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

Heiko Konig,[1] Mhairi Copland,[3] Su Chu,[1] Richard Jove,[2] Tessa L. Holyoake,[3] and Ravi Bhatia[1]

[1]Department of Hematopoietic Stem Cell and Leukemia Research, City of Hope National Medical Center and [2]Division of Molecular Medicine, Beckman Research Institute of the City of Hope, Duarte, California; and [3]Section of Experimental Haematology, Cancer Division, University of Glasgow, Scotland, United Kingdom

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 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 IC_{50} of <1 nmol/L (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 CP 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.

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 μmol/L, and Imatinib was added at a concentration of 5 μmol/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 Biotech) 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% CO_2 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 [LTBMC; 200 pg/mL granulocyte-macrophage colony-stimulating factor (CSF); 1 ng/mL G-CSF; 200 pg/mL stem cell factor; 50 pg/mL leukemia inhibitory factor; 200 pg/mL macrophage-inflammatory 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-erythroid and colony-forming unit-granulocyte and macrophage were counted after 14 d.

Long-term culture-initiating cells. To assess primitive progenitors, CD34+ cells were plated in LTBMC medium on M2-10B4 murine fibroblast feeders subcultured in 96-well plates. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 and fed at weekly intervals. After 6 wk, wells were overlaid with colony forming cells (CFC) growth–supporting medium and scored as positive or negative for the presence of CFC after 2 wk. The frequency of long-term culture-initiating cells (LTC-IC) was calculated with L-Calc software (StemCell Technologies). Results from the CFC and LTC-IC were reported as percentage of growth inhibition versus control.

Flow Cytometry

$CD34^+CD38^+$ and $CD34^+CD38^-$ progenitor cells were labeled with 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) as described previously (7). CFSE-labeled cells were cultured for 96 h in the presence or absence of inhibitors. At the end of the culture period, cells were labeled with Annexin V-PE (BD Pharmingen). Cell division was analyzed on the basis of CFSE fluorescence measured by flow cytometry (FACScalibur; Becton Dickinson). The percentage of cells in different generations was enumerated and a proliferation index was generated using ModFit software (Verity). Apoptotic cells were defined as Annexin $V\text{-}PE^+$. Intracellular phospho-Src (P-Src Alexa-Fluor 488; Upstate) and phospho-Crk-like (P-CrkL; Cell Signalling) staining were performed and analyzed by flow cytometry using methods described previously (14).

Western Blot Analysis

$CD34^+$ cells were cultured in medium containing low concentrations of GFs, with or without inhibitors, for 16 h. Cells were lysed in buffer containing 0.5% Nonidet P-40 (Sigma Diagnostics) and 0.5% sodium deoxycholate supplemented with phenylmethylsulfonyl fluoride (1 mmol/L), protease inhibitor mixture, and phosphatase inhibitors (50 mmol/L Sodium Fluoride, 1 mmol/L Sodium Vanadate; all from Sigma Diagnostics). Proteins were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. Membranes were sequentially reprobed with primary and secondary antibodies. Primary antibodies used were as follows: anti-CrkL rabbit polyclonal antibody (sc-319), anti-Phosphotyrosine mouse monoclonal antibody (mAb; 4G10; Millipore) anti-phosphorylated p42/44 mitogen-activated protein kinase (P-MAPK) mouse mAb (sc-7383), anti-p42/44 MAPK rabbit polyclonal antibody (sc-94), anti-STAT5 rabbit polyclonal antibody (sc-835), anti–Bcl-2 (100) mouse mAb (sc-509), anti–Mcl-1 (S-19) rabbit polyclonal antibody (sc-819; all from Santa Cruz Biotechnology), anti-phosphorylated STAT5 (P-STAT5; Tyr694) rabbit polyclonal antibody, anti-phosphorylated Akt (P-Akt; Ser473) rabbit polyclonal antibody, anti-Akt rabbit polyclonal antibody, anti-phosphorylated Src Family (Tyr416) rabbit polyclonal antibody and anti-Src rabbit polyclonal antibody (all from Cell Signaling Technology), anti-actin mouse mAb (clone AC-15), anti-Bim (BOD) rabbit polyclonal antibody (all from Sigma), and anti–Bcl-X_L rabbit polyclonal antibody (BD Biosciences). Horseradish peroxidase– or alkaline phosphatase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. Antibody detection was performed using the Superfemto kit (Pierce Biotechnology). The protein expression levels were determined by densitometry using Image-Quant software (Amersham Pharmacia Biotech).

Fluorescent *In situ* Hybridization Analysis

Fluorescence *in situ* hybridization (FISH) analysis was performed as previously described (23). Colonies from CFC assays were harvested, pooled, and washed. Cells were resuspended in hypotonic KCl solution, centrifuged, and fixed using Carnoy fixative. Hybridization using the LSI dual-labeled Bcr-Abl DNA probe was performed in accordance with the manufacturer's instructions (Vysis). Lymphocytes from a healthy individual served as a Bcr-Abl negative control; SD-1 cell lines, derived from an acute lymphoblastic leukemia (ALL) patient, served as a Bcr-Abl positive control. A total of 200 nuclei were scored for each sample.

