A Single Amino Acid Exchange Inverts Susceptibility of Related Receptor Tyrosine Kinases for the ATP Site Inhibitor STI-571*

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The tyrosine kinase inhibitor STI-571 potently blocks BCR-Abl, platelet-derived growth factor (PDGF) α- and β-receptors, and c-Kit kinase activity. Flt3, a receptor tyrosine kinase closely related to PDGF receptors and c-Kit is, however, not inhibited by STI-571. Sequence alignments of different kinases and indications from the crystal structure of the STI-571 Abl kinase complex revealed amino acid residues that are probably crucial for this activity profile. It was predicted that Flt3 Phe-691 in the β strand may sterically prevent interaction with STI-571. The point mutants Flt3 F691T and PDGFβ-receptor T681F were constructed, and kinase assays showed that the Flt3 mutant but not the PDGFβ-receptor mutant is inhibited by STI-571. Docking of STI-571 into computer models of the PDGFβ-receptor and Flt3 kinase domains and comparison with the crystal structure of the STI-571 Abl kinase complex indicated very similar binding sites among the three nonphosphorylated kinases, suggesting corresponding courses of their Asp-Phe-Gly motifs and activation loops. Accordingly, we observed reduced sensitivity of preactivated compared with nonactivated PDGFR-β for the inhibition by STI-571. Courses of the activation loop that collide with STI-571 binding explain its inactivity at other kinases as the insulin receptor. The binding site models of PDGFR-β and Flt3 were applied to predict structural approaches for more selective PDGFR-β-receptor inhibitors.

Tyrosine kinase inhibitors have a great pharmacological potential for the treatment of various forms of cancer and other diseases. Most of the recent leads competitively bind at the ATP site of the kinase domain but are nevertheless fairly selective. Some crystal structures of kinase-inhibitor complexes, e.g., of Hck from the c-Src family with the ATP site inhibitor CGP57148B (1) and of the fibroblast growth factor receptor-1 (FGFR-1) tyrosine kinase with different inhibitors (2, 3), indicate molecular determinants of inhibitor selectivity. Because of its pharmacological profile and therapeutic potential, the phenylaminopyrimidine STI-571 (Gleevec™, formerly CGP57148B) has received much attention. This compound inhibits Abelson tyrosine kinases (c-Abl, BCR-Abl) platelet-derived growth factor (PDGF) α- and β-receptor and c-Kit kinase activity with similar potency (4). Based on inhibition of BCR-Abl, STI-571 has recently been introduced successfully into the treatment of chronic myelogenous leukemia (5). Further clinical applications of STI-571 may rest on the inhibition of c-Kit and PDGF receptors and on the established role of these kinases in certain forms of cancer. Two crystal structures of the murine Abl kinase in complex with STI-571 and with a smaller variant (6) suggest binding of the inhibitor to the inactive kinase state with nonphosphorylated Tyr-393, as observed earlier by others for PP1/AGL1872 and Hck (1).

PDGFR-α and -β are members of the class III receptor tyrosine kinases. Abrupt activation of PDGF receptors has been linked to several disease states including certain malignancies and atherosclerosis, restenosis, and fibrotic conditions (7). Selective PDGF receptor tyrosine kinase inhibitors have therefore been developed. These include phenylaminopyrimidines (8) such as STI-571, phenylbenzimidazoles (9, 10), quinoxalines (11, 12), 6,7-dimethoxyquinolines (13), and bis(1H-2-indolyl) methanones (14).

Flt3 (Flk2, STK1) is structurally closely related to the PDGF receptor kinases and c-Kit. It is overexpressed in various types of leukemia, including B-lineage acute lymphoblastic leukemia and acute myeloid leukemia as well as T-lineage acute lymphoblastic leukemia and chronic myelogenous leukemia blast crisis cells (15–17). Different activating mutations in the Flt3 gene have been detected in acute myeloid leukemia patients. Flt3 may, therefore, be a suitable target for therapy of Flt3-dependent leukemias. Despite its close homology to PDGF receptors, Flt3 kinase is not inhibited by STI-571.

