

Dasatinib (BMS-354825) inhibits Stat5 signaling associated with apoptosis in chronic myelogenous leukemia cells

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Abstract

Dasatinib (BMS-354825) is a novel, oral, potent, multi-targeted kinase inhibitor of Bcr-Abl and Src family kinases (SFK) and is a promising cancer therapeutic agent. Preclinical data indicate that dasatinib is 325-fold more potent than imatinib against cells expressing wild-type Bcr-Abl, and that dasatinib is active against 18 of 19 Bcr-Abl mutations known to cause imatinib resistance. Phase I clinical data show that dasatinib is well tolerated and highly effective for the treatment of imatinib-resistant/imatinib-intolerant chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive acute lymphoblastic leukemia. However, the molecular mechanism of action of dasatinib is not fully understood. In this study, we confirm that dasatinib inhibits tyrosine phosphorylation of SFKs, including Src, Hck, and Lyn, in K562 human CML cells. Significantly, downstream signal transducer and activator of transcription 5 (Stat5) signaling is also blocked by dasatinib as shown by decreases in levels of phosphorylated Stat5 and Stat5 DNA-binding activities. In addition, dasatinib down-regulates expression of Stat5 target genes, including *Bcl-x*, *Mcl-1*, and *cyclin D1*. Consistent with these results, blockade of Stat5 signaling by dasatinib is accompanied by inhibition of cell proliferation and induction of apoptosis. Surprisingly, Stat5

DNA-binding activities are enhanced with increasing cell density, which is associated with resistance to apoptosis by dasatinib. Our findings indicate that inhibition of Stat5 signaling downstream of Bcr-Abl/SFKs contributes to the action of dasatinib, and, conversely, that increasing cell density up-regulates Stat5 activation and confers resistance to dasatinib. Moreover, the level of phosphorylated Stat5 in CML cells represents a mechanistically relevant biomarker for monitoring inhibition of Bcr-Abl signaling by dasatinib in CML patients using convenient immunocytochemical assays. [Mol Cancer Ther 2007;6(4):1400–5]

Introduction

Signal transducer and activator of transcription (STAT) proteins have been shown to have a major role in survival, proliferation, angiogenesis, and immune evasion of tumors (1–7). One STAT family member (Stat5) is often persistently activated in blood malignancies by non-receptor tyrosine kinases such as Bcr-Abl, Src, and other Src family kinases (SFK; refs. 1, 8–11). Furthermore, constitutive activation of Stat5 up-regulates the expression of genes, including *Bcl-x*, *Mcl-1*, and *cyclin D1/2*, which are associated with survival and proliferation in chronic myelogenous leukemia (CML) cells (11–14). Taken together, increasing evidence indicates that persistent activation of Stat5 and consequent deregulation of downstream gene expression contribute to malignant progression in CML (1, 7, 15).

Dasatinib is a potent tyrosine kinase inhibitor that targets Bcr-Abl and SFKs (16, 17). Preclinical data indicate that dasatinib is ~2 orders of magnitude more potent than imatinib against cells expressing wild-type Bcr-Abl (18). Furthermore, phase I clinical trials indicate that dasatinib is a promising therapeutic agent for imatinib-resistant/imatinib-intolerant CML patients. Recently, dasatinib has been approved by the Food and Drug Administration and the European Union in all stages of CML and Philadelphia chromosome-positive acute lymphoblastic leukemia resistant or intolerant to prior therapy. However, the molecular mechanism by which dasatinib induces growth arrest and apoptosis of CML cells downstream of Bcr-Abl is not fully understood. In the present study, we report that dasatinib blocks SFK and Stat5 signaling and down-regulates *Bcl-x*, *Mcl-1*, and *cyclin D1* expression associated with inhibition of proliferation and induction of apoptosis in CML cells. In addition, increasing cell density up-regulates Stat5 activity and confers resistance to dasatinib. Taken together, our data indicate that inhibition of Stat5 signaling downstream of Bcr-Abl/SFKs contributes to the mechanism of action of dasatinib in CML.

