



Dissecting the binding landscape of four cKIT inhibitors through an integrated multifaceted approach

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ABSTRACT

The receptor tyrosine kinase cKIT plays a pivotal role in a variety of physiological processes and is implicated in a broad spectrum of pathological conditions. Its activity is controlled by phosphorylation-dependent conformational changes, which also influence the binding mode of small-molecule inhibitors. We employed an integrated experimental and computational strategy to characterize the conformational landscape of cKIT and to elucidate the binding mechanisms of four inhibitors, Avapritinib, Olverembatinib, Labuxtinib and Ceniseritib. A combination of Surface Plasmon Resonance (SPR), enzymatic assay, Molecular Dynamics (MD) simulations, stability energy evaluation, Limited Proteolysis coupled to Mass Spectrometry (LiP-MS) and X-Ray crystallography was used to investigate both active and inactive kinase states. Avapritinib preferentially binds the active form of cKIT, as supported by SPR kinetics, LiP-MS patterns, and MD results. Structural data further confirm that this compound occupies the ATP-binding pocket, consistent with a Type I inhibitor. Olverembatinib and Labuxtinib exhibit high affinity for the inactive kinase, showing stronger binding to the inactive form by SPR and inducing extensive protection of residues spanning the ATP-binding pocket in LiP-MS experiment. MD analysis reveals the burial of key pocket residues, supporting a Type II inhibition mode. Ceniseritib exhibits a more complex behavior. While SPR indicates binding to both kinase states, LiP-MS, MD and X-Ray crystallography analyses reveal distinct interaction patterns depending on phosphorylation. Overall, this work highlights how distinct inhibitors exploit different conformational states of cKIT and demonstrates the value of integrating structural analyses, biophysical measurements, calculations and molecular simulations to define the mechanism of kinase inhibition.

1. Introduction

Protein kinases are enzymes that regulate several cell signaling pathways, transferring the phosphate group of ATP to the hydroxyl group of a specific residue of the substrate proteins [1]. The largest part of protein kinases is classified as either intracellular or transmembrane proteins. The latter are often included in the category of Tyrosine Kinase Receptors. The activation of these enzymes has been shown to modulate most signaling pathways via the phosphorylation of downstream targets that are pivotal for the homeostasis of the organism [2]. The human proteome expresses more than five hundred members of the kinase

superfamily, showing a peculiar highly conserved fold, with the occurrence of two main structural domains, commonly indicated as N- and C-lobes. A linker connects these two domains [3,4]. The N-lobe consists predominantly of β -sheets (I-V), while the C-lobe comprises largely of α -helices (α D- α F), and both include many conserved structural elements: a G-rich motif loop, located between β – sheets I and II, containing three highly conserved Glycine residues (GxGxxG motif); an α C-helix, a helix in the N-terminal lobe of the kinase fold; a hinge region, a segment utilized as an anchor point by ATP; a DFG motif, situated at the beginning of the catalytic loop and involved in the regulation of the activation state of the protein kinase; a HDR motif, a highly conserved

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segment in the catalytic loop involved in catalysis; a DFG motif, situated at the beginning of the catalytic loop and involved in the regulation of the activation state of the protein kinase; a catalytic and an activation loops [3,5].

Kinases play a crucial role in numerous cell signaling pathways, which are influenced by variations in the cellular context, the presence of cofactors, and protein-protein interactions [6–8]. The activation occurs primarily through post-translational modifications such as autophosphorylation, usually upon binding with ligands. In all cases, the biological message passes through structural rearrangements and conformational variations induced by the above-mentioned modifications. Autophosphorylation is undoubtedly one of the most common mechanisms through which kinases are activated [9]. When stimulated, these enzymes have been observed to undergo self-phosphorylation, primarily on serine, threonine, or tyrosine residues within the activation loop, thus inducing a conformational change that facilitates substrate binding and enhances kinase activity [10]. Kinase activation requires the occurrence of multiple phosphorylation events, carried out in predefined sequences both in space and in time, thus continuously exploring conformational changes [11]. The starting and the ending points of this process consist of two main different conformations showing the lowest and the highest enzymatic activity, commonly indicated as the active (highly phosphorylated) and the inactive (poorly phosphorylated) forms. One of the consequent conformational changes induced by autophosphorylation occurs at the level of the domain including the DFG motif and is well-known as DFG triplet switch [12]. In almost all cases, the initial kinase activation event is followed by an amplified cascade of further phosphorylations involving other downstream kinases, leading to the modulation of many cell processes, including DNA replication, apoptosis, cell cycle progression, cytoskeletal rearrangement, differentiation and cell energy metabolism [13].

