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Biochemical and Cellular Effects of c-Src Kinase-Selective Pyrido[2,3-*d*]pyrimidine Tyrosine Kinase Inhibitors

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ABSTRACT. Increased expression or activity of c-Src tyrosine kinase has been associated with the transformed phenotype in tumor cells and with progression of neoplastic disease. A number of pyrido[2,3-*d*]pyrimidines have been characterized biochemically and in cells as part of an assessment of their potential as anti-tumor agents. The compounds were ATP-competitive inhibitors of c-Src kinase with IC_{50} values < 10 nM and from 6 to >100-fold selectivity for c-Src tyrosine kinase relative to basic fibroblast growth factor receptor (bFGFr) tyrosine kinase, platelet-derived growth factor receptor (PDGFr) tyrosine kinase, and epidermal growth factor receptor (EGFr) tyrosine kinase. The compounds yielded IC_{50} values < 5 nM against Lck. Human colon tumor cell growth in culture was inhibited, as was colony formation in soft agar at concentrations < 1 μM. Phosphorylation of the c-Src cellular substrates paxillin, $p130^{cas}$, and Stat3 was also inhibited at concentrations < 1 μM. Autophosphorylation of EGFr tyrosine kinase or PDGFr tyrosine kinase was not inhibited by c-Src inhibitors, thus showing the selective nature of the compounds in cells. In a mitogenesis assay measuring thymidine incorporation stimulated by specific mitogens, the c-Src tyrosine kinase inhibitors reduced incorporated thymidine in a manner consistent with previously reported roles of c-Src in mitogenic signaling. Progression through the cell cycle was inhibited at G_2/M in human colon tumor cells treated with two of the c-Src-selective compounds, which is also consistent with earlier reports describing a requirement for active c-Src tyrosine kinase for G_2 to M phase progression. The compounds described here are selective inhibitors of c-Src tyrosine kinase and have antiproliferative effects in tumor cells consistent with inhibition of c-Src. BIOCHEM PHARMACOL **60**;7:885–898, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. tyrosine kinase inhibition; c-Src kinase; pyridopyrimidine; antitumor; phosphorylation; EGFr; PDGFr; bFGFr

Protein tyrosine kinases have been characterized as participating in a number of cellular signaling events including those important in mitogenesis. Many tyrosine kinases have been shown to be elevated in either expression or activity in a large percentage of tumor types, and increased tyrosine phosphorylation has been associated with progression of disease and poor prognosis. One class of tyrosine kinases among several that have been associated with the transformed phenotype in human disease is the c-Src kinase family of proteins, which is composed of at least 10 members (reviewed in Refs. 1 and 2). Elevated levels of c-Src kinase activity [3] and of the kinase protein itself [4] have been described in breast tumor cell lines and malignant breast tissue. Further strengthening the association of c-Src kinase and cancer is the description of increasing c-Src kinase activation with progression of colon cancer from adenomatous polyps through metastatic disease [5]. Still further, in a human colon carcinoma cell line, c-*src* antisense transfection results in decreased growth rate *in vitro* and decreased tumorigenicity in a xenograft system, suggesting that in this cell line, c-Src activity is required for maintenance of the transformed phenotype [6]. Additional support for the association of c-Src kinase with cancer may be found in the characterization of elevated expression of c-Src kinase with adenomatous mucosa in resected human specimens [7]. Examination of c-Src levels and activity in human colon tumor cell lines derived from liver metastases has shown increases relative to poorly metastatic cells along with an apparent EGFr‖ family stimulation of c-Src kinase

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‖ *Abbreviations*: EGF, epidermal growth factor; EGFr, epidermal growth factor receptor; PDGF, platelet-derived growth factor; PDGFr, platelet-derived growth factor receptor; bFGF, basic fibroblast growth factor; bFGFr, basic fibroblast growth factor receptor; STAT, signal transducer and activator of transcription; and MEM, minimal essential medium.

activity [8]. A truncating mutation in the C-terminus of the c-Src protein has been identified in nine late-stage, metastatic human colon tumor samples. When this mutated gene was transfected into rat fibroblasts, the mutation was found to be kinase-activating, transforming, tumorigenic in nude mice, and associated with increased metastasis [9]. Other systems where elevated levels of c-Src kinase or activity have been described include head and neck tumors [10] and pancreatic carcinoma [11].

In addition to a correlative association of c-Src with cancer, a mechanistic association is apparent as well. A number of mitogenic receptor tyrosine kinases whose activity has been linked closely to transformation and tumorigenesis either modulate or are themselves modulated by c-Src kinase [12–23]. Because of the association of c-Src kinase with cancer and its activation or overexpression in progression of this disease, it may be a potential target for therapeutic intervention.

Various approaches have been taken to address the inhibition of c-Src as a mechanistic target. An ATP-competitive small-molecule approach led to the characterization of pyrazolo[3,4-*d*]pyrimidines that were described as nanomolar inhibitors of c-Src family kinases [24]. A different approach building on the pyrazolopyrimidines and utilizing an engineered (site-directed mutant) kinase generated 1.5-nM inhibitors with 100-fold or greater selectivity toward the mutant kinase versus wild-type kinase, although activity against kinases other than wild-type or mutant v-Src was not reported [25]. Pyrrolopyrimidines have been reported as selective and potent inhibitors of c-Src with inhibitory potency at concentrations as low as 10 nM and 20-fold selectivity toward a panel of other tyrosine kinases [26]. Other efforts directed at c-Src kinase inhibitors include quinolinones, which have potencies for *in vitro* c-Src inhibition of 500 nM [27], iminochromenes, with potency of 2 μM for c-Src inhibition [28], and isothiazolones, with 4 μM inhibition of the c-Src family kinase $p56^{lck}$ [29]. Peptide or protein substrate-competitive approaches have also been taken for the discovery of inhibitors of c-Src kinase, yielding compounds of somewhat lower potency [30–33].

In light of this body of data, a program to discover novel, selective, and effective small molecule inhibitors of c-Src kinase as anti-tumor agents was undertaken. In this study, a series of substituted pyrido[2,3-*d*]pyrimidines has been characterized against a panel of *in vitro* enzyme assays to determine selectivity toward c-Src kinase as well as the mechanism of enzyme inhibition. Selective inhibitors of the c-Src kinase have been characterized further in cellular models of growth and cytotoxicity to determine their potential as anti-tumor agents. In addition, these kinase inhibitors have been assessed in cells to measure the effects on c-Src-mediated phosphorylation events, mitogen-stimulated DNA synthesis, and cell cycle progression as further measures of c-Src selectivity.

MATERIALS AND METHODS

Chemicals and Reagents

The small-molecule tyrosine kinase inhibitors were synthesized according to published descriptions [34, 35]. [^{14}C]Thymidine and the extended chemiluminescence western blot kit were obtained from Amersham Pharmacia Biotech. [^{32}P]ATP was purchased from either Amersham or New England Nuclear. PDGF, bFGF, and EGF came from Intergen. Antiphosphotyrosine and anti-$p130^{cas}$ antibodies were obtained from Upstate Biotechnology, Inc. The anti-Stat3 antibody was purchased from Santa Cruz Biotechnology. The antibody against paxillin was obtained from Transduction Laboratories/Pharmingen. The antibody for the western blot of EGFr was purchased from the Sigma Chemical Co. Calbiochem/Oncogene Science Research was the source of the immunoprecipitation antibody for the EGFr and of protein A/protein G–Sepharose.

