in apoptotic fraction was associated with large increases in caspase 3 and 9 activities in both PANC1 and PANC1\textsuperscript{GemRes} (Fig. 5). Changes in caspase 2 and 8, although statistically significant, were of a lesser magnitude.

PANC1 (10 \( \mu \text{M} \text{ versus} 50 \text{ nm} ; P < 0.05 \)). This gemcitabine resistance persisted after five passages in gemcitabine-free medium. After 4 days of exposure, the gemcitabine IC\textsubscript{50} for PANC1\textsuperscript{GemRes} was 10 \( \mu \text{M} \), compared with 50 nm for PANC1 (\( P < 0.05 \); Fig. 1). Cell lines with greater gemcitabine resistance demonstrated increased Src kinase activity, as determined by Src tyrosine 416 phosphorylation, a marker of Src kinase activation, and by \textit{in vitro} kinase assay. PANC1\textsuperscript{GemRes} demonstrated 5-fold higher Src phosphorylation levels and 7-fold higher Src kinase activity as compared with parental PANC1 (Fig. 2). Src levels did not differ significantly between PANC1\textsuperscript{GemRes} and PANC1. To confirm that increased Src kinase activity was an independent determinant of gemcitabine resistance, cells were transiently transfected with a constitutively activated Src(Y529F) construct or control empty vector. The constitutively active Src construct increased Src kinase activity in all four native cell lines (Fig. 3A). The empty vector had no effect. Induction of increased Src activity was associated with increased gemcitabine resistance in all four cell lines (Fig. 3B). Transfection with either construct had no significant effect on cellular proliferation under standard culture conditions, consistent with findings reported previously for a similar construct in PANC1 cells (22).

Src Inhibition Enhances Gemcitabine-Induced Cytotoxicity. Inhibition of Src tyrosine phosphorylation and kinase activity by 10 \( \mu \text{M} \) PP2 was confirmed in all cell lines (Fig. 2). Src kinase inhibition with PP2 sensitized all native cell lines to gemcitabine (Table 1). In addition, acquired gemcitabine resistance was attenuated in PANC1\textsuperscript{GemRes}, with the gemcitabine IC\textsubscript{50} decreasing from 10 \( \mu \text{M} \) to 6 \( \mu \text{M} (P = 0.02\); Table 1; Fig. 1). PP2 is reported to suppress proliferation of human colon, liver, and breast cancer cell lines (23). We therefore determined the effect of PP2 on pancreatic adenocarcinoma cellular proliferation in the absence of gemcitabine. At 10 \( \mu \text{M} \), the effect of PP2 on cellular proliferation was not significant, indicating that, at this low concentration, the effect of PP2 on gemcitabine cytotoxicity does not simply reflect a direct antiproliferative effect, but rather a potentiation of gemcitabine-induced cytotoxicity. Above 20 \( \mu \text{M} \), growth was increasingly suppressed, a finding consistent with reports in other human cancer cell lines (23). Although 10 \( \mu \text{M} \) PP2 was used in our study, at higher concentrations PP2 is reported to inhibit other intracellular kinases (14). To confirm that inhibition of Src kinase was sufficient to impair gemcitabine resistance, cells were transiently transfected with a dominant negative Src construct or control empty vector. Suppression of Src kinase activity by the dominant negative construct, but not the empty vector, was confirmed \textit{in vitro} kinase assay. Expression of dominant negative Src impaired gemcitabine chemoresistance in all unmodified cell lines (Fig. 3). In addition, dominant negative Src decreased the gemcitabine IC\textsubscript{50} of PANC1\textsuperscript{GemRes} from 10 \( \mu \text{M} \) to 5 \( \mu \text{M} (P < 0.05 \)). Transfection of the empty vector had no effect on kinase activity or the gemcitabine IC\textsubscript{50}. Transfection with either construct had no significant effect on cellular proliferation under standard culture conditions.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_1.png}
\caption{PANC1\textsuperscript{GemRes} is a gemcitabine-resistant subline of PANC1. \( 4\text{-Amino-5-(4-chlorophenyl)-7-(4-buty-3,4-dipyrazolo[3,4-d]pyrimidine (PP2) enhances gemcitabine-induced cytotoxicity in PANC1 and reverses acquired gemcitabine resistance in PANC1\textsuperscript{GemRes}. Growth curves for (A) PANC1 and (B) PANC1\textsuperscript{GemRes} show the effect of gemcitabine and different concentrations of PP2 on cellular growth, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 4 days. The mean IC\textsubscript{50} of gemcitabine was 50 \( \mu \text{M} \) for PANC1 and 10 \( \mu \text{M} \) for PANC1\textsuperscript{GemRes}. Ten \( \mu \text{M} \) PP2 reduced the gemcitabine IC\textsubscript{50} to 4 \( \mu \text{M} (P < 0.05 \) in PANC1 and 6 \( \mu \text{M} \) (\( P < 0.05 \) in PANC1\textsuperscript{GemRes}. Data are means of three determinations \pm SD.}
\end{figure}
Treatment with the caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone (100 nM), which has been shown to inhibit caspase-mediated apoptosis in human pancreatic cancer cell lines (24, 25), abrogated the increase in apoptotic fraction induced by the combination of gemcitabine and PP2 (Fig. 4).

