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**REVIEW ARTICLE**

# Molecular mechanisms of drug resistance in tyrosine kinases cAbl and cKit

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**Abstract**
The inhibition of protein kinases has gained general acceptance as an effective approach to treat a wide range of cancers. However, in many cases, prolonged administration of kinase inhibitors often leads to acquired resistance, and the therapeutic effect is subsequently diminished. The wealth of recent studies using biochemical, kinetic, and structural approaches have revealed the molecular basis for the clinically observed resistance. In this review, we highlight several of the most common molecular mechanisms that lead to acquired resistance to kinase inhibitors observed with the cAbl (cellular form of the Abelson leukemia virus tyrosine kinase) and the type III receptor tyrosine kinase cKit, including a newly identified mechanism resulting from accelerated kinase activation caused by mutations in the activation loop. Strategies to overcome the loss of drug sensitivity that represents a challenge currently facing the field and the emerging approaches to circumvent resistance are discussed.

**Keywords:** Kinase, drug resistance, imatinib, sunitinib, dasatinib, sorafenib, KIT, GIST, BCR-Abl



## Introduction

The clinical use of tyrosine kinase inhibitors for the treatment of a variety of cancers has markedly increased treatment options available to patients. Inhibition of specific kinase signaling pathways can result in decreased tumor size and time to disease progression (recent reviews of the clinical aspects of tyrosine kinase inhibition on the treatment of cancer can be found in Natoli et al., 2010; Agrawal et al., 2010; Liegl-Atzwanger et al., 2010). Unfortunately, with continued administration of kinase inhibitors, resistance often occurs. In particular, recent work with cKit and cAbl tyrosine kinase has increased our understanding of the molecular mechanisms that underlie resistance to the small-molecule inhibitors imatinib (Gleevec) and sunitinib (Sutent).

Currently, there are multiple options for patients with cancer with disease linked to abnormal activation of the cKit and cAbl signaling pathways (Table 1). In general, small-molecule kinase inhibitors are composed of three common classes based on their mechanism of inhibition. Well known are the conventional adenosine triphosphate (ATP)-competitive inhibitors that bind to the ATP-binding pocket of the kinase. These molecules, frequently referred to as type I inhibitors or classical kinase inhibitors (CKIs), are the earliest generation of kinase inhibitors and represent many approved kinase inhibitor drugs that are currently in clinical use (Figure 1A). Molecules in this class bind to kinases through several key interactions similar to adenosine binding to the pocket, including the critical hydrogen bonding with the hinge region of the kinase. Because type I kinase inhibitors need to compete with endogenous ATP in cells that is normally between 1 and 10 mM, 100- to 1000-fold reduction in potency was frequently observed between biochemical and cellular functional assays.

Significant medicinal chemistry effort has been invested to achieve a better selectivity for type I kinase inhibitors. Although various degrees of selectivity can be attained through precise refinement of the interactions between an inhibitor and the residues in the ATP-binding pocket, the selectivity of this class of molecules is low, in general, because of the highly conserved nature of the ATP-binding region. Consequently, type I kinase inhibitors tend to inhibit a wide variety of kinases in

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the kinome (for a review of approaches to the design of selective inhibitors see Zhang et al., 2009). Among the type I kinase inhibitors, a subgroup of inhibitors, e.g., sunitinib (Sutent, Pfizer Inc.), is shown to bind to the ATP site preferably at an unactivated state of certain kinases such as cKit (Gajiwala et al., 2009; Simard et al., 2009; Namboodiri et al., 2010). Sunitinib bound to the ATP site of cKit has been shown to effectively prevent the enzyme from autoactivation (Gajiwala et al., 2009). The therapeutic effectiveness of inhibiting an activated kinase versus preventing the targeted kinase from activation has been a subject of rigorous research and has led to the development of type II or non-CKIs (NCKIs).

In contrast to the type I inhibitors, type II kinase inhibitors specifically target an inactivated form of the kinase via binding to the hydrophobic back pocket of the ATP-binding site formed by the Asp-Phe-Gly (DFG) triad in the "out" conformation (DFG-Asp out). Birb796 (Pargellis et al., 2002; Regan et al., 2002, 2003), imatinib (Gleevec, Novartis, Figure 1B), and sorafenib (Nexavar, Bayer Healthcare) are three representative compounds of this class, among which imatinib and sorafenib are currently in clinical use (Simard et al., 2009; Namboodiri et al., 2010). Although the DFG-Asp out hydrophobic pocket has been observed in numerous kinases such as cKit, cAbl and cSrc, it seems to offer a higher degree of structural diversity than does the ATP-binding site, hence allows the design of inhibitors with higher specificity. Birb796 and imatinib have been shown to have significantly improved kinase selectivity compared with type I inhibitors (Cumming et al., 2004; Fabian et al., 2005; Kubo et al., 2005; Jung et al., 2006), whereas sorafenib remains potent across a wide variety of kinases (Simard et al., 2009; Namboodiri et al., 2010).

A third type of kinase inhibitors (type III) is characterized by binding to a pocket adjacent to the ATP-binding cleft formed by the activation loop (A-loop) in the inactivated conformation or in its transition to the active conformation. Examples of type III kinase inhibitors have been observed for mitogen-activated protein kinase (MEK) (Tecle et al., 2009) and p38α (Ahn et al., 2010; Liu et al., 2010). Comprehensive selectivity screenings across the kinase family for compounds targeting this region indicate that indeed they exhibit highly selective inhibition of the targeted kinase that further exceeds type II inhibitors. Such a high specificity is achieved because of the additional structural diversity of the inhibitor-binding pocket provided by distinct A-loop structures and activation mechanisms in different kinases (Mittag et al., 2010; Grant et al., 2010).

When kinase inhibitors are used clinically, almost without exception, patients develop resistance after continuous treatment. In this article, we review the most commonly observed resistant mutations emerged in patients treated with drugs targeting the cKit and BCR-Abl kinases and their resistance mechanisms. Table 2 illustrates mutants identified in patients that have been treated with imatinib and/or sunitinib. Many of these mutations are located at the drug-binding site where key interactions between the drug and the target kinase are impeded. In some cases, mutations at the site cause steric incompatibility for the drugs to bind. There are also mutations located at the juxtamembrane (JM) domain and A-loop of these enzymes. These mutations are distal to the drug-binding pocket, and their resistance mechanism is not immediately obvious. Because these mutations are located in the regions involved in the regulation of kinase activation, understanding how the kinase transitions from an inactivated to the active state and how the mutations affect this transitioning may elucidate the molecular basis of the drug resistance caused by these mutations.