Tyrosine Kinase Assays

The isolated enzyme assays were performed as previously described, and they made use of recombinant enzymes expressed in a baculovirus-infected insect cell system [36]. The c-Src enzyme was immunopurified using an antibody directed at the N-terminal 2–17 amino acid residues (Quality Biotech) and was homogeneous when assessed by Coomassie-stained gel electrophoresis. The enzyme (approximately 20–100 ng) was assayed in a buffer comprised of 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.1% Triton X-100. Under standard assay conditions, the ATP concentration was 40 μM, and the final concentration of the random copolymer substrate of glutamate and tyrosine (4:1 molar ratio) was 200 μg/mL. These conditions resulted in maximal activity of the enzyme. The phosphorylation reaction was carried out for 10 min in a total volume of 125 μL at 25° (using [^{32}P]ATP, 4 μCi/mL) and was terminated by the addition of 125 μL of 30% trichloroacetic acid containing 0.1 M sodium tetraphosphate, precipitated on ice for a minimum of 30 min, and then washed and filtered through a 0.65-μm polyvinylidine filter (Millipore) using 15% trichloroacetic acid/0.1 M sodium pyrophosphate.

Baculovirus-infected insect cell lysate was the source of the PDGFr and bFGFr tyrosine kinases and was used without further purification. Control experiments demonstrated that vector-only baculovirus-infected insect cell lysates possessed no tyrosine kinase activity under the conditions of the assay. The lysates, containing 60–750 ng enzyme, were assayed in 25 mM HEPES buffer, pH 7.4, that contained 150 mM NaCl, 10 mM $MnCl_2$, 0.2 mM Na_3VO_4, 215 μg/mL of glutamate/tyrosine peptide substrate, 1 mM phenylmethylsulfonyl fluoride, 40 μM ATP (4 μCi/mL). The assay was carried out as described for c-Src. These assay conditions resulted in maximal enzyme activity for these preparations.

Lck was obtained from Agouron Pharmaceuticals and was pure as supplied. Enzyme at a concentration of 1 or 5

nM was assayed in a buffer containing 100 mM HEPES, pH 7.5, 0.125% bovine serum albumin, 25 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM dithiothreitol, 50 μM Na_3VO_4, 1 μM biotinylated peptide substrate (Peptide 2, Pierce), and 200 μM ATP. These reaction conditions resulted in maximal activity of the enzyme. The reaction was carried out in 96-well polypropylene plates and was terminated by the addition of 200 mM EDTA in 1% bovine serum albumin. The reaction mixture was transferred to streptavidin-coated plates (Roche Molecular), washed with DELFIA wash buffer (Wallac), incubated with Eu-labeled antiphosphotyrosine antibody (Wallac), and read on a fluorimeter (Wallac) after the addition of DELFIA enhancement solution (Wallac).

EGFr tyrosine kinase was prepared and assayed as previously described [37]. One nanogram of EGFr tyrosine kinase was assayed in a total volume of 100 μL of 50 mM HEPES buffer, pH 7.4, containing 10 mM $MnCl_2$, 10 μM ATP (0.5 μCi [^{32}P]ATP), 20 μg of a random copolymer of glutamate, alanine, and tyrosine (molar ratio of 6:3:1). The reaction mixture was precipitated with cold 15% trichloroacetic acid containing 0.1 M sodium pyrophosphate and filtered as described above.

Cell Growth and Clonogenic Assays

The cell lines used were obtained from the American Type Culture Collection and were maintained in a 1:1 mixture of Dulbecco's MEM and Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (Gibco/BRL) at 37° in 5% CO_2 in air. The cell growth assays were performed as previously described, and sulforhodamine B was used to assess cell density [37, 38]. The clonogenic assays were also performed as described [37, 38] with the following modification for the pretreated version. Cells were seeded into 24-well culture plates, allowed to attach overnight, and then treated with the indicated concentration of compounds for 48 hr. After that time, culture medium was removed, and the cells were washed free of compound, trypsinized, and counted on an electronic particle counter (Coulter). Trypan blue dye exclusion was done to assess non-viable cells. Equivalent numbers of viable cells then were plated in drug-free soft agar as described and allowed to form colonies for 10–14 days.

Immunoprecipitation and Whole Cell Lysates

Cells treated as described in the figure legends were subjected either to immunoprecipitation of target proteins and subsequent western blotting or to western blotting of whole cell lysates as has been described [37, 38].

Thymidine Incorporation Assays

NIH 3T3 cells were plated at 6000/well or A431 cells were seeded at 10,000/well into scintillating microplates (Amersham Pharmacia Biotech) and allowed to attach overnight. Labeled thymidine at 0.1 μCi/well was added, and the cells were treated as described in the figure legend.

Cell Cycle Distribution Determination

HT-29 cells were trypsinized from 6-well cell culture plates, washed in PBS, pH 7.4, resuspended at 2×10^6 cells/mL in 70% ethanol, and allowed to fix for at least 1 hr. Fixed cells were centrifuged for 10 min (700 *g*) and resuspended in 50 μg/mL of propidium iodide in 0.15 M NaCl. Thirty units per mL of RNase was added to the samples, which then were incubated for 1 hr at 37°. The samples were examined by flow cytometry on an EPICS Elite cytometer (Coulter), and the data were analyzed with the Modfit LT software package (Verity Software House).

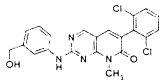
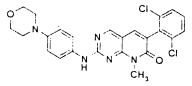
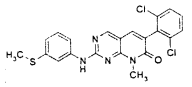
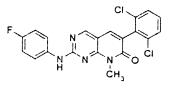
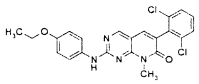
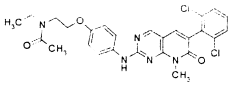
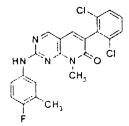
RESULTS

Biochemical Characterization of c-Src Selective Inhibitors

Examination of a series of pyrido[2,3-*d*]pyrimidines that were synthesized as tyrosine kinase inhibitors [34, 35] in a panel of *in vitro* kinase assays led to the characterization of the c-Src kinase selective compounds shown in Table 1. The five enzymes utilized in this study were PDGFr-β, FGFr-1, and c-Src kinase, as previously described [37, 39], the c-Src family kinase Lck, and human EGFr. The c-Src kinase inhibitor compounds share a common structural core with a range of substitutions at the 3′- and 4′-positions on the anilino ring at the 2 position of the pyridopyrimidine. As is evident from the ratio of *in vitro* IC_{50} values shown in Table 1, selectivity of compounds toward c-Src kinase based upon potency ranged from 10-fold to greater than 100-fold. The compounds all possessed substantial inhibitory activity toward Lck as well, with greater than 50% inhibition at 5 nM. Under the assay conditions for Lck used here, the compounds inhibited the enzyme present in the assay completely and thus did not allow for calculation of an IC_{50} value. Reducing the enzyme concentration further (to quantitate the inhibitory activity of the potent compounds) resulted in loss of measurable enzyme activity. Although not every compound was examined against EGFr kinase, those that were assayed showed selectivity toward c-Src *in vitro* relative to EGFr kinase ranging from 6- to 23-fold.

