Effects of Dasatinib on Src Kinase Activity and Downstream Intracellular Signaling in Primitive Chronic Myelogenous Leukemia Hematopoietic Cells

Heiko Konig, Mhairi Copland, Su Chu, Richard Jove, Tessa L. Holyoake, and Ravi Bhatia

Abstract

Bcr-Abl tyrosine kinase inhibitors (TKI) are effective in inducing remissions in chronic myelogenous leukemia (CML) patients but do not eliminate primitive CML hematopoietic cells. There is a need to identify mechanisms that contribute to retention of CML progenitors. Src family tyrosine kinases have been identified as potential mediators of Bcr-Abl–induced leukemogenesis. Dasatinib (BMS-354825) is a potent dual Abl/Src kinase inhibitor approved for clinical use in CML patients. We evaluated Src activity in primitive human CML progenitors from different stages of disease and investigated the effects of Dasatinib on Src activity and downstream signaling pathways. P-Src expression was increased in CD34+ cells and CD34+CD38- cells in all phases of CML. Dasatinib showed potent Src inhibitory activity in CML progenitors, inhibiting both Bcr-Abl–dependent and –independent Src activity. In contrast, Imatinib inhibited only Bcr-Abl–dependent Src activity. Dasatinib inhibited P–mitogen-activated protein kinase (MAPK), P-Akt, and P-STAT5 levels in CML progenitors in the absence of growth factors but not in the presence of growth factors. A marked increase in P-MAPK levels seen in the presence of growth factors with Imatinib was much less prominent with Dasatinib. Dasatinib significantly suppressed CML colony-forming cells and long-term culture-initiating cells but did not significantly alter the level of apoptosis-regulating proteins in CML CD34+ cells. Our results indicate that Dasatinib, in addition to potent anti–Bcr-Abl kinase activity, effectively inhibits Src kinase activity and downstream signaling pathways in CML progenitors but does not induce a strong proapoptotic response. These observations argue against a prominent role for Src kinases in persistence of primitive CML cells in TKI-treated patients. [Cancer Res 2008;68(23):9624–33]

Introduction

The Philadelphia (Ph) chromosome and the resulting BCR-ABL fusion gene represent the pathogenetic hallmark of chronic myelogenous leukemia (CML). The deregulated tyrosine kinase activity of the Bcr-Abl protein alters cellular homeostatic mechanisms in primitive hematopoietic cells, resulting in increased proliferation, decreased apoptosis, and disturbed interaction with the extracellular matrix. The natural course of CML is an inevitable progression from an initial chronic phase (CP) to an accelerated phase (AP) and a fatal blast crisis (BC). Treatment with Imatinib mesylate (Imatinib), results in remarkably improved outcomes for CML patients. The majority of CP CML patients receiving Imatinib achieves and maintains major cytogenetic responses and substantial molecular responses (1, 2). However, it is also known that primitive CML hematopoietic cells escape elimination by Imatinib and that discontinuation of drug results in disease relapse (3). Previous studies suggest that effective inhibition of Bcr-Abl kinase activity by different tyrosine kinase inhibitor (TKI) is not sufficient to induce apoptosis in CML progenitors (4–7). These results indicate the importance of identifying the intracellular signaling mechanisms that are responsible for retention of CML progenitors despite Bcr-Abl kinase inhibition, and that could be targeted to enhance elimination of CML progenitor cells.

