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Article

Structural Rigidification and Entropy Loss Underlie D816V-Driven c-Kit Activation and Type II Inhibitor Resistance

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Highlights

- D816V is predicted to reduce conformational entropy in the juxtamembrane latch, N-lobe wedges, and activation loop.
- A Val816-Asn819 hydrophobic seam replaces the wild-type Asp816-centered hydrogen-bond network.
- Type II inhibitor binding is partly retained but uncoupled from DFG-out conformational induction.
- D816 and N819 substitutions define opposing rigid/stabilizing and flexible/destabilizing thermodynamic regimes.
- Residual local disorder and mutant-wild-type dimer energetics generate testable biophysical predictions.

Abstract

Activation-loop mutations in receptor tyrosine kinases alter catalytic-state equilibria by changing the balance between local flexibility, global allostery, and inhibitor-induced conformational selection. Here we analyze the c-Kit D816V substitution as a model for how mutation-induced entropy loss can convert a regulated kinase into a rigidified active-like enzyme that resists type II inhibition. Using vibrational-entropy mapping, normal-mode analysis, molecular dynamics simulations, docking, dimer-interface energetics, and in silico saturation mutagenesis, we identify a conformational rigidification mechanism centered on Val816. The substitution replaces the wild-type Asp816-centered hydrogen-bond network with a Val816-Asn819 hydrophobic seam that propagates rigidity through the juxtamembrane latch, N-lobe wedges, and activation loop. This architecture is predicted to pre-organize the catalytic spine and restrict access to the inactive DFG-out state. Although imatinib and dasatinib retain favorable calculated binding energies, D816V imposes a larger predicted energetic penalty on imatinib than on dasatinib, and the rigidified pocket is predicted to impair the conformational collapse required for productive type II inhibition, thereby uncoupling binding from functional shutdown. Saturation mutagenesis separates hydrophobic D816 substitutions into a rigid/stabilizing thermodynamic regime and N819 substitutions into a flexible/destabilizing regime, indicating that activation-loop variants can be classified by the conformational states they impose rather than by position alone. Residual local disorder near residue 816 and energetically permissive mutant-wild-type heterodimerization suggest additional mechanisms for signaling adaptability. These results support a testable biophysical model in which conformational entropy loss, rather than increased flexibility, drives D816-centered c-Kit activation and type II inhibitor resistance.

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Introduction

Protein kinases are regulated by coupled changes in local packing, activation-loop order, catalytic-spine alignment, and long-range allosteric communication [1]. Receptor tyrosine kinases provide a particularly useful framework for examining these principles because extracellular ligand binding, juxtamembrane autoinhibition, dimerization, and activation-loop switching are physically linked to catalytic output [2,3]. The c-Kit receptor tyrosine kinase (CD117) is activated by stem cell factor (SCF) and controls survival, proliferation, and migration in hematopoietic, melanocytic, and interstitial lineages [4] (Figure 1). In the regulated wild-type receptor, SCF-induced dimerization promotes rearrangement of the juxtamembrane (JM) latch and activation loop (A-loop), enabling transition from the inactive Asp-Phe-Gly-out (DFG-out) state to the catalytically competent DFG-in state [5].

Figure 1. | Structural classification and functional overview of c-Kit signaling and D816V activation. Type III receptor tyrosine kinase family members, including c-Kit, share a conserved extracellular immunoglobulin-like region and intracellular kinase module. The schematic summarizes SCF-dependent c-Kit signaling through mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and stress-activated kinase pathways, and contrasts ligand-regulated wild-type c-Kit with the constitutively active-like D816V mutant. The panel provides biological context for the conformational and thermodynamic analyses that follow.

