Inhibition of Src Kinases by a Selective Tyrosine Kinase Inhibitor Causes Mitotic Arrest

Mark M. Moasser, Mary Srethapakdi, Komal S. Sachar, Alan J. Kraker, and Neal Rosen


ABSTRACT
src kinase activity is elevated in some human tumors, including breast and colon cancers. The precise cellular function of the src family kinases is not clearly understood, but they appear to be involved in numerous signaling pathways. We studied the effects of PD173955, a novel src family-selective tyrosine kinase inhibitor, on cancer cell lines and found that it has significant antiproliferative activity due to a potent arrest of mitotic progression. The mitotic block occurs after chromosome condensation in prophase, before spindle assembly and without loss of cyclin A and B kinase activities. This effect is seen in cancer cell lines of all types with low or high activities of src kinases as well as in untransformed cell lines. In MDA-MB-468 breast cancer cells, this drug produces a rapid inhibition of cellular src and yes kinase activities as well as suppression of the mitotic hyperactivity of these kinases. This compound defines a novel class of antimitotic drugs that work through inhibition of src kinases and possibly other protein kinases that are required for progression through the initial phases of mitosis.

INTRODUCTION
Phosphorylation of proteins on tyrosine plays a crucial role in regulating a range of cellular processes. The importance of tyrosine phosphorylation is reflected in the large array of structurally diverse cellular protein tyrosine kinases that participate in a wide variety of cellular activities including proliferation, secretion, adhesion, and responses to mitogens and stress (reviewed in Refs. 1 and 2). Because the deregulation of tyrosine phosphorylation seems to be a critical abnormality in malignant transformation, the inhibition of tyrosine phosphorylation represents an attractive strategy for controlling unregulated growth and aberrant malignant behavior. However, target specificity is an important part of this strategy. High-throughput pharmaceutical technology has led to the development of families of small molecules that selectively inhibit specific tyrosine kinases. These agents will facilitate the study of the precise cellular functions of particular tyrosine kinases and could lead to new strategies for the treatment of cancer. We have been studying the activities of a tyrosine kinase inhibitor which shows selectivity toward the src family of protein kinases.

The src-related kinases are members of the nonreceptor family of protein tyrosine kinases, c-src was originally identified as the cellular homologue of v-src, the transforming protein encoded by the Rous sarcoma virus (3, 4). Nine other src-related cellular protein kinases have subsequently been identified that constitute a family of closely related tyrosine kinases including c-yes, fyn, c-fgr, lyn, lck, hck, blk, and yrk (reviewed in Ref. 5). Members of the src family are Mr, 55,000–62,000 myristylated proteins that share a common structural organization consisting of an SH2, an SH3, and a kinase domain that are highly homologous within the family, as well as NH2-terminal sequences that are unique to the individual members of the family. The tyrosine kinase activity of src-related kinases is regulated by complex intra- and intermolecular interactions as well as phosphorylation at a COOH-terminal tyrosine by the csk (6).

The activity of src is thought to play an important role in mediating cell proliferation and transformation. A number of primary tumors and tumor cell lines from patients with breast cancer, colon cancer, melanoma, and sarcoma have been shown to have elevated src kinase activity (reviewed in Ref. 7), and activating src mutations are seen in some advanced colon cancers (8). The development of mammary tumors in polyoma mT oncogene transgenic mice depends on activation of c-src, and Her2/Neu transgenic mice develop breast tumors with high src activity (9–11).

The precise cellular function of src family kinases has remained elusive, and they may in fact be involved in diverse pathways. Numerous lines of investigation suggest that src family kinases function as second messenger molecules in response to activated growth factor receptors (2). The mitogenic response to certain growth factors in quiescent fibroblasts is inhibited if the activities of src, yes, and fyn are blocked by microinjection of neutralizing antibodies or transfection of kinase inactive mutants (12, 13). src is found in plasma membrane caveolae, where it associates with and phosphorylates caveolin (14). The presence of multiple signaling molecules including Gα subunits and H-ras in these large membrane complexes suggests a signaling function; however, the role of src kinases in these complexes is only beginning to be studied.

