Characterization of Potent Inhibitors of the Bcr-Abl and the c-Kit Receptor Tyrosine Kinases

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ABSTRACT

The early stage of chronic myelogenous leukemia (CML) is caused by the tyrosine kinase Bcr-Abl. Imatinib mesylate (also known as STI-571 and Gleevec), a tyrosine kinase inhibitor, has shown encouraging results in CML clinical trials and has become a paradigm for targeted cancer therapies. Recent reports of resistance to imatinib argue for further development of therapies for CML. During studies of signal transduction, we observed that the pyrido[2,3-d]pyrimidine src tyrosine kinase inhibitor PD173955 inhibited Bcr-Abl-dependent cell growth. Subsequently, a related compound, PD180970, was reported as a potent inhibitor of Bcr-Abl. We have compared the potency of these two compounds and four other analogues with imatinib on Bcr-Abl-dependent cell growth, cytokine-dependent cell growth, and tyrosine kinase inhibition. PD173955 inhibited Bcr-Abl-dependent cell growth with an IC50 of 2–3 nM in different cell lines. Fluorescence-activated cell-sorting analyses of cells treated with PD173955 showed cell cycle arrest in G1. PD173955 has an IC50 of 1–2 nM in kinase inhibition assays of Bcr-Abl, and in cellular growth assays it inhibits Bcr-Abl-dependent substrate tyrosine phosphorylation. Of the six pyrido[2,3-d]pyrimidine analogues studied, PD166326 was the most potent inhibitor of Bcr-Abl-dependent cell growth. PD173955 inhibited kit ligand-dependent cell proliferation (IC50 = ∼25 nM) and kit ligand-dependent proliferation of M07e cells (IC50 = 40 nM) but had a lesser effect on interleukin 3-dependent (IC50 = 250 nM) or granulocyte macrophage colony-stimulating factor (IC50 = 1 μM)-dependent cell growth. These compounds are potent inhibitors of both the Bcr-Abl and c-kit receptor tyrosine kinases and deserve further study as potential treatments for both CML and for diseases in which c-kit has a role.

INTRODUCTION

CML is a clonal disease involving the pluripotent hematopoietic stem cell compartment and is associated with the Philadelphia chromosome, a reciprocal translocation between chromosomes 9 and 22 (1–3). This translocation links the c-abl tyrosine kinase oncogene on chromosome 9 to the 5′ half of the bcr gene on chromosome 22 and creates the fusion gene bcr/abl (4, 5). The fusion gene produces a chimeric 8.5-kb transcript that codes for the p210bcr/abl protein (6). It is well established that the p210bcr/abl gene product is an activated protein tyrosine kinase (7) and that mutation or inhibition of the tyrosine kinase domain in vitro abolishes the transforming potential of p210bcr/abl in model systems (8). Indeed, overexpressed recombinant Bcr-Abl can cause CML-like leukemia in animal models (9, 10) and growth factor independence in tissue culture systems (11, 12). In contrast, cultures of primary chronic-phase CML leukemia cells are dependent on the addition of exogenous cytokines and colony-stimulating factors for cell growth and retain a program of maturation (13, 14). However, when compared with normal hematopoietic progenitor cells, alterations have been detected in response to factors such as KL (15, 16). This finding suggests that although Bcr-Abl clearly has oncogenic potential and is the pathogenic agent of CML, the observable changes in primary leukemic cells are subtle.

Signal transduction through phosphorylating pathways has been studied extensively, and tyrosine phosphorylation has been linked to multiple cell growth and differentiation pathways (17–22). Altered or elevated tyrosine kinase activity arising from mutation or abnormal protein structure (for example, translocation) may lead to constitutive activation of both growth and antiapoptotic pathways and has been implicated in many human cancers. Because the observed leukemic state of CML is dependent on the intact Bcr-Abl tyrosine kinase activity, extensive work has been done to identify substrates of Bcr-Abl and thus possible mechanisms leading to a myeloid expansion. Many groups, including our own, have characterized prominent tyrosine-phosphorylated protein substrates (23) in both CML blasts and Bcr-Abl-expressing cell lines, including SHP1, SHP2, c-bcr, p62dok, p56dok, SHC, and Crkl (24–31). In addition, key signal transduction pathways involving phosphoinositide 3-kinase, ras, myc, c-jun kinase, and STAT5 (signal transducers and activators of transcription 5) are also activated in a Bcr-Abl kinase-dependent manner (32). Thus, whereas Bcr-Abl clearly acts on multiple aspects of cell growth and activation, the exact mechanisms by which the leukemic state is achieved require further study.

From a therapeutic standpoint, because CML arises from a single genetic lesion, a research goal has been to develop kinase-specific inhibitors. The recently developed imatinib (STI-571) has become a paradigm for tyrosine kinase-inhibiting targeted therapeutics (33). Imatinib was originally shown to be a selective inhibitor for the abl and platelet-derived growth factor tyrosine kinases (34). More recently, it was observed that imatinib also inhibited the c-kit receptor tyrosine kinase (35, 36). In vitro, treatment of Bcr-Abl-positive cell lines with imatinib abolishes kinase-dependent substrate phosphorylation and leads to growth arrest and apoptosis (37). In recently reported clinical trials, imatinib has shown great promise in the chronic phase (38) and some promise in the accelerated and blastic phases of CML as well as in Bcr-Abl-expressing acute lymphoblastic leukemias (39). Moreover, the finding of inhibition of the c-kit signal transduction pathway by imatinib has shown potential efficacy in treating GISTs, which contain activating mutations in the c-kit receptor (40). These successful clinical results provide a proof of principle...
for targeting a causal agent in cancer. As frequently happens with cancer therapeutics, resistance to imatinib has been reported in Bcr-Abl-expressing cell lines and in patients with CML (41–47). Such observations argue for the continued development of specific therapeutic agents for this disease.