The Asp816 to Val (D816V) substitution provides an instructive example of how a single activation-loop mutation can remodel the conformational ensemble of a kinase. D816V is common in systemic mastocytosis and occurs in additional KIT-driven malignancies, including gastrointestinal stromal tumors, acute myeloid leukemia, chronic myelomonocytic leukemia, and rare melanomas [6–8]. It also confers resistance to imatinib and alters sensitivity to other kinase inhibitors, including dasatinib, depending on the conformational state favored by the mutant kinase [9–11]. These inhibitors depend on access to an inactive DFG-out-compatible state; therefore, resistance cannot be understood from binding affinity alone. It must be interpreted in terms of the conformational states available to the kinase after ligand engagement.

Earlier structural models proposed that D816V activates c-Kit by increasing activation-loop mobility and freeing the loop to sample an active conformation [12]. Although this explanation accounts for ligand-independent activation, it does not fully explain why type II inhibitors fail. A highly mobile activation loop should, in principle, remain accessible to inhibitor-induced DFG-out stabilization. Nor does a simple flexibility model explain why mutant receptors can influence signaling through mixed wild-type/mutant dimers. These inconsistencies motivate an alternative hypothesis: D816V may activate c-Kit by reducing key degrees of freedom, stabilizing an active-like regulatory architecture and preventing inhibitor-induced inactive-state selection.

We evaluated this rigidification hypothesis using an integrated computational strategy. Normal-mode vibrational-entropy mapping was used to identify mutation-induced changes in local mobility; molecular dynamics simulations assessed conformational stability; docking and binding-energy estimates tested whether drug engagement is preserved; dimer-interface energetics evaluated the physical plausibility of mixed receptor complexes; and in silico saturation mutagenesis at positions 816 and 819 tested whether the predicted mechanism extends beyond the single D816V substitution. Together, these analyses define a biophysical model in which D816-centered hydrophobic packing causes entropy loss and active-state pre-organization, whereas N819-centered substitutions favor a contrasting flexible regime. The study therefore frames c-Kit D816V resistance as a problem of conformational-state control, allosteric coupling, and mutation-induced entropy redistribution.

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Results

D816V Produces Entropy Loss in Regulatory Regions that Control Autoinhibition

Vibrational entropy analysis ($\Delta\Delta S_{vib}$) using DynaMut predicted that D816V rigidifies three discrete regulatory blocks in c-Kit: the juxtamembrane-proximal segment (residues 547-551), two proximal kinase-domain wedges (~592-601 and ~625-631), and the activation loop (~813-831) (Figure 2). These regions are positioned to couple autoinhibition, N-lobe organization, and activation-loop switching. Their predicted entropy loss is therefore consistent with stabilization of an active-like kinase architecture rather than simple loosening of the activation loop. The A-loop showed the most pronounced rigidification, consistent with its central role in controlling access to DFG-in and DFG-out states.

Residue-level analysis showed that wild-type Asp816 participates in a polar network involving Lys818, Asn819, and Asp820, a set of interactions positioned to support the inactive DFG-out-compatible state. D816V abolishes this Asp-centered network and introduces a Val816-Asn819 hydrophobic contact that reshapes the 816 microenvironment (Figure 3A). Despite this rewiring, key catalytic residues remain aligned (Figure 3B-C), suggesting that the mutant kinase can preserve catalytic competence while reducing conformational access to inactive-state geometries. Thus, the mutation is predicted to destabilize autoinhibitory regulation through entropy loss and local packing rather than through generalized disorder (Table 1).

Figure 2. | Entropy fingerprint of the D816V mutant. Vibrational-entropy differences ($\Delta\Delta S_{vib}$) calculated with DynaMut and mapped onto AlphaFold2-guided structural models reveal three discrete rigid blocks in c-KitD816V: the juxtamembrane-proximal segment (547-551), two N-lobe wedges (592-601 and 625-631), and the activation loop (813-831) [28]. Residues that lose entropy, consistent with rigidification, are colored blue; residues that gain flexibility are colored red. Val816 is shown as sticks for spatial reference. The concerted rigidification is consistent with pre-organization of the catalytic spine and reduced access to DFG-out-compatible states.

