Inhibition of Wild-Type and Mutant Bcr-Abl by Pyrido-Pyrimidine-Type Small Molecule Kinase Inhibitors

Nikolas von Bubnoff, Darren R. Veach, W. Todd Miller, Wanqing Li, Jana Sänger, Christian Peschel, William G. Bornmann, Bayard Clarkson, and Justus Duyster

Department of Internal Medicine III, Technical University of Munich, Munich, Germany [N. v. B., J. S., C. P., J. D.]; Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [D. R. W., G. B., B. C.]; and Department of Physiology and Biophysics, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794 [W. T. M., W. L.]

ABSTRACT

Imatinib mesylate (STI571, Gleevec), a 2-phenylaminopyrimidine small-molecule ATP-competitive-type kinase inhibitor, proved to be active in Philadelphia-positive leukemias. Resistance toward imatinib develops frequently in advanced-stage Philadelphia-positive leukemia, and is even observed in chronic-phase chronic myelogenous leukemia. Point mutations within the BCR-ABL kinase domain emerged as a major mechanism of resistance toward imatinib. Mutations occur at positions that determine specific contacts of imatinib to the ATP-binding site. We aimed to examine whether pyrido-pyrimidine-type kinase inhibitors were capable of inhibiting both wild-type and mutant forms of BCR-ABL. We screened 13 different pyrido-pyrimidine with cells expressing wild-type and mutant BCR-ABL. All of the substances specifically suppressed the Bcr-Abl dependent phenotype and inhibited Bcr-Abl kinase activity with higher potency than imatinib. Two of the most active compounds were PD166326 and SKI-DV-M016. Interestingly, these compounds suppressed the activation loop mutant Bcr-Abl H396P as effectively as wild-type Bcr-Abl. In addition, nucleotide-binding loop mutations (Y253H, E255K, and E255V) were selectively and potently inhibited. In contrast, T315I, a mutant located at a position that makes a direct contact with imatinib, was not affected. This observation is consistent with the hypothesis that unlike imatinib, pyrido-pyrimidine inhibitors bind Bcr-Abl regardless of the conformation of the activation loop. We conclude that pyrido-pyrimidine-type kinase inhibitors are active against different frequently observed kinase domain mutations of BCR-ABL that cause resistance toward imatinib. Resistance as a consequence of selection of mutant BCR-ABL by imatinib may be overcome using second-generation kinase inhibitors because of their higher potency and their ability to bind Bcr-Abl irrespective of the conformation of the activation loop.

INTRODUCTION

The discovery of the Ph1 chromosome, a reciprocal translocation between the long arms of chromosomes 9 and 22 (1, 2), is the characteristic molecular abnormality present in ~95% of cases of CML and up to 20% of adult ALL (3, 4). Bcr-Abl, the resulting fusion protein, is a deregulated, constitutively active oncogenic protein tyrosine kinase. When Bcr-Abl is introduced in mice, it causes CML (5), and transformation is strictly dependent on its tyrosine kinase activity (6). Thus, Bcr-Abl constitutes an attractive target for molecular-based therapy of Ph+ ALL and CML.

A high-throughput screen of compound libraries at Ciba-Geigy (now Novartis) identified imatinib mesylate, a derivative of the initial 2-phenylaminopyrimidine lead compound, to inhibit several tyrosine kinases, among them the protein tyrosine kinase Abl (7, 8). Imatinib was demonstrated to inhibit the corresponding oncogenic fusion proteins Bcr-Abl p210 (9) and p185 (10), and displayed activity in a murine model of Bcr-Abl-induced leukemia (8, 11). Impressive and encouraging response rates resulted from the conducted Phase I and II clinical studies demonstrating the activity of imatinib in chronic phase CML (12, 13) but also in advanced-phase CML, such as accelerated phase and blast crisis CML, and Ph+ ALL (14–17).

