Chronic myeloid leukemia (CML), gastrointestinal stromal tumors (GISTs), and idiopathic hypereosinophilic syndrome are associated with pathological deregulation of the tyrosine kinases BCR-ABL, KIT, and PDGFRA, respectively. Patients who become resistant to imatinib treatment often develop secondary mutations, the most common of which results in a substitution of isoleucine for threonine at the same location in the ATP-binding domain in all three kinases (in KIT this occurs at amino acid 670). We sought to determine why Thr is always replaced by Ile.

**Methods**
All possible point mutations in the DNA triplet codon that could result in amino acid substitutions at Thr670 (Thr670Arg, Thr670Ile, Thr670Lys, Thr670Ala, Thr670Ser, Thr670Pro) were introduced by site-specific mutagenesis of the complementary DNA for a constitutively active, imatinib-sensitive form of the KIT receptor, Δ559/KIT. The resulting mutant KIT proteins were transiently expressed in COS1 African green monkey kidney cells grown with and without imatinib, and cell extracts were analyzed for KIT activation by immunoprecipitation and immunoblotting to determine autophosphorylation levels. We also performed molecular modeling to estimate the relative affinities of wild-type (Thr670) KIT and the KIT mutants for ATP and imatinib.

**Results**
Like the parental strain, Thr670Ala, Thr670Ser, and Thr670Lys mutants were inhibited by 5 µM imatinib, but in comparison, they were only weakly active and Thr670Pro and Thr670Arg were not active at all. Only the Thr670Ile mutant was fully active (autophosphorylated) and resistant to imatinib. These findings were consistent with computer modeling predictions that ranked these mutants Thr ~ Ile > Ala, Ser > Lys > Pro according to their affinity for ATP but Thr > Ala, Ser > Lys > Pro ~ Arg ~ Ile according to their affinity for imatinib.

**Conclusions**
This combination of in vitro and molecular modeling analyses shows why, among all possible amino acid substitutions at position 670 of KIT, only Ile is naturally selected as a resistance mutant in imatinib-treated GIST patients.

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imatinib has not been uniformly successful (10–16). Imatinib resistance in GIST usually results from selection for the preferential growth of cells that have acquired additional, second-site point mutations in the part of the gene that encodes the KIT kinase domain (10–15).

Among these imatinib-resistant KIT mutants, only one shared amino acid substitution affecting the ATP-binding pocket of KIT and PDGFRA has been reported, that is, Thr670Ile in KIT and Thr674Ile in PDGFRA (3,14). Both amino acid substitutions have been reported to induce a change in the conformation of the ATP-binding site (17,18), which results in a dramatically lower affinity of the mutant receptors for imatinib (11–14,16,17). Interestingly, an alignment of the KIT and PDGFRA primary sequences revealed that Thr670 in KIT and Thr674 in PDGFRA occur at positions homologous to Thr315 in BCR-ABL, a residue reported to be mutated in imatinib-resistant CML patients (19–23). Surprisingly, in all imatinib-resistant cases involving any of these tyrosine kinases, threonine was substituted exclusively by isoleucine at this position.

To understand why, in the presence of imatinib, only isoleucine is naturally selected to replace threonine over all possible amino acid substitutions that can be generated by point mutation at the Thr670 codon, we introduced other possible mutations of the Thr670 triplet codon into a sequence encoding a constitutively active, imatinib-sensitive form of the KIT receptor, Δ559/KIT, which remains active in the absence of ligand due to a one-amino acid deletion in the juxtamembrane domain. These double-mutant receptors mimic those observed in the tumors of GIST patients, in which secondary point mutations have often been detected on the same KIT allele that carried an activating mutation at Val559 (14). We transiently transfected constructs expressing these mutant receptors and the Δ559/KIT parental receptor into COS1 cells and used immunoprecipitation and immunoblot analysis of the resulting cell lysates to evaluate tyrosine kinase expression and phosphorylation. Also, cells expressing each KIT mutant were incubated with imatinib to examine the capacity of the drug to inhibit the activity of each variant. Finally, molecular simulations using the theoretical framework of the molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) method were used to analyze the binding affinities of the wild-type (Thr670) receptor and of all receptors mutated at position 670 for both ATP and imatinib (24).

