A Novel Pyridopyrimidine Inhibitor of Abl Kinase Is a Picomolar Inhibitor of Bcr-abl-driven K562 Cells and Is Effective against STI571-resistant Bcr-abl Mutants

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Abstract

Inhibition of the constitutively active Bcr-abl tyrosine kinase (TK) by STI571 has proven to be a highly effective treatment for chronic myelogenous leukemia (CML). However, STI571 is only transiently effective in blast crisis, and drug resistance emerges by amplification of or development of mutational changes in Bcr-abl. We have screened a family of TK inhibitors of the pyrido[2,3-d]pyrimidine class, unrelated to STI571, and describe here a compound with substantial activity against STI-resistant mutant Bcr-abl proteins. This compound, PD166326, is a dual specificity TK inhibitor and inhibits src and abl in vitro with IC_{50}s of 6 and 8 nM, respectively. PD166326 inhibits the growth of K562 cells with IC_{50} of 300 pM, leading to apoptotic G1 arrest, whereas non-Bcr-abl cell types require 1000 times higher concentrations. We tested the effects of PD166326 on two of the clinically observed STI571-resistant Bcr-abl mutants. PD166326 potently inhibits the E255K mutant Bcr-abl protein and the growth of Bcr-abl/E255K-driven cells. The T315I mutant Bcr-abl protein, which is mutated within the ATP-binding pocket, is resistant to PD166326; however, the growth of Bcr-abl/T315I-driven cells is partially sensitive to this compound, likely through the inhibition of Bcr-abl effector pathways. These findings show that TK drug resistance is a structure-specific phenomenon and can be overcome by TK inhibitors of other structural classes, suggesting new approaches for future anticancer drug development. PD166326 is a prototype of a new generation of anti-Bcr-abl compounds with picomolar potency and substantial activity against STI571-resistant mutants.

Introduction

CML is a myeloproliferative disorder of hematopoietic stem cells. The pathologic hallmark of this disease is the Philadelphia chromosome, which is seen in >90% of CML patients. The Philadelphia chromosome is the result of a t(9;22) chromosomal translocation that results in the juxtaposition of 3' sequences of the Ab1 TK proto-oncogene located on chromosome 9 with 5' sequences of the Bcr gene on chromosome 22. The resulting Bcr-abl fusion gene encodes a novel cytoplasmic protein with constitutive TK activity (1). Expression of this fusion gene is seen in 90% of patients with CML, and experimental evidence confirms that Bcr-abl expression is sufficient to induce CML-like disease in mice (2). The oncogenic potency of the Bcr-abl oncoprotein is quantitatively related to its TK activity (3).

Efforts to develop novel treatments for CML have focused on targeting the TK activity of Bcr-abl and have led to the development of STI571, a 2-phenylaminopyrimidine compound that is potent, and selective inhibitor of Abl, c-kit, and platelet-derived growth factor-receptor TKs (4). This compound selectively inhibits the growth of Bcr-abl-positive but not -negative cell lines (5, 6). STI571 has potent antileukemic activity in clinical studies producing complete hematological responses in 98% of patients in chronic phase CML, including complete cytogenetic responses in >50% (7, 8). Clinical response is associated with inhibition of Bcr-abl TK activity in vivo (7). STI571 also produces hematological remissions in 50–70% of patients with blast crisis, although these responses are frequently not durable, and patients relapse within 2–6 months of therapy (9). Relapse during STI571 therapy is associated with drug-resistant Bcr-abl TK activation. This is associated with amplification of the Bcr-abl gene, mutations within the ATP-binding pocket, and other regions of Bcr-abl (10, 11). Effective treatment of patients in initial blast crisis or relapse after STI571 therapy awaits novel strategies to more effectively inhibit Bcr-abl signaling.