## cKit structure

Figure 2A shows a crystal structure of cKit in an inactive conformation complexed with sunitinib and may be used to demonstrate the general kinase structure. The catalytic domain is arranged in a common bilobed architecture consisting of a small N-terminal lobe composed of predominantly β-strands, followed by a large C-terminal domain composed of α-helices. The two lobes of the kinase are connected by a short hinge region. In the apo structure of cKit bound to adenosine diphosphate (ADP), two hydrogen bonds are formed with the amide backbone of the hinge region—one with the adenine ring and Glu671 and another with the exocyclic $NH_2$ group of the adenine and Cys673. The α- and β-phosphates of the bound ATP make contact with a flexible glycine-rich loop (or P-loop) located

Table 1. Current Food and Drug Administration–approved inhibitors of cKit and BCR-Abl in clinical use.

| | | | Target Kinase | | |
|---|---|---|---|---|---|
| Inhibitor | Class | Manufacturer | cKit | BCR-Abl | Indication |
| Imatinib (Gleevec) | II | Novartis | X | X | Chronic mylogenous leukemia (CML), acute lymphoblastic leukemia |
| Sunitinib (Sutent) | I | Pfizer | X | | Gastrointestinal stromal tumors (GIST), renal cell carcinoma (RCC) |
| Lapatinib (Tykerb) | II | GSK | X | | HER2+ breast cancer |
| Sorafenib (Nexavar) | II | Bayer | X | | Hepatocellular carcinoma (HCC), RCC |
| Dasatinib (Sprycel) | I | Bristol-Myers Squibb | X | X | CML, acute lymphoblastic leukemia |
| Nilotinib (Tasigna) | II | Novartis | X | X | CML |
| Pazopanib (Votrient) | I | GSK | X | | RCC |

on the N-terminal lobe. The catalytic pocket is at the interface of the two lobes where the substrate binds in an extended conformation. cKit, being a member of the platelet-derived growth factor (PDGF) receptor tyrosine kinase family, contains an N-terminal JM domain that folds into the space between the N- and C-terminal lobes and keeps the kinase autoinhibited. The C-terminal lobe of cKit contains a kinase insert domain (KID) of 76 amino acids in length, including three phosphorylated tyrosines that serve as docking sites for downstream

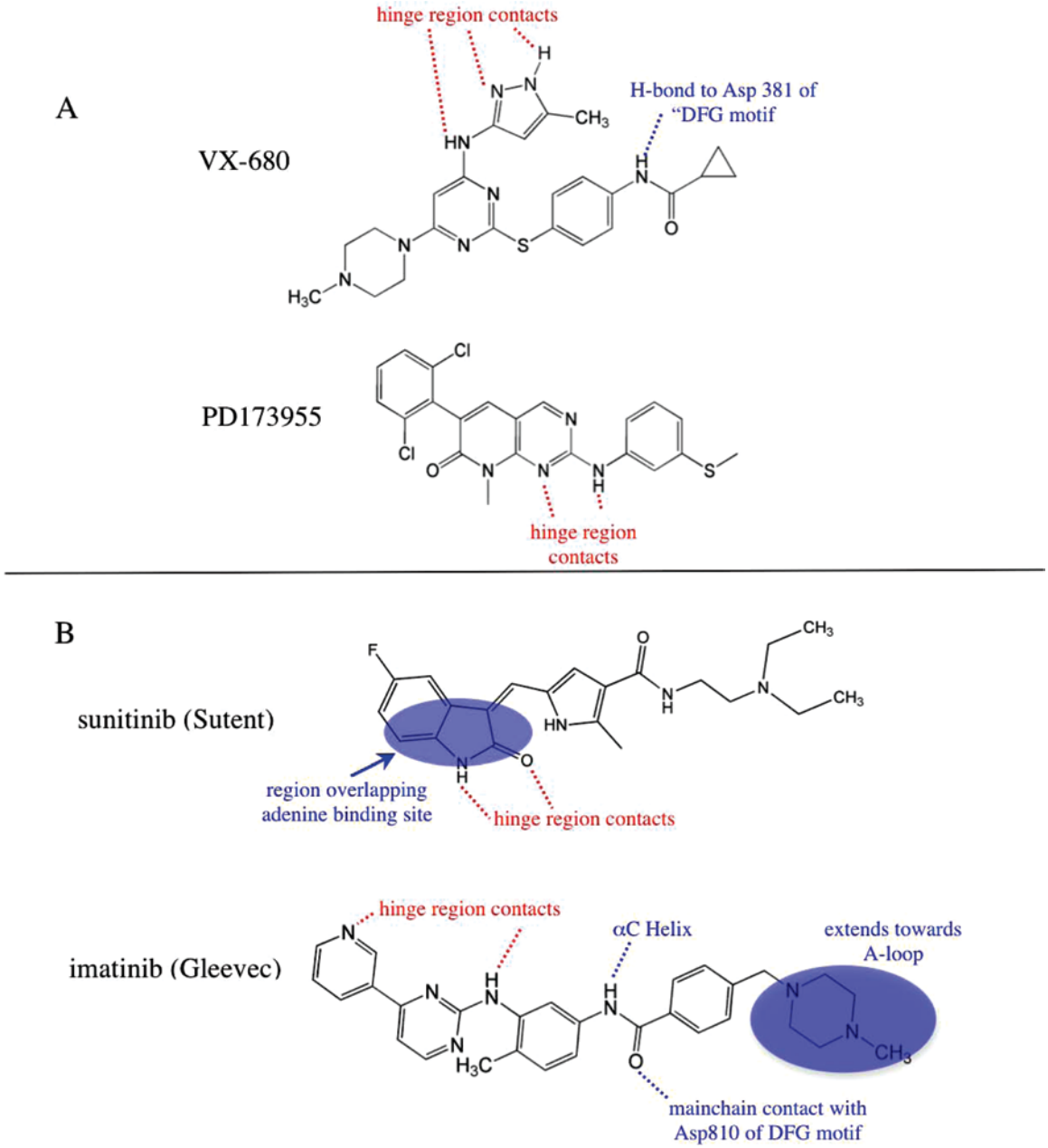


Figure 1. Binding interactions for type I and type II cKit and cAbl inhibitors. Contacts with specific regions of either cKit or cAbl are indicated based on crystal structure data. (a) Binding interaction observed between the type I inhibitors VX-680 (Young et al., 2006) and PD173955 with cAbl (Nagar et al., 2002). The VX-680 makes three hydrogen bonds to main chain atoms in the hinge region, two through carbonyl and one to the amide. An additional hydrogen bond is formed between the nitrogen of the amide group that connects the phenyl group to the cyclopropyl substituent and the side chain of the aspartate of the DFG motif (Asp381). This interaction is most likely stabilizing the active conformation of the DFG motif. In the structure of PD173955 bound to cAbl, the A-loop is in the open conformation resembles the active form; however, the DFG motif varies in the conformation compared with other kinases in the active form such as Lck. The majority of the interactions with cAbl are Van der Waals interactions with the ATP-binding site, with the exception of the two hydrogen bonds to the hinge region as indicated. (b) The cKit type I inhibitor sunitinib and the type II inhibitor imatinib. The binding of sunitinib to cKit is mediated by contacts with the hinge region and dihydroxyindole ring, which partially overlaps the binding region for the adenine ring observed in the apo cKit structure bound to ADP (Mol et al., 2003). In the cKit-imatinib complex, hinge region contacts are present and additional contacts with the αC helix and main chain nitrogen of the Asp 810 in the DFG motif. A similar hydrogen bonding pattern is observed in the cAbl–imatinib complex structure with the hinge and αC helix (Nagar et al., 2002; Mol et al., 2004).

Table 2. Aquired mutations in cKit and BCR-Abl that confer resistance to small-molecule inhibitors.

| Mutation | Resistance conferred | Location |
|---|---|---|
| cKit | | |
| V654A | Imatinib | ATP-binding pocket |
| T670I | Imatinib | Gatekeeper |
| D816V/H | Imatinib, sunitinib | A-loop |
| N822K | Imatinib, sunitinib | A-loop |
| Y823D | Imatinib | A-loop |
| A829P | Imatinib, sunitinib | A-loop |
| BCR-Abl | | |
| Y253H/F | Imatinib | Glycine-rich loop |
| E255K/V | Imatinib | Glycine-rich loop |
| T315I | Imatinib, dasatinib, nilotinib | Gatekeeper |
| M351T | Imatinib | C-term lobe |
| E355G | Imatinib | C-term lobe |
| F359C/V | Imatinib | C-term lobe |
| L387F/M | Imatinib | A-loop |
| M388L | Imatinib | A-loop |
| H396R | Imatinib | A-loop |
| A397P | Imatinib | A-loop |

signaling molecules Grb2 (Sun et al., 2007), phosphatidylinositol (PI)-3 kinase (Lev et al., 1992; Shearman et al., 1993; Serve et al., 1994), and phospholipase Cγ (Gommerman et al., 2000; Maddens et al., 2002).

