Analysis of the Structural Basis of Specificity of Inhibition of the Abl Kinase by STI571*

STI571, a selective inhibitor of Bcr-Abl, has been a successful therapeutic agent in clinical trials for chronic myelogenous leukemia. Chronic phase chronic myelogenous leukemia patients treated with STI571 have durable responses; however, most responding blast phase patients relapse despite continued therapy. Co-crystallization studies of Abl kinase and an STI571-related compound identify specific amino acid residues as critical to STI571 binding, one of which, T315, has been characterized as an acquired Thr to Ile mutation in relapsed patients. Other studies, however, suggest that mutations other than these predicted contact points are capable of conferring STI571 resistance in relapsed patients. Using a variety of models of STI571 binding to the Abl kinase, we have performed an extensive mutational analysis of sites that might alter the sensitivity of the Abl kinase to STI571. Although mutation of many of the predicted contact points between Abl and STI571 result in a kinase-inactive protein, additional mutations that render the Abl kinase less sensitive to STI571 demonstrate a broad range of possibilities for clinical resistance that are now becoming evident.

Chronic myelogenous leukemia is a clonal hematopoietic stem cell disorder characterized by the presence of the Philadelphia chromosome. Clinically, the disease progresses through distinct phases referred to as chronic or stable, accelerated, and blast. The chronic or stable phase of the disease is characterized by excess numbers of myeloid cells that differentiate normally. Within an average of 4–6 years, the disease transforms through an “accelerated phase” to an invariably fatal acute leukemia, also known as blast crisis. Disease progression is likely due to the accumulation of molecular abnormalities that lead to a progressive loss of the capacity for terminal differentiation of the leukemic clone (1, 2).

A variety of experimental evidence has established Bcr-Abl, the product of the (9;22) translocation that produces the Philadelphia chromosome as the causative agent of chronic myelogenous leukemia (3, 4). The critical role of this constitutively activated tyrosine kinase in the survival of the leukemic clone has been validated by the development and significant clinical activity of STI571, a specific inhibitor of the Bcr-Abl tyrosine kinase (5). Clinical studies with STI571 have demonstrated a durable response in patients in the chronic phase of the disease, whereas most responding patients in blast phase relapse despite continued therapy (6, 7).

Recent investigations suggest that one of the most common mechanisms of relapse is a mutation of amino acid 315 of the Abl kinase domain, a predicted site of contact between an STI571-related compound and the ATP binding site of the Abl kinase (8, 9). However, additional study centers have reported that kinase domain mutations may occur at additional sites (10, 11, 14). To examine a broader spectrum of sites of mutation that might be observed in clinical materials, we have generated numerous mutations in the Abl kinase domain based on three models of STI571 bound to the Abl kinase domain and assessed these mutant kinases for sensitivity to STI571.

EXPERIMENTAL PROCEDURES

Mutation Construction—The Abl kinase domain consisting of c-Abl amino acids 220–498 was subcloned into the BamHI site of pGEX KG (Amersham Biosciences). A hemagglutinin (12CA5) antibody recognition tag was inserted at the 5′ end of the Abl kinase domain sequence both as an Abl phosphorylation site and for detection of protein expression. All mutations within the kinase domain were constructed using polymerase chain reaction amplification of the Abl kinase pGEX KG plasmid with primers containing appropriate point mutations.