The inhibitory mechanism of these compounds toward c-Src kinase was assessed using two methods of data analysis of assays done by varying the concentration of ATP in the presence of a fixed concentration of peptide substrate. Figure 1 shows a Lineweaver–Burk plot of data from inhibition of c-Src kinase by PD0166326. The intercept at $x = 0$ of the curves from the assay carried out in various concentrations of inhibitor and in varying concentrations of ATP indicated competitive inhibition with respect to ATP. The other method of analysis used a nonlinear regression to fit data to equations describing mechanisms of inhibition [40] refined by comparisons of $K_{i(slope)}$ versus

TABLE 1. Enzyme inhibition by c-Src kinase inhibitors

PD Number		c-Src	bFGFr	PDGFr	bFGFr/ c-Src	PDGFr/ c-Src	Sum of selectivity	Lck	EGFr
		IC_{50} (M)							
0166326		5.68×10^{-9}	6.15×10^{-8}	1.39×10^{-7}	10.8	24.5	35.3	$< 5 \times 10^{-9}$	8.2×10^{-8}
0173952		8.35×10^{-9}	9.88×10^{-8}	6.36×10^{-7}	11.8	76.2	88.0	$< 5 \times 10^{-9}$	ND
0173955		2.45×10^{-8}	1.25×10^{-6}	1.66×10^{-6}	50.9	67.6	118.5	$< 5 \times 10^{-9}$	ND
0173956		6.63×10^{-8}	9.33×10^{-7}	1.25×10^{-6}	14.1	18.8	32.9	$< 5 \times 10^{-9}$	3.8×10^{-7}
0173958		1.63×10^{-8}	4.51×10^{-7}	2.34×10^{-6}	27.7	144.0	171.7	$< 5 \times 10^{-9}$	ND
0179483		9.03×10^{-9}	2.44×10^{-7}	4.63×10^{-7}	27.0	51.2	78.2	$< 5 \times 10^{-9}$	ND
0180970		1.68×10^{-8}	9.34×10^{-7}	1.43×10^{-6}	55.6	84.9	140.5	$< 5 \times 10^{-9}$	3.9×10^{-7}

The selectivity ratio was calculated by dividing the IC_{50} values as indicated. The sum of selectivity is the sum of the ratios for the two different enzyme pairs. Values reported are the means of at least three separate determinations done in duplicate. ND = not determined.

$K_{i(intercept)}$. This approach yielded $K_{i(intercept)}$ values larger than $K_{i(slope)}$ for each of the compounds in Table 1, which was indicative as well of ATP-competitive inhibition under these conditions.

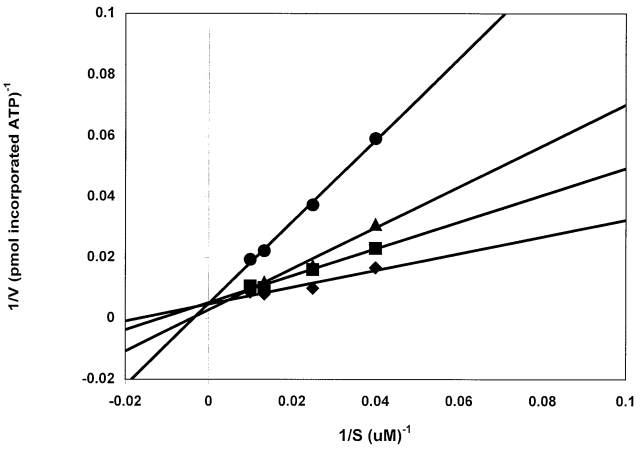


FIG. 1. Double-reciprocal plot for the inhibition of c-Src kinase by PD0166326. The assay made use of a random peptide copolymer of glutamate and tyrosine in a molar ratio of 4:1. Activity was measured as incorporation of ^{32}P-labeled phosphate from ATP into acid-precipitable substrate. Key: (♦) no inhibitor; (■) 3 nM PD0166326; (▲) 20 nM PD0166326; and (●) 50 nM PD0166326.

Growth Delay and Effects of Kinase Inhibitors on Transformed Phenotype in Human Tumor Cell Lines

Two measures of cellular effect were employed to assess the kinase inhibitors. The first was a determination of growth delay of tumor cells over 72 hr when treated continuously with a compound. Table 2 shows the IC_{50} values for three different human colon carcinoma cell lines. The potency of the compounds ranged from 156 nM (PD0179483 in SW620 cells) to 1.8 μM (PD0166326 in SW620 cells). Cells treated in this fashion were examined for measures of nonspecific cytotoxicity by their ability to exclude trypan blue dye (0.4% in 0.15 M NaCl). At the end of 48 hr, the viability of treated cells by this measure was greater than 90%.

The second measure of cellular effect was determination of colony-forming ability in soft agar, which is one measure of maintenance of the transformed phenotype of a tumor cell line (as opposed to growth delay, which is a measure of cellular proliferation only). Cells were plated into soft agar, and colony formation was assessed 10–14 days after seeding. With the exceptions of PD0179483 in HCT-8 cells and PD0166326 in HT-29 cells, the potency of the kinase inhibitors ranged from 260 nM to 3.2 μM.

To examine the reversibility of the growth inhibition of the compounds, HCT-8 cells in culture were exposed to the kinase inhibitors as a function of time. Cells were treated

TABLE 2. Effect of tyrosine kinase inhibitors on cell growth and cytotoxicity

	Growth delay (IC_{50}, μM)			Clonogenic assay (IC_{50}, μM)					
PD Number	HCT-8	SW620	HT-29	HCT-8	HCT-8 pretreat	SW620	SW620 pretreat	HT-29	HT-29 pretreat
0166326-0000	1.11	1.8	0.526	<5	<5	<5	ND	10.3	7.96
0173952-0000	1.53	0.174	0.2	1.26	1.38	0.47	0.53	1.37	1.21
0173955-0000	1.13	0.924	0.81	1.3	2.52	0.98	1.13	1.29	1.21
0173956-0000	0.662	0.619	0.617	1.15	0.9	0.402	1.55	1.54	1.53
0173958-0000	1.15	0.66	0.579	1.26	3.22	0.36	0.46	1.01	0.75
0179483-0000	1	0.156	0.266	>5	>5	ND	ND	<5	1.74
0180970-0000	0.45	0.285	0.461	0.26	0.61	ND	ND	1.52	0.464
0166285-0002B	0.42	0.27	0.279	0.778	ND	0.326	ND	1.87	0.439

Cells were treated with a range of concentrations of compounds and the concentration of compound required to inhibit cell growth or colony-forming ability by 50% compared with the untreated control was determined. For growth delay, cells were treated continuously for 72 hr. For the pretreat clonogenic assay, cells were treated for 48 hr on plastic prior to placement into soft agar without drug. Values are the means of at least three separate determinations done in duplicate. ND = not determined.

with a range of compound concentrations from 25 μM to 100 nM for different time periods of 4–72 hr. After compound exposure, cells were washed extensively and allowed to grow for a total combined time (with and without compound) of 72 hr. The IC_{50} values for growth delay calculated for each compound for an initial short exposure (4 hr) were compared with the values obtained from increasingly longer exposure times and found to be from 5 to 24 times greater than the conventional 72-hr growth delay IC_{50} value. These data suggest that continuous exposure of cells to compound is required to effect potent growth delay and that brief exposure (4 hr) of cells followed by washing does not result in persistent growth delay effects. This relative lack of potent effect at early times of exposure is not likely to be due to cellular transport and uptake, since other intracellular effects of the compounds were apparent at 2 hr of incubation (see below).

Effects of c-Src Kinase Inhibitor on c-Src Kinase-Mediated Phosphorylation Events in Cells

Because of difficulties resulting from potential loss of reversibly bound inhibitors during washes and subsequent analysis in accurately assessing cellular kinase activity from immunoprecipitated kinase complexes, phosphotyrosine levels on physiological substrates of c-Src were used as a measure of kinase activity. Three different substrates were evaluated as indicators of inhibition of c-Src kinase.

