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Selective pyrrolo-pyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr–Abl signal transduction and oncogenesis

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Chronic myelogenous leukemia (CML) is defined by the presence of the Philadelphia (Ph) chromosome, which results in the expression of the 210 kDa Bcr–Abl tyrosine kinase. Bcr-Abl constitutively activates several signaling proteins important for the proliferation and survival of myeloid progenitors, including the Src family kinases Hck and Lyn, the Stat5 transcription factor and upstream components of the Ras/Erk pathway. Recently, we found that kinase-defective Hck blocks Bcr–Abl-induced transformation of DAGM myeloid leukemia cells to cytokine independence, suggesting that activation of the Src kinase family may be essential to oncogenic signaling by Bcr–Abl. To investigate the contribution of Src kinases to Bcr–Abl signaling *in vivo*, we used the pyrrolo-pyrimidine Src kinase inhibitors PP2 and A-419259. Treatment of the Ph^+ CML cell lines K-562 and Meg-01 with either compound resulted in growth arrest and induction of apoptosis, while the Ph^- leukemia cell lines TF-1 and HEL were unaffected over the same concentration ranges. Suppression of Ph^+ cell growth by PP2 and A-419259 correlated with a decrease in Src kinase autophosphorylation. Both inhibitors blocked Stat5 and Erk activation, consistent with the suppressive effects of the compounds on survival and proliferation. In contrast, the phosphotyrosine content of Bcr–Abl and its endogenous substrate CrkL was unchanged at inhibitor concentrations that induced apoptosis, blocked oncogenic signaling and inhibited Src kinases. These data implicate the Src kinase family in Stat5 and Erk activation downstream of Bcr–Abl, and identify myeloid-specific Src kinases as potential drug targets in CML.
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Introduction

Chronic myelogenous leukemia (CML) is a human malignancy that affects hematopoietic progenitor cells and accounts for 15% of all adult leukemias (Sawyers, 1999). The clinical course of CML progresses through three phases, becoming more resistant to treatment in each successive phase. The initial chronic phase is associated with the clonal expansion of multiple myeloid lineages that retain the potential for normal differentiation. Within 4–6 years, patients enter an accelerated phase in which immature blast cells begin to accumulate in the blood. Patients inevitably progress to blast crisis, which is characterized by the rapid and uncontrolled expansion of bone marrow progenitor cells and is ultimately fatal.

The cytogenetic hallmark of CML is the Philadelphia chromosome (Ph^+), which arises from a translocation between the c-*abl* locus on chromosome 9 and the *bcr* locus on chromosome 22 (Nowell and Hungerford, 1960; Rowley, 1973). The Ph^+ translocation results in the expression of Bcr–Abl, a 210 kDa oncogenic fusion protein with constitutive tyrosine kinase activity. Bcr–Abl transforms both fibroblasts and hematopoietic cells in culture and causes a CML-like condition in mouse models, providing strong evidence that it is directly responsible for disease initiation (Lugo and Witte, 1989; McLaughlin *et al*., 1987; Daley *et al*., 1990).

Bcr–Abl has been shown to constitutively activate numerous signal transduction pathways that are normally regulated by growth factors and cytokines in hematopoietic cells. Activation of these pathways can lead to growth factor independence, increased proliferation, altered differentiation, and resistance to apoptosis. For example, tyrosine phosphorylated Bcr–Abl can directly interact with the Grb-2/Sos guanine nucleotide exchange factor (GEF) complex, providing a link to Ras/Erk activation (Pendergast *et al*., 1993; Puil *et al*., 1994; Goga *et al*., 1995; Cortez *et al*., 1996). Bcr–Abl can also activate Ras through the Shc adaptor protein, which links many growth factor and cytokine receptors to Grb-2/Sos (Goga *et al*., 1995). A related adaptor protein, CrkL, is constitutively phosphorylated in Ph^+ cells and may also provide a link to several small-G-protein signaling pathways including Ras (Feller, 2001; Sattler and Salgia, 1998). Bcr–Abl directly binds to the p85

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subunit of phosphatidylinositol 3-kinase (PI-3K), which leads to activation of Akt downstream and subsequent survival signaling (Skorski *et al*., 1997; Neshat *et al*., 2000). Bcr–Abl also induces constitutive activation of Stat transcription factors, particularly Stat5 (Carlesso *et al*., 1996; Nieborowska-Skorska *et al*., 1999; Shuai *et al*., 1996; de Groot *et al*., 1999, 2000; Sillaber *et al*., 2000; Gesbert and Griffin, 2000; Hoover *et al*., 2001). Stat5, in turn, upregulates transcription of several genes necessary for the growth and anti-apoptotic effects observed in CML cells, including Bcl-X_L and cyclin-D1 (de Groot *et al*., 2000; Gesbert and Griffin, 2000).

Although Bcr–Abl possesses a constitutively active tyrosine kinase domain, recent work suggests that it may initiate signaling by activating other non-receptor tyrosine kinases, particularly members of the Src family. Bcr–Abl binds both Hck and Lyn following expression in the murine 32D myeloid cell line and in primary CML cells, and this association correlates with increased activity of these myeloid Src family members (Danhauser-Riedl *et al*., 1996). Moreover, Hck phosphorylates Bcr–Abl on tyrosine 177 (Y177) within the Bcr region, providing a docking site for the Grb2 SH2 domain and thus a possible link to the Ras pathway (Warmuth *et al*., 1997). Recent work from our laboratory has shown that Bcr–Abl can bind to the SH2 and SH3 domains of Hck through kinase-dependent and kinase-independent mechanisms, respectively (Lionberger *et al*., 2000). Furthermore, several regions of the Abl portion of Bcr–Abl were found to bind Hck, Lyn and Fyn through the Abl SH2, SH3, and kinase domains as well as the distal portion of the Abl C-terminal tail (Lionberger *et al*., 2000; M Wilson and T Smithgall, unpublished data). Lastly, a kinase-defective mutant of Hck blocked Bcr–Abl-induced transformation of the murine myeloid cell line DAGM to cytokine independence, suggesting that Src family kinase activation may be necessary for Bcr–Abl transformation signaling (Lionberger *et al*., 2000).

Because Bcr–Abl is necessary for the initiation and maintenance of the CML phenotype and is not found in normal cells, it represents an ideal target for specific small-molecule drug therapy. The novel tyrosine kinase inhibitor, STI-571 (Gleevec), potently inhibits Bcr–Abl kinase activity, induces apoptosis in Ph^+ cells, and reduces tumor formation by Bcr–Abl-transformed cells *in vivo* (Druker *et al*., 1996). STI-571 is a 2-phenylaminopyrimidine that acts as a competitive inhibitor for the ATP binding pocket within the kinase region of Bcr–Abl (Schindler *et al*., 2000; Nagar *et al*., 2002). Previous studies have shown that STI-571 selectively inhibits Ph^+ leukemia cells, leaving normal cells largely unaffected (Druker *et al*., 1996; Oetzel *et al*., 2000; Waller *et al*., 2000). A phase I study with STI-571 showed that 51 of 53 patients with chronic phase disease exhibited complete hematological remission after 265 days (Druker *et al*., 2001). A larger, more recent study confirmed these initial findings, showing that the drug induces high rates of cytogenetic and hematological responses in chronic phase CML patients (Kantarjian *et al*., 2002). Recent phase II studies show that STI-571 is also effective in CML accelerated phase and blast crisis, although some individuals relapse with drug-resistant disease (Talpaz *et al*., 2002; Sawyers *et al*., 2002). These studies provide important evidence that targeting an oncogenic signaling protein with a highly selective inhibitor is very effective in the management of cancer.

Although STI-571 has shown promise in chronic phase CML, patients with more advanced disease can develop resistance to the drug and relapse. Initial findings by Gorre *et al*. (2001) showed that patients resistant to STI-571 had either a single amino acid substitution in the kinase domain of Bcr–Abl which rendered it unable to bind to the drug, or developed resistance through progressive Bcr–Abl gene amplification. More recent studies have reported a wider range of Abl kinase domain mutations, many of which map to the ATP binding region (Branford *et al*., 2002; Roumiantsev *et al*., 2002). Other groups have found that Bcr–Abl gene and protein levels are increased in cells resistant to STI-571 (Weisberg and Griffin, 2000) and that plasma proteins in a murine model can inactivate the drug (Gambacorti-Passerini *et al*., 2000).

The identification of additional therapeutic targets remains critical in improving the long-term survival of patients with CML, particularly those in the accelerated phase or blast crisis. Our recent work suggests that Src family kinases play a role in Bcr–Abl-mediated transformation and may serve as potential drug targets for blocking CML progression. In the present study, we show that the Src kinase inhibitors PP2 and A-419259 block Ph^+ leukemia cell proliferation and induce apoptosis, but do not affect the growth or survival of Ph^- myeloid cell lines. Moreover, we show that these cellular effects are due to disruption of Src family kinase activity and not direct inhibition of Bcr–Abl. Finally, we observed that inhibition of Src family kinases correlates with the suppression of Ras/Erk signaling and Stat5 activation. These data provide new evidence that Src kinases expressed in myeloid cells, such as Hck, Lyn, Fyn and Fgr, are essential intermediates coupling Bcr–Abl to Stat and Ras/Erk signaling and represent rational targets for anti-CML therapy either alone or in combination with STI-571.