In light of their crucial role in the regulation of so many vital processes, the dysregulation of these enzymes has been observed in a variety of pathologies, such as cancer [14,15], autoimmune and inflammatory disorders [16], neurodegenerative diseases [17,18] and cardiovascular problems [19], rendering them one of the principal pharmaceutical targets.

According to the different binding sites of potential kinase inhibitors, orthosteric inhibitors are defined as those molecules that block the enzymes' activity by competing with ATP for binding to the specific pocket. Conversely, allosteric inhibitors are molecules that bind to other portions of the enzyme, more or less distant from the ATP-binding pocket. More in detail, four different classes of kinase inhibitors can be defined as follows: i) Type I: molecules that are ATP-competitive kinase inhibitors (orthosteric inhibitors) with a highest affinity for kinases active conformers [20]; ii) Type II: molecules binding the ATP site, stabilizing the inactive conformation known as "DFG-out", in which a conserved DFG (Asp-Phe-Gly) motif partially blocks the ATP pocket [21]. These kinds of inhibitors often extend their structure into the hydrophobic back pocket formed in the "DFG-out" state [22]; iii) Type III: molecules bind exclusively to an allosteric pocket close to the ATP site in the active kinase conformation [23,24]; iv) Type IV: molecules bind to allosteric sites further away from the ATP site [23,24].

cKIT, also known as CD117, is an ubiquitarian protein kinase receptor [25], largely expressed in mast cells, where its interaction with its natural ligand, the Stem Cell Factor (SCF) is essential for their survival and maturation [26]. Structurally speaking, the intracellular domain of cKIT is composed of different regions. The JM region (residues 544–581) functions as an inhibitor of cKIT kinase by positioning itself between the N- and C-lobes, thereby maintaining the enzyme in an inactive state. The kinase domain (582–935) consists of a smaller N-lobe (582–671), which includes a glycine-rich P-loop (596–603) and an α C-helix (631–647) that modulates catalytic activity. The larger C-lobe (672–935) encompasses the activation loop (810–835), essential for substrate binding and catalysis. Collectively, these domains facilitate ligand-induced activation and determine sensitivity to inhibitors [27].

Under physiological conditions, when not bound to SCF, cKIT resides in the cell membrane as a monomer [27,28]. Upon ligand stimulation, cKIT undergoes auto/transphosphorylation of selected tyrosine residues (Y568, Y570 and Y823). The full activation of cKIT arises when Y703, Y721 and Y730 and the ones in the C-terminal tail (Y900 and Y936) are completely phosphorylated [29–31]. The resultant phosphotyrosines become docking sites for associating critical signaling molecules, including Src kinase, Lyn and Fyn, phospholipase C γ , phosphoinositide 3-kinase (PI3K) and the adaptor molecules Gfb2 and Shc [32–39]. However, the dysregulation of its activity has been linked to various pathologies, such as pulmonary [40], and myocardial fibrosis [41], and keloid [42], and several types of tumors, such as gastrointestinal stromal tumor [43,44], acute myelogenous leukemia [45], small-cell lung carcinoma [46].

Since this kinase is considered a primary pharmacological target for many pathologies, the identification of new potential specific inhibitors is an urgent request to be fulfilled.