Activity of the phosphatidylinositol 3'-kinase/Akt pathway is of functional importance in pancreatic cancer cell proliferation (26, 27), apoptosis (28, 29), and susceptibility to chemotherapeutic agents including gemcitabine (30–33). Activation of Akt, which requires phosphorylation of serine 473 (18), has been shown to inhibit activation of caspases 9 and 3 at the postmitochondrial level (34). It has recently been recognized that Src activates Akt directly, through an interaction between its SH3 domain and a conserved proline-rich motif in the COOH-terminal regulatory region of Akt (35).

For these reasons, we assessed the effect of PP2 on Akt (Ser-473) phosphorylation. PP2, alone and in combination with gemcitabine treatment, decreased Akt phosphorylation (Fig. 6) in PANC1 and PANC1 GemRes. Total Akt levels were unchanged by this treatment. This observation may account, at least in part, for the markedly increased caspase 3 and 9 activities observed when gemcitabine administration is combined with Src inhibition by PP2.

 Src overexpression does not universally sensitize cancer cells to all chemotherapeutic agents. Increased sensitivity to taxanes after artificial overexpression of Src has been reported recently (36). We therefore sought to determine whether the effect of Src inhibition on gemcitabine resistance was specific to gemcitabine by assessing the effect of PP2 on the IC50 for the widely used agent 5-FU. Having determined the 5-FU IC50 for...
each cell line, cells were exposed to 5-FU in the presence or absence of 10 μM PP2, and the IC50 was determined under these conditions. PP2 induced relatively minor changes in the 5-FU IC50 and, in the cases of BxPC3 and Capan2, PP2 increased the 5-FU IC50 (Table 2).

Overexpression of the RRM2, a Potential Mechanism of Acquired Gemcitabine Resistance in PANC1GemRes, Is Suppressed by PP2. Increased expression of RRM2 is reported to potentiate gemcitabine chemoresistance and malignant cellular phenotypes (13, 37–39). We therefore investigated RRM2 ex-

Fig. 3 Src kinase activity is a determinant of gemcitabine chemosensitivity. Transfection with a dominant negative Src construct decreased Src kinase activity (A) and decreased the gemcitabine IC50 (B). Conversely, overexpression of a constitutively active Src construct increased Src kinase activity levels (A) and increased the gemcitabine IC50 (B). Data are means of three determinations ± SD. *, P < 0.05 versus empty vector control.