Multiple sequence alignments and the three-dimensional structure of the Abl kinase STI-571 complex (6) indicate possible reasons for selectivity. In some chronic myelogenous leukemia patients who responded initially to STI-571 but then relapsed, the resistance to the drug was associated with a single T315I mutation in the β strand of the Abl kinase domain (18). The side chain of Thr-315 mutation has been demonstrated to disrupt the hydrogen bond with the pyrimidinylamino group of STI-571. Replacement of Abl Thr-315 by IRK Met-1076 has been sug-
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FIG. 1. Sequence alignment of the targets PDGFR-β and Flt3 with the four tyrosine kinases c-Abl, FGFR-1, IRK, and VEGFR-2. The alignment results from the COMPOSER algorithm implied in SYBYL 6.8, considering three-dimensional superposition (similarity matrix was constructed from inter-Cα distances). The numbering of residues in the second column corresponds to the PDB files (see "Experimental Procedures"). Bars delineate secondary structure elements and functional loops. SCR, structurally conserved regions used for construction of the model frames in COMPOSER. Bold (additionally, Abl is underlined) residues depict the STI-571 binding site (3 Å around STI-571 according to the liep crystal structure with hydrogens added). An asterisk denotes the position of the critical residue, Thr-681, in PDGFR-β.

Experimental Procedures

Synthesis of STI-571—Synthesis was done according to published procedures (21), which contain, however, no details of the reaction conditions for the individual steps. Reaction conditions were described empirically and are given in the synthesis scheme (found in the Supplemental Material, available online at http://www.jbc.org). DNA Constructs—Human Flt3 and Flt3-ITD cDNAs were cloned as described previously (22, 23) and subcloned into the pcDNA3.1 (Invitrogen) eukaryotic expression vector. Versions with a C-terminal HA tag were constructed and kindly provided by D. Schmidt-Arras (Friedrich Schiller University, Jena, Germany). cDNAs of the human PDGFβ receptor (M21616) and human FGFR-1 were kindly provided by Drs. C. H. Heldin and L. Claesson-Welsh (Uppsala, Sweden), respectively, and subcloned into pcDNA3.1 (Invitrogen) eukaryotic expression vector. Versions with a C-terminal HA tag were introduced into the PDGFR-β sequence as described previously (24). A cDNA of the human insulin receptor in pRK5RS was kindly provided by Dr. A. Ullrich (Martinsried, Germany). Point mutations were introduced with the Quick-Change kit (Stratagene) according to the instructions of the manufacturer and were verified by DNA sequencing (MWG Biotech).

Kinase Assays—The expression constructs of Flt3 and PDGFβ receptor and the corresponding mutants were transfected into HEK293 cells as described previously. The transfected cells were starved overnight in 0.5% fetal calf serum/Dulbecco’s modified Eagle’s medium. Incubation with STI-571 (or with Me2SO as solvent control, final concentration 1%) was performed at 37 °C for 30 min, and then the cells were stimulated with Flt3 ligand (FL, PeproTech) or PDGF-BB (PeproTech) at room temperature in a concentration of 100 ng/ml for 5 min or 50 ng/ml for 10 min, respectively. The cells were lysed in buffer containing 20 mm HEPES, pH 7.4, 5 mm MnCl2, and 0.1 mm sodium orthovanadate. The immunoprecipitates of each dish were suspended, divided into six aliquots in new tubes, resedimented, and resuspended in 5 μl of kinase buffer. STI-571 or Me2SO solvent control (1% final concentration) was added (2 μl/sample) and the samples were incubated for 1 h at 30 °C. The samples were boiled with SDS-PAGE sample buffer and analyzed by SDS-PAGE or by autoradiography.