Although the etiologic role of Bcr-abl in the pathogenesis of CML is now well established, the signaling pathways by which it transforms myeloid cells is much less understood.
Numerous substrates are tyrosine phosphorylated by Bcr-abl. These include adapter molecules (Crk, Shc, and p62\textsuperscript{DOK}), proteins associated with cytoskeletal functions (Paxillin, Fak, and Talin), proteins with catalytic functions (phosphatidylinositol 3’ kinase, peritoneal lymphocyte-\(\gamma\), Ras-GTPase-activating protein, and Syk), and other proteins, including Bap-1, Cbl, and Vav (reviewed in Ref. 12). Bcr-abl also has autophosphorylation activity. In fact, Bcr-abl may be signaling through several pathways. Autophosphorylation provides docking sites for adapter proteins, including Grb2 and Shc, which can lead to proliferation through activation of the Ras/MAPK pathway; recruitment of CrkI, which can lead to altered cell adhesion; recruitment of phosphatidylinositol 3’ kinase, leading to activation of the Akt pathway and antiapoptotic signaling; and phosphorylation of Stat1 and Stat5 with activation of the Stat pathway (reviewed in Ref. 12). Bcr-abl also activates other nonreceptor TKs, including the src family kinases Hck and Lyn. Bcr-abl directly associates with Hck and Lyn and results in their increased activities (13).

Novel therapies for CML need to address the emerging problem of clinical resistance to STI571. Because tumor progression in patients receiving STI571 seems to be mediated by amplification of or mutations in the Bcr-abl gene that cause the TK to be less efficiently inhibited by the drug, newer TK inhibitors may be susceptible to the same mechanisms of resistance. We report here that STI571 resistance can be overcome by a novel TK inhibitor of a different class. We have been studying a family of structurally unrelated TK inhibitors selective for src kinases. A member of this family of TK inhibitors was recently reported to have substantial activity against Bcr-abl and Bcr-abl-driven cells (14). In this study, we have screened this family of TK inhibitors, designated previously as src-selective inhibitors, for activity against Bcr-abl, and found varying degrees of anti-abl activities, and identified a compound with the most potent anti-Bcr-abl activity to date. This compound shows picomolar antileukemic activity specifically in Bcr-abl-driven cell lines, with activity against some STI571-resistant Bcr-abl mutants, and provides a prototype for the next generation of CML therapies.

Materials and Methods

Cell Culture and Growth Assays. Cells were cultured in RPMI medium supplemented with 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 4 mM glutamine, and 10% heat-inactivated fetal bovine serum and incubated at 37°C in 5% CO\(_2\). For growth assays, cells were seeded in 12-well clusters at 10,000–20,000 cells/well. Cells were placed in media containing various concentrations of the drugs with vehicle (DMSO) never contributing >0.1%. After 4–7 days, cells were counted using a Coulter counter. All experiments were performed in duplicate, and results were averaged. PD166326 was stored in a 10 mg/ml DMSO solution and stored at \(-70°C\). The derivation and chemical structure of PD166326 has been published previously (15).

Cell Cycle Assays. Cells were treated with indicated concentrations of PD166326 or vehicle (DMSO) for the indicated times. For synchronization, cells were incubated in media containing 5 \(\mu\)g/ml aphidicolin for 24 h, washed twice in PBS, and replaced in growth media. At the time of harvest, cells were washed once in PBS, cell nuclei were prepared by the method of Nusse (16), and cell cycle distribution was determined by flow cytometric analysis of DNA content using red fluorescence of 488 nm excited ethidium bromide-stained nuclei.

Protein Extraction and Western Blotting. Cells were washed in PBS once and lysed in modified RIPA buffer (10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% NP40, 1% Na deoxycholate, 1 mM Na Vanadate, and protease inhibitors). Total cellular protein (50 \(\mu\)g) was separated by SDS-PAGE, transferred to membrane, and immuno-blotted using antibodies to phosphotyrosine (Santa Cruz Biotechnology), c-abl (E89), phospho-Hck (Santa Cruz Biotechnology), MAPK (Santa Cruz Biotechnology), and phospho-MAPK (Promega).

In Vitro Kinase Assay. C-abl kinase assays were performed using purified recombinant c-abl and peptide substrate (New England Biolabs). Kinase assays were performed in 50 \(\mu\)l Tris-Cl (pH 7.5), 10 mM MgCl\(_2\), 1 mM EGTA, 2 mM DTT, 0.2% Triton-X, 100 \(\mu\)M ATP, and 40 \(\mu\)M peptide substrate, in 100-\(\mu\)l reaction volumes containing 50 units of c-abl enzyme and 10 \(\mu\)Ci [\(^{32}\)P]γ-ATP. Reactions were allowed to proceed for 10 min at 30°C and stopped by the addition of EDTA and boiling. Reaction products were spotted on phosphocellulose paper, washed several times with phosphoric acid and then acetone, and counted in scintillation fluid. Pilot experiments were initially performed to establish that these reaction conditions were in linear range.