## The inactive to active transitions in cKit tyrosine kinase

Abnormal activation of cAbl and cKit has been shown to associate with chronic mylogenous luekemia (CML) and gastrointestinal stromal tumors (GIST), respectively (Hantschel et al., 2003; Duensing et al., 2004; Conca et al., 2009). Rigorous studies have elucidated that several key regulatory elements in these kinases play key roles in controlling the transition of the enzymes from an inactivated to an active conformation state (Nagar et al., 2002; Hantschel et al., 2003; Mol et al., 2003; Nagar et al., 2003; Mol et al., 2004; Levinson et al., 2006; Gajiwala et al., 2009; DiNitto et al., 2010). cKit and other type III receptor tyrosine kinase family members are normally in an autoinhibited or inactivated state until extracellular ligands trigger dimerization that subsequently allows transactivation of the cytosolic kinase domain. Full activation of the cKit requires a series of phosphorylation at certain specific sites across the entire kinase domain (DiNitto et al., 2010). Figure 2A and 2B show the structural movement from an inactivated to an active conformation.

Activation of cKit in the absence of ligand (stem cell factor (SCF)) binding to the extracellular domain has been observed in some clinical variants that have mutations in the JM domain, e.g., V560D (Rubin et al., 2001). Dimerization and subsequent kinase autophosphorylation of the dimerized V560D cKit molecules can readily take place without SCF. As a result, cell growth and proliferation occur in an uncontrolled manner, which consequently leads to tumorigenesis and the formation of GIST (Nakahara et al., 1998).

But how is cKit kinase activated via phosphorylation? For a cKit molecule to be capable of catalyzing the kinase reaction, the enzyme needs to assume an active conformation as shown in Figure 2B, where the active site blocker JM domain stays away from the active site entry, and the (DFG) triad is at an DFG-Asp in position (DFG-Asp in) so that the Asp can participate in catalysis by binding $Mg^{2+}$, which in turn binds to ATP. In the DFG-Asp out conformation, this interaction cannot occur. In addition, the control helix (αC helix) along with the N-lobe has to be in close proximity to the C-lobe, thereby enabling the active site to assume the catalytic conformation. Proximal to the ATP-binding region is the A-loop, a region of 30 amino acids in length (29 amino acids in cAbl) that begins with the conserved DFG triad and ends with Ala-Pro-Glu. On activation, the A-loop transitions from an inactivated or closed position to an activated or open state. In doing so, it allows substrates to access the catalytic site and the DFG triad to rotate about 120° to situate the carboxyl side chain of the Asp to a catalytically competent position.

For the A-loop to be in the open configuration, the JM domain needs to move out to an extended position. Recent solution-phase hydrogen–deuterium exchange studies (Zhang et al., 2010a) indicate that cKit is constantly in equilibrium among many conformers, including a catalytically active conformation and a large number of inactive states (Figure 3). Before the activation, the majority of the cKit molecules remain in inactive conformations where the autoinhibitory JM domain is inserted into the cleft between the N- and C-lobe of the kinase and interacts with the αC helix. On initiation of autophosphorylation, residues in the KID domain and JM domain tyrosines Y547, Y553, Y568, and Y570 are phosphorylated (DiNitto et al., 2010). Incorporation of phosphates to these tyrosine residues induces the JM domain to move from its autoinhibitory position and shifts the equilibrium to the right in Figure 3. Consequently, the population of cKit molecules in the active conformation is enriched and thus the overall kinase activity is increased.

In a cell-free system, phosphorylation of JM domain is a slow process relative to phosphorylation of the KID domain. This process is dependent on cKit protein concentration (DiNitto et al., 2010); hence, the reaction must undergo an intermolecular mechanism rather than an intramolecular autophosphorylation. The latter is expected to be kinase concentration independent. From a structural point of view, it is difficult to explain how the JM domain in an autoinhibitory position is phosphorylated by a second cKit molecule. It is likely that among various conformational states in the equilibrium, only a small population of molecules exist with the JM domain in the extended position. The JM domain of these molecules would be more exposed to a second cKit molecule that assumes the catalytically active conformation, thus making intermolecular