Kinase Assays—Glutathione S-transferase (GST) fusion proteins of the Abl kinase domain mutations as well as wild type Abl kinase were generated by inducing exponentially growing transformed DH5α bacteria with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were lysed via sonication in MT phosphate-buffered saline (150 mM NaCl, 16 mM NaHPO₄, 4 mM Na2HPO₄, pH 7.3) containing 1% Triton X-100, 10 μg/ml aprotinin, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μM 5′-mercaptoethanol. The GST-Abl kinase mutants were purified from the lysate by binding to glutathione-Sepharose overnight at 4 °C. Bound proteins were washed twice with 0.5 M LiCl, thrice with phosphate-buffered saline, pH 7.5, and once with Abl kinase wash buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂). Bound protein concentrations were determined by SDS-PAGE followed by Coomasie Blue staining. All Abl kinase proteins and mutations were expressed and purified in this manner. 500 ng of bound protein was used in each kinase reaction. Kinase reactions were performed in 30 μl of Abl kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂, 10 μM sodium vanadate, 1 μM dithiothreitol, 1% dimethyl sulfoxide (Me₂SO)). STI571 was dissolved in 3% Me₂SO prior to addition to the kinase reaction. The Abl kinase mutations were incubated with concentrations of STI571 ranging from 0 to 1 μM for 10 min, after which 10 μCi of [γ-32P]ATP (111 nM total ATP) was added and the kinase reaction allowed to proceed for 30 min. The reactions were terminated by boiling in SDS loading dye, and samples were analyzed by SDS-PAGE. Abl autophosphorylation signal intensity was quantitated with a PhosphorImager (Molecular Dynamics).

Determining ATP Affinity and Vmax Values—Protein preparation and kinase assay conditions were the same as described for the IC₅₀ determination in the absence of STI571. ATP was added to final concentrations ranging from 20 to 100 μM, and the kinase reactions were allowed to proceed for 30 min. As indicated by time-course analysis of wild type

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Abl (data not shown) the reaction rate of the kinase is linear for reaction times <60 min; therefore, °P incorporation over a 30-min period of time is proportional to the initial rate of reaction. Reactions were analyzed by SDS-PAGE as described above, and signal intensity was quantitated. The ATP concentrations resulting in 50% maximum signal ([ATP]50% maximum signal) for wild type Abl and each of the mutations that showed a change in STI571 sensitivity were determined from the slope and y-intercept of double reciprocal plots of 1/[ATP] versus 1/signal. ATP affinities were determined from the average of four experimental repeats. As the graphs to determine kinetic parameters represent several experiments and signal intensity cannot be easily converted into molar °P incorporation or normalized between separate gels, absolute V max values cannot be reported in the context of this assay. Instead, relative V max values of wild type Abl and the mutants were determined by comparing the relative signal intensity of the proteins at 100 nM ATP. Relative V max values were calculated using the average ATP affinities in the following equation: V max = signal [ATP]50% maximum signal/[ATP] + 1 with the values reported relative to wild type Abl = 1.0.

Immunoblots—Gels were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) for 4 h at 0.45 A. The membrane was blocked in 5% non-fat milk in TBS (10 mM Tris, pH 8.0, 150 mM NaCl) at 25 °C for 1 h. The GST-Abl kinase domain mutations were detected by incubation overnight with an anti-Abl kinase domain antibody, Ab-2 (Santa Cruz) at 2 µg/ml in TBS containing 0.5% Tween 20. Antibody interaction was detected using enhanced chemiluminescence with a lumi-imager (Roche Molecular Biochemicals). Identical procedures were used for all immunoblots unless specified.

RESULTS

Mutation of STI571 Contact Points Predicted by the Abl Kinase Crystal Structure—Co-crystallization of the Abl kinase domain with an STI571-related 2-phenylaminopyrimidine Abl-specific inhibitor identified a number of amino acid residues within the ATP binding pocket predicted to contact the inhibitor (Fig. 1A) (9). The potential STI571 contact points are illustrated in Fig. 1B. They include hydrogen bonds with Thr-315, Met-290, Glu-286, Lys-271, and the peptide backbone at Asp-381 and Met-318. Hydrophobic interactions are predicted with Ile-313, Phe-382, Val-256, Tyr-253, and Leu-370. mutations were generated to eliminate the potential for hydrogen bonding or hydrophobic interactions between Abl and the STI571-related compound. Hydrogen bonds are predicted with Thr-315, Met-290, Glu-286, Lys-271, and the peptide backbone at Asp-381 and Met-318. Hydrophobic interactions are predicted with Ile-313, Phe-382, Val-256, Tyr-253, and Leu-370.