Methods

Construction and Transfection of Mutated KIT

An expression vector carrying wild-type human complementary DNA (cDNA) for KIT (the kind gift of Professor Y. Yarden, Weizmann Institute, Rehovot, Israel) and a commercially available site-directed mutagenesis kit (Promega, Madison, WI) were used to generate all mutated forms of KIT. DNA sequencing revealed that the template plasmid insert was identical to KIT variant 1 (NCBI accession number NM_000222), which encodes a 976-amino acid protein. We constructed mutant Δ559, in which amino acid 559 is deleted from the juxtamembrane region (amino acids 550–591) of the cytoplasmic domain of KIT (amino acids 550–976) by deleting nucleotides 1696–1698 from the portion of the wild-type cDNA that was derived from exon 11. For the Thr670Ile mutant, cytosine 2030, the second base of the Thr670 triplet codon, ACA, was mutated to a thymine. All other mutants were obtained by making different changes in the first or second bases of the Thr670 triplet codon: Thr670Arg (ACA to AGA), Thr670Lys (ACA to AAA), Thr670Ala (ACA to GCA), Thr670Ser (ACA to TCA), and Thr670Pro (ACA to CCA), using Δ559 KIT as template. All plasmid inserts were sequenced after mutagenesis to verify their identity.

Each of the above mutated forms of Δ559 KIT (in the same expression vector above) was then transiently transfected into COS1 African green monkey kidney cells (American Type Culture Collection, Manassas, VA) by diethylaminoethyl–dextran and chloroquine treatment as previously described (14). Briefly, 8 × 10^4 exponentially growing cells per 100-mm dish were incubated with 2 µL of serum-free medium (Dulbecco’s Modified Eagle Medium; Invitrogen, Carlsbad, CA) containing 500 µg of DEAE–dextran (D-9885; Sigma, St Louis, MO), 5 µg of each KIT expression vector DNA, and 20 µg of pRC/CMV plasmid DNA as carrier. After incubation at 37°C for 30 minutes, cells were washed with serum-free medium, treated with 100 µM chloroquine (Sigma) in medium supplemented with 10% fetal bovine serum (FBS; Invitrogen) for
3 hours, and then refed with medium containing 10% FBS. For studies of KIT inhibition, cell cultures were treated at 72 hours after transfection for 8 hours with a final concentration of 5 μM imatinib mesylate (Novartis, Basel, Switzerland), which roughly corresponds to the concentration of the imatinib found in the blood of patients who are treated with the highest dosage regimen (ie, 800 mg/d) (25). Our 10 mM stock solutions of imatinib were dissolved in dimethyl sulfoxide (DMSO), and DMSO alone was used for negative controls. Cell cultures were treated with a tyrosine phosphatase inhibitor, 5 mM orthovanadate, for 2 hours before the protein extraction to detect very low levels of KIT phosphorylation (data not shown).

Immunoprecipitation and Immunoblotting of KIT
The expression and phosphorylation of each mutant form of KIT were studied using immunoprecipitation and immunoblotting of whole-cell lysates prepared from transiently transfected cells. The cells were lysed at 4°C in a buffer composed of 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM Na2P2O7, and 100 mM NaF, supplemented with 1 mM phenylmethylsulfonylfluoride and with 1:100 dilutions of Protease Inhibitor Cocktail and of Phosphatase Inhibitor Cocktail (Promega, Madison, WI) and exposure to ECL Hyperfilm (Amersham Biosciences) according to the manufacturer’s instructions. Protein bands detected on immunoblots from each lysate were imaged in the linear range with the ImageMaster VDS Software (Pharmacia Biotech, Piscataway, NJ) and exposure to ECL Hyperfilm (Amersham Biosciences) according to the manufacturer’s instructions.

Computational Analysis
All molecular dynamics (MD) simulations were carried out by using the Sander module within the AMBER 9 suite of programs [available from http://amber.scripps.edu/ (26) and the parm94 all-atom force field by Cornell et al. (27)]. These analyses were performed using the 32 processors of the Tartaglia cluster at the University of Trieste (Trieste, Italy). Molecular surface areas were estimated with the MSMS software package [available at http://www.scripps.edu/~sanner/html/msms_home.html (28)]. The crystallographic coordinates of both the active KIT structure in complex with the ADP [Protein Data Bank entry 1PKG.pdb (29), structure resolution 2.9 Å] and those of the inactive KIT structure in complex with imatinib [Protein Data Bank entry 1T46.pdb (30), structure resolution 1.6 Å] were used as starting geometries for both the wild-type and the mutant protein simulations in complex with the nucleotide and the inhibitor, respectively.