Statistical Analysis

Data obtained from independent experiments were reported as the mean ± SE. Student's *t* test analysis was performed to determine statistical significance.

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. 1*A*–*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. 1*A* 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. 1*B*) 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. 1*D*). 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 more primitive CML $CD34^+CD38^-$ cells ($P < 0.001$) compared with no drug controls (Fig. 2*A*). 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. 2*B*). 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. 2*C*, 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. 2*D*). 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. 3*A*). Incubation of CML CD34+ cells with Dasatinib in the presence of GF did not lead to a significant change in P-Akt and P-STAT levels in CML CD34+ cells (Fig. 3*B* 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. 4*A*). 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,

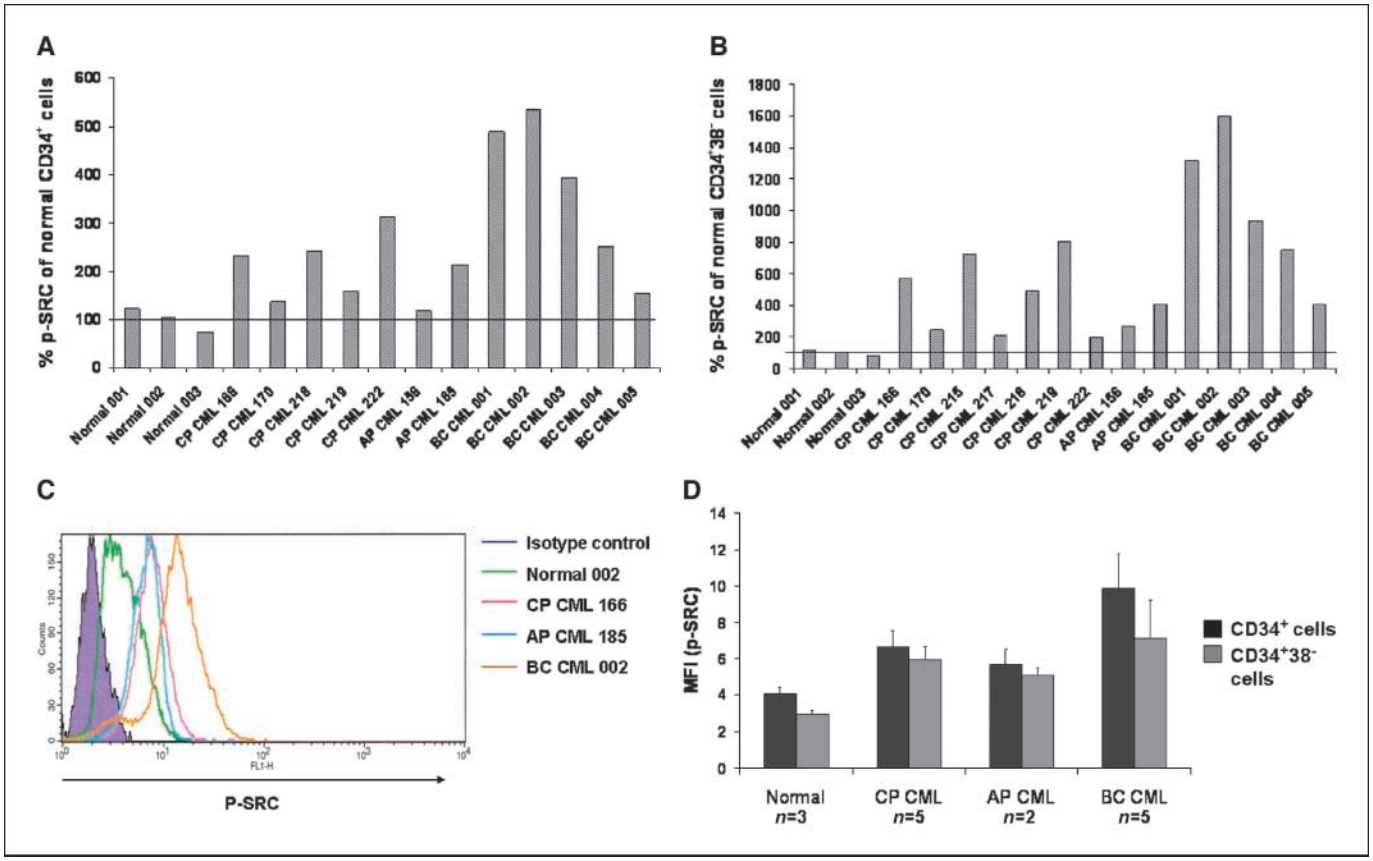

Figure 1. Assessment of P-Src expression in $CD34^+$ and CD34+38− cells from patients with CP, AP, and BC CML. P-Src expression as assessed by flow cytometry in (*A*) $CD34^+$ and (*B*) CD34+38− CML cells compared with normal progenitor cells. *C,* a representative fluorescence-activated cell sorting (FACS) histogram plot of P-Src in the different phases of CML compared with normal $CD34^+$ cells is shown. *D,* histograms showing P-Src expression in total $CD34^+$ compared with the more primitive $CD34^+38^-$ subpopulation; *MFI,* mean fluorescence intensity.