The Src family of nonreceptor tyrosine kinases have been identified as potential mediators of Bcr-Abl–induced leukemogenesis (8–10). Overexpression of Src family kinases has been implicated in Imatinib resistance and CML progression (11–13). Imatinib does not inhibit Src activity in mouse leukemic cells, suggesting that Src activation may also occur independently of Bcr-Abl kinase activity (14–16). Dasatinib (BMS-354825), a highly potent dual Abl/Src kinase inhibitor, which is active against most Imatinib-resistant mutants, has been approved for clinical use in CML patients who fail Imatinib (17–19). Dasatinib inhibits wild-type Bcr-Abl and all members of the Src family, with an IC50 of <1 nM/mL (20, 21). However, it is not clear from previous studies whether Src kinase activity is elevated in primary progenitors from CML patients. In addition, the effects of Dasatinib on Src kinase activity in primary CML progenitor cells and on downstream signaling activities and apoptosis-regulating mechanisms have not been studied. In this study, we evaluated Src activity in primitive human CML progenitors from different stages of disease and investigated the effects of Dasatinib on Bcr-Abl and Src kinase activity and downstream growth signaling pathways in CML progenitors.

Patients, Materials, and Methods

Subjects

Peripheral blood samples were obtained from newly diagnosed CML patients. Peripheral blood stem cell (PBSC) and umbilical cord blood samples were obtained from healthy donors. This study was approved by the Institutional Review Boards at City of Hope Cancer Center, in accordance with an assurance filed with and approved by the Department of Health and Human Services, and the North Glasgow University Hospital Division of NHS Greater Glasgow and Clyde, and met all requirements of the Declaration of Helsinki.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Ravi Bhatia, Department of Hematopoietic Stem Cell and Leukemia Research, City of Hope National Medical Center, Duarte, CA 91010. Phone: 626-359-8111, ext. 62705; Fax: 626-301-8973; E-mail: rbhatia@coh.org.

©2008 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-08-1131
Inhibitors
Ten–micromole per liter stock solutions of Dasatinib (Bristol-Myers Squibb) and Imatinib (Novartis Pharmaceuticals) were prepared in DMSO and stored at −20°C. Dasatinib was added to cell cultures at concentrations ranging between 0.01 and 0.15 μM/L, and Imatinib was added at a concentration of 5 μM/L, corresponding to plasma concentrations in patients receiving these agents (18, 22).

Selection of CD34+ Progenitors
Mononuclear cells were isolated by Ficoll-Hypaque (Sigma Diagnostics) density gradient centrifugation (specific gravity, 1.077) for 30 min at 400 g. CD34+ cells were selected by means of immunomagnetic column separation (Miltenyi Biotec) following the manufacturer’s instructions.

Cell Culture and Exposure to Inhibitors
CD34+, CD34+CD38−, or CD34+CD38− cells were cultured with or without addition of Dasatinib or Imatinib at the indicated concentrations at 37°C in a humidified atmosphere with 5% CO2 in serum-free medium (SFEM; StemCell Technologies) supplemented with growth factors (GF) at concentrations similar to that found in stroma-conditioned medium from long-term bone marrow cultures [LTBM; 200 pg/mL granulocyte-macrophage colony-stimulating factor (G-CSF); 1 ng/mL G-CSF; 200 pg/mL stem cell factor; 50 pg/mL leukemia inhibitory factor; 200 pg/mL macrophage-inhibitory protein-1α; and 1 ng/mL interleukin 6]. Cells were harvested after 96 h and assayed in progenitor, proliferation, and apoptosis assays.

Progenitor Assays
 Colony-forming cells. To assess committed progenitors, CD34+ cells were plated in methylcellulose progenitor culture, and burst forming unit-granulocyte and macrophage were counted after 14 d.

Results
Src phosphorylation is enhanced in primitive and committed progenitor cells from CML patients. P-Src expression was assessed in CD34+ and more primitive CD34+CD38− CML cells from patients with CP, AP, and BC CML and compared with normal CD34+ cells using intracellular antibody labeling and flow cytometry (Fig. 1A–D). A P-Src antibody capable of measuring phosphorylation status on the same tyrosine residue (Tyr416) of all members of the Src kinase family was used. Although there was considerable interpatient variability in expression of P-Src, CML CP and BC CD34+ cells showed significantly increased levels of P-Src compared with normal CD34+ cells (P = 0.02 and 0.022, respectively; Fig. 1A and C). As with total CD34+ cells, CML CP and BC CD34+CD38− cells also showed significantly increased levels of P-Src (P = 0.032 and 0.013, respectively; Fig. 1B) compared with normal CD34+CD38− cells. There was again a trend toward higher P-Src levels in the BC compared with CP samples. There was also a trend toward higher P-Src levels in total CD34+ cells compared with CD34+CD38− cells (Fig. 1D). These results indicate that P-Src expression is increased in CD34+ cells and CD34+CD38− cells in all phases of CML.