Each amino acid substitution was introduced into the corresponding wild-type KIT structure using the Biopolymer module of Insight II (v. 2001; Accelrys, Inc., San Diego, CA) (31). The best residue rotamer (ie, the residue side chain conformation characterized by the most favorable values of the torsion angles) was chosen according to a validated procedure (17,32,33). Briefly, each possible rotamer structure was generated, and then, each one was subjected to energy minimization. A systematic search for 100 possible conformations was carried out for each mutant residue. Each conformation was first checked to confirm that would avoid a serious steric clash between the mutant residue and any other amino acid in the molecule. If any atom of the mutant amino acid was closer than 1 Å to any atom of any other amino acid, that conformation for the mutant amino acid was discarded. Conformations that survived these selection criteria were then minimized for energy using the Sander module of AMBER 9 with steepest descent algorithm for the first 100 steps and then subsequently with a conjugate gradient approach. First, the mutated residue was minimized for a maximum of 500 steps, whereas the rest of the system was held fixed; second, only the hydrogen atoms were minimized for a maximum of 500 steps; finally, all atoms were minimized for 1000 steps. The structure whose rotamer led to the best set of torsion angles and, hence the lowest final energy value, was selected for the subsequent modeling. Each ATP- or imatinib-bound KIT protein complex was then solvated by placing it in a virtual box filled with water molecules, and a suitable number of counterions (Cl– and Na+) were added to ensure electroneutrality at neutral pH. All MD simulations were run using periodic boundary conditions, and the particle mesh Ewald approach was used to introduce long-range electrostatic effects.

MD simulations were carried out using 6 nanoseconds of constant pressure–constant temperature (P = 1 atm, T = 37°C) to equilibrate each complex system, followed by 2 nanoseconds in the canonical ensemble (ie, under constant volume–constant temperature conditions). The atomic equations of motion were integrated using a time step of 2 femtoseconds, and the SHAKE algorithm was also adopted to improve simulation computational efficiency.

All energetics analyses were done for only a single MD trajectory of each KIT–ligand complex considered, with unbound protein and substrate snapshots taken from the snapshots of that trajectory. The reported energy values for each molecular complex
were averaged over the entire 2-nanosecond MD data collection. According to the MM/PBSA method (24), the binding free energy between ATP (or imatinib) and KIT (ΔGbind) can be calculated as

\[ ΔG_{\text{bind}} = ΔG_{\text{MM}} + ΔG_{\text{el}} - TΔS_{\text{MM}} \]

where \( ΔG_{\text{MM}} = ΔG_{\text{el}} + ΔG_{\text{NP}} \) and \( ΔG_{\text{el}} \) denotes the sum of MM energies of the molecules, that is, the sum of the electrostatic (\( ΔE_{\text{el}} \)) and van der Waals (\( ΔE_{\text{vdW}} \)) energies:

\[ ΔE_{\text{el}} = ΔE_{\text{el}}^{\text{NP}} + ΔE_{\text{el}}^{\text{SASA}}. \]

To examine the individual contribution of the protein residues to \( ΔE_{\text{el}}^{\text{MM}} \), the 2000 collected MD frames were further processed, and the interaction energy between ATP or imatinib and the binding site of each of the mutant KIT proteins was calculated on a residue-by-residue basis.

In the above equations, \( ΔG_{\text{el}} \) represents the solvation free energy. The polar solvation process represented by \( ΔG_{\text{el}}^{\text{NP}} \) can be imagined as the energy of transfer of a protein from one medium with dielectric constant equal to that of the interior of the protein to another medium with dielectric constant equal to that of the exterior of the protein, or in other words, the work done to reversibly charge the solute and polarize the solvent. The free energy of nonpolar solvation, \( ΔG_{\text{el}}^{\text{SASA}} \), can be imagined as the energy of transfer of a nonpolar molecule with the shape of the protein from vacuum to water: It includes cavity creation in water and van der Waals interactions between the modeled nonpolar protein and the water molecules. The polar component of \( ΔG_{\text{el}} \) was evaluated with the Poisson–Boltzmann approach (34), whereas the nonpolar contribution to the solvation energy was calculated as \( ΔG_{\text{NP}} = γ(SASA) + β \), in which \( γ = 0.00542 \text{kcal/Å}^2 \), \( β = 0.92 \text{kcal/mol} \), and SASA is the solvent-accessible surface area estimated with the MSMS program (28).

Finally, we used the normal-mode analysis approach (35) to estimate the change in solute entropy upon association, \( -TΔS_{\text{MM}} \).

**Statistical Analysis**

The 95% confidence intervals were calculated using bootstrap estimation of SEs (100 bootstrap samples). The null hypothesis of percentage of KIT inhibition upon imatinib addition equal to 0 was tested by two-sided Wald test. Stata 10 statistical software (StataCorp 2007 Stata Statistical Software: Release 10; StataCorp LP, College Station, TX) was used for statistical analyses.

