

## Interleukin-3 protects Bcr-Abl-transformed hematopoietic progenitor cells from apoptosis induced by Bcr-Abl tyrosine kinase inhibitors

JF Dorsey<sup>1,2,3</sup>, JM Cunnick<sup>1,2</sup>, R Lanehart<sup>1,2</sup>, M Huang<sup>1,2</sup>, AJ Kraker<sup>4</sup>, KN Bhalla<sup>1,2</sup>, R Jove<sup>1,2,5</sup> and J Wu<sup>1,2,3</sup>

<sup>1</sup>Molecular Oncology and Experimental Therapeutics Programs, H Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, Tampa, FL, USA; <sup>2</sup>Department of Interdisciplinary Oncology, University of South Florida College of Medicine, Tampa, FL, USA; <sup>3</sup>Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa, FL, USA; <sup>4</sup>Cancer Pharmacology, Pfizer Global Research and Development, Ann Arbor Laboratories, Ann Arbor, MI, USA; and <sup>5</sup>Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, FL, USA

**Bcr-Abl tyrosine kinase has been validated as a molecular target for the treatment of chronic myelogenous leukemia (CML). More recently, it has been reported that CML patients could develop resistance to the Bcr-Abl tyrosine kinase inhibitor, imatinib (STI571, Gleevec), pointing to the need for development of additional Bcr-Abl tyrosine kinase inhibitors or other therapeutic strategies. It was also found that a significant proportion of patients who received the Bcr-Abl inhibitor did not achieve complete cytogenetic response. Mechanisms for incomplete cytogenetic response to Bcr-Abl inhibition are not entirely clear. We report here three new pyrido[2,3-*d*]pyrimidine Bcr-Abl tyrosine kinase inhibitors, PD164199, PD173952, PD173958, that induced apoptosis of Bcr-Abl-dependent hematopoietic cells. An interleukin-3 (IL-3) autocrine loop was observed previously in primitive CD34<sup>+</sup>/Bcr-Abl<sup>+</sup> leukemic cells in CML patients. Using 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells as models, we tested whether IL-3 might protect Bcr-Abl-transformed, IL-3-responsive cells from apoptosis caused by Bcr-Abl tyrosine kinase inhibition. Results of trypan blue exclusion, fluorescein isothiocyanate-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (FITC-VAD-FMK), and Annexin-V/7-aminocincomycin D (7-AAD) binding assays indicate that IL-3 could protect Bcr-Abl-transformed, IL-3 responsive hematopoietic progenitor cells from apoptosis induced by Bcr-Abl tyrosine kinase inhibitors. This finding raises the possibility that the IL-3 autocrine loop found in primitive CD34<sup>+</sup>/Bcr-Abl<sup>+</sup> cells in CML patients could contribute to the incomplete eradication of Bcr-Abl<sup>+</sup> cells by Bcr-Abl inhibition.**

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### Introduction

Chronic myelogenous leukemia (CML) is a malignant disorder of hematopoietic stem cells.<sup>1,2</sup> A cytogenetic characteristic of CML is the presence of the Philadelphia chromosome, which arises from the reciprocal t(9;22) chromosomal translocation and contains the Bcr-Abl fusion gene. The Bcr-Abl oncogene encodes either the p210<sup>Bcr-Abl</sup> tyrosine kinase that is found in most cases of CML or the p185<sup>Bcr-Abl</sup> tyrosine kinase that is found mostly in acute lymphoblastic leukemia.<sup>1</sup> Bcr-Abl confers cytokine-independent proliferative and anti-apoptotic activities to hematopoietic cells.<sup>2</sup> Genetic and biochemical studies have demonstrated that the tyrosine kinase activity of Bcr-Abl is essential for its transforming activity.<sup>3–6</sup> Moreover, the wild-type c-Abl tyrosine kinase does not appear to be essential for normal cell survival and proliferation. Therefore,

Bcr-Abl tyrosine kinase represents an excellent, rational molecular target for therapy of CML. This concept has been validated by demonstration of the clinical efficacy of the Bcr-Abl tyrosine kinase inhibitor, imatinib (formerly CGP57148, STI571, Gleevec), in CML.<sup>7–11</sup>

While imatinib has remarkable clinical efficacy in CML, primary and acquired resistance in some patients have been observed as a result of Bcr-Abl gene amplification, Bcr-Abl tyrosine kinase mutation or other unknown changes.<sup>8,12–18</sup> Several mutations in the tyrosine kinase domain of Bcr-Abl have been identified from patients with acquired resistance to imatinib.<sup>12–17</sup> Laboratory experiments have confirmed that Bcr-Abl gene harboring these mutations become resistant to inhibition by the Bcr-Abl tyrosine kinase inhibitor.<sup>12,16</sup> Therefore, while imatinib represents a major therapeutic advance for CML, further development of new, more potent Bcr-Abl tyrosine kinase inhibitors or other therapeutic strategies are warranted.