Results

The Src family kinase inhibitors, PP2 and A-419259, block proliferation and induce apoptosis in Ph^+ cells

Recent studies have shown that a large percentage of patients in the blast crisis phase of Ph^+ leukemia develop resistance to the anti-CML drug and Bcr–Abl inhibitor STI-571, highlighting the need for additional therapeutic approaches (Warmuth *et al*., 1999). Previous work from our laboratory and others suggests that the Src family of tyrosine kinases plays an essential role in Bcr–Abl-mediated transformation and CML progression and may prove to be an attractive therapeutic target (Lionberger *et al*., 2000; Warmuth *et al*., 1997; Danhauser-Riedl *et al*., 1996). To test this hypothesis, we used the pyrrolo-pyrimidine Src kinase inhibitors, PP2 and A-419259. PP2 was

initially described as a potent inhibitor of the Src family of tyrosine kinases (Hanke *et al*., 1996), with lower activity toward Jak2 and the EGF receptor kinase and virtually no activity toward Ser/Thr kinases. To determine the effects of Src inhibition on CML cell growth, the Ph^+ myeloid leukemia cell lines K-562 (Klein *et al*., 1976; Lozzio *et al*., 1981) and Meg-01 (Ogura *et al*., 1985) were treated with various concentrations of PP2 and cell proliferation was measured using a 96-well plate-based assay. Figure 1 shows that incubation with PP2 resulted in a dose-dependent decrease in cell proliferation in both K-562 and Meg-01 cells over the 4 day incubation period. Specifically, K-562 cell proliferation was inhibited by PP2 with an IC_{50} of approximately 10 μM (Figure 1a). Similarly, PP2 inhibited cellular proliferation of Meg-01 cells with an IC_{50} between 3 and 10 μM (Figure 1b).

Since PP2 has been reported to inhibit other tyrosine kinases at higher concentrations (Blake *et al*., 2000; Hanke *et al*., 1996), we sought to confirm these results with a more specific Src family kinase inhibitor. A-419259 is a second-generation pyrrolo-pyrimidine designed to enhance selectivity towards the Src family relative to other cytoplasmic tyrosine kinases, similar in design to those reported by Arnold *et al*. (2000). Importantly, A-419259 exhibited greater than 1000-fold selectivity towards Lyn and Lck compared to c-Abl in an *in vitro* kinase assay (Table 1), suggesting that this compound would allow us to assess the effect of Src family kinase inhibition on CML cell growth without direct effects on Bcr–Abl. Surprisingly, A-419259 inhibited the growth of the same CML cell lines with a 30- to 100-fold increase in potency compared to PP2. Specifically, A-419259 inhibited K-562 cells with an IC_{50} between 0.1 and 0.3 μM (Figure 1c), and Meg-01 proliferation with an IC_{50} of approximately 0.1 μM (Figure 1d).

To determine if Src inhibitor-induced growth arrest was specific to CML cells, the Ph^- GM-CSF-dependent monocytic precursor cell line, TF-1 (Kitamura *et al*., 1989), and the Ph^- erythroleukemic cell line, HEL (Martin and Papayannopoulou, 1982), were tested in the same assay. PP2 had no effect on the proliferation of TF-1 cells at 10 μM, although growth was slightly diminished in the presence of 30 μM PP2. However, inhibition was only observed at this high PP2 concentration and was significantly less than that of either Ph^+ cell line (Figure 2a). PP2 had no effect on HEL cell proliferation over the entire concentration range (Figure 2b). A-419259 was also without effect on

Table 1 Comparison of IC_{50} values of A-419259 for the Src family members Src, Lck, and Lyn with c-Abl and PKC

Kinase	*IC_{50}, (μM)*
Src	0.009
Lck	<0.003
Lyn	<0.003
Abl	3.0
PKC	>33

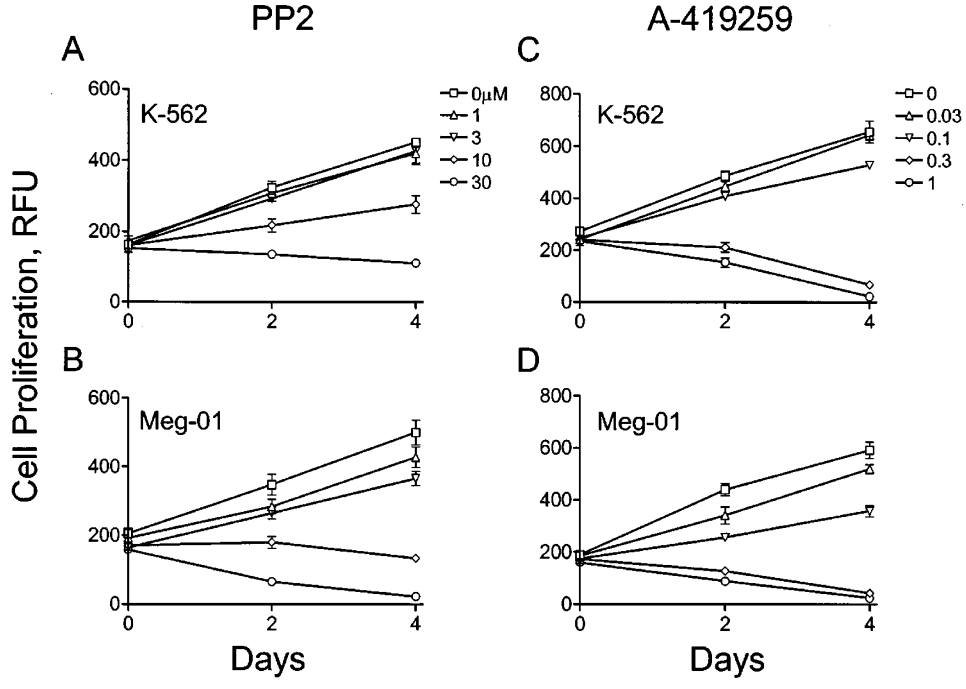

Figure 1 The pyrrolo-pyrimidine Src family kinase inhibitors PP2 and A-419259 block Ph^+ leukemia cell proliferation in a dose-dependent fashion. K-562 (**a**,**c**) and Meg-01 (**b**,**d**) cells were treated with the indicated concentrations of PP2 (**a**,**b**) or A-419259 (**c**,**d**) over the course of 4 days. On days 0, 2, and 4, viable cell counts were determined using the fluorescent microplate assay described under Materials and methods. Five wells were plated for each time and inhibitor concentration, and the relative mean fluorescence is shown in arbitrary units (RFU)$\pm$s.d. Growth curves were repeated at least three times with comparable results and a representative experiment is shown

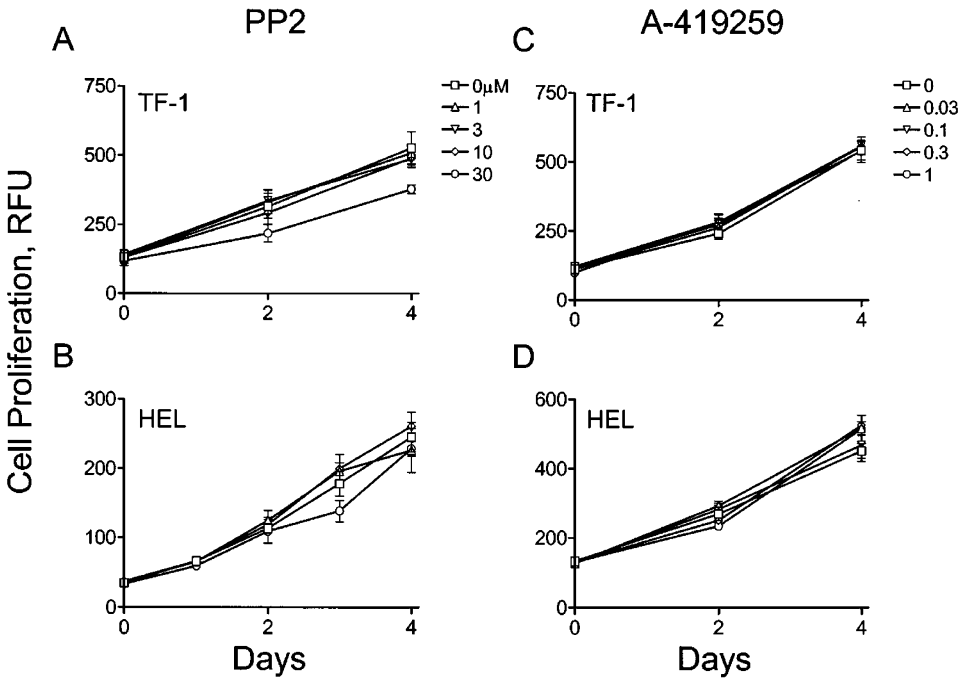

Figure 2 PP2 and A-419259 do not markedly affect proliferation of Ph^- myeloid leukemia cells. TF-1 (**a**,**c**) and HEL (**b**,**d**) cells were treated with the indicated concentrations of PP2 (**a**,**b**) or A-419259 (**c**,**d**) over the course of 4 days. On days 0, 2, and 4, viable cell counts were determined using the fluorescent microplate assay described under Materials and methods. Five wells were plated for each time and inhibitor concentration, and the relative mean fluorescence is shown in arbitrary units (RFU) $\pm$ s.d. Growth curves were repeated at least three times with comparable results and a representative experiment is shown

either TF-1 or HEL cell growth at the highest dose tested (1 μM; Figure 2c,d, respectively).