Phosphorylation of the cytoplasmic transcription factor Stat3 (*s*ignal *t*ransducer and *a*ctivator of *t*ranscription) has been associated with its activation and has been shown to result from the action of c-Src kinase [41–47]. A431 cervical carcinoma cells in culture were serum-starved overnight, treated with either 1 μM or 250 nM PD0180970 for 2 hr, and then stimulated by the addition of 100 ng/mL of EGF. After 5 min, the cells were lysed in immunoprecipitation lysis buffer, and Stat3 was immunoprecipitated using a rabbit polyclonal antibody directed against Stat3. Figure 2 shows the resulting anti-phosphotyrosine western blot, in which phosphotyrosine content of Stat3 is apparent after EGF stimulation of the cells (lane 1) in contrast to unstimulated and serum-starved cells (lane 2). Treatment of cells with either 1 μM or 250 nM PD0180970 caused a reduction in the phosphotyrosine content of the Stat3 band (lanes 3 and 4) under circumstances where the total amount of Stat3 was relatively constant (lower panel). In addition to immunoprecipitated phospho-Stat3, another prominent phosphorylated protein co-immunoprecipitated with Stat3 that was shown by electrophoretic mobility and by western blot (data not shown) to be the EGFr. The presence of the EGFr band here supports similar reported results in this cell line, in which immunoprecipitation of the EGFr resulted in co-immunoprecipitation of Stat3 [47].

The cytoskeletal protein paxillin is another protein reported to be a c-Src kinase substrate [48–50], and its phosphorylation was examined in HT-29 colon carcinoma cells in culture. Figure 3 depicts the results of an anti-paxillin immunoprecipitation that was western-blotted

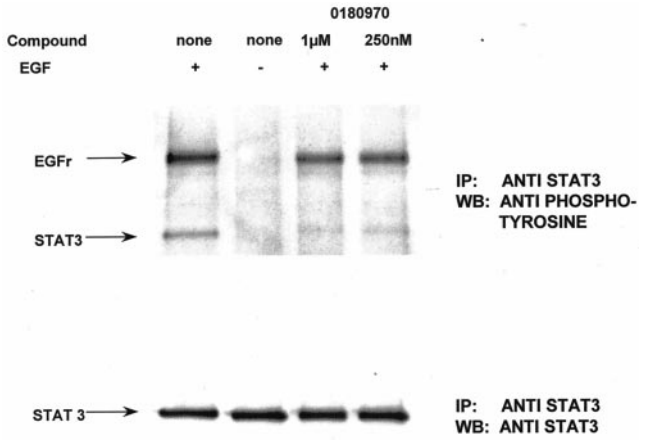


FIG. 2. Immunoprecipitation (IP) of Stat3 from A431 cells treated with c-Src kinase inhibitor. Cells at 7.75 × 10^5/well were serum-starved, treated with the indicated concentration of compound for 2 hr prior to the addition of 20 ng/mL of EGF, lysed, and immunoprecipitated using anti-Stat3 antibodies as described in Materials and Methods. The samples were split and run on separate gels that were subjected to western blotting (WB) as indicated. The protein bands were visualized using an extended chemiluminescence kit according to the manufacturer's instructions.

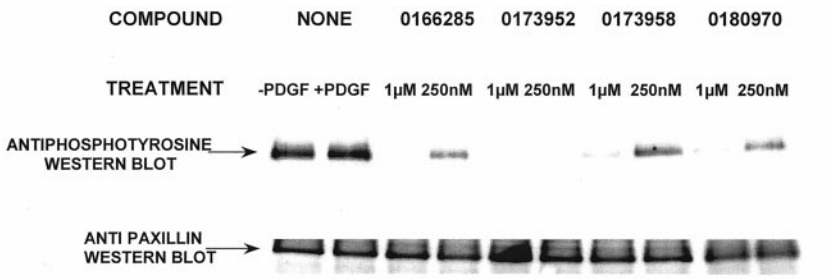


FIG. 3. Immunoprecipitation of paxillin from HT-29 cells treated with c-Src kinase inhibitors. Serum-starved cells at 1 × 10^6/well were treated with the indicated concentrations of compounds for 2 hr prior to the addition of 20 ng/mL of PDGF, followed by lysis and immunoprecipitation with an anti-paxillin antibody. The samples were split and run on duplicate gels that were subjected to western blotting as indicated. The protein bands were visualized using an extended chemiluminesence kit according to the manufacturer's instructions.

with either anti-phosphotyrosine or anti-paxillin antibodies. Three different c-Src kinase inhibitors (Tables 1 and 2) in a concentration-dependent fashion reduced the amount of tyrosine-phosphorylated paxillin that was immunoprecipitated from the cell lysates. To determine whether upstream tyrosine kinase receptor activation had an effect on paxillin tyrosine phosphorylation, cells were serum-starved overnight and then stimulated with PDGF in the presence or absence of c-Src kinase inhibitors. The addition of 20 ng/mL of PDGF 5 min prior to cell lysis had no effect on the tyrosine phosphorylation state of paxillin, suggesting that PDGF stimulation is not a physiological inducer of paxillin phosphorylation in this cell line.

The third substrate examined as an indicator of c-Src kinase activity was $p130^{cas}$ (Crk-*a*ssociated *s*ubstrate) [51, 52], which is a 130-kDa focal adhesion protein originally found by virtue of extensive phosphorylation in v-Src- and v-Crk-transformed cells [53, 54]. Antiphosphotyrosine and anti-$p130^{cas}$ western blots of lysates from HT-29 colon carcinoma cells are shown in Fig. 4A. The cells were serum-starved overnight, then treated for 2 hr with solvent alone or solvent containing the indicated concentration of PD0180970 (Tables 1 and 2), followed by a 5-min treatment of cells with 20 ng/mL of EGF. Cells were lysed, and the lysates were immunoprecipitated with an antibody to $p130^{cas}$. The phosphotyrosine content of the immunoprecipitated $p130^{cas}$ was reduced substantially in the lysates from compound-treated cells (upper panel) under conditions where the total amount of the protein was essentially identical (lower panel). Stimulation of the cells with EGF 5 min prior to lysis had no effect on the phosphotyrosine content of $p130^{cas}$ (data not shown).

To assess the selectivity of inhibition of phosphorylation of $p130^{cas}$ relative to other phosphorylated proteins, PD0173952 or PD0173955 was used to treat HT-29 cells as above. The immunoprecipitate was subjected to an antiphosphotyrosine western blot as shown in Fig. 4B and quantitated by laser densitometry. The amount of phosphotyrosine in the molecular mass range from 120 to 85 kDa ranged from 97% of the control treatment for 250 nM PD0173952, to 74% at 500 nM and 60% of control for a 1 μM concentration of compound. By way of comparison, the