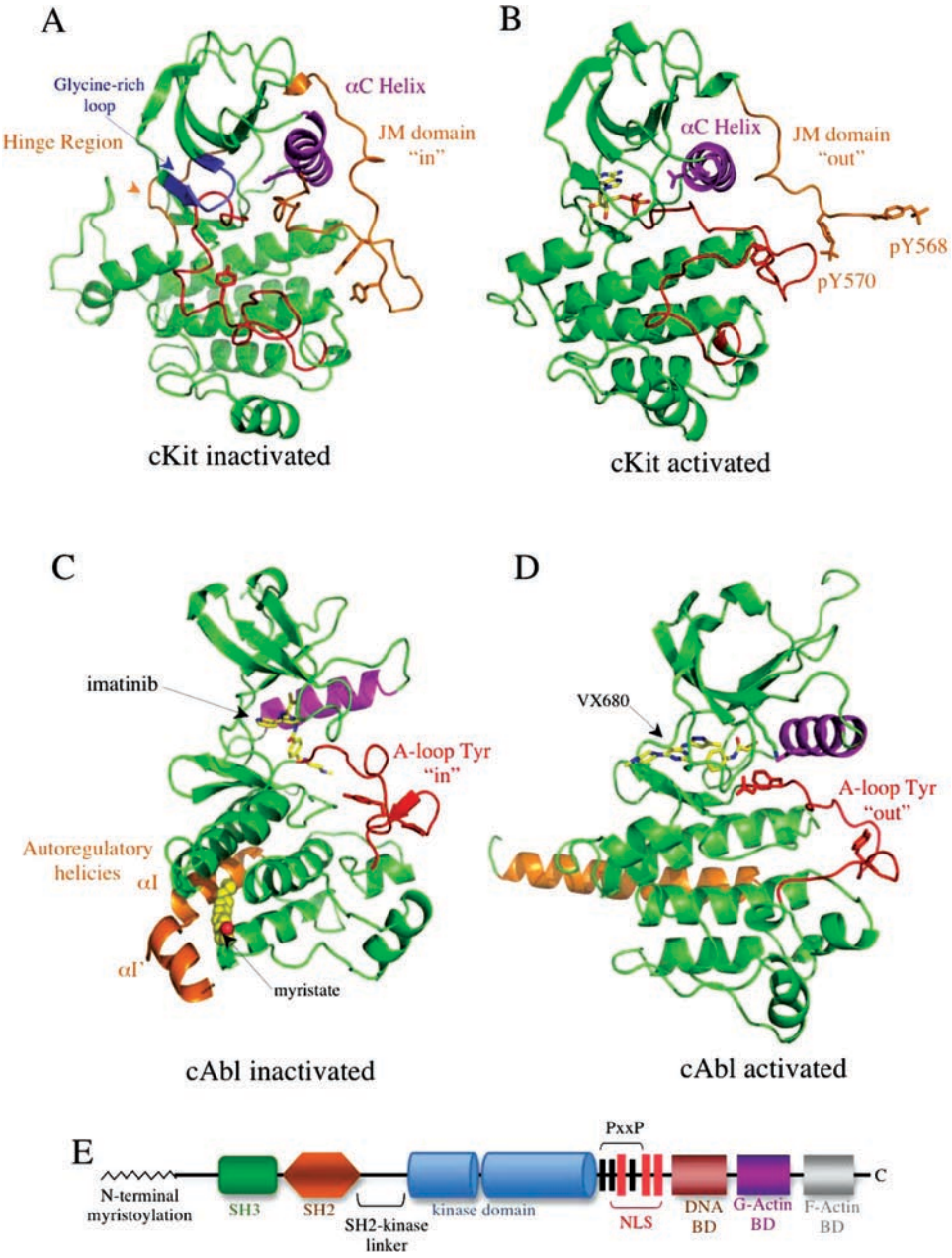


Figure 2. Structures of activated and inactivated state of cAbl and cKit tyrosine kinases. (a) cKit in the activated state (PDB code 1PKG, Mol et al., 2003) showing autoinhibitory JM domain (orange), A-loop in red, αC helix in magenta. (c) cAbl kinase domain (PDB code 1OPJ, Nagar et al., 2003) in the inactivated state bound to imatinib. Structural elements that change conformation on myristoyl binding are indicated in orange (αI and αI′). Yellow spheres represent myristoyl group. A-loop and αC helix are colored as in panel A. (d) Active conformation of cAbl imatinib-resistant mutant H396P bound to VX-680 (Young et al., 2006). (e) Domain organization of cAbl kinase. The catalytic core kinase domain is flanked by SH3 and SH2 domains on the N-terminus. The C-terminus contains DNA-, G-actin–, and F-actin–binding domains (BD). Several nuclear localization signals (NLS) and PxxP motifs are present between the DNA BD and the kinase domain. Autoinhibitory interactions occur between the SH3 domain and the proline-rich region in the SH2-kinase domain linker, and the N-terminal myristoylation and the C-terminal lobe of the kinase domain (Pluk et al., 2002; Nagar et al., 2003). Structure figures were generated using PyMol (Schrödinger, LLC).

phosphorylation possible. Once the JM domain is phosphorylated and displaced from the autoinhibitory position, the A-loop can move out, and DFG motif can position to the DFG-Asp in conformation. Consequently, the kinase assumes the active conformation. In the inactivated 'DFG-Asp out' orientation, the DFG Phe is oriented near the ATP-binding pocket, whereas in the activated DFG-Asp in conformation, the Phe is pointed away from the ATP-binding pocket and the A-loop is in an extended conformation. Among various kinases, A-loop is controlled by phosphorylation at single or multiple Ser/Thr and/or Tyr residues on the loop. Phosphorylation at Ser, Thr, or Tyr is one of the common mechanisms that kinases use to switch

on or switch off their activities. But how does phosphorylation of the A-loop switch on or switch off the kinase activity? Because the Asp of the DFG triad has to assume the "in" position for a kinase to be catalytically active, a mechanism that allows the A-loop to lock into a conformation that maintains the DFG motif in the active position has to exist for each kinase. Phosphorylation of the A-loop, which subsequently interacts with Arg and/or Lys at appropriate locations in the molecule to stabilize the active conformation, is one such mechanism. This mechanism is observed in many kinases and has been demonstrated in protein kinase A (PKA) (Adams et al., 1995; Grant and Adams, 1996), insulin receptor kinase (IRK) (Schmid et al., 1999; Pautsch et al., 2001), p38α (Raingeaud et al., 1995; Enslen et al., 1998), and p38γ. When cKit is activated in a purified enzyme assay system containing 4 mM ATP, 17–22 amino acids are phosphorylated. Although many phosphorylation sites have been shown to be critical for kinase activity or downstream functions, not all of the phosphorylated sites, including the A-loop Y823, have apparent roles (Mol et al., 2003; Zou et al., 2008; DiNitto et al., 2010). It has been shown that Y823 is phosphorylated late in the cKit autoactivation reaction where full activity can be obtained before this tyrosine is phosphorylated (DiNitto et al., 2010). Furthermore, experiments using a Tyr823 →Phe mutant show that this substitution does not significantly impede the enzyme activity (<2×). This observation confirms that phosphorylation of this tyrosine is not required for cKit kinase activation.

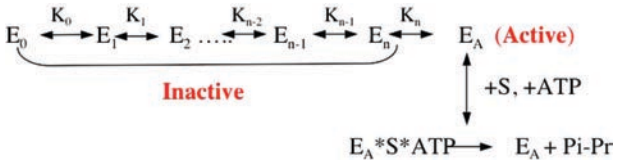


Figure 3. Kinase conformational equilibrium. Kinases equilibrate among a large number of conformational states where $E_0$, $E_1$...and $E_n$ represent various enzymatically inactive conformers of the kinase, and $E_A$ is the only active conformation that is capable of catalysis. In a sample of kinase that is not phosphorylated, the majority of the enzyme population remains at the most stabilized $E_0$ state, some at $E_1$–$E_n$ states, and a few molecules assume the $E_A$ conformation. Thus, the overall kinase activity of this sample is low, but not equal to 0. When activation occurs, the equilibrium shifts to the $E_A$ direction. In the example of cKit, this is driven by the shift in the JM domain to the "out" position followed by the change in the A-loop to the active DFG-Asp in conformation. The effect of these transitions is to stabilize the $E_A$ conformation, thus providing sufficient free energy to drive the majority of phosphorylated kinase molecules to the $E_A$ conformation. An inhibitor could target any one of the inactive states where the assembly of an optimized catalytically competent kinase form is not yet established. With a sufficient binding affinity and time to equilibrate, the inhibitor will slowly drive the kinase from all conformations to $E_{n-1} \times I$ or $E_n \times I$. However, drug-resistant forms of the kinase can shift the population of E states to those that are closer to the $E_A$ form, thus eliminating the target conformation that the inhibitor can bind to.

## cAbl structure

cAbl is a member of the Src family of nonreceptor tyrosine kinases and is involved in regulation of cell growth, survival, and morphogenesis. The kinase domain of cAbl has a similar overall architecture to cKit (compare Figure 2A and 2C). In addition to the kinase domain, cAbl contains several domains that serve regulatory functions (Figure 2E). The N-terminus of cAbl contains a consensus motif for myristoylation followed by an SH3 domain and an SH2 domain. A short linker sequence connecting the SH2 domain with the kinase domain contains a proline-rich motif. Three important functional domains after the kinase domain are DNA-, G-actin–, and F-actin–binding domains. In the inactive state, cAbl forms a closed conformation where the SH3 domain binds to the proline-rich motif on the linker. The SH2 domain and the N-terminal myristoyl group dock into a hydrophobic pocket in the C-lobe of the kinase domain through interaction with helices αI and αI′ (Pluk et al., 2002; Nagar et al., 2003). Thus, the kinase is autoinhibited in an intramolecular manner by the SH2, SH3, and the myristoylated N-terminus (Figure 2C).

## Conformational transition to the active form of cAbl tyrosine kinase

On stimulation with extracellular signals, cAbl is activated. Several conformational transitions accompany the activation of cAbl with extracellular signals. On a molecular level, the activation of cAbl leads to a shift in the αI and αI′ helical regions that form the binding site for the N-terminal SH2 domain from a kinked conformation to a single long helical region of 25 amino acids in length (Figure 2D). The SH3 domain also dissociates from the linker proline-rich motif. This results in an open conformation of cAbl and allows the SH3 domain to interact with PxxP motifs in other regulatory proteins and the SH2 domain to interact with phosphotyrosine residues. Activated cAbl has been shown to have multiple phosphorylation sites in the N-terminal half of the protein and in the kinase domain that stabilize the activated state. The roles of these phosphorylation sites in cAbl kinase activity and signal transduction are the subject of active investigation in several laboratories (Tanis et al., 2003; Goss et al., 2006).

