Inhibition of Phosphotyrosine Phosphatase 1B Causes Resistance in BCR-ABL-Positive Leukemia Cells to the ABL Kinase Inhibitor STI571

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of BCR-ABL-mediated transformation in vitro and in vivo. To investigate whether PTP1B modulates the biological effects of the abl kinase inhibitor STI571 in BCR-ABL-positive cells, we transfected Philadelphia chromosome–positive (Ph+) chronic myeloid leukemia cell-derived K562 cells with either wild-type PTP1B (K562/PTP1B), a substrate-trapping dominant-negative mutant PTP1B (K562/D181A), or an empty vector (K562/mock). Cells were cultured with or without STI571 and analyzed for its effects on proliferation, differentiation, and apoptosis. In both K562/mock and K562/PTP1B cells, 0.25 to 1 μmol/L STI571 induced dose-dependent growth arrest and apoptosis, as measured by a decrease of cell proliferation and an increase of Annexin V-positive cells and/or of cells in the sub-G1 apoptotic phase. Western blot analysis showed increased protein levels of activated caspase-3 and caspase-8 and induction of poly(ADP-ribose) polymerase cleavage. Low concentrations of STI571 promoted erythroid differentiation of these cells. Conversely, K562/D181A cells displayed significantly lower PTP1B-specific tyrosine phosphatase activity and were significantly less sensitive to STI571-induced growth arrest, apoptosis, and erythroid differentiation. Pharmacologic inhibition of PTP1B activity in wild-type K562 cells, using bis(N,N-dimethylhydroxramido)hydroxooxovanadate, attenuated STI571-induced apoptosis. Lastly, comparison of the STI571-sensitive Ph+ acute lymphoblastic leukemia cell line SupB15 with a STI571-resistant subline revealed significantly decreased PTP1B activity and enhanced BCR-ABL phosphorylation in the STI571-resistant SupB15 cells. In conclusion, functional PTP1B is involved in STI571-induced growth and cell cycle arrest, apoptosis, and differentiation, and attenuation of PTP1B function may contribute to resistance towards STI571.

Philadelphia chromosome–positive (Ph+) chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) have become model diseases for the effectiveness of targeted drug therapy. The p185bcr-Abl and p210bcr-Abl tyrosine kinase proteins are formed by the reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome). STI571 (imatinib) is a selective BCR-ABL tyrosine kinase inhibitor that has significantly improved the prognosis of CML. Whereas 80% of patients with newly diagnosed CML achieve a complete cytogenetic response to STI571, acquired (secondary) resistance to STI571 occurs with an annual incidence of ~4% (1–3). Primary resistance to STI571 is considerably more frequent in patients with advanced CML or Ph+ ALL and secondary resistance develops rapidly in the majority of responding patients (4, 5). At relapse, most patients display enhanced BCR-ABL kinase activity, reflecting loss of kinase inhibition by STI571 (6). Several mechanisms of resistance against STI571 have been reported in patients with CML, including point mutations in the tyrosine kinase domain and amplification of BCR-ABL at the genomic or transcriptional level (7, 8). However, neither mutation nor amplification of BCR-ABL is detected in a significant number of patients (7, 8).

Protein tyrosine phosphatases (PTP) encompass a large family of enzymes that catalyze the dephosphorylation of tyrosyl-phosphorylated proteins. In conjunction with protein tyrosine kinases, they regulate the level of protein tyrosine phosphorylation and are important for fundamental physiological processes including cell growth and differentiation (9). PTPs have been shown to be implicated in a wide range of cancers including breast, prostate, and brain cancer, juvenile