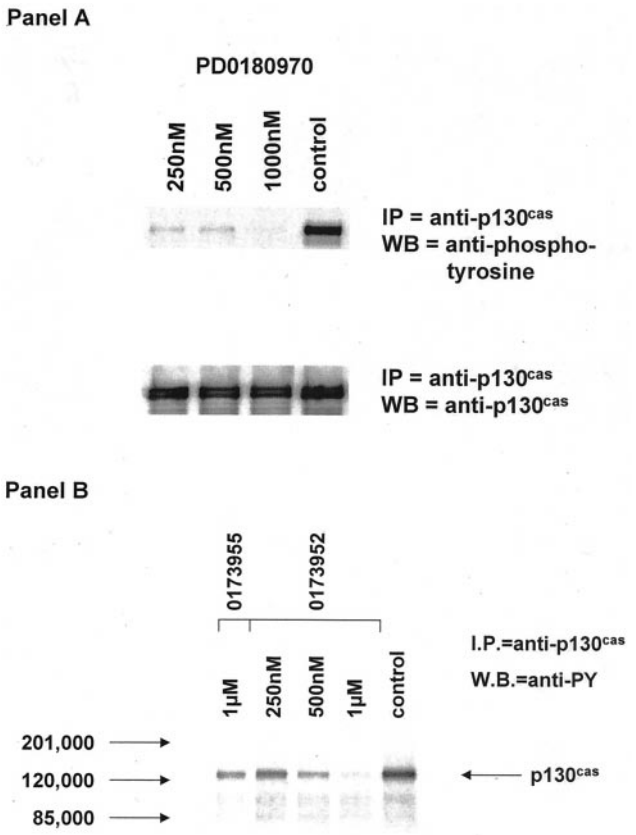


FIG. 4. Immunoprecipitation (IP) of $p130^{cas}$ from HT-29 cells treated with a c-Src tyrosine kinase inhibitor. Panel A: Serum-starved cells at 1 × 10^6 cells/well were treated with the indicated concentrations of PD0180970 for 2 hr prior to the addition of 20 ng/mL of EGF. Cells were lysed in immunoprecipitation buffer, and the protein was precipitated by use of an anti-$p130^{cas}$ antibody. The samples were split and run on duplicate gels that were subjected to western blotting (WB) as indicated. The protein bands were visualized using an extended chemiluminescence kit according to the manufacturer's instructions. Panel B: Serum-starved cells (1.3 × 10^6/well) were treated as in panel A with PD0173955 or PD0173952 as indicated. The $p130^{cas}$ immunoprecipitate was western-blotted with an anti-phosphotyrosine antibody and visualized as in panel A.

p130^{cas} phosphotyrosine content was 61% of control for a 250 nM concentration of compound, 32% for 500 nM, and 11% for 1 μM. Although the compound does cause some decrease in phosphotyrosine content of non-p130^{cas} proteins (especially at concentrations well above those for growth delay effects; Table 2), the phosphotyrosine content of p130^{cas} decreased to a greater extent than did the phosphotyrosine content of other proteins that co-immunoprecipitated with p130^{cas}, demonstrating a measure of selectivity of this compound toward inhibiting phosphorylation of a substrate of c-Src relative to a population of other phosphotyrosine-containing proteins.

Selectivity of c-Src Kinase Inhibitors Toward Receptor Tyrosine Kinase Phosphorylation

Having demonstrated inhibition of phosphorylation of cellular substrates of c-Src, we examined the effects of two compounds on the EGF-stimulated autophosphorylation of the EGFr to address the question of selectivity in a cellular system. HT-29 (see above) and the human epidermoid carcinoma A431 [39, 55] were two human cell lines used to characterize these effects. Cells growing in logarithmic phase were serum-starved overnight and then treated with either 1 μM or 250 nM concentrations of inhibitor for 2 hr prior to a 5-min stimulation with 20 ng/mL of EGF followed immediately by lysis in immunoprecipitate buffer. The EGFr was immunoprecipitated from the lysates, and the resulting precipitates were split, electrophoretically resolved on polyacrylamide gradient slab gels, transferred to nitrocellulose membranes, and probed by western blotting for either phosphotyrosine or the amount of total EGFr. Included in these experiments as a positive control was the selective EGFr kinase inhibitor PD0158780 [55]. As is shown in panels A and B of Fig. 5, the addition of EGF stimulated the phosphorylation of tyrosine on the EGFr in both cell lines relative to no addition (lanes 1 and 2). The EGFr tyrosine kinase selective inhibitor PD0158780 completely (HT-29) or substantially (A431) inhibited EGF-stimulated phosphorylation of the EGFr (lanes 5 and 6). Neither of the c-Src-selective kinase inhibitors inhibited phosphorylation relative to the uninhibited control in these cells (lanes 3, 4, 7, and 8). These data demonstrate the selective nature of these c-Src kinase inhibitors relative to the EGFr tyrosine kinase.

The effects of three c-Src kinase-active compounds on PDGFr phosphorylation were examined in a similar fashion in HT-29 cells. HT-29 cells were serum-starved overnight, then treated with compounds at the indicated concentrations for 2 hr prior to a 5-min stimulation with 20 ng/mL of PDGF. The cells were treated as described above, and the lysates were immunoprecipitated by an anti-PDGFr antibody. The resulting western blot is shown in Fig. 5C, where no apparent difference is seen in the phosphotyrosine content of PDGFr from cells treated with PD0173952, PD0173956, or PD0173958. These data suggest that the compounds do not inhibit PDGF-stimulated phosphorylation of the PDGFr.

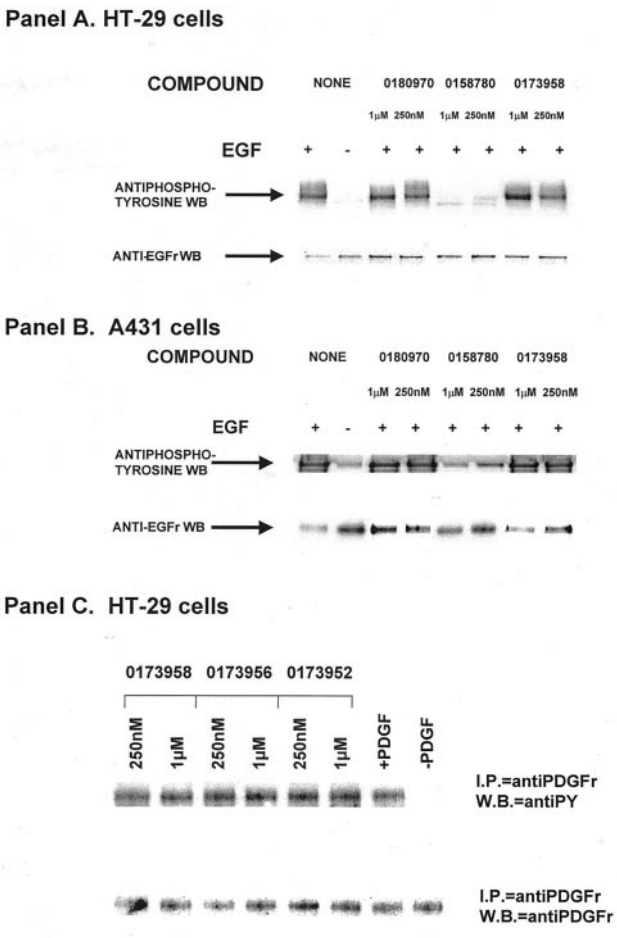


FIG. 5. Effect on tyrosine phosphorylation of EGFr or PDGFr by inhibitors of c-Src and EGFr tyrosine kinases. Serum-starved cells were treated for 2 hr with the indicated concentration of compounds prior to a 5-min stimulation with 20 ng/mL of EGF or PDGF. The cells were lysed, and the EGFr or PDGFr was immunoprecipitated (IP) and subjected to western blotting (WB) as described for either phosphotyrosine content or total EGFr or PDGFr content. PD0158780 is an EGFr kinase-specific inhibitor [55]. Panel A, 1.75 × 10^6 HT-29 cells/well; panel B, 9.3 × 10^5 A431 cells/well; panel C, 8.9 × 10^5 HT-29 cells/well.