**Results**

**Constitutive Activation of KIT Thr670 Mutants**

To examine why Thr670 in the ATP-binding pocket of the KIT receptor tyrosine kinase is only mutated to Ile among imatinib-resistant GIST patients, we created all other genetically probable amino acid substitutions at this position and examined the abilities of the mutant proteins to be activated or inhibited by imatinib. All of the amino acids that can substitute for the wild-type residue, threonine, at position 670 of KIT were determined on the basis of the genetic triplet code (Figure 1, A). Accordingly, an expression vector carrying the gene for Δ559/KIT, a constitutively active deletion mutant of KIT, was further mutagenized to introduce Arg, Ile, Lys, Ala, Ser, or Pro at position 670. All of these mutated KIT constructs were then transfected into COS1 cells, with Δ559/KIT, the gene for the activated receptor with a wild-type (ie, Thr670) ATP-binding pocket, used as a positive control. Cell lysates were examined on immunoblots probed with anti-KIT or anti-phosphotyrosine antibodies.

As shown in Figure 1, B, Δ559/KIT protein was expressed and autophosphorylated in transiently transfected COS1 cells, and both the mature (145 kDa) and partially glycosylated (125 kDa) forms of the receptor could be detected by immunoblotting of immunoprecipitated lysates. Comparison of lysates from KIT-transfected cells with COS1 cell lysate, the negative control, confirmed the absence of detectable endogenous KIT expression. The KIT protein that contained the Thr670Ile substitution in the Δ559 background was strongly autophosphorylated as previously described (14), similar to its Thr670, Δ559/KIT parent. Interestingly, Δ559/KIT proteins containing the other substitutions at position 670 were expressed, but only weakly phosphorylated (Thr670Ala, Thr670Ser, and Thr670Lys), very weakly phosphorylated (Thr670Pro), or not phosphorylated at all (Thr670Arg). The same trend was also observed when the transfected cells were incubated 2 hours before the protein extraction with orthovanadate, a protein tyrosine phosphatase inhibitor, although in this case, mutant Thr670Arg was also found to be very weakly phosphorylated (data not shown).

Based on the densitometry results (Figure 1, B, bottom), we ranked the degree of phosphorylation of all mutants as follows: Thr670Ile >> Thr670Ser > Thr670Ala > Thr670Lys > Thr670Pro > Thr670Arg. We concluded that all potential secondary point mutations that caused amino acid substitutions in the ATP pocket of KIT except Thr670Ile were able to attenuate the activating effect of the Δ559 deletion in the juxtamembrane domain of the KIT receptor.

**Effect of Imatinib on Autophosphorylation of KIT Mutants**

We next examined the effect of imatinib on the activation of the KIT mutants described above. COS1 cells that were transiently transfected with the Thr 670 or mutated KIT expression constructs were cultured for 8 hours in the absence or presence of 5 µM imatinib, followed by immunoblotting of cell lysates with anti-KIT or anti-phosphotyrosine antibodies. Because autophosphorylation of Thr670Pro and Thr670Arg was difficult to detect by immunoblotting, we excluded these two mutants from these experiments (Figure 1, C).

In the presence of 5 µM imatinib, autophosphorylation of Thr670, Δ559/KIT was completely abolished. However, the Thr670Ile mutant in the Δ559 background continued to be strongly phosphorylated, consistent with an earlier report (14). Autophosphorylation of the remaining mutant receptors (Thr670Ser, Thr670Ala, and Thr670Lys-Δ559/KIT) was weakly inhibited by 5 µM imatinib (Figure 1, D); however, their basal levels of phosphorylation (ie, in the absence of imatinib) were much lower than those of Thr670, Δ559 KIT. These results were quantified by densitometry (Figure 1, C, bottom). Of all that variants tested, Thr670Ile, Δ559 KIT was least sensitive (ie, the most resistant) to inhibition of phosphorylation by imatinib.