Recently, a family of small molecule inhibitors with specificity for tyrosine kinases and based on pyrido[2,3-d]pyrimidines has been developed (48, 49). Derivatives of this family show great specificity for individual subgroups of receptor tyrosine kinases including epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor. Other members of the family inhibit non-receptor tyrosine kinases such as the c-src family (50), which has been implicated in the downstream signal transduction pathways of several mitogenic cytokines. Some of these compounds are being investigated as potential therapeutic agents, and others have been useful as tools for dissection of signal transduction pathways. For example, the addition of one of these compounds, the src inhibitor PD173955, causes cell cycle arrest of mammary carcinoma cells in vitro (51).

The work of several laboratories suggests that src may be involved either directly or indirectly in signal transduction by Bcr-Abl (52–56). Thus, we originally set out to determine whether PD173955 might act synergistically with imatinib to inhibit Bcr-Abl-dependent growth. In addition, given our previous observations that the tyrosine phosphorylation targets of Bcr-Abl resemble those detected during c-kit signal transduction, we set out to dissect src family kinase-dependent target phosphorylation in the c-kit signal transduction pathway.

We observed that PD173955 profoundly inhibited Bcr-Abl-dependent cell growth, whereas the Src-specific kinase inhibitor PP1 had essentially no effect. Shortly after we started our studies, it was reported that 500 nM PD180970, a compound closely related to PD173955, strongly inhibited the growth of and induced subsequent apoptosis of the CML cell line K562 (58). That report also provided evidence that the observed growth inhibition of K562 cells by PD180970 was largely attributable to inhibition of Bcr-Abl tyrosine kinase and not to inhibition of Src kinases, as PP1 and the closely related compound PD180970 were shown to have very little effect. The study also showed that PD180970 directly inhibits the kinase activity of Bcr-Abl in the low nanomolar range; however, because the investigators used only one concentration of PD180970 (500 nM) in their cell growth assays, it was not determined whether low nanomolar concentrations of PD180970 could inhibit the growth of K562 cells.

In our report we extend the findings of Dorsey et al. (58) by presenting characterization of the effects of several of the pyrido[2,3-d]pyrimidines including PD173955, PD180970, and PD166326 (50) on Bcr-Abl-dependent cell growth, Bcr-Abl tyrosine kinase activity, the c-kit signaling pathway, and other hematopoietic cytokine pathways.

We observe that pyrido[2,3-d]pyrimidines profoundly inhibit cell lines strictly dependent on Bcr-Abl for growth as well as primary progenitor cells derived from patients with CML. In addition, these PD compounds were found to potently inhibit both autophosphorylation of the c-kit receptor tyrosine kinase and c-KL-dependent cell growth.

MATERIALS AND METHODS

Cells and Cytokines. We previously described a cell line culture system that proved useful for analyzing the contributions of Bcr-Abl and c-kit to the growth of hematopoietic cells and for screening small molecule compounds for Bcr-Abl inhibitory activity (12). Briefly, the megakaryocytic cell line M07e is cytokine dependent for growth and expresses receptors for c-kit, IL-3 and GM-CSF. Bcr-Abl was transfected into this parental line creating M07ep210, which is growth factor independent (Ref. 26; kindly supplied by Dr. Brian Druker; Oregon Health Sciences Center). Subsequent analysis of the resulting transduced cells in our laboratory revealed two distinct populations that could be distinguished by the R10 (glycophorin A) erythroid marker. These two populations were separated by subcloning, and additional studies showed that the erythroid R10(+) cells had acquired extensive karyotypic abnormalities, expressed hemoglobin, and had lost expression of the c-kit receptors (12). In contrast, R10(−) cells retained the karyotype and cytokine receptor expression of the parental M07e cell line. In terms of cell growth, M07e is entirely cytokine dependent, whereas both R10(+) and R10(−) cells are cytokine independent.

Imortalized human hematopoietic Philadelphia chromosome-positive [R10(+), R10(−), K562, RWHu4, and 16BT1] and Philadelphia chromosom-negative (HL60, MANCA, and 16BN1) cell lines were routinely maintained in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc., Grand Island, NY) containing 10% FCS (HyClone, Logan, UT). The M07e megakaryoblastic cell line was maintained as described previously (11, 12). Recombinant IL-3, GM-CSF, and KL that were used for these assays were very kindly provided by Kirin (Gunma, Japan). Six human neuroblastoma cell lines [SK-N-ER, BE(2)/C, LAN1, NMB-7, BE(2)M17, and BE(1)N (59)], seven Ewing’s sarcoma cell lines (SK-N-MC, SK-E51, A673, MMH-E51, RD-E51, Barbato, and TC32), four glioblastoma cell lines (A172, U1183M, U87MG, and T98G), and three sarcoma cell lines (HS-46, HS-16, and H1080) were studied. Adherent solid tumor cell lines were maintained in appropriate culture conditions and passaged by trypsinization.