Effect of Kinase Inhibitors on Mitogen-Stimulated Thymidine Incorporation

Because the tyrosine kinase inhibitors showed c-Src selectivity relative to a mitogenic receptor tyrosine kinase, the functional effects of c-Src kinase inhibition were examined in mitogen-stimulated thymidine incorporation assays in two different cell lines in culture. A431 epidermoid carcinoma cells and NIH 3T3 fibroblasts were chosen for these studies since their responsiveness to serum starvation and subsequent stimulation by serum or individual growth factors had been determined previously [39, 55].

The IC_{50} values for 72-hr growth delay in serum-contain-

TABLE 3. Effect of tyrosine kinase inhibitors on A4341 and NIH3T3 cell growth

PD Number	A431 $IC_{50}(\mu M)$	NIH 3T3 $IC_{50}(\mu M)$
0158780	2.31	20.9
0166285	0.455	0.056
0173952	0.245	0.17
0173958	0.445	0.3825
0180970	0.215	0.17

Cells were plated into 96-well tissue culture plates and allowed to attach and enter logarithmic phase growth. Cells were treated with a range of different concentrations of compounds for 72 hr at which point cells were fixed and stained with sulforhodamine B as described in Materials and Methods. Values are the means of duplicate determinations.

ing medium for these two cell lines treated with c-Src-selective inhibitors (PD0173952, PD0173958, PD0180970), an EGFr-selective inhibitor (PD0158780), and a broadly active kinase inhibitor (PD0166285) are shown in Table 3. Each of the c-Src-selective kinase inhibitors were comparably potent, whereas the EGFr inhibitor was substantially less potent, and the broadly active kinase inhibitor more potently inhibited cell growth of the NIH 3T3 fibroblasts.

To further characterize the cellular response, the concentration effects of a c-Src-selective kinase inhibitor (PD0180970), and for purposes of comparison, both an EGFr kinase-selective inhibitor (PD0158780) and a broadly active tyrosine kinase inhibitor (PD0166285), were examined over a time course of 3–4 days in the two cell lines. Cells were seeded in scintillating microplates [56, 57] and treated as described in Materials and Methods. These culture plates allow for repeated sampling of thymidine incorporation over time for the same cellular sample. In A431 cells (Fig. 6A), the c-Src inhibitor PD0180970 reduced fetal bovine serum-stimulated thymidine incorporation to nearly the basal, unstimulated level. Thymidine incorporation in A431 cells stimulated by EGF treatment was inhibited substantially by 500 nM PD0180970, reaching levels well below the basal, unstimulated incorporated amount. The broadly active kinase inhibitor PD0166285 at 500 nM also reduced EGF-stimulated thymidine incorporation to sub-basal levels by the end of the 4-day evaluation period. The EGFr kinase inhibitor PD0158780 had relatively modest effects on fetal bovine serum-stimulated thymidine incorporation as well as on EGF-stimulated thymidine incorporation.

A full concentration range of each of the three compounds was examined in NIH 3T3 cells and is shown in Fig. 6, B–I. As before, cells were plated, allowed to attach in fetal bovine serum-containing medium for 24 hr, serum-starved overnight, treated with compounds for 2 hr prior to stimulation, and then stimulated with 20 ng/mL of individual growth factors or 10% fetal bovine serum, or left unstimulated. In the case of each treatment except for stimulation by EGF, the EGFr-selective kinase inhibitor was not effective in reducing thymidine incorporation in cells. PD0180970 at 500 nM had a greater inhibitory effect in EGF- and PDGF-stimulated NIH 3T3 cells relative to serum- or bFGF-stimulated cells. Therefore, the c-Src-selective compound PD0180970 was a somewhat more potent inhibitor of EGF- or PDGF-stimulated thymidine incorporation than of bFGF or serum stimulation. The broadly active kinase inhibitor PD0166285 at 250 nM reduced thymidine incorporation to basal levels in all three sets of growth factor-stimulated cells, but not under conditions of serum stimulation.

Effects of c-Src-Selective Tyrosine Kinase Inhibitors on Cell Cycle Distribution

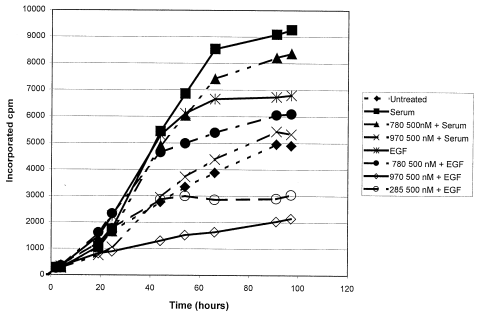
In light of data showing that intact and functional c-Src kinase is required for progression through the G_2M phase of the cell cycle in fibroblasts [58], HT-29 colon carcinoma cells growing in log phase were treated with selected c-Src kinase inhibitors for 24 hr, and the cell cycle distribution was determined using flow cytometry. Single-parameter analysis of propidium iodide-stained nuclei of cells from a representative experiment gave the data shown in Fig. 7. Relative to untreated control cells, the highest concentration of two of the five compounds examined here caused an increase in the fraction of cells found in the G_2M phase. Concentrations of PD0173952 or PD0180970 lower than 1 μM or any concentration of the three other compounds did not result in an accumulation of cells in the G_2M phase of the cell cycle.

DISCUSSION

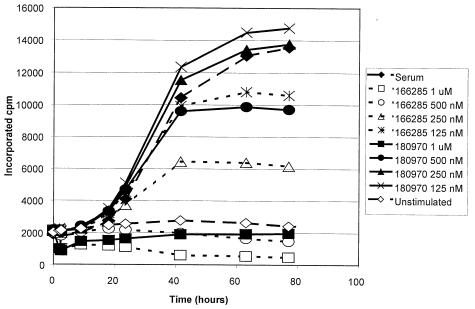
Use of small-molecule tyrosine kinase inhibitors as an approach to cancer therapy has been under investigation for a number of years and has followed a number of paths regarding selection of targets and classes of molecules [37, 59, 60]. c-Src kinase appears to be an attractive therapeutic target for cancer by virtue of its close association with the transformed phenotype in a number of human tumors and established cell lines [5–9]. Along with any approach that

FIG. 6. Effects of kinase inhibitors on thymidine incorporation stimulated by mitogens. Cells were seeded into 96-well scintillating microtiter plates (6000 cells/well for A431 and 10,000 cells/well for NIH 3T3) and allowed to attach to the plate and divide in serum-containing medium for 36–48 hr. Serum was removed for 24 hr. A431 cells (panel A) were labeled with 0.1 μCi [^{14}C]thymidine/well and treated with compounds and mitogens as indicated in the figure. NIH 3T3 cells (panels B–I) were labeled at the time of serum removal and treated with mitogens and compounds as indicated 24 hr after addition of label. Cells were unstimulated, treated with mitogen alone, or treated with mitogen plus the indicated compound. Data shown are the means of triplicate determinations. In all cases, the SD was less than 5% of the value; error bars are smaller than the size of the symbols.

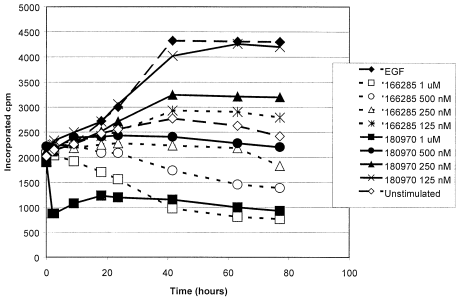
Panel A.



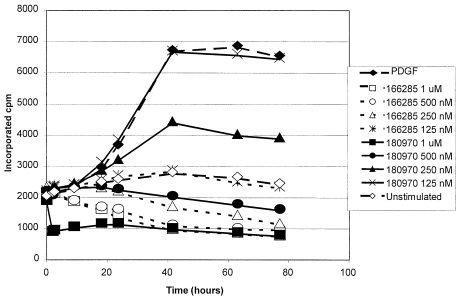
Panel B.