In the active conformation of cAbl, phosphorylation of the A-loop tyrosine (Y416) stabilizes the DFG-Asp in conformation of the kinase (Schindler et al., 2000). Similar to that in cKit, when the A-loop Y416 of cAbl is phosphorylated, the activated cAbl becomes insensitive to imatinib with a >100-fold reduction in binding affinity compared with the inactive form of the enzyme (Seeliger et al., 2007). This is due to the selectivity of imatinib, which only binds to inactive cAbl in the DFG-Asp out conformation. Figure 2D shows the active conformation of cAbl kinase domain observed from a crystal structure

of the H396P imatinib-resistant mutant complexed with VX-680 (Vertex Pharmaceuticals) (Young et al., 2006). The active conformation displays the DFG motif in the Asp-in position as expected, despite the lack of A-loop phosphorylation that is required for activating wild-type cAbl. It seems that VX-680 can substantially stabilize H396P cAbl in the active conformation without A-loop phosphorylation. This is because VX-680 can form a hydrogen bond with Asp381 of the DFG motif when it is in the DFG-Asp in configuration, thereby stabilizing the active conformation. Indeed, superposition of the A-loop α-carbon atoms of cAbl in the VX-680 structure with that of a related tyrosine kinase Lck containing a phosphorylated A-loop is nearly superimposable with an root mean square deviation (RMSD) of 0.5 Å. In addition, a catalytically essential glutamate (E286) on the αC helix located on the N-terminal lobe forms a salt bridge with Lys271 in the N-terminal lobe. This ionic interaction effectively locks the kinase in the active state. A similar type of interaction is observed in the cKit kinase/product complex structure, in which the αC helix glutamate residue forms ionic interactions with a lysine side chain and positions this amino acid for bridging the α and β phosphates of ATP (Mol et al., 2003). In inactivated cAbl, the αC helix is rotated away, and E286 forms ionic interaction with R362 in the catalytic loop instead of Lys271 in the N-lobe. As such, it allows the DFG motif to adopt the "out" conformation. Like most kinases, cAbl has a high degree of conformational plasticity. Several intermediate conformers of inactivated cAbl have been obtained in various crystals, whereby the majority of the αC helix is surface exposed and an ion pair is formed between E286 and R386. Such intermediate states of conformation are structurally similar to those observed in the c-Src kinase domain (Hantschel et al., 2003; Levinson et al., 2006).

In cells, the kinase activity of cAbl is kept under strict control by several regulatory mechanisms as described above. Dysfunction of the regulatory mechanisms has been linked to a variety of disease states (Sirvent et al., 2008; reviewed in Colicelli, 2010). A common disease associated with dysfunctional regulation of cAbl has been CML where the aberrant fusion of the *Bcr* (breakpoint cluster region) gene with the cAbl kinase domain was observed. Indeed, more than 90% of patients with CML express the BCR-Abl fusion protein (Erikson et al., 1986). The fusion of BCR to the N-terminus of cAbl results in an active kinase with a loss of the critical autoinhibitory function of the N-terminus. In wild-type cAbl, the N-terminal myristoyl modification inhibits the kinase by binding to the C-lobe of kinase domain (Figure 2C). Imatinib has been approved for use in the treatment of patients with CML. In cell assays, imatinib effectively inhibits both cAbl and the BCR-Abl fusion protein with a similar $IC_{50}$ value of approximately 300–600 nM (La Rosee et al., 2002; Azam et al., 2003). Treatment of patients with this type II NCKI results in remission of nearly 100% of cases that are in the early stages of CML (Druker et al., 1996; Druker and Lydon, 2000; Druker et al., 2001).

## Acquired resistance caused by steric incompatibility or loss of drug–target interaction

Despite the success of small-molecule kinase inhibitors in the clinical setting, acquired resistance is an ongoing problem. In general, most of the resistance mutations are located at the drug-binding site and cause a steric clash or a loss of critical interaction with the drug. An example of this can be seen in patients with GIST where the activation of cKit receptor tyrosine kinase is critical in the pathogenesis of the disease (Demetri et al., 2002). In the treatment of GIST patients with the first-line inhibitor imatinib, 40–50% of patients develop resistance within 2 years of treatment (Verweij et al., 2004; Van Glabbeke et al., 2005; Heinrich et al., 2008). Subsequent treatment of these patients with sunitinib is often effective, although a subset of imatinib-resistant tumors remain refractory to both drugs. Sequence determination of cKit revealed mutations in the drug-binding pockets for the two drugs, e.g., V654A in the ATP pocket and T670I (the gatekeeper residue) in the back pocket. Both these variants are resistant to imatinib yet retain sensitivity to sunitinib. In addition, mutations in the A-loop (D816H/V) and JM domain are observed (Heinrich et al., 2008; Table 2), and the resistance mechanism of these variants is not immediately clear. The V654A substitution results in the loss of a key hydrophobic interaction with the diaminophenyl ring of imatinib (McLean et al., 2005). Several excellent reviews have discussed the V654A mutation and the well-characterized imatinib-resistant gatekeeper T670I mutation in detail (Gramza et al., 2009; Krishnamurty and Maly, 2010; Wang et al., 2011).

In the treatment of CML, mutations in BCR-Abl arise from continued use of imatinib. These mutants have been extensively studied, and a large number of these mutations can be mapped to the kinase catalytic domain and, specifically, the kinase glycine-rich loop (Soverini et al., 2006). This loop engages the α- and β-phosphates of ATP and is a highly ordered region of the cAbl kinase domain that has restricted conformation. In the imatinib-bound structure, the specific conformation of the glycine-rich loop blocks the pyridine and pyrimidine moieties of imatinib from solvent (Figure 4). Residues frequently mutated in the glycine-rich loop are Y253 and E255. The variants Y253F or Y253H result in a loss of side chain interaction with the pyrimidine ring of imatinib that is oriented perpendicular to the edge of the aromatic ring. Mutation of E255 to either Val or Lys, although not making direct contact with the drug, disrupts a hydrogen bond network that stabilized the conformation of the glycine-rich loop.

The most common observed resistance mutation in tyrosine kinases cKit and cAbl involves replacement of the gatekeeper threonine residue with a larger hydrophobic amino acid. These mutations cause a steric clash for the drug binding to the site. The cKit T670I gatekeeper variant leads to imatinib resistance in patients with GIST; similarly, the BCR-Abl T315I mutant observed in patients

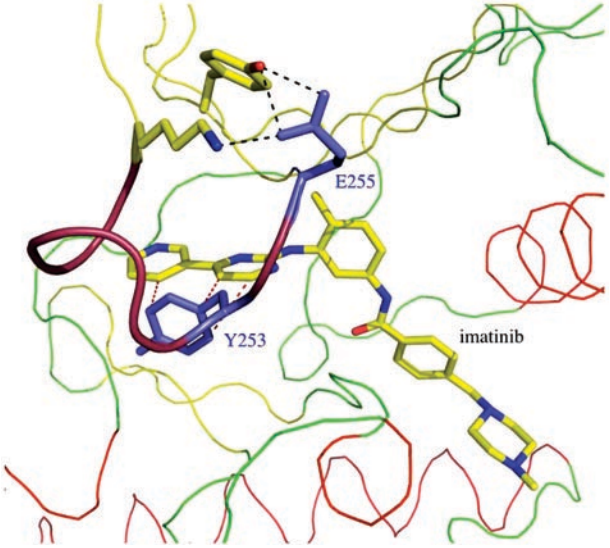


Figure 4. Role of glycine-rich loop residues in imatinib binding and stabilization of the loop conformation. The structure of the imatinib–cAbl complex (pbd code 1IEP, Nagar et al., 2002). Stabilization of the glycine-rich loop conformation by H-bonding interactions with E255 is indicated (black dashed lines). The face-to-edge aromatic interaction between Y253 and the purine and pyrimidine rings of the drug are indicated with dashed lines (red).

with CML confers resistant to imatinib (Tamborini et al., 2001; Pao et al., 2005; Kobayashi et al., 2005; Tamborini et al., 2006; Azam et al., 2008). Substitution of the gatekeeper threonine with isoleucine in BCR-Abl and cKit results in a loss of hydrogen bond interaction with the threonine side chain hydroxyl and the nitrogen that connects the pyrimidine ring with the m-diaminophenyl ring in imatinib. This loss of critical interaction, as a result of the steric clash caused by the bulky side chain of isoleucine, leads to imatinib resistance.

