Investigating the Role of JAK/STAT Pathway on Dasatinib-Induced Apoptosis for CML Cell Model K562

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Abstract

We aimed to evaluate the cytotoxic and apoptotic effects of dasatinib (BMS-354825) on K562 chronic myeloid leukemia (CML) cells and to examine the roles of *STAT* genes on dasatinib-induced apoptosis. The results showed that dasatinib decreased proliferation and induced apoptosis in K562 cells in a dose- and time-dependent manner. mRNA and protein levels of *STAT5A* and *STAT5B* genes were significantly reduced in dasatinib-treated K562 cells. These data indicated that *STAT* inhibition by dasatinib might be therapeutic in JAK/STAT pathway–associated malignancies after confirmation with clinical studies.

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Introduction

Chronic myeloid leukemia (CML) is a malignant disorder of hematopoietic stem cells that arises from the reciprocal translocation between the breakpoint cluster region (*BCR*) gene on chromosome 22, and the Abelson (*ABL*) murine leukemia virus gene on chromosome 9, t(9;22)(q34;q11), resulting in the formation of Philadelphia (Ph) chromosome. The Ph chromosome encodes the BCR-ABL fusion protein, which has constitutive tyrosine kinase activity, leading to leukemogenesis.[1]

Imatinib mesylate, a selective inhibitor of the ABL tyrosine kinase, has demonstrated a remarkable efficacy in the treatment of CML by inducing cytogenetic remissions in over 75% of chronic-phase patients as first-line therapy.[2] However, drug resistance and early relapses frequently occur in a considerable proportion of patients—a main limitation for prolonged survival.[3] Resistance to imatinib is caused primarily by point mutations in the kinase domain of BCR-ABL that block drug binding.[4]

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In general, imatinib resistance can be subdivided into BCR-ABL-dependent and -independent mechanisms. BCR-ABL-dependent mechanisms include over expression or amplification of the BCR-ABL gene and point mutations within the BCR-ABL kinase domain that interfere with imatinib binding. BCR-ABL-independent mechanisms include factors influencing the concentration of imatinib within the cell—for example, by alterations in drug influx and efflux and activation of BCR-ABL-independent pathways, such as members of the Src kinase family.[5]

To overcome this problem, more selective second-generation ABL tyrosine kinases have been developed. For these patients, other tyrosine kinase inhibitors (TKIs) such as nilotinib and dasatinib (BMS-354825) are used as second-generation therapy; these are approved for the treatment of CML after imatinib failure or intolerance. Currently marketed second-generation drugs include nilotinib, dasatinib, bosutinib, and ponatinib.

Dasatinib is a thiazolylaminopyrimidine developed as the hydrochloride salt. It was discovered with a program directed toward immunosuppressive drugs and is 325-fold more potent against cells expressing wild-type BCR-ABL compared to imatinib.[6,7] Dasatinib is a multitargeted inhibitor of BCR-ABL and the Src kinase family and also has inhibitory activity against additional downstream kinases.[7,8] Dasatinib exclusively binds the active conformation of ABL kinase, in contrast to most TKIs.[9]

Dasatinib prevents the gained imatinib resistance, responsible for multidrug-resistant genes' overexpression and BCR-ABL kinase region mutations by activating signaling pathways of the Src kinase family (LYN, HCK) and by inhibiting BCR-ABL, Src kinase

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family (SRC, LCK, YES, FYN), c-KIT, EPHA2, and PDGFR kinases.

BCR-ABL activates multiple signalling pathways, including rat sarcoma (Ras), myelocytomatosis (myc), phosphoinositide 3-kinase/Akt (PI3K/Akt), and Janus kinase/signal transducers and activator of transcription (JAK/STAT), which leads to uncontrolled proliferation and inhibition of apoptosis. Because the critical role of JAK/STAT pathway has been demonstrated in myeloid differentiation, novel therapeutic strategies have been focused on targeting this pathway.

STAT proteins are a family of latent cytoplasmic transcription factors that are involved in several cellular processes, such as proliferation, survival, apoptosis, and differentiation.[10] Constitutive or aberrant activation of STATs was hypothesized to cause cellular transformation, in particular leukemogenesis.[11,12] Seven mammalian STAT proteins have been discovered: STAT1 to STAT4, STAT5A, STAT5B, and STAT6.[13]

After dimerization and phosphorylation, STATs migrate into the nucleus, where they are activated, thus effecting cellular processes such as transcriptional regulations of several growth factors and oncoproteins.[14] In the pathway, constitutive JAK activation leads to persistent activation of STATs, and in consequence, several cancer cells exhibit permanent STAT activity.[15] Among STATs, *STAT5A* and *STAT5B* have also been shown to be necessary for the development of malignancy, and they exhibit regulatory roles in the development of leukemia.[16]

Until now, the efficacy of dasatinib in inducing apoptosis of leukemic cells has not been widely investigated in a point of JAK/STAT pathway. The aim of this study was to investigate the apoptotic case of leukemic cells and to evaluate the transcription and translation levels of *STAT5A* and *STAT5B*, which are the potential targets of JAK/STAT pathway after dasatinib treatment on the model CML K562 cell line in order to clarify the underlying mechanism of dasatinib.