To determine the necessity of the wild type residue to STI571 function, we analyzed mutations that would remove the potential functionality of the original amino acid side chain. Some of the mutations require single amino acid substitutions that might be observed in patients. Additional mutations require two nucleotide changes and therefore may not represent likely amino acid substitutions to be seen in patient material. From this information we could speculate about the likelihood that the site would be a target for resistance. As Met-318 and Asp-381 are predicted to form hydrogen bonds with STI571 via the peptide backbone, mutations at these sites would not be informative. The relative IC50 values of the mutations at fixed ATP concentrations are summarized in Table I. Consistent with previous reports, the IC50 value for wild type Abl kinase was 0.025 µM in this assay (Fig. 2A). K271R was previously reported as a kinase-inactive mutation. Additionally, E286L, M290A, and I313G were kinase-inactive suggesting that many sites involved in STI571 binding are also critical for ATP binding and kinase activity. Mutation of L370G did not change the sensitivity to STI571. In contrast, both T315V and T315I demonstrated a decreased sensitivity to STI571. The IC50 value of the T315V mutation averaged to 0.30 µM, ~10-fold higher than that of wild type Abl kinase (Fig. 2B), whereas T315I showed no significant inhibition at STI571 concentrations 200-fold higher than the IC50 value of wild type Abl kinase. The decreased sensitivity of these mutations to STI571 is consistent with the crystal structure predictions that illustrate a critical hydrogen bond between the secondary amino group of the inhibitor and the T315 side chain.

Because the residues analyzed are within the ATP binding pocket of the enzyme, the kinetic parameters for ATP binding were determined in the absence of STI571 to examine the effects of these mutations on ATP binding and the overall function of the Abl kinase. ATP affinity was determined for wild type Abl and each of the mutations that demonstrated a decreased sensitivity to STI571 (Fig. 3A). Relative V max values were also determined (Fig. 3B). Because gel-based assays that measure signal intensity rather than molar phosphate incorporation were used in this kinetic analysis, relative V max values of the mutations and not absolute V max values are reported here. ATP affinities and relative V max values are summarized...
in Table I. Both the T315V and T315I mutations demonstrate a 2-fold increase in their ATP affinities relative to wild type, suggesting that these mutations may not only eliminate a critical hydrogen bond with STI571 but also increase the affinity of ATP binding relative to STI571, contributing to resistance. The $V_{\text{max}}$ values of both Thr-315 mutants are lower than the wild type enzyme, with T315V retaining only 28% of its kinase activity and the T315I mutation retaining 61% of its kinase activity. Regardless of a decrease in kinase activity, the significant decrease in sensitivity of mutations at Thr-315 to STI571 make this residue a likely target for clinical resistance, consistent with a mutation seen in resistant patients (8, 11, 14).

Additionally, the Y253F mutation showed a decreased sensitivity to STI571 with an IC$_{50}$ value of 1.8 $\mu$M, 72-fold higher than wild type Abl kinase. This result is also consistent with the crystal structure that predicts Tyr-253 to be a critical STI571 contact point. The Y253F mutation demonstrated an identical $V_{\text{max}}$ value to wild type; however, it showed a 2-fold decrease in ATP affinity, suggesting that this residue functions in ATP binding as well as STI571 binding. The huge increase in the IC$_{50}$ value of this mutation, therefore, suggests a decrease in the binding affinity for STI571, making it also a likely candidate for clinical resistance as seen in patient samples (11, 14).

**TABLE I**

IC$_{50}$ values of Abi mutants based on the crystal structure

Mutations were made to residues lacking either hydrogen bond-forming capabilities or hydrophobicity. STI571 was predicted to form hydrogen bonds with the peptide backbone at Met-318 and Asp-381; therefore mutations of these residues could not be made. Many of the mutations resulted in a kinase-inactive Abl. Mutation of L370G did not change the sensitivity to STI571. Mutation of T315V, T315I, and Y253F decreased the sensitivity to STI571. ATP affinities (reported as ATP concentrations necessary to achieve 50% of the maximum signal) and relative $V_{\text{max}}$ values are listed for the mutations that showed a decrease in sensitivity to STI571.