Furthermore, while complete hematologic responses in peripheral blood was achieved in virtually all patients with CML in the chronic phase when they were given a sufficiently high dose of imatinib, complete cytogenetic remissions in bone marrow was observed in 41% of these patients.<sup>9</sup> This observation implies that a majority of these patients have one or more sub-populations of Bcr-Abl<sup>+</sup> hematopoietic cells in bone marrow that are resistant to the Bcr-Abl tyrosine kinase inhibitor. Interleukin-3 (IL-3) is a known growth and survival factor for hematopoietic stem/progenitor cells. Expression of IL-3 receptor is repressed when hematopoietic progenitor cells differentiate. Interestingly, it was observed that primitive (CD34<sup>+</sup>) leukemic cells isolated from Bcr-Abl<sup>+</sup> CML patients have an autocrine loop of IL-3 production and response.<sup>19</sup>

In this study, we identified three new Bcr-Abl tyrosine kinase inhibitors, which are among the most potent Abl tyrosine kinase inhibitors *in vitro*. Using two p210<sup>Bcr-Abl</sup>-transformed, IL-3 responsive hematopoietic progenitor cells as models, we investigated the possibility that IL-3 might protect Bcr-Abl-transformed, IL-3 responsive cells from apoptosis caused by Bcr-Abl tyrosine kinase inhibitors. We found that while Bcr-Abl tyrosine kinase inhibitors induced apoptosis of these cells in the absence of IL-3, they were ineffective in the presence of IL-3. This finding suggests that IL-3 could play a critical role in protecting Bcr-Abl<sup>+</sup> hematopoietic cells from Bcr-Abl tyrosine kinase inhibitors and may contribute to the incomplete cytogenetic response to Bcr-Abl inhibition observed in CML patients.

## Materials and methods

### Reagents and cells

PD159373, PD164199, PD173952, PD173958, and PD180970 (Table 1) were synthesized by Pfizer Global Research and Development.<sup>20,21</sup> Imatinib (STI571) was from Novartis Pharmaceuticals (East Hanover, NJ, USA). Recombinant Abl and Lyn tyrosine kinases were obtained from New England Biolab (Beverly, MA, USA) and Upstate Biotechnology (Lake Placid, NY, USA), respectively. A plasmid for GST-Csk was kindly provided by Dr Torkel Vang (University of Oslo, Oslo, Norway).<sup>22</sup> GST-Csk protein was expressed in *E. coli* DH5 $\alpha$  and affinity purified by standard procedure. Baculovirus-expressed human insulin receptor tyrosine kinase was a gift of Coralia Rivas (Memorial Sloan-Kettering Cancer Center, New York, USA).<sup>23</sup> Murine IL-3 was obtained from PeproTech (Rocky Hill, NJ, USA). K562 is a human CML cell line that contains p210<sup>Bcr-Abl</sup>. 32Dp210<sup>Bcr-Abl</sup> is a p210<sup>Bcr-Abl</sup>-transformed mouse promyeloid 32D cell line, while Baf3p210<sup>Bcr-Abl</sup> is a p210<sup>Bcr-Abl</sup>-transformed mouse Baf3 pre-B cell line.<sup>24</sup> All cell lines were grown in RPMI 1640 containing 10% fetal bovine serum and 100  $\mu$ g/ml penicillin-streptomycin.

### In vitro tyrosine kinase assays

Abl tyrosine kinase activity was determined using a synthetic peptide (EAIYAAPFAKKK) as substrate. The activities of Lyn and Csk tyrosine kinases were assayed using a Src substrate peptide (KVEKIGEGTYGVVYK). The activity of a recombinant insulin receptor tyrosine kinase was measured using Raytide (Calbiochem, La Jolla, CA, USA) as substrate. The conditions

for the *in vitro* kinase assays were as provided previously.<sup>25</sup> Kinase reactions were performed in the presence of various concentrations of test compound (1  $\mu$ l in DMSO, total reaction mixture volume was 40  $\mu$ l). IC<sub>50</sub> was obtained as the concentration of compound required to inhibit tyrosine phosphorylation of substrate by 50%.