## Resistance due to alteration of conformational dynamics

The two resistance mechanisms described above do not account for all the resistance mutations observed. Another group of resistant variants have mutations located distal to the drug-binding site. The molecular mechanism of resistance for this group of mutants involves a shift in the dynamic equilibrium of the kinase to one that is not preferred by the drug. For example, in cKit, the D816 position in the A-loop, which is not in the binding site for sunitinib and imatinib, is a hotspot for missense mutation to histidine and valine that leads to resistance to both drugs. Structural and biochemical data indicate that the mutation D816H results in a 184-fold increase in autoactivation rate (Gajiwala et al., 2009). Thus, when cKit is activated, the mutated enzyme changes from a sunitinib-preferred inactive conformation to a sunitinib-insensitive active conformation with a much faster rate than wild-type cKit. Consequently, the inhibitory activity of sunitinib is significantly reduced, and the $IC_{50}$ of sunitinib in a cKit activation assay increased from 40 nM for wild type to >10 μM for the D816H mutant. But why would a mutation at D816 in the A-loop result in such a marked acceleration in the activation? Crystallographic data indicate that the conformational equilibrium of D816H mutant is significantly shifted, and the autoinhibitory JM domain is in the "out" conformation, similar to the apo structure of cKit (Figure 5). The D816H mutation destabilizes the JM domain at its autoinhibitory position and allows the domain to be more solvent exposed and accessible to efficient phosphorylation. This consequently promotes the kinase to adopt the active conformation.

Similarly, other mutations that could destabilize the JM domain from its autoinhibitory position such as the primary mutation V560D in patients with GIST would also accelerate phosphorylation. Hydrogen–deuterium exchange studies indicate that the JM domain of the V560D mutant prefers the extended position and thus shifts the conformational equilibrium of JM domain to the open position as indicated by a higher rate of hydrogen–deuterium exchange. Studies with the V560D mutant also indicate that the kinase samples two predominant conformations, a flexible conformer that resembles the D816H mutant, and the other is less flexible, and is similar to the wild-type cKit in the autoinhibited state. This result is consistent with the observation that V560D mutant remains sensitive to sunitinib, although it is less sensitive than wild-type cKit. This V560D mutant shows a markedly accelerated rate of JM domain phosphorylation and nearly instant activation of the kinase on exposure to ATP. Because of its critical role in maintaining cKit in the inactivated state, the JM domain is often found to be harboring gain-of-function activating mutations (Rubin et al., 2001; Heinrich et al., 2003). Furthermore, kinetic analysis also indicates that in solution, the conformation of the JM domain in both the D816H and V560D cKit mutants is shifted from its autoinhibitory position to one that is more exposed to solvent and disordered, such that the on-rate for imatinib binding to these mutants is significantly increased compared with wild-type cKit. The binding of imatinib to cKit requires the JM to be displaced from its autoinhibitory conformation (Gajiwala et al., 2009). Taken together, these results indicate that the conformational sampling of resistant mutant D816H and the gain-of-function V560D mutant (Zhang et al., 2010a) is altered compared with wild type, which leads to an increased population of molecules with JM domain in the extended position and allows efficient phosphorylation of this domain that ultimately results in accelerated autoactivation and drug insensitivity to imatinib and sunitinib. As such, the conformation of the JM domain is a critical determinant for imatinib and sunitinib sensitivity. Mutations on the A-loop such as D816H that cause the JM domain to move out from its autoinhibited position would expedite the phosphorylation and lead to insensitivity to the drugs.

In addition to JM domain phosphorylation, A-loop phosphorylation is another important event that is

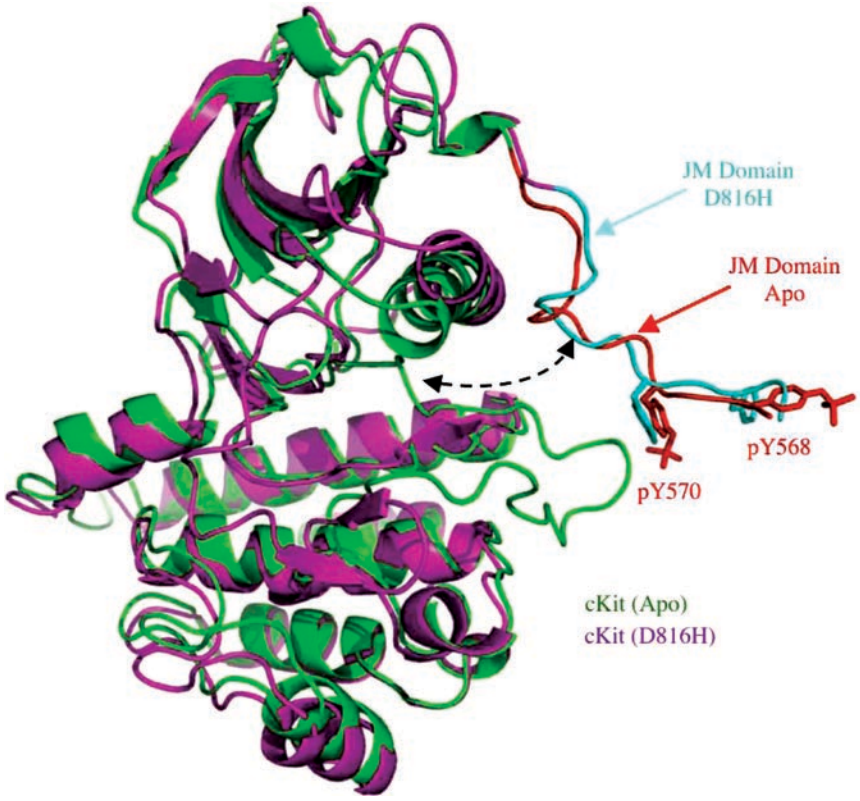


Figure 5. Conformation of JM domain mimics apo cKit JM domain conformation. (a) Superposition of wild-type ADP-bound apo form of cKit (PDB code 1PKG, Mol et al., 2003) and the D816H–sunitinibcomplex (PDB code 3GOF, Gajiwala et al., 2009). Superposition of the kinase domain excluding the JM domain results in an RMSD of 1.89 Å. The JM domain is displaced from the autoinhibitory position in both structures (dashed arrow).

often required for activation of catalytic activity of tyrosine kinases (Murray et al., 2001; Furdui et al., 2006; Wu et al., 2008). Autoactivation of cKit analyzed by liquid chromatography/mass spectrometry indicates that initial phosphorylation occurs on residues in the KID domain such as Y703, Y721, S729, and Y747 and in the JM domain such as Y547, Y553, Y568, and Y570. At a much later stage in the autoactivation process, the phosphorylation of the single A-loop tyrosine (pY823) is observed after full catalytic activity is obtained. The crystal structure of the apo form of cKit with bound ADP shows that Y823 is unphosphorylated. However, the electropositive environment surrounding Y823 suggests that phosphorylation of Y823 might further stabilize or lock the enzyme in the active conformation (Mol et al., 2003). Although not required for enzyme activity, without a phosphorylated A-loop at position Y823, cKit is much less stable in conformation than the Y823-phosphorylated cKit and can shift back to adopt inactive conformation. As such, cKit with unphosphorylated Y823 remains sensitive to imatinib and sunitinib. Because both drugs only bind to cKit in the inactive conformation, on the Y823 phosphorylation, the activated cKit is no longer inhibited. When Y823 is mutated to Phe, phosphorylation at this position is not possible; however, the mutant Y823F cKit exhibits only minimal loss of kinase activity and is able to retain sensitivity to both sunitinib and imatinib. The $IC_{50}$ values for the activated form of Y823F are decreased 100-fold for sunitinib and 39-fold for imatinib compared with activated wild-type cKit (DiNitto et al., 2010). The ability of pY823 to stabilize the active form is also supported by a clinical observation that the variant of cKit with the Y823D substitution is associated with testicular seminomas and that, when expressed in Chinese hamster ovary (CHO), the mutated cKit is subject to ligand-independent activation (Kemmer et al., 2004).