Figure 3. | Active-site rewiring and affinity-inhibition uncoupling produced by D816V. Residue-level comparisons identify replacement of the Asp816-centered hydrogen-bond network by a Val816-Asn819 hydrophobic seam. Superimposition of imatinib-bound wild-type and D816V c-Kit indicates that the mutant pocket remains compatible with ligand binding, while local activation-loop rigidification is predicted to restrict DFG-out collapse. Close-ups of the ATP site highlight loss of the Asp816-Lys818/Asn819/Asp820 hydrogen-bond network and formation of a compensatory Val816-imatinib contact. Binding free energies from AutoDock Vina are shown for imatinib and dasatinib; additional dasatinib interaction comparisons and ranked docking-energy values are provided in Supplementary Figure S1 and Supplementary Table S1. The central interpretation is that inhibitor binding is retained but no longer productively coupled to inactive-state induction.

Table 1. | Structural, interaction, and binding-energy summary for c-KitWT and c-KitD816V.

Property	c-KitWT	c-KitD816V
Key hydrogen bonds	Asp816-Lys818, Asp816-Asn819, Asp816-Asp820	Val816-Lys818 (weaker), Val816-Asn819
Hydrophobic contacts	Limited	Val816-Lys818, Val816-Asn819 (enhanced)
$\Delta\Delta S_{vib}$ rigidification	None detected in equivalent regulatory blocks	Juxtamembrane-proximal region, activation loop, N-lobe wedges
Imatinib ΔG (kcal mol^{-1})	-9.4	-8.6
Dasatinib ΔG (kcal mol^{-1})	-10.2	-9.7
Homodimerization ΔG (kcal mol^{-1})	-39.1	-20.5
Heterodimerization ΔG (kcal mol^{-1})	N/A	-29.3

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Conformational Rigidification Provides an Alternative to Flexibility-Centered Models

Previous models proposed that c-Kit D816V activation arises from increased activation-loop flexibility [12]. The present analysis suggests a different physical mechanism. If enhanced mobility were the dominant feature, the mutant activation loop would be expected to remain accessible to type II inhibitors that stabilize the inactive DFG-out state. Instead, the predicted entropy profile indicates rigidification of the activation loop, proximal kinase-domain wedges, and juxtamembrane-proximal region. The Val816-Asn819 seam provides a local packing explanation for this transition: although Asn819 is polar, its side-chain methylene group can engage in van der Waals packing with Val816 in the hydrophobic environment of the activation loop. This interaction creates a rigidifying seam that was not emphasized in flexibility-centered models [12]. The resulting picture is one of conformational locking, in which the mutation narrows the ensemble toward an active-like state that is less able to undergo inhibitor-induced deactivation.

Active-Site Rewiring Uncouples Binding Affinity from Inhibitory Conformational Selection

Docking with AutoDock Vina predicted that both inhibitors retained favorable binding energies in wild-type and D816V c-Kit, but the calculated energetic penalty was greater for imatinib than for dasatinib. In the top-ranked poses shown in Figure 3D, imatinib shifted from −9.4 to −8.6 kcal mol^{-1}, whereas dasatinib shifted from −10.2 to −9.7 kcal mol^{-1}. Structural comparison of dasatinib-bound wild-type and D816V c-Kit showed that dasatinib binding was less perturbed by the D816V substitution than imatinib binding (Supplementary Figure S1). Across the ten ranked docking predictions, imatinib shifted from a mean of −8.6 to −7.4 kcal mol^{-1}, whereas dasatinib shifted from −8.0 to −7.6 kcal mol^{-1} (Supplementary Table S1). This difference is consistent with the model that imatinib is more vulnerable to D816V-induced restriction of the DFG-out inactive state, whereas dasatinib binding is less dependent on the same inactive-state collapse. Because the absolute D816V docking energies for imatinib and dasatinib are similar, the dasatinib comparison should be interpreted as evidence for a smaller mutation-induced energetic penalty rather than as evidence of preserved functional inhibition. In the wild-type receptor, imatinib stabilizes the inactive state through interactions that are coupled to DFG-out geometry. In D816V, a compensatory contact between Val816 and the imatinib carbonyl is predicted to preserve engagement, but the surrounding rigidified pocket restricts the conformational reorganization required for productive type II inhibition.