### Cell viability, poly-(ADP-ribose) polymerase (PARP) cleavage, fluoroisothiocyanate-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (FITC-VAD-FMK) and Annexin-V/7-amino-actinomycin D (7-AAD binding assays)

Cells ( $1 \times 10^5$  cells/ml) were plated in 2 ml RPMI/10% FCS. Tyrosine kinase inhibitors, IL-3 (10 ng/ml), or DMSO (solvent control) were added at the time of cell plating. The volume of DMSO was kept at 0.1% of the medium volume. Cell viability was determined by the trypan blue exclusion assay.<sup>25</sup> PARP cleavage assay and flow cytometric analysis of apoptosis using the Annexin-V/7-AAD binding assay were performed as described<sup>25</sup> except that 7-AAD (Via-Probe, Pharmingen, San Diego, CA, USA), instead of propidium iodide, was used.

For flow cytometric analysis of apoptosis using the CaspACE FITC-VAD-FMK In Situ Marker (Promega, Madison, WI, USA), cells ( $1.5 \times 10^5$  in 0.5 ml) were incubated with 10  $\mu$ M FITC-VAD-FMK in the dark at 37°C, 5% CO<sub>2</sub> for 20 min. After incubation, cells were washed twice with phosphate-buffered saline, resuspended in 0.5 ml phosphate-buffered saline, and analyzed immediately. At least  $1 \times 10^4$  gated-cells in each sample were analyzed. Untreated cells stained with FITC-VAD-FMK were used to compensate for the flow cytometric analysis.

**Table 1** Inhibitory activities of pyrido[2,3-*d*]pyrimidine derivatives on protein tyrosine kinases *in vitro*

Inhibitor	Structure	Inhibition of protein tyrosine kinases [IC <sub>50</sub> (nM)]			
		Abl	Lyn	Csk	IR
PD159373		>50	>25	>100	>10 000
PD164199		0.6 ± 0.3	0.6 ± 0.5	7.3 ± 1.7	>10 000
PD173952		1.7 ± 1.2	0.3 ± 0.1	6.6 ± 1.2	>10 000
PD173958		1.3 ± 0.6	0.8 ± 0.8	10.8 ± 7.8	>5 000
PD180970		2.8 ± 1.9	2.8 ± 1.8	9.0 ± 1.9	>10 000

Data are derived from three independent experiments. IR, insulin receptor kinase.

## Other assays

Immunoprecipitation and immunoblotting for analysis of tyrosine phosphorylation of Bcr-Abl and CrkL were performed as described.<sup>25,26</sup>

## Results

### *Analysis of pyrido[2,3-d]pyrimidine derivatives for Bcr-Abl tyrosine kinase inhibitory activities in vitro*

We previously found that a pyrido[2,3-d]pyrimidine derivative, PD180970, was a potent Abl tyrosine kinase inhibitor.<sup>25</sup> To test whether other pyrido[2,3-d]pyrimidine derivatives also inhibit the Abl tyrosine kinase, we analyzed the Abl tyrosine kinase inhibitor activity of four other derivatives available to us (Table 1). As shown in Table 1, PD164199, PD173952 and PD173958 have a similar potency as PD180970 in inhibiting the Abl tyrosine kinase activity *in vitro*. The IC<sub>50</sub> of these three compounds for Abl tyrosine kinase inhibition *in vitro* was in the 0.6–1.7 nM range. On the other hand, PD159373 had only a small effect on Abl tyrosine kinase activity *in vitro* at the concentrations that we have tested. PD164199, PD173952, PD173958 and PD180970 also showed similar inhibitory activities towards the Lyn tyrosine kinase (IC<sub>50</sub>: 0.3–2.8 nM), which is a hematopoietic cell Src-family kinase, consistent with these compounds as Src tyrosine kinase inhibitors. The Csk tyrosine kinase, which is a negative regulator of the Src family kinases, was inhibited by PD164199, PD173952, PD173958 and PD180970 at higher concentrations (IC<sub>50</sub>: 6.6–10.8 nM), (Table 1). All five compounds did not inhibit the insulin receptor tyrosine kinase (Table 1).

