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Phosphorylation of tyrosine 393 in the kinase domain of Bcr-Abl influences the sensitivity towards imatinib *in vivo*

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The Bcr-Abl fusion protein arising through the t(9;22)(q34;q11) reciprocal translocation is the causative agent in chronic myeloid leukemia and a subset of acute lymphocytic leukemia. Imatinib mesylate is a specific inhibitor of the Bcr-Abl kinase and has shown promising results in clinical studies. The structural relation between the Bcr-Abl oncogene and the tyrosine kinase inhibitor imatinib has recently been elucidated by an elegant crystal structure analysis, emphasizing the importance of dephosphorylated tyrosine 393 (Tyr393) in Bcr-Abl for access of the inhibitor to the kinase domain. By mutating this tyrosine to phenylalanine and thereby mimicking a constitutively dephosphorylated state, we now show that Ba/F3 cells transformed by this mutant demonstrate an increased sensitivity towards imatinib *in vivo*. This effect is not due to an impaired kinase activity of Bcr-Abl Y393F, since a synthetic substrate is phosphorylated with similar kinetics. Treatment of Ba/F3 cells transfected with Bcr-Abl wild type with a phosphatase inhibitor diminished the effect of imatinib, but did not influence the growth of Ba/F3 cells transfected with Bcr-AblY393F. The results support the findings of the crystal structure and indicate that Tyr393 indeed plays a significant role for the sensitivity of Bcr-Abl towards imatinib *in vivo*. These data implicate the regulation of Tyr393 phosphorylation as a potential mechanism of imatinib resistance.
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Introduction

The tyrosine kinase inhibitor imatinib has recently emerged as an important new treatment option in patients with chronic myeloid leukemia (CML) and Philadelphia chromosome-positive (Ph^+) acute lymphocytic leukemias (ALL) carrying the t(9;22) translocation.[1,2] Unfortunately, many patients with advanced CML and nearly all patients with ALL quickly develop resistance to imatinib treatment.[3–5] Imatinib has been shown to bind specifically to the nucleotide-binding pocket in the catalytic domain of Bcr-Abl.[6]

Tyrosine 393 (Tyr393) is located in the activation loop of the Bcr-Abl kinase domain and has been proposed to stabilize the activation loop in the open formation when phosphorylated, thereby restricting the access of imatinib to the catalytic region and compromising its inhibitory function. Thus, phosphorylation of Tyr393 may function as a switch, regulating accessibility of imatinib to its binding site in the Bcr-Abl protein. Therefore, other molecules that are able to 'flick the switch' may influence imatinib sensitivity in a Bcr-Abl tranformed cell, and overexpression of kinases or downregulation of phosphatases targeting Tyr393 may induce imatinib resistance. As the importance of this amino acid for imatinib binding has been shown *in vitro*, we aimed at examining the relevance of phosphorylation of Tyr393 *in vivo*. We demonstrate here that a mutant Bcr-Abl imitating a constitutively dephosphorylated Tyr393 renders Ba/F3 cells more sensitive towards imatinib inhibition.

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Materials and methods

DNA constructs, cells and transfections

The mutation of Tyr393 (the numbering is based on murine Type I Abl) to phenylalanine in the p185 wild type (wt) Bcr-Abl cDNA was performed utilizing the Quick-Change Kit as described previously.[7] Ba/F3 cells grown in RPMI supplemented with 10% FCS and 1 ng/ml mIL-3 were electroporated with Bcr-Ablwt and Bcr-Abl185Y393F mutant cDNAs in the MigRI vector and subsequently grown without growth factors as described previously.[8]

Western blot analysis

Imatinib in 0.1 and 0.5 μM concentrations was added to 2×10^6 Ba/F3 transformed by either p185wt or p185Y393F growing factor independent. After 2 h, cells were lysed and Western blot with antibodies against Abl (8E9) or antiphosphotyrosine (4G10) was performed as described previously.[7] Monoclonal antiphospho-STAT5 antibodies were a kind gift from Tom Wheeler (Hamilton, New Zealand),[9] the hybridoma supernatant was diluted 1:200. Polyclonal anti-STAT5 antibodies were purchased from Santa Cruz (Heidelberg, Germany). Bands were quantitated utilizing the Image Quant© software (Molecular Dynamics, Krefeld, Germany).