We next investigated whether the dose-dependent inhibition of Ph^+ cell proliferation by Src family kinase inhibitors was associated with the induction of apoptosis. To answer this question, K-562 (Ph^+) and TF-1 (Ph^-) cells were treated in parallel with various concentrations of each Src inhibitor over the course of 4 days and apoptosis was measured using fluorescence-activated cell sorting assays for annexin-V binding and caspase-3 activation. Measurable induction of apoptosis in K-562 cells was evident 24 h after incubation with either inhibitor (data not shown) and increased over the 3-day time course. Figure 3 shows the results of PP2 and A-419259 treatment of K-562 cells after 72 h. Treatment with PP2 increased the apoptotic population in a dose-dependent fashion to a maximum of 67% at the highest dose tested (30 μM; Figure 3a, top). The caspase-3 activation assay correlated well with these findings, ranging from 8% in untreated cells to 48% at 30 μM (Figure 3a, bottom). A-419259 also potently induced apoptosis in K-562 cells beginning at 0.1 μM and increasing in a dose-dependent manner (Figure 3b). In contrast, neither compound induced appreciable apoptosis in TF-1 cells at 72 h, although there was a small increase in the apoptotic population at 30 μM PP2 (Figure 4). This finding is consistent with the small growth suppression observed with this high PP2 concentration (Figure 2a), and may result from the non-specific inhibition of Jak2 or other tyrosine kinases linked to the GM–CSF receptor in this cell line.

PP2 and A-419259 inhibit Src family kinase activity in Ph^+ leukemia cells at concentrations that causes growth arrest and apoptosis

We next determined the effects of PP2 and A-419259 on Src family kinase activity in the CML cell lines shown to respond to these inhibitors. Cells were treated for 20 h with the same inhibitor concentrations used in the proliferation and apoptosis assays. Lysates from control and treated cells were then probed with an antibody specific for the active, tyrosine-phosphorylated forms of myeloid Src family members. Using recombinant Src family kinase proteins, we found that this antibody specifically recognizes the autophosphorylated activation loop peptide sequence found in Hck, Lyn, Fyn, and Fgr, the predominant members of the Src family expressed in myeloid cells (data not shown). Therefore, this antibody allowed us to assay the effects of the inhibitors on the entire population of Src family kinases within the cells simultaneously. Figure 5a shows that PP2 inhibited Src kinase autophosphorylation in both Ph^+ cell lines (K-562 and Meg-01) with an IC_{50} between 3 and 10 μM, while A-419259 blocked kinase activation between 0.1 and 0.3 μM. This dose-response for Src kinase inhibition closely correlates with the effects of both agents on CML cell growth and survival (Figures 1 and 3).

As a control, Ph^- TF-1 cells were treated with the same concentrations of PP2 and A-419259 under identical conditions (Figure 5a). Although both compounds inhibited Src family kinase autophos-

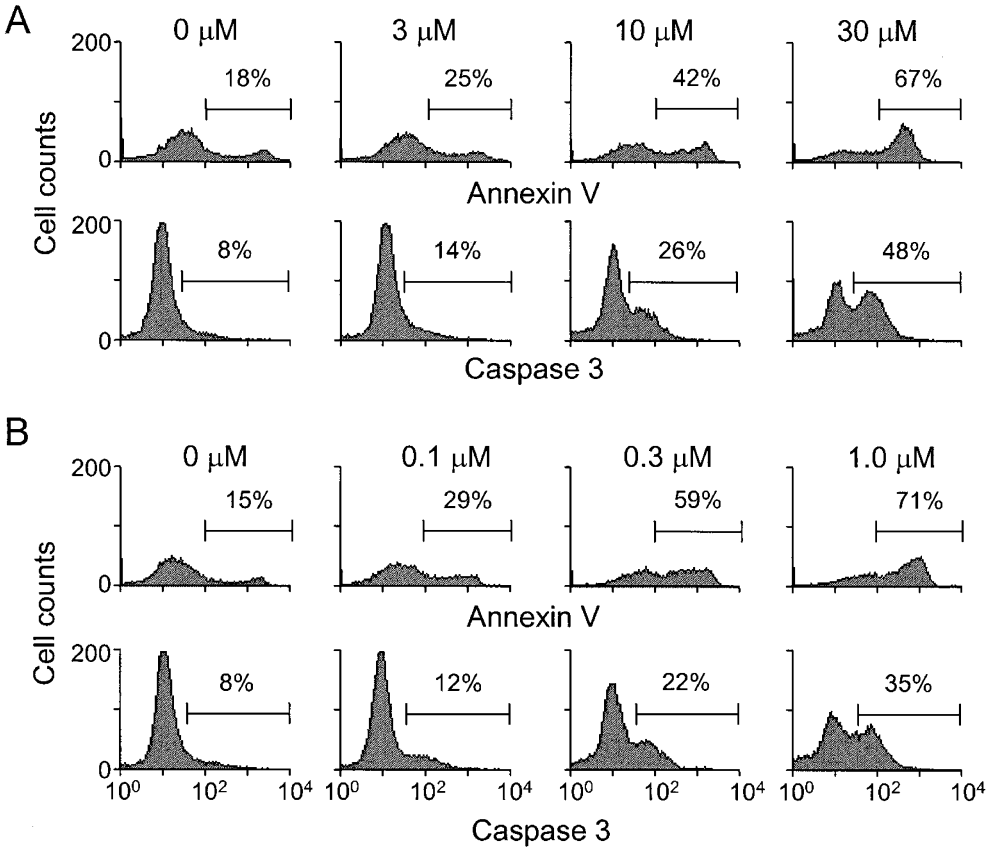

Figure 3 PP2 and A-419259 induce apoptosis in Ph^+ leukemia cell lines. K-562 cells were incubated for 72 h with the indicated micromolar concentrations of PP2 (**a**) or A-419259 (**b**) and apoptosis was measured by either annexin-V binding, or caspase-3 activity using flow cytometry as indicated. Per cent apoptotic cells are shown above the bar in each panel. Experiments were performed at least twice and produced comparable results in each case; a representative assay is shown

phorylation in TF-1 cells with about the same potency observed in the Ph^+ leukemia cell lines, the growth suppressive and apoptotic effects were strictly limited to the Ph^+ leukemia cells. These results strongly suggest that CML cells are dependent on Src family kinase activity for growth and survival, whereas cytokine-dependent cells such as TF-1 are not.

We next determined the effects of PP2 and A-419259 on Bcr–Abl phosphotyrosine content in treated and untreated CML cells. K-562 and Meg-01 cells were treated overnight with the same concentrations of PP2 or A-419259 used in the proliferation and apoptosis assays. Lysates from the cells were then probed using an anti-phosphotyrosine antibody, which readily detected the 210 kDa Bcr–Abl phosphoprotein. Figure 5b shows that inhibitor concentrations that caused growth arrest and apoptosis had little impact on Bcr–Abl phosphotyrosine content. PP2 inhibited cell proliferation in K-562 cells with an IC_{50} of approximately 10 μM (Figure 1a). Bcr–Abl remains tyrosine phosphorylated to nearly the same extent at this inhibitor concentration compared to untreated control cells when Bcr–Abl protein levels are taken into account (Figure 5b, top left). Similarly, Meg-01 cell growth was inhibited by PP2 with an IC_{50} between 3 and 10 μM, and Bcr–Abl remains tyrosine phosphorylated within this dose range (Figure 5b, bottom left). At the highest concentration of PP2 tested (30 μM), Bcr–Abl phosphotyrosine content was reduced compared to untreated cells. Similar results were obtained with A-419259, although a small reduction in Bcr–Abl phosphotyrosine content was observed at lower concentrations of this compound. However, whether these effects were due to direct inhibition of Bcr–Abl kinase activity or inhibition of transphosphorylation of Bcr–Abl by Src kinases remains to be determined (see Discussion).