Enrichment and Culture of Primary CD34+ CML GM Progenitor Cells. We obtained fresh light-density normal bone marrow mononuclear cells, CML bone marrow, or peripheral blood mononuclear cells. These were incubated with a mixture of CD36, CD41, and anti-glycoprotein monoclonal antibodies (Beckman Coulter, Brea, CA) for 30 min on ice, washed twice, and incubated with sheep antimesoglobulin IgG-conjugated magnetic beads (DYNA Beads; Dynal, Oslo, Norway). The antibody-positive cells were removed using a magnetic particle concentrator (Dynal). CD34+ cells were then positively selected using the MACS CD34 isolation kit (Miltenyi Biotec, Inc., Auburn, CA) according to the manufacturer’s instructions. The final cell population was routinely >95% CD34+ with a CD13+CD15+ phenotype, characteristic of GM progenitor cells. Isolated CD34+ cells were cultured in Iscove’s modified Dulbecco’s medium containing 20% FCS (HyClone) and 10 ng/ml recombinant G-CSF and GM-CSF.

Measurement of Cell Growth. Cell growth was determined by two methods. For the [3H]thymidine assay, cells (10^4 cells/well) were cultured in 96-well, round-bottomed plates (Fisher Scientific) with diluted DMSO (control) or with varying concentrations of a specific compound that was resuspended in DMSO for 48 h at 37°C. [3H]Thymidine was added at a concentration of 1 μCi/well, and cells were incubated for an additional 18 h. Cells were harvested with the Unifilter system, scintillation fluid (25 Ci/well), and cells were incubated for an additional 18 h. Cells were harvested with the Unifilter system, scintillation fluid (25 μl/well) was added to each well, and [3H]thymidine incorporation was determined on a Packard Scintillation Counter. Data points for all assays were obtained in triplicate, and background incorporation from cell-free wells was determined and subtracted from all data points. For cell viability, control and drug-treated harvested cells were counted on a hemocytometer using the trypan blue dye exclusion method.
Antibodies, Immunoprecipitation, and Western Blot Analysis. Cell lysates were prepared and immunoprecipitated as described previously in detail (31). Anti-c-kit was obtained from Santa Cruz Biotechnology (product number C-19). SDS-PAGE and Western transfer were performed as described previously (31), and anti-pTyr monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) was used for Western blots. The secondary antibody was horseradish peroxidase-conjugated affinity-purified donkey antirabbit IgG and sheep antimouse IgG (Jackson Immuno-Research, West Grove, PA). All blots were developed using the enhanced chemiluminescence system (Amersham Life Science) and visualized by exposure to X-ray film (Hyperfilm; Amersham Life Science).

Flow Cytometry Cell Cycle Analysis. Cells were harvested, and cell suspensions were washed twice in PBS and then fixed in 80% ice-cold ethanol and stored at −20°C until analysis. Before analysis, cell suspensions were digested by DNAse-free RNase (Boehringer Mannheim) for 20 min at 37°C and then stained in a propidium iodide solution (50 μg/ml) containing 0.1% Triton X-100 overnight in the dark. Cell cycle analysis was performed with a FACScan equipped with a FACSStation running CellQuest software (Becton Dickinson, San Jose, CA). Debris was eliminated from analysis using a forward angle light scatter threshold trigger. Cell doublets and other clumps were removed using analysis gates on either fluorescence pulse width or height versus pulse area (integral). Data were collected for 1–2 × 10^6 single cells/sample. G1–S–G2 values were obtained by standard histogram analysis. Cell cycle analysis of DNA frequency histograms was performed with MultiCycle (Phoenix Flow System, San Diego, CA).

Drugs Studied. PD173955 [6-(2,6-dichlorophenyl)-2-(3-methylsulfonylphenylamino)-8H-pyrido[2,3-d]pyrimidin-7-one], PD180970 [6-(2,6-dichloro-phenyl)-2-(4-flouro-3-methyl-phenylamino)-8H-pyrido[2,3-d]pyrimidin-7-one], PD173952 [6-(2,6-dichlorophenyl)-8-methyl-8H-pyrido[4,3-b]pyrrolo[2,3-c]pyrimidin-7-one], PD173956 [6-(2,6-dichlorophenyl)-2-(4-flourophenylamino)-8H-pyrido[2,3-d]pyrimidin-7-one], PD173958 [6-(2,6-dichlorophenyl)-2-(4-ethoxyphenylamino)-8H-pyrido[2,3-d]pyrimidin-7-one], and PD166326 [6-(2,6-dichlorophenyl)-2-(3-...
Table 1 Summary of the effects on the growth of Bcr-Abl-positive and -negative cell lines of PD173955, PD180970, PP1, and STI-571

<table>
<thead>
<tr>
<th></th>
<th>PD173955 (nM)</th>
<th>PD180970 (nM)</th>
<th>STI-571 (nM)</th>
<th>PP1 (nM)</th>
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</thead>
<tbody>
<tr>
<td>Bcr-Abl (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R10(−)</td>
<td>1–2.5</td>
<td>5–7.5</td>
<td>10–20</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>R10(+)</td>
<td>1–5</td>
<td>4–7.5</td>
<td>50</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>K562</td>
<td>35</td>
<td>N.D.</td>
<td>&gt;500</td>
<td>N.D.</td>
</tr>
<tr>
<td>RWLeu4</td>
<td>10</td>
<td>N.D.</td>
<td>250</td>
<td>N.D.</td>
</tr>
<tr>
<td>Bcr-Abl (−)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td>200</td>
<td>N.D.</td>
<td>&gt;500</td>
<td>N.D.</td>
</tr>
<tr>
<td>SK-N-ER′</td>
<td>375</td>
<td>N.D.</td>
<td>&gt;10,000</td>
<td>N.D.</td>
</tr>
<tr>
<td>SK-N-MC′</td>
<td>1000</td>
<td>N.D.</td>
<td>&gt;10,000</td>
<td>N.D.</td>
</tr>
<tr>
<td>U138MG′</td>
<td>400</td>
<td>N.D.</td>
<td>&gt;10,000</td>
<td>N.D.</td>
</tr>
<tr>
<td>HS-16′</td>
<td>600</td>
<td>N.D.</td>
<td>&gt;10,000</td>
<td>N.D.</td>
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</tbody>
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Data are presented as the mean values for each curve representing at least 20,000 analyzed events. In parallel, cell growth and viability were evaluated by trypan blue dye exclusion and microscopy (inset).