**In Silico Study of Mutant KITs**

To examine the binding affinities of native and mutated KITs for ATP and imatinib, we performed extensive MD simulations of the KIT intracellular domain (amino acids 546–976) using a protocol based on the MM/PBSA approach (24). ATP binding to KIT results in an active complex conformation capable of...
Figure 1. Biochemical analysis of all possible products of point mutations at codon 670 in the ATP-binding pocket of the kinase domain of KIT. A) Amino acid substitutions possible at the Thr670 tripeptide codon, ACA. The mutations that were made are shown in boxes. B) Expression and phosphorylation of KIT mutants in COS1 cells. Plasmids encoding all mutated forms of activated Δ559/KIT that were studied (Thr670Ile, Thr670Ser, Thr670Ala, Thr670Lys, Thr670Pro, and Thr670Arg; here labeled Ile, Ser, Ala, Lys, Pro, and Arg, respectively) were transiently transfected into COS1 cells in parallel with Δ559/KIT Thr670 (Thr); untransfected cells (−) were used as a negative control. Cell lysates were evaluated for protein content, and, in each case, 1 mg total protein was immunoprecipitated with a monoclonal antibody (K45) directed against the KIT receptor. Washed immunoprecipitates were examined for KIT expression and activation on immunoblots using a polyclonal anti-KIT antibody (sc-5535) or a monoclonal anti-phosphotyrosine antibody (4G10) and the appropriate horseradish peroxidase–linked secondary antibodies followed by enhanced chemiluminescence assay. Both antibodies detected the mature (145 kDa) and partially glycosylated (125 kDa) forms of the KIT receptor. This blot is representative of at least three independent determinations. Mean levels of tyrosine-phosphorylated KIT vs total levels of KIT protein were quantified by densitometry of X-ray film images from three independent experiments and were normalized relative to Thr670Δ559/KIT, for which the level was set at 100%. The panel below the blot shows the estimated percentage of activated KIT and the 95% CI for each mutant. C) Inhibition of autophosphorylation of KIT mutants by imatinib. COS1 cells that were transiently transfected with plasmids encoding Thr670Δ559/KIT or with the mutants Thr670Ile, Thr670Ala, Thr670Ser, and Thr670Lys that were derived from it were incubated at 72 hours after transfection with 0 or 5 µM imatinib for 8 hours. Cell lysates containing 1 mg total protein were immunoprecipitated as described in panel B, and immunoblots were used to evaluate KIT expression (anti-KIT antibody) and KIT autophosphorylation (anti-phosphotyrosine antibody) in the presence and absence of imatinib. Antibody binding on three independent immunoblots was quantified by densitometry as described above, and estimated percentages of activated KIT compared with total KIT in the presence and absence of imatinib are reported. The lower table lists the estimated percentage of KIT inhibition obtained upon imatinib addition \[ \%\text{inhib} = \frac{\%\text{KIT phos} - \%\text{KIT phos + drug}}{\%\text{KIT phos + drug}} \]. For each determination, the corresponding 95% CI and the $P$ value of the two-sided Wald test of the null hypothesis ($H_0: \%\text{inhib} = 0$) are reported. CI = confidence interval.

autophosphorylation, substrate binding, and kinase activity (Figure 2, A). This portion of the KIT protein is characterized by a two-lobed structure typical of the tyrosine and serine/threonine kinases: The smaller, amino-terminal N-lobe extends from amino acid Arg586 to Asn680 and is composed mainly of β-strands, and the larger, carboxyl-terminal C-lobe, which begins at amino acid Asp765, is mostly α-helical. The N-lobe includes a single α-helix, also called the control or C-helix (from residues His630 to Gly648). One ATP molecule binds in an interdomain cleft between the N- and C-lobes and is stabilized by the concerted conformation of four structural motifs: the C-helix, the phosphate-binding P-loop (residues Gly596 to Gly601), the adenine-recognition hinge loop (residues Tyr672 to Gly676), and the kinase activation A-loop (from residues Ile808 to Glu839).

The upper half of Table 1 lists the total free energy of ATP binding and related thermodynamic values that were calculated for the kinase domains of the wild-type (Thr670) KIT receptor and mutant KIT proteins with amino acid substitutions at position 670. The value of $\Delta G_{\text{bind}}$ for wild-type KIT with respect to ATP was found to be −24.2 kcal/mol, in agreement with published findings (36).
Figure 2. Molecular modeling of KIT–ATP complexes. A) Overall ribbon structure of the active form of KIT in complex with ATP (shown as a stick representation), showing the positions of the amino- and carboxyl-terminal lobes, with α-helices in dark red, β-sheets in blue, and loops in gray. The C-helix (amino acids His630 to Gly648) is highlighted in orange-red, the phosphate-binding P-loop (residues Gly595 to Gly601) is colored dark green, the adenine-recognition hinge loop (from Tyr672 to Gly676) is depicted in gold, and the kinase activation A-loop (amino acids Ile808 to Glu839) is portrayed in light green [Protein Data Bank entry 1PKG (26)]. B) Overlay ribbon representation of the molecular dynamics – equilibrated structures for the active, ATP-bound kinase domain (residues from Asn567 to Gln927) of wild-type vs Thr670Arg KIT obtained from the application of the MM/PBSA. The wild-type structure is shown in blue, and the Thr670Arg structure is shown in pink. C) The structural environment surrounding the ATP nucleotide in a superimposed view of the Thr670Ile KIT/ATP (yellow) and Thr670Arg KIT/ATP (purple) complexes. ATP, residue Ile670, and residue Arg670 are depicted in stick representation (i.e., thick lines), whereas the main kinase residues involved in interactions with the nucleotide are shown as thinner lines. Hydrogen bonds are visualized as broken lines. Hydrogen atoms, water molecules, and counterions are omitted for clarity. D) Overlay ribbon representation of the molecular dynamics – equilibrated structures for the active, ATP-bound kinase domain (residues from Asn567 to Gln927) of wild-type vs Thr670Ile KIT obtained from the application of the MM/PBSA. The wild-type structure is shown in cyan, and the Thr670Ile structure is shown in yellow. MM/PBSA = molecular mechanics/Poisson–Boltzmann surface area.