Proliferation assays

p185wt and p185Y393F Ba/F3 cells were plated in 96-well plates at a density of 2×10^5 cells/ml and different concentrations of either imatinib (a kind gift from H Gschaidmeier, Novartis, Nürnberg, Germany) or sodium orthovanadate (Sigma, Deisenhofen, Germany) or both were added. Viable cells were measured utilizing the CellTiter 96© (Promega, Mannheim, Germany) at 0, 24 and 48 h. In some experiments, 9×10^4 cells per well were plated in a six-well plate, imatinib and/or sodium orthovanadate were added to some wells and cells were counted every 24 h.

Kinase assay

Kinase assays were performed with a nonradioactive method utilizing a green fluorescent protein (GFP)-based substrate. The Abl-kinase substrate was produced by PCR-mediated addition of an Abl-specific phosphorylatable peptide sequence to the EGFP

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protein as described by Yang *et al*.[10] The cDNA was subcloned into a glutathione-*S*-transferase coupled expression vector (pGEX-4 T, Amersham Biosciences, Braunschweig, Germany), and the substrate was prepared by expression in *Escherichia coli* and subsequent purification and removal of glutathione–agarose beads by dialysis. Lysates of Ba/F3 cells were immunoprecipitated with anti-Abl antibody (K-12, Santa Cruz, Heidelberg, Germany) or isotype control, and kinase reactions were performed as described previously.[11] The probes were visualized by Western blotting with antiphosphotyrosine antibody (4G10) and quantitated with Image Quant© software.

Results

The Bcr-AblY393F mutant is more sensitive towards imatinib treatment than Bcr-Ablwt

In order to exchange Tyr393 with phenylalanine, site-directed mutagenesis was performed by PCR and the correct mutation was confirmed by automatic sequencing (data not shown). The Bcr-Ablwt and Y393F mutant cDNAs were cloned into the MigRI bicistronic expression vector coexpressing the EGFP, simplifying identification of transfected cells by FACS analysis. After growth factor withdrawal, expression of both p185wt and p185Y393F rapidly led to the outgrowth of stably transfected cells reaching 100% by day 10 after electroporation as determined by FACS analysis (data not shown). Subsequently, we incubated the cells for 48 h with different concentrations of imatinib ranging from 0 to 1 μM and viability was measured with a tetrazolium assay. Cells expressing Bcr-AblY393F displayed an enhanced sensitivity towards imatinib compared to Bcr-Ablwt, as shown in Figure 1a. The IC_{50} values for Ba/F3 cells transfected by Bcr-Ablwt and Bcr-AblY393F were approximately 0.85 and 0.31 μM, respectively ($P = 0.03$). We then analyzed the effect of imatinib on autophosphorylation of Bcr-Ablwt and Bcr-AblY393F. A total of 2×10^6 cells were incubated with up to 0.75 μM imatinib for 2 h and subsequently analyzed by Western blot. Again, the cells carrying the mutant Bcr-Abl showed a more rapid decrease in autophosphorylation (Figure 1b). Additionally, phosphorylation of other proteins likely representing known substrates of the Bcr-Abl kinase such as p120Cbl and p56DOK was also significantly more inhibited by imatinib in Bcr-AblY393F-transfected cells (Figure 1c).

In order to further delineate imatinib effects on downstream targets of Bcr-Abl, we examined STAT5 activation in Bcr-Ablwt- and Bcr-AblY393F-transfected Ba/F3 cells. Also in the case of STAT5, imatinib treatment led to a more pronounced response in the cells transfected by the Tyr393 mutant, as shown in Figure 2 by antiphospho-STAT5 and anti-STAT5 antibodies recognizing the phosphorylated and total amount of both STAT5 isoforms STAT5a and STAT5b.