Results

PD166326 Inhibits c-Abl in Vitro. In screening a compound library for inhibitors of c-src TK activity, a number of pyrido[2,3-d]pyrimidines were described previously that are ATP-competitive inhibitors of c-src with IC\(_{50}\)s < 20 nM and varying degrees of selectivity for c-src (15). We screened this group of compounds for activity against c-abl using purified recombinant c-abl and peptide substrate in in vitro kinase assays. The most potent compound was PD166326 with an IC\(_{50}\) of 8 nM (against c-abl) and 6 nM (against src; Table 1). The src family kinase Lck is inhibited with IC\(_{50}\) < 5 nM. This compound also has activity against basic fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor receptor TKs in vitro with IC\(_{50}\)s of 62, 139, and 80 nM, respectively (15). PD166326 shows no significant activity against c-Jun-NH\(_2\)-terminal kinases, cyclic AMP-dependent PKA, PKB-β, PKC-α, rho-dependent PK, casein kinase-2, and phosphorylase kinase. In comparison with PD166326, STI571 is a weaker inhibitor of Abl in vitro with an IC\(_{50}\) = 50 nM (Table 1).

PD166326 Inhibits Bcr-abl in Vivo. PD166326 also inhibits Bcr-abl activity in cells as determined by Western blot.
reveals that PD166326 inhibits cell proliferation specifically in the G1 phase of the cell cycle. At concentrations that fully inhibit the growth of Bcr-abl-positive cells but not other cell types, PD166326 leads to accumulation of cells in the G1 phase accompanied by a significant increase in the number of apoptotic cells (Fig. 2). Additional phases of the cell cycle are not affected by this compound as shown by experiments with synchronized cells. K562 cells were synchronized at the G1–S boundary with aphidicolin and released into PD166326 or vehicle, and cell cycle progression was studied over the following 24 h. These data show that PD166326 treatment does not interfere with progression through the S, G2, or mitotic phases of the cell cycle, but PD166326-treated cells are unable to exit the G1 phase (Fig. 3). Similar experiments with nocodazole-synchronized cells also confirm that PD166326 blocks G1 progression (data not shown). The inhibition of G1 progression and induction of apoptosis in K562 cells are similar to the effects reported previously for STI571 (17). These data show that PD166326 is a potent inhibitor of Bcr-abl kinase activity and inhibits Bcr-abl-driven cell growth through the inhibition of G1 progression, leading to apoptotic cell death.

**PD166326 Inhibits Cells Driven by STI571-resistant Mutant Bcr-abl.** Resistance to STI571 treatment is associated with mutations in the Bcr-abl oncoprotein that render it refractory to STI571 inhibition (10). Because PD166326 inhibits both Src and Abl, whereas STI571 only inhibits Abl, it may bind Bcr-abl differently than STI571. This difference raises the possibility that it may be effective against some mutant Bcr-abl proteins. We compared the activities of PD166326 and STI571 against two such mutant Bcr-abl proteins derived from patients who have relapsed on STI571 therapy. The T315I mutation is frequently seen in relapsed patients, eliminates a critical Thr residue within the ATP-binding pocket of Abl, and greatly reduces the binding affinity of STI571. The E255K mutation also lies within a region of Bcr-abl commonly mutated in relapsed patients; however, the structural basis for STI571 resistance conferred by mutations in this region is not currently understood. BaF3 mouse hematopoietic cell lines were stably transfected with either the wild-type p210Bcr-abl cDNA or the T315I or E255K mutant versions as described previously (10). The expression of Bcr-abl renders BaF3 cells interleukin-3 independent, whereas control cells transfected with vector alone require interleukin-3 for growth. Although STI571 inhibits the wild-type p210Bcr-abl cells with IC50 = 500 nM, the T315I and E255K mutant p210Bcr-abl cells are highly resistant. However, resistance to STI571 does not appear to confer cross-resistance to PD166326. PD166326 inhibits the autophosphorylation of p210Bcr-abl in vivo as effectively as the autophosphorylation of the wild-type p210Bcr-abl, whereas this mutant is highly resistant to inhibition by STI571 (Fig. 4). However, the p210Bcr-ablT315I mutant is resistant to PD166326 as it is to STI571. This is not surprising, considering the critical role of Thr (315) within the ATP-binding pocket (see “Discussion”).