As suggested by the last row of the top half of Table 1, which shows the difference in the free energy change for ATP binding and compares wild-type (Thr670) with mutant KIT proteins \(\Delta \Delta G_{\text{bind}} = \Delta G_{\text{bind}}(\text{wild type}) - \Delta G_{\text{bind}}(\text{mutant})\), all of the KIT mutants that we tested except Thr670Ile showed much less affinity than the wild-type receptor for ATP. In fact, the negative values calculated for \(\Delta \Delta G_{\text{bind}}\) indicate that these amino acid substitutions at position 670 were unfavorable in terms of ATP affinity, whereas the positive \(\Delta \Delta G_{\text{bind}}\) value for Thr670Ile indicates that this mutation was favorable.

Inspection of the modeled structures of most KIT mutants in complex with ATP revealed slight distortions in the ATP-binding
site induced by the mutated residues, such as those that were apparent in the case of Thr670Ala, Thr670Ser, and Thr670Lys. By contrast, in the case of Thr670Pro and Thr670Arg, the conformation of the ATP-binding pocket was drastically altered. These findings revealed the structural reason behind the lower affinity of these mutants for the ATP (see Table 1) and were also in keeping with our experimental results for the mutant proteins. For example, as depicted in the ribbon structure of the kinase domain of Thr670Arg KIT (Figure 2, B), an Arg residue at position 670 is too big to be accommodated without causing a distortion of the entire ATP-binding pocket. This conformational rearrangement results in a direct steric clash and strong electrostatic repulsion between the Arg670 and the Lys 623 side chains. In this configuration, the KIT control C-helix, which directly contacts the nucleotide-binding site, is in a conformation that is incompatible with ATP binding. In fact, the conserved amino acid Glu640 that belongs to this helical domain is no longer able to maintain a favorable interaction with the side chain of Lys623 (see Figure 2, C), which is critical for correct positioning of the Lys623 side chain to bridge the α- and β-phosphates of the bound ATP (29). This evidence is quantitatively supported by the lower steric and electrostatic energies, ∆E_{vdw} and ∆G_{el}, respectively, that we calculated for the interaction of Glu640 with ATP in the case of the Thr670Arg mutant as compared with those for the wild-type KIT kinase domain (see Table 2). By contrast, in the Thr670Ile mutant, the smaller dimensions and the neutral character of the Ile side chain allow less distortion of

Table 1. Free energies of ATP and imatinib binding, ∆G_{bind} (kcal/mol), and energy contributions to ∆G_{bind} calculated for WT (Thr670) KIT and for all amino acid substitution mutants at position 670*.

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* The interaction energy terms ∆E_{vdw} and ∆G_{el} represent the steric and electrostatic contributions to binding, respectively, although here they refer to the contribution to ATP binding that is brought about by each single amino acid residue that is listed. Both ∆E_{vdw} and ∆G_{el} are expressed in kcal/mol.
the ATP-binding pocket (see Figure 2, D). Hence, a favorable interaction between Glu640 and Lys623 is maintained (as demonstrated in Figure 2, C), and undiminished ATP binding is confirmed by interaction energy values for Glu640 and ATP that are similar for Thr670Ile and wild type (Table 2).

Certain bond lengths also influence the affinity of KIT for ATP binding. In MD models of the kinase domain of Thr670Ile KIT, the hydrogen bond between the adenine N1 atom of ATP and the backbone -NH moiety of Cys673 has an average dynamic length (ADL) of 3.73 Å. The hydrogen bond between the exocyclic N6 atom of ATP and the backbone -CO moiety of Glu671 has an ADL of 3.17 Å (Figure 2, C). This difference is reflected by the higher energies, $\Delta E_{\text{cov}}$ and $\Delta E_{\text{int}}$ in Table 2, for the interaction of residues Cys673 and Glu671 with ATP in Thr670Ile compared with wild-type KIT. By contrast, in the Thr670Arg mutant KIT protein, the ATP ligand tilts within the protein cleft in order to accommodate the longer Arg side chain, the exocyclic N6 atom of the ATP still hydrogen bonds to the backbone -CO group of Glu671 (ADL = 3.35 Å), whereas the ribose 3′-OH hydrogen bonds with the backbone -CO group of Arg796 (ADL = 2.60 Å) (Figure 2, C). All of these interactions are supported by the predicted energy values listed in Table 2. In the case of the Thr670Arg mutant, the MD model still includes the hydrogen bond between the ATP ribose 3′-OH and the Arg796 -CO (ADL = 2.80 Å), whereas the hydrogen bond between the ATP ribose 2′-OH and the Asp677′ -COOH is no longer detected due to the counter clockwise rotation of the sugar ring and the side chain of Asp677 (Figure 2, C).