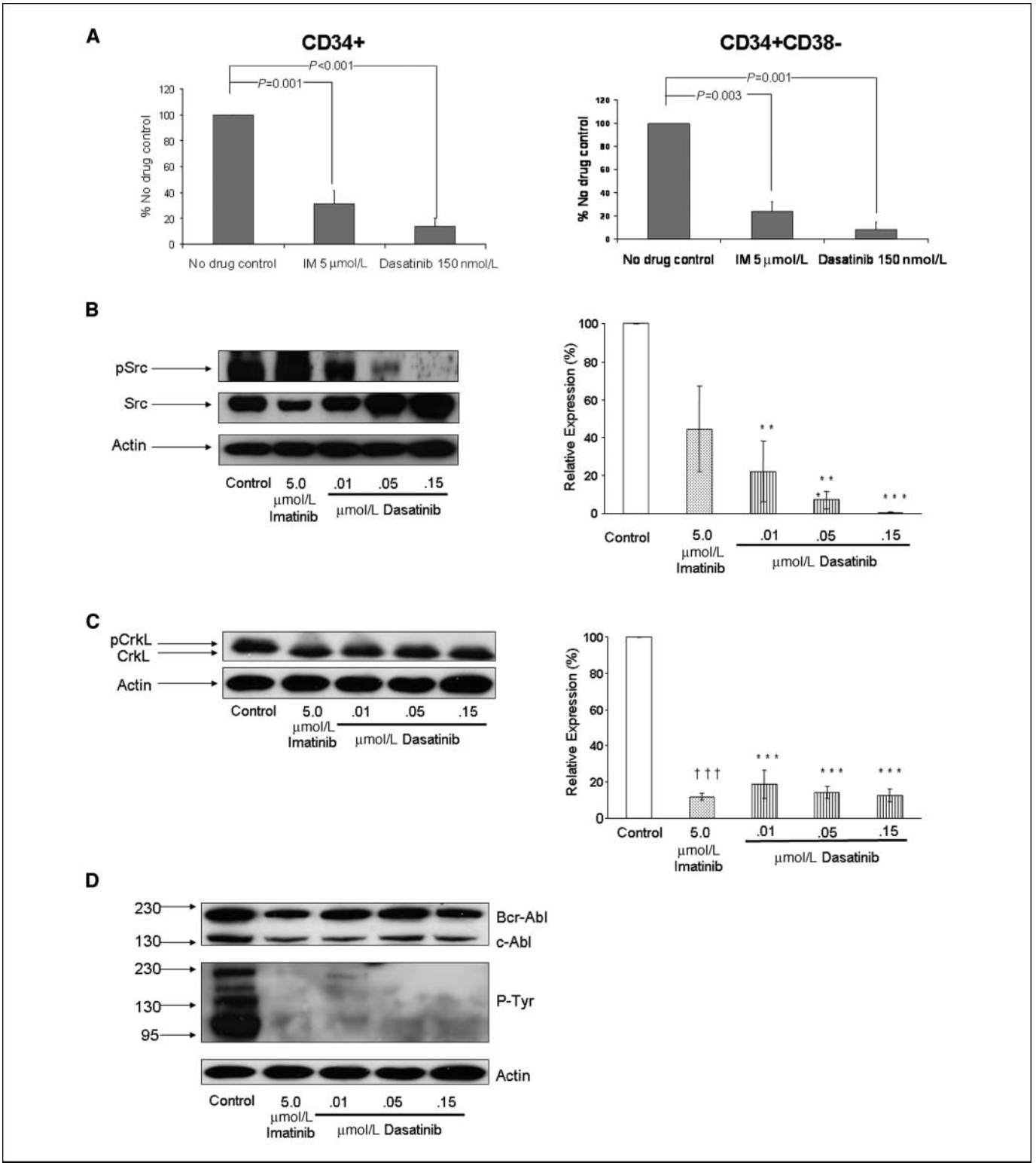

Figure 2. Effects of Imatinib and Dasatinib on P-Src and P-CrkL expression in CML CD34+ and CD34+CD38− cells. The effect of Imatinib and Dasatinib on P-Src expression was assessed by flow cytometry in total $CD34^+$ (*left*) and more primitive $CD34^+38^-$ (*right*) CML cells at 16 and 72 h ($n = 6$; 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 (***, $P < 0.001$). 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*).