These findings support an affinity-inhibition uncoupling model. In this model, drug binding and catalytic shutdown are distinct physical events. A ligand may still occupy the ATP-binding pocket, yet fail to impose the inactive conformational state if the kinase ensemble has been narrowed by mutation-induced rigidification. For c-Kit D816V, the relevant resistance mechanism is therefore predicted to be conformational: the mutant is not simply unable to bind inhibitor, but is less able to convert the bound state into a DFG-out inhibited state. This interpretation places inhibitor resistance within the broader biophysical framework of conformational selection and allosteric state stabilization.

Local Disorder Coexists with a Rigidified Catalytic Core

Despite the predicted entropy loss in the catalytic regulatory blocks, IUPred2A and DisEMBL identified a narrow region of intrinsic disorder near residue 816 in both wild-type and D816V c-Kit (Figure 4A-B). This result indicates that D816V does not render the kinase uniformly rigid. Instead, the mutant appears to combine a rigidified catalytic core with residual local adaptability adjacent to the rigidifying seam. Such coexistence may be biophysically important: a rigid core can stabilize catalytic output, whereas a local disordered segment may preserve interaction flexibility and allow context-dependent protein-protein interactions [13].

Figure 4. | Residual intrinsic disorder flanking Val816. IUPred2A/DisEMBL disorder-propensity traces for wild-type and D816V c-Kit show a narrow intrinsically disordered window centered near residue 816. The

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disorder threshold is indicated by a dashed line. This local disorder persists even though adjoining regions rigidify, suggesting that the mutant receptor may combine a rigid catalytic core with a residual adaptable interaction surface.

Dimer-Interface Energetics Suggest a Route for Conformational Signal Amplification

HADDOCK-modeled dimers evaluated with PRODIGY revealed asymmetric interface energetics (Figure 5). Wild-type homodimers showed the most favorable predicted interface energy (ΔG = -39.1 kcal mol^{-1}), whereas D816V homodimers were weaker (ΔG = -20.5 kcal mol^{-1}). Mixed wild-type-D816V dimers showed intermediate stability (ΔG = -29.3 kcal mol^{-1}), indicating that the mutant receptor may remain physically compatible with recruitment of wild-type protomers. This energetic pattern suggests a mechanism for signal amplification that does not require unusually strong mutant homodimerization. Instead, a rigidified mutant kinase could recruit wild-type receptors into mixed complexes, expanding the set of active signaling assemblies.

Figure 5. | Energetic asymmetry in c-Kit dimer formation. HADDOCK-generated dimers are shown for WT/WT, WT-D816V, and D816V/D816V complexes. Binding free energies were estimated with PRODIGY: WT/WT homodimer, ΔG = -39.1 kcal mol^{-1}; WT-D816V heterodimer, ΔG = -29.3 kcal mol^{-1}; D816V/D816V homodimer, ΔG = -20.5 kcal mol^{-1}. The intermediate stability of mixed dimers suggests that D816V may recruit wild-type protomers into signaling complexes, providing a possible route for conformational signal amplification.

The dimerization analysis should be interpreted cautiously because interface predictions do not reproduce the full membrane environment of receptor activation. Nevertheless, the energetic asymmetry is consistent with a model in which ligand-independent signaling arises from intramolecular conformational locking combined with permissive intermolecular coupling. In this sense, D816V may act not by strengthening every dimeric interaction, but by changing the distribution of receptor states that can support signaling.