In light of the aforementioned observation on the effects of resistant mutations on the conformational plasticity of kinases, enzyme kinetic assays that allow for quantitative monitoring of how an inhibitor prevents the kinase from activation are essential for understanding the molecular basis of drug resistance. Setting up such assays seems to be straightforward as demonstrated in Figure 6, whereby the ability of the inhibitor to act on inactivated versus activated kinase can be easily evaluated by kinetic analysis (Gajiwala et al., 2009; DiNitto et al., 2010). In principle, the ability of an inhibitor to act on the unactivated kinase can be measured quantitatively with this method for kinases that are autoactivated or activated in trans by other enzymes or cofactors, such as PI-3 kinase, which has been shown to be activated by peptides corresponding to insulin receptor substrate-1 phosphopeptide (Yu et al., 1998).

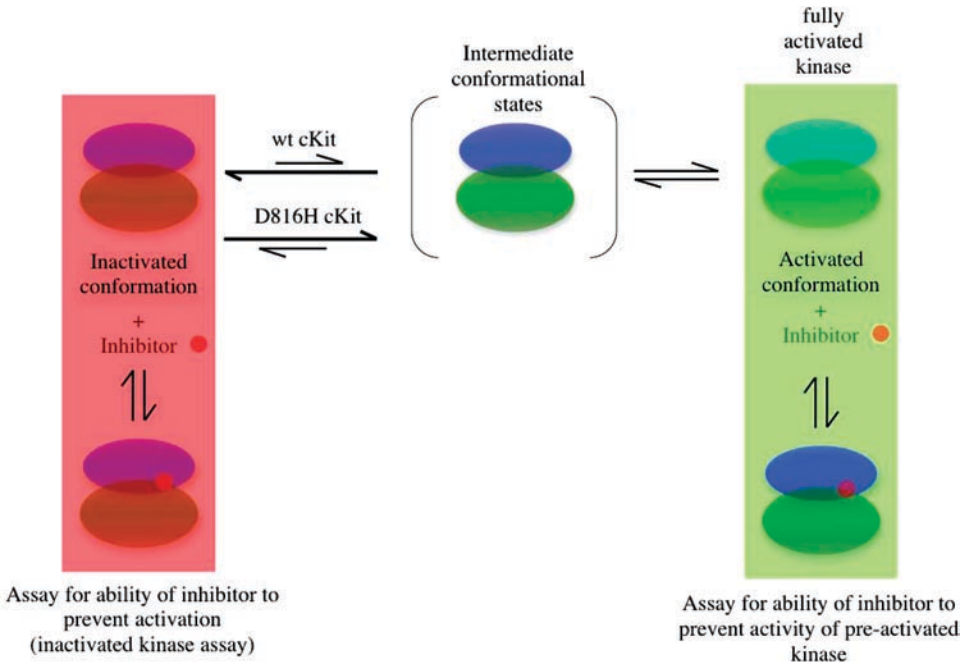


Figure 6 .Biochemical assay for kinase inhibitor conformational selectivity. Assays for selectivity of small-molecule inhibitors can be performed with the inactivated kinase by first incubating the kinase with inhibitor before addition of ATP to initiate autoactivation. Mutations that confer resistance to the drug shift the ensemble of conformational states to those that are incompatible with binding inhibitor, such as the sunitinib D816H variant of cKit in patients with GIST. The ability of the molecule to inhibit preactivated kinase is assessed by first activating the kinase by preincubation with ATP followed by addition of the small molecule of interest.



## Strategies to overcome resistance

Since the identification of drug-resistant mutants in patients, significant effort has been made to overcome resistance to imatinib and sunitinib. Recently, motesanib, which targets the vascular endothelial growth factor receptors PDGF and cKit, has been shown to be effective in inhibiting autophosphorylation of the several imatinib-resistant mutants. These include cKit with double mutations at the JM and glycine-rich loop region (V560D/V654A, $IC_{50}$ = 77 nM) and mutations in the JM and hinge region (V560D/T670I, $IC_{50}$ = 277 nM) as well. However, motesanib is unable to inhibit the A-loop mutant D816V (Caenepeel et al., 2010). Interestingly, the cKit D816V mutant that is not effectively inhibited by imatinib, sunitinib, and motesanib can be readily inhibited by dasatinib (BMS-354825) (Shah et al., 2006; Schittenhelm et al., 2006). Dasatinib is an ATP-competitive inhibitor that targets the BCR-Abl, Src, PDGF, and cKit kinases. Crystallographic and computer simulation data of dasatinib-bound BCR-Abl suggest that the inhibitor is capable of recognizing multiple conformational states of the BCR-Abl kinase, including the active conformation (Tokarski et al., 2006). Thus, the drug is also effective on mutants that cause accelerated autoactivation. Dasatinib inhibits cKit D816V effectively with 37 nM $IC_{50}$ *in vitro* and displayed high nanomolar efficacy in cell-based assays (Shah et al., 2006). Dasatinib has been proposed to have the ability of recognizing certain resistant mutants compared with imatinib and sunitinib, which have more stringent conformational requirements for binding as is observed for D816V variant of cKit.

Many kinase inhibitors in clinical use today interact through specific hydrogen bonds to the hinge region. Substitution to a large hydrophobic moiety in this region blocks these interactions. As such, considerable effort has been undertaken recently to develop molecules that can overcome the resistance caused by the gatekeeper mutation. The second-generation BCR-Abl inhibitors nilotinib (Weisberg et al., 2005), bosutinib (Puttini et al., 2006), and dasatinib (Shah et al., 2004) are capable of inhibiting most imatinib-resistant mutants, with the exception of the T315I gatekeeper mutation (O'Hare et al., 2005). Recently, there have been newer BCR-Abl inhibitors developed that display prominent potency against the T315I mutant. By appending onto a 3,4-dihydropyrimido[4,5-d]pyrimidin-2(1H)-one scaffold (Figure 7A), Choi et al. (2010) have reported an inhibitor that is effective against both wild type and T315I mutant BCR-Abl *in vitro* and show *in vivo* efficacy in xenograft mouse models. Structural data indicate that the scaffold region of the inhibitor binds cAbl in a DFG-Asp out conformation that is identical to that recognized by imatinib (Liu and Gray, 2006; Okram et al., 2006). In both structures, the same hydrogen bonding pattern with Asp in the DFG motif and the conserved Glu in the αC helix was observed. Such a scaffold should serve as a lead in the design of improved BCR-Abl inhibitors.