In vitro Bcr-Abl Kinase Assays. We have previously demonstrated that a significant proportion of Bcr-Abl is complexed to SHIP2 (via SH3 domain interactions) in the K562 erythroleukemia cell line (31). Bcr-Abl complexed to SHIP2 was immunoprecipitated from cell lysates of K562 cells maintained in log-phase culture conditions. Complexes were collected on protein A-Sepharose, and complexes were washed three times in lysis buffer and then washed twice in abl kinase buffer [50 mM Tris (pH 8.0), 10 mM MgCl2, 1 mM DTT, 2 mM β-nitrophenylphosphate, and 2 μM ATP; New England Biolabs Buffer and protocol]. Kinase assays were performed with 10 μM [γ-32P]ATP/sample for 15–60 min at 30°C in the presence or absence of the indicated concentrations of drug. The reaction was stopped by the addition of SDS-PAGE sample buffer and heated at 100°C for 10 min. Proteins were separated on 7.5% SDS-polyacrylamide gels, gels were dried under vacuum, and phosphorylation was visualized by autoradiography on X-ray film.

Assessment of Autophosphorylation of c-Kit Receptor. M07e cells were maintained in IL-3/GM-CSF for 24 h before the experiment. Cells were washed and cytokine-starved for 90 min before the experiment. During this period, they were incubated with solvent vehicle, imatinib, or PD173955 at the various concentrations indicated. Cells were then stimulated with KL (100 ng/ml) for 5 min at 37°C. Cells were washed and lysed, and c-kit was isolated using rabbit antiserum to c-kit (Santa Cruz Biotechnology). Lysates were resolved by SDS-PAGE and transferred for immunoblotting. Blots were probed for phosphotyrosine expression and c-kit receptor expression.

Computational Modeling of the Structure of PD173955 and PD166326 Complexed to the abl Kinase Domain. The kinase domain of abl (AbiK) was crystallized in complex with PD173955 (60). The Protein Data Bank coordinate file for the AbiK:PD173955 cocrystal structure was subjected to molecular modeling and graphics renderings using the SYBYL 6.7.2 (Tripos Associates Inc., St. Louis, MO) software package on a Silicon Graphics Octane2 R12000 work station. The atom types for the inhibitor were specified, hydrogen atoms were added to the protein, and the C and N end groups were fixed using the SYBYL/BIOPOLYMER module. Protein and inhibitor atomic charges were calculated using MMFF94 force field. The energy of the complex for force field was minimized using the SYBYL gradient convergence method with a MMFF94s force field and 0.05 kcal/mol Å root mean square gradient as the convergence criterion. All heavy atoms (inherent to the crystal structure) were constrained in an aggregate during minimization. To create the AbiK:PD166326 model, the inhibitor in the AbiK:PD173955 cocrystal structure was replaced with PD166326. PD166326 was aligned to PD173955, and all of the hydroxymethylphenylamino)-8-methyl-8/H/-pyrido[2,3-d]/pyrimidin-7-one were synthesized as described previously (34) in the Core Synthesis Laboratory at Memorial Sloan-Kettering Cancer Center (Figs. 1 and 8). Crystalline compounds were synthesized as described previously (48), and imatinib (STI-571) was synthesized by liquid scintillation. Data represent the means of triplicate wells.

Fig. 3. PD173955 induces strong accumulation in G1 phase of the cell cycle. Log-phase R10(−) cells were treated with either DMSO (solvent vector) or PD173955 at 5, 10, or 25 nM. Cells were harvested at 6, 24, 48, and 72 h; fixed in ethanol; stained with propidium iodide, treated with RNase; and analyzed by FACS. Graphed data of the FACS profiles are presented as the mean values for each curve representing at least 20,000 analyzed events. In parallel, cell growth and viability were evaluated by trypan blue dye exclusion and microscopy (inset).

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atoms of the terminal phenylamino group were included in the minimization as described for AblK:PD173955. Several conformers of the terminal phenylamino group were minimized, but only one positioned the hydroxymethyl group such that an additional hydrogen bond was formed with the protein (Thr-319); this conformer was virtually identical to that of the 3-methylthiophenyl group in PD173955.

RESULTS

PD173955 Inhibits Bcr-Abl-dependent Cell Proliferation in the Low Nanomolar Range. In initial studies, dose-response assays of PD173955 were performed on the Bcr-Abl-dependent cell lines R10(+) and R10(–). The Bcr-Abl tyrosine kinase inhibitor imatinib (STI-571) was used as a positive control for inhibition in each experiment. For cell viability studies, cells were maintained in drug for 96 h and evaluated for viability at 24-h intervals. For [3H]thymidine uptake inhibition experiments, cells were preincubated with drug for 48 h and then maintained in both drug and [3H]thymidine for an additional 18 h. As observed in Fig. 2, A and B, this analysis revealed that for [3H]thymidine incorporation (Fig. 2A) and cell viability (Fig. 2B) the Bcr-Abl-dependent cell line R10(–) had IC50s for PD173955 of approximately 2.5 and 1 nM, respectively, suggesting a high degree of inhibition of Bcr-Abl-dependent cell growth. The IC50s for imatinib (STI-571) were ~35 nM for both thymidine incorporation (Fig. 2A) and cell viability (Fig. 2B). For the Bcr-Abl-containing erythroid cell line R10(+), the IC50s for PD173955 and imatinib (STI-571) as judged by [3H]thymidine incorporation were 2 and 50 nM, respectively (data not shown). Over the course of multiple experiments, the difference in inhibition of PD173955 compared with imatinib ranged from 10- to 25-fold with a mean value of 20-fold, demonstrating that PD173955 is substantially more inhibitory than imatinib (STI-571) toward Bcr-Abl-dependent cell growth.