Since the mutation of Tyr393 in Bcr-Abl might have compromised the kinase activity and thus induce an increased imatinib effect towards the mutant molecule, we compared the kinase activity of the two proteins *in vitro*. We found that Bcr-Abl wt and the Y393F mutant phosphorylated a synthetic substrate with similar kinetics and efficiency (Figure 3).

Proliferation of Ba/F3 cells transfected with Bcr-AblY393F is more inhibited by imatinib than growth of Bcr-Abl wt-expressing cells

We then examined, whether the differential effect of imatinib on Bcr-Abl activity in these cell lines would also translate into

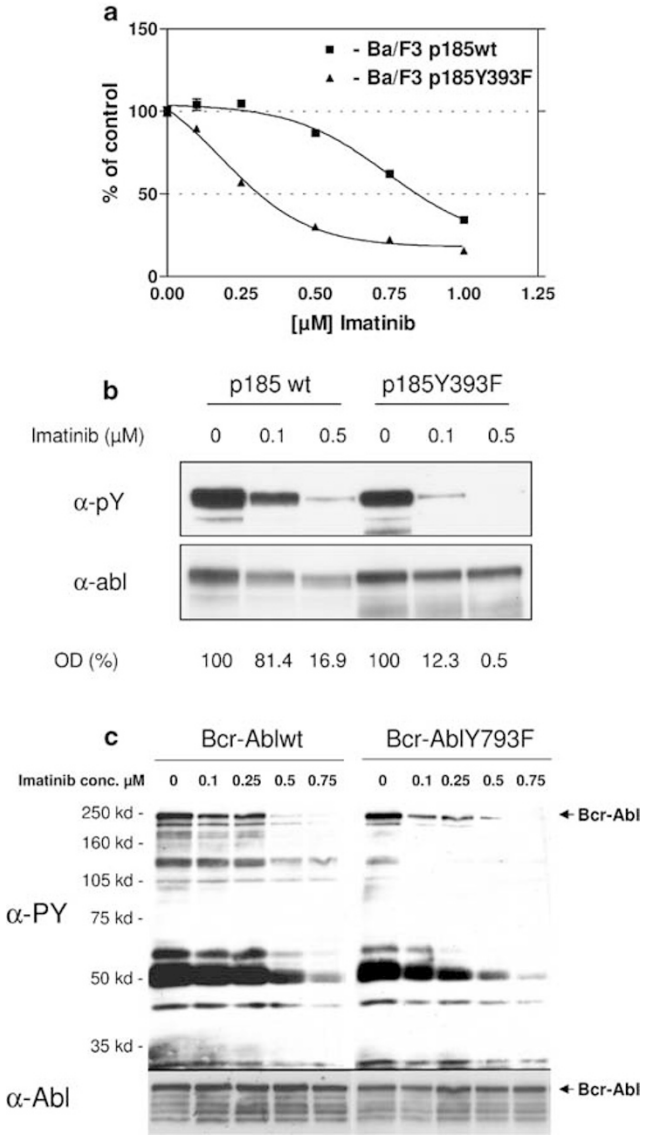

Figure 1 (a) Ba/F3 cells transfected with Bcr-Abl wt or Bcr-Abl Y393F were incubated for 48 h with different concentrations of imatinib and viability was measured in a MTS assay. The relative inhibition compared to untreated controls was calculated and plotted against the imatinib concentrations. (b) Bcr-Ablwt and Y393F protein autophosphorylation in the presence of rising concentrations of imatinib. (c) The effect of imatinib on substrate phosphorylation. After incubation of transfected Ba/F3 cells for 2 h with the indicated concentrations of imatinib, cells were lysed and Western blot was performed. The blots were probed with antibodies against phosphotyrosine and Abl.