There is increasing evidence that c-src plays an important role in regulating mitotic events. src shows changes in activity and localization during mitosis, which suggests a mitotic function (15, 16). src kinase activity is stimulated in fibroblasts undergoing mitosis (15), and the NH2-terminal phosphorylation of src on serine and threonine residues is at least partially responsible for this increased activity (15, 17). Catalytic activity of src kinases is necessary for initiation of mitosis because microinjection of antibodies that neutralize src, yes, and fyn in NIH3T3 cells inhibit entry into mitosis (18). Although gene inactivation experiments in mice fail to demonstrate an essential mitotic role for either of the src kinases alone, the possibility of functional redundancy between src, yes, and fyn is supported by the high neonatal lethality seen in double knock-out experiments (19).

The precise functional activity of src kinases in initiating mitosis remains to be worked out. At least one mitotic target of src has been described. This is an RNA-binding and SH3-binding protein named SAM68 that both associates with and is phosphorylated by src specifically during mitosis (20, 21). Because src and SAM68 are partitioned into separate cytoplasmic and nuclear compartments during interphase, it is unlikely that their association is important in regulating the entry into mitosis, and it may be more important for mitotic progression after nuclear envelope breakdown. Work is in progress to

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3 The abbreviations used are: csk, c-src kinase; RIPA, radioimmunoprecipitation assay; FACS, fluorescence-activated cell-sorting.
shows selectivity for src kinases in vitro is a pyrido-[2,3-d]pyrimidine, a tyrosine kinase inhibitor that inhibits the biological activities of the src family of tyrosine kinases. This inhibition is mediated through inhibition of src kinases or possibly other protein kinases; however, it defines a novel class of antimitotic drugs that identifies the functional targets of src kinases at the G2-M-phase transition.

We have used a src-selective tyrosine kinase inhibitor to study the biological activities of the src family of tyrosine kinases. This inhibitor is a pyrido-[2,3-d]pyrimidine, a tyrosine kinase inhibitor that shows selectivity for src kinases in in vitro assays. This compound inhibits src and yes activity in tumor cells and shows antiproliferative and antimitotic activity in a broad panel of tumor cell lines. It inhibits mitosis in early prophase, consistent with existing data that suggest a mitotic function for src. The biological effects of this compound may be mediated through inhibition of src kinases or possibly other protein kinases; however, it defines a novel class of antimitotic drugs that work through inhibition of tyrosine phosphorylation.

**MATERIALS AND METHODS**

**Cell Culture and Cell Cycle Assays.** All cell lines were obtained from the American Type Culture Collection; maintained in a 1:1 mixture of DMEM: Ham’s F-12 supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM glutamine, and 10% heat-inactivated fetal bovine serum; and incubated at 37°C in 5% CO2. PD173955 was maintained as a 10 mM frozen stock solution in DMSO. For determination of cell cycle effects, cells were seeded at approximately 2 million cells/10-cm dish and placed the next day in media containing 5% FBS. MDA-MB-468 cells were seeded in 10-cm dishes and treated for 24 h with 5 mM PD173955 or DMSO. The cell cycle distribution of cells was determined by FACS analysis and listed under the G1, S, and G2-M columns. In addition, mitotic indices were manually counted under fluorescence microscopy of bis-benzimide-stained cells and listed under the M column.

<table>
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<th>% G1</th>
<th>% S</th>
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<td>DMSO</td>
<td>49</td>
<td>30</td>
<td>21</td>
<td>2</td>
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<tr>
<td>PD173955</td>
<td>1</td>
<td>3</td>
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Table 1 Cell cycle activity of PD173955

MDA-MB-468 cells were seeded in 10-cm dishes and treated for 24 h with 5 mM PD173955 or DMSO. The cell cycle distribution of cells was determined by FACS analysis and listed under the G1, S, and G2-M columns. In addition, mitotic indices were manually counted under fluorescence microscopy of bis-benzimide-stained cells and listed under the M column.