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myelomonocytic leukemia, myelodysplastic syndromes, acute myeloid leukemia, and B-cell lymphomas (10–13). PTP1B is a widely expressed nonreceptor PTP that has been shown to be a negative regulator of multiple tyrosine kinases, including receptor tyrosine kinases such as the insulin receptor, epidermal growth factor receptor, and erythropoietin receptor, as well as of cytoplasmic tyrosine kinases such as Src family kinases (14–20). PTP1B was shown to bind to and dephosphorylate BCR-ABL and to antagonize BCR-ABL-mediated signaling and transformation in vivo and in vitro (14–17). Overexpression of PTP1B antagonized BCR-ABL-mediated transformation of Rat-1 fibroblasts, reducing their capacity to generate colonies in soft agar and their ability to form tumors in nude mice (15). In addition, both PTP1B overexpression and inhibition of BCR-ABL by STI571 induced erythroid differentiation of BCR-ABL-positive K562 cells (15). Interestingly, BCR-ABL induces expression of PTP1B (14, 21), suggesting that PTP1B is implicated in a negative feedback loop of BCR-ABL. Because STI571 preferentially binds to the unphosphorylated conformation of the ABL kinase domain (22), we hypothesized that attenuation of PTP1B activity would enhance phosphorylation of BCR-ABL and thereby promote abnormal phosphorylation of its downstream signals and STI571 resistance. We therefore investigated whether suppression of PTP1B activity would cause STI571 resistance in p210BCR-ABL positive K562 cells and whether PTP1B activity is altered in an STI571-resistant subline of p185BCR-ABL positive SupB15 cells (15). To select for the STI571-resistant phenotype, SupB15 cells were cultured with gradually increasing concentrations of STI571 for 6 months. Cells were then further maintained in the presence of 1 μmol/L STI571. The antiproliferative effects of STI571 were examined using WST-1 reagent (Roche, Mannheim, Germany) according to the protocol of the manufacturer.

Overexpression of full-length or mutant PTP1B in K562 cells. Human PTP1B cDNA from K562 cells was amplified by PCR and cloned into the BamHI and EcoRI site of pcDNA3 (Invitrogen) after ligation of two PstI-restricted fragments. The following primers were used: 5'-ATTG-GATCCATGGGAGATGGAAAAGGAGTTCGAGCAGATC (PTP1B sense 1 BamHI) and 5'-GGCTGTTGAACAGGAA (PTP1B antisense 1), as well as 5'-GGTGTGTGTCCTGCGTGGCGAGCAT (PTP1B sense 2) and 5'-ATTGACCTTACTAGTTGTGGTGCACTGCAGTGCAGG (PTP1B antisense 2 EcoRI). The aspartate 181 mutant PTP1B (PTP1B-D181A) was generated using a mutagenesis kit (Clontech, Heidelberg, Germany). All constructs were confirmed by DNA sequencing. K562 cells were transfected with 1 μg of either pcDNA3 alone (K562/mock), PTP1B-pcDNA3 (K562/PTP1B), or PTP1B/D181A-pcDNA3 (K562/D181A) using lipofectamine as described by the manufacturer (Invitrogen), and positive clones were selected under 1 mg/mL G418 (Invitrogen). All experiments were done using early passage cells to avoid changes of the cell lines over time.

PTP1B phosphatase activity assay. PTP1B- and T-cell PTP-specific tyrosine phosphatase activities were analyzed through dephosphorylation of a tyrosine phosphatase-specific synthetic phosphorylated peptide using the Universal tyrosine phosphatase assay Kit (Takara Bio, Kyoto, Japan) following the protocol of the manufacturer. For

Materials and Methods

**Cells and reagents.** K562 cells (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were propagated in RPMI 1640 supplemented with 1% l-glutamine (Invitrogen, Karlsruhe, Germany) and 10% FCS (Serva, Heidelberg, Germany). SupB15 cells were cultured in RPMI 1640 supplemented with 15% FCS and 1% l-glutamine. To select for the STI571-resistant phenotype, SupB15 cells were cultured with gradually increasing concentrations of STI571 for 6 months. Cells were then further maintained in the presence of 1 μmol/L STI571. The antiproliferative effects of STI571 were examined using WST-1 reagent (Roche, Mannheim, Germany) according to the protocol of the manufacturer.

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**Results**

**PTP1B-mutant (D181A) transfected K562 cells display altered PTP1B activity.** To investigate whether PTP1B modulates the biological effects of STI571, BCR-ABL-positive K562 cells were transfected with expression plasmids harboring either wild-type human PTP1B (K562/PTP1B), a substrate-trapping PTP1B-D181A-mutant (K562/D181A) that retains substrate binding ability but is catalytically impaired (24), or the vector alone (K562/mock), and stable cell lines were generated. Western blot analysis showed that PTP1B and PTP1B-D181A proteins were overexpressed 2- and 3-fold as compared with K562/mock cells, respectively (Fig. 1A). As expected, phosphatase activity was significantly lower in K562/D181A cells than in K562/mock cells whereas K562/PTP1B cells displayed a trend towards higher PTP activity as compared with K562/mock cells (Fig. 1B). Overexpression of wild-type PTP1B or PTP1B-D181A-mutant had no obvious effect on cell proliferation over 8 weeks (data not shown).