In Silico Saturation Mutagenesis Defines Opposing Activation-Loop Thermodynamic Regimes

DynaMut2-based mutagenesis of positions 816 and 819 revealed two distinct thermodynamic regimes **(Supplementary Data S1)**.Hydrophobic substitutions at Asp816, including Val, Phe, Trp, Tyr, Leu, and Ile, reduced predicted vibrational entropy and increased predicted stability ($\Delta\Delta G$ approximately +0.6 to +1.4 kcal mol^{-1}; $\Delta\Delta S_{vib} < 0$), forming a rigid/stabilizing class. This pattern indicates that the D816V mechanism is not unique to valine but reflects a broader physical property of hydrophobic replacement at this activation-loop position. In contrast, substitutions at Asn819 tended to lower $\Delta\Delta G$ and increase $\Delta\Delta S_{vib}$, defining a flexible/destabilizing class. Clustering of the $\Delta\Delta G$ and $\Delta\Delta S_{vib}$ landscape separated these regimes, supporting the idea that activation-loop variants can be classified by the thermodynamic states they impose on the kinase **(Supplementary Data S1)**. Structurally, the rigid D816 class is linked to loss of the Asp816-Lys818-Asn819-Asp820 hydrogen-bond network and replacement by compensatory hydrophobic packing. This packed nucleus is predicted to propagate entropy loss into the juxtamembrane region and N-lobe wedges. By contrast, N819 substitutions disrupt local contacts without generating an equivalent stabilizing seam, leaving the activation loop more flexible. These opposing regimes suggest that inhibitor response may depend less on whether a mutation sits in the activation loop per se, and more on whether the substitution narrows or expands access to DFG-out-compatible conformations. Direct biochemical and cellular validation will be required to test this thermodynamic-pharmacologic relationship.

Discussion

This study supports conformational rigidification and entropy loss as central biophysical mechanisms by which D816-centered activation-loop substitutions alter c-Kit regulation. The combined computational evidence indicates that D816V does not primarily increase activation-loop

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flexibility. Instead, it replaces an Asp816-centered polar network with a Val816-Asn819 hydrophobic seam that stabilizes an active-like regulatory architecture and restricts access to the DFG-out inactive state. This conclusion is consistent across vibrational-entropy mapping, structural contact analysis, docking, dimer-interface energetics, and mutational thermodynamic profiling.

The most important implication is that kinase resistance can arise from failure of conformational coupling rather than from loss of drug binding. Type II inhibitors require not only pocket occupancy, but also productive stabilization of an inactive state. The D816V model shows how a mutation can preserve apparent binding while impairing the conformational transition required for inhibition. This distinction is especially relevant for biophysical studies of kinase inhibitors because docking energies alone can overestimate functional inhibition when the conformational ensemble is constrained. Consistent with the importance of inhibitor mechanism beyond pocket occupancy alone, midostaurin has shown clinical activity in advanced systemic mastocytosis, although such responses cannot be inferred directly from the present computational model [14].

The mutation-wide analysis extends the mechanism from a single variant to a broader thermodynamic class. Hydrophobic substitutions at position 816 share a rigid/stabilizing signature, whereas substitutions at Asn819 are more flexible and destabilizing. This result argues that activation-loop mutations should be interpreted by their effect on the conformational energy landscape, not only by their sequence position or steric footprint. It also generates experimentally testable predictions: hydrophobic 816 variants should preferentially populate active-like, DFG-in-compatible ensembles, whereas mutations that increase entropy may retain greater access to inactive-state geometries.

The coexistence of a rigidified catalytic core with residual local disorder near residue 816 adds another layer to the model. Rather than behaving as a uniformly rigid object, D816V c-Kit may combine a locked catalytic architecture with a small adaptable interface that can support context-dependent interactions [13]. This combination may help explain how constitutive activity and signaling versatility coexist. Similarly, the predicted intermediate stability of wild-type-D816V heterodimers suggests that intermolecular signaling amplification may arise from physical compatibility between mutant and wild-type protomers rather than from unusually stable mutant homodimers.