**Kinase Assays.** For src kinase assays, total cellular lysates were harvested in modified RIPA buffer [1% sodium deoxycholate, 1% NP40, 0.1% SDS, 150 mg NaCl, and 10 mM sodium phosphate (pH 7.2)] supplemented with 10 µM aprotinin, 10 µM leupeptin, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Total cellular lysates (300 µg) were incubated with antibodies specific for each of the src family kinases and immunoprecipitated using protein G-Sepharose (Pharmacia). Immunoprecipitates were washed twice in cold lysis buffer and once in kinase buffer and added to an in vitro kinase reaction consisting of 50 mM PIPES (pH 7.0), 10 mM MnCl2, 10 mM DTT, 10 µM ATP, 2 µg of acid-denatured enolase, and 5 µCi of [γ-32P]ATP. Reactions were allowed to proceed at 30°C for 5 min and then stopped immediately by boiling in sample buffer, products separated on a 10% SDS-PAGE gel, transferred to membrane, and exposed to film. Monoclonal antibodies specific for src (m327; Calbiochem), c-yes (1B7; Wako), lyn (LO5620; Transduction Laboratories), and polyclonal anti-fyn antibodies (SC-16; Santa Cruz Biotechnology) were used in immunoprecipitations and in immunoblotting, using the appropriate secondary immunological reagents.

For cyclin A- and B-dependent kinase assays, 100 µg of total cellular NP40 fraction were incubated with 100 µCi of [γ-32P]ATP. Reactions were allowed to proceed at 30°C for 5 min and then stopped immediately by boiling in sample buffer, products separated on a 10% SDS-PAGE gel, transferred to membrane, and exposed to film. Monoclonal antibodies specific for cyclin A (C-17; Santa Cruz Biotechnology) and B (SC-8; Santa Cruz Biotechnology) were used in immunoprecipitations and in immunoblotting, using the appropriate secondary immunological reagents.

**Nocodazole release**

T=0

DMSO PD173955

T=1 hr

T=2 hrs

T=4 hrs

T=6 hrs

T=10 hrs

Fig. 2. MDA-MB-468 cells were treated with 200 ng/ml nocodazole for 24 h. The mitotic cells were collected by shaking off, washed twice in PBS, and reseeded in 6-cm dishes in media containing 5 µM PD173955 or DMSO. At the indicated time points after nocodazole release, cells were harvested, and cell cycle distribution was determined by FACS analysis.
lysates were incubated with polyclonal anti-cyclin A or monoclonal anti-cyclin B antibodies (Santa Cruz Biotechnology) for 2 h at 4°C and immunoprecipitated with protein A-Sepharose (Pharmacia). Immune complexes were washed four times in cold lysis buffer and twice in kinase buffer [20 mM Tris-Cl (pH 7.4), 7.5 mM MgCl2, and 1 mM DTT]; added to 40 μl of kinase buffer containing 2 μg of histone H1, 300 μM ATP, and 10 μCi of [γ-32P]ATP; and incubated for 10 min at 37°C. Reactions were stopped by boiling in sample buffer, products separated on SDS-PAGE, transferred to membrane, and exposed to film or phosphorimaging screen. Kinase activity was quantitated using a FUJIX phosphorimager and MacBAS program software.

Immunofluorescence Analysis. Harvested cells were washed in PBS, fixed in methanol for 20 min at −20°C, washed again in PBS, and blocked for 30 min in PBS with 2% BSA. Cells were stained first with mouse monoclonal anti-α-tubulin (Sigma) and human polyclonal anti-centromere (a gift of Dr. J. D. Rattner, University of Calgary, Alberta, Canada) antibodies and then stained with rhodamine conjugated antimouse and FITC-conjugated antihuman secondary antibodies as well as 2 μg/ml bis-benzimide. Results were visualized and imaged under confocal microscopy.