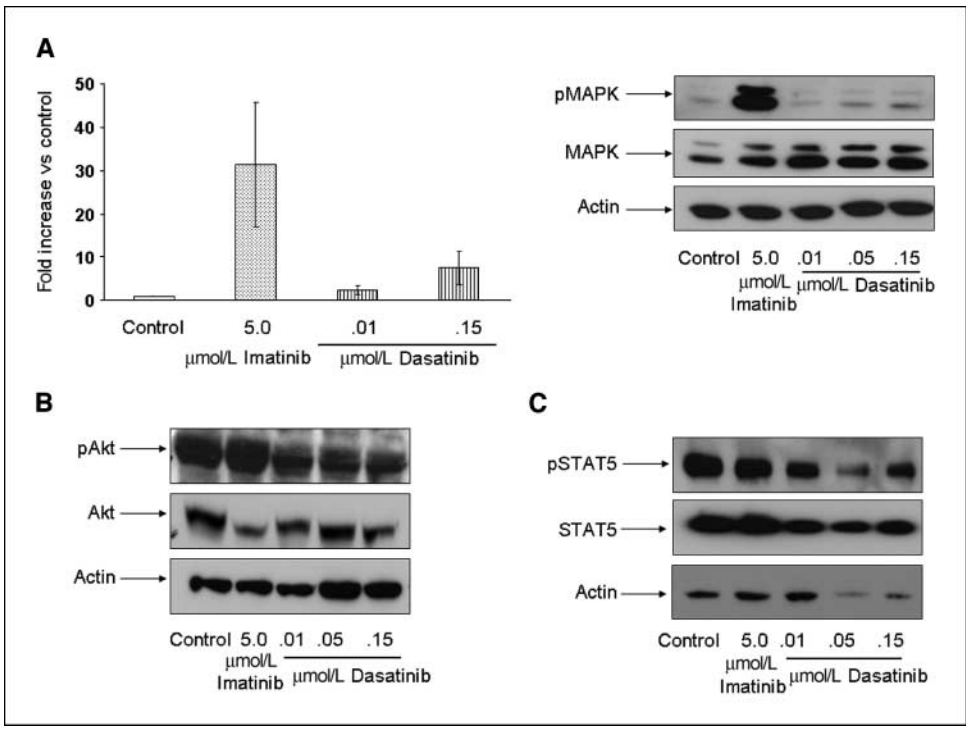

Figure 3. Effects of Dasatinib on MAPK, Akt, and STAT5 signaling in CML $CD34^+$ cells. CML $CD34^+$ cells were incubated with Dasatinib or Imatinib for 16 h in low GF–containing medium as described in the Materials and Methods. Cell lysates were prepared and Western blotting was performed using anti–P-MAPK, anti-MAPK, and anti-actin antibodies. Representative blots for one CML sample are shown. Densitometry analysis was performed and the ratio of phosphorylated protein/actin was calculated. *Columns,* mean fold increase of phosphorylated protein compared with untreated control based on replicate experiments ($n = 4$; *A*); *bars,* SE. Western blotting was performed using anti–P-Akt and anti-Akt (*B*), and anti–P-STAT5 and anti-STAT5 (*C*), and anti-actin antibodies. The results shown are representative of $n = 4$ to 5 experiments using different CML patient samples.

the additional Src inhibition by Dasatinib does not further inhibit signaling through the MAPK, PI-3K, and STAT5 pathways in cells exposed to GF.

Dasatinib treatment does not alter apoptosis-regulating proteins and has only modest effects on CML progenitor cell apoptosis in the presence of GFs but has significant antiproliferative effects. CML, cord blood, and normal PBSC CD34+ cells were cultured for 96 hours in low GF conditions with or without Dasatanib or Imatinib, and the number of CFC (committed progenitors) and LTC-IC (primitive progenitors) present after culture was assessed. Dasatinib resulted in dose-dependent suppression of CML LTC-IC compared with untreated controls ($P = 0.0008$; Fig. 5*A*). Dasatinib treatment also resulted in a significant, dose-dependent suppression of CML CFC ($P = 0.0001$; Fig. 5*A*). 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. 5*A* 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 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. 5*B*). Dasatinib also inhibited normal PBSC CFC to a lesser extent than CML CFC (Fig. 5*C*).

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. 6*A* and *B*). Treatment with Dasatinib resulted in only a modest increase in apoptosis of CML primitive and committed progenitors. Similar results were seen 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. 6*A* 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-X_L, and Bim after adjusting for protein loading based on actin (Fig. 6*C*). 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. S1*A*). Dasatinib also inhibited proliferation of cord blood primitive progenitors (Supplementary Fig. S1*B*) and normal PBSC primitive and committed progenitors (Supplementary Fig. S1*C*) 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 S1*D*; 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. 6*D*).