However, it became clear that the majority of patients suffering from advanced-phase CML and Ph+ ALL experience a relapse of their disease despite continued treatment with the drug (14, 16, 17). Kinase domain mutations that interfere with drug binding while retaining ATP binding and catalytic activity constitute the major cause of resistance (18–24), detected in at least 70% of patients with CML or Ph+ ALL relapsing after an initial hematological response to imatinib (25). The causality between mutations in BCR-ABL and refractoriness of Ph+ leukemia toward imatinib was proved by introducing mutated BCR-ABL into cell lines, thereby conferring resistance to the drug (18, 20) and additionally substantiated by crystal structure analysis indicating that some of the mutations observed in resistant patients occur at positions that were predicted to be critical for binding imatinib (26, 27). Specific binding of imatinib to Abl implies a complex interaction to various residues within the ATP-binding site (26). In addition to the initially described exchange of threonine at position 315 to isoleucine (18), a number of residues within the BCR-ABL kinase domain were found to be mutated in cases of refractory or relapsed Ph+ leukemia (19–24, 28–30). The resulting amino acid exchanges can be divided into different categories. First, there are residues that make direct contact to imatinib where an exchange presumably causes a steric clash with imatinib, such as F311, T315, and F317 (23, 26, 31), with T315I being the most frequent exchange (23, 32). Second, there are mutations affecting the nucleotide-binding (P) loop that have to adopt a specific conformation to allow imatinib to bind (23, 26, 31). The most frequent exchange falling into this category is E255K/V, followed by Y253H/F (23, 32). The third includes mutations that are located within the activation loop, with H396P/R being the most frequent one (23, 32). An in vitro screen of randomly mutagenized variants of BCR-ABL demonstrated recently that mutations involving critical interdomain contact sites beyond the kinase domain as well can cause resistance to imatinib in vitro (33).

Pyrido-[2,3-d]pyrimidines were synthesized as small molecule tyrosine kinase inhibitors of ATP-binding (34, 35), and were initially identified as selective and potent inhibitors of src family kinases (36, 37). It was found recently that PD166326, PD173955, and PD180970 are potent inhibitors of Bcr-Abl (27, 38–42). PD173955 has been cocrystallized with the kinase domain of murine c-Abl (residues 229–515) (27), and PD166326 was crystallized in complex with larger Abl constructs, residues 1–531 of human c-Abl, and residues 46–534 of murine c-Abl (43, 44). Crystal structure analysis has
demonstrated differences in the mode of binding of pyrido-pyrimidines versus imatinib, but at the same time suggested that positions critical for binding of either drug may also overlap (26, 27). Thus, we intended to examine whether pyrido-[2,3-d]pyrimidine derivatives perform superior to imatinib in the potency of inhibition of Bcr-Abl and whether activity is maintained against clinically relevant mutant forms of \textit{BCR-ABL}, which cause resistance toward imatinib.

**MATERIALS AND METHODS**

**Inhibitors.** Pyrido-[2,3-d]pyrimidine analogues were synthesized by Darren R. Veach and William G. Bornmann (Memorial Sloan-Kettering Cancer Center). The compounds were dissolved at 10 mM in DMSO and stored at −20°C.

**Generation of Cell Lines.** Mutations within the kinase domain of Abl in cases of Ph+ leukemia resistant to imatinib were identified as described (20). Briefly, reverse transcription-PCR fragments derived from clinical samples were cloned into pBluescript SK+ p185\textsuperscript{v-abl} (Stratagene, Amsterdam, the Netherlands), and subcloned into pcDNA3.1/Zeo (+; Invitrogen, Leek, the Netherlands) and Mig EGFP (45). Presence of single point mutations was verified by sequencing. Ba/F3 cells were maintained in RPMI 1640 growth medium (Life Technologies, Inc., Karlsruhe, Germany) containing 10% FCS (Life Technologies, Inc.) and P/S (Life Technologies, Inc.; 200 units penicillin/ml and 200 µg streptomycin/ml). Parental cells were cultured in the presence of 2 ng/ml IL-3 (R&D, Wiesbaden, Germany). IL-3-independent Ba/F3 cells expressing wild-type and mutant \textit{BCR-ABL} were generated by electroporation and transformed on withdrawal of IL-3. Expression of Bcr-Abl was confirmed by Western blot and flow-cytometric analysis.