RESULTS

Antimitotic Activity. PD173955 is an ATP-competitive tyrosine kinase inhibitor with a pyrido-[2,3-d]pyrimidine structure (Fig. 1). It was selected from a group of compounds because of selectivity for c-src kinase inhibition. Selectivity was determined in in vitro assays that measure [32P]ATP incorporation into peptide substrates. This compound inhibits src kinase activity in vitro with an IC50 of 22 nM. In comparison, it inhibits α-fibroblast growth factor receptor and platelet-derived growth factor receptor with IC50s of 1.6 μM and is inactive against the insulin receptor and protein kinase C in this range of concentrations (data not shown). It inhibits both src and yes kinase activities equally in vitro. To determine its in vivo activities, we first studied the effects of this compound on growth in tissue culture of MDA-MB-468 and MCF-7 breast cancer cells. This compound inhibits the growth of these cells with IC50s of 500 nM and 1 μM, respectively, with an accumulation of suspended cells (data not shown). To determine whether the growth-inhibitory effects of PD173955 are due to a phase-specific inhibition of cell cycle progression, cells were treated for 24 h, and cell cycle analysis was performed by flow...
cytometry of ethidium bromide-stained nuclei to measure DNA content and fluorescence microscopy of bis-benzimide-stained cells to study the nuclear chromatin patterns. Cells treated with PD173955 show a near complete redistribution to the G2-M phase of the cell cycle in comparison with control cells, and quantitation of mitotic indices by immunofluorescence microscopy shows an accompanying accumulation of mitotic cells (Table 1). These data are consistent with an arrest of mitotic progression. Cells released from a nocodazole block into PD173955 remain blocked in mitosis, whereas control cells complete mitosis and progress into G1, confirming the antimitotic activity of this drug (Fig. 2). Increasing drug concentrations up to 50 μM produce the same cell cycle profile, confirming that this drug does not have a dose-dependent G1- or S-phase blocking activity (data not shown). This mitotic block is accompanied by an accumulation of a significant number of cells with sub-G1 DNA content, suggestive of apoptotic cell death (data not shown). Experiments were performed to determine whether this mitotic block is reversible. MDA-MB-468 cells were treated with 5 μM PD173955 for 24 h, and mitotic cells were washed with PBS and replated in media. These cells had poor ability to resume proliferation compared with control cells released from a nocodazole block (data not shown). Although a mitotic block is evident at a concentration of 1 μM, a concentration of 5 μM was chosen for additional studies because this is the lowest concentration that causes maximal antimitotic activity.

The antimitotic activity of PD173955 was further evaluated in a panel of eight breast cancer cell lines. This drug causes mitotic arrest in each of these cell lines (Fig. 3). The activities of the different members of the src-related kinases were determined in these cells by in vitro assays using antibodies specific for src, yes, fyn, and lyn kinases. Most of these cells have high total src and yes kinase activities, comparable to those of colon cancer cells (Fig. 4). Only a few of these cell lines have low fyn and lyn kinase activities, and no significant expression of lck, hck, fgr, and blk is seen in these cells (data not shown). Therefore PD173955 shows antimitotic activity in breast cancer cells with high or low src and yes kinase activities. An analysis of additional cancer cell lines from other tumor types as well as nontransformed cell lines (MCF10A and NIH-3T3) shows that the antimitotic activity of PD173955 is independent of cell type or malignant transformation (Fig. 3). In fact, no cell line tested to date is resistant to the antimitotic activity of this agent.

**Phase of Mitotic Block.** To further characterize the phase of the mitotic block, we examined mitotic spindle assembly and chromosome migration in drug-treated cells. PD173955-arrested mitotic cells have condensed chromatin, consistent with the initiation of the mitotic phase, but have no spindle assembly or chromosome movement or alignment to indicate the initiation of metaphase (Fig. 5). These features are consistent with a block in prophase similar to that seen in nocodazole-treated cells. We then assayed biochemical markers related to mitotic phase. The onset of mitosis is characterized by high cyclin A- and B-related kinase activities, and mitotic progression is characterized by a fall in cyclin A-related kinase activity in metaphase and a subsequent fall in cyclin B-related kinase activity in later mitosis. PD173955-blocked mitotic cells have cyclin A- and B-related kinase activities that are comparable to those of nocodazole-blocked mitotic cells and consistent with an early mitotic block (Fig. 6).

