Characteristics of Dasatinib- and Imatinib-Resistant Chronic Myelogenous Leukemia Cells

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Abstract

Purpose: Although dual src-family kinase/BCR/ABL inhibitor, dasatinib (BMS-354825), provides therapeutic advantages to imatinib-resistant cells, the mechanism of dasatinib resistance was not fully known.

Experimental Design: We used TF-1 BCR/ABL cells, by introducing the BCR/ABL gene into a leukemia cell line, TF-1 and K562, and established dasatinib- (BMS-R) and imatinib-resistant (IM-R) cells. We characterized chronic myelogenous leukemia drug-resistant cells and examined intracellular signaling.

Results: The IC₅₀ of dasatinib was 0.75 nmol/L (TF-1 BCR/ABL), 1 nmol/L (K562), 7.5 nmol/L (TF-1 BCR/ABL IM-R), 10 nmol/L (K562 IM-R), 15 μmol/L (TF-1 BCR/ABL BMS-R), and 25 μmol/L (K562 BMS-R). The number of BCR/ABL copies in resistant cell lines was the same as the parental cell line by fluorescence in situ hybridization analysis. There was no mutation in Abl kinase. We found that protein levels of BCR/ABL were reduced in dasatinib-resistant cell lines. BCR/ABL protein was increased by treatment of an ubiquitin inhibitor. The Src kinase, Lck, as well as mitogen-activated protein kinase and Akt were activated, but p21^{WAF}, phosphatase and tensin homologue was reduced in K562 BMS-R cells. Removal of dasatinib from the culture medium of K562 BMS-R cells led to apoptosis, and activated caspase 3 and poly (ADP-ribose) polymerase.

Conclusion: These results suggest that the expression and protein activation signatures identified in this study provide insight into the mechanism of resistance to dasatinib and imatinib and may be of therapeutic chronic myelogenous leukemia value clinically.

The development of imatinib has redefined the management of chronic myelogenous leukemia (CML; refs. 1, 2). Most newly diagnosed patients with chronic-phase disease, treated with imatinib, achieve durable complete cytogenetic responses (3). An update of the IRIS study after 5 years of follow-up shows a hematologic remission rate of 98%, a major cytogenetic response rate of 92%, a rate of complete cytogenetic response of 87%, and a progression-free survival rate in 84% of the patients (4). However, because only a minority of patients achieves undetectable levels of BCR/ABL transcripts, it is suggested that imatinib could not kill the all BCR/ABL expressing cells (5, 6). Moreover there were some patients who experienced relapse during imatinib treatment, especially in the advanced phase, and were resistant to imatinib. This is clinically problematic (7, 8). Various mechanisms may contribute to imatinib resistance. The leading cause of acquired resistance to imatinib is reactivation of BCR/ABL kinase activity via kinase domain mutations (9–11). Mutations in the kinase domain of BCR/ABL impair binding of imatinib. However, only 35% to 45% of imatinib resistance arises from mutations in the ABL kinase domains (10, 12), suggesting that other mechanisms may also be identified in many cases, although this is not fully evaluated at this time.

Dasatinib (SPRYCEL, formally BMS-354825) is an oral, multitargeted kinase inhibitor of BCR/ABL and Src kinases, and is now used in the treatment for CML patients resistant or intolerant to previous therapy, including imatinib (13). Dasatinib inhibits BCR/ABL kinase activity in the low-nanomolar range and inhibits all clinically relevant imatinib-resistant forms with the exception of the T315I mutation. Dasatinib is also being tried in a phase II clinical study of CML and Philadelphia chromosome–positive acute lymphoblastic leukemia patients (START-A, START-C, START-R, and START-L; refs. 14–17). Although dasatinib, a second generation of tyrosine kinase inhibitor, is another promising new clinical candidate for CML treatment, the mechanism of dasatinib resistance has also not been evaluated.

In this study, we first established the dasatinib- and imatinib-resistant cell lines and examined the intracellular signaling in CML cell lines for insight into the resistance to two BCR/ABL inhibitors. Our results indicate that resistance to dasatinib can
be identified on the basis of intracellular protein levels such as BCR/ABL. Understanding BCR/ABL-mediated resistance to imatinib and dasatinib in these cell lines may lead to additional insights into the treatment of CML.