| Potential contact sites | Mutations | Sensitivity to STI571 | ATP affinity: [ATP]$_{50%}$ maximum signal | Relative $V_{\text{max}}$ |
|------------------------|-----------|-----------------------|------------------------------------------|--------------------------|
| WT                     |           | IC$_{50}$ = 0.025 $\mu$M | 62nm | 1.00 |
| Tyr-253                | Phe       | IC$_{50}$ = 1.8 $\mu$M | Kinase-inactive | 1.01 |
| Val-256                | Glu       | IC$_{50}$ = 0.30 $\mu$M | 38nm | 0.28 |
| Lys-271                | Arg       | IC$_{50}$ > 5.0 $\mu$M | 37nm | 0.61 |
| Glu-286                | Leu       | Kinase-inactive        |           |    |
| Met-290                | Ala       | Kinase-inactive        |           |    |
| Ile-313                | Glu       | Kinase-inactive        |           |    |
| Thr-315                | Val       | IC$_{50}$ > 5.0 $\mu$M | 37nm | 0.61 |
| Ile                    | Gly       | Same as WT             |           |    |

**FIG. 2.** IC$_{50}$ values of wild type Abl kinase and the T315V mutation. A, kinase assays performed in duplicate produced an IC$_{50}$ value of 0.025 $\mu$M for STI571 binding to wild type Abl. B, the IC$_{50}$ value for T315V was 0.25 $\mu$M in the gel shown and averaged to 0.30 $\mu$M in four repeats. Equal protein loading is demonstrated by anti-Abl immunoblots.

32216
kinases, thereby explaining the high selectivity of the inhibitor for Abl. The IC₅₀ values of the mutations are summarized in Table II. The majority were either kinase-inactive or displayed no change in STI571 sensitivity. These results support predictions from the crystal structure that suggests that topological differences rather than amino acid sequence differences in the ATP binding pockets of the Abl kinase and the Src family kinases are responsible for the specificity of STI571. Interestingly, mutation of A380T raised the IC₅₀ for STI571 binding 10-fold over that of wild type Abl kinase. A kinetic analysis of the A380T mutation shows a 2-fold decrease in the \( V_{\text{max}} \) relative to wild type Abl and little change in the ATP affinity (Table II). This suggests that the decrease in sensitivity to STI571 is due to a decreased affinity for the inhibitor with little effect on ATP binding. Although this residue is not a predicted contact point in the Schindler et al. model (9) the adjacent residue Asp-381 forms a critical hydrogen bond with STI571. Because the mutation of Ala-380 to the smaller glycine did not alter the sensitivity to STI571 (data not shown), it is likely that a slightly larger residue either introduces steric effects or small conformational changes in the binding surface accounting for the decreased sensitivity. An alternative explanation for the decreased sensitivity of the A380T mutation is that Ala-380 makes van der Waals interactions with Phe-382, another residue that directly contacts STI571. Thus, the A380T mutation would be predicted to alter the conformation of the ATP binding pocket by disrupting this interaction. Regardless, all of the predictions from the Lck kinase can be explained by the Abl crystal structure.

Mutation of Predicted STI571 Contact Points Based on a Fibroblast Growth Factor Receptor Model of Inhibitor Binding—A third model of STI571 binding to the Abl kinase was extrapolated from computer modeling of the structure of the fibroblast growth factor receptor (FGFR) (Fig. 4A) (13). In this model STI571 binds to the active form of Abl kinase in reverse orientation relative to that seen in the Schindler et al. model.
Despite this, several of the predicted contact points are similar to those predicted from the crystal structure (Fig. 4B), including Val-256 and Leu-370. Most of the mutations resulted in either no change in sensitivity to STI571 or a kinase-inactive Abl. Mutations of A380T decreased the sensitivity to STI571 10-fold relative to wild type Abl kinase. This is likely due to steric effects or conformational changes in the STI571 binding surface of the mutated Abl. ATP affinities (reported as ATP concentrations necessary to achieve 50% of the maximum signal) and relative $V_{\text{max}}$ values are reported for the mutations that showed a decrease in sensitivity to STI571.