Dasatinib effectively inhibits Src and Bcr-Abl kinase activity in CML primitive and committed progenitor cells. The effects of Dasatinib and Imatinib on Src and Bcr-Abl kinase activity were assessed after 16 hours of exposure in culture. On assessment by intracellular flow cytometry, Dasatinib significantly reduced P-Src expression in both CML CD34+ (P < 0.001) and CD34+CD38− cells (P < 0.001) compared with no drug controls (Fig. 2A). Imatinib also inhibited P-Src expression in CML CD34+ (P < 0.001) and CD34+CD38− cells (P = 0.003) but to a lesser extent...
than Dasatinib. We also assessed P-Src levels by performing Western blot analysis for P-Src on protein extracts from CD34+ cells treated with Dasatinib and Imatinib. As was seen with flow cytometry assays, Western blot analysis also indicated that P-Src levels were effectively suppressed in response to Dasatinib (0.01–0.15 μmol/L) treatment (P < 0.001; Fig. 2B). P-Src levels were only partially suppressed after treatment with Imatinib (5 μmol/L; P = 0.06). To study the effect of Dasatinib on Bcr-Abl kinase activity, we performed Western blotting for P-CrkL, which can be distinguished from nonphosphorylated CrkL by its slower migration on Western blots. As shown in Fig. 2C, treatment with Dasatinib at doses as low as 0.01 μmol/L effectively suppressed P-CrkL protein levels (P < 0.001). Increasing the Dasatinib concentration to 0.15 μmol/L resulted in further suppression of P-CrkL levels. P-CrkL levels were also suppressed after treatment with 5 μmol/L Imatinib (P < 0.001). We also preformed Western blotting for phosphorylated Bcr-Abl and Abl (Fig. 2D). Membranes were sequentially probed with anti-Phosphotyrosine and anti-Abl antibodies to detect phosphorylated and total Bcr-Abl. Potent inhibition of Bcr-Abl phosphorylation was observed, consistent with the results of anti-CrkL blotting.

Dasatinib inhibits MAPK, Akt, and STAT5 phosphorylation in CML progenitors in the absence of GFs, but phosphorylation is maintained in the presence of GFs. The MAPK, Akt, and STAT5 signaling pathways are activated downstream of Bcr-Abl and may contribute to abnormal proliferation and survival of CML progenitors. We assessed the activity of these signaling pathways in CML CD34+ cells after 16 hours of exposure to Imatinib (5 μmol/L) and Dasatinib (0.01–0.15 μmol/L) with or without exogenous GF. Consistent with our previous observations, treatment with Imatinib, in the presence of GF, resulted in increased MAPK activity in CML CD34+ cells (31.4 ± 14.4-fold; n = 4). Increased MAPK activity was less prominent with Dasatinib treatment than with Imatinib treatment and was only seen at the highest concentrations of Dasatinib (7.6 ± 4.0-fold at 0.15 μmol/L Dasatinib; Fig. 3A). Incubation of CML CD34+ cells with Dasatinib in the presence of GF did not lead to a significant change in P-Akt and P-STAT5 levels in CML CD34+ cells (Fig. 3B and C). Similar results were obtained with Imatinib.