Materials and Methods

Reagents and antibodies. Imatinib and dasatinib were kindly provided by Novartis Pharmaceuticals and Bristol-Myers Squibb. Stock solutions were dissolved in distilled water or DMSO. Anti-phosphotyrosine antibody (Ab) was from Upstate Biotechnology. Anti-phosphorylated Akt (Ser473), mitogen-activated protein kinase (Thr202/Tyr204), Src (Tyr416), Lyn (Tyr507), phosphatase tensin homologue, Anti-phospho Akt (Ser473), mitogen-activated protein kinase (Thr202/Tyr204), Src (Tyr416), Lyn (Tyr507), phosphatase tensin homologue, phosphatase and tensin homolog, cleaved caspase 3, and poly (ADP-ribose)polymerase (PARP) Abs were from Santa Cruz Biotechnology. p21WAF Ab was from Transduction Laboratories. Antibody Microarray was from Lab Vision Corporation. Other reagents were from Sigma.

Cell culture and transfection. The CML cell line, K562, was obtained from American Type Culture Collection. The TF-1 BCR/ABL (p210) cell line was created as described previously (18). K562 and TF-1 BCR/ABL cells were incubated with increasing concentrations of dasatinib and imatinib (starting at 0.1 nmol/L and 0.1 μmol/L), and surviving cells were collected and treated with 2-fold higher concentrations (up to 10 μmol/L). The resistant cells were cloned by limiting dilution in the presence of 1 μmol/L dasatinib.

Cell proliferation assay. Cell proliferation assay was done as described previously (19).

Ab microarray assay. Protein microarray assay was done according to the manufacture protocol. Briefly, total protein was extracted from

![Fig. 1. Fluorescence in situ hybridization analysis of dasatinib- and imatinib-resistant K562 cell lines. Interphase fluorescence in situ hybridization for fusion of the ABL oncogene and BCR (breakpoint cluster region) of K562, K562 BMS-R, and K562 IM-R cells.](image-url)
and resistant cell lines (TF-1 BCR/ABL BMS-R, IM-R, K562 BMS-R, and IM-R) were immunoblotted with the indicated Ab. We found that tyrosine phosphorylation was reduced in K562 BMS-R cells (Fig. 4A). We also found that the protein level of BCR/ABL was reduced in dasatinib-resistant two cell lines (Fig. 4A). We next examined whether ubiquitin is involved in the degradation of BCR/ABL in K562 BMS-R cells. After 24 hours of culture with or without 10 ng/mL of lactacystin, cell lysates were immunoprecipitated with Abl Ab and blotted with Abl Ab. Figure 4B and C show that the protein level of BCR/ABL was enhanced after lactacystin.

Fig. 2. Proliferation of dasatinib- and imatinib-sensitive and imatinib-resistant cell lines. K562, TF-1 BCR/ABL, K562 IM-R, TF-1 BCR/ABL IM-R, K562 BMS-R, and K562 IM-R cells exposed to dasatinib (A) or imatinib (B) for 72 h were quantified by cell proliferation assay as described in “Materials and Methods.” Each result is presented as the mean percentage of proliferation of unexposed control cultures, and three independent experiments are represented. Points, mean; bars, SD.

Fig. 3. Protein expression profile in a dasatinib-resistant cell line. Dasatinib-resistant and control cells were cultured in a drug-free medium, and protein expression data from three independent Ab microarray hybridizations were analyzed as described in “Materials and Methods.” Tyrosin kinase and a tumor suppressor, a cell cycle related protein, and the transcription factors are shown.
treatment within 24 hours. Moreover, treatment with lactacystin also inhibited the induction of apoptosis in K562 BMS-R cells. These results indicate that the degradation of BCR/ABL was mediated by the ubiquitin-proteasome system. We also found that one of the Src family kinases, Lck, was greatly enhanced in K562 BMS-R cell, but p21WAF and phosphatase tensin homologue were reduced (Fig. 4D). Erk and Akt are activated principally in response to external stimuli and regulate cell growth. We found that Erk and Akt were activated in dasatinib- and imatinib-resistant cells. Although enhanced phosphorylation of Lyn was detected in TF-1 BCR/ABL and K562 IM-R cells (Fig. 4D). Determination of the loading of equal amounts of protein in all lanes was accomplished by stripping and blotting with actin Ab.

Removal of Dasatinib for 24 hours induces apoptosis in K562 BMS-R cells. We examined caspase activation 24 hours after the removal of dasatinib or imatinib in K562 BMS-R cells. Cleaved caspase 3 and cleaved PARP were detected 24 hours after the removal of dasatinib in K562 BMS-R cells (Fig. 5A). We also found that activation of PARP was detected from 6 to 24 hours after the removal of dasatinib in K562 BMS-R (Fig. 5B). These results indicate that apoptosis of K562 BMS-R cells after the removal of dasatinib involves activation of caspases.