To determine whether cell growth sensitivity to PD166326 correlates with inhibition of the mutant Bcr-abl oncoproteins, we also determined the sensitivity of the BaF3 cells driven by the wild-type and mutant Bcr-abl proteins. BaF3/p210Bcr-abl cells are very sensitive to PD166326 (IC50 = 6 nM; Table 3). The effective inhibition of p210Bcr-ablT315I activity at dose ranges that inhibit the growth of these cells is additional evidence that STI571-resistant leukemic cells are driven by persistent activity of the mutated Bcr-abl oncoprotein. In comparison, the T315I mutant cells are partially resistant to PD166326, although not fully resistant.

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**Table 2** Cell growth IC50 for PD166326 (all values in nM)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Bcr-abl</th>
<th>PD166326 IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>+</td>
<td>0.3</td>
</tr>
<tr>
<td>BaF3 p210Bcr-abl</td>
<td>+</td>
<td>6.0</td>
</tr>
<tr>
<td>MCF-7 (breast cancer)</td>
<td>–</td>
<td>1000.0</td>
</tr>
<tr>
<td>MDA-MB-468 (breast cancer)</td>
<td>–</td>
<td>2000.0</td>
</tr>
<tr>
<td>32D (immortalized myeloid)</td>
<td>–</td>
<td>800.0</td>
</tr>
<tr>
<td>BaF3 (immortalized myeloid)</td>
<td>–</td>
<td>10000.0</td>
</tr>
</tbody>
</table>

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![Figure 1](image_url)  
Fig. 1 Log phase K562 cells were treated with STI571 or PD166326 for 24 h at the indicated concentrations and harvested in RIPA buffer, and total cellular lysates were separated by SDS-PAGE, transferred to membrane, and immunoblotted using antiphosphotyrosine, anti-abl, anti-MAPK, or antiphosphoMAPK antibodies. The concentrations of PD166326 shown here are considerably lower than shown for STI571, because Bcr-abl autophosphorylation is much more sensitive to PD166326. These concentrations are within dose ranges that produce growth inhibition with these two drugs.

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**Discussion**

The biological activity and potency of PD166326 was initially evaluated in cell growth assays using K562 cells. This compound inhibits K562 cell growth with IC50 ~ 0.3 nM compared with 100 nM for STI571 (Fig. 1). Bcr-abl autophosphorylation correlates with Bcr-abl-signaling activity as shown by the parallel decline of MAPK activity with inhibition of Bcr-abl in these assays (Fig. 1, bottom panel).

**PD166326 Inhibits Bcr-abl-driven Cell Growth.** The biological activity of PD166326 was evaluated in cell growth assays using K562 cells. This compound inhibits K562 cell growth with IC50 = 0.3 nM (Table 2). Another Bcr-abl-driven cell line, BaF3-p210Bcr-abl, is also extremely sensitive to PD166326 with an IC50 of 6 nM. The potent biological activity of PD166326 is highly specific for Bcr-abl-driven cells as additional hematopoietic and epithelial cell lines are only inhibited at two to three log higher concentrations and IC50s in the 0.8–2 μM range (Table 2).