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Materials and Methods

Cell Lines and Reagents

K562 human CML and HL-60 human promyelocytic leukemia cells were obtained from the American Type Culture Collection (Manassas, VA). HL-60/Bcr-Abl (expressing ectopic Bcr-Abl protein) and Re-HL-60/Bcr-Abl (imatinib-resistant cells expressing Bcr-Abl protein) were described previously (19). All cells were cultured in RPMI 1640 containing 10% fetal bovine serum. Monoclonal antibodies to Abl, phosphorylated tyrosine, and cyclin D1 were obtained from BD Biosciences (San Diego, CA). Polyclonal antibodies to phosphorylated Stat5 (p-Stat5; Tyr⁶⁹⁴) and phosphorylated Src (Tyr^{416/419}) were obtained from Cell Signaling Technologies (Cambridge, MA). Polyclonal antibodies to Stat5, Bcl-x, Mcl-1, phosphorylated Hck, Hck, and β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to c-Src was obtained from Upstate Biotechnology (Lake Placid, NY).

Immunoprecipitation and Western Blot Analyses

Immunoprecipitations and Western blots were done as described previously, with minor modifications (4, 11). Briefly, cell lysates (500 μ g) were incubated with Abl antibody at 4°C followed by protein A/G-agarose beads (Pierce, Rockford, IL). Immunoprecipitates or whole-cell lysates were resolved by SDS-PAGE and immunoblotted with specific antibodies. Primary phospho-specific antibodies were incubated in TBS (pH 7.5) with 0.1% Tween 20 and 5% bovine serum albumin with gentle agitation overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were incubated in TBS (pH 7.5) with 5% nonfat milk and 0.1% Tween 20 at a 1:2,000 dilution for 1 h at room temperature. Positive immunoreactive proteins were detected using the ECL system (Pierce).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays were done as described in detail previously (11). To assess Stat5 DNA-binding activity, 8 μ g of nuclear protein extract was incubated with ³²P-radiolabeled oligonucleotide probe containing the mammary gland factor element, derived from the bovine β -casein gene promoter (5-AGATTCTAG-GAATCAA-3'; ref. 11). For supershifts, 1 μ L of antibody to Stat5 was preincubated with nuclear extract for 30 min before addition of the ³²P-labeled mammary gland factor element probe. Resolution of protein-DNA complexes was done by 5% nondenaturing PAGE and detected by autoradiography.

Viability and Apoptosis Assays

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays were done for cell viability as described by the supplier (Promega, Madison, WI). Cells were seeded in 96-well plates (10,000 per well), incubated overnight at 37°C in 5% CO₂, and exposed to dasatinib for the indicated times. DMSO was used as the vehicle control. Viable cell numbers were determined by tetrazolium conversion to its formazan dye, and absorbance was measured at 490 nm using an automated ELISA plate reader. Apoptosis assays based on loss of membrane

integrity were carried out using Annexin V-FITC as described by the supplier (BD Biosciences Pharmingen, San Diego, CA). Cells were analyzed using a FACScan flow cytometer to quantify fluorescence.

Statistical Analysis

We compared the means of our two-sample assays by using Dunnett's two-sided *t* tests for controlling the type I error rate under multiple comparisons. For all analyses, *P* < 0.01 was considered statistically significant.

Results and Discussion

Dasatinib Inhibits Tyrosyl Phosphorylation of SFKs in CML Cells

Previous studies showed that dasatinib reduced the levels of phosphorylated Abl in imatinib-resistant mouse Ba/F3 cells expressing recombinant Bcr-Abl (17, 18). To confirm that dasatinib inhibits tyrosyl phosphorylation of endogenous Bcr-Abl in human CML cells, immunoprecipitates were prepared from lysates of K562 cells treated with dasatinib for 4 h. Dasatinib substantially reduced the levels of phosphorylated Bcr-Abl at 1 nmol/L after 4 h treatment (Fig. 1A), in agreement with a prior study showing that dasatinib inhibits Abl kinase activity at 1 to 10 nmol/L in Ba/F3 mouse cells expressing Bcr-Abl (17).

Originally, dasatinib was selected as a small molecule inhibitor of SFKs (16). We also showed that dasatinib directly inhibits the kinase activities of Src and other SFKs in human prostate cancer cells both *in vitro* and *in vivo* (20).