| Potential contact sites | Mutations | Sensitivity to STI571 | ATP affinity: $[\text{ATP}]_{50\%}$ maximum signal | Relative $V_{\text{max}}$ |
|------------------------|-----------|-----------------------|-----------------------------------------------|----------------------|
| WT                     |           | $\text{IC}_{\text{50}} = 0.025 \mu\text{M}$ | 62nM | 1.00 |
| Leu-248                | Ala       | Kinase-inactive       | 62nM | 1.00 |
| Tyr-320                | Lys       | Same as WT            | 62nM | 1.00 |
| Asn-322                | Ser       | Same as WT            | 62nM | 1.00 |
| Glu-375                | Asn       | Same as WT            | 62nM | 1.00 |
| His-375                | Leu       | Kinase-inactive       | 62nM | 1.00 |
| Ala-380                | Cys       | Same as WT            | 62nM | 1.00 |
|                        | Thr       | $\text{IC}_{\text{50}} = 0.34 \mu\text{M}$ | 59nM | 0.48 |
|                        | Leu       | Kinase-inactive       | 62nM | 1.00 |

(9). Despite this, several of the predicted contact points are similar to those predicted from the crystal structure (Fig. 4B), including Val-256 and Leu-370. However, several new potential contact points are identified including Glu-258, Met-318, and Leu-248 (Fig. 4B). Mutations of these residues were examined for their sensitivity to STI571 and summarized in Table III. As with many of the previous mutations, several were kinase-inactive indicating the necessity of these residues for ATP binding to the Abl kinase. Interestingly, E258G demonstrated an $\text{IC}_{\text{50}}$ value of 0.18 $\mu\text{M}$, 8-fold higher than that of wild type Abl. There are several possible explanations for this result. The model based on STI571 binding to the FGFR suggests that the hydrogen bonding capabilities of Glu-258 may be critical to interactions between STI571 and Abl. This model is also consistent with our data that STI571 is capable of inhibiting the activated form of Abl (data not shown). The problem with the FGFR model, however, is that it cannot adequately explain other mutations generated above, specifically the Thr-315 mutations. In the Schindler et al. model, Glu-258 is 8 Å from the closest atom of the STI571-related compound (9). Although this is not close enough to effectively interact with the inhibitor, Glu-258 is directly adjacent to Phe-317 and Leu-248, both of which have hydrophobic interactions with STI571. Mutation of Glu-258 might alter the conformation of these residues, which could alter the conformation of the binding pocket. This mutation showed a large decrease in $V_{\text{max}}$, indicating that a high

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**FIG. 4.** Model of STI571 bound to the ATP binding pocket of the active form of the Abl kinase domain. A, a three-dimensional model of STI571 binding based on a FGFR model of inhibitor binding. B, a two-dimensional model of STI571 binding illustrating critical hydrogen bonds (dashed lines) and hydrophobic interactions between Abl and STI571. A hydrogen bond is predicted with Glu-258. Hydrophobic interactions are predicted with Met-318, Val-256, Leu-248, and Leu-370. Val-256 and Leu-370 are also predicted contact points in the Abl kinase crystal structure.

**TABLE III**

IC$_{50}$ values of Abl mutants based on an FGFR model of inhibitor binding.