Fig. 4 4-Amino-5-(4-chlorophenyl)-7-((t)-butyl)pyrazolo[3,4-d]pyrimidine (PP2) augments the apoptotic response of PANC1 and PANC1GemRes to gemcitabine. Control samples were grown in DMEM with 10% fetal bovine serum and treated with DMSO vehicle. Cells were treated with 10 μM PP2, 1 μM gemcitabine, 10 μM PP2 and 1 μM gemcitabine, or 10 μM PP2, 1 μM gemcitabine, and 100 μM of the caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone for 4 days. PP2 caused only a small increase in apoptotic fraction (PANC1, 0.2% to 5%; PANC1GemRes, 0.1% to 2%; P < 0.01). PANC1GemRes exhibited a lower apoptotic fraction in response to gemcitabine than PANC1 (4% versus 15%; P < 0.01). PP2 and gemcitabine cotreatment increased the apoptotic fraction from 15% to 35% in PANC1 and from 4% to 24% in PANC1GemRes (P < 0.01), a response that was suppressed by caspase inhibition with Z-Val-Ala-Asp-fluoromethyl ketone.
expression in PANC1GemRes. Higher levels of RRM2 transcript and protein expression were observed in PANC1GemRes compared with PANC1 (Fig. 7). Forty-eight h of PP2 exposure decreased RRM2 transcript levels in PANC1GemRes (Fig. 7A). Western blot confirmed that RRM2 subunit levels were significantly decreased relative to control cells (Fig. 7B). Next, we investigated the effect of PP2 treatment on activity of the transcription factor E2F1, which has been shown to regulate ribonucleotide reductase expression (40). Exposing PANC1GemRes to 1 μM gemcitabine for 48 h had minimal effect on E2F1 activity in PANC1GemRes nuclear extract. In contrast, 10 μM PP2, both alone and in combination with gemcitabine, suppressed E2F1 activity (Fig. 8).

**Tumor Growth and Metastasis.** All mice survived the duration of the study, and necropsy was performed 4 weeks after orthotopic implantation with PANC1GemRes. The tumor growth inhibition rate was 25% in the PP2 treatment group and 5% in the gemcitabine treatment group ($P < 0.05$). When administered in combination, PP2 and gemcitabine produced a tumor growth inhibition rate of 98% ($P < 0.05$; Fig. 9). Hepatic metastasis occurred in 100% of control and gemcitabine-treated groups; 88% of the PP2-treated group developed liver metastases. There were no detectable metastases in the group treated with PP2 and gemcitabine in combination ($P < 0.05$).

**Table 2.** PP2 does not decrease the mean IC$_{50}$ for 5-FU in pancreatic adenocarcinoma cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>5-FU IC$_{50}$ (±SD)</th>
<th>5-FU + PP2 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC1</td>
<td>62 ± 2</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>PANC1GemRes</td>
<td>115 ± 2</td>
<td>108 ± 5</td>
</tr>
<tr>
<td>MiaPaCa2</td>
<td>38 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>BxPC3</td>
<td>46 ± 2</td>
<td>62 ± 2*</td>
</tr>
<tr>
<td>Capan2</td>
<td>8 ± 2</td>
<td>31 ± 2*</td>
</tr>
</tbody>
</table>

*PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; 5-FU, 5-fluorouracil.
Discussion

This study identifies increased Src tyrosine kinase activity as a potential chemoprotective mechanism adopted by gemcitabine-resistant pancreatic adenocarcinoma cells. Increased Src kinase activity correlates with inherent gemcitabine resistance in pancreatic adenocarcinoma cells. Furthermore, PANC1\textsuperscript{GemRes}, a cell line specifically selected for its gemcitabine resistance, demonstrates markedly elevated Src kinase activity. Inhibition of Src kinase activity by PP2 promotes gemcitabine-induced apoptosis in human pancreatic adenocarcinoma cell lines and reverses acquired gemcitabine resistance in PANC1\textsuperscript{GemRes}. These proapoptotic effects were associated with decreased Akt phosphorylation. Expression of dominant negative Src confirms that Src inhibition enhances gemcitabine cytotoxicity. Con-
versely, transfection with a constitutively active Src construct promotes gemcitabine resistance. In addition, we have demonstrated that RRM2, which is emerging as an enzyme of functional importance in chemoresistance, is overexpressed by PANC1GemRes. We have also shown for the first time that Src inhibition by PP2 suppresses RRM2 at the transcriptional level and that PP2-mediated inhibition of RRM2 expression is associated with decreased activity of E2F1, a known transcriptional regulator of RRM2.