In vitro kinase assays for PDGF activity against an exogenous protein substrate were performed in a similar manner to those described previously (25). HEK293 cells, stably transfected with the HA-tagged PDGFR-β (kindly provided by Dr. B. Markova), were starved overnight in medium containing 0.5% fetal calf serum, stimulated with PDGF-BB (for obtaining preactivated receptor) or left unstimulated, and then were extracted. PDGFR-β was immunoprecipitated with monoclonal anti-phosphotyrosine antibodies (4G10, Upstate Biotechnology, Inc.). Receptor loading was controlled by immunoblotting with anti-Flt3 antibody C-20 (Santa Cruz Biotechnology) or anti-HA-antibody (BabCO, Richmond, CA). For in vitro kinase assays, the lysates of transfected HEK293 cells were subjected to immunoprecipitation (5 μg of anti-HA antibody or 5 μg of anti-Flt3 and 100 μl of protein A-Sepharose/10-cm dish), and the immunoprecipitates were washed three times in lysis buffer and once with kinase buffer containing 50 μM HEPES, pH 7.4, 5 mM MnCl2, and 0.1 mM sodium orthovanadate. The immunoprecipitates of each dish were suspended, divided into six aliquots in new tubes, resedimented, and resuspended in 20 μl of kinase buffer. STI-571 or Me2SO solvent control (1% final concentration) was added for 30 min on ice, and then [γ-32P]ATP was added (2–3 μCi/sample in 5 μl) and the kinase reaction was allowed to proceed at 30 °C for 20 min. The samples were boiled with SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography.

In vitro kinase assays for PDGFR activity against an exogenous protein substrate were performed in a similar manner to those described previously (25). HEK293 cells, stably transfected with the HA-tagged PDGFR-β (kindly provided by Dr. B. Markova), were starved overnight in medium containing 0.5% fetal calf serum, stimulated with PDGF-BB (for obtaining preactivated receptor) or left unstimulated, and then were extracted. PDGFR-β was immunoprecipitated with an-
ti-HA antibodies. The immunoprecipitate from PDGF-stimulated cells was treated under shaking with 1.2 mM ATP at 30 °C for 15 min in 50 mM Hepes, pH 7.5, 5 mM MgCl₂, 0.1 mM sodium orthovanadate (kinase buffer). The immunoprecipitate from nonstimulated cells was treated likewise in buffer without ATP. The immunoprecipitates were washed three times in ice-cold kinase buffer and were then aliquoted. Aliquots were incubated with STI-571 at different concentrations on ice for 20 min. The peptide (25) was added to a final concentration of 2 mM, and the kinase reaction was initiated by the addition of [γ-32P]ATP (2.5 μCi/sample) and allowed to proceed for 20 min at 30 °C. The reaction was stopped by adding EDTA to a final concentration of 100 mM, and peptide phosphorylation was evaluated as described (25).

Modeling—Initial computer models of the PDGFR-β kinase and the Flt3 F691T mutant, excluding the kinase insert regions, were generated using the program COMPOSER (26), part of the molecular modeling package SYBYL version 6.8 (Tripos Inc., St. Louis, MO). Eight crystal structures from the SYBYL binary Protein Data Bank (PDB) library were selected as templates by overall sequence identity: VEGFR-2 (PDB code 1yv2, chain A (27), identity 54.6% with PDGFR, 52.9% with Flt3, PDB: 1fgk; chain A (28), identity 51.4–53.6% with PDGFR and 49.3–51.8% with Flt3); murine c-Abl kinase (PDB code 1fpu, chain A (6), 1iep, chain A (20), identity 37.7% with PDGFR and 40.4% with Flt3), inactive insulin receptor kinase (1irk (29), identity 36.1% with PDGFR and 40.4% with Flt3), and Flt3 Phe-691 mutants (0.56 (FGFR 1fgi) to 1.51 Å (1irk)).