**Proliferation.** Proliferation was measured using a MTS tetrazolium-based method by absorption of formazan at 490 nm (CellTiter 96; Promega, Madison, WI). Measures were taken as triplicates after 24 and 48 h of culture without and in the presence of inhibitor at the indicated concentrations.

**Western Blot.** Ba/F3 cells were cultured for 2.5 h without and in the presence of inhibitor at the indicated concentrations. Cell lysis, SDS-PAGE, and immunoblotting were done as described previously (46). Abl antibodies were obtained from PharMingen (8E9; BD Biosciences, Heidelberg, Germany) and Calbiochem-Novabiochem (Ab3; Schwalbach, Germany). Antibodies to phosphotyrosine were purchased from Upstate Biotechnology (4G10; Biozol, Eching, Germany) and Transduction (PY20; BD Biosciences). Anti-phospho-Stat5 (Tyr694) was obtained from Cell Signaling (New England Biolabs,
RESULTS

Pyrido-Pyrimidines Specifically Inhibit the Growth of Bcr-Abl Transformed Cells. Thirteen pyrido-[2,3-d]pyrimidine analogues were examined that differed in the substituents located at position 2 of the pyrimidine-ring (see Fig. 1). Tetrazolium-based proliferation assays with Ba/F3 cells transformed with Bcr-Abl wild-type were performed. PD166326, SKI DV 2–43, and SKI DV-M016 were the most inhibitory derivatives tested and exhibited IC_{so} values of 9 nM, 9 nM, and 12 nM, and IC_{50} values of 45 nM, 42 nM, and 75 nM, respectively, after 48 h of culture (see Fig. 2, A and B). IC_{50} values for growth inhibition covered a range from 9 nM (SKI DV 2–43 and PD166326) to 115 nM (SKI DV 1–10 biotinyl) with a mean IC_{50} of 32.5 nM. Most of the compounds displayed IC_{50} values in the range of 10 nM to 100 nM in wild-type Bcr-Abl expressing cells after 48 h of culture (Fig. 3A, front row, purple area). Ninety-five percent of growth inhibition in Bcr-Abl wild-type expressing Ba/F3 cells required a 3.8–7.7-fold (mean 5.45-fold) increase in concentrations of inhibitors compared with IC_{so} values, resulting in IC_{50} values for growth inhibition that were between 42 nM (SKI DV 2–43) and 515 nM (SKI DV 1–10 biotinyl, see Fig. 3B, front row). Large similar results were obtained in 32D cells expressing Bcr-Abl wild-type (data not shown). Specific inhibition of cells expressing wild-type Bcr-Abl occurred at many concentrations. The most active compounds exhibited unspecific inhibition of parental cells with IC_{so} values that were at least 100-fold higher than in cells transformed with wild-type Bcr-Abl.

Differential Growth Inhibition of Wild-Type versus Mutant Bcr-Abl. When Bcr-Abl wild-type and the activation loop mutant H396P were compared, both IC_{so} and IC_{50} values were identical for all of the pyrido-pyrimidines tested (see Fig. 2, first and second rows; Fig. 3, A and B; and Fig. 4, A and B, first two columns). In contrast, an IC_{so} of the activation loop mutant H396P required a 10-fold increase in imatinib concentrations (Fig. 4C, second column; Ref. 20). Specific inhibition of a Bcr-Abl-dependent phenotype was also observed when nucleotide-binding loop (P loop) mutations were introduced in Ba/F3 cells. All of the compounds exhibited an efficient and specific suppression of growth in cells expressing the P loop mutants E255K (Fig. 2; Fig. 3, A and B, third row), Y253H (Fig. 2; Fig. 3, A and B, fourth row), and E255V (Fig. 2; Fig. 3, A and B, fifth row), whereas IC_{so} values for inhibition of P loop mutants with imatinib were not reached even with a 40-fold increase in imatinib concentrations (Fig. 4C, columns 4, 6, and 8; Ref. 20). In opposition to activation loop and P loop mutants, none of the substances was capable of suppressing growth in Ba/F3 cells expressing T315I (see Fig. 2). When the IC_{so} and IC_{50} values for growth inhibition in wild-type and mutant BCR-ABL-expressing cells were compared, the mean fold increase in inhibitor concentration necessary to compensate the lower sensitivity of P loop mutants was 3.9/2.5-fold for IC_{so} and IC_{50}, and 11.5/10-fold for E255K, 4.7/3.6-fold for Y253H, and 5.5/4.9-fold for E255V (see Fig. 4A). When a subgroup of five inhibitors was analyzed (SKI DV-M016, SKI DV 2–87, PD173958, PD173956, and SKI DV 1–10-biotinyl), the mean fold increase in substance concentration corresponding to IC_{50} values of the least sensitive mutant E255V compared
with wild-type decreased to three (range, 1.5–4.7; see Fig. 4B, column 7). Comparing this subgroup with the remaining inhibitors, the smaller increment in IC_{95} values from wild-type to P loop mutants is represented by a lower slope along the Z-axis and represented by insections along the X-axis in Fig. 3B. In comparison to the most active pyrido-pyrimidines, PD180970 required higher concentrations for inhibition of growth and displayed unspecific inhibition of parental Ba/F3 cells at concentrations that were necessary for effective growth suppression of P loop mutants (Fig. 2C).