increased effects of the inhibitor on proliferation. Therefore, Bcr-Abl wt- and Bcr-AblY393F-expressing Ba/F3 cells were treated with different imatinib concentrations and proliferation was measured employing an assay for cell proliferation based on a tetrazolium compound. Remarkably, also in this assay the Bcr-AblY393F mutant displayed a higher sensitivity (Figure 4). Since phosphatases might counter the effect of imatinib by preserving the Tyr393 in a phosphorylated state, we examined possible effects of the nonspecific tyrosine phosphatase inhibitor sodium orthovanadate on imatinib-treated Ba/F3 cells transformed by Bcr-Abl. Concentrations between 0.1 and 10 μM were tested (Figure 4a), and 5 μM had the strongest effect. At this

concentration, orthovanadate significantly reduced the effects of imatinib on Bcr-Ablwt, but did not rescue proliferation of the Y393F mutant (Figure 4c). This indicates that the effect of orthovanadate in this assay may be due to dephosphorylation of Tyr393. There was no effect of imatinib or sodium orthovanadate on Ba/F3 cells transfected with a control vector growing factor dependent (data not shown).

Discussion

Up to now, an already broad spectrum of possible mechanisms of resistance against imatinib has been described. These comprise overexpression, amplification or mutation of Bcr-Abl as well as increased expression of imatinib binding proteins in the blood plasma of patients.[12–16] Recent studies indicate that point mutations in the Abl-kinase domain may play a prominent role in resistance development, especially in patients with resistant ALL.[13,17] A crystal structure analysis, resolving the structural conditions of the interaction between imatinib and Abl, highlights the importance and the impact of structural data for studying drug effects on a molecular level.[6] As the same study suggested a critical influence of the phosphorylation status of Tyr393 in Bcr-Abl for imatinib response, this prompted us to examine the relevance of this amino-acid modification *in vivo*.

By examining Bcr-Abl kinase activity in Ba/F3 cells transformed with either Bcr-Ablwt and Bcr-AblY393F, we found a heightened sensitivity of Bcr-Abl towards imatinib when Tyr393 was changed to phenylalanine, mimicking a constitutively dephosphorylated state of this amino acid. The results were corroborated by our investigations examining the proliferation of p185wt- and p185Y393F-transformed Ba/F3 cells in the presence of imatinib, indicating that the point mutant is more sensitive towards imatinib-mediated proliferation inhibition. The growth inhibition through imatinib was partially reversible by the addition of a nonspecific tyrosine phosphatase inhibitor in the p185wt, but not in the p185Y393F-transformed Ba/F3 cells, suggesting that the phosphorylation status of this tyrosine may indeed influence the inhibitory effects of imatinib (Figure 5).

Recently, phosphorylation of Tyr393 (Tyr412 in c-abl) has been shown to activate the c-abl kinase *in vitro*.[18,19] In our experiments, Bcr-Abl auto- and substrate phosphorylation was similar in wt and mutant Bcr-Abl, and, in agreement with a previous report,[20] mutation of the tyrosine of the major autophosphorylation site of Bcr-Abl to phenylalanine did not have a significant effect on *in vitro* tyrosine kinase activity and transforming capacity in Ba/F3 murine pre-B cells.

The identification of a post-translational regulatory mechanism of imatinib binding affinity to Bcr-Abl may represent a further mechanism of resistance. The upregulation of kinases specifically phosphorylating Bcr-Abl at Tyr393 or the downregulation of phosphatases responsible for removing the phosphoryl group from this tyrosine could lead to an increased resistance of Bcr-Abl-expressing cells. Until now, the kinases and phosphatases responsible for phosphorylation/dephosphorylation of Tyr393 *in vivo* are not known, although a range of phosphatases influencing Bcr-Abl signalling have been described.[21–24] *In vitro*, the Hck tyrosine kinase, a member of the Src family, has been shown to phosphorylate Abl on Tyr393.[6] Most interestingly, a recent report describing that Src-kinases are overexpressed in imatinib-resistant patients which may lend further support to a possible role of Tyr393 in the development of resistance at least in some patients.[25] Finally, the synergistic effects of the imatinib related small molecule tyrosine kinase inhibitor PP1 targeting Src kinases may in part be explained through this mechanism.[26,27] The differential expression of factors influencing the phosphorylation of the Tyr393 in Bcr-Abl thus might contribute to the already diverse mechanisms of resistance in patients refractory to imatinib treatment.