**Effects on Cellular src and yes Activity.** To determine the cellular biochemical effects of PD173955, we examined src and yes kinase activities after drug treatment. Because src kinases are substrates for autophosphorylation, we also studied changes in the tyrosine phosphorylation of src and yes after drug treatment. Treatment of cells with 5 μM PD173955 produces a rapid decline in the in vitro kinase activities of cellular src and yes proteins (Fig. 7, bottom panels). This effect is rapid and is much more pronounced with yes than with src. Quantitative analysis of enolase phosphorylation shows a 37% loss of src kinase activity and a 76% loss of yes kinase activity at 15 min after drug treatment. This is associated with a rapid loss of tyrosine phosphorylation of both src and yes proteins (Fig. 7, middle panels), but no significant changes in cellular src and yes expression (Fig. 7, top panels). These data confirm the rapid in vivo activity of the drug. It should be noted that substrate-specific activities of src and yes...
may not necessarily parallel the autophosphorylation and enolase phosphorylation activities that are assayed here. However, although these assays do not attempt to differentiate the activities of PD173955 with regard to various src substrates, they do confirm a rapid in vitro activity, at least with regard to enolase and autophosphorylation.

The inhibition of src and yes kinase activities appears to persist through an immunoprecipitation followed by several washes, leading to the results seen in Fig. 7 (bottom panels), suggesting that in addition to the competitive inhibition, there is a more stable inhibition of src and yes kinase activities in response to drug treatment. We then performed an in vitro experiment to see whether exposure to this drug has an irreversible effect on src and yes kinases (Fig. 8). Exposure of src and yes immune complexes to 100 nM PD173955 for 10 min in vitro significantly inhibits their kinase activities, as expected (Lane 2); however, washing these complexes several times after drug exposure does not relieve this inhibition (Lane 4), which suggests that exposure to this drug persistently inhibits src and yes kinases.

At the time of mitotic arrest of PD173955-treated cells, src and yes proteins are not tyrosine phosphorylated, and both proteins have low in vitro kinase activities (Fig. 9). This differs slightly from the immediate effects of PD173955 exposure, which produces a pronounced inhibition of in vitro yes kinase activity but only a slight inhibition of in vitro src kinase activity (Fig. 7).

To study the effects of PD173955 on cell cycle-associated protein tyrosine phosphorylation, total cellular phosphotyrosine content was studied by immunoblot analysis. Entry into mitosis is associated with an increase in tyrosine phosphorylation of a number of cellular proteins, most notably the Mr 110,000, Mr 120,000, and Mr 85,000 bands, and treatment with PD173955 results in significant decreases in tyrosine phosphorylation of these proteins as well as that of other cellular proteins that are not tyrosine-phosphorylated in a mitosis-specific manner (Fig. 10).

DISCUSSION

PD173955 is an inhibitor of protein tyrosine kinase activity with selective activity against src-related kinases. It potently inhibits mitotic progression in cells of all types and induces varying degrees of apoptotic cell death, comparable to tubulin-binding antimitotic agents such as vinblastine or Taxol. The block is in early mitosis with no apparent spindle formation or chromosome migration and with active cyclin A- and B-related kinases, consistent with an early phase of mitosis. These data suggest that PD173955 represents a new class of antimitotic drugs.

The mitotic target of this drug is not yet clear. Although the in vitro specificities of this agent suggest that it is a specific inhibitor of

Fig. 6. Cyclin A (A)- and B (B)-associated kinase activities were assayed in drug-treated mitotic cells. MDA-MB-468 cells were treated for 24 h with 200 ng/ml nocodazole (Noc), 5 μM PD173955, or DMSO. Mitotic cells were collected by shake-off for the nocodazole and PD173955 arms, whereas the total cell population was used in the DMSO arm. Kinase activities of cyclin A and B immunoprecipitates were assayed as described in “Materials and Methods” along with mouse IgG control immunoprecipitates. Results were recorded by exposure to film and quantitated by densitometry using arbitrary units (A.U.) to show the relative signal intensities. Both the photographic image and the quantitated values are depicted.
studies of src-related kinases, it remains possible that other protein kinases are also inhibited. However, the importance of such unknown activities is difficult to speculate at this time. The data presented in this study confirm potent in vivo inhibition of src and yes kinase activities, suggesting that the inhibition of src-related kinases may be mediating its biological effects. However, our data show similar antimitotic activity in cells with very low src and yes kinase activities. This can mean that even low src kinase activity in such cells is necessary for mitotic progression or, alternatively, that the inhibition of mitosis by PD173955 is mediated through the inhibition of proteins other than the known src family kinases, including possible unidentified family members or other homologous protein kinases.