Integrated Conformational-Energy Model

The integrated model in Figure 6 summarizes the proposed conformational-energy mechanism. In wild-type c-Kit, the juxtamembrane region, N-lobe regulatory elements, and activation loop form a dynamic apparatus that supports reversible switching between inactive and active kinase conformations. This plasticity is required both for SCF-dependent activation and for type II inhibitor action, because type II inhibitors depend on access to and stabilization of a DFG-out-compatible inactive state. D816V is predicted to shift this equilibrium by replacing the Asp816-centered polar network with a Val816-centered hydrophobic seam involving Asn819, thereby pre-organizing the catalytic spine and favoring an active-like DFG-in-compatible state.

Figure 6. | D816V remodels c-Kit conformational thermodynamics through structural rigidification and affinity–inhibition uncoupling. (A) Wild-type c-Kit is shown as a ligand-regulated kinase with conformational plasticity that permits reversible DFG-in/DFG-out switching. D816V favors a ligand-independent, active-like state in which the juxtamembrane latch and activation loop are rigidified and pre-organized. (B) In the proposed rigidification mechanism, the wild-type Asp816-centered polar network is replaced by a Val816-Asn819 hydrophobic seam that propagates entropy loss through the juxtamembrane latch, N-lobe wedges, and activation loop. (C) Imatinib can still occupy the D816V ATP-binding pocket, but rigidification prevents the DFG-out conformational collapse required for productive type II inhibition, uncoupling binding from inhibitory conformational selection. (D) Mutation-wide thermodynamic profiling separates hydrophobic D816 substitutions into a rigid/stabilizing regime and N819 substitutions into a flexible/destabilizing regime **(Supplementary Data S1)**. (E) The model predicts that active-state/type I inhibitor strategies may better match the rigidified DFG-in-compatible state, while residual local disorder and WT-D816V heterodimerization remain

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potential biophysical contributors to signaling adaptability. These predictions require direct experimental validation.

Within this framework, inhibitor binding and inhibitor function are separated. Imatinib can occupy the ATP-binding pocket, but the rigidified activation loop and associated regulatory blocks restrict the conformational collapse required for durable type II inhibition. The saturation-mutagenesis landscape generalizes the model by showing that hydrophobic D816 substitutions impose a rigid/stabilizing regime, whereas N819 substitutions impose a more flexible/destabilizing regime. Thus, the key physical variable is not only residue identity but the thermodynamic ensemble imposed on the kinase regulatory architecture.

Figure 6 also emphasizes that D816V does not eliminate all mobility. A residual disordered segment near residue 816 and a permissive heterodimerization landscape may allow conformational locking to coexist with interaction adaptability. The model therefore reconciles ligand-independent activation, type II inhibitor resistance, residual local disorder, and mixed-dimer signaling within a single physical framework. In the context of Biophysical Chemistry, the broader message is that mutation-induced redistribution of conformational entropy can be sufficient to rewire enzyme regulation and drug response even when ligand binding remains energetically favorable.

Several limitations should be emphasized. The analysis is computational and requires experimental validation by kinase activity assays, inhibitor-response profiling, hydrogen-deuterium exchange mass spectrometry, nuclear magnetic resonance relaxation, cryo-electron microscopy or crystallography, and cellular studies of selected D816 and N819 variants. The 100 ns molecular dynamics trajectories may not sample rare transitions, and binding free-energy estimates from docking, PRODIGY, and MM/GBSA are approximate. The disorder-mediated interaction hypothesis and mixed-dimer amplification model are particularly important to evaluate in membrane-proximal cellular systems. Nevertheless, the convergence of independent computational approaches supports rigidification as a mechanistically plausible and experimentally testable model for D816-centered c-Kit activation and type II inhibitor resistance.

Methods

Protein Modeling and Mutation Analysis

The amino acid sequence of human c-Kit (UniProt P10721) was used to model wild-type c-Kit and c-KitD816V. The inactive-state crystal structure (PDB: 1T45) served as template; D816V was introduced with PyMOL 2.5 (Schrödinger, LLC), and resulting structures were energy-minimized in Molecular Operating Environment (MOE) to relieve steric clashes [15]. Structure quality was assessed with MolProbity [16] and PROCHECK [17]. Vibrational entropy changes ($\Delta\Delta S_{vib}$) were calculated with DynaMut using normal mode analysis, focusing on the activation loop, juxtamembrane region, and proximal kinase domain [18].