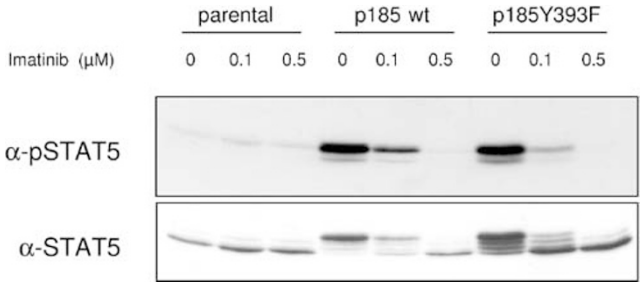

Figure 2 Stat5a/b phosphorylation was measured by Western blotting Ba/F3 cells transfected with Bcr-Ablwt or Bcr-Abl Y793F treated with different concentrations of imatinib with a phospho-specific antibody. The blot was stripped and reprobed with a Stat5 antibody showing total Stat5a/b protein below, which is shifted by Bcr-Abl-induced phosphorylation.

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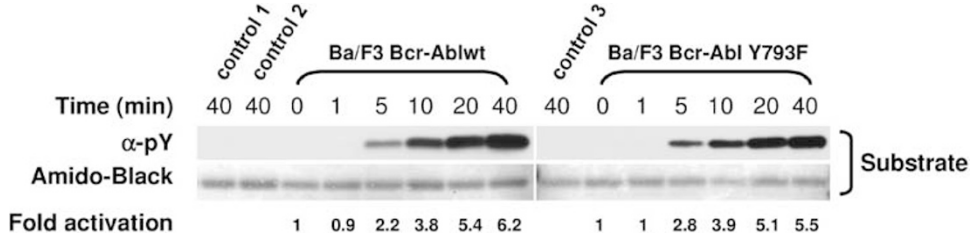

Figure 3 Kinase assay with immunoprecipitated proteins from Ba/F3 cells transformed by Bcr-Ablwt or Bcr-AblY393F. Kinase reactions were allowed to proceed for the indicated time at 30°C, then the substrate was blotted and probed with an antiphosphotyrosine antibody. The membrane was stained with amido black stain to show equal amounts of substrate protein had been used. Controls included immunoprecipitation from nontransfected Ba/F3 cells (control 1), as well as controls with isotype antibody used for IP (controls 2 and 3).

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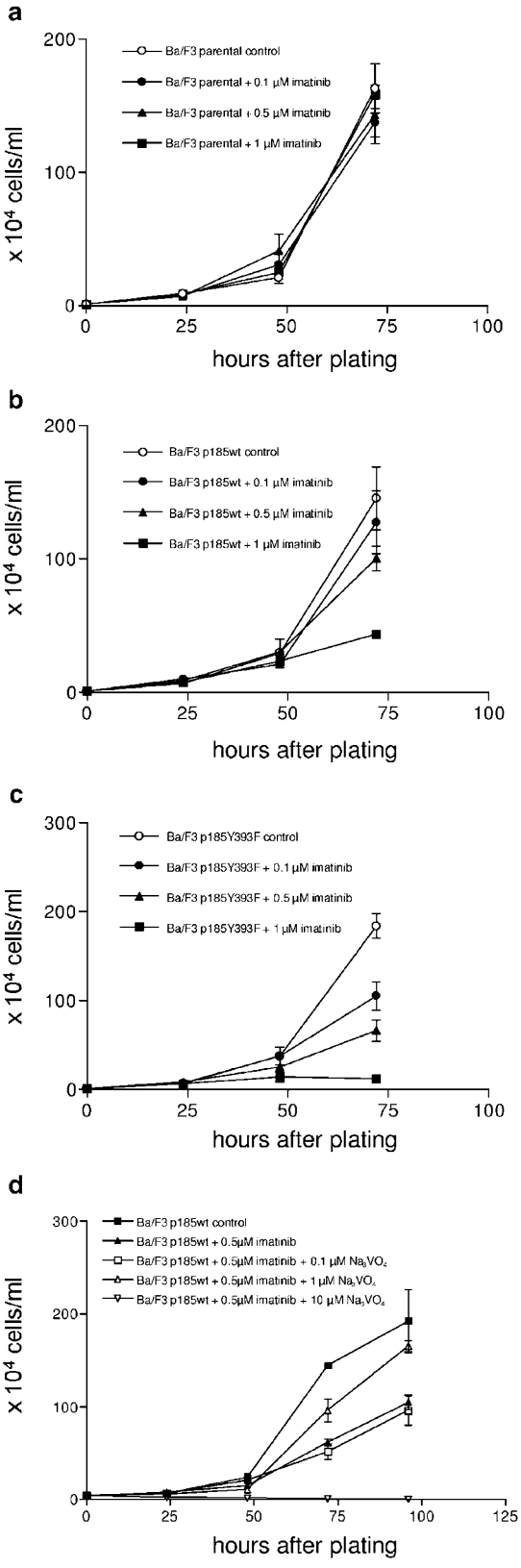