Therefore, the lower interaction energy terms for Asp677 in the Thr670Arg mutant reflect the lower binding affinity of the Thr670Arg mutant for ATP compared with Thr670 (Table 2).

For both the Thr670 wild-type KIT and the Thr670Ile mutant, the active conformation of the A-loop is further supported by a hydrogen bond between the -COOH group of the Tyr823 side chain and the side chain -NH moiety of Arg815 (ADL = 1.95 Å) (see also the favorable values for the interaction energy of both residues [Tyr823 and Arg815] with ATP in Table 2). Interestingly, Tyr823 is one of the amino acids at which tyrosine autophosphorylation takes place. Although Tyr823 was not phosphorylated in the crystal structure of active KIT used for this work (29), if this residue would become phosphorylated in vivo, the proximity of the positively charged side chain (the two H,N⁺ groups) of Arg791 to the Tyr823′ -OH group (ADL = 3.35 Å) that is detected in the Thr670Ile mutant should neutralize the eventual presence of a negatively charged phosphate group at Tyr823 and maintain the A-loop in the active conformation. When Arg replaces Thr at position 670 of KIT, a conformation similar to that described above for Tyr823, Arg815 and the A-loop was detected in the corresponding MD trajectory, but the length of the hydrogen bond between Tyr823 and Arg815 was longer than the one detected in the Thr670Ile mutant KIT (ADL = 2.43 Å), and hence, the strength of interaction of residues Tyr823 and Arg815 with ATP was somewhat lower in the Thr670Arg mutant than in the wild-type protein (Table 2).

When the effects of the remaining amino acids substituted at position 670 of KIT were modeled, the Ala, Ser, and Lys mutant proteins exhibited an intermediate behavior between the two extremes discussed above in terms of ATP-binding affinity (see $\Delta \Delta G_{\text{bind}}$ values in the last rows of top half of Table 1), in accordance with the evidence obtained from our immunoblotting experiments (Figure 1, B and C). The missense mutation Thr670Pro was associated with a calculated free energy of ATP binding ($\Delta G_{\text{bind}}$) of $-14.6$ kcal/mol, which is practically equal to that for the Thr670Arg mutant ($-14.9$ kcal/mol). Proline residues are known to induce strong structural perturbations by virtue of their peculiar cyclic structures; accordingly, the Thr670Pro KIT mutant was expected to have a lower affinity for ATP than the wild-type protein, and this finding was fully supported by the numerical value of its $\Delta G_{\text{bind}}$.

We next used molecular modeling to explore the energetics of imatinib binding among KIT mutants with amino acid substitutions at Thr670. Imatinib mesylate binds to the inactive conformation of KIT, in which the A-loop adopts an inactive, folded conformation that is quite different from the active, extended conformation and interferes with the access of ATP to the kinase catalytic site (Figure 3, A). The inactive structure may also normally be regulated by the juxtamembrane motif that is amino-terminal to the kinase domain, which has been reported to insert into the kinase active site and thereby disrupt ATP binding and the formation of the activated structure (29,30), thus preventing the transfer of phosphate groups to substrate molecules, thereby inhibiting downstream signaling cascades (9). Binding of imatinib at the ATP-binding site locks the enzyme into the inactive position. Because the Thr670Ile mutation in KIT is prevalent among imatinib-resistant patients, we wanted to know why this mutant had lower affinity for imatinib than wild-type KIT or other mutants.

The estimated affinities for imatinib of wild-type KIT and of all position 670 mutants considered are shown in lower half of Table 1. Whereas the mutants Thr670Ala, Thr670Ser, and Thr670Lys still maintain some affinity for imatinib, mutants Thr670Pro and Thr670Arg are no longer able to bind the drug efficiently because their $\Delta G_{\text{bind}}$ values are $3$–$4$ kcal/mol lower than that of the wild-type receptor (Table 1). It is important to remember that a 1.4-kcal/mol difference in free energy of binding results in an order of magnitude decrease in the corresponding value of the binding constant. In other words, these mutants are not able to bind imatinib because their binding affinities are approximately two to three orders of magnitude lower than those of the wild-type protein.