**Growth Suppression Is Accompanied by Inhibition of Bcr-Abl Kinase Activity.** To investigate whether the observed effects on cell growth resulted from inhibition of Bcr-Abl kinase activity, we cul-
fig. 5A, top panel), paralleling the observed effects on growth (compare Fig. 2A). In cells expressing the activation loop mutant H396P, the effects of PD166326 again were identical to wild-type (Fig. 5A, second panel). As for Bcr-Abl wild-type and H396P, tyrosine phosphorylation of the P loop mutants E255K, Y253H, and E255V disappeared at concentrations that were in between IC_{50} and IC_{95} values for growth inhibition. At 125 nm in E255K, and at 250 nm in Y253H and E255V, respectively (Fig. 5A). As expected from the growth assays, activity of Bcr-Abl/T315I was not affected (Fig. 5A, bottom panel). SKI DV-M016 performed similarly, but inhibition of Bcr-Abl tyrosine phosphorylation required slightly higher concentrations compared with PD166326 (Fig. 5B). This was again in line with the effects of SKI DV-M016 on cell growth (compare Fig. 2B). Compatible with its lower activity in cellular growth suppression (see Fig. 2C), the application of PD180970 necessitated higher concentrations for Bcr-Abl inhibition. Thus, complete disappearance of autophosphorylated Bcr-Abl occurred at 250 nm for wild-type and H396P, 1 μM for E255K, and 2 μM for Y253H and E255V, respectively. As observed for PD166326 and SKI DV-M016, PD180970 did not impair activity of Bcr-Abl/T315I (Fig. 4C).

Inhibition of wild-type Bcr-Abl was additionally validated by in vitro kinase assays using purified Abl catalytic domain. The resulting IC_{50} values for SKI DV-M016, PD166326, and SKI DV 2–43 (see Table 1) were in accordance with the observed cellular effects. Phosphorylation of Stat5 representing activation of a known target protein of Bcr-Abl correlated well with inhibition of Bcr-Abl autophosphorylation and cellular effects elicited by PD166326 (Fig. 5D). Inhibition of Stat5 phosphorylation occurred at lower concentrations of PD166326 than inhibition of Bcr-Abl autophosphorylation. It has been observed previously that inhibition of Bcr-Abl autophosphorylation required higher concentrations of imatinib than phosphorylation of Bcr-Abl substrates (47, 48), and this may be a general feature of competitive protein tyrosine kinase inhibitors (47).