Figure 4 Growth curves of Ba/F3 cells (a) with 1 ng/ml IL-3 or Ba/F3 cells transformed either by Bcr-Ablwt (b) or Bcr-AblY393F (c) in the presence of 0.1, 0.5 and 1 μM concentrations of imatinib. After trypan blue staining, viable cells were counted on three consecutive days. (d) The effect of sodium orthovanadate on imatinib-treated Ba/F3 cells transformed by Bcr-Ablwt was analyzed by counting cells after the addition of 0.1, 1 and 10 μM sodium orthovanadate.

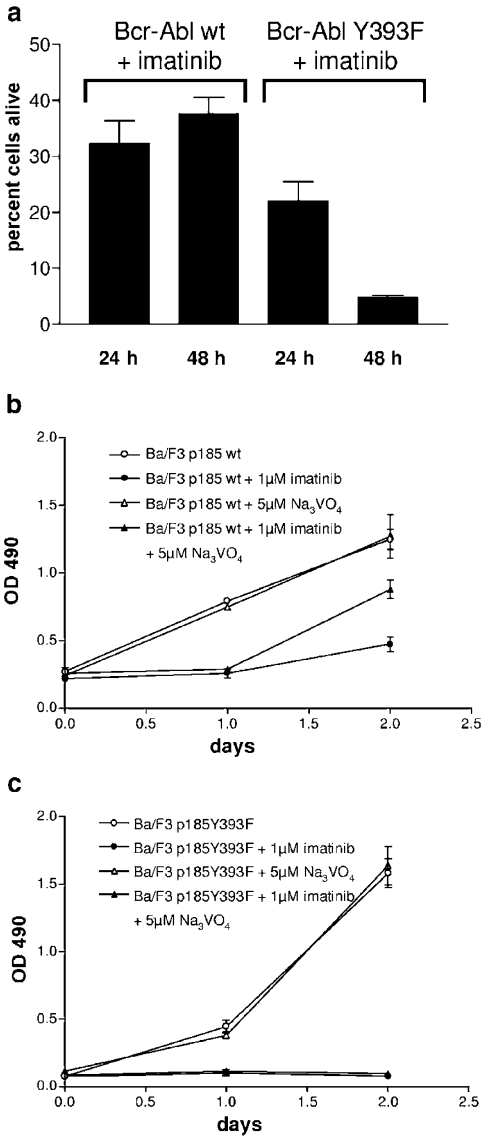

Figure 5 (a) Proliferation of Ba/F3 cells transformed with Bcr-Ablwt or Bcr-AblY393F in the presence of imatinib. Viable cells were measured in a tetrazolium assay (MTT) at 0, 24 or 48 h after plating. The relative effect of imatinib on cell growth was calculated by dividing the OD490 for cells treated with 1 μM imatinib by the OD490 of untreated cells after the indicated time. Growth curves for Bcr-Ablwt (b) or Bcr-AblY393F cells (c) either with or without the addition of 1 μM imatinib and 5 μM sodium orthovanadate were determined by the MTT assay.

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