The Bcr region of Bcr–Abl is phosphorylated on Tyr 177 and possibly other non-regulatory sites (Puil *et al*., 1994; Pendergast *et al*., 1993; Liu *et al*., 1993), and these phosphorylation events may be due to transphosphorylation by Hck in addition to Bcr–Abl autokinase activity (Warmuth *et al*., 1997). Thus, changes in overall Bcr–Abl phosphotyrosine content may not necessarily reflect direct actions of PP2 or A-419259 on the Bcr–Abl kinase domain *in vivo*. Therefore, we investigated tyrosine phosphorylation of the CrkL adaptor protein as an additional measure of Bcr–Abl kinase activity in the inhibitor-treated cultures. CrkL is a direct substrate for Bcr–Abl in CML cells and serves as useful endogenous reporter of Bcr–Abl tyrosine kinase activity (Senechal *et al*., 1996;

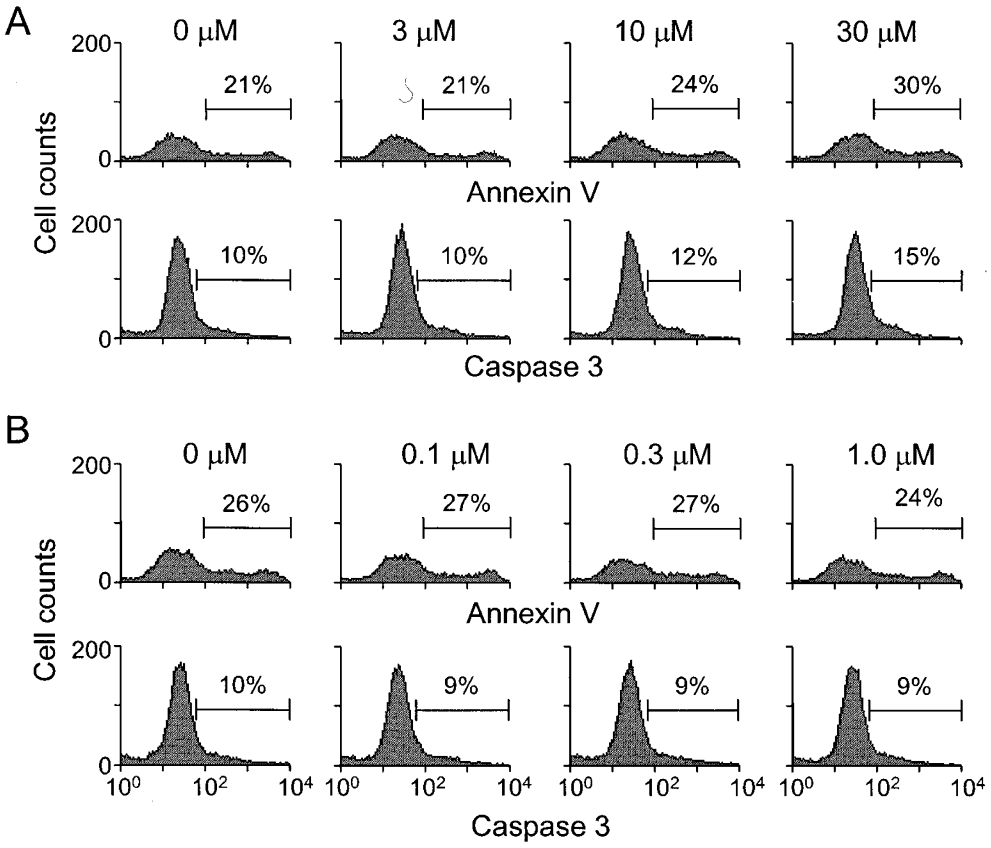

Figure 4 PP2 and A-419259 do not induce apoptosis in Ph^- myeloid leukemia cells. TF-1 cells were incubated for 72 h with the indicated micromolar concentrations of PP2 (**a**) or A-419259 (**b**) and apoptosis was measured by either annexin-V binding, or caspase-3 activity using flow cytometry as indicated. Per cent apoptotic cells are shown above the bar in each panel. Experiments were performed at least twice and produced comparable results in each case; a representative assay is shown

Sattler and Salgia, 1998; Gorre *et al*., 2001). When phosphorylated by Bcr–Abl, CrkL migrates more slowly on SDS-polyacrylamide gels, providing a rapid assay for its phosphorylation status (Gorre *et al*., 2001). Using this method, we probed the lysates from inhibitor-treated K-562 and Meg-01 cells for phosphorylation-induced changes in CrkL migration. Interestingly, 10 μM PP2 did not affect the levels of phospho-CrkL in either Ph^+ cell line, suggesting that Bcr–Abl remained active in the presence of this inhibitor. At 30 μM PP2, CrkL phosphorylation was reduced in both cell lines, although the levels of phosphorylated CrkL remained higher than those observed following direct inhibition of Bcr–Abl with STI-571 (Figure 6a). Similarly, A-419259 did not affect CrkL phosphorylation in either cell line at the fully growth-inhibitory concentration of 0.3 μM (Figure 6b), although phosphorylation was partially reduced at the highest concentration tested (1.0 μM). Most importantly, CrkL remained significantly phosphorylated in both Ph^+ cell lines in the presence of PP2 and A-419259 concentrations that caused growth arrest and induction of apoptosis, suggesting that the growth-inhibitory and pro-apoptotic effects of these compounds are primarily due to the inhibition of Src family kinases. However, we cannot exclude the possibility that high concentrations of these compounds may inhibit both Src kinases and Bcr–Abl, or that inhibition of Src kinases indirectly reduces CrkL phosphorylation by affecting Bcr–Abl kinase activity (see Discussion).

A-419259 inhibits proliferation of myeloid cells following transformation with Bcr–Abl

Data presented so far demonstrate that CML-derived leukemia cell lines are sensitive to growth arrest and apoptosis induced by PP2 and A-419259, whereas Ph^- cell lines are not. This observation suggests that transformation by Bcr–Abl may sensitize myeloid cells to the anti-proliferative and apoptotic effects of Src kinase inhibitors. To test this idea, we transformed the IL-3-dependent myeloid leukemia cell line DAGM with a p210 Bcr–Abl retrovirus (DAGM/Bcr–Abl) or a drug selection marker as a negative control (DAGM/Neo). As observed previously (Lionberger *et al*., 2000; Goga *et al*., 1995), the DAGM/Bcr–Abl cells grew in the absence of IL-3, exhibited increased cellular protein tyrosine phosphorylation, and demonstrated enhanced Stat5 tyrosine phosphorylation (data not shown). Both populations of DAGM cells were then incubated with A-419259 and proliferation was measured over a 4-day

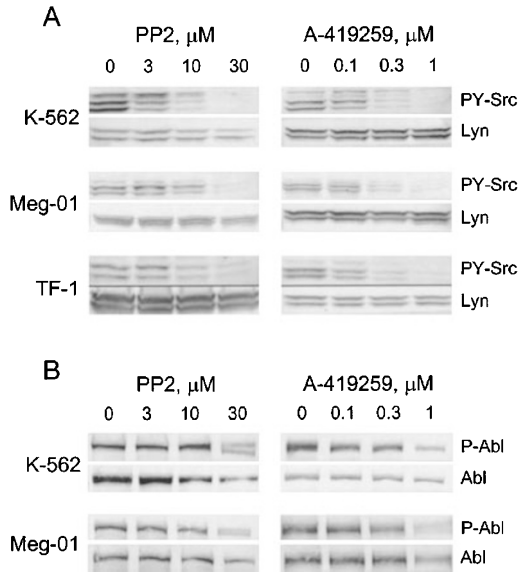

Figure 5 PP2 and A-419259 inhibit Src family kinase activity but not Bcr–Abl tyrosine phosphorylation at concentrations that inhibit cell growth and induce apoptosis. (**a**) The Ph^+ cell lines K-562 and Meg-01 and Ph^- cell line TF-1 were incubated with the indicated concentrations of PP2 (left) or A-419259 (right) for 20 h. Cell lysates were prepared and probed with an antibody specific for the autophosphorylated, active forms of Src family kinases via immunoblotting (PY-Src). Duplicate blots were probed for Lyn expression as a loading control (Lyn). (**b**) K-562 and Meg-01 cells were treated with PP2 (left) or A-419259 (right) and Bcr–Abl phosphotyrosine content was measured via immunoblotting with an anti-phosphotyrosine antibody (P-Tyr) or an Abl antibody as a loading control (Abl). Experiments were repeated at least three times with similar results and a representative experiment is shown

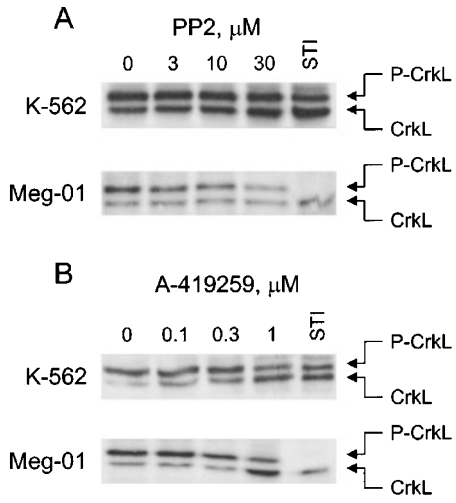

Figure 6 Phosphorylation of the endogenous Bcr–Abl substrate CrkL is not affected by growth-inhibitory concentrations of PP2 or A-419259. The Ph^+ leukemia cell lines K-562 and Meg-01 were treated for 20 h with the indicated concentrations of PP2 (**a**) or A-419259 (**b**) and cell lysates were probed for CrkL by immunoblotting. Cells were also treated with the Bcr–Abl inhibitor STI-571 (5 μM) as a positive control. The upper, slower migrating band represents the tyrosine phosphorylated form of CrkL (P-CrkL), while the lower band represents the unphosphorylated form (CrkL) (Gorre *et al*., 2001). Experiments were repeated at least three times with comparable results

period either with or without IL-3. As shown in Figure 7, A-419259 strongly inhibited DAGM/Bcr–Abl cell proliferation in the absence of IL-3 with an IC_{50} between 0.1 and 0.3 μM. This result is strikingly similar to that observed with K-562 and Meg-01 CML cells (Figure 1). Interestingly, when the DAGM/Bcr–Abl cells were incubated with the same A-419259 concentrations in the presence of IL-3, the effect of the inhibitor was partially reversed. Finally, A-419259 had no effect on the growth of the untransformed DAGM/Neo cells, consistent with results obtained from HEL and TF-1 cells. These results suggest that Bcr–Abl-induced proliferation and survival of DAGM cells requires Src kinase activity, whereas IL-3-dependent growth and survival does not.