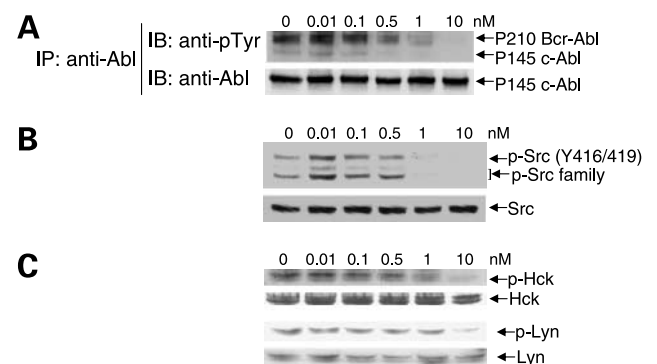


Figure 1. Dasatinib blocks Bcr-Abl and SFK activities. **A**, inhibition of Bcr-Abl phosphorylation in intact cells. K562 human CML cells were treated with dasatinib in a dose-dependent manner for 4 h. Cell lysates (500 μ g) were immunoprecipitated with specific antibody to Abl (anti-Abl) at 4°C and then incubated with protein A/G-agarose beads. After bead complexes were washed with immunoprecipitation (IP) buffer, samples were boiled, resolved by SDS-PAGE, and immunoblotted with antibodies to phosphorylated tyrosine (*anti-pTyr*) or c-Abl protein. **B**, dasatinib reduces phosphorylation levels of c-Src (Y416/419) and other SFKs in intact cells. CML cells were treated with dasatinib in a dose-dependent manner for 4 h. Western blot analysis was done with specific antibodies to phosphorylated Src (*p-Src*) family and total Src protein. **C**, dasatinib reduces levels of phosphorylated Hck (*p-Hck*) and phosphorylated Lyn (*p-Lyn*). Cell lysates used in (**B**) were immunoblotted with antibodies to phosphorylated Hck and total Hck protein. Lysates were immunoprecipitated with antibody to Lyn and then blotted with antibody to phosphorylated Src family (Y416/419), which cross-reacts with tyrosyl autophosphorylated p-Lyn (Y396).

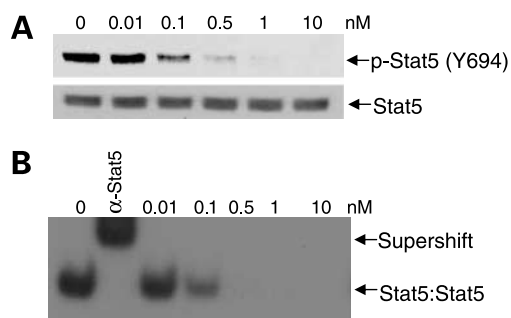


Figure 2. Dasatinib inhibits Stat5 signaling in K562 CML cells. **A**, whole-cell lysates were immunoblotted with specific antibodies to p-Stat5 (Y694) and total Stat5. **B**, dasatinib inhibits Stat5 dimer (Stat5:Stat5) DNA-binding activity. EMSA was done to assess Stat5 DNA-binding activity in nuclear extracts. Supershift with anti-Stat5 antibodies (α -Stat5) confirms that the DNA-binding activity corresponds to Stat5.

To examine the effects of dasatinib on autophosphorylation levels of Src and other SFKs in CML cells, Western blot analyses were done. A dose-dependence study using whole-cell lysates prepared 4 h after treatment with dasatinib revealed significant decreases in autophosphorylation levels of Src and other SFKs (Fig. 1B, *top*) at 1 nmol/L, whereas dasatinib did not affect total Src protein levels (Fig. 1B, *bottom*). Previous studies showed that the Src family members Hck and Lyn bind to Bcr-Abl in CML cells and have an important role in cell growth and survival of hematopoietic cells (9, 10). To identify the SFKs in K562 cells inhibited by dasatinib, we did immunoprecipitation and Western blot analyses with cell lysates used in Fig. 1B. A dose-response study showed a decrease in levels of phosphorylated Hck at 1 nmol/L of dasatinib (Fig. 1C, *top*), whereas dasatinib reduced levels of phosphorylated Lyn at 10 nmol/L (Fig. 1C, *bottom*). These results indicate that dasatinib is a potent inhibitor of Src and Hck kinases and, to a lesser extent, Lyn kinase in CML cells.