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

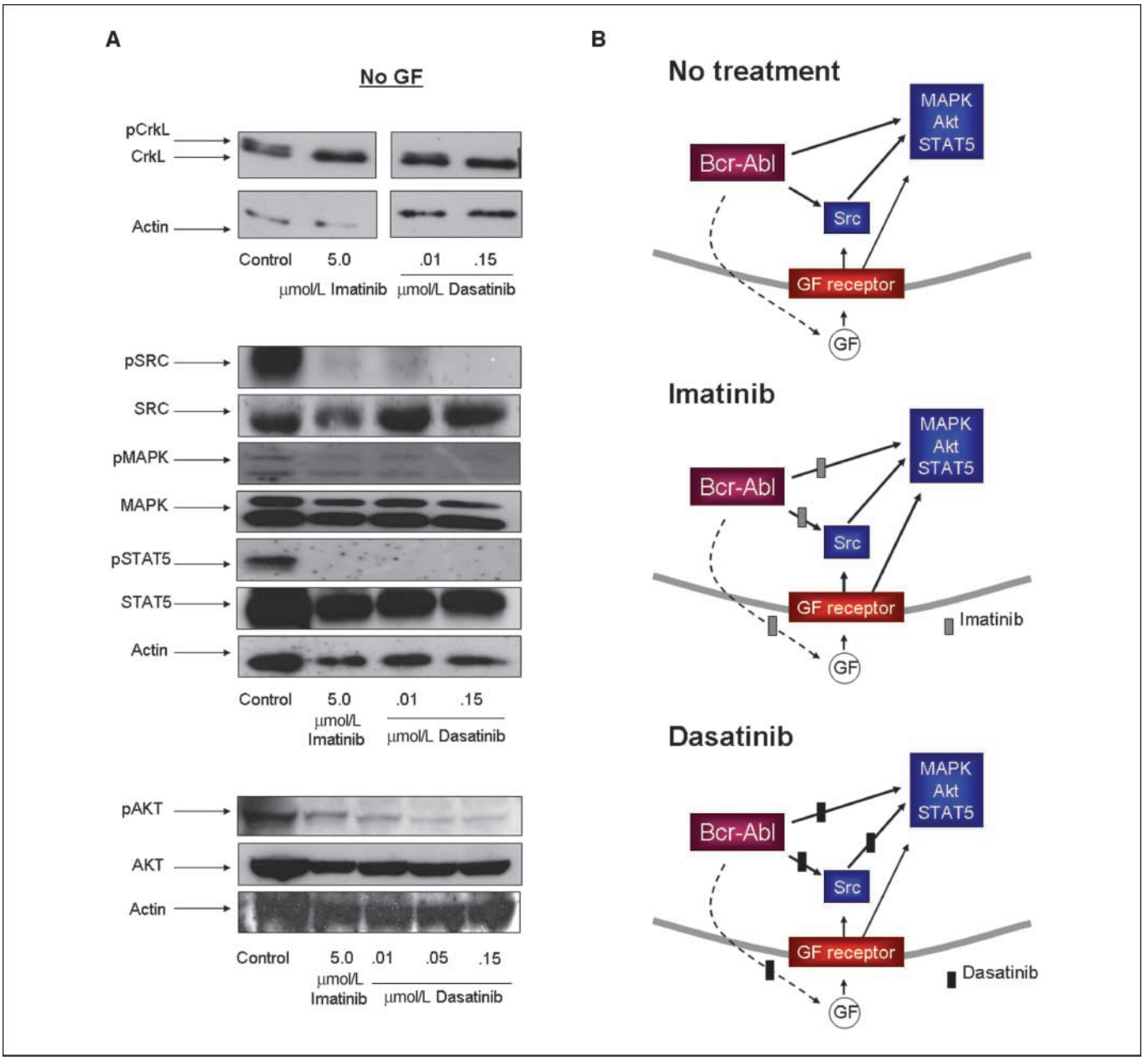

Figure 4. Effects of Dasatinib on cell signaling pathways in CML CD34$^+$ cells in the absence of GFs. CML CD34$^+$ cells were incubated with Dasatinib or Imatinib for 16 h in SFEM without addition of GF, followed by preparation of cell lysates and Western blotting with the indicated antibodies. The 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.