A recent report suggested that 500 nM PD180970 induced apoptosis in the Bcr-Abl-expressing cell line K562 (58). However, it was unclear from that study whether low nanomolar concentrations of the PD180970 are growth inhibitory as we have observed with PD173955 or whether our observations were unique for PD173955 as described in Fig. 2. Thus, the effects of PD173955, PD180970, imatinib (STI-571), and the src kinase inhibitor PP1 were evaluated on Bcr-Abl-dependent cell growth (Table 1). Because both PD173955 and PD180970 inhibit src kinase activity with high specificity (50, 58), PP1 was used to distinguish src-dependent cell growth. The results in Table 1 clearly show that PD173955 and PD180970 are relatively similar inhibitors of Bcr-Abl-dependent cell growth and that this inhibition is independent of the activity of c-src because PP1 has no effect on cell growth in this system.

We tested two other Bcr-Abl-positive cell lines as well as other leukemic lines that were Bcr-Abl negative. Dose titration assays of PD173955 performed on the Bcr-Abl-positive erythroleukemia cell line K562 and the Bcr-Abl-positive myelomonocytic cell line RW4 revealed IC50s of 35 and 10 nM, respectively (Table 1). By contrast, imatinib (STI-571) had IC50s of >500 nM for K562 and 250 nM for RW4. We also tested the Bcr-Abl-negative myeloid cell line HL-60. The IC50 for HL-60 was 200 nM (PD173955) and >500 nM (imatinib, STI-571).

Previous work by others using pyrido[2,3-d]pyrimidine compounds investigated their effects on the growth of breast, colon, ovarian, lung, and prostate cancer cell lines (50, 51). The relative IC50s in these studies ranged from 200 nM to 1 μM. We also set out to screen several other human tumor cell lines from different origins to determine
whether any other class of tumor exhibits sensitivity to these compounds. As judged by cell viability, we determined the average inhibitory activity of PD173955 for sarcomas \( N = 3; IC_{50} \approx 500 \text{ nM} \), glioblastomas \( N = 4; IC_{50} \approx 450 \text{ nM} \), neuroblastomas \( N = 6; IC_{50} = \approx 400 \text{ nM} \) and Ewing’s sarcomas \( N = 7; IC_{50} \approx 1 \mu M \); Table 1).

Thus, when compared with Bcr-Abl-negative cell lines, it is clear that all Bcr-Abl-containing cell lines are 100- to 200-fold more sensitive to PD173955.

**PD173955 Induces Accumulation in the G1 Phase of the Cell Cycle in the Low Nanomolar Range.** The PD173955 compound clearly inhibits Bcr-Abl-dependent cell growth *in vitro*. Dorsey *et al.* (58) reported that the PD180970 analogue induces apoptosis of K562 cells using 500 nM drug concentrations. We have observed similar results with both drugs at concentrations higher than 250 nM (data not presented). However, because we observed IC_{50}s in a much lower dose range, the question arose as to whether the inhibitory effects of this compound were on cell cycle progression or were merely the result of increased apoptosis by a distinct mechanism in the pool of cycling cells. Moasser *et al.* (51) reported that PD173955 used at concentrations of \( \geq 1 \mu M \) induces a src-related G2-M arrest in epithelial tumors. Thus, a dose-response titration was performed to determine the effects of 5, 10, and 25 nM PD173955 (approximately the IC_{50}, IC_{90}, and IC_{99}) on the cell cycle progression of R10(−) cells using FACS analysis and propidium iodide staining. As shown in Fig. 3, there is little change in cell cycle within 6 h after the addition of drug as compared with control. However, within 24 h of addition of drug, there is a dose-dependent increase in cells in G1 phase and a concomitant decrease in cells in S-phase and G2-M as compared with untreated control cells. The peak G1 accumulation occurs between 24 and 48 h.

The accumulation of cells in G1 phase is consistent with the observation that oncogenic Abl influences the G1 progression and G1-S-phase transition of the cell cycle (61). A low percentage of cells in the sub-G1 phase of the cell cycle was observed in these experiments, suggesting that apoptosis cannot solely account for the overall growth inhibition seen in tissue culture (data not presented). In addition, there is a decrease in G2-M cells in these cultures, which suggests that whereas PD173955-dependent inhibition of G2-M transition occurs at higher doses (1 \( \mu M \); Ref. 51), at low doses, Bcr-Abl inhibition only influences G1-phase transition, further confirming the specificity of activity on Bcr-Abl.