GF receptor engagement may also contribute to signaling through the MAPK, phosphatidylinositol-3-OH kinase (PI-3K)/Akt and STAT5 pathways. Dasatinib exposure in the presence or absence of GF stimulation resulted in similar inhibition of P-CrkL. However, inhibition of P-Src in response to low levels of Dasatinib (0.01 μm) was enhanced in the absence of GF. Similarly, Imatinib effectively inhibited Src signaling in the absence of GF but resulted in partial inhibition of P-Src levels in the presence of GF. These results suggest a role for GF stimulation in residual Src signaling in cells exposed to low levels of Dasatinib and to Imatinib. Exposure to Dasatinib in the absence of GF resulted in complete inhibition of P-STAT5 and reduction in P-MAPK, P-Akt, and P-STAT5 levels (Fig. 4A). Similar effects were seen with Imatinib. Because signaling in the absence of GF is likely to be mainly Bcr-Abl driven, these results suggest that Dasatinib effectively inhibits Bcr-Abl–mediated activation of the MAPK, PI-3K, and STAT5 pathways. In contrast,
Figure 2. Effects of Imatinib and Dasatinib on P-Src and P-CrkL expression in CML CD34+ and CD34+CD38− cells. The effect of Imatinib and Dasatinib on P-Src expression was assessed by flow cytometry in total CD34+ (left) and more primitive CD34+CD38− (right) CML cells at 16 and 72 h (n = 6; 4 CP, 2 BC; A). Results are expressed as a percentage of the no drug control (± SE). There was no difference between CP and BC CML in the samples assessed. The effect of inhibitors on P-Src, total Src, and Actin was also assessed by Western blotting (B). A representative blot for one CML sample is shown. Expression of P-Src (P-Src/Actin ratio) after inhibitor treatment expressed as a percentage of P-Src expression in the absence of inhibitors is shown; columns, mean of three replicate experiments; bars, SE. Significant differences in protein expression levels for treated cells compared with untreated controls are indicated for Dasatinib (***, P < 0.001; **, P < 0.01). To assess the effect of Dasatinib or Imatinib on Bcr-Abl kinase activity, Western blotting was performed using anti-CrkL antibodies (C). Representative blots for one CML sample are shown. P-CrkL (P-CrkL/total CrkL ratio) expressed as percentage of P-CrkL in the absence of inhibitors is shown; columns, mean (n = 5); bars, SE. Significant differences in protein expression levels for treated cells compared with untreated controls are indicated for Imatinib (***, P < 0.001) and Dasatinib (**, P < 0.01). The effect of Dasatinib or Imatinib on Bcr-Abl kinase activity was also assessed by Western blotting for phosphorylated Bcr-Abl with anti-Phosphotyrosine and total BCR-ABL with anti-ABL antibodies. Potent inhibition of Bcr-Abl phosphorylation was observed (D).
Dasatinib also inhibited normal PBSC CFC to a lesser extent than CML progenitors (Fig. 5). Suppression of CML LTC-IC by Dasatinib was comparable with that observed with 5 µmol/L Imatinib treatment (P = 0.0002 and 0.001, respectively). We have shown that Imatinib (94.8%) similarly inhibits CML progenitors. FISH analysis of colonies generated in CFC culture revealed that 98.3% of cells derived from CFC from untreated cells were BCR-ABL positive. Suppression of CML CFC and LTC-IC by Dasatinib was comparable with that observed with 5 µmol/L Imatinib treatment (P = 0.0002 and 0.001, respectively). We have shown that Imatinib shows higher potency of inhibition of CML CFC compared with LTC-IC (7). Results shown in Fig. 5A indicate that Dasatinib similarly inhibits CML CFC more effectively than LTC-IC at low concentrations (0.01 µmol/L). This is consistent with a greater effect of these inhibitors on committed progenitors compared with primitive progenitors. FISH analysis of colonies generated in CFC culture revealed that 98.3% ± 0.4% (n = 4) of cells derived from CFC from untreated cells were BCR-ABL positive. CFC remaining after Dasatinib or Imatinib treatment were also predominantly BCR-ABL positive (0.15 µmol/L Dasatinib: 84.8% ± 12.7%, n = 3; 5 µmol/L Imatinib: 94.8% ± 4.3%, n = 4), indicating persistence of BCR-ABL-positive cells. Dasatinib treatment suppressed cord blood LTC-IC and CFC to a lesser extent than CML progenitors (Fig. 5B). Dasatinib also inhibited normal PBSC CFC to a lesser extent than CML CFC (Fig. 5C).