The present study has been focused on analyzing 3 known orthosteric cKIT inhibitors, Avapritinib [47], Olverembatinib [48], Labuxtinib [49], and a not well described cKIT inhibitor Cenisertib [50], in order to characterize their binding mode within the intracellular domain, encompassing residues from 535 to 976, taking into account their different chemical properties (Fig. 1). The characterization of these interactions is crucial in the optimization of drug design and synthesis procedures, with the aim of maximizing specificity and therapeutic efficacy. To achieve the highest level of molecular detail, a multifaceted approach encompassing complementary techniques such as biochemical assays, Surface Plasmon Resonance (SPR), Molecular Dynamics (MD), stability energy evaluation, Limited Proteolysis coupled to Mass Spectrometry (LIP-MS) and X-Ray crystallography was employed. This comprehensive strategy was applied to the analysis of protein-drug complexes including both the active, which had been pre-incubated with ATP and consequently phosphorylated, and the inactive form of the protein, enabling the identification of the binding site(s) within the intracellular domain of cKIT and its detailed molecular characterization.

2. Results

2.1. Enzymatic assay and SPR results

Preliminary tests of enzymatic assay and SPR were performed to confirm the inhibition of these compounds and their affinity for cKIT. In particular, for the enzymatic assay, the evaluation of inhibition of compounds on the activity of the cKIT kinase was quantified by measuring the phosphorylation of the substrate Ulight-TK peptide using a human recombinant enzyme and the LANCE® detection method. The enzymatic analysis was performed in triplicate, and the results are reported as logIC₅₀ (Table 1). All the compounds showed a pIC₅₀ between 7 and 8, confirming their capability of inhibiting cKIT.

Furthermore, each of the four compounds was incubated with both forms of the intracellular domain of cKIT, the hyper- and the hypophosphorylated one, corresponding to the activated and inactivated conformational states of the receptor, respectively. The affinity values and kinetics were reported in Table 2. Labuxtinib is the tightest inhibitor, showing in both states an affinity within nM range. The slow dissociation rate results in long residence times ($t_{1/2}$ of ~4300–6000 s), typical of the type II inhibitor and reflects a deep pocket engagement [51]. Avapritinib binds both states of cKIT with similar, sub-micromolar affinity: activated K_d: $6.63 \times 10^{-7} \pm 1.85 \times 10^{-5}$ M, inactivated K_d: $4.41 \times 10^{-7} \pm 1.58 \times 10^{-5}$ M. Kinetics are fast and lead to short residence times, typical of type I inhibitors [52]. Cenisertib shows stronger affinity for the inactivated form (K_d = $8.04 \times 10^{-7} \pm 2.20 \times 10^{-4}$ M), compared to a moderate affinity for the activated form (K_d = $2.37 \times 10^{-6} \pm 5.81 \times 10^{-7}$ M). This gap suggests that Cenisertib stabilizes inactive kinase conformations. Although no cKIT-Cenisertib cocrystal exists to univocally assign its type of inhibition, a shift toward the inactivated state is