**PD173955 Directly Inhibits Bcr-Abl Kinase Activity *in Vivo* and *in Vitro*.** We have previously characterized the major cellular substrates for Bcr-Abl tyrosine kinase including SHIP1, SHIP2, c-CBL, and p62dok (31). These four phosphotyrosine proteins can be observed in CD34+ primary primitive progenitor cells as well as in Bcr-Abl-expressing cell lines. We performed anti-pTyr immunoblot analysis on extracts of these cell lines cultured normally (control) or in the presence of various concentrations of PD173955 or imatinib (STI-571) for 6 h. The tyrosine phosphorylation of all major Bcr-Abl substrate proteins and autophosphorylation of Bcr-Abl were progressively diminished in extracts from K562 cells (Fig. 4A) in cells treated with 10 nM (*Lane 3*) and 100 nM (*Lane 4*) PD173955 as compared with untreated controls (*Lane 1*). Similarly, Bcr-Abl kinase activity was greatly diminished in extracts of R10(−) cells (Fig. 4B) treated with PD173955 (100 nM; *Lane 8*) as compared with imatinib (STI-571; 100 nM)-treated cells (*Lane 7*) or untreated controls (*Lane 1*). In other studies, we have observed that this decrease in phosphorylation is not due to decreases in protein expression for each substrate.\(^5\) These

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\(^5\) A. Strife, D. Wisniewski, C. Liu, C. L. Lambek, and B. Clarkson. Direct evidence that Bcr-Abl tyrosine kinase activity disrupts normal synergistic interactions between kit ligand and cytokanes in primary primitive progenitor cells, submitted for publication.
results indicate that PD173955 directly inhibited the Bcr-Abl kinase in vivo.

We previously reported (31) that Bcr-Abl is associated with the SHIP2 inositol phosphatase, most likely through an SH3-dependent interaction. We performed an in vitro kinase analysis of immunofinity-purified Bcr-Abl to determine the relative IC₅₀ of PD173955 as compared with STI-571. This analysis showed (Fig. 5A) that the IC₅₀ of PD173955 is approximately 1–2 nM, which is in very close agreement with previous observations (58) for PD180970. The IC₅₀ of imatinib (STI-571) is higher by a factor of 25–50. Indeed, we did a direct comparison of PD173955 and PD180970 and found their IC₅₀ to be nearly identical (Fig. 5B). Similar IC₅₀ results were observed for substrate phosphorylation of SHIP2 in these experiments. Thus, the pyrido[2,3-d]pyrimidines are the most potent inhibitors of Bcr-Abl tyrosine kinase activity yet reported.

**PD173955 Inhibits the c-Kit Receptor and KL-dependent Cell Proliferation.** The recent observation that imatinib (STI-571) is a direct inhibitor of the c-kit tyrosine kinase receptor (35, 36) with an IC₅₀ of ~100 nM suggests that there is structural similarity between the abl and kit tyrosine kinase domains. To determine whether PD173955 also inhibited c-kit or other cytokine pathways, dose-response analysis was performed on M07e cells growing in the presence of KL, GM-CSF, or IL-3. For M07e grown in KL (Fig. 6A), PD173955 had an IC₅₀ of approximately 40–50 nM, suggesting that PD173955 inhibited some aspect of the KL-induced growth pathway. The IC₅₀ of imatinib (STI-571) falls between 50 and 250 nM, which is consistent with previous reports (35, 36). In contrast, when M07e was maintained in either IL-3 (Fig. 6B) or GM-CSF (Fig. 6C), the observed IC₅₀ values were 250–500 nM or 1 μM, respectively, for PD173955 and ≥5 μM for imatinib (STI-571). PD180970 had very similar observed IC₅₀ values when compared with PD173955 in inhibition of cell growth by IL-3, GM-CSF, or KL (data not presented), suggesting that these compounds have the same range of activity.

Table 2 summarizes our evaluation of the effect of these compounds on cytokine-dependent cell growth. These results, taken together, suggest that PD173955 had a higher degree of specific inhibition toward KL-dependent cell growth as compared with other cytokines.

We then determined whether the inhibition of KL-induced cell growth by PD173955 was due to direct inhibition of the c-kit receptor or to inhibition of a kinase(s) downstream of activation of the kit receptor. As judged by anti-pTyr analysis of autophosphorylation of the c-kit receptor protein (Fig. 7), the apparent IC₅₀ of PD173955 for c-kit autophosphorylation in vitro is 25–50 nM. This result is in direct agreement with in vitro cell growth data (Fig. 6C) and indicates that PD173955 is a direct inhibitor of the c-kit tyrosine kinase receptor.

An analysis of our results suggests that the selectivity of PD173955-dependent inhibition of Bcr-Abl versus c-kit is a factor of approximately 25–50 (1–2 nM for Bcr-Abl versus 25–50 nM for c-kit). In contrast, we observed the selectivity for imatinib (STI-571) to be 2–5 fold, whereas it was recently suggested that the therapeutic window for imatinib (STI-571) for these two kinases approached identity (36). In other studies, we have observed that primary primitive progenitor cells obtained from normal bone marrow cultured with KL and colony-stimulating factors grow equally well in the presence or absence of 10 nM PD173955. In contrast, primary primitive progenitor cells derived from patients with CML show marked inhibition of cell growth when cultured in the presence of 10 nM PD173955 and 2 nM PD166326 (see below).³

**Analysis of Several Pyrido[2,3-d]pyrimidine Analogues Reveals PD166326 to be the Most Potent Inhibitor of Bcr-Abl-dependent Cell Growth.** We have previously reported the structure of the abl kinase domain in complex with an analogue of STI-571 (62). In collaboration with other investigators, we studied the structure of the abl kinase domain in complex with PD173955 (60). One result of this analysis revealed that the methylthioether side chain of PD173955 appeared to play no role in the direct interaction of the compound with the protein. In the original report (50), several pyrido[2,3-d]pyrimidine src family inhibitors were described, and these varied at this side chain. We recently synthesized several of the reported pyrido[2,3-d]pyrimidine analogues (PD173953, PD166326, PD173956, and PD173958; Fig. 8) and evaluated their effect on Bcr-Abl-driven cell growth of R10(−) and the growth of the human lymphoma line MANCA and the EBV-transformed lymphoblastoid cell line 16BN1 (Table 3). Whereas most of these analogues were found to be no more potent than PD173955, PD166326 was observed to be the most potent inhibitor of Bcr-Abl-dependent cell growth.
inhibitory to Bcr-Abl-dependent cell growth than was PD173955, one compound, PD166326, was observed to be about 4-fold more inhibitory toward Bcr-Abl cell growth with an IC_{50} of 400 pM for R10(−) cells (Fig. 9). As further support of this finding, we performed the Bcr-Abl kinase inhibition assay and found that PD166326 had an IC_{50} of 100–200 pM, which is in agreement with the cell growth findings (Fig. 10).

