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Clinical resistance to the kinase inhibitor STI-571 in chronic myeloid leukemia by mutation of Tyr-253 in the Abl kinase domain P-loop

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The Abl tyrosine kinase inhibitor STI-571 is effective therapy for stable phase chronic myeloid leukemia (CML) patients, but the majority of CML blast-crisis patients that respond to STI-571 relapse because of reactivation of Bcr-Abl signaling. Mutations of Thr-315 in the Abl kinase domain to Ile (T315I) were previously described in STI-571-resistant patients and likely cause resistance from steric interference with drug binding. Here we identify mutations of Tyr-253 in the nucleotide-binding (P) loop of the Abl kinase domain to Phe or His in patients with advanced CML and acquired STI-571 resistance. Bcr-Abl Y253F demonstrated intermediate resistance to STI-571 *in vitro* and *in vivo* when compared with Bcr-Abl T315I. The response of Abl proteins to STI-571 was influenced by the regulatory state of the kinase and by tyrosine phosphorylation. The sensitivity of purified c-Abl to STI-571 was increased by a dysregulating mutation (P112L) in the Src homology 3 domain of Abl but decreased by phosphorylation at the regulatory Tyr-393. In contrast, the Y253F mutation dysregulated c-Abl and conferred intrinsic but not absolute resistance to STI-571 that was independent of Tyr-393 phosphorylation. The Abl P-loop is a second target for mutations that confer resistance to STI-571 in advanced CML, and the Y253F mutation may impair the induced-fit interaction of STI-571 with the Abl catalytic domain rather than sterically blocking binding of the drug. Because clinical resistance induced by the Y253F mutation might be overcome by dose escalation of STI-571, molecular genotyping of STI-571-resistant patients may provide information useful for rational therapeutic management.

A new era of targeted cancer therapy was inaugurated with the approval of STI-571 (Gleevec/imatinib mesylate) for the treatment of chronic myeloid leukemia (CML). STI-571 is a phenylaminopyrimidine compound initially identified from a high-throughput screen for inhibitors of protein kinase C and subsequently found to be a potent and selective inhibitor of the Abl, platelet-derived growth factor β receptor, and Kit tyrosine kinases (1). STI-571 binds to the ATP-binding site of the Abl catalytic domain and effectively inhibits Abl kinase activity *in vitro* and *in vivo* at concentrations of 0.1–1.0 μ M (2). In phase I trials, STI-571 was remarkably effective as a single agent in IFN-resistant CML chronic-phase patients, inducing durable hematologic remissions in 90% and major cytogenetic responses in 55% of patients (3). In patients with advanced disease, including accelerated phase, myeloid and B-lymphoid blast crisis, and those with *de novo* Philadelphia-positive (Ph⁺) B-lymphoblastic leukemia, STI-571 is less effective. Although 50–70% of such patients initially respond to the drug, 60% of myeloid blast-crisis patients and all B-lymphoid leukemia patients relapse within 3–6 months of starting therapy (4).

Clinical resistance to STI-571 is likely to be complex and may include extrinsic causes such as increased plasma levels of α 1 acid glycoprotein that bind the drug and decrease its effective concentration (5) as well as intrinsic resistance of the leukemia cells to the drug. Two distinct types of intrinsic resistance to STI-571 can be envisioned: cases in which the leukemic cells have

become independent of Bcr-Abl kinase activity for survival and proliferation and those that still require Bcr-Abl but somehow have escaped the inhibitory effect of STI-571. To distinguish between these various mechanisms, we previously analyzed the phosphorylation of the adapter protein Crkl, which is a direct substrate of Bcr-Abl, in primary leukocytes from CML patients. Leukemic cells from patients that respond to STI-571 have significantly lower Crkl phosphorylation during therapy, whereas the 11 patients in our study with acquired resistance exhibited increased cellular Crkl phosphorylation at the time of relapse (6). In all five evaluable patients, Crkl phosphorylation was resistant to STI-571 when leukemic cells obtained at relapse were incubated with the drug *ex vivo*. Although larger numbers of patients must be studied before definitive conclusions can be reached, these results suggest that cell-intrinsic mechanisms that restore Bcr-Abl kinase activity and signaling are a major cause of acquired resistance to STI-571 in advanced CML.

Because Bcr-Abl signaling is reactivated in the resistant leukemia cells, Bcr-Abl is still a valid therapeutic target in these patients, and understanding the molecular mechanisms of intrinsic STI-571 resistance will be important for designing clinical strategies to avoid and overcome resistance. In our previous study (6) we found evidence of amplification of the *BCR-ABL* gene in three patients, whereas six of nine evaluable patients were found to have point mutations at *ABL* nucleotide 944, resulting in substitution of isoleucine for threonine at position 315 of c-Abl (T315I). In a crystal structure of the Abl catalytic domain complexed with a homologue of STI-571, Thr-315 makes a critical hydrogen bond with the secondary amino group of the drug (7). The side chain of isoleucine both lacks the ability to form this hydrogen bond and would be predicted to sterically clash with the central phenylaminopyrimidine group of STI-571 and interfere with drug binding (6). Indeed, the Bcr-Abl T315I mutant is highly resistant to STI-571 *in vivo* (6) yet retains the ability to transform IL-3-dependent hematopoietic cells to IL-3 independence and induce CML-like disease in mice in a retroviral bone marrow transduction/transplantation model (S.R. and R.A.V., unpublished data).

To extend these results, we searched for mutations in additional patients with advanced CML and acquired resistance to STI-571 and report here on the biochemical characterization of mutations at Tyr-253 in the Abl kinase domain nucleotide-binding loop.