PD166326 Specifically Induces Apoptosis in Ba/F3 Cells Expressing Wild-Type and Mutant Bcr-Abl. We next examined whether inhibition of Bcr-Abl by a pyrido-pyrimidine gives rise to apoptotic cell death in a Bcr-Abl-dependent phenotype expressing wild-type and mutant forms of Bcr-Abl. PD166326 initiated apoptosis in Ba/F3 cells expressing wild-type (Fig. 6A), as well as activation loop (Fig. 6B) and P loop (Fig. 6C, D–E) mutant forms of Bcr-Abl, as determined by detection of Annexin V-positive cells. Viability of parental Ba/F cells (Fig. 6G) and cells expressing Bcr-Abl/T315I (Fig. 6F) was not affected. The content of Annexin V-positive cells was in accordance with the fraction of dead cells as measured by trypan exclusion (data not shown). Relative sensitivity corresponded to the activity of PD166326 seen in growth and phosphorylation assays, and concentrations that blocked autophosphorylation of Bcr-Abl and inhibited cellular proliferation, at the same time instituted apoptosis.

**DISCUSSION**

Like imatinib, pyrido-pyrimidines bind to the ATP-binding site, a highly conserved nucleotide-binding pocket within the kinase domain of protein tyrosine kinases, thereby blocking access of ATP (27). Crystal structure analysis of Abl in complex with PD173955 and imatinib suggested that the mode of binding to Abl may differ between 2-phenylaminopyrimidine-type (imatinib) and pyrido[2,3-d]pyrimidine-type compounds (27). The activation loop (residues 381–402 in Abl), a highly conserved region in most kinases located at the NH2-terminal of the ATP-binding site, controls catalytic activity by switching between different states in a phosphorylation-dependent manner. Imatinib exclusively binds the closed conformation (26, 27). In this conformation, the activation loop folds Y393, the major auto-
phosphorylation site of Abl, toward the active site, superseding bound substrate, and the kinase is inactive. If Y393 gets phosphorylated, the activation loop adopts the open conformation, and the active site is accessible for substrate phosphorylation (26, 27). In contrast to imatinib, the pyrido-pyrimidine PD173955, one of the compounds tested here, was cocrystalized with Abl with the activation loop in an open conformation, and inhibited Abl independent of the phosphorylation state of Abl, suggesting that PD173955 binds irrespective of the conformation of the activation loop (26, 27).

This observation can explain several findings. A much more potent inhibition of wild-type Abl than with imatinib is obviously a common feature of pyrido-pyrimidines. The most active pyrido-pyrimidines were by a factor of 28 more active in suppressing the growth of, and by a factor of 50 more active in blocking Bcr-Abl autophosphorylation in cells expressing wild-type Bcr-Abl than imatinib (IC50 proliferation: PD166326 and SKI DV 2–43: 9 nM, imatinib: 250 nM; IC50 Bcr-Abl autophosphorylation: PD166326 and SKI DV 2–43: 4 nM, imatinib: 200 nM). Imatinib, although making more interactions with Abl, may lose some of its binding energy for stabilizing the activation loop in the closed conformation (27).

The activation loop mutant H396P, whereas causing an increase in IC50 for imatinib by a factor of 10 (see Fig. 4C), is inhibited as effectively as wild-type Bcr-Abl by all of the pyrido-pyrimidines tested here (Fig. 4A). As we have proposed earlier, an exchange of the histidine at position 396 may lead to an extended conformation of the activation loop that impairs binding of imatinib (20, 25). A pyrido-pyrimidine-type inhibitor does not require a specific conformation of the activation loop to bind. Therefore, inhibition of H396P by pyrido-pyrimidines is possible in the presence of H396P. In a previous study, cellular IC50 values for PD166326, PD173955, and PD180970 in cells expressing wild-type Bcr-Abl were demonstrated to be lower than the IC50 values reported here (38). In that report, the IC50 value for [3H]thymidine incorporation of glycophorin A negative (R10 negative) MO7e/p210 cells with PD166326 was 0.4 nM, with PD173955 1–2 nM, and with imatinib 35–40 nM; in our study, the respective IC50 values were 9 nM, 30 nM, and 250 nM, respectively. Several reasons may explain this finding. First, different assays were used. We used tetrazolium-based proliferation assays. A direct comparison of tetrazolium- and [3H]thymidine-based proliferation assays with R10(−) cells and PD166326 revealed that incorporation of [3H]thymidine was 3–4-fold more sensitive in comparison with

| IC50 values of Abl kinase assays for the most active pyrido-pyrimidinesa |
|-------------------------------|-----------------|-----------------|
| SKI DV-M016                   | 4.2 ± 0.6       | 4.3 ± 0.8       |
| PD166326                      | 4.3 ± 0.5       | 4.3 ± 0.5       |