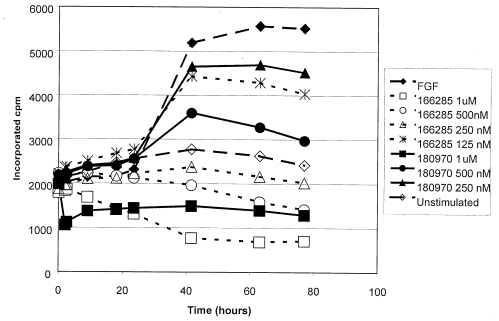
Panel C.



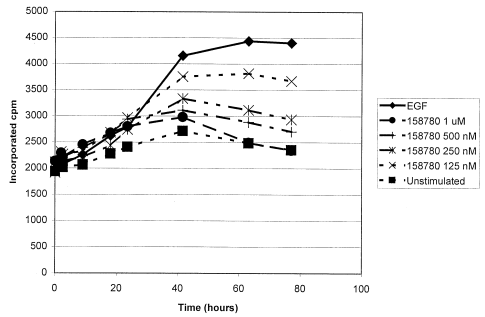
Panel D.



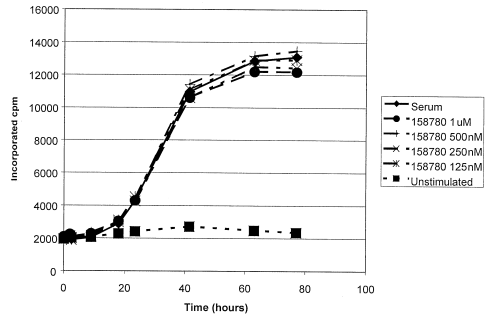
Panel E.



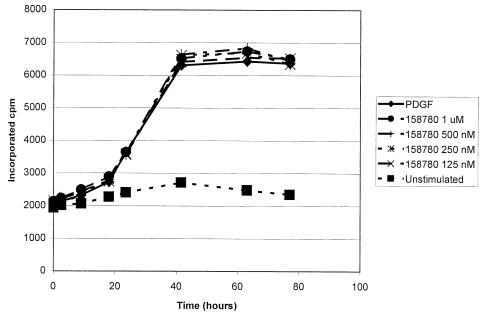
Panel G



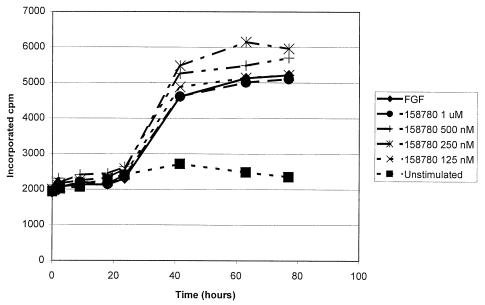
Panel F



Panel H



Panel I



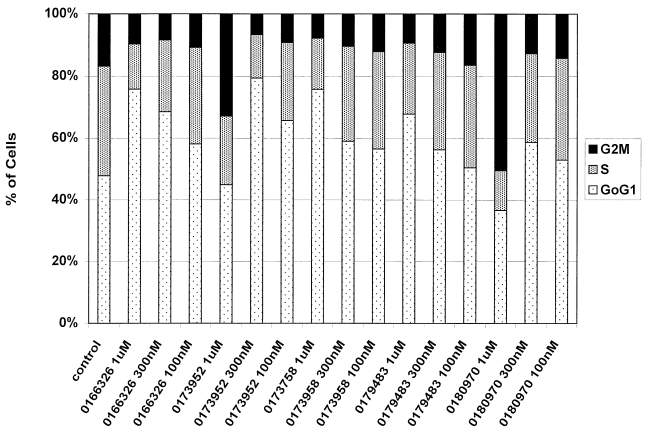


FIG. 7. Cell cycle distribution effects of c-Src kinase inhibitors. HT-29 cells in log-phase growth were treated with the indicated concentrations of compounds for 24 hr. The cells were collected, fixed in 70% ethanol, stained with propidium iodide, and examined by flow cytometry (see Materials and Methods). The distributions shown are the means of two separate determinations.

utilizes inhibitors of tyrosine kinases comes the concern about selectivity of inhibition of the target kinase relative to other kinases whose inhibition may not be desirable, but previous studies have demonstrated that selective ATP-competitive inhibitors are achievable [39, 61, 62].

Comparisons of *in vitro* c-Src tyrosine kinase inhibition by the pyrido[2,3-*d*]pyrimidines in this study have shown 10- to 50-fold selectivity for c-Src relative to bFGFr kinase and approximately 20- to nearly 150-fold selectivity relative to PDGFr kinase. This selectivity compares favorably with previously reported compounds [24, 26]. All of the compounds were also potent ($IC_{50} < 5$ nM) inhibitors of the c-Src family kinase Lck. Three of the compounds were examined for selectivity relative to EGFr tyrosine kinase and were found to possess 6- to 23-fold selectivity *in vitro*. One of the compounds (PD01809780) has been evaluated in v-Src kinase-transformed NIH 3T3 cells for its inhibition of Jak-1 kinase autophosphorylation and was found not to be an inhibitor of Jak-1 in cells at concentrations up to 10 μM.* These data confirm Src kinase selectivity against yet another kinase. Furthermore, the c-Src kinase inhibitors reported here demonstrated cellular activity as assessed by growth delay and effects on the transformed phenotype (clonogenicity in soft agar) at concentrations from 200–400 nM up to the micromolar range, showing that these compounds are able to penetrate the cellular membrane.

The concentration at which cellular effects are seen from the kinase inhibitors in this study is at least an order of magnitude greater than that required for inhibition of isolated enzyme. This phenomenon has been seen in other tyrosine kinase systems as well [26, 55]. Whereas kinase inhibition is apparent at low nanomolar concentrations, phosphorylation events in cells are seen at 250 nM to 1 μM, and cell growth delay or clonogenic cytotoxicity are apparent from 200 nM up to 10 μM. The *in vitro* conditions under which the enzymes are assayed, the subcellular localization of enzyme, and the subcellular partitioning of substrate and drug all may contribute to the differences in potency that are seen.

Cellular studies on the actions of the compounds were carried out by examination of phosphorylation states of physiologic substrates of c-Src kinase because of the labile nature of an immunoprecipitated complex of c-Src kinase and an inhibitor. Previous experiments had shown that immunocomplexed c-Src kinase isolated from cells treated with inhibitor retained essentially full activity upon *in vitro* assay, so following the effects of kinase inhibitors in this fashion would not be useful.