Materials and Methods

Identification of Patient Mutations. Peripheral blood and/or bone marrow samples were collected after obtaining appropriate

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Abbreviations: CML, chronic myeloid leukemia; Ph⁺, Philadelphia-positive; WT, wild type; GST, glutathione S-transferase; SH, Src homology.

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informed consent from a cohort of 20 patients with CML myeloid blast crisis undergoing STI-571 treatment at University of California Los Angeles Medical Center in multicenter clinical trials sponsored by Novartis Pharmaceuticals (East Hanover, NJ). Before initiating STI-571 therapy, all patients had >30% bone marrow blasts and responded to treatment based on reduction of bone marrow blasts to <15% (partial) or <5% (complete) as described (3). Relapse was defined as an increase in the percentage of marrow blasts after an initial response despite continued STI-571 treatment. Total RNA was isolated from mononuclear cells, and reverse transcription-PCR was used to specifically amplify a 1.32-kb fragment containing the *BCR-ABL* junction and entire Abl catalytic domain as described (6). PCR products were cloned into pCR2.1 TA or pBluescript II KS(+) vectors and between 5 and 10 individual subclones bidirectionally sequenced. The frequency of mutant clones for the three patients with the Y253F mutation was 3/10, 2/10, and 1/6, whereas the two patients with Y253H had 1/10 and 1/7 mutant clones. Assessment of cellular Crkl phosphorylation (6) at relapse was not done for these patients because of a lack of sufficient material. In addition to the mutations at Tyr-253, other patients in this cohort had mutations at different Abl residues and will be reported elsewhere.

DNA Constructs. The numbering of amino acids in this paper is according to the type Ia human c-Abl sequence; the mutation P112L corresponds to P131L in the type Ib Abl sequence (8), whereas Y393F and Y226F correspond to Y412F and Y245F, respectively (9). The Y253F mutation was generated in Bcr-Abl and c-Abl by PCR-mediated mutagenesis of a subfragment that was sequenced to confirm the presence of the solitary mutation before subcloning into the corresponding cDNA. Wild-type (WT) and mutant cDNAs were cloned in the retroviral vector MSCVneo (10) for expression by transduction and in the vector pcDNA3/His-6 (9) for protein purification.

Ba/F3 Cell Viability Assay. Ba/F3 cells were plated at 5×10^3 per well in 96-well plates in RPMI medium 1640 media lacking IL-3. STI-571 (Novartis Pharmaceuticals) was included in media at increasing concentrations. Viable cell number was assessed 48 h postplating by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay as described (11). Quadruplicate assays were averaged, and absorbance at 570 nm (A_{570}) versus concentration of STI-571 was graphed as a best-fit sigmoidal curve by using a single-site, nonlinear curve-fitting algorithm (ORIGIN 5.0, OriginLab, Northampton, MA).

Purification of c-Abl Proteins. Abl proteins with a hexahistidine tag at the COOH terminus were expressed by transient transfection in 293 cells and purified by single-step affinity chromatography on nickel-agarose (Talon resin, CLONTECH-Becton Dickinson Biosciences, Palo Alto, CA) as described (9). To purify unphosphorylated Abl proteins, STI-571 was included in the culture medium at 50 μ M and increased to 100 μ M at 6 h before harvest. To remove residual phosphotyrosine, purified proteins in storage buffer (50 mM Tris-HCl, pH 7.5/50 mM NaCl/0.1 mM EDTA/0.01% Brij35/1 mM DTT) were treated with 1:20 volume T-cell phosphatase (New England Biolabs) at 30°C for 30 min and terminated by the addition of 0.1 mM vanadate. Dephosphorylated kinases were stored on ice and used in kinase assays within 24 h. T-cell phosphatase treatment did not affect kinase activity or STI-571 sensitivity of c-Abl when purified from STI-571-treated cells (data not shown). In some experiments, phosphorylated Abl proteins were purified from cells grown without STI-571, with 0.1 mM vanadate added to the medium 6 h before harvest.

In Vitro Kinase Assays and STI-571 Resistance. Immune complex kinase assays of immunoprecipitated Bcr-Abl and c-Abl proteins using a glutathione *S*-transferase (GST)-Crk substrate were performed as described (12). 32 P incorporation into GST-Crk was determined by PhosphorImager analysis (Molecular Dynamics STORM 850, Amersham Pharmacia). *In vitro* kinase assays of purified Abl proteins using a biotinylated peptide substrate were performed in triplicate as described (9). For STI-571 dose-response assays, Abl kinases were preincubated with drug for 10 min at 30°C before the addition of peptide substrate and ATP. IC₅₀ values were determined by using the ORIGIN 5.0 program as described above.

Results and Discussion

Mutation of Tyr-253 to Phe in STI-571-Resistant CML Patients. We investigated a cohort of 20 patients with CML myeloid blast crisis and acquired resistance to STI-571. All patients had an initial hematologic response to STI-571, although none achieved a major cytogenetic response, and all subsequently exhibited hematologic progression while on the drug. We searched for mutations in this population by direct sequencing of the *ABL* kinase domain region after reverse transcription-PCR amplification of *BCR-ABL* mRNA from primary leukemia samples. Three of the 19 patients had a single change at *ABL* nucleotide A758 to T, whereas two other patients had mutation of the adjacent nucleotide T757 to C, predicted to alter the Tyr at amino acid position 253 (all numbering based on the human type Ia c-Abl sequence) to Phe or His, respectively. None of the five patients had evidence of *BCR-ABL* gene amplification as assessed by fluorescence *in situ* hybridization of the leukemic cells (6).