*a In vitro kinase assays were performed using 10 nM purified Abl catalytic domain. Initial rates were measured, and IC50 values were determined by non-linear regression analysis.
Fig. 6. Inhibition of Bcr-Abl autophosphorylation and suppression of growth is followed by induction of apoptosis in a Bcr-Abl-dependent phenotype. Parental Ba/F cells (G) and Bcr-Abl transformed Ba/F3 cells (A: Bcr-Abl wild-type; B: Bcr-Abl H396P; C: Bcr-Abl E255K; D: Bcr-Abl Y253H; E: Bcr-Abl E255V; and F: Bcr-Abl T315I) were measured for induction of apoptosis when cultured in the presence of PD166326 at different concentrations. Annexin V-positive cells at indicated time points are depicted. Two experiments per cell line were performed. Results of one representative experiment are shown.
tetrazolium-based assays (data not shown). Second, compared with other Bcr-Abl-positive cell lines, sublines of MO7e/p210 cells seem to be particularly sensitive to inhibition of Bcr-Abl (38).

In marked contrast with Bcr-Abl wild-type, pyrido-pyrimidines had no advantage over imatinib for inhibition of Bcr-Abl/T315I. None of the compounds inhibited proliferation of Ba/F3 or 32D cells transformed by Bcr-Abl/T315I or inhibited autophosphorylation of Bcr-Abl/T315I in Ba/F3 cells. Both imatinib and the pyrido-pyrimidine PD173955 interact with the threonine at position 315 of c-Abl, and imatinib forms an H-bond with T315 not present with I315 (26, 27). An exchange to isoleucine at this position adds an extra hydrocarbon group in its side chain presumably causing a steric clash with both types of compounds (see Fig. 7). Therefore, Bcr-Abl/T315I may be insensitive not only to the compounds tested here, but as well to other pyrido-pyrimidines carrying a dichlorophenyl substituent at the same position. Whether a modification of the dichlorophenyl substituent would be sufficient to circumvent the clash with isoleucine side chain of T315I remains to be shown. Alternatively, presence of T315I may necessitate inhibition of biologically relevant proteins other than Bcr-Abl. Inhibition of the molecular chaperone HSP90 by geldanamycin and its derivative 17-AAG was demonstrated recently to induce proteasomal degradation of Bcr-Abl and apoptosis in cells expressing wild-type Bcr-Abl (49–51), and instituted apoptosis in primary cells derived from patients with blast crisis CML and resistance to imatinib (52). Moreover, geldanamycin and 17-AAG were active in cells expressing E255K and T315I (53). Thus, proteasomal degradation of Bcr-Abl may serve as a strategy for the treatment of patients harboring particular inhibitor-resistant mutants.

Parallel to our investigations, another group reported that one of the most active compounds tested here, PD166326, was active in Bcr-Abl E255K, whereas Bcr-Abl T315I was largely resistant (54). This is in line with our results for PD166326 in those two particular mutants. However, that report described some activity of PD166326 in growth assays of Ba/F3 Bcr-Abl T315I, with an IC₅₀ value of 150 nM. In contrast, as stated above, we were not able to see any difference between Bcr-Abl T315I and their parental counterparts looking at proliferation, Bcr-Abl autophosphorylation, and apoptosis in different cell lines.

The region that is most frequently mutated in cases of imatinib-resistant Ph+ leukemia is the nucleotide-binding (P) loop, a highly conserved region of the kinase domain involved in ATP binding, with Y253 and E255 being affected in the majority of cases (23, 32, 55). The P loop mutations examined here are highly resistant to imatinib with cellular IC₅₀ values >40-fold higher than wild-type Bcr-Abl (see Fig. 4C; Ref. 20) that by far exceed concentrations measured in vivo (56). P loop mutations in patients with resistance toward imatinib were associated with a particular poor prognosis (55). Moreover, Y272F in c-Abl, corresponding with Y253F in Bcr-Abl, activates transformation by c-Abl (57), and these mutations were shown to give rise to an increase of catalytic activity of c-Abl and Bcr-Abl in vivo (30, 57, 58). In contrast, T315I has been demonstrated to decrease Bcr-Abl kinase activity to 60% of wild-type (58).