To assess the effects of Dasatinib on apoptosis, CML CD34+38− and CD34+38+ cells were selected using flow cytometry and incubated with Dasatinib or Imatinib under low GF conditions for 96 hours and then labeled with Annexin V-PE and analyzed by flow cytometry for apoptosis (Annexin V-positive cells; Fig. 6A and B). Treatment with Dasatinib resulted in only a modest increase in apoptosis of CML primitive and committed progenitors. Similar results were reported after Imatinib treatment. Treatment of cord blood and normal PBSC CD34+CD38− and CD34+CD38+ cells with Dasatinib or Imatinib did not result in significant increase in apoptosis in the tested dose range (Fig. 6A and B). We also evaluated the effect of Dasatinib treatment on the expression of proteins known to be important in regulating apoptosis and reported to be regulated by Bcr-Abl, including the antiapoptotic proteins Mcl-1, Bcl-2, and Bcl-xL and the proapoptotic protein Bim. Treatment with Dasatinib in the presence of GF did not result in alteration in the expression of Mcl-1, Bcl-2, Bcl-xL, and Bim after adjusting for protein loading based on actin (Fig. 6C). These results suggest that maintenance of signaling through the GF-receptors is sufficient to prevent alterations in these apoptosis regulatory mechanisms after Dasatinib treatment.

The effect of Dasatinib on cell division was evaluated by labeling CML and normal CD34+CD38− committed and CD34+CD38+ primitive progenitors with CFSE before culture and tracking cell division using flow cytometry. Treatment with Dasatinib or Imatinib resulted in a significant inhibition of CML CD34+CD38− and CD34+CD38+ progenitor growth (Supplementary Fig. S1A). Dasatinib also inhibited proliferation of cord blood primitive progenitors (Supplementary Fig. S1B) and normal PBSC primitive and committed progenitors (Supplementary Fig. S1C) but to a lesser extent than CML progenitors. An increased proportion of undivided progenitors were seen after Dasatinib treatment, as has been previously described for Imatinib (Supplementary Fig S1D; refs. 14, 24). Annexin V labeling indicated that apoptosis was largely restricted to dividing cells and that nondividing CML progenitors were resistant to apoptosis after Dasatinib and Imatinib treatment (Fig. 6D).
Discussion

Imatinib treatment has been shown to be highly effective in all phases of CML with most patients achieving substantial and prolonged reduction in levels of Bcr-Abl–positive cells. However, low levels of residual Bcr-Abl expressing stem and progenitor cells can be detected in most CML patients in remission on Imatinib (25). Imatinib does not effectively induce apoptosis in primitive CML progenitors, despite inhibiting Bcr-Abl tyrosine kinase activity in these cells (5, 14). The mechanisms that contribute to preservation of CML progenitors in patients receiving Bcr-Abl TKI treatment are unclear because previous studies indicate that Imatinib and other TKI can effectively inhibit Bcr-Abl kinase activity in CD34+ cells. Here, we evaluated Src kinase activity and the effect of blocking Src signaling with Dasatinib on primitive human CML progenitors.