### *Inhibition of p210<sup>Bcr-Abl</sup> and CrkL tyrosine phosphorylation in human K562 cells by the pyrido[2,3-d]pyrimidine derivatives*

To determine whether PD159373, PD164199, PD173952 and PD173958 inhibit Bcr-Abl tyrosine kinase activity in human CML cells, we treated K562 cells with various concentrations of these compounds (Figure 1a and b). Cells were also treated with the previously characterized PD180970 as a positive control. p210<sup>Bcr-Abl</sup> and its substrate CrkL were immunoprecipitated from cell lysates and analyzed by immunoblotting with an anti-phosphotyrosine antibody (PY20). As shown in Figure 1a and b, PD164199, PD173952, PD173958 and PD180970 inhibited tyrosine phosphorylation of p210<sup>Bcr-Abl</sup> and CrkL in K562 cells in a concentration-dependent manner. Consistent with the *in vitro* kinase assay, PD159373 did not inhibit p210<sup>Bcr-Abl</sup> and CrkL tyrosine phosphorylation in K562 cells (Figure 1a and b). In additional experiments, we found that inhibition of the p210<sup>Bcr-Abl</sup> tyrosine kinase correlated well with loss of STAT5 DNA binding activity and down-regulation of Bcl-xL expression in K562 cells but not in the Bcr-Abl-negative HEL leukemic cells that also have constitutively active STAT5 (data not shown).

### *Induction of K562 and MEG-01 cell death by pyrido[2,3-d]pyrimidine derivatives*

We next analyzed the effects of pyrido[2,3-d]pyrimidine derivatives on cell viability of K562 cells. K562 cells were

incubated with pyrido[2,3-d]pyrimidine derivatives (0.5 μM) or DMSO (solvent) for 1–4 days and cell viability was determined. Figure 1c shows that incubation of K562 cells with PD164199, PD173952, PD173958 and PD180970 caused cell death, whereas PD159373 and the solvent control had no effect. Incubation with PD164199, PD173952, PD173958 and PD180970, but not PD159373, also induced cell death of another human Bcr-Abl<sup>+</sup> leukemic cell line, MEG-01 (data not shown). In comparison, PD173952 and PD173958 had no effect on cell viability of the Bcr-Abl-negative HL60 and HEL leukemic cells as reported previously for PD180970,<sup>25</sup> whereas PD164199 did reduce cell viability of HL60 and HEL cells (data not shown).

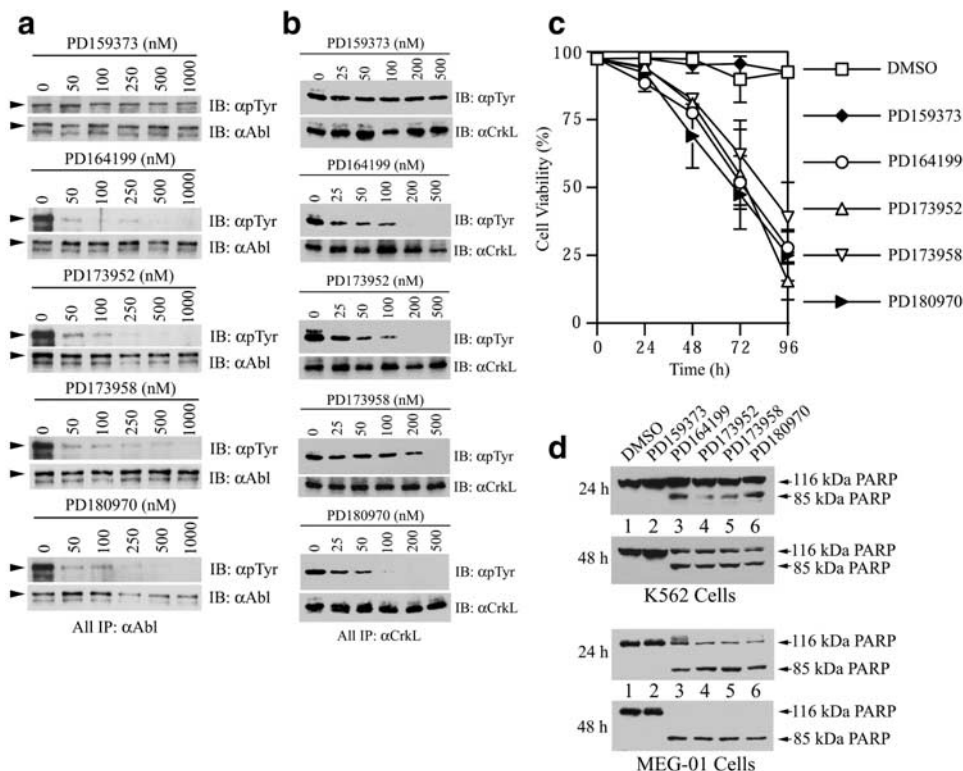
The 116-kD PARP is cleaved specifically into 85-kDa and 25-kDa fragments during apoptotic cell death.<sup>25</sup> Figure 1d shows that no 85-kDa PARP fragment was detected in K562 and MEG-01 cells treated with DMSO or PD159373, whereas the 85-kDa fragment was present in K562 and MEG-01 cells treated for 24 or 48 h with PD164199, PD173952, PD173958 and PD180970. These results suggest that PD164199, PD173952, PD173958 and PD180970, but not PD159373, induced apoptosis of K562 and MEG-01 cells.