Bcr-Abl Y253F Has Intermediate Resistance to STI-571 *In Vivo* and *In Vitro*, Relative to Bcr-Abl T315I. To determine the effect of the Tyr-253 to Phe (Y253F) substitution on Bcr-Abl, site-specific mutagenesis was used to introduce the Y253F mutation into p210 Bcr-Abl. Based on antiphosphotyrosine immunoblot analysis in transfected 293 cells, Bcr-Abl Y253F clearly was resistant to STI-571 relative to WT Bcr-Abl but nonetheless was inhibited significantly at STI-571 concentrations of 5 μ M or greater, whereas the previously described Bcr-Abl T315I mutant (6) was absolutely resistant to STI-571 up to concentrations as high as 40 μ M (Fig. 1A). Both the Bcr-Abl Y253F and T315I mutants efficiently transformed IL-3-dependent Ba/F3 cells to IL-3 independence (data not shown). The proliferation of Ba/F3 cells expressing WT p210 Bcr-Abl was impaired by STI-571 treatment with 50% inhibition (IC₅₀) achieved at 0.80 μ M, where cells expressing the Bcr-Abl Y253F mutant exhibited significant resistance to STI-571 with an IC₅₀ of 6.99 μ M (Fig. 1B and Table 1). Cells expressing the Bcr-Abl T315I mutant were very resistant to STI-571 (IC₅₀ = 16.88 μ M). Although decreased proliferation of Bcr-Abl T315I-expressing cells was observed at STI-571 concentrations of 30 μ M and higher, this inhibition probably reflects nonspecific cytotoxicity, because a similar degree of inhibition of parental and WT Bcr-Abl-expressing Ba/F3 cells growing in the presence of IL-3 was observed at this dose (Fig. 1B and data not shown). To determine the relative resistance of the Bcr-Abl mutants in a more quantitative fashion, Bcr-Abl proteins were immunoprecipitated from the Ba/F3 cells and analyzed in an immune complex kinase assay with a GST-Crk substrate (Fig. 1C and D and Table 1). The results confirmed that the *in vitro* kinase activity of Bcr-Abl Y253F was significantly resistant to STI-571 compared with WT Bcr-Abl (IC₅₀ of 1.31 versus 0.35 μ M, respectively), whereas the Bcr-Abl T315I mutant was extremely resistant to the drug (IC₅₀ = 30.1 μ M).

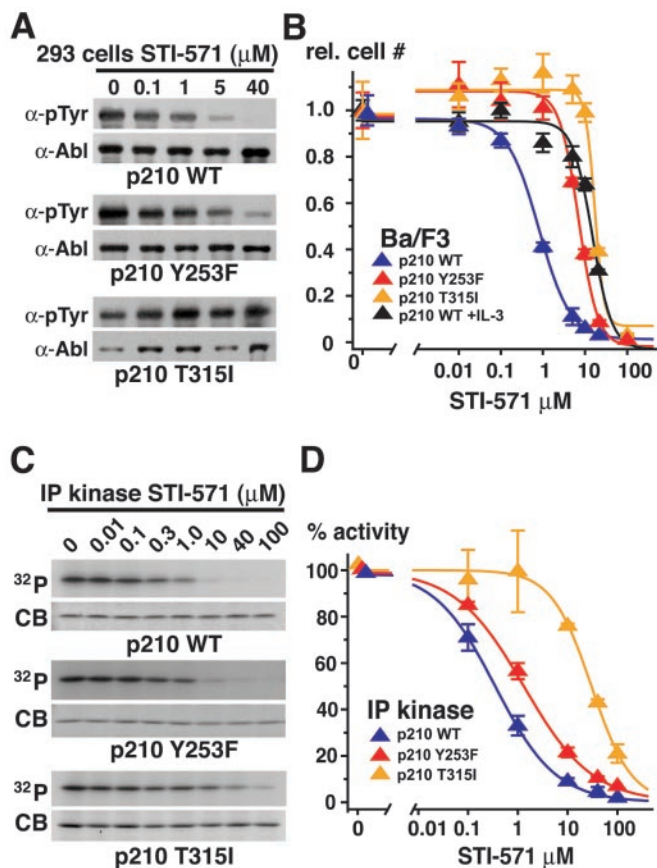


Fig. 1. The Bcr-Abl Y253F mutant has intermediate *in vivo* resistance to STI-571 relative to the T315I mutant. (A) 293 cells transfected with Bcr-Abl WT or the Y253F or T315I mutants were treated for 2 h with increasing doses of STI-571, and cell lysates were analyzed by immunoblotting with anti-Abl (Top) and antiphosphotyrosine (Bottom) antibodies. (B) Ba/F3 cells expressing Bcr-Abl WT (blue symbols), Bcr-Abl Y253F (red symbols), or Bcr-Abl T315I (orange symbols) were incubated for 24 h in the indicated concentrations of STI-571 in the absence of IL-3, and viable cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye-reduction assay (11). Results are plotted as percentage of cells incubated without STI-571. As a control for toxicity, Bcr-Abl WT-expressing cells grown in the presence of IL-3 (black symbols) are included. (C) Anti-Abl immunoprecipitates (IP) from the cell lines in B were subjected to *in vitro* kinase assay with a GST-Crk substrate in the presence of the indicated concentration of STI-571 as described (12); incorporation of ³²P into the substrate was determined by autoradiography (Top and Middle), whereas the GST-Crk protein levels were demonstrated by Coomassie blue (CB) staining (Bottom) in a representative of three independent experiments. (D) Data from C were quantitated by PhosphorImager and densitometric analysis, and sigmoidal plots were generated for the calculation of IC₅₀ values as described in Materials and Methods. Error bars indicate standard deviation.