In this work, we have assayed both the kinase activities of src and yes as well as the tyrosine phosphorylation of these proteins. The relationship between these tyrosine phosphorylations and kinase activity has been investigated for many years and appears to be complex. src kinases undergo tyrosine phosphorylation at two sites. Phosphorylation of Tyr416 in the COOH-terminal tail by csk represses its activity, and mutations or truncations of this residue result in unregulated and transforming activity (23). There is also in vitro evidence for autophosphorylation of Tyr527, although the physiological importance of this possible autoregulatory mechanism has not yet been established (24). The regulatory function of Tyr416 phosphorylation in the catalytic domain is less well understood, but it is a site of autophosphorylation, and its phosphorylation correlates with the active state of the enzyme and is inversely correlated with the inhibitory Tyr527 phosphorylation (reviewed in Refs. 25–27). Down-regulation of src kinase activity by csk could inversely affect phosphorylations of Tyr416 and Tyr527, and total src phosphotyrosine content may not change significantly. However, inhibition by an inhibitor such as PD173955 may reduce Tyr416 autophosphorylation without increasing Tyr527 phosphorylation. This would be consistent with our data, which show decreased total tyrosine phosphorylation of src and yes soon after drug treatment. Additional studies are required to definitively characterize the effects of this drug on the phosphorylation of specific tyrosine residues.

Our data show that PD173955 treatment causes a rapid and persistent inhibition of src and yes kinase activities. Whereas in vitro kinase assays in the presence of increasing ATP concentrations show that PD173955 inhibition is competitive with respect to ATP (data not shown), our data also show that it is not readily reversible. The mechanism of this extended inhibition is difficult to speculate at this time but may include high binding affinity of PD173955 for src and yes or possibly the induction of conformational changes in src and yes proteins with a resulting loss of kinase activities.

PD173955 produces a rapid down-regulation of yes kinase activity that is much more pronounced than that of src kinase activity. However, with entry and arrest in mitosis, there is a marked down-regulation of both src and yes kinase activities. The precise mechanism of regulation of src kinase during mitosis is not clearly understood; however, activating serine/threonine phosphorylations by p34cdc2 (28–30) and inactivating Tyr527 tyrosine phosphorylation by csk have been described during mitosis (31). PD173955 could effect...
this regulation either through inhibition of src and yes autoregulatory feedback loops, or possible cross-member regulatory effects such as the effects of src on yes activity, or, alternatively, due to effects of PD173955 on other protein kinases that are not yet recognized. Our data suggest differences in the mitotic regulation of src and yes because the mitotic activation of src is associated with increased tyrosine phosphorylation and stable expression, whereas the mitotic activation of yes is associated with decreased tyrosine phosphorylation and decreased expression (Fig. 9). Little is known about the mitotic regulation of yes kinase, and a better understanding of any differences in the mitotic functions of these two related protein kinases awaits additional studies.

The inhibition of mitotic progression by PD173955, a pharmacological inhibitor of src-related kinases, shows some differences with existing data in microinjection experiments. Microinjection studies using antibodies that neutralize src, yes, and fyn function show failure of G2 fibroblasts to enter mitosis (18). This occurs before chromatin condensation in G2, whereas PD173955 treatment induces arrest after chromatin condensation in prophase. These differences are likely due to the different inhibitory activities. src kinases have biochemical functions in addition to the known catalytic kinase function, and these functions are not fully understood (32, 33). A small molecule ATP competitive inhibitor and a neutralizing antibody can be expected to have different effects on these kinase-independent functions as well as different characteristics with regard to unintended inhibition of other proteins leading to observed differences in biological effects.