On the basis of optimal sequence alignments, the structurally conserved regions (SCR) (see Fig. 1) and an average Ca framework structure of the template SCRs were determined by an iterative approach, improving both the multiple alignment and the subsequent SCR framework by pairwise Needleman and Wunsch dynamic programming procedures with a similarity matrix constructed from inter-Ca distances. The backbone of each SCR of the PDGFR-β kinase and the Flt3 F691T mutant was then built by fitting the corresponding SCR from one of the known homologs (namely that with the highest block sequence identity, mostly VEGFR-2) to the appropriate region of the framework. The least-squares fits are inversely weighted by the variation of the residue positions across the known structures. The average r.m.s. distance of the eight templates (pairwise fits of SCR with corresponding SCR from one of the known homologs (namely that with the highest block sequence identity, mostly VEGFR-2) to the appropriate region of the framework) is 0.67 Å (2fgi) to 1.65 Å (1irk) and 0.56 Å (FGFR 1fgk) to 1.51 Å (1irk). The backbone of each SCR of the PDGFR-β kinase and the Flt3 F691T mutant is shown in Fig. 1 and an average Ca framework structure of the template SCRs were determined by an iterative approach, improving both the multiple alignment and the subsequent SCR framework by pairwise Needleman and Wunsch dynamic programming procedures with a similarity matrix constructed from inter-Ca distances. The backbone of each SCR of the PDGFR-β kinase and the Flt3 F691T mutant was then built by fitting the corresponding SCR from one of the known homologs (namely that with the highest block sequence identity, mostly VEGFR-2) to the appropriate region of the framework. The least-squares fits are inversely weighted by the variation of the residue positions across the known structures. The average r.m.s. distances of the eight templates (pairwise fits of SCR with corresponding SCR from one of the known homologs (namely that with the highest block sequence identity, mostly VEGFR-2) to the appropriate region of the framework) is 0.67 Å (2fgi) to 1.65 Å (1irk) and 0.56 Å (FGFR 1fgk) to 1.51 Å (1irk).

RESULTS AND DISCUSSION

Flt3 Phe-691/PDGFR-β Thr-681 as a Critical “Switch” for Inhibitor Sensitivity—According to the sequence alignment (Fig. 1) and the crystal structures of the Abl kinase, Phe-691 of PDGF-receptor β kinase and the F691T mutants were fitted by fitting the corresponding SCR from one of the known homologs (namely that with the highest block sequence identity, mostly VEGFR-2) to the appropriate region of the framework. The least-squares fits are inversely weighted by the variation of the residue positions across the known structures. The average r.m.s. distances of the eight templates (pairwise fits of SCR with corresponding SCR from one of the known homologs (namely that with the highest block sequence identity, mostly VEGFR-2) to the appropriate region of the framework) is 0.67 Å (2fgi) to 1.65 Å (1irk) and 0.56 Å (FGFR 1fgk) to 1.51 Å (1irk).
Flt3 may prevent binding of STI-571. To test this prediction, mutant variants of Flt3 and PDGFR-β/H9252 were generated and analyzed with respect to inhibition by STI-571. Phe-691 in Flt3 was replaced by Thr, the corresponding residue in the PDGFR-β/H9252 kinase. When expressed in HEK293 cells, the Flt3 F691T variant has a somewhat reduced kinase activity compared with wild-type Flt3. Importantly, STI-571 inhibits Flt3 F691T with an IC₅₀ of 0.1–0.3 μM (Fig. 2A, lower panel), which is very close to the known IC₅₀ of PDGFR-β kinase inhibition in intact cells (Ref. 4; see also Fig. 3). As shown previously (4), Flt3 wild type is refractory to STI-571 inhibition (Fig. 2A, upper panel). The in vitro immunocomplex assays (Fig. 2B) confirmed these results, although higher concentrations of STI-571 are required to obtain complete inhibition of Flt3 F691T. The strong difference in susceptibility compared with Flt3 wild type is still obvious. Corresponding results were obtained with a pathologically relevant, constitutively active Flt3 variant harboring an internal tandem duplication (ITD) in the juxtamembrane domain. Although Flt3ITD is resistant to inhibition (Fig. 2C, upper panel), the Flt3ITD F691T variant was potently inhibited by STI-571 (Fig. 2C, lower panel). In agreement with the suggested Abl-like binding mode, the replacement of Phe-691 with Thr removes the steric constraints for binding of STI-571. As an additional indication of this mechanism, replacing Thr-681 by Phe in the corresponding position of the PDGFR-β/H9252 kinase should lead to inactivity of STI-571. This was indeed the case; although the wild-type PDGFR-β/H9252 kinase was potently inhibited by STI-571 (Fig. 3A, upper panel) with an IC₅₀ of 0.1–0.3 μM, the PDGFR-β T681F mutant had an unaltered kinase activity but was unresponsive to STI-571 inhibition (Fig. 3A, lower panel). These findings were reconfirmed by in vitro kinase assays with corresponding immunoprecipitates (Fig. 3B).