The Y253F Mutation both Dysregulates c-Abl and Causes Resistance to STI-571. The Y253F mutation was shown previously to activate c-Abl to transform Ba/F3 cells to IL-3 independence and complement some NH₂-terminal deletion mutations in Bcr-Abl in the same assay (13). Transforming Abl proteins such as Bcr-Abl and c-Abl Y253F exhibit increased tyrosine kinase activity *in vivo*, raising the possibility that dysregulation of Abl kinase activity by the Y253F mutation might contribute directly to STI-571 resistance. Alternatively, the sensitivity of Abl to STI-571 might be independent of the regulatory state of the kinase. We chose to examine this issue in the context of c-Abl, because Bcr-Abl is already dysregulated relative to c-Abl, and the regulatory mechanism of c-Abl is better understood. Mutational studies suggest that, similar to Src family kinases (14, 15), c-Abl is regulated in part through an intramolecular interaction of the Abl Src homology (SH)3 domain with a single proline (Pro-223) in the SH2-catalytic domain linker region, such that mutation of either SH3 (9) or Pro-223 (16, 17) dysregulates Abl kinase activity *in vivo* and results in constitutively increased kinase activity *in vitro*. To clarify the relationship between altered regulation of Abl and drug resistance, we compared the STI-571 response of WT murine c-Abl type Ib (myristoylated form) and a c-Abl Y253F mutant with that of c-Abl P112L, which contains a point mutation in the Abl SH3 domain that disrupts binding of SH3 ligands and dysregulates Abl kinase activity *in vivo* (8) and *in vitro* (9). c-Abl WT can be overexpressed stably in Ba/F3 cells (18) but lacks detectable phosphotyrosine *in vivo* (Fig. 2A) and does not induce IL-3 independence, whereas both c-Abl mutants efficiently transformed Ba/F3 cells to IL-3-independent growth (8, 13) and were highly tyrosine-phosphorylated *in vivo* (Fig. 2A). In the immune complex kinase assay (Fig. 2 B and C), c-Abl Y253F was significantly more resistant to STI-571 than c-Abl WT (IC₅₀ 12.42 versus 1.53, respectively). Although Bcr-Abl is dysregulated by the addition of Bcr and perhaps through loss of Abl first exon sequences (19), these results demonstrate that the Y253F mutation induces a similar relative increase in STI-571 resistance in the context of both c-Abl and Bcr-Abl (4–8-fold, Table 1), although Bcr-Abl seems intrinsically more sensitive to the drug than c-Abl. The STI-571 response of c-Abl P112L in this assay was intermediate between c-Abl WT and Y253F.

We also determined the *in vivo* dose response of hematopoietic cells and fibroblasts expressing the c-Abl mutants to STI-571. Ba/F3 cells transformed by c-Abl Y253F were significantly more resistant to STI-571 than those expressing c-Abl P112L (IC₅₀ = 1.52 versus 0.22 μM, respectively; Fig. 2D). Both c-Abl mutants also efficiently transformed NIH 3T3 cells to anchorage-independent growth (data not shown). c-Abl P112L-expressing NIH 3T3 cells reverted from transformed to flat morphology at 0.25–0.5 μM STI-571, whereas c-Abl Y253F-expressing cells required 2.5–5.0 μM STI-571 for

Table 1. Summary of IC₅₀ values for inhibition of Bcr-Abl and c-Abl by STI-571

Abl protein	IP kinase	IC ₅₀ <i>in vitro</i> , μM		IC ₅₀ <i>in vivo</i> , μM
		Unphosphorylated	Phosphorylated	
Bcr-Abl WT	0.35	ND	ND	0.80
Bcr-Abl Y253F	1.31	ND	ND	6.99
Bcr-Abl T315I	30.1	ND	ND	16.88
c-Abl WT	1.53	1.56	8.51	ND
c-Abl P112L	4.12	0.14	1.79	0.22
c-Abl Y253F	12.42	8.79	29.6	1.52
c-Abl Y253/393F	ND	9.73	ND	ND

ND, not determined.