The mitotic substrates of src and yes that could mediate mitotic arrest are not currently known. Whereas the phosphorylation of SAM68 by src has been described, its requirement in initiating mitosis has not been demonstrated (20, 21). Inhibition of SAM68 phosphorylation is not likely to be the mechanism by which PD173955 arrests mitotic progression because we have thus far been unable to detect an appreciable mitotic tyrosine phosphorylation of SAM68 in MDA-MB-468 cells (data not shown). src has been shown to associate with tubulin in osteoclasts in response to substrate recognition (34) and to phosphorylate α- and β-tubulins in nerve growth cone membranes (35); however, the mitotic phosphorylation of tubulin has not been demonstrated, and a function in regulating mitotic microtubule dynamics remains to be shown. src has been shown to phosphorylate numerous other cellular substrates; however, none of these substrates are currently recognized to be important for mitotic progression (reviewed in Refs. 2 and 36). The precise regulation of mitotic events is only beginning to be understood; however, the importance of protein phosphorylation, including tyrosine phosphorylation and dephosphorylation, has become evident in recent years, and work is under way to identify and study these mitotic phosphoproteins (reviewed in Refs. 37 and 38). src kinases may be involved in the phosphorylation of one or more of these mitotic phosphoproteins.

In addition to a role in mitotic progression, src-related kinases seem to be involved in other diverse cellular pathways. In some experimental systems, src kinases seem to function as second messenger molecules in response to mitogenic signals (Ref. 18; reviewed in Refs. 1, 2, and 39). In other systems, there is evidence of a role in cell adhesion (40) and motility (41, 42). In this study, we present the cell cycle phenotype associated with inhibition of src kinases. Additional biological activities such as the possible inhibition of mitogenic signaling, cell adhesion, or motility were not investigated in this report and await additional studies specifically examining these activities.

Whereas the mitotic block induced by PD173955 is consistent with existing data regarding a mitotic function for src kinases, the absence of a G1 block is more unexpected. Our data show that the tyrosine kinase activity of src kinases is not required for G1 progression in proliferating cells. However, this does not rule out src kinase regulation of redundant pathways involved in G1 progression. Mitogenic control of cell proliferation is known to be mediated through growth factor receptors in G1, and the association of src family kinases with a number of these receptors and the src induction of cyclin D1 suggest a second messenger or regulatory function for src family kinases in these pathways (43–46). The physical association of c-src with HER1 and HER2 proteins in some human and murine breast tumors also suggests a second messenger function in breast cancers, although the functional role of this association with regard to cell cycle regulation is unclear (47–49). To date, we have not detected an effect of PD173955 treatment on mitogenic signaling in our system. Treatment of MDA-MB-468 cells with PD173955 does not inhibit the epidermal growth factor-induced phosphorylation of the epidermal growth factor receptor or the activation of mitogen-activated protein kinase (data not shown). It should be noted that our studies, which are designed to inhibit src using PD173955, differ from studies that inhibit src through neutralizing antibodies or dominant negative constructs in an important way. Whereas PD173955 inhibits the kinase activity of src proteins, there are src protein functions that are independent of its catalytic kinase activity, as demonstrated by experiments involving kinase-defective src mutants (32, 33). Such functions may be important in G1 regulation and would likely be unaffected by a pure kinase inhibitor. The precise role of src family members in G1 regulation awaits further definition of the kinase-dependent and -independent functions of these proteins in mitogenic signal transduction pathways.

PD173955 represents a novel class of antimitotic drugs. Additional studies are necessary to further characterize its target specificities and mechanism of action and to design compounds with even more selective substrate affinities. These are promising tools for future studies of the physiological and oncogenic functions of src kinases. The possible role of PD173955 in cancer therapeutics awaits additional studies to better characterize its bioavailability and pharmacokinetic properties, and additional structural modifications may serve to optimize these parameters so that its true therapeutic index and potential may be determined in preclinical and clinical studies.
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