### *IL-3 protects p210<sup>Bcr-Abl</sup>-transformed 32D and Baf3 cells from apoptosis induced by Bcr-Abl tyrosine kinase inhibitors*

During our preliminary studies with PD180970, we found that the viability of 32D cells was not affected by PD180970. 32D cells are IL-3 dependent and were grown in medium supplemented with IL-3. This observation suggested that PD180970, or a Bcr-Abl tyrosine kinase inhibitor in general, does not inhibit the IL-3-induced cell survival pathway. It was also observed previously that IL-3 could increase viable cell numbers of Bcr-Abl-transformed 32D cells when treated with imatinib.<sup>4,27</sup> These observations raise the possibility that IL-3 could protect IL-3 responsive, Bcr-Abl-transformed hematopoietic cells from apoptosis induced by Bcr-Abl tyrosine kinase inhibitors.

To test this possibility, we determined the effects of PD180970, PD164199 and imatinib on 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells. These two cell lines were derived from IL-3-dependent 32Dcl3 and Baf3 cells by p210<sup>Bcr-Abl</sup> transformation.<sup>24</sup> They can grow in medium without IL-3, but remain IL-3 responsive. In the absence of IL-3, incubation of 32Dp210<sup>Bcr-Abl</sup> cells (Figure 2a) with PD180970 (50 nM), PD164199 (50 nM) or imatinib (1 μM) resulted in time-dependent decreases in cell viability. Similar results were obtained using Baf3p210<sup>Bcr-Abl</sup> cells (Figure 2b). Cells treated with solvent (DMSO) remained viable through the time course (Figure 2a and b). Remarkably, the effects of PD180970, PD164199 and imatinib on viability of 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells were completely blocked when these cells were cultured in the presence of IL-3 (10 ng/ml) (Figure 2a and b). Additional experiments performed with PD173952 and PD173958 gave the same results (data not shown).

To determine whether PD180970, PD164199 and imatinib induce apoptosis of 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells and to confirm that IL-3 protects these cells from Bcr-Abl tyrosine kinase inhibitor-induced apoptosis, we first performed flow cytometric analysis using the CaspACE FITC-VAD-FMK Marker (Promega). This cell permeable compound binds to activated caspases and fluorescently labels apoptotic



**Figure 1** Inhibitory effects of pyrido[2,3-d]pyrimidine derivatives on Bcr-Abl and on viability of Bcr-Abl positive cells. (a) and (b) K562 cells were treated with DMSO (0) or the indicated concentrations of inhibitors for 12 h. p210<sup>Bcr-Abl</sup> (a) or CrkL (b) were immunoprecipitated from cell lysates of  $6 \times 10^6$  cells/each. One half of each immunoprecipitate was analyzed by immunoblotting with an anti-phosphotyrosine antibody (upper panels), the other half of each immunoprecipitate was analyzed by immunoblotting with an anti-Abl antibody (a, lower panels) or anti-CrkL antibody (b, lower panels). Arrowheads indicate p210<sup>Bcr-Abl</sup>. A repeated experiment gave the same results. (c) K562 cells ( $1 \times 10^5$  cells/ml, 2 ml/each) were treated with the indicated compounds (0.5  $\mu$ M/each) and cells viability was determined every 24 h. The data were derived from two duplicate experiments. (d) K562 and MEG-01 cells were treated with the indicated compounds (0.5  $\mu$ M/each) for 24 and 48 h. Cell lysates (40  $\mu$ g protein/each lane for K562 cells, 30  $\mu$ g protein/each lane for MEG-01 cells) were analyzed by immunoblotting with an anti-PARP antibody.

cells. Figure 2c shows that about 5% of solvent-treated 32Dp210<sup>Bcr-Abl</sup> cells were labeled positive with the apoptosis marker. In the absence of IL-3, 32Dp210<sup>Bcr-Abl</sup> cells treated with PD180970 (100 nM), PD164199 (100 nM) or imatinib (1  $\mu$ M) for 18 h were 42.9%, 70.9% or 41.8% positive, respectively, for the apoptosis marker (Figure 2c). Strikingly, when IL-3 was included in the medium, these numbers returned to the basal 5% level. Experiments performed in Baf3p210<sup>Bcr-Abl</sup> cells gave similar results (not shown).