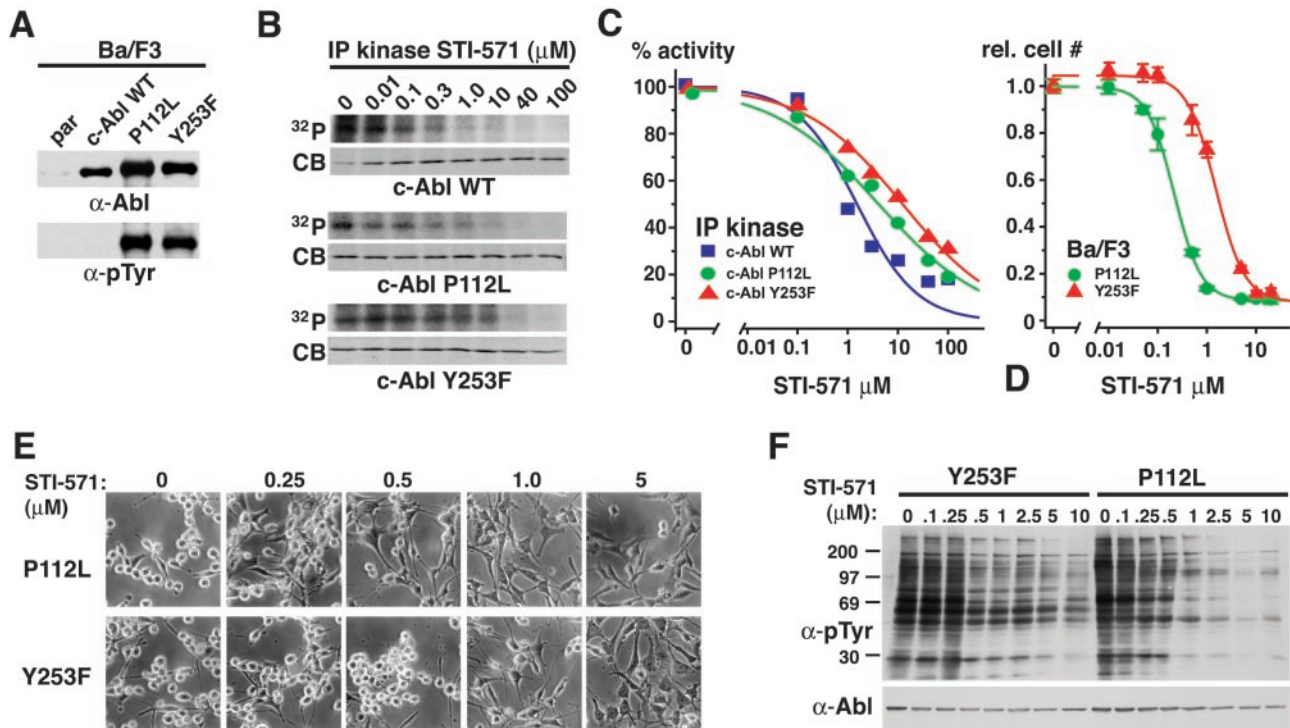


Fig. 2. The Y253F mutation dysregulates c-Abl and induces resistance to STI-571 *in vitro* and *in vivo*. (A) Anti-Abl (Upper) and antiphosphotyrosine (Lower) immunoblots of lysates from parental Ba/F3 cells (par) and populations stably overexpressing c-Abl WT, P112L, and Y253F proteins. (B) Anti-Abl immunoprecipitates (IP) from the lysates in A were subjected to *in vitro* kinase assay as described for Fig. 1C, with incorporation of ³²P into the Crk substrate determined by autoradiography (Upper) and GST-Crk protein levels demonstrated by Coomassie blue (CB) staining (Lower) in a representative of two independent experiments. (C) Data from B were quantitated by PhosphorImager and densitometric analysis, and sigmoidal plots were generated for the calculation of IC₅₀ values. (D) Ba/F3 cells expressing c-Abl P112L (green symbols) or c-Abl Y253F (red symbols) were assessed for their response to STI-571 as described for Fig. 1B. (E) Photomicrographs of transformed NIH 3T3 cells stably expressing c-Abl P112L (Upper) and c-Abl Y253F (Lower), incubated 24 h in the indicated concentration of STI-571. (Magnification, ×250.) (F) Antiphosphotyrosine (Upper) and anti-Abl (Lower) immunoblots of lysates from the cells in E. Molecular mass markers are shown on the left.

a similar effect (Fig. 2E). Analysis of intracellular phosphotyrosine levels in the two fibroblast populations confirmed that the *in vivo* kinase activity of c-Abl Y253F was relatively resistant to STI-571 (Fig. 2F). Together, these results demonstrate that the Y253F mutation renders c-Abl resistant to STI-571 *in vitro* and *in vivo*.

The Response of Abl to STI-571 Is Influenced by the Regulatory State of the Kinase. Quantitating the kinase activity and STI-571 response of immunoprecipitated Abl proteins is difficult, because differences in tyrosine phosphorylation of Abl proteins (Fig. 2A) affect both the catalytic activity (9) and the STI-571 response (see below), and Abl undergoes rapid autophosphorylation in an immune complex (8). In addition, the presence of both Bcr-Abl and c-Abl in the reaction tends to reduce the apparent resistance of the Y253F mutant. To avoid these problems, we purified hexahistidine-tagged Abl proteins from transfected 293 cells by affinity chromatography on nickel-agarose (9). To isolate proteins in their unphosphorylated state, we treated the 293 cells with high doses of STI-571 to prevent autophosphorylation *in vivo* and removed residual phosphotyrosine from c-Abl Y253F by using T-cell phosphatase. The kinetic parameters of the purified proteins, which were devoid of detectable phosphotyrosine by immunoblot assay (Fig. 3A, zero time points), were determined in a peptide-based phosphorylation assay. As observed previously (9), c-Abl P112L (Fig. 3B, green symbols) demonstrated peptide kinase activity that was ≈5-fold higher than that of WT c-Abl (Fig. 3B, blue symbols), whereas the activity of c-Abl Y253F was very close to that of c-Abl (Fig. 3B, red symbols). These results demonstrate that the

Y253F mutation, although it activates Abl kinase activity *in vivo*, does not significantly disrupt the intramolecular regulation of c-Abl as a purified polypeptide.