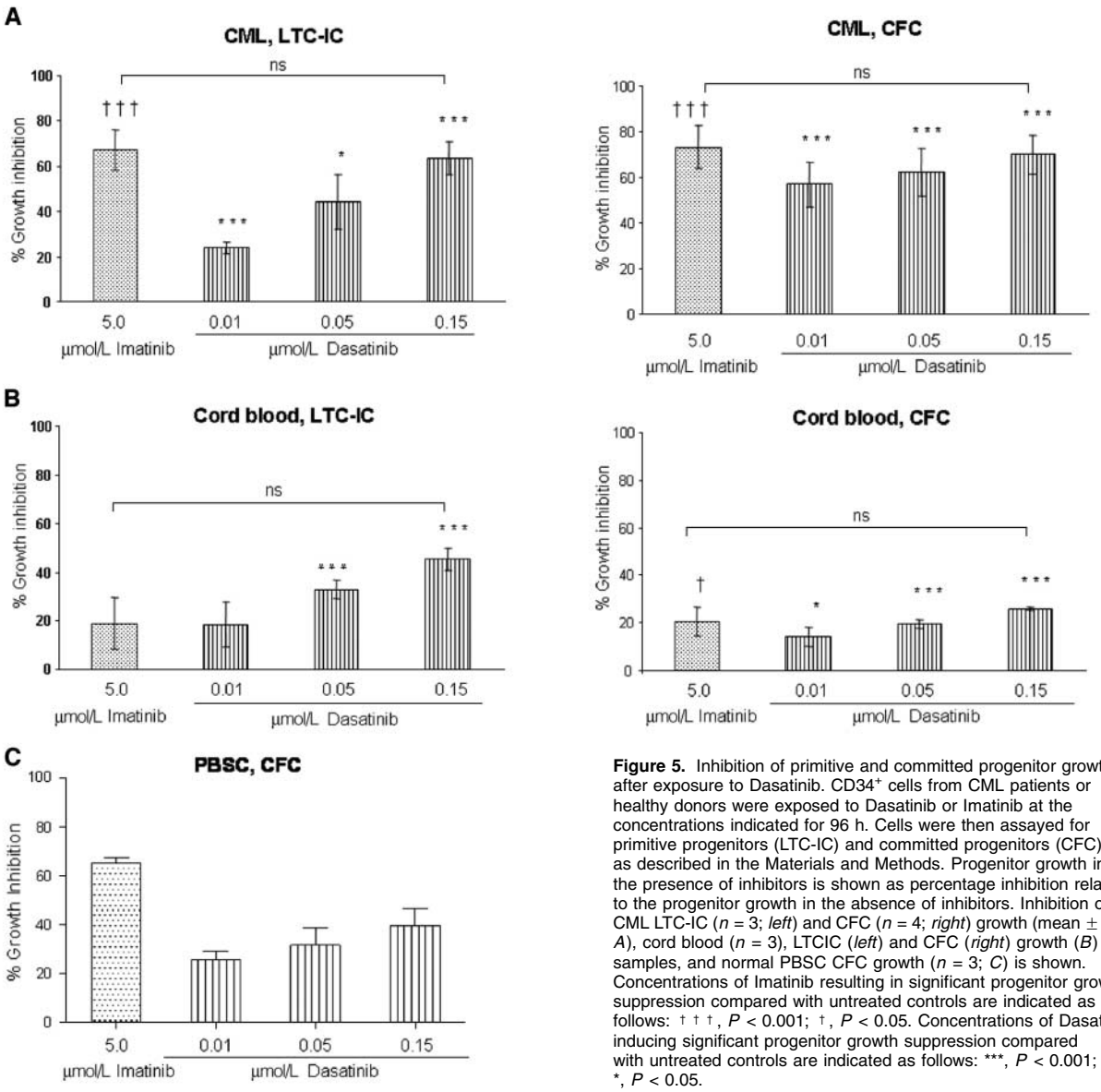

Figure 5. Inhibition of primitive and committed progenitor growth after exposure to Dasatinib. CD34$^+$ cells from CML patients or healthy donors were exposed to Dasatinib or Imatinib at the concentrations indicated for 96 h. Cells were then assayed for primitive progenitors (LTC-IC) and committed progenitors (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: ***, $P < 0.001$; *, $P < 0.05$.

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− 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