**PTP1B regulates STI571-induced changes of phosphotyrosine proteins in K562 cells.** First, we examined whether alterations of PTP1B phosphatase activity in K562 cells interfered with the effects of STI571 on BCR-ABL-mediated signaling processes. Within 8 hours of exposure to STI571, phosphorylation levels of BCR-ABL in K562/mock and K562/PTP1B cells had

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decreased whereas they were only marginally altered in K562/D181A cells (Fig. 2A). Of note, the amount of constitutively phosphorylated BCR-ABL was elevated in K562/D181A cells compared with K562/mock and K562/PTP1B cells. Furthermore, the amount of constitutively tyrosine-phosphorylated STAT5 was decreased in K562/PTP1B as compared with K562/mock cells whereas that of AKT was increased in K562/D181A cells as compared with K562/mock cells (Fig. 2B). Neither STAT5 nor AKT phosphorylation levels were altered by 8-hour incubation with STI571.

The extent and kinetics of ERK dephosphorylation and hyperphosphorylation may critically dependent on the dose of STI571, and different STI571 dosages as well as time points analysis may explain seemingly discrepant observations (25, 26). We observed that STI571 reduced the level of ERK phosphorylation as early as 2 hours after onset of STI571 treatment in all three transfectants. However, after 4 hours, STI571 provoked a significant increase of ERK phosphorylation in K562/mock to roughly control levels and an increase to above-control levels in K562/PTP1B cells whereas total ERK protein expression remained unchanged. This reactivation of ERK was not observed in K562/D181A cells and the level of ERK phosphorylation declined to each parental control level after 48 hours (data not shown). Our results suggest that whereas STI571 induces apoptosis and cell cycle arrest in all three cell lines, attenuation of PTP1B activity causes K562 cells to be less sensitive to inhibition by STI571.

Overexpression of PTP1B-mutant (D181A) counteracts STI571-induced growth inhibition, cell cycle arrest, and erythroid differentiation. To evaluate the role of PTP1B in this process, we assessed the effects of STI571 on glycophorin-A expression, a marker of erythroid differentiation. After 4 days, K562/D181A cells showed no significant increase of glycophorin-A positive cells in response to 0.25 μmol/L STI571 whereas the percentage sensitivity to STI571, transfected cells were cultured with and without STI571 and cell growth, cell cycle distribution, and erythroid differentiation were investigated. As shown by the dose-response curve in Fig. 3A, K562/D181A cells were significantly more resistant to the antiproliferative effects of STI571 than K562/mock and K562/PTP1B cells (Fig. 3A). In addition, the number of viable cells decreased to significantly greater extent in cultures of STI571-treated K562/mock and K562/PTP1B cells than in K562/D181A cultures exposed to 0.25 μmol/L STI571 (data not shown). When the effects of PTP1B on the STI571 modulated cell cycle distribution were analyzed by flow cytometry (Table 1), STI571 increased the fraction of sub-G1 apoptotic population in a dose-dependent manner in all of the transfectants. However, the percentage of sub-G1 apoptotic cells following exposure to either 0.25 or 0.5 μmol/L STI571 was significantly smaller in cultures of K562/D181A cells than of K562/mock cells (P < 0.05). In addition, the percentage of cells G2-M phases remained significantly higher in 0.5 μmol/L STI571–treated K562/D181A cultures than in K562/mock cultures (P < 0.05). K562/PTP1B did not differ from K562/mock cultures in cell cycle analysis assays except that they displayed a lower percentage of cells in G2-M phase (P < 0.05). These results suggest that whereas STI571 induces apoptosis and cell cycle arrest in all three cell lines, attenuation of PTP1B activity causes K562 cells to be less sensitive to inhibition by STI571.