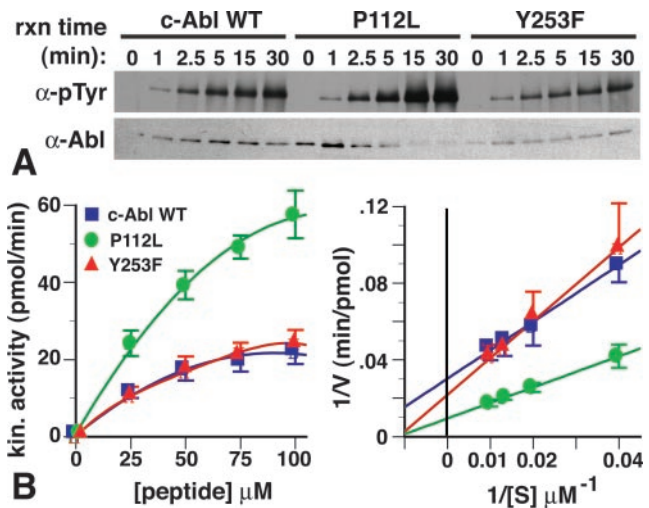


Fig. 3. The Y253F mutation does not dysregulate the catalytic activity of c-Abl *in vitro*. (A) Purified c-Abl WT, P112L, and Y253F proteins have undetectable phosphotyrosine but rapidly autophosphorylate upon incubation in the presence of ATP and magnesium for the indicated reaction (rxn) time (9). (Upper) Antiphosphotyrosine blot. (Lower) Anti-Abl blot. (B) Michaelis-Menten (Left) and Lineweaver-Burke (Right) plots of the catalytic activity of c-Abl WT (blue), P112L (green), and Y253F (red) in solution in the peptide kinase assay.

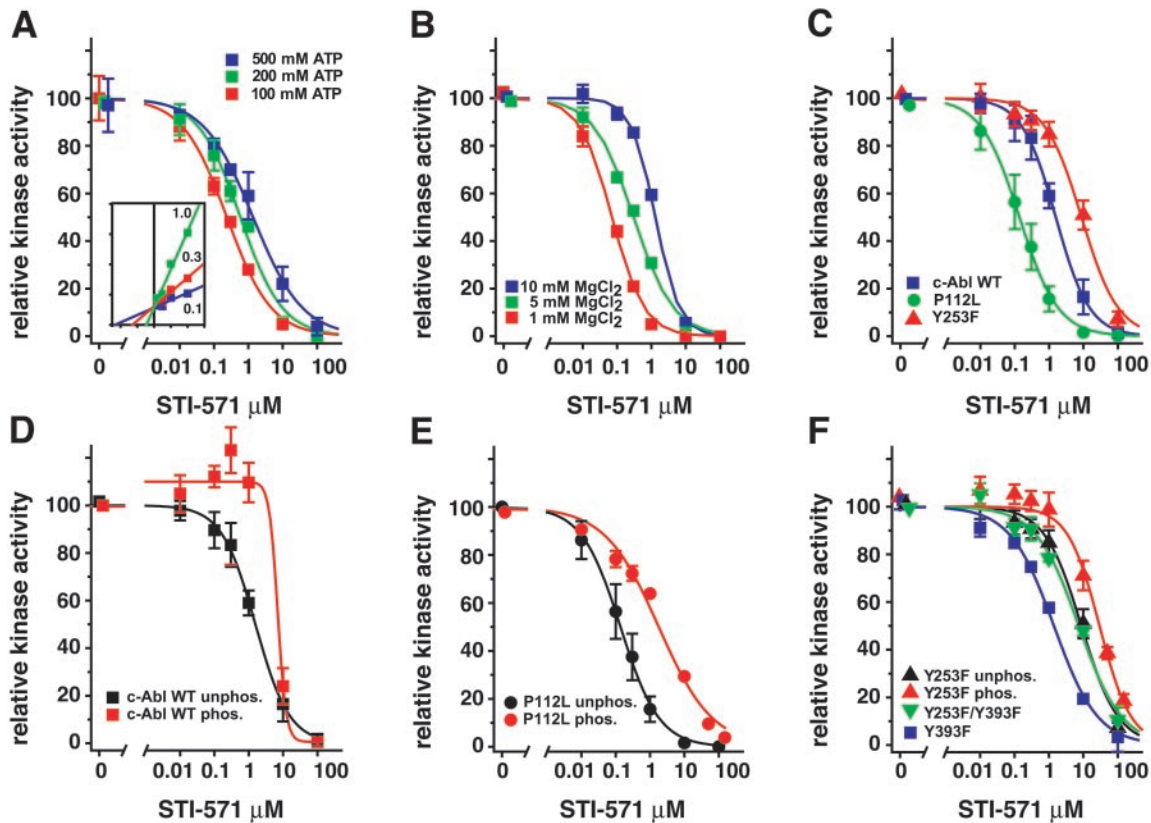


Fig. 4. The Abl Y253F mutant has intrinsic resistance to STI-571 that is independent of phosphorylation of Tyr-393. (A) Sensitivity of purified unphosphorylated c-Abl WT kinase to STI-571 at different concentrations of ATP (red, 100 μM ; green, 200 μM ; blue, 500 μM). (Inset) Data from these ATP concentrations and a 50 μM ATP series (not shown) were plotted in a double-reciprocal format [x axis: $1/(\text{ATP})(\mu\text{M}^{-1})$; y axis: $1/v(\text{min}\cdot\text{pmol}^{-1})$] for STI-571 concentrations of 0.1, 0.3, and 1.0 μM (as indicated). (B) Sensitivity of c-Abl to STI-571 at different concentrations of magnesium chloride (red, 1 μM ; green, 5 μM ; blue, 10 μM). (C) Sensitivity of purified unphosphorylated c-Abl WT (blue), P112L (green), and Y253F (red) to STI-571. (D–F) Comparison of the STI-571 sensitivity of unphosphorylated (black symbols) and tyrosine-phosphorylated (red symbols) c-Abl WT (D), P112L (E), and Y253F (F). The Y393F mutation alone did not alter the STI-571 sensitivity of c-Abl significantly ($\text{IC}_{50} = 1.40$, blue symbols in F), and the resistance of c-Abl Y393F to STI-571 was not increased by phosphorylation (data not shown). A modest stimulatory effect of STI-571 on peptide kinase activity of phosphorylated c-Abl and c-Abl Y253F was reproducibly observed at lower drug concentrations.