Src kinase activity is increased in a variety of malignancies (41), including pancreatic adenocarcinoma (9), and overexpression of Src is associated with malignant cellular behavior (7, 8). For these reasons, Src appears to be a rational therapeutic target. Inhibition of Src has precedence as an anticancer strategy. PP2 is reported to reduce colorectal liver metastasis in a severe combined immunodeficient mouse model (23). It has been reported that antisense-mediated suppression of Src has antitumor effects in nude mouse xenograft model of colon (42) and ovarian carcinoma (43). In our study, augmentation of gemcitabine-induced cytotoxicity by PP2 and by dominant negative Src was apparent at clinically relevant micromolar and nanomolar gemcitabine concentrations (44). The augmentation of cytotoxicity induced by gemcitabine at and below these concentrations indicates that PP2 promotes cytotoxicity at gemcitabine concentrations that would otherwise be subtherapeutic. This effect would be of benefit in pancreatic tumors, which are by nature relatively avascular (45) and may receive subtherapeutic levels of cytotoxic agents. PP2, at a concentration of 10 μM, potentiates gemcitabine cytotoxicity rather than being directly cytotoxic. This is evidenced by the marked increase in apoptotic fraction and caspase activity induced by gemcitabine and PP2 coadministration, an effect that is not induced by PP2 treatment alone. Neither 10 μM PP2 nor dominant negative Src significantly suppressed proliferation under standard culture conditions. Similarly, constitutively active Src did not significantly affect proliferation in standard culture. These observations are consistent with those of Flossmann-Kast et al. (22). After transfection of cells with a similar Src expression construct, no effect on proliferation in standard culture was observed, although insulin-like growth factor I-dependent proliferation was increased (22).

The current paradigm for Akt activation involves phosphatidylinositol 3'-kinase-dependent membrane localization and

Fig. 8  PP2 treatment suppresses activity of the E2F1 transcription factor in PANC1GemRes nuclear extracts. Representative E2F1 electrophoretic mobility shift assay. Lane 1, nuclear extract omitted (negative control). Lane 2, control (gemcitabine-free media). Lane 3, E2F1 supershift using anti-E2F1 monoclonal antibody. Lane 4, 1 μM gemcitabine. Lane 5, 10 μM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Lane 6, 1 μM gemcitabine + 10 μM PP2. Lane 7, unlabeled competing E2F1 oligonucleotide.

Fig. 9  4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) enhances gemcitabine cytotoxicity in a nude mouse orthotopic xenograft model of gemcitabine-resistant pancreatic adenocarcinoma. Gemcitabine-resistant PANC1GemRes cells (10^6) were orthotopically implanted into the pancreata of nude mice. After 4 weeks of treatment with PP2 (3 × 2 mg/kg/week; n = 8), gemcitabine (3 × 100 mg/kg/week; n = 8), the same doses of PP2 and gemcitabine combined (n = 8), or control (equal volume of DMSO vehicle; n = 8), necropsy was performed. Primary tumors were identified, and their masses, normalized to total body mass, were recorded. The tumor growth inhibition rates (percentage; equal to (1 − M_T/M_C) × 100, where M_T and M_C are the mean normalized tumor masses of treatment and control groups, respectively) were calculated. PP2 caused a numerical but nonsignificant reduction in mean tumor mass; gemcitabine alone had no significant effect on PANC1GemRes. In contrast, combination of PP2 and gemcitabine produced a marked inhibition of tumor growth, with a tumor growth inhibition rate of 98% (*, P < 0.05).
activating phosphorylation of residues including Ser-473 at the COOH terminus (17, 18). In PANC1 and PANC1\textsuperscript{GemRes}, PP2 causes a decrease in Akt (Ser-473) phosphorylation. Activated Akt directly phosphorylates initiator caspase 9, preventing its activation (46). Akt has also been shown to inhibit caspases by posttranslational modification of a cytosolic factor downstream of cytochrome c and upstream of caspase 9 activation (34). Decreased Akt phosphorylation may therefore represent one mechanism contributing to the increased caspase 9 activity we observed in cells treated with PP2 in combination with gemcitabine.

Ribonucleotide reductase is becoming recognized as a promoter of malignant cellular phenotypes including chemoresistance (13, 38, 39). Enhanced gemcitabine resistance is observed after artificial overexpression of ribonucleotide reductase (13). Gemcitabine itself inhibits ribonucleotide reductase (47) and depletes the deoxynucleotide triphosphate pool, which is important for effective DNA repair (48). It has been suggested that increased ribonucleotide reductase activity exerts a chemoprotective effect in a number of ways: increased ribonucleotide reductase activity causes expansion of the deoxynucleotide triphosphate pool, decreasing deoxycytidine kinase activity through negative feedback. Decreased deoxycytidine kinase activity reduces phosphorylation of gemcitabine, a step required for its activity. Excess deoxynucleotide triphosphate, resulting from increased ribonucleotide reductase activity, also competes with gemcitabine triphosphate derivatives for incorporation into DNA and may also increase activity of dCMP deaminase, which metabolizes gemcitabine (13).