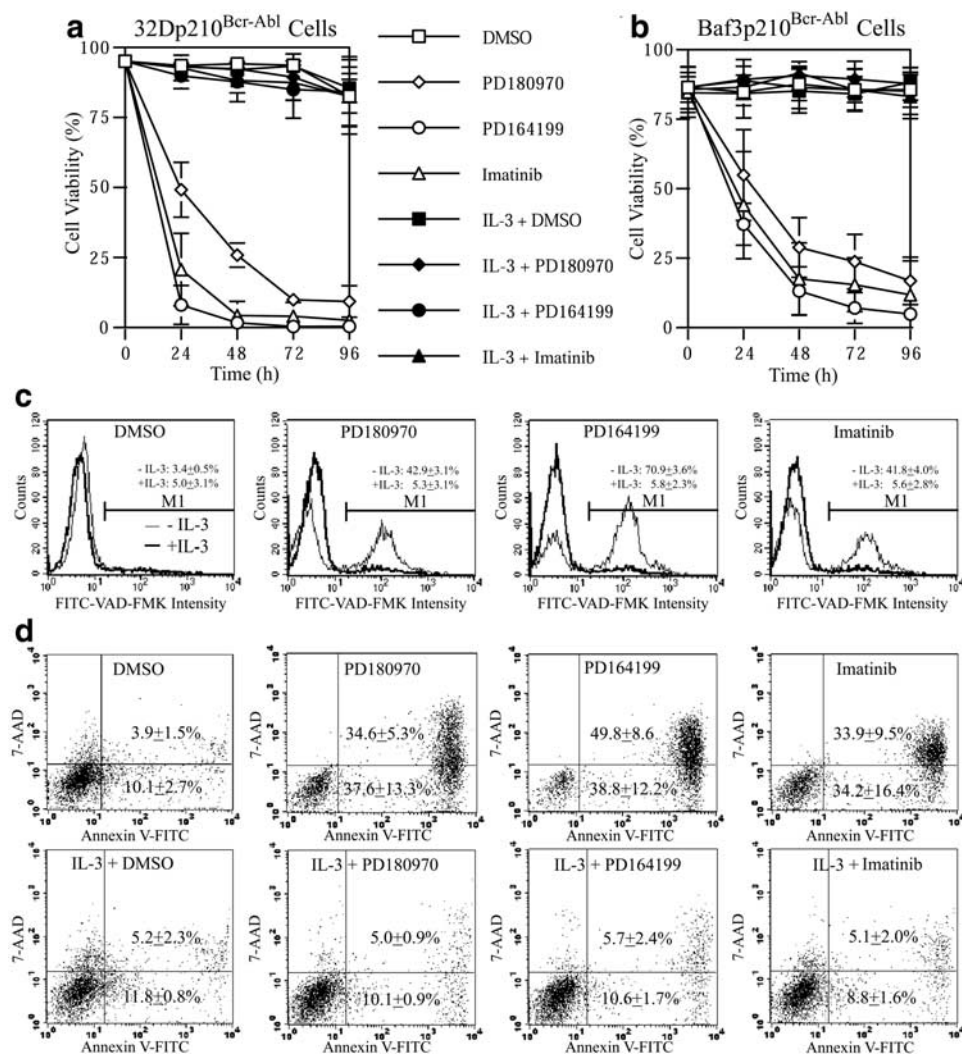
To further verify the above results, an Annexin-V/7-AAD binding assay was performed (Figure 2d). In solvent-treated cells, 10.1% cells were stained Annexin-V positive (early apoptotic cells) and 3.9% cells were stained Annexin-V/7-AAD double-positive (late apoptotic and necrotic cells). In the absence of IL-3, incubation with PD180970 (100 nM), PD164199 (100 nM) or imatinib (1  $\mu$ M) for 24 h increased the Annexin-V-positive population to 37.6%, 38.8% or 34.2% and the double-positive population to 34.6%, 49.8% or 33.9%, respectively (Figure 2d, upper panels). Again, the effects of PD180970, PD164199 and imatinib on induction of apoptosis of these cells disappeared when IL-3 was present in the medium (Figure 2d, lower panels). Therefore, although the FITC-VAD-FMK and Annexin-V/7-AAD binding assays gave slightly different basal staining levels, both assays clearly show that PD180970, PD164199 and imatinib did not induce apoptosis of 32Dp210<sup>Bcr-Abl</sup> cells when IL-3 was present.