To evaluate whether the formation of a hydrogen bond between PDGFR-β Thr-681 and the STI-571 pyrimidinylamino group is important for STI-571 binding, we also tested a PDGFR-β T681A mutant. Interestingly, this mutant was not
less, but even somewhat more, susceptible to STI-571 inhibition than the PDGFR-β wild type (mean IC_{50} 0.18 μM in five experiments versus 0.34 μM in six experiments, respectively), indicating no independent contribution of the hydrogen bond at least in the case of PDGFR-β. Possibly, there is no net gain of binding energy because the H-bond formation may be preceded by the displacement of a water molecule H-bonded to Thr-681. This is not the case in the Ala mutant, which, however, enables a similar or even better spatial fit of STI-571. It seems that the residue in β5 corresponding to PDGFR-β Thr-681 critically determines the susceptibility to STI-571 and probably other phenylaminopyrimidines, mainly by sterical constraints. Taken together with previous observations (31, 32), the atypical variability of this position in different kinases suggests that it may be a general key switch for obtaining selective inhibitors. We, therefore, tested whether other receptor tyrosine kinases could also be sensitized to STI-571 by mutating the corresponding residue. However, as shown in Fig. 4, neither the FGFR-1 V561T mutant (Fig. 4A) nor the insulin receptor M1103T mutant (Fig. 4B) was inhibited by STI-571. Thus, further structural determinants must prevent binding of STI-571 to these kinases.

The Activation Loop, Another Critical Determinant of Inhibitor Selectivity—The STI-571 binding site of murine c-Abl (6, 20) spans the whole core between both kinase domains and contains 23 residues within 3 Å around the ligand. The perfect complementary fit of STI-571 to the inactive, nonphosphorylated state of Abl (6) indicates in particular that little spatial scope is left for the most flexible functional regions, the activation and the nucleotide binding loop. Activation of Abl by pre-phosphorylation greatly reduced the STI-571 sensitivity of the kinase (6). We therefore tested whether phosphorylation and activation of the PDGFR-β would likewise affect STI-571 susceptibility. This was indeed the case. When assayed against an exogenous peptide substrate, preactivated PDGFR-β required a one order of magnitude higher concentration of STI-571 for inhibition than unstimulated PDGFR-β (IC_{50} 0.63 μM versus 5.05 μM, respectively, Fig. 5). In line with these experiments, the PDGFR-β Y857F mutant, which lacks the tyrosine whose phosphorylation is critical for kinase activation, is slightly better inhibited than the wild type when expressed in intact cells (IC_{50} 0.25 versus 0.34 μM, respectively). It should be noted here that effective inhibition of the stimulated wild-type kinase in intact cells occurs because the susceptible, inactive kinase conformation is regenerated by the action of protein tyrosine phosphatases (33). This is most likely the reason for the relatively small difference in inhibition of wild type and the PDGFR-β Y857F mutant in intact cells.

The conformation of the activation loop is a feature that distinguishes not only between the phosphorylated and the inactive state of a given species but also between different tyrosine kinases. Binding of STI-571 at Abl kinase involves interactions with Asp-381 and Phe-382 in the highly conserved N-terminal anchor region (the Asp-Phe-Gly motif) of the loop. Schindler et al. (6) have demonstrated that in the complex between STI-571 and the natural, autoinhibitory Abl conformation, Tyr-393 mimics a tyrosine residue of substrate peptides but is not phosphorylated because of the displacement of Asp-Phe-Gly. Inactivity of STI-571 at tyrosine kinases of the Src family (Hck, Lck) in both the inactive and active states follows from collision with this motif. Thus, an appropriate Asp-Phe-Gly course seems to be essential for STI-571 binding. To model activation loops of the PDGF-β receptor kinase and Flt3, this assumption was checked by analyzing the course of additional kinases.