Dasatinib Inhibits Stat5 Signaling

Constitutive activation of Bcr-Abl and SFKs results in persistent tyrosyl phosphorylation of Stat5, which translocates to the nucleus and binds to the promoters of genes (1, 15). Western blot analysis using a specific antibody to tyrosyl p-Stat5 revealed that levels of p-Stat5 at Tyr⁶⁹⁴ were substantially reduced by 1 nmol/L dasatinib, whereas levels of total Stat5 protein remained unchanged (Fig. 2A). Consistent with these results, nuclear Stat5:Stat5 dimer DNA-binding activity, as measured by electrophoretic mobility shift assay, was also inhibited in a dose-dependent manner (Fig. 2B). SFKs are associated with Bcr-Abl and cooperate with Bcr-Abl in activation of Stat5 signaling during hematopoietic cell transformation (9, 10, 21). Thus, it is likely that dasatinib blocks Stat5 signaling by inhibiting both Bcr-Abl and SFKs.

It was important to assess if inhibition of the Stat5 signaling pathway by dasatinib is associated with reduced cell viability in CML and Bcr-Abl/Stat5-positive leukemia cells. Human promyelocytic leukemia cells HL-60 (lacking

Bcr-Abl), HL-60/Bcr-Abl (expressing ectopic Bcr-Abl), and Re-HL-60/Bcr-Abl (imatinib-resistant cells expressing ectopic Bcr-Abl) were used to investigate if dasatinib activity is dependent on Bcr-Abl/Stat5 signaling in human leukemia cells. Previous studies showed that HL-60 cells lack detectable levels of p-Stat5, consistent with low expression of total endogenous Stat5 proteins in these cells (22, 23). In this study, we show that K562, HL-60/Bcr-Abl, and Re-HL-60/Bcr-Abl cells contain high levels of constitutively active Stat5 protein, whereas HL-60 cells lack activated Stat5 protein (Fig. 3A and B). Dasatinib reduced levels of p-Stat5 and inhibited Stat5 DNA-binding activity in Bcr-Abl/Stat5-positive leukemia cells (Fig. 3A and B). Consistent with inhibition of Stat5 activation, dasatinib significantly inhibited cell viability in Bcr-Abl/Stat5-positive leukemia cells, but not in HL-60 cells lacking Stat5 expression and activity (Fig. 3C). These findings indicate that blockade of the Stat5 signaling pathway by dasatinib

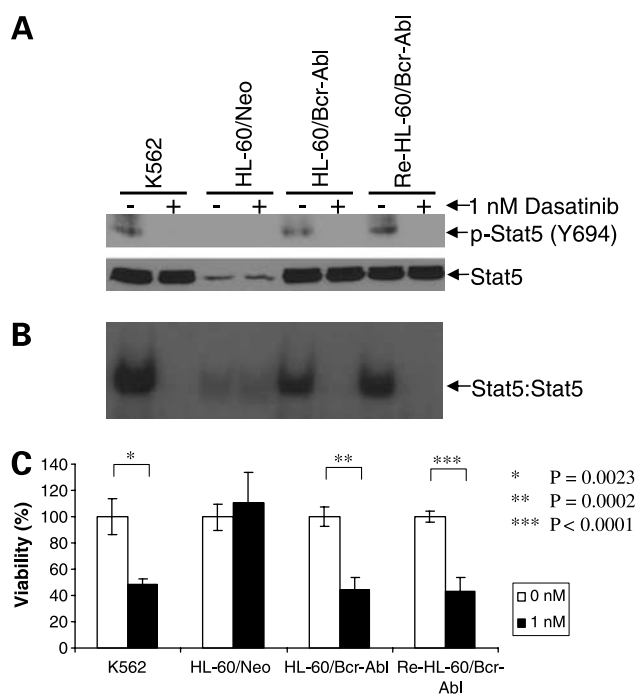


Figure 3. Dasatinib inhibits Stat5 signaling and reduces cell viability in Bcr-Abl-positive cells. K562 CML, HL-60/Neo (lacking Bcr-Abl), HL-60/Bcr-Abl (expressing ectopic Bcr-Abl), and Re-HL-60/Bcr-Abl (imatinib-resistant cells expressing Bcr-Abl) cells were treated with 1 nmol/L dasatinib for 4 h. **A**, Bcr-Abl induces Stat5 activation and dasatinib reduces levels of p-Stat5 in Bcr-Abl-positive cells. Whole-cell lysates were immunoblotted with specific antibodies to p-Stat5 (Y694) and total Stat5. **B**, dasatinib inhibits Stat5 dimer (Stat5:Stat5) DNA-binding activity in Bcr-Abl-positive cells. EMSA was done with nuclear extracts. **C**, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assays were done to determine effects of dasatinib on cell viability. Cells were treated with 1 nmol/L dasatinib for 48 h. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was added to each well for 1 h, and absorbance was measured at 490 nm. Cell viability was normalized to DMSO vehicle. *Columns*, mean for five independent experiments; *bars*, SD. *P* values (DMSO vehicle versus 1 nmol/L dasatinib in each cell line) are shown.