### IL-3 does not prevent inhibition of p210<sup>Bcr-Abl</sup> tyrosine kinase by its inhibitors

To assess if Bcr-Abl tyrosine kinase inhibitors inhibited p210<sup>Bcr-Abl</sup> in 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells when IL-3 was present, we treated these cells with Bcr-Abl inhibitors in the presence or absence of IL-3, and analyzed tyrosine phosphorylation of p210<sup>Bcr-Abl</sup>. As shown in Figure 3, p210<sup>Bcr-Abl</sup> was phosphorylated on tyrosine in solvent-treated cells. PD180970, PD164199 and imatinib inhibited tyrosine phosphorylation of p210<sup>Bcr-Abl</sup> in 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells regardless of whether IL-3 was in the cell culture medium (Figure 3). These results rule out the possibility that p210<sup>Bcr-Abl</sup> tyrosine kinase was not inhibited in 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells in the presence of IL-3, and suggest that the protective effect of IL-3 is mediated by an IL-3-induced signal transduction pathway that does not require Bcr-Abl tyrosine kinase activity.

### Discussion

Our *in vitro* kinase assays indicate that PD164199, PD173952 and PD173958, like the previously described PD180970,<sup>25</sup>



**Figure 2** IL-3 protects 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells from apoptosis induced by PD180970, PD164199 and imatinib. (a) and (b) 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells ( $7 \times 10^4$  cells/ml, 2 ml/each) were plated in medium with or without IL-3 (10 ng/ml) and incubated with PD180970 (50 nM), PD164199 (50 nM), imatinib (1  $\mu$ M), or DMSO (0.1%) for up to 96 h. Cell viability was determined every 24 h. Data were from two (a) or three (b) duplicate experiments. (c) Flow cytometric analysis of 32Dp210<sup>Bcr-Abl</sup> cells using the FITC-VAD-FMK marker. Each panel contains two representative flow cytometric graphs from cells cultured with (thick line) and without (thin line) IL-3. M1 indicates labeled cells. The percentage labeled cells in each sample from two duplicate experiments ( $n = 4$ ) are given. (d) 32Dp210<sup>Bcr-Abl</sup> cells were analyzed by the Annexin-V/7-AAD binding assay. The graphs are representatives of two duplicate experiments and the percentage of Annexin-V positive (lower-right quadrant) and Annexin-V/7-AAD double positive (upper-right quadrant) cells from these experiments are given.

are among the most potent Abl tyrosine kinase inhibitors that have been reported. p210<sup>Bcr-Abl</sup> and CrkL tyrosine phosphorylation assays in K562 cells demonstrate that these compounds are able to inhibit Bcr-Abl tyrosine kinase activity and cause apoptotic cell death of Bcr-Abl-dependent cells. However, it remains to be determined which of these Bcr-Abl inhibitors has better pharmacokinetic properties *in vivo*.

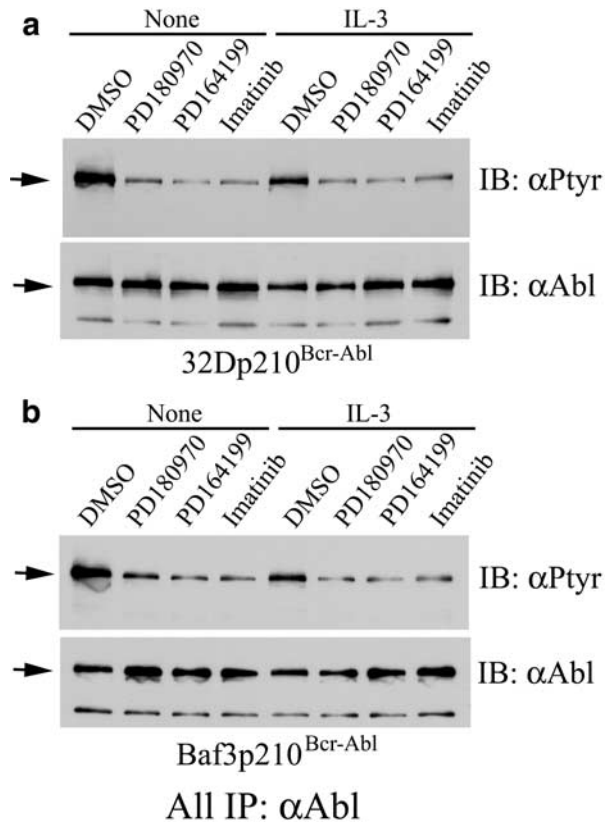
Although it inhibits Bcr-Abl kinase, PD164199 has an additional undefined activity that also affects viability of Bcr-Abl-independent HL60 and HEL leukemic cells (data not shown). Nevertheless, this undefined activity does not appear to interfere with the IL-3-induced cell survival signaling pathway because PD164199 was unable to cause 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cell death in the presence of IL-3.