**PD166326 Inhibits G1 Progression.** Further analysis reveals that PD166326 inhibits cell proliferation specifically in the G1 phase of the cell cycle. At concentrations that fully inhibit the growth of Bcr-abl-positive cells but not other cell types, PD166326 leads to accumulation of cells in the G1 phase accompanied by a significant increase in the number of apoptotic cells (Fig. 2). Additional phases of the cell cycle are not affected by this compound as shown by experiments with synchronized cells. K562 cells were synchronized at the G1–S boundary with aphidicolin and released into PD166326 or vehicle, and cell cycle progression was studied over the following 24 h. These data show that PD166326 treatment does not interfere with progression through the S, G2, or mitotic phases of the cell cycle, but PD166326-treated cells are unable to exit the G1 phase (Fig. 3). Similar experiments with nocodazole-synchronized cells also confirm that PD166326 blocks G1 progression (data not shown). The inhibition of G1 progression and induction of apoptosis in K562 cells are similar to the effects reported previously for STI571 (17). These data show that PD166326 is a potent inhibitor of Bcr-abl kinase activity and inhibits Bcr-abl-driven cell growth through the inhibition of G1 progression, leading to apoptotic cell death.
PD166326 inhibits BaF3p210T315I cells with IC50 of 150 nM (Table 3). Although this is 25-fold weaker than the inhibition of the wild-type BaF3p210 cells, it may still be of therapeutic value because it is 8-fold more potent than the inhibition of the BaF3-vector controls and non-Bcr-abl-driven cells (Tables 2 and 3). Although PD166326 inhibits the growth of BaF3p210Bcr-ablT315I cells with IC50 of 150 nM, it fails to inhibit the autophosphorylation of the T351I Bcr-abl mutant at doses ≥1 μM (Fig. 4), suggesting that its antiproliferative effects are mediated in part through mechanisms other than the inhibition of Bcr-abl (see “Discussion”).

**PD166326 Inhibits the Bcr-abl-induced Activation of src Kinases.** PD166326 is also active against src kinases, and its antileukemic effects may be in part related to its inhibition of the src kinases Hck and Lyn, which function downstream of Bcr-abl. The src kinases Hck and Lyn are activated by Bcr-abl and may mediate some of the transforming functions of Bcr-abl. Phosphorylation of tyr (416) in the catalytic domain is required for activation of src kinases, although the mechanism by which Bcr-abl activates Hck and Lyn is not understood (see “Discussion”). Inhibition of Bcr-abl by STI571 results in a parallel inhibition of Hck activation in K562 cells (Figs. 1 and 5). In these cells, PD166326 also inhibits Bcr-abl and Hck activation, although at 100-fold lower doses than seen with STI571 (Figs. 1 and 5). Hck is also activated by mutant forms of Bcr-abl, and in the mutant BaF3p210Bcr-ablE255K cells, PD166326 inhibits Hck activation; this correlates with the observed inhibition of Bcr-ablE255K autophosphorylation (Figs. 4 and 6) and inhibition of cell growth (Table 3). In contrast, the activation of Hck by the Bcr-ablT315I mutant is not inhibited by PD166326 (Fig. 6), and this correlates with the observed resistance of Bcr-ablT315I activity to PD166326 (Fig. 4). However, despite failure to inhibit Bcr-abl activity and the consequent activation of Hck, PD166326 inhibits the growth of BaF3p210Bcr-ablT315I cells with IC50 of 150 nM, likely through additional mechanisms (see “Discussion”). The antiphospho-Hck antibodies used in these experiments specifically recognize the phosphorylated tyrosine residue in the catalytic domain of Hck. This epitope is highly homologous, and in some cases identical, among most members of the src family, and therefore, these immunoblots reflect the activation of any or multiple members of the src family.

**Discussion**

Although STI571 has revolutionized the treatment of CML, the problem of TK resistance is now emerging as a clinical reality. Resistance to STI571 appears to have a structural basis, and newer TK inhibitors may also be susceptible to similar mechanisms of resistance. However, TK inhibitors of a different structural class may have more favorable binding characteristics. Dorsey et al. (14) initially reported that a src-selective TK inhibitor of the pyrido[2,3-d]pyrimidine class has substantial activity against Bcr-abl kinase. We have extended this finding by screening a family of src-selective pyrido[2,3-d]pyrimidines and identified a compound with the most potent activity against abl kinase. Here, we report the characterization of this compound, PD166326, a novel dual specificity TK inhibitor that is >100-fold more potent than STI571 in vitro and inhibits K562 cells with an IC50 of 300 pm. It is unlikely that the potent growth inhibitory activities of PD166326 are related to nonspecific activities because the potency of this compound appears to be specific for cell types driven by Bcr-abl kinase. Although Bcr-abl-driven cells are inhibited with IC50 of the 0.3–6 nM range, other cell types, including the hematopoietic cells BaF3 and 32D, as well as epithelial cancer cells, including MCF-7 and MDA-MB-468 cells, which are driven by epidermal growth factor receptor overactivity, are inhibited with IC50 in the 0.8–2 μM range (Table 2). The micromolar activity of PD166326 against the growth of non-Bcr-abl-driven cells is most likely mediated through inhibition of additional cellular targets because unlike Bcr-abl-positive cells, the growth of Bcr-abl-negative cells is inhibited during the S phase of the cell cycle (data not shown). The picomolar potency and cellular selectivity of PD166326 are significantly superior to STI571 in vitro.