Table I presents the ϕ and ψ backbone angles of the Asp-Phe-Gly motif and the preceding residue from seven tyrosine kinase crystal structures. The courses are determined mainly by the Asp ϕ angles, discriminating between inactive states of Abl and insulin receptor kinase (IRK) on the one hand, of Hck, VEGFR-2, and FGFR-1 kinase, as well as active states of IRK and Lck on the other hand. In the second group, typical Asp ϕ and Phe ψ values separate active (IRK, Lck) from inactive conformations (Hck, VEGFR-2) and indicate, together with specific Gly angles, an individual course for the FGFR-1 activation loop, corresponding to a special autoinhibitory mecha-

![Fig. 5. Preactivation of PDGFR-β reduces sensitivity to STI-571 inhibition. HEK293 cells overexpressing PDGFR-β were stimulated with PDGF-BB, PDGFR-β was immunoprecipitated, and auto-phosphorylation was allowed in the presence of unlabeled ATP. Excess ATP was removed by washing, and a kinase assay was performed with the synthetic peptide KY751 and [γ-^32P]ATP as substrates. To obtain nonactivated PDGFR-β, cell stimulation was omitted, and prephosphorylation was replaced by mock treatment. Inhibition of kinase activity against the KY751 peptide is depicted (means of three independent experiments performed in duplicate).](image-url)
nism (28). Although the autoinhibitory activation loops of Abl and IRK, both with a substrate-mimicking tyrosine, are alike, IRK Gly-1149 preceding Asp induces an individual course of the Asp-Phe-Gly motif because the \( \phi \) angle of 170° is possible only in glycine residues.

Fig. 6 illustrates the Asp-Phe-Gly courses of the seven kinases together with a volume contour of STI-571 bound to Abl. The model is based on an alignment of Ca atoms of \( \beta \) and \( \beta' \) (r.m.s. distance 0.25–0.36 Å in pairwise fits to Abl). Three principal groups become obvious: 1) Abl and inactive IRK; 2) inactive FGFR-1, VEGFR-2, and Hck; 3) active IRK and Lck. However, the course of inactive IRK is markedly steeper than that of Abl, leading to a complete overlap of the Phe-1151 side chain with the pyrimidinylamino moiety of STI-571. This overlap might contribute to the inactivity of the inhibitor at IRK. The courses of the other five kinases are completely incompatible with STI-571 docking because they all cross the benzylpiperazinyl moiety of the ligand.

Computer Models of the PDGFR-β Kinase and the Flt3 F691T Mutant—The simple reversal of the wild-type PDGFR-β kinase selectivity of STI-571 into Flt3 selectivity of the F691T mutant with potencies close to those for inhibition of Abl requires more detailed investigations and interpretations by means of three-dimensional computer models of the complexes. PDGFR-β kinase and Flt3 F691T mutant models were derived from template crystal structures and the alignment in Fig. 1 as described (see “Experimental Procedures”). The kinase insert regions were ignored. The SCRs (see Fig. 1) and, except for the nucleotide binding and the activation loops, the remaining regions of the models (structurally variable regions) were inserted from one of the eight templates.

As derived previously, the Asp-Phe-Gly courses must be similar to those in c-Abl to enable binding of STI-571. Modeling approaches based on this assumption, however, have demonstrated that even the entire activation loop of the targets may follow an Abl-like course. This implies an autoinhibitory mechanism with Tyr-857 (PDGFR-β) and Tyr-842 (Flt3) pointing inward toward the catalytic site and mimicking substrate binding (see Fig. 7). The reduced sensitivity of preactivated PDGFR-β to STI-571 inhibition discussed above further supports such a structure. The predicted PDGFR-β activation loop conformation may be stabilized by four intramolecular interactions: H bonds or electrostatic forces between the side chains of Arg-853 and Asp-691 (αD), Asn-856 and Arg-830 (catalytic loop), and Tyr-857 and Asp-826 (catalytic loop) and a hydrophobic cluster of Leu-847, Ile-851, and Met-852. The first interaction is not possible in Flt3 (Ser-838 instead of Arg). In the
The binding site describes a nearly complete lock-and-key activation loop. The separating surface between STI-571 and final models (Criegee (20), and refined by energy minimization. Fits of the surrounding water molecules from the Abl kinase crystal structure show higher levels of tyrosine phosphorylation. Thus, the inactive state of the activation loop, which protects the substrate-mimicking tyrosine from phosphorylation. Thus, the inactive state of the activation loop, which protects the substrate-mimicking tyrosine from phosphorylation.