We next assessed Src kinase activity in the DAGM cells. DAGM/Bcr–Abl cells were treated overnight with various concentrations of A-419259 either in the presence or absence of IL-3 and cell lysates were probed for activated Src family kinases with phosphospecific antibodies as described in Figure 5. Incubation with A-419259 caused a partial inhibition of Src kinases at 0.1 μM and nearly complete inhibition at 0.3 μM (Figure 8a, top). In contrast to the Src kinases, only modest changes in Bcr–Abl phosphotyrosine content were observed at these A-419259 concentrations, although both the expression and phosphorylation of Bcr–Abl were diminished at 1 μM (Figure 8a, bottom). Nearly identical patterns of Src family kinase inhibition were observed in the presence of IL-3 (Figure 8b), suggesting that the compensatory effects of IL-3 on cellular sensitivity to the inhibitor were not due to Src reactivation. Similarly, Bcr–Abl phosphorylation in the presence of IL-3 was nearly identical to that of cells grown without cytokine (Figure 8b, bottom). As a control, Src family kinase activity was measured in the DAGM/Neo cells after incubation with A-419259. The activated Src kinases present in these cells were partially inhibited at 0.3 μM and displayed complete inhibition at 1 μM (Figure 8c). These results show that A-419259 inhibits the Src kinases present in both DAGM cell populations, but the growth-suppressive effect of this inhibitor is limited to DAGM cells expressing Bcr–Abl in the absence of IL-3.

PP2 and A-419259 block Stat5 and Erk activation in Ph^+ leukemic cells

We next used PP2 and A-419259 to assess the contribution of the Src kinase family to the activation of growth and survival pathways downstream of Bcr–Abl. Several recent studies have shown that constitutive activation of Stat5 contributes to Bcr–Abl-induced leukemogenesis through upregulation of Bcl-X_L and cyclin-D1, and possibly other survival and proliferative genes (de Groot *et al*., 2000; Hoover *et al*., 2001; Gesbert and Griffin, 2000). Moreover, other work has implicated small G protein/MAP kinase pathways in Bcr–Abl signaling, including the Ras/Erk

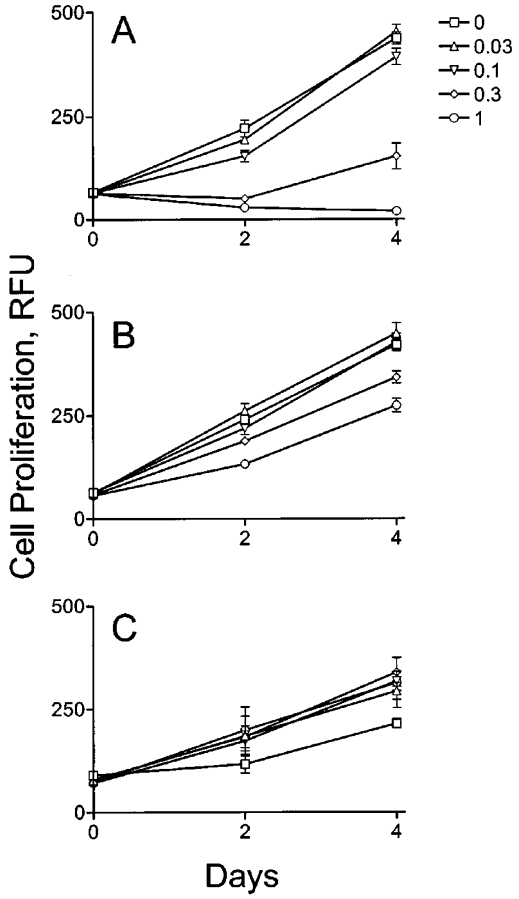

Figure 7 Transformation with Bcr–Abl sensitizes DAGM myeloid leukemia cells to growth arrest by A-419259. The IL-3-dependent myeloid leukemia cell line DAGM was transformed to cytokine independence by infection with a recombinant Bcr–Abl retrovirus (DAGM/Bcr–Abl). DAGM cells infected with a retrovirus carrying only a drug selection marker served as a negative control (DAGM/Neo). DAGM/Bcr–Abl cells were treated with the indicated concentrations of A-419259 in the absence (**a**) or presence of IL-3 (**b**) and proliferation was measured over the course of 4 days. The DAGM/Neo control population was similarly treated in the presence of IL-3 (**c**); these cells undergo rapid apoptosis in the absence of IL-3 (data not shown). On days 0, 2, and 4, viable cell counts were determined using the fluorescent microplate assay described under Materials and methods. Five wells were plated for each time and inhibitor concentration, and the relative mean fluorescence is shown in arbitrary units (RFU) $\pm$ s.d. Growth curves were repeated at least three times with comparable results and a representative experiment is shown

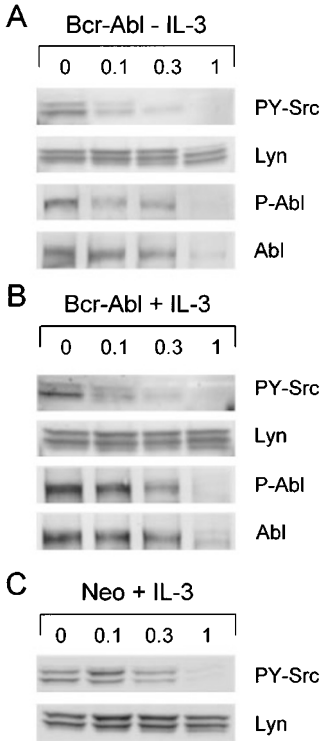

Figure 8 A-419259 inhibits Src kinase activity but not Bcr–Abl autophosphorylation at growth-inhibitory concentrations in Bcr–Abl-transformed DAGM cells. DAGM/Bcr–Abl cells were treated with the indicated concentrations of A-419259 in the absence (**a**) or presence (**b**) of IL-3, and cell lysates were prepared and probed with an antibody specific for the autophosphorylated, active forms of Src family members via immunoblotting (PY-Src). Duplicate blots were probed for Lyn expression as a loading control (Lyn). Bcr–Abl phosphotyrosine content was determined by immunoblotting with an anti-phosphotyrosine antibody (P-Tyr) and with an Abl antibody as a loading control (Abl). (**c**) DAGM/Neo cells were treated with A-419259 in the presence of IL-3 and lysates were probed with Src phosphospecific and Lyn antibodies as in **a**. Each experiment was repeated at least twice with comparable results

(Puil *et al*., 1994; Pendergast *et al*., 1993; Goga *et al*., 1995; Sawyers *et al*., 1995) and c-Jun kinase (Jnk) pathways (Raitano *et al*., 1995). Other studies have shown that Src family kinases can also activate these pathways directly in other cellular contexts (McGlade *et al*., 1992; Li and Smithgall, 1998; Turkson *et al*., 1998, 1999). Therefore, we focused our attention on Stat5 and Ras/Erk signaling as possible effectors for Src kinases downstream of Bcr–Abl.

An electromobility shift assay (EMSA) was used to examine DNA binding of activated Stat5 (Skorski *et al*., 1998). K-562 cells were treated overnight with concentrations of either PP2 or A-419259 shown above to cause growth arrest and induce apoptosis. Nuclear extracts were prepared and incubated with a radio-labeled DNA probe containing the FcγR1 promoter GAS consensus sequence for Stat5 binding. Both PP2 and A-419259 potently inhibited Stat5/DNA complex formation (Figure 9a). PP2 partially inhibited complex formation at 3 μM, with complete inhibition at 10 μM, while A-419259 disrupted the DNA/Stat5 complex partially at 0.1 μM and completely at 0.3 μM. Figure 9b shows that both compounds also inhibited Stat5 tyrosine phosphorylation, consistent with the gel-shift data. Importantly, inhibition of both the DNA-binding and tyrosine phosphorylation of Stat5 correlates very well with inhibition of Src family kinases but not Bcr–Abl. This result supports the idea that Src kinases couple Bcr–Abl to Stat5 activation *in vivo*. Neither compound affected Stat5 DNA binding or tyrosine phosphorylation in Ph$^-$ TF-1 cells, suggesting that Src kinases are not involved in Stat5