Because Bcr-abl signaling is known to involve the src...
family kinases Hck and Lyn, and because PD166326 is also a potent inhibitor of src family kinases, it is plausible that the biological potency of this compound is related to dual inhibition of these two functionally related TKs. Hck associates with and phosphorylates Bcr-abl on Tyr 177, leading to recruitment of Grb2/Sos and activation of the Ras pathway (18). Kinase-defective Hck mutants suppress Bcr-abl-induced transformation, suggesting that Hck-mediated signaling is essential for the transforming activity of Bcr-abl (19). The role of Lyn in Bcr-abl signaling is less well studied. However, Lyn activity is also elevated in acute myeloid leukemia cell lines, and in these cells, inhibition of Lyn expression using antisense molecules leads to decreased proliferative activity; inhibition of Lyn kinase activity using src family-selective pharmacological inhibitors leads to potent inhibition of cell growth and colony formation (20). It is also possible that the potency of PD166326 is mediated through the inhibition of other, yet undiscovered cellular proteins, and our data do not exclude this possibility. However, the role of currently unknown cellular targets in mediating the growth inhibitory effects of this compound in Bcr-abl-driven cells is difficult to know until such candidate targets are identified and studied.

Because relapse on STI571 is associated with mutations in Bcr-abl that alter the binding of STI571, understanding the nature of the STI571 interaction with Abl is of fundamental importance to overcome drug resistance. The crystal structure of a variant STI571 in complex with the catalytic domain of Abl was recently solved by Schindler et al. (21). STI binds within the ATP-binding pocket of Abl in its inactive conformation. This interaction is critically affected by the conformation of the Abl activation loop. When phosphorylated, this activation loop favors an open and activating conformation, which, by virtue of its NH$_2$-terminal anchor, interferes with STI571 binding to the ATP-binding pocket. Consistent with this model, the binding of STI571 is selective for the inactive conformation of Abl, and
An Inhibitor of STI571-resistant Bcr-abl Mutants

catalytic domain of most members of the src family. This antibody recognizes the phosphorylated tyrosine residue within the catalytic domain of most members of the src family.

Inhibition of overall enzyme activity that we see and active conformations of Abl, leading to the more effective selectivity for the inactive conformation is postulated to confer STI571, it may not bind selectively to the inactive conformation of Abl, because in its active conformation, Abl bears considerable structural homology to the src kinases (21). Although selectivity for the inactive conformation is postulated to confer a high degree of molecular specificity to STI571, this may be at the price of potency. PD166326 may be binding to both inactive and active conformations of Abl, leading to the more effective inhibition of overall enzyme activity that we see in vitro (Table 1). In addition, phosphorylation of the activation loop of Abl is catalyzed by the src family member Hck in Bcr-abl-transformed cells. Because PD166326 also inhibits Hck, this may prevent phosphorylation of the activation loop, destabilizing the Abl active conformation. This allosteric mechanism, in addition to the direct binding of PD166326 to the ATP-binding pocket, could provide dual mechanisms for its inhibition of Abl activation and provide the basis for its increased potency. Validation of these hypotheses awaits crystallographic studies of PD166326 bound to Abl.