Our studies show that human CML stem and progenitor cells display increased Src kinase activity. Although studies in myeloid cell lines have shown that Bcr-Abl can directly and indirectly

![Diagram of cell signaling pathways](image)

**Figure 4.** Effects of Dasatinib on cell signaling pathways in CML CD34+ cells in the absence of GFs. CML CD34+ cells were incubated with Dasatinib or Imatinib for 16 h in SFEM without addition of GF, followed by preparation of cell lysates and Western blotting with the indicated antibodies. The results shown are representative of two experiments using different CML patient samples (A). The effect of Imatinib and Dasatinib on Bcr-Abl and Src signaling is illustrated in B. Bcr-Abl and GF receptors both signal through the MAPK, PI-3K/Akt, and STAT5 pathways through Src-dependent and -independent mechanisms. In addition, BCR-ABL–transformed cells and primary CML progenitors show enhanced autocrine secretion of GF (dashed line). Imatinib-mediated inhibition of Bcr-Abl kinase activity results in reduced downstream signaling through Src-dependent and Src-independent mechanisms in addition to inhibition of Bcr-Abl–mediated autocrine GF signaling. However, Src signaling through GF receptor engagement and other Bcr-Abl–independent mechanisms is not affected. Dasatinib in addition to inhibiting Bcr-Abl kinase–dependent signaling such as Imatinib also inhibits Bcr-Abl–independent Src signaling. Although the additional Src inhibition leads to reduced elevation of P-MAPK levels in response to exogenous GF, Src-independent GF-activated signals can persist in Dasatinib-treated cells.
interact with and activate Src family kinases (10), previous studies have not directly evaluated Src kinase expression and activity in primary CML cells. Other studies have shown that Bcr-Abl retrovirus–transduced marrow from mice lacking Src kinases efficiently induced CML but not B-ALL in transplant recipients, and Src kinase inhibitors prolonged survival of mice with B-ALL but not with CML (26). These studies suggested an important role for Src in Ph+ALL, whereas its activity and role in CML is less clear. We show here that levels of P-Src are significantly increased in CD34+ and CD34+CD38−/C0 cells from patients with CP CML. Increased Src activity was associated with disease progression with a trend toward increased P-Src in cells from patients with BC compared with CP CML. Interestingly, P-Src levels were higher in CD34+ cells compared with CD34+CD38− cells, indicating maturation stage–related changes in Src activity. We further show that Imatinib treatment only partially inhibited P-Src levels in CML progenitors, whereas Dasatinib potently inhibited Src kinase activity under these conditions. These studies were conducted in cells exposed to exogenous GF. Because Src kinases can be activated by signaling from GF receptors, we also studied the effects of inhibitors in the absence of GF. Dasatinib and Imatinib were both highly effective in inhibiting Src signaling in the absence of GF, suggesting that incomplete inhibition of Src in CML cells exposed to exogenous GF may be related to GF receptor–mediated activation of Src. These results indicate that both Bcr-Abl and non–Bcr-Abl kinase–dependent mechanisms contribute to Src activation in CML progenitor cells and that whereas Imatinib only inhibits Bcr-Abl kinase mediated Src activation, both Bcr-Abl kinase-dependent and kinase-independent Src activation are inhibited by Dasatinib. These observations help clarify the relationship of Bcr-Abl kinase Src activity in human CML progenitors.

Our studies elucidate the relative contribution of Src and Bcr-Abl kinases to the activity of important downstream signaling pathways.