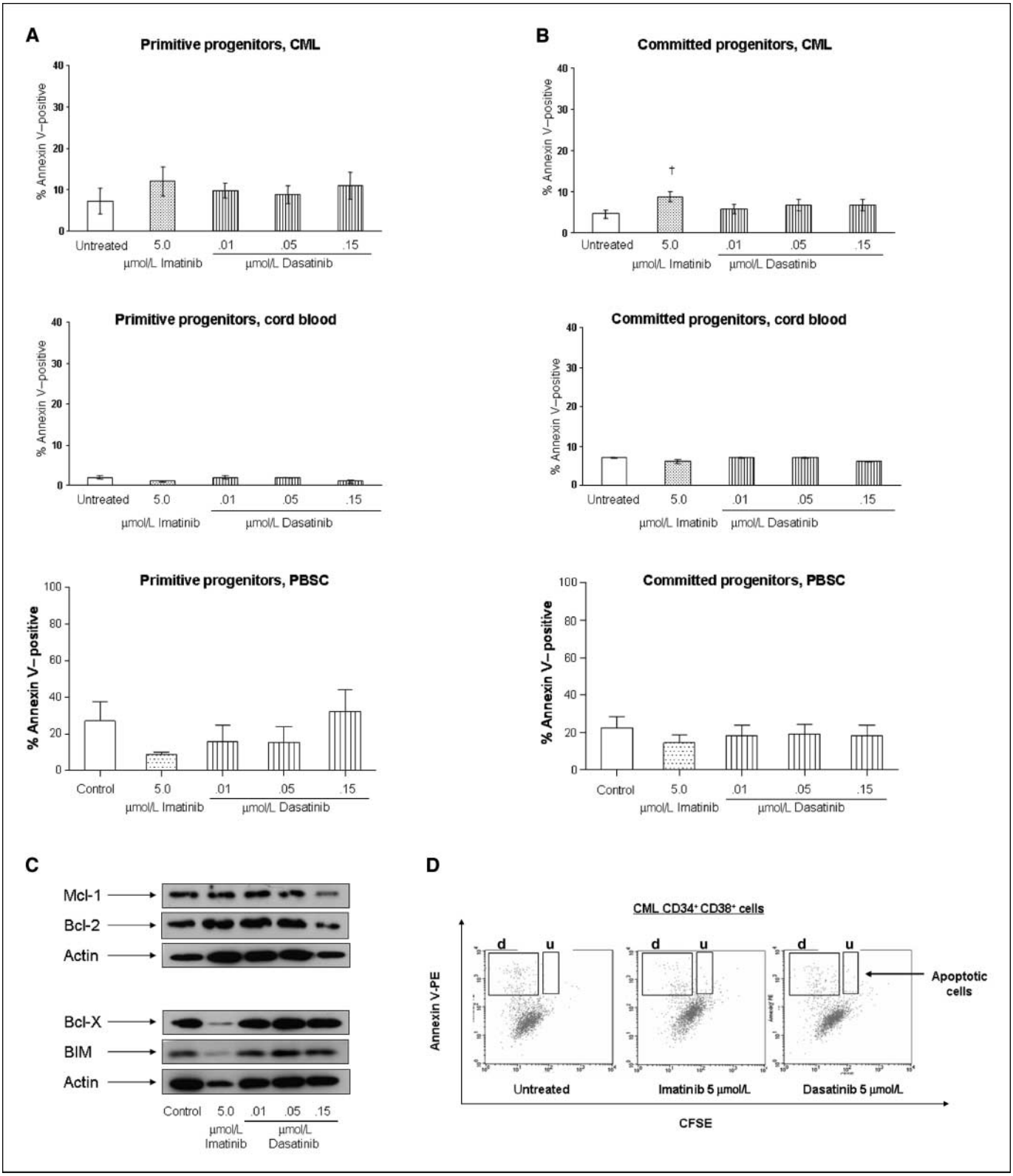

Figure 6. Effect of Dasatinib on apoptosis in CML primitive and committed progenitors. CML CD34+CD38− primitive and CD34+CD38+ committed progenitors were exposed to Dasatinib or Imatinib at indicated concentrations for 96 h. Apoptosis was analyzed by FACS after Annexin V-PE labeling. The percentage of 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 (†, $P < 0.05$) 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-X_L, 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 Abl 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 (G84/6317) 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 Bedell in 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

1. Hochhaus A, Druker B, Sawyers C, et al. Favorable long-term follow-up results over 6 years for response, survival, and safety with imatinib mesylate therapy in chronic-phase chronic myeloid leukemia after failure of interferon-α treatment. Blood 2008;111:1039–43.
2. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med 2006;355:2408–17.
3. Rousselot P, Huguet F, Rea D, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. Blood 2007;109:58–60.
4. Holtz MS, Bhatia R. Effect of imatinib mesylate on chronic myelogenous leukemia hematopoietic progenitor cells. Leuk Lymphoma 2004;45:237–45.
5. Konig H, Holtz M, Modi H, et al. Enhanced BCR-ABL kinase inhibition does not result in increased inhibition of downstream signaling pathways or increased growth suppression in CML progenitors. Leukemia 2008;22:748–55.