STI571 has been shown to promote differentiation of K562 cells (15, 27, 28). To evaluate the role of PTP1B in this process, we assessed the effects of STI571 on glycophorin-A expression, a marker of erythroid differentiation. After 4 days, K562/D181A cells showed no significant increase of glycophorin-A positive cells in response to 0.25 μmol/L STI571 whereas the percentage
of glycophorin-A positive cells increased significantly in K562/mock and K562/PTP1B cells exposed to 0.25 μmol/L STI571 (P = 0.007 and P = 0.001, respectively; Fig. 3B). Together, these results indicate that reduction of PTP1B activity confers resistance against STI571-induced growth inhibition, apoptosis, cell cycle arrest, and erythroid differentiation of K562 cells.

**Table 1.** Cell cycle analysis of PTP1B-overexpressing stable K562 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>STI571 treatment, 48 h (μmol/L)</th>
<th>Sub-G1</th>
<th>G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562/mock</td>
<td>None</td>
<td>4.4 ± 2.6</td>
<td>39.0 ± 1.8</td>
<td>27.5 ± 7.7</td>
<td>18.4 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>30.9 ± 3.3</td>
<td>36.1 ± 3.0</td>
<td>22.1 ± 1.8</td>
<td>8.8 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>36.9 ± 2.0</td>
<td>37.1 ± 2.1</td>
<td>15.5 ± 12</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>K562/PTP1B</td>
<td>None</td>
<td>6.0 ± 2.1</td>
<td>40.5 ± 3.5</td>
<td>28.0 ± 6.2</td>
<td>20.2 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>33.6 ± 4.0</td>
<td>32.1 ± 2.3</td>
<td>22.3 ± 3.5</td>
<td>5.1 ± 0.5*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>38.6 ± 3.8</td>
<td>32.5 ± 2.9</td>
<td>20.6 ± 31</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td>K562/D181A</td>
<td>None</td>
<td>8.0 ± 3.0</td>
<td>42.4 ± 5.7</td>
<td>25.7 ± 4.2</td>
<td>16.0 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>18.8 ± 3.9*</td>
<td>40.0 ± 3.6</td>
<td>22.3 ± 1.1</td>
<td>12.7 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>25.2 ± 3.9*</td>
<td>33.5 ± 2.7</td>
<td>24.3 ± 21</td>
<td>9.9 ± 0.8*</td>
</tr>
</tbody>
</table>

*P < 0.05 versus K562/mock in the presence of the respective concentration of STI571 (t test).
was generated from maternal SupB15 cells as described in Materials and Methods. Treatment of wild-type SupB15 (SupB15-WT) cells with 1 μM STI571 resulted in a profound reduction of BCR-ABL phosphorylation (Fig. 5A). In contrast, the level of BCR-ABL phosphorylation in the presence of 1 μM STI571 remained higher in SupB15-RT cells as compared with SupB15-WT cells (Fig. 5A). To determine whether impaired PTP1B function mediates this effect, we examined PTP1B-specific phosphatase activity in SupB15-WT and SupB15-RT cells. As shown in Fig. 5B, PTP1B-specific tyrosine phosphatase activity was significantly lower in SupB15-RT cells as compared with SupB15-WT cells (P < 0.05). To further show that attenuation of phosphatase activity was specific for PTP1B, we assessed T-cell PTP-specific phosphatase activity in SupB15-WT and SupB15-RT cells. No significant difference was observed in T-cell PTP-specific phosphatase activity between these cells (Fig. 5C). There was no difference in protein expression levels of PTP1B and T-cell PTP in both cell lines regardless of treatment with 1 μmol/L STI571 (Fig. 5D). To exclude the possibility that ABL tyrosine kinase domain mutations were responsible for STI571 resistance of SupB15-RT cells (29, 30), we sequenced exons 4 to 7 for all previously described mutations of BCR-ABL. No mutation was detected in either SupB15-WT or SupB15-RT cells (data not shown). Together, these results suggest that STI571 resistance of p185BCR-ABL positive SupB15 cells may at least, in
part, be due to enhanced phosphorylation of BCR-ABL and reduction of PTP1B activity.