Figure 5. Inhibition of primitive and committed progenitor growth after exposure to Dasatinib. CD34+ cells from CML patients or healthy donors were exposed to Dasatinib or Imatinib at the concentrations indicated for 96 h. Cells were then assayed for primitive progenitors (LTC-IC) and committed progenitors (CFC) as described in the Materials and Methods. Progenitor growth in the presence of inhibitors is shown as percentage inhibition relative to the progenitor growth in the absence of inhibitors. Inhibition of CML LTC-IC (n = 3; left) and CFC (n = 4; right) growth (mean ± SE; A), cord blood (n = 3), LTCIC (left) and CFC (right) growth (B) samples, and normal PBSC CFC growth (n = 3; C) is shown. Concentrations of Imatinib resulting in significant progenitor growth suppression compared with untreated controls are indicated as follows: ***, P < 0.001; *, P < 0.05. Concentrations of Dasatinib inducing significant progenitor growth suppression compared with untreated controls are indicated as follows: ***, P < 0.001; *, P < 0.05.
Figure 6. Effect of Dasatinib on apoptosis in CML primitive and committed progenitors. CML CD34+CD38− primitive and CD34+CD38+ committed progenitors were exposed to Dasatinib or Imatinib at indicated concentrations for 96 h. Apoptosis was analyzed by FACS after Annexin V-PE labeling. The percentage of apoptotic cells is shown as columns for each concentration of Dasatinib or Imatinib. Columns, mean values for apoptosis of CML (n = 5), cord blood (n = 3), and normal PBSC (n = 3) primitive progenitors (A) and CML, cord blood, and normal PBSC committed progenitors (B); bars, SE. Significant increase in apoptosis in response to Imatinib (c, P < 0.05) is indicated. CML CD34+ cells were exposed to Dasatinib or Imatinib at indicated concentrations for 16 h. C, Western blotting for apoptosis-regulating proteins (Mcl-1, Bcl-2, Bcl-XL, Bim) and actin expression was performed. The results shown are representative of two experiments using different CML patient samples. Representative FACS dot plot for CFSE and Annexin V labeling of CML CD34+CD38+ cells, demonstrating restriction of apoptosis to the divided (d) and not undivided (u) cell populations (D).
pathways in CML progenitors. Src kinases are known to play an important role in regulating mitotic events and, such as the Bcr-Abl kinase, can activate the STAT5, PI-3K/Akt, and MAPK signaling pathways (27, 28). We show here that exposure to Dasatinib in the absence of GF resulted in almost complete suppression of P-STAT5 expression and reduced P-MAPK and P-Akt expression. However, Imatinib resulted in similar suppression of P-STAT, P-Akt, and P-MAPK, suggesting that combined inhibition of Src and Bcr-Abl kinase activity did not result in increased suppression of these signaling pathways. Although GF signaling from autocrine mechanisms has been observed in primitive CML cells even in the absence of exogenous GF (29), autocrine GF production and signaling is Bcr-Abl kinase dependent and rapidly inhibited with Imatinib treatment (30). On the other hand, treatment with Dasatinib in the presence of GF did not inhibit P-STAT5 or P-Akt expression in CML CD34+ cells. This indicates that inhibition of Src activity did not suppress GF-activated signaling through these pathways. In contrast, a dose-dependent increase in MAPK activity observed in CD34+ progenitor cells treated with Imatinib in the presence of GF was much less apparent after Dasatinib treatment, suggesting that Src signaling may contribute to increased MAPK activity under these conditions.

Importantly, inhibition of Src signaling in combination with Bcr-Abl kinase inhibition by Dasatinib did not induce proapoptotic signals in CML progenitors. This is consistent with our previous and current observations that primitive CML CP cells are resistant to induction of apoptosis with Dasatinib (4, 14). Primitive leukemic cells from mice with Bcr-Abl retrovirus–induced B-ALL and CML have also been shown to be insensitive to both Imatinib and Dasatinib treatment (31). These observations suggest that combined Src and Bcr-Abl kinase inhibition does not enhance targeting of primitive progenitors from CP CML patients. It remains possible that Src inhibition may have a role in advanced phase or Imatinib-resistant CML. Overexpression of Src family kinases has been implicated in Imatinib resistance and CML progression, and short interfering RNA targeting the Src kinase Lyn can induce apoptosis in CML BC cells (11, 13). An adaptive increase in GF production and Jak2 signaling may contribute to Imatinib resistance, and inhibition of Src signaling may also be beneficial in this context (28, 32).