Molecular Dynamics Simulations

Molecular dynamics simulations were performed with GROMACS 2021 using the CHARMM36 force field. Proteins were solvated in TIP3P water with 0.15 M NaCl. Simulations were run for 100 ns at 310 K and 1 atm after NPT and NVT equilibration using a V-rescale thermostat. RMSD, RMSF, and secondary-structure trajectories were analyzed to assess conformational stability [19,20].

Molecular Docking

Imatinib and dasatinib coordinates were retrieved from the ZINC database and prepared in MOE by optimizing protonation states at physiological pH followed by energy minimization. AutoDock Vina was used to dock ligands into the ATP-binding pocket. Ten conformations were generated per ligand, and the lowest-energy pose was selected for analysis. Hydrogen bonds, van der Waals contacts, and hydrophobic interactions were analyzed with LigPlot+ [21,22].

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Intrinsic Disorder Analysis

Disorder propensity was predicted with IUPred2A and DisEMBL using primary sequence. Predicted disordered regions were compared with DynaMut $\Delta\Delta S_{vi\beta}$ profiles and molecular-dynamics-derived RMSF values to evaluate the relationship between local disorder and global rigidification [23,24].

Dimerization and Binding-Energy Calculations

Homodimeric and heterodimeric c-Kit complexes were modeled using HADDOCK 2.4. Interface binding free energies (ΔG) and dissociation constants (Kd) were estimated with PRODIGY. Interface residues and structural deviations were analyzed with PyMOL [25,26].

In Silico Saturation Mutagenesis

The apo-state c-Kit kinase-domain model was used as template. Single-residue substitutions to all 19 alternatives were introduced at positions 816 and 819 using DynaMut2, which combines normal mode analysis with graph-based structural signatures to estimate ΔΔG (kcal mol^{-1}) and $\Delta\Delta S_{vi\beta}$ (kcal mol^{-1} K^{-1}) [27]. Negative $\Delta\Delta S_{vi\beta}$ values were interpreted as reduced conformational mobility. K-means clustering (k = 2) of ΔΔG and $\Delta\Delta S_{vi\beta}$ vectors was performed in R, and cluster robustness was assessed by silhouette analysis. The complete substitution-level mutagenesis dataset, including predicted ΔΔG, $\Delta\Delta S_{vi\beta}$, and thermodynamic class assignments, is provided in **Supplementary Data S1**.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Supplementary Figure S1 | Dasatinib binding is less perturbed by D816V than imatinib binding. Supplementary Table S1 | D816V produces a larger predicted docking-energy penalty for imatinib than for dasatinib. Supplementary Data S1 | CKit activation-loop mutagenesis dataset.

Author Contributions: N.A.P. and M.C. conceived the study. M.C. and H.P. performed structural modeling and molecular dynamics simulations. Z.M. and L.A.M. conducted molecular docking and MM/GBSA calculations. A.E., E.D., Z.D., and J.L. performed in silico saturation mutagenesis and clustering analyses. N.A.P. supervised all aspects of the work. M.C. and N.A.P. wrote the manuscript with contributions from all authors. All authors reviewed and approved the final manuscript.

Institutional Review Board Statement: No human participants, human data, or animal experiments were involved in this study. All analyses were performed using publicly available structural databases and computational simulations.

Data availability: The structural models, docking results, supplementary docking-energy table, and activation-loop saturation-mutagenesis dataset generated in this study are available in the supplementary material or from the corresponding author upon reasonable request. All computational tools and databases used are publicly available: PDB, UniProt, ZINC, DynaMut, DynaMut2, HADDOCK, PRODIGY, IUPred2A, and DisEMBL.

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Conflicts of Interest: The authors declare no competing interests.

Code Availability: Custom analysis scripts are available from the corresponding author upon reasonable request.

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