In contrast to imatinib, all of the pyrido-pyrimidines tested here were potent inhibitors of P loop mutants. Cellular IC₅₀ values for inhibition with the most active compound SKI DV 2–43 were 110 nM for inhibition of E255K, 125 nM for Y253H, and 200 nM for inhibition of E255V. Concentrations of PD166326 that resulted in a complete growth inhibition and suppression of Bcr-Abl tyrosine phosphorylation at the same time induced apoptosis of Ba/F3 cells transformed with either of the P loop mutant forms of BCR-ABL (see Fig. 6).

Our results for PD180970, one of the pyrido-pyrimidines tested here, are in line with a recent report showing activity of PD180970 in wild-type and several mutant forms of Bcr-Abl that were examined here as well (59). Relative activity of PD180970 for growth inhibition of Ba/F3 cells expressing wild-type Bcr-Abl and Bcr-Abl H396P, E255K, Y253H, and T315I is in full compliance with our results with cellular IC₅₀ values that were similar. However, compared with the most active compounds examined here, PD180970 required 5-fold higher concentrations for equivalent suppression of growth and inhibition of Bcr-Abl autophosphorylation, and displayed a narrow range of concentration where specific inhibition of a Bcr-Abl-dependent phenotype occurred (see Figs. 2, 3, and 5). Using PD180970, cellular IC₅₀ values for Y253H (770 nM) and E255V (935 nM) are in the range or above the concentration where unspecific growth inhibition of parental Ba/F3 cells (IC₅₀ 830 nM) occurs.

Some of the pyrido-pyrimidines that were used here were initially described as potent inhibitors of src family kinases (36, 37). It may be argued that inhibition of wild-type and mutant forms of Bcr-Abl is attributable to inhibition of src kinases. However, several observations clearly argue against this possibility. First, pyrido-pyrimidines are active against purified Abl and Bcr-Abl kinase in the low nanomolar range (Refs. 27, 38–41; Table 1). Ba/F3 and 32D cells transfected with wild-type or mutant Bcr-Abl display a transformed phenotype upon withdrawal of IL-3 that is strictly dependent on active Bcr-Abl. The biological activities of pyrido-pyrimidines described here selectively occurred in cells that depend on active Bcr-Abl, but not in parental cells, and emerged at concentrations that inhibit purified Bcr-Abl and impede Stat5 activation. Second, it has been demonstrated previously that src kinase-specific inhibitors PP1 and PP2 had only marginal effects on a Bcr-Abl-dependent phenotype (38, 39). Finally, Bcr-Abl wild-type, P loop, and activation loop mutant forms of Bcr-Abl were potently inhibited by pyrido-pyrimidines, whereas Bcr-Abl/T315I, a mutation that is critical for binding of imatinib and pyrido-pyrimidines to Bcr-Abl, was completely unresponsive. Taken together, the observed effects of pyrido-pyrimidines on Bcr-Abl-dependent cells are attributable to inhibition of Bcr-Abl, not src family kinases.

We conclude that pyrido-pyrimidine-type small molecule ATP-
competitors have two important advantages over imatinib. First, all pyrido-pyrimidines differ in their mode of binding to the kinase domain of Abl and, therefore, are active where imatinib fails. They are capable of suppressing some of the most frequently detected mutations of Bcr-Abl that cause resistance toward imatinib and may, therefore, be used not only to treat imatinib-resistant disease, but also to prevent resistant disease clones to emerge. These properties of pyrido-pyrimidines advise therapeutic application in clinical trials. Therefore, ongoing and future studies will determine the pharmacokinetic properties of pyrido-pyrimidines.

REFERENCES


INHIBITION OF BCR-ABL


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