Materials and Methods

Culturing Conditions of Cells

Human chronic myelogenous leukemia cell line K562 were purchased from ECACC (European Collection of Cell Cultures). Cells were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 100 U of penicillin—streptomycin per milliliter, and 1% L-glutamine at 37°C in humidified air containing 5% CO_2. Cells with 95% survival rates and 80% confluence were used in experimental analyses.

Dasatinib Treatment

Dasatinib (BMS-35482) was provided by Bristol-Myers-Squibb (Princeton, NJ) and dissolved in dimethyl sulfoxide. Serial dilutions were prepared using serum-free RPMI 1640 medium. The experimental setup was generated with 3.3 nM of dasatinib treatment with IC_{50} (drug concentration causing 50% inhibition) value of K562 cells for 24 to 96 hours' time course as well as with untreated control group cells. After 48 hours' dasatinib treatment, cells were collected for apoptosis, gene expression, and Western blot analyses. All experiments were performed in triplicate, and the average of the results was taken.

Cell Proliferation Assay

Cell viability and proliferation of untreated controls and dasatinib-treated cells were assessed by the Cell Proliferation Kit II (XTT; Roche Applied Science, Mannheim, Germany). K562 cells were seeded into 96-well plates at a density of 30×10^3 cells in 100 µL RPMI 1640 medium per well. Then cells were treated with increasing doses of dasatinib for a duration of 72 hours. After the proliferation assay, absorbance of each sample was measured spectrophotometrically with an enzyme-linked immunosorbent assay reader (ELISA; Thermo, Vantaa, Finland). All experiments were performed in triplicate. The obtained results were evaluated with the GraphPad Prism 5.01 (GraphPad Software, La Jolla, CA); cell proliferation curves were generated, and IC_{50} value was calculated for K562 cells.

Quantitative Real-Time Reverse Transcriptase—Polymerase Chain Reaction (qRT-PCR) Assay

In expression analyses experiments, dasatinib was reverse transfected onto the cells, and at the end of 96 hours, each day's cells were collected, including untreated control cells. In this manner, after 24 to 96 hours, total RNA extraction was performed from dasatinib-treated and -untreated control cells according to the manufacturer's protocol (MagnaPure LC RNA Isolation Kit; Roche Applied Science). The amount and quality of RNAs were measured by a nanoDrop spectrophotometer, and 100 ng RNA was reverse transcribed into cDNA via Transcriptor High Fidelity cDNA Synthesis kit (Roche Applied Science) after the instructions. *STAT5A* and *STAT5B* mRNA expression levels were assessed by the LightCycler Fast Start DNA Master Hybridization Probes Kit in the qRT-PCR instrument LightCycler v2.0 in accordance with the G6PDH housekeeping gene set (Roche Applied Science). The primers and hybridization probes used for the amplification of *STAT5A* (NM_003152) and *STAT5B* (NM_012447) were as follows: *STAT5A*, F: 5′- GAAGCTGAACGTGCACATGAATC-3′, R: 5′-GTAGGGACAGAGTCTTCACCTGG-3′, FL: ACAGGA CTGTGAACTTCTCCTCTGTCACGG-FL, LC: CTCTGCA CCCCGCCGGTCAG-p. *STAT5B*, F: 5′-AGTTTGATTCTCA GGAAAGAATGT-3′, R: 5′-TCCATCAACAGCTTTAGCAGT-3′, FL: TTGGGAGACTTGAATTACCTTATCTACGT-FL, LC: TT CCTGATCGGCCAAAAGATGAA-p. The relative expression levels of target genes were determined by the proportion of the target value to reference value.

Western Blot Analyses

Cells were lysed in Complete Lysis-M Buffer containing Protease Inhibitor Cocktail Tablets (Roche Applied Science), and the supplied protein amounts were assessed by the Bradford method by use of bovine serum albumin concentration standards ranging between 0.25 and 2 mg/mL. Finally, 35 µg of each protein extract was resolved at 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to polyvinyl difluoride membranes using the iBlot dry transfer system (Invitrogen, Carlsbad, CA). The used primer antibody concentrations were 1:1000 diluted STAT5A (06-968; Upstate Biotechnology, Lake Placid, NY), STAT5B (06-969; Upstate), and β-actin (#4967; Cell Signaling Technology, Danvers, MA). Primary antibody incubation, blotting, and