PD166326 is noncross-resistant with STI571 and has substantial activity against the T315I and E255K STI571-resistant Bcr-abl mutants. This finding has important implications for the future design and use of TK inhibitors of all kinds, because it is the first report showing that TK inhibitor resistance can be overcome by another TK inhibitor of a different structural class. It is difficult to speculate on whether the development of resistance to PD166326 will be just as likely as with STI571, but because these compounds are structurally unrelated, resistance to PD166326 will likely involve a different structural basis than resistance to STI571. This distinction creates the opportunity for strategies to prevent or overcome resistance, such as sequential or combination therapies. However, understanding drug sensitivity and resistance is of fundamental importance in this regard. Although more studies are necessary to understand the structural and cellular basis underlying STI571 resistance and PD166326 sensitivity, existing data at least partly explain our findings. A number of amino acid residues mediate the binding of STI571 within the ATP-binding pocket, and among these, Thr (315) is critical for hydrogen bond formation with the drug (21). The T315I mutation, seen in STI571-resistant CML, precludes hydrogen bonding with STI571 and results in a steric clash attributable to the extra hydrocarbon group in Ile (10). Likewise, PD166326 does not inhibit the activity of Bcr-ablT315I in vivo (Fig. 4), suggesting that Thr (315) is also important for its binding within the ATP pocket of abl. However, PD166326 has some activity against BaF3p210T315I cells and inhibits their growth with IC50 of 150 nM (Tables 2 and 3). This activity is related to Bcr-abl-driven growth because growth inhibition of non-Bcr-abl-driven cell types requires 5–15-fold higher concentrations. Because PD166326 is a potent inhibitor of src kinases, and because the src kinases Hck and Lyn mediate some of the transforming activities of Bcr-abl, it is possible that PD166326 inhibits the growth of BaF3p210T315I cells through the inhibition of Hck and Lyn. However, seemingly inconsistent with this hypothesis, we fail to see inhibition of Hck Y416 phosphorylation in these cells at growth inhibitory concentrations (Fig. 6). However, this does not disprove the hypothesis because of limitations in assaying Hck activity in vivo. If PD166326 binds with and inhibits the active Y416 phosphorylated conformation of Hck, then this catalytically inactive drug-Hck complex may remain stably in this phosphorylated conformation, and phospho-Y416 Hck antibodies will be unable to demonstrate the in vivo inhibition of Hck catalytic function. In vitro kinase assays do not help in this regard either, because during the process of cell lysis and immunoprecipitation, the Hck-PD166326 interaction is lost (data not shown). Therefore, in BaF3 p210T315I cells, where Bcr-abl activity is resistant to PD166326, inhibition of Hck activity may be responsible for the observed growth inhibitory effects at IC50 = 150 nM despite persistent phosphorylation of Hck at these doses. In addition, although Y416 is a site of autophosphorylation in src kinases, it may also be a substrate for phosphorylation by other kinases. In fact, in our experiments, Hck Y416 phosphorylation status parallels Bcr-abl activity (compare Figs. 4 with 6), which suggests that Hck Y416 may also be a substrate for Bcr-abl. Although the activity of PD166326 against src kinases would suggest that it inhibits BaF3 p210T315I cells through a src family member, these experiments do not rule out the possibility that this cellular sensitivity is mediated through the inhibition of other, yet unknown, kinases.

The structural basis for the STI571 resistance of the E255K-mutated Bcr-abl is less clear because the functional significance of this residue is currently unknown. Interestingly, this mutation confers little resistance to PD166326 (Table 2 and Fig. 4). PD166326 shows no loss of activity against Bcr-ablE255K autophosphorylation in vivo (Fig. 4) and only 2.5-fold less activity against the growth of BaF3Bcr-ablE255K cells (Table 3) compared with wild-type Bcr-abl controls. The cellular IC50 of PD166326 against BaF3Bcr-ablE255K cells (15 nM) is much lower than its activity in non-Bcr-abl-driven cell types (0.8–2 μM) and much greater than the activity of STI571 against this mutant. If the basis for Bcr-abl E255K resistance to STI571 is destabilization of the inactive conformation, and if PD166326 in fact binds to the active conformation, then this would explain why PD166326 is effective in inhibiting Bcr-ablE255K. However, validation of these hypotheses requires crystal structure
data to better define the function of the Glu (255) residue and binding of PD166326 to Bcr-abl.

Although the clinical success of STI571 has validated the importance of Bcr-abl as a therapeutic target for the treatment of CML, the emerging problem of Bcr-abl TK resistance has highlighted the need for continued efforts to develop more effective treatments for this universally fatal disease. More effective inhibitors of Bcr-abl signaling may eventually lead to highly effective treatments even for patients in blast crisis or patients who have relapsed on STI571 therapy. PD166326 is a prototype of a new generation of anti-Bcr-abl compounds with greater activity than STI571. The development of this class of compounds awaits further optimization of the pharmacological parameters and in vivo studies of safety and efficacy.

References
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