Because we had assayed most of these compounds on Bcr-Abl–dependent cell lines, we sought to test PD173955 and PD166326 on primary CML leukemic cells. CML CD34+ primitive progenitor cells were isolated from the peripheral blood of a patient with CML in chronic phase and cultured in the presence of G-CSF and GM-CSF in the absence or presence of PD166326 or PD173955 in the concentration range of 0.1–10 nM (Fig. 11). We had previously observed that this concentration range has no effect on the growth of normal CD34+ primitive progenitors. The results of these experiments demonstrated that PD166326 had an IC_{50} of 2 nM and that PD173955 had an IC_{50} of 7.5 nM, suggesting that both compounds inhibit Bcr-Abl–positive CML leukemic cells and further confirming that PD166326 is approximately 4-fold more active than PD173955 at inhibiting Bcr-Abl–dependent cell growth.

PD166326 was also more active in inhibiting blast cells from a patient with CML in blastic phase (Fig. 12). The greater inhibition observed for PD166326 may be due several factors, including relative solubility, greater half-life within the cell, or additional interactions with the abl kinase domain.

In an accompanying article (60), we present the full crystal structure of the PD173955/Bcr-Abl kinase complex. In a detail from that study, it is observed that PD173955 forms hydrogen bonds with the peptide backbone of the abl kinase domain at Met-318 (Fig. 13A). We have modeled the structure of PD166326 with the existing model of the PD173955/abl kinase domain complex and observed that the hydroxymethyl side chain may form an additional hydrogen bond with the peptide backbone around Thr-319 of the abl kinase domain (Fig. 13B). The additional hydrogen bond might increase the overall stability of the inhibitor kinase interaction and thus partially explain the increased potency of the PD166326 compound. Crystallization studies are planned to determine whether this interaction is observed.

### DISCUSSION

One of the major goals of recent cancer research is to identify rationally derived, disease-specific therapeutic agents. Tyrosine kinases have been strongly linked to multiple aspects of carcinogenesis, and several cancers including CML have clearly defined, activated tyrosine kinases. Many studies have been undertaken using combinatorial chemistry and tyrosine kinase domain targets to define classes of compounds that show both kinase inhibition and specificity to families of kinases. The first report of a Bcr-Abl kinase inhibitor (34) from the 2-phenylaminopyrimidine class of compounds, which also inhibits the c-kit and platelet-derived growth factor receptors, raised the possibility that a therapeutic agent could be targeted to CML. The recent successful clinical trials with this Bcr-Abl inhibitor, imatinib (STI-571), in the treatment of CML and c-kit-expressing GISTs (38–40) validate targeted therapies and open the door for extensive application of this approach to cancer research. However, as with much of cancer therapy, caution must be applied when evaluating the success of a particular approach or drug. There were

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**Table 3 Summary of the IC_{50}s of pyrido[2,3-d]pyrimidine analogues on Bcr-Abl-dependent and -independent cell growth**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PD173955 (nM)</th>
<th>PD166326 (nM)</th>
<th>PD173952 (nM)</th>
<th>PD173956 (nM)</th>
<th>PD173958 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R10</td>
<td>1–2</td>
<td>0.4</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>MANCA</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>250</td>
<td>1000</td>
</tr>
<tr>
<td>EBV LCL</td>
<td>250</td>
<td>−175</td>
<td>250</td>
<td>250</td>
<td>1000</td>
</tr>
</tbody>
</table>

* Assays were performed as described in Table 1.

* Bcr-Abl-negative cell line.

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**Fig. 9.** Comparison of the effects of STI-571 (imatinib), PD173955, and PD166326 on growth inhibition of the R10(−) Bcr-Abl–positive cell line. R10(−) cells were treated with solvent vector (DMSO; control), STI-571, PD173955, or PD166326 at the concentrations indicated. Cell viability was determined as described in “Materials and Methods.” Viable and total cell numbers were determined by trypan blue dye exclusion at 24, 48, 72, and 96 h. Each time point was performed in triplicate, and data are presented as the arithmetic means. Similar results were obtained in multiple experiments.

**Fig. 10.** Comparison of the relative inhibition of p210^{Bcr-Abl} autophosphorylation by PD173955 and PD166326. Bcr-Abl tyrosine kinase assays were performed as described in Fig. 5. Top row, autophosphorylation of Bcr-Abl in the presence of various concentrations of PD173955 and PD166326. Bottom row, substrate phosphorylation of SHIP2. A 70-kDa non-specific phosphorylated substrate was detected after autoradiography in each immunoprecipitate and functioned as a loading control for each lane (data not shown).
several reported mechanisms of drug resistance to imatinib in vitro, including protein overexpression, gene amplification, multidrug resistance, and binding to serum proteins (41–46). In recent studies the leukemic cells of patients with CML who have entered late-stage blast crisis during the course of imatinib (STI-571) therapy were found to contain either point mutations in the abl kinase domain or Bcr-Abl gene amplifications, both of which confer drug resistance (41). These results argue for the exploration of multimodality therapy using existing chemotherapeutic approaches and for the development of additional agents specifically targeted to Bcr-Abl and CML.