Three proteins that had been characterized as substrates of c-Src were evaluated [43, 49, 51]. Phosphorylation of Stat3 is required for its activity as a transcriptional activator, and under at least some conditions, c-Src activates Stat3 [63]. A431 epidermoid carcinoma cells treated with the c-Src inhibitor PD0180970 and stimulated with EGF show a substantial reduction in phosphorylation of Stat3. The possibility that PD0180970 inhibited another kinase that phosphorylates Stat3, such as Jak1, can be ruled out here, based on other studies.* Further examination of this experiment also suggested that the compound is not fully active toward the EGFr tyrosine kinase, based on the presence of the phosphorylated form of the EGFr that co-immunoprecipitated with Stat3 (Fig. 2, top bands). Paxillin phosphorylation in HT-29 human colon tumor cells was also inhibited by the tyrosine kinase inhibitors used in this study. Since c-Src has been implicated in the phosphorylation of paxillin [48–50, 64], inhibition of phosphorylation of this protein may indicate inhibition of c-Src. As with phosphorylation of Stat3, the possibility that inhibition of another kinase led to reduction of phosphotyrosine on paxillin cannot be excluded. The phosphorylation of $p130^{cas}$, yet another reported substrate of c-Src [51], was inhibited as well in cells treated for 2 hr with PD0173952. Examination of other proteins that co-immunoprecipitated with $p130^{cas}$ showed at least partial selectivity toward this c-Src substrate relative to a range of other cellularly phosphorylated proteins (Fig. 4B) whose phosphorylation state was less affected than was the phosphorylation of $p130^{cas}$. The marked reduction in phosphotyrosine content for all three of these c-Src substrates over the time course of a 2-hr treatment by inhibitors also suggests that the phosphorylation state of each protein is quite dynamic—in a 2-hr period where the kinase is inhibited and total protein levels of the substrates do not change appreciably, existing phosphotyrosine levels are diminished substantially, presumably by the action of phosphatases.

Selectivity of two c-Src kinase inhibitors was characterized in two different cell lines by examination of the phosphotyrosine content of EGFr tyrosine kinase after

* Zhang Y, Turkson J, Carter-Su C, Smithgall T, Levitzki A, Kraker A, Krolewski J, Medveczky P and Jove R, Manuscript submitted for publication.

stimulation by its ligand, which leads to autophosphorylation. Functional interactions between EGFr and c-Src kinase in mitotic signaling have been described [22], and this pathway, therefore, is appropriate to sort out contributions c-Src may make in transmitting growth signals. The two c-Src-selective kinase inhibitors did not modulate the EGF-stimulated signal on EGFr under conditions where an EGFr-selective compound did inhibit phosphorylation (Fig. 5, A and B), thus demonstrating the selective nature of these two compounds toward c-Src kinase relative to EGFr kinase. The lack of effect of the c-Src compounds on EGFr phosphotyrosine in these experiments appears at first inspection to be different than the effect on EGFr phosphotyrosine associated with Stat3 (Fig. 2), but in that experiment (where EGFr phosphotyrosine appears to be reduced relative to the uninhibited control) the EGFr was coimmunoprecipitated with Stat3 as opposed to a direct immunoprecipitation of EGFr in the experiment depicted in Fig. 5.

Three compounds were also examined in HT-29 cells for their effects on inhibition of PDGF-stimulated phosphorylation of the PDGFr. The compounds did not inhibit this phosphorylation event, lending further support to the selective nature of inhibition of phosphorylation in cells by these compounds.

EGF or serum-stimulated thymidine incorporation into A431 cells was inhibited by the c-Src-selective kinase PD0180970 (Fig. 6A) supporting the model of EGFr kinase signaling through c-Src [12]. Paradoxically, thymidine incorporation in A431 cells stimulated by either serum or EGF was not inhibited by concentrations of PD0158780 (an EGFr-selective kinase inhibitor) that did inhibit EGFr kinase phosphorylation stimulated by EGF. The reason for this apparent inconsistency is not clear but may be explained partially by the earlier finding of continued DNA synthesis and lack of cell division in this cell line treated with that compound, resulting in multinucleated cells [38].

Inhibition of growth factor- or serum-stimulated thymidine incorporation by c-Src-selective compounds in NIH 3T3 fibroblasts (Fig. 6, B–I) also is consistent with models describing the interaction of c-Src with EGFr, where c-Src phosphorylates EGFr and thus, directly or indirectly, may contribute to signal transduction and subsequent events [13, 22, 23]. The inhibition of PDGF-stimulated thymidine incorporation by the c-Src inhibitor is consistent as well with data that describe a role for c-Src downstream of PDGF-stimulated mitogenic signaling in the cell type used here [12, 65], although the role of c-Src in this signaling pathway is not clearly defined in all cases for every cell type or cell line [66, 67]. The relative lack of effect of PD0180970 on bFGF-stimulated thymidine incorporation may suggest that c-Src does not participate in downstream signaling by this mitogen in this cell line, unlike the circumstance for EGF and PDGF. Since serum contains a number of mitogens that likely act through pathways in addition to those examined here, the loss of inhibition of thymidine incorporation as a result of treatment of cells with a selective concentration of PD0180970 ($\leq$500 nM) is not surprising. As the concentration was increased up to 1 μM, inhibition of thymidine incorporation stimulated by all of the mitogens was achieved. These data show that the selective range of concentrations for inhibition of mitogen stimulation by PD0180970 in NIH 3T3 cells is from 250 nM to less than 1 μM. Reduction of thymidine incorporation below the level of initial unstimulated incorporation (Fig. 6, C–E) was seen at 1 μM of PD0180970 but not to an appreciable extent at the next lower concentration of 500 nM. These data may indicate that nonspecific effects may begin to become apparent at levels of this drug approaching 1 μM.

The cell cycle distribution effects of c-Src kinase inhibitors were consistent with a role for c-Src kinase in progression from G_2 to M phase [58, 68] for two of the five compounds examined. No readily apparent characteristic of the two compounds with cell cycle effect (G_2M accumulation) is distinguishable compared with the three compounds that did not cause G_2/M accumulation. All five were comparably selective for c-Src relative to PDGFr and bFGFr; all five were similarly potent in cell growth and cytotoxicity effects. One possible difference may lie within the inhibitory profile these compounds have for different members of the c-Src kinase family. A pan-Src family antibody microinjected into cells blocked progression, whereas a c-Src-specific antibody had less effect [58]. A compound structurally related to the two active compounds in this experiment inhibited Yes kinase as well as c-Src kinase itself [68]. Whether the two kinase inhibitors that caused a block in cell cycle progression here inhibit other c-Src family kinases besides Lck, and whether the three compounds that did not cause the block do not inhibit family members other than Lck, remain to be determined. The difference in potency for the effect of PD0180970 on cell cycle distribution (1 μM) compared with thymidine incorporation (selective concentration of 250–500 nM) must be considered in the context of two different cell lines (NIH 3T3 versus HT-29), two different times of exposure (2 hr prior to stimulation with mitogen versus 24 hr in log-phase cycling cells), and two different end points.

Selective inhibitors of c-Src tyrosine kinase are described here that reduce *in vitro* tumor cell growth by mechanisms other than nonselective cytotoxicity and cause loss of transformed phenotype as measured by ability to grow in soft agar, in at least one cell line for which intact and functioning c-Src kinase appears to be required for growth and survival [6] as well as in two other human tumor cell lines. Phosphorylation of three different cellular substrates of c-Src kinase was inhibited by these compounds at concentrations consistent with inhibition of cell growth. In a cell line overexpressing the EGFr tyrosine kinase, c-Src kinase inhibitors did not inhibit EGFr-stimulated phosphorylation of the EGFr, indicating functional selectivity toward c-Src kinase-mediated events. In another cell line containing both EGFr and PDGFr kinase, the c-Src compounds did not inhibit ligand-mediated receptor phosphor-

ylation events, also indicating a selective effect as opposed to a general kinase inhibition. Inhibition of c-Src kinase also inhibited mitogenesis stimulated by individual mitogenic growth factors. Progression through the cell cycle was blocked by c-Src kinase inhibitors in a manner not inconsistent with that seen by microinjection of cells with anti-Src antibodies [58]. Examination of *in vivo* activity of c-Src-selective compounds from the described series will be another indication of potential anti-tumor activity and is currently ongoing.

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