is associated with loss of cell viability. In addition, these observations suggest that inhibition of Bcr-Abl/Stat5 signaling is a relevant biomarker for assessing dasatinib activity in CML patients.

Dasatinib Down-regulates Bcl-x, Mcl-1, and Cyclin D1

Stat5 signaling was shown to be directly involved in cell proliferation and survival in CML by up-regulating the expression of antiapoptotic and proliferation-specific genes such as *Bcl-x*, *Mcl-1*, and *cyclin D1/2* (1, 11–14). As shown in Fig. 4, inhibition of Stat5 signaling was accompanied by down-regulation of expression of Stat5 target gene products, including *Bcl-x*, *Mcl-1*, and *cyclin D1*, following treatment with 1 nmol/L dasatinib. The concentration range of dasatinib for down-regulation of these proteins correlates with that for inhibition of Bcr-Abl kinase activity and Stat5 activation in CML cells.

Dasatinib Induces Apoptosis of CML Cells

To assess the biological consequences of dasatinib treatment on CML cells, we did 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium viability assays and Annexin V apoptosis assays. As shown in Fig. 5A, dasatinib significantly reduced cell viability at 0.5 nmol/L concentration and above in a dose-dependent manner. These results are similar to an earlier study showing that dasatinib inhibits cell growth at <1 nmol/L concentration in CML cells (16). Moreover, dasatinib has antiproliferative activity against all imatinib-resistant mutant Bcr-Abl proteins tested except for the T315I Bcr-Abl mutant (17, 18).

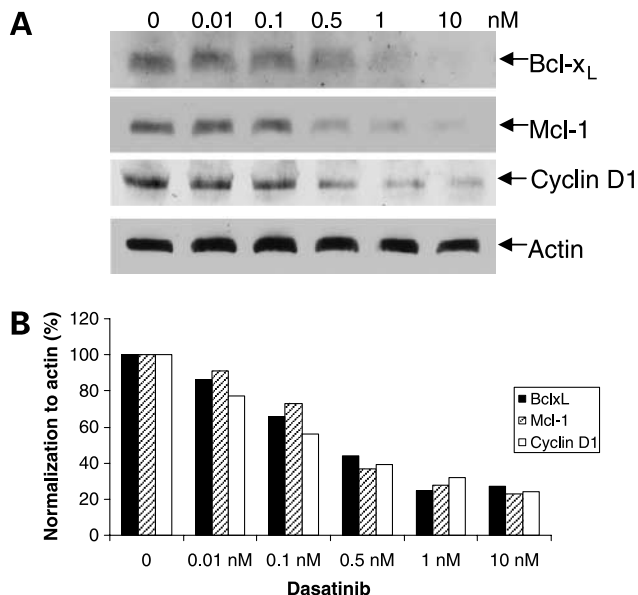


Figure 4. Effects of dasatinib on expression of downstream target genes of Stat5. CML cells were treated with dasatinib in a dose-dependent manner for 24 h. **A**, Western blot analysis was done with specific antibodies to Bcl-x_L, Mcl-1, and cyclin D1 using whole-cell lysates. **B**, normalized protein loading was confirmed by probing with antibodies to actin. Absorbance was analyzed using Quantity One software (Bio-Rad, Hercules, CA), and protein levels were normalized to actin.