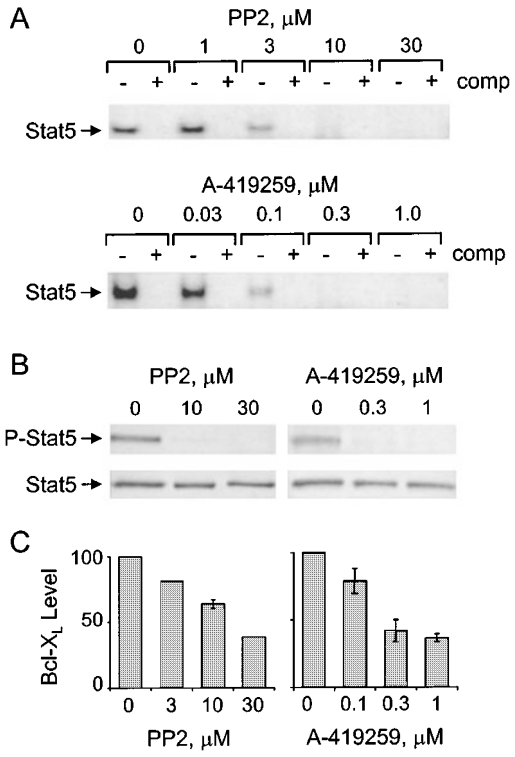

Figure 9 PP2 and A-419259 inhibit Stat-5 activation and Bcl-X_L expression in CML cells. K-562 cells were treated with the indicated concentrations of PP2 and A-419259 for 20 h. (**a**) Nuclear extracts were prepared and tested for the presence of activated Stat5 by electromobility shift assay (EMSA) using a ^{32}P-labeled probe based on the γ-activation sequence (GAS) as described under Materials and methods. To test probe specificity, reactions were incubated in the presence (+) or absence (−) of a 100-fold molar excess of unlabeled oligonucleotide probe. (**b**) Stat5 tyrosine phosphorylation was assessed by immunoprecipitation of Stat5 from clarified cell lysates and immunoblotting with anti-phosphotyrosine antibodies (P-Tyr). Aliquots of the immunoprecipitates were also blotted with anti-Stat5 antibodies to ensure equivalent loading in each lane (Stat5). (**c**) To analyse Bcl-X_L expression, total RNA was isolated and subjected to ribonuclease protection assay using a Bcl-2 family probe set as described under Materials and methods. The intensity of the Bcl-X_L signals were measured by storage phosphor imaging and normalized to levels of the GAPDH housekeeping gene. Normalized Bcl-X_L levels are presented as per cent of maximum signal observed in the absence of inhibitor treatment. The experiment was repeated twice and the mean Bcl-X_L levels ± s.d. are indicated

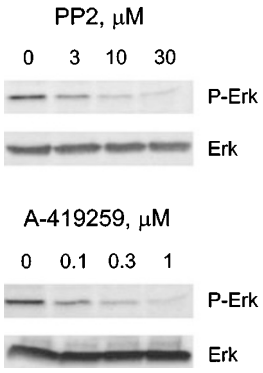

Figure 10 PP2 and A-419259 inhibit the Ras/Erk pathway in CML cells. K-562 cells were treated with the indicated concentrations of PP2 (top) or A-419259 (bottom) for 20 h. Cell lysates were prepared and analysed for the presence of active Erk by immunoblotting with phosphospecific antibodies (P-Erk). Duplicate blots were probed with antibodies to Erk2 protein as a loading control (Erk). Experiments were repeated three times with comparable results

activation in response to GM–CSF treatment in this cell line (data not shown).

As mentioned above, Stat5 regulates transcription of several genes involved in deregulated survival of CML leukemia cells. Bcl-X_L is a member of the Bcl-2 family of anti-apoptotic genes and has been shown to be upregulated by Stat5 in Ph$^+$ cells (de Groot *et al*., 2000). Since the Src inhibitors blocked Stat5 activation, we predicted that one or more members of the Bcl-2 family, particularly Bcl-X_L, would also be affected. To test this prediction, K-562 cells were treated overnight with either PP2 or A-419259 and gene expression was determined using an RNase protection assay with probes specific for Bcl-X_L and other members of the Bcl-2 gene family. Both PP2 and A-429159 caused a dose-dependent decrease in Bcl-X_L expression in K-562 cells with concentrations of compound that correlated well with Stat5 inhibition (Figure 9c). Moreover, the effects of the inhibitors appeared to be limited to Bcl-X_L expression and did not affect Bcl-2 (data not shown). Neither inhibitor had an effect on Bcl-2 family gene expression in TF-1 cells, consistent with the lack of inhibitor action on Stat5 activation in this cell line (data not shown).

To determine if the Src inhibitors affected constitutive activation of the Ras pathway in CML cells, we examined Erk activation using phosphospecific antibodies. K-562 cells were treated overnight with each Src inhibitor, and the cellular lysates were probed for activated Erk using phosphospecific antibodies. Growth inhibitory concentrations of both Src inhibitors caused a dose-dependent decrease in Erk phosphorylation, suggesting that down-regulation of this pathway may contribute to the growth suppressive actions of these compounds as well (Figure 10).

Discussion

Previous work from our laboratory and others has implicated the Src tyrosine kinase family in Bcr–Abl-mediated transformation of myeloid cells (Lionberger *et al*., 2000; Danhauser-Riedl *et al*., 1996; Warmuth *et al*., 1997). In the present study, we have demonstrated a requirement for Src kinase activity in Bcr–Abl transformation and oncogenic signal transduction using a pharmacological approach. The Src-selective pyrrolo-pyrimidines PP2 and A-419259 blocked Ph$^+$ CML cell growth and induced apoptosis in a dose-dependent manner, but had little or no effect on Ph$^-$ myeloid cells. Growth arrest and apoptosis correlated with inhibition of Src kinase activity at drug concentrations that did not markedly affect Bcr–Abl kinase activity. Transformation of IL-3-dependent myeloid

cells with Bcr–Abl sensitized them to the effects of A-419259, while the untransformed parent cells were unaffected. The anti-proliferative actions of both inhibitors correlated with a decrease in Stat5 and Erk activation, but did not impact the tyrosine phosphorylation of the direct Bcr–Abl substrate, CrkL. These results strongly support the hypothesis that Src family kinases are necessary for Bcr–Abl signal transduction as well as the proliferation and survival of CML cells.

The anti-proliferative actions of Src-selective inhibitors on K-562, Meg-01, and DAGM/Bcr–Abl cells are very similar to those observed previously following direct inhibition of Bcr–Abl with STI-571 in Ph^+ cells (Druker *et al*., 1996). Therefore, it was critical to show that the effects of PP2 and A-419259 are due to Src inhibition and not non-specific effects on Bcr–Abl. Although no *in vitro* data have been reported for PP2 with Abl kinases, other studies have shown that the closely-related inhibitor PP1 exhibits only modest (10-fold) selectivity for Src kinases compared to c-Abl *in vitro* (Liu *et al*., 1999) (J Kamens, unpublished data). PP2 differs from PP1 by a substitution of a chlorine atom for a methyl group and both have a similar inhibition profile against Src, Lck, Jak2 and the EGF receptor (Hanke *et al*., 1996). In addition, Abl shares some of the kinase domain residues proposed to determine PP1 selectivity in the Hck-PP1 co-crystal structure (Schindler *et al*., 1999). Despite these caveats, we found that concentrations of PP2 that inhibited Src family kinases and induced growth arrest and apoptosis in CML cells did not markedly reduce Bcr–Abl phosphotyrosine content or levels of phospho-CrkL, suggesting that Src kinases are the target for this compound.

A-419259 is one of a group of second generation pyrrolo-pyrimidines recently developed to exhibit improved selectivity towards the Src kinase family (Arnold *et al*., 2000). This compound was chosen for our study because it exhibited greater than 1000-fold selectivity towards the leukocyte Src family members Lck and Lyn compared to c-Abl *in vitro* (Table 1). A-419259 also inhibited Hck and Lyn with equal potency in immune-complex kinase assays (data not shown), indicating that Hck may be a target for this compound *in vivo* as well. Hck has been strongly implicated as a Bcr–Abl signaling partner in several previous studies (Klejman *et al*., 2002; Lionberger *et al*., 2000; Danhauser-Riedl *et al*., 1996; Warmuth *et al*., 1997). A-419259 inhibited CML cell proliferation and induced apoptosis with 30- to 100-fold greater potency than PP2. Importantly, concentrations of A-419259 responsible for these anti-CML effects also inhibited Src family kinases but did not markedly affect Bcr–Abl or CrkL tyrosine phosphorylation, again suggesting that Src kinases are the primary target for this drug in CML cells. Together with the PP2 data, these findings establish a necessary role for Src family kinases in Bcr–Abl signaling.

At the highest concentrations of PP2 and A-419259 tested (30 and 1 μM, respectively), reductions in Bcr–Abl and CrkL tyrosine phosphorylation were observed in CML cell lines. At least two explanations are possible for this observation. First, pyrrolo-pyrimidines may directly bind and inhibit the Abl kinase domain at higher concentrations. This may be particularly true for PP2, because the Abl and Hck kinase domains share residues important for binding of the related compound PP1 in the Hck crystal structure (Schindler *et al*., 1999). A second possibility is that inhibition of Src kinases may indirectly affect Bcr–Abl phosphotyrosine content and kinase activity. Previous work has shown that Src family kinases can trans-phosphorylate Bcr–Abl, particularly on the Grb2 SH2 domain binding site in the Bcr-derived portion of the molecule (Warmuth *et al*., 1997). More recently, Src family kinases have been shown to trans-phosphorylate c-Abl within its activation loop and SH2-kinase linker region, suggesting that Src kinases may regulate Bcr–Abl kinase activity as well (Plattner *et al*., 1999; Furstoss *et al*., 2002). Thus, selective inhibitors for Src family kinases may block this transphosphorylation event *in vivo*, subsequently reducing Bcr–Abl phosphotyrosine content or kinase activity. Experiments to address these possibilities are currently under way.