The response of purified unphosphorylated c-Abl to STI-571 was assessed in the peptide kinase assay and found to depend on the concentration of both ATP and magnesium in the reaction (Fig. 4 A and B), consistent with a predominantly competitive mechanism of inhibition (Fig. 4A Inset). Under standard conditions (500 μM ATP and 10 mM MgCl_2), the kinase activity of unphosphorylated c-Abl was inhibited by STI-571 with an IC_{50} of 1.56 μM (Fig. 4C, blue symbols, and Table 1). This value is significantly higher than that obtained in previous studies (2, 7) that used dysregulated forms of Abl or the isolated Abl catalytic domain, suggesting that disruption of the autoinhibitory structure of c-Abl actually might increase the sensitivity of Abl to this drug. Indeed, unphosphorylated c-Abl P112L (Fig. 4C, green symbols) was inhibited more potently by STI-571 ($\text{IC}_{50} = 0.14 \mu\text{M}$) than WT c-Abl. These results suggest that dysregulation of Abl kinase activity by SH3 mutation or Bcr fusion increases the sensitivity to STI-571. In contrast, the c-Abl Y253F mutant demonstrated significant intrinsic resistance to STI-571 as an unphosphorylated protein, with an IC_{50} of 8.8 μM (Fig. 4C, red symbols). The kinase dysregulation and STI-571 resistance induced in c-Abl by the Y253F mutation therefore seem to be independent of one another.

Resistance of Abl Y253F to STI-571 Is Independent of Phosphorylation of the Regulatory Tyr-393. After incubation with ATP and magnesium, c-Abl undergoes intermolecular autophosphorylation

(Fig. 3A) at two distinct regulatory tyrosine residues, Tyr-393 in the activation loop of the kinase domain and Tyr-226 in the linker region between the Abl SH2 and kinase domains, resulting in stimulation of intrinsic kinase activity up to 18-fold (9), whereas Tyr-253 is not phosphorylated detectably (13). Comparison of the structure of the isolated Abl catalytic domain bound to a homologue of STI-571 (7) with that of the activated Src family kinase Lck (20) suggests that phosphorylation of Tyr-393 would favor an open conformation of the catalytic domain cleft that would be incompatible with binding of STI-571. In agreement with this prediction, phosphorylated c-Abl and c-Abl P112L were both significantly more resistant to STI-571, with increases in IC_{50} of 5.4- and 13-fold, respectively (Fig. 4 D and E, red symbols, and Table 1). Interestingly, c-Abl Y253F became very resistant ($\text{IC}_{50} = 29.6 \mu\text{M}$) after phosphorylation (Fig. 4F, red symbols). To be certain that the STI-571 resistance of c-Abl Y253F was not caused by residual phosphorylation at Tyr-393, we generated and purified a c-Abl Y253F/Y393F double mutant. The Y393F mutation does not alter the basal catalytic activity of c-Abl or its sensitivity to STI-571 (Fig. 4F, blue symbols) but greatly reduces its activation after autophosphorylation (9). Importantly, the c-Abl Y253F/Y393F double mutant was still resistant to STI-571 (Fig. 4F, green symbols), demonstrating that mutation of Y253 in the nucleotide-binding loop of Abl reduces the intrinsic sensitivity of Abl kinase activity to STI-571. These results indicate that the sensitivity of Abl

proteins to STI-571 is influenced in a complex way by the activation state of the kinase. Activation of Abl by intermolecular tyrosine phosphorylation impairs STI-571 inhibition, whereas disruption of the intramolecular folding of Abl (through SH3 mutation or fusion of Bcr) increases sensitivity to the drug. The *in vivo* response of Ph⁺ leukemic cells to STI-571 reflects the balance between these two factors, where STI-571 acts to trap Abl in the unphosphorylated state and prevent reactivation by autophosphorylation.

Clinical Implications of the Y253F Mutation. The c-Abl Y253F mutant did not exhibit increased catalytic activity *in vitro*, suggesting that this mutation does not affect the intramolecular binding of SH3 to Pro-223. Therefore, the activation of Abl kinase activity by the Y253F mutation is only manifest when the protein is expressed within a cell. Biochemical and genetic evidence suggests that Abl is additionally regulated *in vivo* through binding of a cellular protein inhibitor (21–23), and the Y253F mutation may interfere with binding of this putative factor. It is possible that Y253F may synergize with Bcr to increase the oncogenicity of Bcr-Abl *in vivo* by blocking the action of an endogenous protein inhibitor. In support of this notion, the combination of P112L and Y253F mutations in c-Abl increases Abl kinase and transforming activity *in vivo* relative to either single mutant (data not shown). Similarly, the Bcr-Abl Y253F mutant induced significantly more phosphotyrosine *in vivo* than WT Bcr-Abl when expressed at similar levels (Fig. 1A and data not shown). This finding raises the interesting possibility that some Bcr-Abl mutations that cause resistance to STI-571 also might contribute to the progression of chronic-phase CML to accelerated phase or blast crisis, which may help explain the rapid emergence of drug resistance in these patients.