An alternative strategy for overcoming resistance due to the gatekeeper mutation involves the synthesis of hybrid

molecules containing elements of both type I and type II kinase inhibitors. By hybridizing the portion of nilotinib molecule that interacts with the DGF-Asp out pocket with the portion of dasatinib that interacts with the hinge region of the kinase, Weisberg et al. have reported compounds that are capable of inhibiting wild type, the gatekeeper T315I mutant, and other variants with a large hydrophobic residue at this position (Figure 7B). Selectivity profiles of these hybrid molecules against 400 kinases indicate that a high selectivity across the kinome can be readily achieved using this approach (Weisberg et al., 2010).

Using alternative binding pockets that are involved in autoregulatory interactions presents another promising approach for the development of new inhibitors able to overcome drug-resistant forms of BCR-Abl. One such binding pocket is the binding region for the N-terminal myristate that keeps cAbl in check by a tightly regulated autoinhibitory mechanism. Recently, this has been exploited as an approach for overcoming resistance to inhibitors of BCR-Abl. Cell-based screening has been used to identify small molecules that bind to the myristate-binding site (Deng et al., 2010; Fabbro et al., 2010; Zhang et al., 2010b). One of these non–ATP-competitive small molecules, GNF-2, is able to induce and/or stabilize the clamped inactive conformation of cAbl and prevent kinase activation. A derivative of GNF-2, GNF-5, when

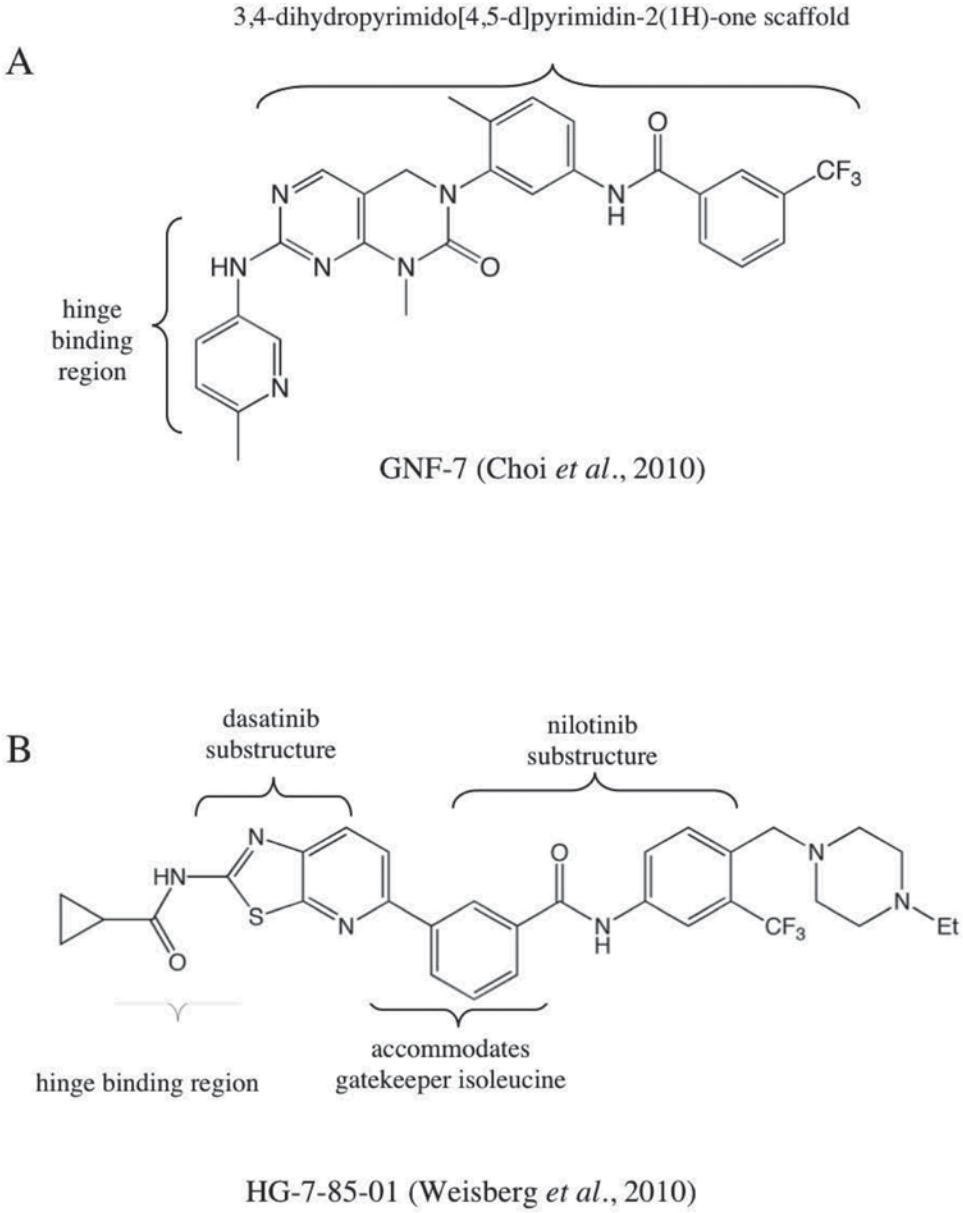


Figure 7 .Structures of type II inhibitors effective against BCR-Abl T315I gatekeeper resistance mutant. (a) Structure of GNF-7 showing the hinge-binding motif. Kinase profiling of GNF-7 *in vitro* in cellular kinase autophosphorylation assays indicate the drug has $IC_{50}$ values <5 nM against wild type BCR-Abl and 61 nM against T315I mutant (Choi et al., 2010). (b) Structure of hybrid type II inhibitor (HG-7-85-01) capable of inhibitory activity against wild type and T315I imatinib-resistant variant of BCR-Abl (Choi et al., 2010). On the basis of cellular proliferation assays, $IC_{50}$ values of 58.5 and 140 nM for wild type and T315I were obtained, respectively. The region of molecule that accommodates the gatekeeper T315I is indicated based on the co-crystal structure of HG-7-85-01 with the closely related kinase cSrc.

used in combination with imatinib, displayed additive inhibitory activity against the T351I gatekeeper mutant in biochemical and cellular assays. Although the exact inhibition mechanism of GNF-5 for the T670I BCR-Abl mutant is yet to be determined, hydrogen–deuterium exchange experiments indicate that the binding of GNF-5 alters the conformational dynamic of the peptide segment containing the T670I mutation. These changes may be related to the additive inhibitory activity imatinib and GNF-5 to inhibit the T670I mutant of BCR-Abl. Thus, it seems that combined use of agents that bind the allosteric myristate-binding site with compounds such as imatinib that crosses over the DFG-Asp out pocket and adenosine site can overcome resistance conferred by the gatekeeper mutation.

## Conclusions

The recent data on the molecular mechanism of kinase inhibitor resistance shed new light on the ways cells can circumvent inhibition. Unfortunately, in addition to the on-target mechanisms involving site-specific mutations discussed here, other mechanisms of drug resistance have also been observed. These mechanisms may involve increased expression of alternate signaling proteins that result in activation of signaling pathways. Different isoforms of the targeted kinase with redundant function may also compensate for the lost function of the inhibited kinase. These drug resistance mechanisms have been reviewed in detail by Rubin and Duensing (2006).

Since the development of the early-generation type I tyrosine kinase inhibitors, a large body of research has been performed with the focus of improving selectivity and potency. With the new type II inhibitors, this has been achieved to some extent. However, the emergence of resistance is an increasing problem facing the field. The rapid development of third-generation inhibitors that take advantage of unique binding pockets outside of the ATP-binding site presents a promising new avenue for the generation of unique chemical matter. A clear understanding of the molecular mechanisms that confer drug resistance will undoubtedly help facilitate the generation of new inhibitors that will lead to effective treatments to combat kinase inhibitor resistance and prolong the effectiveness of treatment in patients.

## Declarations of interest

The authors report no declarations of interest.

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