Studies with the cytokine-dependent cell line DAGM suggest that transformation with Bcr–Abl sensitizes myeloid cells to Src inhibitors. A-419259 potently inhibited the proliferation of Bcr–Abl-transformed DAGM cells in the absence of IL-3 with an IC_{50} similar to that observed with the K-562 and Meg-01 CML cell lines (0.1–0.3 μM). This finding supports the general conclusion that Bcr–Abl requires Src kinases for growth and survival signal transduction. Interestingly, incubation of A-419259-treated DAGM/Bcr–Abl cells with IL-3 partially rescued the cells from the growth-inhibitory effects of this compound. IL-3 treatment did not reactivate Src kinases, however, suggesting that Src activation by IL-3 is not required for proliferation or survival in this cell line. Similar results were obtained with the DAGM/Neo control cells as well as GM-CSF-dependent TF-1 cells, in which proliferation and survival were unaffected despite a complete block of Src kinase activity by the inhibitor. This observation helps to validate Src family kinases as CML drug targets, because it implies that Src activation is only crucial to the proliferation and survival of Bcr–Abl transformed cells and not normal myeloid progenitors. However, the finding that IL-3 can rescue Bcr–Abl-transformed DAGM cells from the toxic effects of A-419259 (Figure 7) may influence the effectiveness of selective Src kinase inhibitors when used as stand-alone agents in CML.

Data presented in this report strongly suggest that Src family kinases may couple Bcr–Abl to various downstream effector molecules, facilitating CML disease progression. Both PP2 and A-419259 blocked Stat5 and Erk activation at concentrations that inhibited Src family kinases but did not affect Bcr–Abl or CrkL tyrosine phosphorylation. Our results agree with recent evidence suggesting a dominant and non-redundant role for Stat5 in mediating resistance of CML cells to apoptosis, while both Ras/Erk and Stat5 signaling drive proliferation (Hoover *et al*., 2001). We also showed that PP2 and A-419259 blocked expression

of the anti-apoptotic Stat5 target gene Bcl-X_L in CML cells, but had no effect on Stat5 activation and Bcl-X_L expression in the Ph^- cell line, TF-1.

Despite mounting evidence that Src kinases are involved in Bcr–Abl-mediated activation of Stat5, the exact mechanism is not clear. The simplest mechanism for Stat5 activation in Ph^+ CML cells is direct tyrosine phosphorylation by Bcr–Abl. However, Nieborowska-Skorska *et al*. (1999) were unable to demonstrate a direct association of Stat5 with Bcr–Abl. These authors also found that the SH2 and SH3 domains of Bcr–Abl are necessary for Stat5 activation in myeloid cells. Interestingly, this region of Bcr–Abl binds tightly to Hck and other myeloid Src family members *in vitro* (Lionberger *et al*., 2000). Furthermore, Hck and Lyn can bind and phosphorylate Stat5 both in Sf-9 insect cells and *in vitro* (Chin *et al*., 1998) (S Schreiner and T Smithgall, unpublished data). A general role for Stats in oncogenesis has emerged in recent years, and the Src kinase family may have a prominent role as an intermediate coupling upstream tyrosine kinases to Stat activation in several cancers including CML (Bowman *et al*., 2000; Garcia *et al*., 1998).

Numerous studies have demonstrated that Ras activation is critical to Bcr–Abl-mediated transformation and that inhibition of Ras or its downstream effectors can block Ph^+ proliferation (Pendergast *et al*., 1993; Sawyers *et al*., 1995; Goga *et al*., 1995; Puil *et al*., 1994; Morgan *et al*., 2001; Woessmann and Mivechi, 2001). Data presented here show that the Src kinase inhibitors PP2 and A-419259 can block Erk activation at concentrations that do not affect Bcr–Abl kinase activity. Several possible mechanisms of Ras/Erk activation in CML have been suggested, including direct interaction with the Grb-2/Sos complex through the Grb2 SH2 domain binding site at Tyr 177 (Puil *et al*., 1994; Pendergast *et al*., 1993) or through the Shc (Goga *et al*., 1995) or CrkL adaptor proteins (Senechal *et al*., 1996; Feller *et al*., 1995). Previous studies have shown that Hck phosphorylates Bcr–Abl on Tyr 177 in a heterologous expression system (Warmuth *et al*., 1997), predicting that Src inhibition would suppress Bcr–Abl interaction with Grb2 in CML cells. However, we did not detect a decrease in Bcr–Abl/Grb-2 complex formation in the presence of either PP2 or A-419259 (data not shown). This result suggests that Bcr–Abl autokinase activity may also contribute to the phosphorylation of this site in CML cells.

In summary, data presented here validate the Src family kinases expressed in myeloid cells as alternative targets for CML drug therapy, particularly in patients shown to be refractory to treatment with STI-571. Treatment of CML patients, especially those in blast crisis, with a combination of Src and Bcr–Abl inhibitors could provide a dramatic therapeutic benefit. Furthermore, like combinational therapy, the rational design of compounds that have dual specificity for both kinases could also provide similar benefit and would potentially prevent drug resistance and subsequent disease relapse. Such an agent has recently been reported by Huang *et al*. (2002) and is a very potent inhibitor of CML cell growth. Future work will address the role that Src kinases play in CML progression *in vivo* and the effects of Src inhibition on Bcr–Abl signaling and oncogenic activity in a whole-animal model of CML.

Materials and methods

Cell culture and proliferation assays

The K-562 (Klein *et al*., 1976) and Meg-01 (Ogura *et al*., 1985) cell lines, both derived from Ph^+ CML patients in blast crisis, as well as the Ph^- erythroleukemia cell line HEL (Martin and Papayannopoulou, 1982) were purchased from the American Type Culture Collection (ATCC). K-562 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), and 50 μg/ml gentamycin. Meg-01 cells were cultured in Vitacell modified RPMI 1640 (ATCC), supplemented with 10% FCS and 50 μg/ml gentamycin. The human GM–CSF-dependent myeloid leukemia cell line TF-1 (Kitamura *et al*., 1989) was obtained from the ATCC and grown in RPMI 1640 supplemented with 10% FCS, 50 μg/ml gentamycin, and 1 ng/ml of recombinant human GM–CSF. DAGM murine myeloid leukemia cells (a gift from Dr Owen Witte, Howard Hughes Medical Institute, UCLA, USA) were cultured in RPMI 1640 supplemented with 10% FCS, 50 μg/ml gentamycin, and 0.5 ng/ml recombinant IL-3. Concentrated stock solutions of PP2 (5 mM; Calbiochem) and A-419259 (2 mM; Abbott Biotech) were prepared in DMSO and stored at -20°C. Cellular proliferation was measured using the Live/Dead growth assay (Molecular Probes) according to the manufacturer's protocol. This assay employs calcein-AM, a fluorogenic esterase substrate that is taken up by viable cells and hydrolyzed intracellularly, releasing a green fluorescent product. Briefly, 10^4 cells were plated per well in 96-well plates for each day of a 4-day time course. Various concentrations of PP2, A-419259 or vehicle control were added to the wells (five wells per concentration per day) and the plates were incubated at 37°C. At each time point, one plate was centrifuged at 1500 *g* for 10 min to pellet the cells. Cells were washed with phosphate buffered saline (PBS), and calcein-AM was added to each well to a final concentration of 1 μM. Plates were incubated in the dark at room temperature for 1 h. The plates were then read at 485/530 nm (excitation/emission) using a SpectraMax Gemini XS fluorescent plate reader and data were analysed with SoftMax Pro software (Molecular Devices).

Apoptosis assays

To measure apoptosis, K-562 and TF-1 cells were treated with PP2, A-419259 or the vehicle control for 72 h at 37°C. Apoptosis was assessed by determining cell-surface annexin-V binding and intracellular caspase-3 activity. For annexin-V binding, K-562 and TF-1 cells were incubated with annexin-V-FITC (BD Pharmingen) according to the manufacturer's protocol. Briefly, 10^6 cells were centrifuged at 1000 *g* for 10 min, and washed twice with cold 1 × PBS. Cells were then resuspended in annexin-V binding buffer and 10^5 cells were pelleted and incubated with 5 μl annexin-V-FITC and 5 μl propidium iodide (50 μg/ml) for 15 min. Apoptosis was measured using a FACSCalibur flow cytometer (Becton-Dickinson) set for two-color acquisition, and data were analysed using CellQuest software. For the caspase-3 assay, 10^6 cells were centrifuged at 1000 *g* for 5 min and media was completely removed. Sixty microliters of 10 μM PhiPhiLux

reagent (OncoImmunin, Inc.) and 5 μl FCS were added to the cell pellet and incubated at 37°C for 45 min. PhiPhiLux is a profluorescent protease peptide substrate that upon cleavage by intracellular caspase-3 emits green fluorescence at 530 nm. Following incubation, cells were washed once with ice-cold flow cytometry dilution buffer provided with the reagent. The cells were resuspended in 1 ml of the same buffer for FACS analysis.