Fig. 8. Models of STI-571 binding to the PDGFR-β kinase and Flt3. A, PDGFR-β kinase STI-571 complex showing 23 residues corresponding to amino acids within 3 Å around the inhibitor in the Abl crystal structure, 1iep. Colors of carbon and some hydrogen atoms: white, STI-571; orange, identical residues; magenta, mutated residues compared with murine Abl kinase. The isolated red balls are suggested water oxygens, and hydrogen atoms marked as balls participate in suggested hydrogen bonds. Transparent tubes: blue, β-strands; green, α-helices; gray, loops. B, docking of STI-571 into Flt3 derived from the model of the Flt3 F691T mutant. The depiction of residues and the coloring scheme are as described in A, except for carbon and some hydrogen atoms: orange, identical residues; green, mutated residues compared with PDGFR-β kinase. The green MOLCAD surface of the Phe-691 side chain is drawn in the two common positions of the χ1 torsion angle: 180°, opaque; −60°, transparent.

Crystal structure of c-Abl kinase, only the corresponding Tyr-393–Asp-363 H bond and hydrophobic Leu-Leu-Met cluster are obvious. Thr-392 (instead of PDGFR-β Asn-856) does not approach Arg-367 but contacts Met-388 and Pro-402 via hydrophobic or van der Waals interactions. Very recent results (34) have indicated that Ala mutants in the human c-Abl kinase at positions corresponding to Met-388 and Thr-392 in the c-Abl crystal structure show higher levels of tyrosine phosphorylation. Thus, the inactive state of the activation loop, which protects the substrate-mimicking tyrosine from phosphorylation, must be stabilized by several intramolecular interactions and/or an inhibitor like STI-571 to freeze the natural flexibility of this region.

The nucleotide binding loops are well ordered in the crystal structures of Abl and FGF1-1 complexed with the inhibitors STI-571 and SU-5402, respectively, and adopt similar, specific conformations induced by ligand fitting. The aromatic side chains of Abl Tyr-253 and FGF1-1 Phe-489 stabilize the downfold of the loops by van der Waals contacts and by a water-mediated hydrogen bond between Tyr-253 and Asn-322 in the case of Abl, or by oxygen-aromatic interactions in the case of FGF1-1. A corresponding fold of the nucleotide binding loops of PDGFR-β and the Flt3 F691T mutant may likewise be stabilized; Phe-611 and Phe-621, respectively, replacing Abl Tyr-253, could be involved in perpendicular van der Waals contacts with the pyridylpyrimidinylamino moiety and in oxygen-aromatic interactions with an Asp residue in place of Abl Asn-322. In conclusion, the similar inhibitory activity of STI-571 at all three kinases, Abl, PDGFR-β, and Flt3 F691T, suggests a resemblance among their nucleotide binding loops.

On the basis of these considerations, the preliminary SCR models of the PDGFR-β kinase and the Flt3 F691T mutant were completed, provided with the STI-571 conformation and surrounding water molecules from the Abl kinase crystal structure 1iep (20), and refined by energy minimization. Fits of the final models (Cα atoms of SCRs) with the templates resulted in r.m.s. distances from 0.87 (FGFR 1fgk) to 1.69 Å (1irk) for PDGFR-β kinase, and from 0.92 (FGFR 1fgi) to 1.72 Å (1irk) for the Flt3 F691T mutant. Fig. 7 presents an overview of the PDGFR-β kinase model and the predicted conformation of the activation loop. The separating surface between STI-571 and the binding site describes a nearly complete lock-and-key shape, with only one edge of the piperazinyl moiety not in contact with site atoms.