6. Konig H, Holyoake TL, Bhatia R. Effective and selective inhibition of chronic myeloid leukemia primitive hematopoietic progenitors by the dual Src/Abl kinase inhibitor SKI-606. Blood 2008;111:2329–38.
7. Holtz MS, Slovak ML, Zhang F, Sawyers CL, Forman SJ, Bhatia R. Imatinib mesylate (STI571) inhibits growth of primitive malignant progenitors in chronic myelogenous leukemia through reversal of abnormally increased proliferation. Blood 2002;99:3792–800.
8. Danhauser-Riedl S, Warmuth M, Druker BJ, Emmerich B, Hallek M. Activation of Src kinases p53/56lyn and p59hck by p210bcr/abl in myeloid cells. Cancer Res 1996;56:3589–96.
9. Lionberger JM, Wilson MB, Smithgall TE. Transformation of myeloid leukemia cells to cytokine independence by Bcr-Abl is suppressed by kinase-defective Hck. J Biol Chem 2000;275:18581–5.
10. Klejman A, Schreiner SJ, Nieborowska-Skorska M, et al. The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. Embo J 2002;21:5766–74.
11. Donato NJ, Wu JY, Stapley J, et al. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. Blood 2003;101:690–8.
12. Wu J, Meng F, Lu H, et al. Lyn regulates BCR-ABL and Gab2 tyrosine phosphorylation and c-Cbl protein stability in imatinib-resistant chronic myelogenous leukemia cells. Blood 2008;111:3821–9.
13. Ptasznik A, Nakata Y, Kalota A, Emerson SG, Gewirtz AM. Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukemia cells. Nat Med 2004;10:1187–9.
14. Copland M, Hamilton A, Elrick LJ, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood 2006;107:4532–9.
15. Li S. Src kinase signaling in leukaemia. Int J Biochem Cell Biol 2007;39:1483–8.
16. Li S. Src-family kinases in the development and therapy of Philadelphia chromosome-positive chronic myeloid leukemia and acute lymphoblastic leukemia. Leuk Lymphoma 2008;49:19–26.
17. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 2004;305:399–401.
18. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354:2531–41.
19. Hochhaus A, Kantarjian HM, Baccarani M, et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. Blood 2007;109:2303–9.
20. O'Hare T, Walters DK, Stoffregen EP, et al. *In vitro* activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. Cancer Res 2005;65:4500–5.
21. O'Hare T, Walters DK, Stoffregen EP, et al. Combined Abl inhibitor therapy for minimizing drug resistance in chronic myeloid leukemia: Src/Abl inhibitors are compatible with imatinib. Clin Cancer Res 2005;11:6987–93.
22. Peng B, Hayes M, Resta D, et al. Pharmacokinetics and pharmacodynamics of imatinib in a phase I trial with chronic myeloid leukemia patients. J Clin Oncol 2004;22:935–42.
23. Bhatia R, Munthe HA, Williams AD, Zhang F, Forman SJ, Slovak ML. Chronic myelogenous leukemia primitive hematopoietic progenitors demonstrate increased sensitivity to growth factor-induced proliferation and maturation. Exp Hematol 2000;28:1401–12.
24. Graham SM, Jorgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 *in vitro*. Blood 2002;99:319–25.
25. Chu S, Xu H, Shah NP, et al. Detection of BCR-ABL kinase mutations in CD34+ cells from chronic myelogenous leukemia patients in complete cytogenetic remission on imatinib mesylate treatment. Blood 2005;105:2093–8.
26. Hu Y, Liu Y, Pelletier S, et al. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. Nat Genet 2004;36:453–61.
27. Smithgall TE, Briggs SD, Schreiner S, Lerner EC, Cheng H, Wilson MB. Control of myeloid differentiation and survival by Stats. Oncogene 2000;19:2612–8.
28. Nam S, Williams A, Vultur A, et al. Dasatinib (BMS-354825) inhibits Stat5 signaling associated with apoptosis in chronic myelogenous leukemia cells. Mol Cancer Ther 2007;6:1400–5.
29. Holyoake TL, Jiang X, Jorgensen HG, et al. Primitive quiescent leukemic cells from patients with chronic myeloid leukemia spontaneously initiate factor-independent growth *in vitro* in association with up-regulation of expression of interleukin-3. Blood 2001;97:720–8.
30. Chalandon Y, Jiang X, Loutet S, Eaves AC, Eaves CJ. Growth autonomy and lineage switching in BCR-ABL-transduced human cord blood cells depend on different functional domains of BCR-ABL. Leukemia 2004;18:1006–12.
31. Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice. Proc Natl Acad Sci U S A 2006;103:16870–5.
32. Wang Y, Cai D, Brendel C, et al. Adaptive secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) mediates imatinib and nilotinib resistance in BCR/ABL+ progenitors via JAK-2/STAT-5 pathway activation. Blood 2007;109:2147–55.
33. Copland M, Pellicano F, Richmond L, et al. BMS-214662 potently induces apoptosis of chronic myeloid leukemia stem and progenitor cells and synergizes with tyrosine kinase inhibitors. Blood 2008;111:2843–53.
34. Strauss AC, Chu S, Holyoake T, Bhatia R. Effective Induction of Apoptosis in Chronic Myeloid Leukemia CD34+ Cells by the Histone Deacetylase Inhibitor LAQ824 in Combination with Imatinib. Blood (ASH Annual Meeting Abstracts) 2007;110:Abstract 1031.

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.

Cancer Res 2008;68:9624-9633.