Tyr-253 makes only a weak van der Waals interaction with

STI-571 but forms a water-mediated hydrogen bond with the side chain of Asp-322 that folds the P-loop down and increases surface complementarity with the drug through an induced-fit mechanism (7). Substitution of Phe for Tyr-253 would not be predicted to interfere directly with drug binding but might decrease the affinity of an induced-fit interaction with the ATP-binding site. In agreement with this model, the Y253F mutation was associated with an intermediate level of resistance to STI-571 *in vivo* and *in vitro*, whereas the T315I mutation that seems to interfere sterically with binding of STI-571 caused nearly complete resistance to the drug. Mutation of other residues in the Abl nucleotide-binding loop including alterations of Glu-255 to Lys or Val have been reported recently in patients with advanced CML or Ph⁺ acute lymphoblastic leukemia and acquired resistance to STI-571 (24–26), suggesting that the Abl P-loop may be a frequent target for acquisition of resistance mutations in this population. Our results demonstrate that at least some P-loop mutants are still sensitive to STI-571 at increased drug concentration, because the Abl Y253F kinase was inhibited significantly by 5 μ M STI-571, a level that theoretically is achievable in patients based on pharmacokinetic data from the phase I clinical trials. We suggest that clinical resistance to STI-571 induced by the Y253F mutation might be overcome by dose escalation of the drug, and molecular genotyping of STI-571-resistant patients could provide information useful for the rational therapeutic management of these patients.

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- Buchdunger, E., Zimmermann, J., Mett, H., Meyer, T., Müller, M., Druker, B. J. & Lydon, N. B. (1996) *Cancer Res.* **56**, 100–104.
- Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J. & Lydon, N. B. (1996) *Nat. Med.* **2**, 561–566.
- Druker, B. J., Talpaz, M., Resta, D. J., Peng, B., Buchdunger, E., Ford, J. M., Lydon, N. B., Kantarjian, H., Capdeville, R., Ohno-Jones, S. & Sawyers, C. L. (2001) *N. Engl. J. Med.* **344**, 1031–1037.
- Druker, B. J., Sawyers, C. L., Kantarjian, H., Resta, D. J., Reese, S. F., Ford, J. M., Capdeville, R. & Talpaz, M. (2001) *N. Engl. J. Med.* **344**, 1038–1042.
- Gambacorti-Passerini, C., Barni, R., le Coutre, P., Zucchetti, M., Cabrita, G., Cleris, L., Rossi, F., Gianazza, E., Brueggen, J., Cozens, R., *et al.* (2000) *J. Natl. Cancer Inst.* **92**, 1641–1650.
- Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N. & Sawyers, C. L. (2001) *Science* **293**, 876–880.
- Schindler, T., Bornmann, W., Pellicena, P., Miller, W. T., Clarkson, B. & Kuriyan, J. (2000) *Science* **289**, 1938–1942.
- Van Etten, R. A., Debnath, J., Zhou, H. & Casasnovas, J. M. (1995) *Oncogene* **10**, 1977–1988.
- Brasher, B. B. & Van Etten, R. A. (2000) *J. Biol. Chem.* **275**, 35631–35637.
- Hawley, R. G., Lieu, F. H. L., Fong, A. Z. C. & Hawley, T. S. (1994) *Gene Ther.* **1**, 136–138.
- Mosmann, T. (1983) *J. Immunol. Methods* **65**, 55–63.
- Li, S., Ilaria, R. L., Million, R. P., Daley, G. Q. & Van Etten, R. A. (1999) *J. Exp. Med.* **189**, 1399–1412.
- Allen, P. B. & Wiedemann, L. M. (1996) *J. Biol. Chem.* **271**, 19585–19591.
- Sicheri, F., Moarefi, I. & Kuriyan, J. (1997) *Nature (London)* **385**, 602–609.
- Xu, W., Harrison, S. C. & Eck, M. J. (1997) *Nature (London)* **385**, 595–602.
- Barila, D. & Superti-Furga, G. (1998) *Nat. Genet.* **18**, 280–282.
- Brasher, B. B., Roumiantsev, S. & Van Etten, R. A. (2001) *Oncogene* **20**, 7744–7752.
- Wetzler, M., Talpaz, M., Van Etten, R. A., Hirsch-Ginsberg, C., Beran, M. & Kurzrock, R. (1993) *J. Clin. Invest.* **92**, 1925–1939.
- Pluk, H., Dorey, K. & Superti-Furga, G. (2002) *Cell* **108**, 247–260.
- Yamaguchi, H. & Hendrickson, W. A. (1996) *Nature (London)* **384**, 484–489.
- Pendergast, A. M., Muller, A. J., Havlik, M. H., Clark, R., McCormick, F. & Witte, O. N. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5927–5931.
- Walkenhorst, J., Goga, A., Witte, O. N. & Superti-Furga, G. (1996) *Oncogene* **12**, 1513–1520.
- Wen, S.-T. & Van Etten, R. A. (1997) *Genes Dev.* **11**, 2456–2467.
- Hochhaus, A., Kreil, S., Corbin, A., La Rosee, P., Lahaye, T., Berger, U., Cross, N. C. P., Linkesch, W., Druker, B. J., Hehlmann, R., *et al.* (2001) *Science* **293**, 2163.
- Hofmann, W.-K., Jones, L. C., Lemp, N. A., de Vos, S., Gscheidmeier, H., Hoelzer, D., Ottmann, O. G. & Koefler, H. P. (2002) *Blood* **99**, 1860–1862.
- von Bubnoff, N., Schneller, F., Peschel, C. & Duyster, J. (2002) *Lancet* **359**, 487–491.