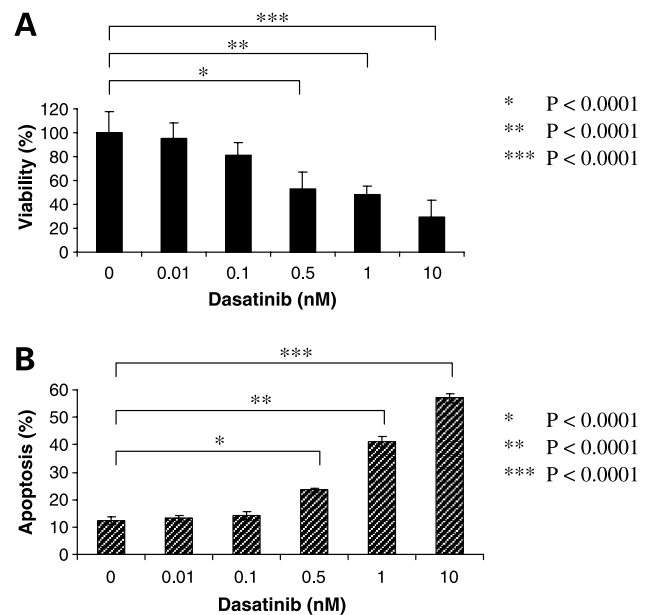


Figure 5. Dasatinib induces apoptosis of K562 human CML cells. **A**, reduction of cell viability. A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay was done following exposure to dasatinib in a dose-dependent manner. After incubation with dasatinib for the indicated times, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was added to each well for 1 h, and absorbance was measured at 490 nm. Cell viability was compared with DMSO vehicle controls. **Columns**, mean for five independent experiments; **bars**, SD. *P* values (DMSO vehicle versus dasatinib dose). **B**, induction of apoptosis. K562 cells (0.1×10^6 /mL) were treated with dasatinib in a dose-dependent manner for 48 h. Annexin V-FITC staining was used as an early marker of apoptosis. Cells were analyzed using a FACScan flow cytometer. **Columns**, mean for four independent experiments; **bars**, SD. *P* values (DMSO vehicle versus dasatinib dose).

Dasatinib also significantly induced apoptosis at 0.5 nmol/L concentration and above in a dose-dependent manner (Fig. 5B) at a cell density of 0.1×10^6 /mL. These findings are consistent with down-regulation of the products of the Stat5 downstream target genes *Bcl-x*, *Mcl-1*, and *cyclin D1* (Fig. 4). The extent of apoptosis is over 50% following 10 nmol/L dasatinib treatment for 48 h (Fig. 5B).

Increasing Cell Density Up-regulates Stat5 DNA-Binding Activity and Confers Resistance to Dasatinib

Recently, Stat3 activity was found to be enhanced with increasing cell-to-cell contact in solid tumor cells (24). To determine whether increasing cell density up-regulates Stat5 activity in K562 CML suspension cultures, Stat5 activity was measured at different cell densities. Time course studies revealed that Stat5 DNA-binding activity increased greatly over time with increasing cell density (Fig. 6A). Levels of p-Stat5 and Stat5 DNA-binding activity were also found to increase as cell density increased at 24 h after cells were seeded (Fig. 6B). To evaluate the ability of dasatinib to inhibit levels of p-Stat5 with increasing cell density, cells were seeded at various cell densities for 24 h

and then exposed to 1 nmol/L dasatinib for 4 h (Fig. 6B, bottom). Results show that increasing cell density decreased the activity of dasatinib against Stat5 signaling (Fig. 6B, bottom).

To determine whether this increase in Stat5 activity with cell density could result in resistance to dasatinib, cells were seeded for 24 h as in Fig. 6B. Following treatment with dasatinib for 48 h, flow cytometry with Annexin V-FITC staining was done. Coincident with up-regulation of Stat5 activity over increasing cell density as shown in Fig. 6B, induction of apoptosis by dasatinib was significantly reduced in a cell density-dependent manner (Fig. 6C). Stat5 up-regulates the expression of survival genes in hematopoietic cells, including *Bcl-x* (9, 25) and *Mcl-1* (26). Associated with the decreased ability of dasatinib to inhibit levels of p-Stat5 at high cell density, dasatinib was less effective in down-regulating the expression of *Bcl-x* with increasing cell densities (data not shown). Although