Discussion

In this study, we established K562 and TF-1 BCR/ABL cell lines that were resistant to the second generation of the BCR/ABL expression, tyrosine phosphorylation, and signaling in dasatinib- and imatinib-resistant cell lines and the ubiquitin inhibitor lactacystin modifies the BCR/ABL protein. Protein tyrosine phosphorylation and abl (A), phosphorylation of Lyn, Lck, p21WAF, Akt, mitogen-activated protein kinase, actin (D) levels were analyzed by an immunoblotting protein (30 μg) from cell lysates. B, K562 BMS-R cells were treated with or without lactacystin for 24 h. Cell lysates were immunoprecipitated (IP) with anti-Abl Ab and immunoblotted (WB) with the ubiquitin or Abl Abs. C, K562 BMS-R cells were treated lactacystin for indicated hours. Cell viability was evaluated through the trypan blue exclusion and cell lysates were immunoblotted with Abl or actin Abs.
ABL kinase inhibitor, dasatinib, in our laboratory. These dasatinib-resistant cell lines are 1,000-fold more resistant than the parental cell lines. These cell lines showed no mutation in the Abl kinase domain, suggesting that other mechanisms were involved in resistance. There are several reports on imatinib resistance, including the amplification of the BCR/ABL gene, overexpression of the multidrug resistance P-glycoprotein, and persistence of tyrosine phosphorylation of specific proteins implying compensatory signaling via BCR/ABL-independent pathways (22). It has also been reported that resistance to imatinib in CML often is associated with specific point mutations in the BCR/ABL kinase domain (23–26).

In the fluorescence in situ hybridization analysis, the BCR/ABL gene was found in K562 BMS-R cells and there was no amplification of BCR/ABL copies compared with the parental cell line, K562. We also showed that BCR/ABL protein expressions were down-regulated in dasatinib-resistant cell lines. These results indicate that inhibition of BCR/ABL occurs in posttranslational. It is known that the ubiquitination-proteasome system is a major tool for extralysosomal cytosolic and nuclear protein degradation in the cell (27). The ubiquitin-proteasome system plays a pivotal role in controlling levels and/or activities of proteins in the cell. Moreover, the ubiquitination-proteasome system functions as a quality control mechanism that selectively removes abnormal and damaged proteins. Proteasome inhibitors represent a powerful tool for detailing the role of the ubiquitination-proteasome system in the cell. We showed that the BCR/ABL protein levels increased in the presence of the proteasome inhibitor, lactacystin, within 24 hours. The treatment with lactacystin also prevent the induction of apoptosis in dasatinib-resistant K562 BMS-R cells. These results indicate that the inhibition of BCR/ABL is induced by protein degradation through the ubiquitination-proteasome system. The removal of dasatinib may affect the activation of the ubiquitination-proteasome pathway in dasatinib-resistant K562 BMS-R cells.

BCR/ABL confers resistance to apoptosis in leukemic cells. Protection from programmed cell death may be mediated, in part, through the activation of STATs, PI3K, Ras, and Src family kinases (28–31). Src kinases are involved in BCR/ABL-mediated leukemogenesis and have been implicated in some cases in imatinib resistance (32). Src family kinases regulate multiple cellular events such as proliferation, differentiation, survival, cytoskeletal organization, adhesion, and migration (33). Using the Ab microarray system, we could show that one of the src family kinases, Lyn, was activated in imatinib-resistant K562 cells. These results are consistent with previous reports (32). Lyn kinase may regulate survival in these imatinib-resistant cell lines. We also found that one of the PI3-K/Akt phosphatases, phosphatase tensin homologue, was reduced in K562-BMS-R cells, and mitogen-activated protein kinase and Akt were activated in dasatinib- and imatinib-resistant K562 cells. Elevation of Akt kinase and mitogen-activated protein kinase activity were also causes leading to the inhibition of apoptosis.

In this report, we could show that K562 BMS-R cells undergo apoptosis after the removal of dasatinib. We found that the removal of dasatinib induces apoptosis after 6 hours. It has been reported that the removal of imatinib leads to the apoptosis of BCR/ABL-overexpressing leukemic cells via a transient activation of the STAT5/8cxl pathway (34). In this study, the BCR/ABL protein was reduced in dasatinib-resistant cells, suggesting that another mechanism was involved in the apoptotic process.

We first established and investigated the dasatinib-resistant cells. Dasatinib is now clinically available and used in CML patient with imatinib resistance or imatinib intolerance. We have also evaluated the primary leukemia cells from imatinib- or dasatinib-resistant patients. The expression of BCR/ABL protein is very low; therefore, it is hard to evaluate the characteristics of primary leukemia cells (data not shown). In this study, we are the first to report dasatinib resistance by degradation of BCR/ABL protein. Our data provides new information regarding the molecular basis of strategy against the dasatinib resistance in CML.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.


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