The Thr670Ile mutation was associated with a $\Delta \Delta G_{\text{bind}}$ for imatinib of $-3.8$ kcal/mol (Table 1). This mutant’s markedly decreased affinity for imatinib compared with wild-type KIT can be ascribed to the molecular dimensions of imatinib, which is too big to fit within the pocket formed at the N- and C-lobe interface of the inactive structure of the kinase (17) (Figure 3, B). As reported for the analogous mutation—Thr315Ile—in the ABL kinase domain (32),
substitution of Ile at position 670 of KIT induces several sometimes substantial modifications in the conformation of other amino acid residues, both at the imatinib-binding site and in surrounding areas, in the presence of the inhibitor. The hydrogen bond that stabilizes the aminopyridine nitrogen of imatinib and the side chain of Thr670 in the wild-type KIT–imatinib complex no longer exists in the mutant Thr670Ile complex, nor is it replaced by any similar, favorable interaction (Figure 3, C). Moreover, the greater molecular volume of the isoleucine side chain compared with that of threonine induces imatinib to assume a slightly modified position within the binding pocket to alleviate sterically unfavorable interferences from the inhibitor’s phenyl and piperazinyl rings with respect to the amino acid residues lining the imatinib-binding pocket (Figure 3, C). From an electrostatic viewpoint, the Thr670Ile
mutant protein is surrounded by a strongly polar environment generated by three nitrogen atoms of imatinib, a situation that clearly disfavors imatinib binding (Table 2). In the case of the Thr670Arg mutation, which is estimated to have a $\sim 3.7$ kcal/mol lower binding affinity ($\Delta G_{\text{unw}}$) with respect to the wild-type kinase, all of the unfavorable conditions explained above for isoleucine are present in addition to greater steric hindrance due to the larger dimensions of the Arg side chain (Figure 3, C) and to unfavorable solvation and entropic terms (Table 1).

Discussion

Various studies have reported the occurrence of secondary mutations among imatinib-resistant patients whose cancers are associated with the expression of oncogenic forms of the tyrosine kinases KIT, PDGFRA, and BCR-ABL (10–16,19–23). The only amino acid substitution responsible for imatinib resistance that is shared by these three oncogenic kinases is the substitution of Thr to Ile in the kinase active site. By contrast to other KIT residues for which many different amino acid substitutions have been reported in GISTs (eg, Val559, Asp816, Asp820, and Asn822) (37,38), to our knowledge, only the Thr670Ile substitution has been described at codon 670. It is also interesting that Thr790, the threonine at a homologous position in the kinase domain of the epidermal growth factor receptor is frequently found to be substituted by a homologous position in the kinase domain of the epidermal growth factor receptor. Accordingly, these mutated KITs can introduce strong structural perturbations to either the active or the inactive form of the receptor. Consequently, these mutated KITs can undergo autophosphorylation, but, at the same time, imatinib can bind and inhibit autophosphorylation, albeit slightly less efficiently than for wild-type KIT. Second, substitutions with proline and arginine result in a loss of function because a highly distorted conformation at the ATP and imatinib binding pocket induced by the isoleucine substitution were clearly evident and resulted in impaired binding of this drug to the target receptor.

Our study had some limitations. For instance, there is a possibility, although extremely small, that two point mutations might occur at the same codon in patients treated with imatinib. This phenomenon has been observed in one imatinib-resistant GIST patient in whom the Thr670Glu substitution (corresponding to the DNA mutation ACA vs GAA) was detected (40). Such an occurrence would widen the spectrum of amino acids that could substitute for the wild-type Thr residue at position 670 of KIT. Although the application of this strategy of combined experiments and simulations to the analysis of such a case would still be feasible, it would necessarily require the involvement of considerable human and computational resources. Furthermore, even though only single point mutations that lead to a single amino acid substitutions have been reported to date in the literature as second-site mutations that confer imatinib resistance, we cannot exclude the possibility that deletions involving the Thr670 residue could also be detected in the future. In this event, however, the application of our methodology to the prediction of the ATP and imatinib affinities of the corresponding mutant would be rather straightforward.

Our results suggest that different amino acid substitutions at position 670 of KIT are likely to have different effects with regard to both ATP and imatinib binding. In particular, the main conclusions of our combined in vitro and in silico efforts can be summarized as follows. First, we find that substitutions with alanine, serine, and lysine do not introduce strong structural perturbations to either the active or the inactive form of the receptor. Accordingly, these mutated KITs can undergo autophosphorylation, but, at the same time, imatinib can bind and inhibit autophosphorylation, albeit slightly less efficiently than for wild-type KIT. Second, substitutions with proline and arginine result in a loss of function because a highly distorted conformation at the ATP-binding site renders the receptor poorly effective in binding ATP and, thus, less prone to autophosphorylation. Third, the substitution with Ile results in gain of function because this is the only substitution able to leave the KIT receptor strongly autophosphorylated and to make it imatinib insensitive.

In summary, these data explain why, among GIST patients who develop imatinib resistance, the mutation that causes the Thr670Ile substitution is the only mutation that is naturally selected at codon 670. Indeed, isoleucine is the only amino acid substitution that can arise through point mutation at that position that is able to yield a selective advantage to tumors under conditions of imatinib treatment. We believe that this study demonstrates that computer-based molecular simulation is a reliable technique that is able to predict...
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