Several laboratories have identified point mutations in the tyrosine kinase domain of Bcr-Abl protein derived from CML patients with acquired resistance to imatinib.<sup>12-17</sup> It was demonstrated recently that these Bcr-Abl mutants could confer

resistance to imatinib in Baf3 cells harboring these mutants.<sup>16</sup> It will be of significant interest to determine if one or more of these imatinib-resistant Bcr-Abl mutants are sensitive to inhibition by PD164199, PD173952, PD173958 or PD180970.

PD164199, PD173952, PD173958 and PD180970 were originally identified as Src tyrosine kinase inhibitors.<sup>20,21</sup> Not surprisingly, our data show that they inhibit Lyn kinase. Interestingly, Csk, a negative regulator of Src family kinases, was inhibited by PD164199, PD173952, PD173958 and PD180970 at slightly higher drug concentrations (Table 1). Because Src family kinases are downstream of Csk in cell signaling and since these compounds are more potent as Src inhibitors, it is predicted that inhibition of Csk will not diminish the effectiveness of these compounds on blocking Src signaling in the cells.

Clinical studies have shown that a high proportion of CML patients do not have complete cytogenetic responses when treated with imatinib.<sup>7,9</sup> Several possibilities may be envi-



**Figure 3** IL-3 does not prevent inhibition of Bcr-Abl tyrosine kinase by Bcr-Abl tyrosine kinase inhibitors in 32Dp210<sup>Bcr-Abl</sup> and Baf3p210<sup>Bcr-Abl</sup> cells. 32Dp210<sup>Bcr-Abl</sup> (a) or Baf3p210<sup>Bcr-Abl</sup> cells (b) were plated in the presence or absence of IL-3 (10 ng/ml) and treated with PD180970 (100 nM), PD164199 (100 nM) or imatinib (1  $\mu$ M) for 10 h. p210<sup>Bcr-Abl</sup> was immunoprecipitated with an anti-Abl antibody and analyzed by immunoblotting with an anti-phosphotyrosine antibody (upper panels) or an anti-Abl antibody (lower panels). Arrows indicate the p210<sup>Bcr-Abl</sup> band.  $\alpha$ Ptyr, anti-phosphotyrosine antibody.  $\alpha$ Abl, anti-Abl antibody.

sioned to explain the incomplete cytogenetic response observed in clinical studies. For example, a small subpopulation of Bcr-Abl inhibitor-resistant leukemic cells may already be present in a CML patient prior to the drug treatment. Importantly, it was observed that primitive CD34<sup>+</sup>/Bcr-Abl<sup>+</sup> cells from CML patients have an IL-3 autocrine loop.<sup>19</sup> Experiments presented in this study illustrate that IL-3 can protect IL-3 responsive, Bcr-Abl-transformed hematopoietic progenitor cells from apoptosis caused by Bcr-Abl tyrosine kinase inhibitors, including imatinib. This finding raises the possibility that the IL-3 autocrine loop found in primitive CD34<sup>+</sup>/Bcr-Abl<sup>+</sup> cells could be one of the mechanisms responsible for incomplete eradication of Bcr-Abl<sup>+</sup> leukemic cells from CML patients by the Bcr-Abl tyrosine kinase inhibitor. If further studies with clinical samples support this notion, one could predict that stimulation of differentiation of Bcr-Abl<sup>+</sup> hematopoietic progenitor cells will lead to a better cytogenetic response to Bcr-Abl inhibition in CML patients.

Further studies are needed to identify the IL-3-dependent survival pathway and to evaluate feasibility of simultaneous inhibition of both IL-3-dependent and Bcr-Abl-dependent cell survival pathways for complete eradication of Bcr-Abl<sup>+</sup> cells from CML patients. In addition, since PD164199, PD173952, PD173958 and PD180970 are also potent Src inhibitors, our finding that IL-3 can protect 32Dp210<sup>Bcr-Abl</sup> and

Baf3p210<sup>Bcr-Abl</sup> cells from apoptosis induced by these compounds suggests that the IL-3-induced cell survival pathway is Src-independent.

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