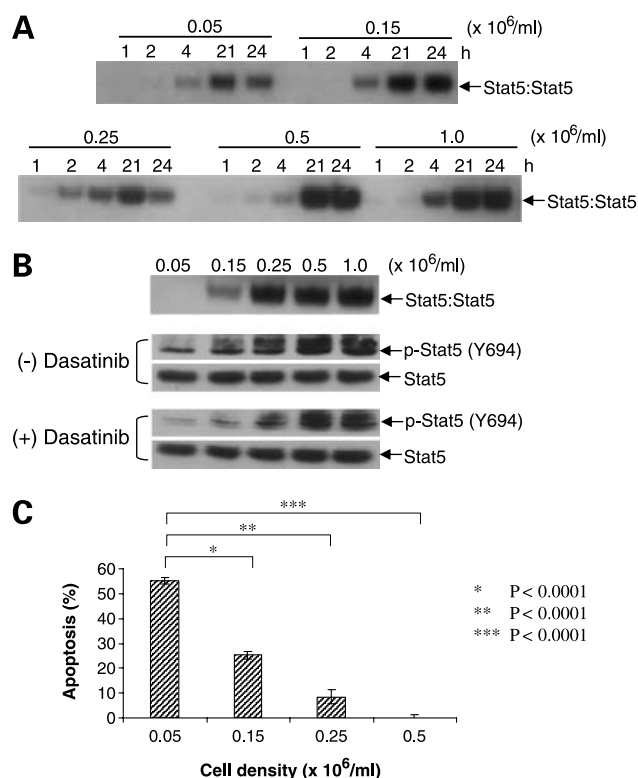


Figure 6. Increasing cell density up-regulates Stat5 DNA-binding activity and confers resistance to dasatinib. **A**, cell densities were increased from $0.05 \times 10^6/\text{mL}$ to $1.0 \times 10^6/\text{mL}$ at seeding, and cells were incubated for various time intervals. EMSA was done to assess Stat5 DNA-binding activity in nuclear extracts. **B**, cells were incubated at different cell densities for 24 h. EMSA was done with nuclear extracts (top). Cells were treated with DMSO or 1 nmol/L dasatinib for 4 h, and Western blot analysis was done with specific antibody to p-Stat5 (bottom). After 24 h of incubation, cells were treated with 1 nmol/L dasatinib for 48 h. **C**, annexin V-FITC staining was used as an early marker of apoptosis. **C**, cells were analyzed using a FACScan flow cytometer. Columns, mean for three independent experiments; bars, SD. *P* values ($0.05 \times 10^6/\text{mL}$ versus each cell density).

dasatinib down-regulates levels of *Mcl-1* as shown in Fig. 4, inhibition of *Mcl-1* by 1 nmol/L dasatinib was not influenced by cell density (data not shown). Thus, up-regulation of *Bcl-x* by Stat5 signaling may contribute to CML cell resistance to dasatinib at high cell densities.

The experiment shown in Fig. 5B was done using a cell density of $0.1 \times 10^6/\text{mL}$. In addition, treatment with 1 nmol/L dasatinib resulted in ~40% apoptosis, whereas the experiment presented in Fig. 6C was done with a range of cell densities (0.05 – 0.5×10^6 cells/mL); hence, treatment with 1 nmol/L dasatinib resulted in a range of apoptosis depending on the cell density. These findings raise the possibility that cell-to-cell interactions increase Stat5 activity and confer resistance to dasatinib in CML cells. Alternatively, higher cell density might reduce the availability of dasatinib to cells, resulting in less dasatinib exposure per individual cell, and therefore contribute to dasatinib resistance. Finally, Stat5 signaling can be activated by cytokine and growth factor stimulation (1, 2, 27), which may be elevated in cell cultures at higher density. Any one or a combination of the above factors may contribute to dasatinib resistance at high cell density.

Therapeutic Implications for CML

In the present study, we provide evidence for a molecular mechanism whereby dasatinib exerts at least part of its therapeutic effects in CML. Our results indicate that dasatinib inhibits Bcr-Abl and SFK activities, thereby blocking Stat5 signaling that is dependent on both Bcr-Abl and SFK activities. This blockade of Stat5 signaling activity is associated with inhibition of downstream survival gene expression, including *Bcl-x* and *Mcl-1*, and induction of apoptosis. Moreover, increasing cell density up-regulates Stat5 activation and results in striking resistance to dasatinib. Although the clinical significance of this latter finding remains to be determined, the condition of high cell density may mimic the tumor microenvironment *in vivo*, resulting in resistance to Bcr-Abl inhibitors. Our findings further suggest that p-Stat5 levels are a mechanistically relevant biomarker for assessing the extent to which dasatinib inhibits Bcr-Abl signal transduction in CML patients.

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