Discussion

Cellular transformation by the BCR-ABL oncogene depends on its constitutive and deregulated tyrosine kinase activity, which results in phosphorylation of a large number of cellular signaling proteins including STAT5, ERK, and phosphatidylinositol 3-kinase/AKT. STI571 inhibits the function of BCR-ABL through competitive binding at the ATP-binding kinase domain of the ABL kinase and is highly effective in most patients with chronic-phase, but not advanced, CML. A serious drawback of this specific treatment, however, is the emergence of drug resistance. PTP1B acts as a negative regulator of multiple signaling pathways, including insulin- and growth factor-mediated processes (9). Interestingly, expression of PTP1B is increased by BCR-ABL (14, 21) and these effects coincide with the ability of PTP1B to antagonize BCR-ABL-induced transformation (15). We hypothesized that PTP1B could be involved in resistance to STI571 in BCR-ABL-positive cells. Our results show that attenuated PTP1B phosphatase activity causes resistance to STI571-induced growth and cell cycle arrest, apoptosis, and differentiation in K562 cells. One possible mechanism by which attenuation of PTP1B results in resistance to STI571 is enhanced activation of the BCR-ABL tyrosine kinase in cells displaying low PTP1B activity. The presence of enhanced expression of BCR-ABL has been reported as a mechanism of STI571 resistance (7, 31, 32). Amplification of the BCR-ABL locus was frequently observed in those cells leading to enhanced activation of its downstream signals. Nevertheless, increased BCR-ABL expression was also observed in the absence of genomic amplification (29). Mutations within the BCR-ABL tyrosine kinase domain have been implicated in resistance to STI571 but their precise role is still controversial. Another mechanism of resistance to STI571 in cells displaying low PTP1B activity may be reduced binding of STI571 to the BCR-ABL protein due to increased BCR-ABL phosphorylation (33). In this context, it was recently shown that binding of STI571 to BCR-ABL requires dephosphorylation of tyrosine 393 of BCR-ABL and that overexpression of a Y393F mutant BCR-ABL, which cannot be dephosphorylated, confers resistance against STI571 (22). We here show that the level of BCR-ABL phosphorylation after treatment with STI571 is enhanced in K562/D181A cells, which express a dominant-negative mutant of PTP1B. These results strongly suggest that activation of BCR-ABL, by way of attenuated PTP1B function, may drive STI571 resistance in these cells.

We further sought to analyze the molecular mechanisms underlying resistance to STI571-induced apoptosis in these cells. In K562/D181A cells, expression and phosphorylation levels of AKT were markedly elevated. Activated AKT has been shown to phosphorylate and inactivate caspase-9, thereby preventing the cleavage and activities of the executioner caspases and apoptosis (28–29). Consistently, we observed that STI571-induced activation of caspase-3 and caspase-8 was decreased in STI571-resistant K562/D181A cells compared with K562/mock and K562/PTP1B cells exposed to STI571. These effects could confer a survival advantage to cells which display low PTP1B activity during exposure to STI571.

Exposure of BCR-ABL-positive cells to STI571 has been shown to down-regulate ERK activation at early time points, followed by a significant increase in ERK activation at later intervals (26, 34). Consistent with these reports, we observed activation of ERK after transient dephosphorylation in K562/mock cells and in K562/PTP1B cells but not in K562/D181A cells, again indicating that inhibition of PTP1B activity alters downstream signaling events of BCR-ABL in response to STI571.

STI571 has been shown to induce erythroid cell differentiation in BCR-ABL expressing cells (35), indicating that BCR-ABL inhibits erythroid differentiation in these cells. Here, we show that inhibition of PTP1B activity interferes with STI571-induced glycophorin-A expression, suggesting that, in addition to control of growth and apoptosis, PTP1B is also involved in the differentiation of hematopoietic progenitor cells.

Consistent with a role of PTP1B in resistance to STI571, we observed attenuated PTP1B activity and enhanced phosphorylation of BCR-ABL in a STI571-resistant subline of SupB15 Ph+ ALL cells (SupB15-RT). Previous studies have indicated that the loss of phosphatase T-cell PTP may contribute to STI571 resistance in STI571-resistant sublines from CML cell line KT-1 (13). In this study, we detected neither decreased expression nor attenuated phosphatase activity of T-cell PTP in STI571-resistant SupB15 cells as compared with wild-type SupB15 cells. These results indicate that attenuated function of PTP1B is likely to be a common feature for STI571-resistance in CML and ALL cells.

In conclusion, our results suggest that functional PTP1B is involved in STI571-induced growth and cell cycle arrest, apoptosis, and differentiation of BCR-ABL-positive leukemia cells and that strategies to enhance BCR-ABL dephosphorylation may delay the development of resistance to STI571 in patients with BCR-ABL associated disease.

References

Inhibition of PTP1B Causes Resistance to STI571


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