Transformation of DAGM cells with Bcr–Abl retroviruses

The pMig-Bcr–Abl/GFP retroviral vector was generously provided by Dr Warren Pear, University of Pennsylvania. This vector encodes the 210 kDa form of Bcr–Abl followed by an internal ribosomal entry sequence (IRES) and the GFP coding sequence under the control of the murine stem cell virus long-terminal repeat (Pear *et al*., 1998). The control pSRαMSVtk*neo* retroviral construct was provided by Dr Owen Witte, Howard Hughes Medical Institute, UCLA (Muller *et al*., 1991). To make retroviral stocks, subconfluent 100 mm dishes of 293T cells were co-transfected with each retroviral vector and an ecotropic packaging vector using a calcium phosphate procedure described elsewhere (Briggs *et al*., 1997). Viral supernatants were collected at 48, 72, and 96 h post transfection, pooled, filtered with 0.45 μm filters, and stored at −80°C. DAGM cells were stably infected as follows: 10^6 cells were incubated with 5 ml of viral supernatant in the presence of 4 mg/ml polybrene. To increase viral uptake, cells were centrifuged during infection at 2400 r.p.m. for 3 h at room temperature (Lionberger *et al*., 2000). DAGM cell populations infected with the pMig-Bcr–Abl/GFP retroviral vector were observed for expression of GFP by fluorescence microscopy, cytokine independent outgrowth, and expression of Bcr–Abl protein by immunoblotting. For cytokine independent outgrowth, cells were washed free of cytokine and resuspended in media lacking IL-3. Proliferation was measured using the Live/Dead assay as described above over the course of 10 days. To confirm expression of Bcr–Abl protein, lysates were prepared from 10^7 cells and probed with anti-Abl (8E9, BD-Pharmingen) or anti-phosphotyrosine (PY99; Santa Cruz) antibodies on immunoblots as described below. Control populations were prepared by infecting DAGM cells with a retrovirus carrying only the *neo* drug resistance marker and selecting with G-418 at 800 μg/ml for 7–10 days in medium plus IL-3.

Analysis of protein expression and tyrosine kinase activity

To monitor Src kinase activity, 10^7 cells were collected by centrifugation, washed once with PBS, and lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) supplemented with the protease inhibitors aprotinin (25 μg/ml), leupeptin (25 μg/ml) and PMSF (1 mM) and the phosphatase inhibitors NaF (10 mM), and Na_3VO_4 (1 mM). Clarified lysates were resolved by SDS–PAGE, transferred to PVDF membranes, and blotted with an antibody to the phosphorylated form of the Src activation loop (Y-418; BioSource International) or an anti-Lyn polyclonal antibody (Santa Cruz) to normalize for loading. Bcr–Abl phosphorylation was determined by probing replicate immunoblots with the anti-phosphotyrosine and anti-Abl monoclonal antibodies. CrkL phosphorylation was assessed by determining the relative levels of the unphosphorylated and phosphorylated forms of the protein following visualization on immunoblots with an anti-CrkL polyclonal antibody (H-62, Santa Cruz) as described (Gorre *et al*., 2001). Erk activation was measured using an anti-phospho-Erk monoclonal antibody (E-4; Santa Cruz) as well as an anti-Erk2 polyclonal antibody (C-14; Santa Cruz) as a loading control. To assess Stat5 tyrosine phosphorylation, 10^7 cells were lysed in RIPA buffer, and Stat5 was immunoprecipitated with 2 μg of the Stat5a polyclonal antibody (L-20; Santa Cruz) and 25 μl protein G-Sepharose (50 : 50 w/v slurry; Amersham-Pharmacia Biotech) by rotation at 4°C overnight. Beads were then collected and washed three times with ice-cold RIPA buffer, resuspended in a final volume of 25 μl, combined with an equal volume of 2 × SDS sample buffer and heated for 5 min at 95°C. Proteins were resolved by SDS–PAGE, transferred to PVDF membranes, and blotted with a Stat5 monoclonal antibody (Transduction Laboratories) or the PY-99 anti-phosphotyrosine antibody. All immunoreactive bands were visualized with goat-anti-rabbit secondary antibodies conjugated to alkaline phosphatase with NBT–BCIP as colorimetric substrate (Southern Biotechnology Associates).

Electromobility shift assays (EMSA)

Following treatment with various concentrations of Src inhibitors for 20 h, K-562 or TF-1 cells (10^7) were centrifuged, washed once with PBS, and nuclear extracts were prepared as described by Skorski *et al*. (1998). Stat5 DNA-binding activity was assessed using a double-stranded DNA probe based on the γ-activation sequence (GAS) (Skorski *et al*., 1998). The probe was prepared by combining 200 pmoles of the complementary single-stranded GAS oligos (5′-AGCTTGTATTTCCCAGAAAAGGG-3′ and 5′-TCCC-TTTTCTGGGAAATAC-3′) with 6.6 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), heating to 70°C, and cooling slowly overnight. For labeling, 1 μl of the duplex GAS oligo (20 pmol) was added to 12 μl H_2O, 4 μl Labeling Mix dATP (0.1 mM dCTP, dGTP, dTTP in 50 mM Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 250 mM NaCl, 25 mM β-mercaptoethanol; AP Biotech), 2 μl [α-^{32}P]dATP (2000 Ci/mmol; New England Nuclear), and 1 μl DNA polymerase I Klenow fragment (0.5 U/μl, Gibco–BRL). Following incubation for 1 h at room temperature, the reaction was diluted with 20 μl H_2O and unincorporated nucleotides were removed using a G-25 Sephadex spin column (AP Biotech) according to the manufacturer's instructions. For each gel-shift reaction, 40 000 c.p.m. of the radiolabeled GAS oligo was combined with 4 μl H_2O, 0.2 μl 0.5 M HEPES, pH 7.9, 0.2 μl 50% glycerol, 0.2 μl 50 mM DTT, 1 μl poly dI-dC (1 μg/μl), and 2.5 μl BSA (2.0 μg/μl, Sigma). Nuclear extracts (5 μl containing equal amounts of protein) were combined with 4 μl of H_2O (3 μl for cold competitor experiments), and 1 μl of 10 × incubation buffer (100 mM HEPES, pH 7.9, 500 mM KCl, 10 mM EDTA). Control reactions for binding specificity were run in parallel and contained 1 μl of a 100-fold molar excess of non-radiolabeled probe. The binding reaction was started by the addition of 10 μl of the labeled probe mixture and incubated at 30°C for 30 min. Reactions were quenched by transferring them to ice, and Stat5-GAS complexes were resolved on 5% non-denaturing polyacrylamide gels pre-run for 30 min at 100 volts in 0.25 × TBE buffer. Gels were fixed with 10% acetic acid/10% methanol, rinsed with H_2O, dried, and radiolabeled bands were visualized by autoradiography.

RNase protection assay (RPA)

For the RPA assay, 10^7 K-562 or TF-1 cells were treated for 20 h with either PP2 or A-419259. Following incubation, total cellular RNA was isolated from each cell line using an

RNA isolation kit (ToTALLY RNA, Ambion Inc.) according to the manufacturer's instructions. To measure expression of Bcl-X_L, a multi-probe set containing cDNA templates of Bcl-2 family members and GAPDH as an internal control was utilized according to the manufacturer's protocol (BD PharMingen). Briefly, ^{32}P-labeled riboprobes of defined length were generated using T7 RNA polymerase and 50 ng of the DNA template in the presence of 150 μCi of [α-^{32}P]UTP (New England Nuclear). Template DNA was digested with RNase-free DNase, followed by precipitation of labeled RNA. Five micrograms of total cellular RNA was mixed with 7.5×10^5 c.p.m. of the ^{32}P-labeled riboprobe in hybridization buffer (40 mM PIPES, 1 mM EDTA, 0.4 M NaCl, and 80% formamide) and incubated for 5 min at 90°C followed by 12 h at 56°C. The hybridized RNA duplexes were then treated with RNase A and RNase T1, followed by proteinase K digestion. RNase-resistant RNA duplexes were extracted with phenol and precipitated by the addition of equal volumes of 4 M ammonium acetate and two volumes of ethanol. Labeled RNA samples were resolved on 6% urea denaturing gels and visualized by autoradiography and PhosphoImager analysis (Molecular Dynamics). The relative expression of Bcl-X_L was normalized to the signal corresponding to GAPDH expression.

In vitro *kinase assays*

In vitro kinase assays were performed on $His_{(6)}$-tagged Lck (residues 62–509) and full-length c-Abl purified from Sf-9 cells, and commercial sources of Lyn, Src (Upstate Biotechnology) and PKC (Calbiochem). Lck, Lyn, Src and Abl activities were measured with an ELISA-based assay. Flat bottom 96-well ELISA plates were incubated with a 200 μg/ml solution of Poly(Glu,Tyr) 4:1 substrate in phosphate buffered saline (PBS) for 1 h at 37°C and then washed with PBS containing 0.1% Tween-20 (PBS-T). Inhibitor dilutions were added to the washed plates already containing the appropriate enzyme in kinase assay buffer (250 mM Mopso, pH 6.75, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 2.5 mM DTT, 0.02% BSA, 2 mM Na_3VO_4, 5% DMSO, 5 μM ATP). After incubation at room temperature for 20 min, plates were washed three times with PBS-T and plate-bound phosphotyrosine was detected with an anti-phosphotyrosine-HRP antibody conjugate and subsequent color development with K-Blue reagents (Neogen Corporation). All assays were optimized to use the least amount of enzyme necessary for a reproducible signal-to-noise ratio. Specifically, the amount of each kinase per reaction was as follows: Src, 0.2 ng; Lck, 12 ng; Lyn, 0.1 ng; and Abl, 0.8 ng.

PKC activity was measured with a radioactive kinase assay. Enzyme (100 ng) and substrate peptide were incubated in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 1 mM EGTA, 10 mM ATP, 8 μM peptide, 5% DMSO and [γ-^{33}P]ATP). Reactions were stopped with 10 μl 5 mM ATP in 75 mM phosphoric acid. The reaction was spotted on phosphocellulose filters and washed in 75 mM phosphoric acid. Incorporation of radiolabel was quantified by liquid scintillation counting.

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