The increased ribonucleotide reductase expression observed in the gemcitabine-resistant cell line PANC1\textsuperscript{GemRes}, relative to its parental cell line PANC1, supports the premise that ribonucleotide reductase overexpression can be chemoprotective. Our findings are supported by those of Goan et al. (13), who reported overexpression of RRM2 in a similarly developed gemcitabine-resistant orpharyngeal epidermoid carcinoma cell line. PP2 decreases RRM2 expression at the transcriptional level, which may be at least one of the mechanisms by which PP2 potentiates gemcitabine-induced cytotoxicity.

RRM2 expression is regulated by E2F1 (40), a transcription factor that is induced by DNA damage (49, 50). Deregulated activity of the E2F family occurs in the majority of human tumors, but E2F1 is unusual in that it has both oncogenic and tumor suppressor functions (51, 52). Although overexpression of E2F1 is reported to promote apoptosis in cultured cells and transgenic mouse models (53, 54), it can also stimulate progression to S phase and DNA synthesis (55) through mechanisms that are discussed in detail elsewhere (51). Our results demonstrate that the increase in gemcitabine-induced apoptosis resulting from PP2 treatment is associated with suppression of E2F1 activity. Our findings are consistent with those of Jung et al. (56), who reported that the synthetic flavone flavopiridol also potentiates gemcitabine-induced apoptosis, in association with suppression of RRM2 expression and reduced E2F1 activity. Although it remains unclear how elevated Src kinase activity increases E2F1 activity, Pasteau et al. (57) reported that E2F1 represents an important component of v-Src signaling in chicken neuroretina cells. Additional studies will be required to clarify the link between Src and E2F1.

Inhibition of Src and ribonucleotide reductase does not, however, universally induce chemosensitization. Boudny and Nakano (36, 58) showed that transfection of v-Src increased sensitivity to taxanes in human gallbladder adenocarcinoma cells by promoting apoptosis, an effect that was inhibited by Src inhibition. Decreased ribonucleotide reductase activity is reported to be associated with increased resistance to agents such as the thymidylate synthase inhibitor 5-FU (59, 60). Taken together with our results, these findings indicate that whereas ribonucleotide reductase and its transcriptional regulation represent potential therapeutic targets, combined therapeutic approaches targeting ribonucleotide reductase will need to be chosen cautiously. It may be necessary to take into account levels of tumor ribonucleotide reductase expression when identifying the optimal treatment strategy.

The observation that combining PP2 with gemcitabine has a marked synergistic effect on tumor growth and metastasis in this orthotopic nude mouse xenograft model of chemoresistant pancreatic adenocarcinoma is consistent with our \textit{in vitro} data and with work of Nam et al. (23). This group reported decreased metastasis after treatment with PP2 alone in a splenic injection severe combined immunodeficient mouse model (23). Whereas we also observed a slight reduction in metastasis with PP2 treatment alone, the effect was relatively minor in comparison with the effect of PP2 in combination with gemcitabine. This may reflect the slightly lower dose of PP2 used in our study. Although it must be emphasized that these results observed in the nude mouse model should be regarded as correlative, they suggest that a combined approach using Src inhibition and gemcitabine may be beneficial.

In summary, this study identifies increased Src tyrosine kinase activity as not only a gemcitabine chemoresistance mechanism but also a potential therapeutic target in chemoresistant pancreatic adenocarcinoma cells. Expression of constitutively active Src potentiates gemcitabine resistance, whereas Src inhibition, by both PP2 and dominant negative transfection, enhances inherent gemcitabine chemoresistance and reverses acquired gemcitabine resistance. Our findings provide further evidence that overexpression of RRM2 can function as a chemoprotective mechanism and that suppression of RRM2 expression, in this case through Src inhibition, contributes to enhancement of gemcitabine cytotoxicity. These \textit{in vitro} data are supported by the synergistic effect of PP2 and gemcitabine treatment observed in the \textit{in vivo} orthotopic xenograft model. Src tyrosine kinase inhibition represents a promising therapeutic strategy warranting further investigation.

Acknowledgments

We gratefully acknowledge the technical assistance of Jan Rounds.

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