Dasatinib significantly suppressed CML primitive and committed progenitor cells in LTC-IC and CFC assays. Dasatinib also significantly reduced the number of dividing cells observed on CFSE tracking experiments. These observations, together with the lack of apoptosis in undivided cells, suggest that Dasatinib suppresses progenitor growth through inhibition of proliferation and a modest increase in apoptosis in dividing progenitors. These effects are very similar to those of Imatinib and again indicate that additional Src inhibition by Dasatinib did not enhance suppression and targeting of CML primitive and committed progenitors. The effects of Dasatinib treatment are similar to those obtained with another dual Bcr-Abl and Src inhibitor, SKI-606. Although less potent than Dasatinib, active concentrations of SKI-606 that effectively inhibit Bcr-Abl and Src kinase activity have similar effects on CML progenitor apoptosis, proliferation, and growth in CFC and LTC-IC assays, with relatively little effect on normal progenitors (6).

In conclusion, our results indicate that Src kinase activity is enhanced in CML progenitor cells and that Dasatinib, although highly effective in inhibiting Src and Bcr-Abl kinase activity in CML progenitor cells, does not show enhanced suppression of important downstream signaling mechanisms compared with Imatinib. The enhanced Src inhibiting activity of Dasatinib does not significantly alter apoptosis regulating proteins in CML progenitors. Although our results indicate that Imatinib and Dasatinib effectively inhibit BCR/ABL kinase activity in primitive CML cell populations, it is important to also consider that there may be considerable heterogeneity in BCR-ABL expression, drug uptake and efflux, and the presence of additional genetic abnormalities within the purified populations studied. Persistence of small populations of malignant stem and progenitor cells despite inhibitor treatment could allow accumulation of additional genetic aberrations leading to drug resistance or evolution to BC. Indeed, we have shown that BCR-ABL kinase mutations can be detected in CD34+ cells from CML patients in CCR on Imatinib, may contribute to persistence of small populations of malignant progenitors, and could be a potential source of relapse or progression (25). Although we cannot exclude the possibility that Bcr-Abl and Src kinase activated is not inhibited in a small subset of CML cells that are not detectable using the assays used here, the lack of apoptosis in the bulk of CML progenitors after TKI treatment cannot be explained by lack of inhibition of Bcr-Abl and Src kinase activity. Therefore, the use of more potent Abi kinase inhibitors or dual Src-Abl kinase inhibitors may not by itself to enhance targeting of residual CML progenitors, and other pathways for CML stem and progenitor cell survival need to be identified and targeted to enhance their elimination. In this respect, our recent observations that farnesyl transferase inhibitors and histone deacetylase inhibitors (33, 34) are capable of effectively inducing apoptosis in quiescent CML primitive progenitors indicate promising areas for further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/28/2008; revised 6/30/2008; accepted 9/29/2008.

Grant support: NIH grant R01 CA95684, a Scholar in Clinical Research award of the Leukemia and Lymphoma Society (R. Bhatia), Medical Research Council (G84/5317) and Leukaemia Research Trust for Scotland funding (M. Copland), and General Clinical Research Center Grant #5M01 RR00043.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Lucy Brown, Claudio Spalla, and Alex Spalla from the Analytical Cytometry Core for excellent technical support, and Dr. Marilyn Slovak and Victoria Bedill on the Cytogenetics Core laboratory for performing the FISH analysis, Emma Hamill for assistance with intracellular P-Src and P-CrkL flow cytometry, and StemCyte for their generous gift of cord blood samples.

References


Effects of Dasatinib on Src Kinase Activity and Downstream Intracellular Signaling in Primitive Chronic Myelogenous Leukemia Hematopoietic Cells

Heiko Konig, Mhairi Copland, Su Chu, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/23/9624

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/11/19/68.23.9624.DC1

Cited articles
This article cites 33 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/23/9624.full.html#ref-list-1

Citing articles
This article has been cited by 13 HighWire-hosted articles. Access the articles at:
/content/68/23/9624.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.