During our studies on the role of src family kinase in both the c-kit signal transduction pathway and the growth of Bcr-Abl-dependent cell lines, it was observed that the pyrido[2,3-d]pyrimidine class src inhibitor PD173955 potently inhibited both Bcr-Abl-dependent and KL-dependent cell growth. After the initiation of our studies, Dorsey et al. (58) reported that another member of this class of compounds (PD180970) was a potent inhibitor of Bcr-Abl kinase. It was demonstrated that PD180970 inhibited Bcr-Abl kinase activity (IC₅₀ = 5 nM) and that high drug concentrations (500 nM) induced apoptosis in Bcr-Abl-expressing erythroleukemia K562 cells. However, it was not determined from those studies what the activity of PD180970 was relative to imatinib (STI-571) or whether the dose range investigated for PD180970 had effects on other cell pathways leading to general toxicity.

Based on our initial findings, we set out to directly compare the relative activities of these two inhibitors with imatinib (STI-571) and to extend the observations in that report by investigating the effects of the PD compounds on cytokine-dependent cell growth. Both compounds target the Bcr-Abl kinase with very high potency (in vitro IC₅₀ = 1–2 nM) and are clearly more potent at inhibiting Bcr-Abl-dependent cell growth than imatinib (STI-571) by a factor of 10-
25-fold. In previous studies, it was observed that oncogenic forms of Abl stimulate passage through G1 into S phase but are not required for the remainder of cell cycle progression (61). Consistent with these observations, we observed that inhibition of Bcr-Abl-dependent cell lines with PD173955 results in some accumulation in the G1 phase of the cell cycle. More importantly, it was found that the parent cell line, M07e, grows normally in this dose range (i.e., 1–2 nM), confirming that the growth inhibition was most likely due directly to Bcr-Abl inhibition. This latter observation indicates that the compound is freely diffusible and accessible to its target.

Our results are relevant from a number of perspectives. Based on this work and previously published data, it appears that several of the pyrido[2,3-d]pyrimidine compounds of this class target both src and Bcr-Abl in the low nanomolar range (50, 58). We have recently presented the crystal structure of the abl kinase domain complexed to an analogue of imatinib (STI-571) (62). That work depicted the first detailed structure of the abl kinase domain and revealed its strong similarity to the src family tyrosine kinase domains. The structural homology between the proteins was fortunate because several elegant biochemical and crystallographic studies of the c-src molecule have created the paradigm for the mechanics of tyrosine kinase function. It has been observed that src requires dephosphorylation of the COOH-terminal tyrosine and subsequent phosphorylation of the activation loop tyrosine to acquire kinase activity. This phosphorylation repositions the activation loop from a closed (inactive) conformation to an open (active) conformation. By analogy and based on these extensive studies of src, it was clear that the imatinib (STI-571) analogue binds to the

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Fig. 13. Modeling reveals that PD166326 may form an additional hydrogen bond with threonine 319 of the abl kinase domain. The crystal structure of the abl kinase domain complexed to PD173955 was determined (60). A model of the complex is presented in A. PD173955 forms a hydrogen bond with methionine 318 of abl kinase. When PD166326 was modeled into the abl structure defined by PD173855, it was observed that the hydroxymethyl side chain formed an additional hydrogen bond with threonine 319 (B).
abl kinase domain when the activation loop is in the inactive conformation (62).

In a companion article in this issue (60), crystallographic studies are presented for imatinib (STI-571) and for PD173955 complexed to the abl kinase domain. Similar to its analogue, compound 15 (62), imatinib (STI-571) binds to the abl kinase domain with the activation loop in the closed conformation. In further work, it was observed that based on the crystal structure, PD173955 binds more favorably to the abl kinase domain with the activation loop in the open conformation. Furthermore, modeling studies suggest that PD173955 can also potentially bind to the abl kinase domain with the activation loop in the closed conformation. Overall, the data suggest that the activation loop of the abl kinase exists in dynamic equilibrium between the open and closed conformations. Nagar et al. (60) thus suggest that the relative greater inhibition that is observed for PD173955 versus STI-571 against Bcr-Abl in vivo may be the result of PD173955 targeting both conformations of the abl kinase domain.

Given what is presently known about abl and Bcr-Abl, other phenomena may play a role in the biological results. Such cis factors as alternate protein folding caused by the fusion protein and Bcr-dependent tetramerization and trans factors such as increased phosphorylation of the Bcr-Abl molecule and decreased phosphatase activity within the leukemic cell may all contribute to the increased potency of the PD compounds against Bcr-Abl. The recent studies of Brasher and Van Etten (63) support the notion that c-abl has multiple states of tyrosine kinase activation with intrinsic tyrosine kinase activity in the unphosphorylated state and tyrosine phosphorylation-dependent relative increases in kinase activity. The sum of their work suggests that in contrast to src, which has an apparent on/off “toggle switch” mechanism of kinase activation, c-abl has a basal activation state and that the relative kinase activity is modulated through phosphorylation and trans-mediated protein interactions.

We have presented evidence that PD173955 inhibits KL-dependent cell growth through direct inhibition of the c-kit receptor. This inhibition is more potent than imatinib [STI-571 (35, 36)] and certainly more potent than previously reported inhibitors of the c-kit receptor. This in vitro inhibition is more potent than imatinib (STI-571) and certainly more potent than previously reported inhibitors of the c-kit receptor. This in vitro inhibition is more potent than imatinib (STI-571) and certainly more potent than previously reported inhibitors of the c-kit receptor.

REFERENCES


Characterization of Potent Inhibitors of the Bcr-Abl and the c-Kit Receptor Tyrosine Kinases

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