The binding sites of the PDGFR-β kinase and the Flt3 F691T mutant were defined by the 23 amino acids aligned to the corresponding Abl kinase residues (PDB code 1iep) within 3 Å around STI-571 (see Fig. 1). Fits of the backbones of these 23 residues resulted in r.m.s. distances of 1.01 (PDGFR-β versus 1iep), 0.92 (Flt3 versus 1iep), and 0.54 Å (PDGFR-β versus Flt3). Fig. 8 shows models of the binding sites, pointing to essential interactions and to putative targets for selectivity. The discussion can be generalized in terms of the PDGFR-β kinase model (Fig. 8A), because only 2 of the 23 residues are different in the Flt3 F691T mutant (Ile-654 versus Flt3 Met-664, Ile-679 versus Flt3 Leu-689). The network of hydrogen bonds in the Abl STI-571 complex is preserved in the models. The pyridine nitrogen interacts with the backbone NH of Cys-684 after β5 (Abl Met-318) like the N1 nitrogen of ATP. The side chain oxygen of Thr-681 (β5) is attached to the pyrimidinylamino NH of STI-571. Fig. 8B demonstrates that Phe-691 interferes in all reasonable conformations with STI-571 binding to the Flt3 wild type. Equal inhibition of the PDGFR-β wild type and the T681A mutant (see above) indicates that indeed sterical hindrance is the main detemrinant of inactivity. Glu-651 (αC) of the PDGFR-β kinase forms a hydrogen bond with the amide NH of STI-571 and an ion pair with Lys-634 typical of many tyrosine kinases. The backbone oxygen of Val-823 in the catalytic loop may be attached to the protonated N4 of the piperazinyl ring, and the backbone NH of Asp-844 (Asp-Phe-Gly motif) is involved in a hydrogen bond with the amide oxygen of the ligand.

Van der Waals interactions in particular contribute to the complementary fit of the 4-pyridin-3-yl-pyrimidin-2-ylaminophenyl moiety, involving aromatic and aliphatic side chains from different regions, e.g. Leu-606 (β1), Phe-611 (nucleotide binding loop), Val-614 (β2), Ile-679 (β5), Tyr-683 (after β5), Leu-833 (β7), and Phe-845 (Asp-Phe-Gly motif), as well as the alkyl chain of Lys-634 (β3). The piperaizin-1-ylmethylbenzamide moiety aligns with three residues in αC (Ile-654, Met-655, and Leu-658) and with Cys-822 (catalytic loop), Cys-843 (after β3), and Asp-844 (Asp-Phe-Gly motif).

In summary, the predicted interactions of STI-571 with the PDGFR-β kinase and the Flt3 F691T mutant closely resemble
those in the complex with the Abl kinase. This is not self-evident because, apart from the activation and the nucleotide binding loop (see above), no regions of the c-Abl kinase crystal structures were explicitly used for model building. The fact that similar binding sites resulted without additional constraints is again an indication of the perfect surface complementarity with STI-571. Its minimized conformation in the kinase model strongly corresponds to that in the Abl IleP structure (r.m.s. distance of 0.64 Å when fitting the heavy atoms). Fig. 8 shows that only 7 of the 23 binding site residues differ between the PDGF-β and the Abl kinase. Some of these residues are potential targets to obtain selectivity of STI-571-like inhibitors for PDGF-β. For example, the side chain of PDGF-β Ile-654 (Abl Val-289, Flt3 Met-664) may be in close van der Waals contact with the methylpiperazinyl moiety, which is aligned perpendicularly with the SH group of Cys-822 (Abl Phe-359, Flt3 Cys-807) in the model. This cysteine should provide various reagents and also to Antje Tru

Drs. Claesson-Welsh, Heldin, Lammers, Markova, and Ullrich for pro-

Abl Ala-380, Flt3 Cys-828) could interact with providing various reagents and also to Antje Trümbler for generating some of the receptor mutants.

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