| Potential contact sites | Mutations | Sensitivity to STI571 | ATP affinity: $[\text{ATP}]_{50\%}$ maximum signal | Relative $V_{\text{max}}$ |
|------------------------|-----------|-----------------------|-----------------------------------------------|----------------------|
| WT                     |           | $\text{IC}_{\text{50}} = 0.025 \mu\text{M}$ | 62nM | 1.00 |
| Leu-248                | Ala       | Kinase-inactive       | 62nM | 1.00 |
| Val-256                | Gly       | Kinase-inactive       | 42nM | 0.18 |
| Glu-258                | Gly       | $\text{IC}_{\text{50}} = 0.18 \mu\text{M}$ | 42nM | 0.18 |
| Met-318                | Ala       | Kinase-inactive       | 62nM | 1.00 |
| Leu-370                | Gly       | Same as WT            | 62nM | 1.00 |
percentage may be in an inactive state, and an ATP affinity that was slightly higher than wild type Abl, suggesting that rather than a reduction of STI571 affinity, the increased affinity of the mutation for ATP is capable of overcoming the potency of the inhibitor. This explanation is consistent with the modest yet noticeable increase in IC50. Thus, it is not necessary to postulate an alternative binding mode of STI571 to the Abl kinase to account for our mutational data.

**DISCUSSION**

Some common themes seem to emerge from this data. It is clear that many of the predicted contact points between STI571 and the Abl kinase are essential to ATP binding and that mutation of these residues produces a kinase-inactive protein. Thus, these mutations are unlikely to produce clinical resistance. It is also possible that mutation of these residues introduces structural instability that leads to kinase inactivity. This would indicate that they are unlikely mutations for clinical resistance; however, more conservative amino acid substitutions might, in some instances, yield different results. An example of this is apparent in the results from an array of mutations of Ala-380. While A380T decreased the sensitivity to STI571, A380L was kinase-inactive, suggesting that only a conservative change at that site is tolerated. It is possible that additional relevant mutations could be identified through analysis of different amino acid substitutions in some of the sites described in this study.

Despite this, there are predicted contact points that do render the kinase less sensitive to STI571. Two of these, Thr-315 and Tyr-253, predicted by the Schindler et al. (9) model, have been found as an acquired mutation in patients who developed resistance to STI571 (8, 11, 14). There are, however, additional residues in the Abl kinase, not immediately obvious from the crystal structure that, when mutated, result in decreased sensitivity to STI571 and therefore may be clinically relevant.

In a recent report, a mutation at amino acid 255 was found in one of 32 patients who were resistant to STI571, whereas no mutations at residue 315 were found (10). Although amino acid 255 is not a predicted contact point in any of the models presented above, in the FGFR and Schindler et al. models this amino acid is immediately adjacent to 256, a predicted site of contact. The decreased sensitivity to STI571 caused by mutation at this residue may be due to alterations in the STI571 binding surface induced by mutation of a residue adjacent to a contact point.

Our data suggests that residues in close proximity to the predicted points of contact may also alter the sensitivity of Abl to STI571, possibly due to subtle structural alterations that either decrease the affinity for STI571 or increase the affinity of Abl for ATP. One such mutation is exemplified by the mutation at amino acid 255, present in a patient with clinical resistance to STI571. Our data also suggests that mutations in the Abl kinase domain other than those currently reported are likely to be observed and that these mutations are likely to be more diverse than originally described.

Many of the STI571-insensitive mutations also demonstrated a reduction in kinase activity. We hypothesize that STI571-resistant mutations may exist in patients prior to therapy and that they are selected for by treatment with STI571. The decrease in kinase activity of the T315I mutation, for example, suggests that in the absence of STI571, the mutation would confer no growth advantage and therefore would not be readily observed in untreated patients. Under selective pressure from STI571 therapy, however, it would emerge as a predominant clone. This result is consistent with a failure to detect kinase domain mutations in patients prior to STI571 therapy and may explain why an initial response to STI571 prior to the development of resistance is common in patients with kinase domain mutations (8, 11).

A number of study centers have already identified an array of acquired mutations in clinically resistant patients beyond those initially identified (10, 11, 14). Until further data is obtained on a larger group of patients, we advocate sequencing of the entire kinase domain to screen for mutations in patients who develop resistance to STI571. It is also imperative that mutations identified in clinical samples are analyzed for their sensitivity to STI571 to clarify their role in development of drug resistance. As we learn more about which mutations frequently confer STI571 resistance in patients, new specific inhibitors can be targeted against STI571-resistant variations of Abl to combat the difficulty of drug resistance in disease treatment.

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