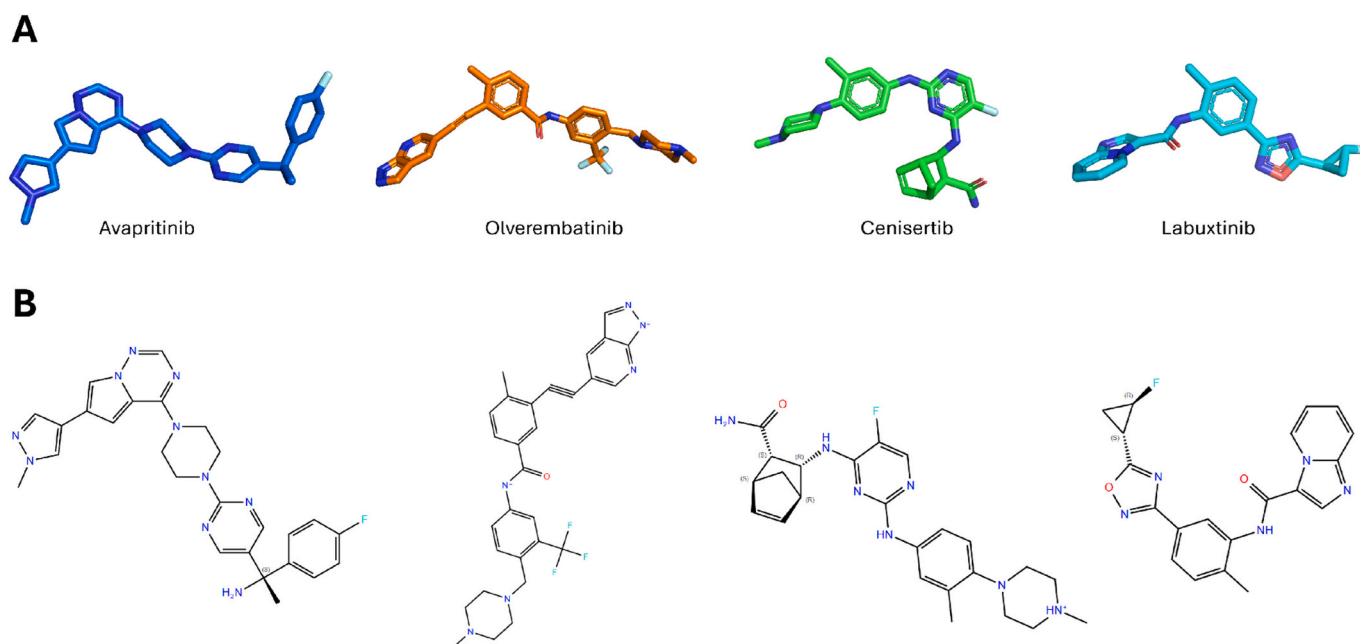


Fig. 1. A) 3D and B) 2D chemical structures of the 4 cKIT inhibitors.

Table 1
pIC₅₀ of the four tested compounds.

Compound	pIC ₅₀
Labuxtinib	6.59 ± 0.18
Cenisertib	8.57 ± 0.28
Avapritinib	8.85 ± 0.28
Olverembatinib	8.45 ± 0.04

consistent with DFGout/Type II engagement.

Olverembatinib binds weakly to the inactive state of cKIT ($K_D = 1.84 \times 10^{-5} \pm 1.04 \times 10^{-5}$ M) and does not show any clear binding properties with the active state.

2.2. Molecular dynamics

In silico studies were performed to provide a comprehensive analysis of the conformational changes in protein residues associated with cKIT in both its active and inactive states, as well as to investigate the extent to which ligand binding affects residue dynamics. Molecular Dynamics (MD) approach was applied to analyze the Solvent Accessible Surface Area (SASA) and the Root Mean Square Fluctuation (RMSF) to characterize the residue exposure in the different conditions and to quantify the mobility of the amino acids according its time-averaged position throughout a molecular dynamics' simulation trajectory, respectively.

Table 2

Binding kinetics and binding constant of selected cKIT inhibitors on activated and inactivated cKIT. Association rate constants (k_{on}), dissociation rate constants (k_{off}), equilibrium dissociation constants (K_D), and derived parameters (half-life $t_{1/2}$) were determined using 1:1 binding model by SPR assays. Data are shown for four inhibitors: Labuxtinib, Avapritinib, Cenisertib, and Olverembatinib. For Cenisertib and Olverembatinib, K_D values were calculated with a steady-state affinity model. Comparisons highlight differences in binding affinity and kinetic profiles between activated and inactivated cKIT conformations.

Inhibitor	cKIT State	k_{on} (1/M·s)	k_{off} (1/s)	K_D (M)	$t_{1/2}$ (s)
Labuxtinib	Activated	2.20×10^3	1.61×10^{-4}	$7.29 \times 10^{-8} \pm 5.61 \times 10^{-7}$	4315
	Inactivated	2.38×10^3	1.16×10^{-4}	$4.87 \times 10^{-8} \pm 4.77 \times 10^{-7}$	5976
Avapritinib	Activated	1.41×10^4	9.32×10^{-3}	$6.63 \times 10^{-7} \pm 1.85 \times 10^{-5}$	74.36
	Inactivated	1.48×10^4	6.54×10^{-3}	$4.41 \times 10^{-7} \pm 1.58 \times 10^{-5}$	106
Cenisertib	Activated	—	—	$2.37 \times 10^{-6} \pm 5.81 \times 10^{-7}$	—
	Inactivated	7.01×10^4	5.64×10^{-2}	$8.04 \times 10^{-7} \pm 2.20 \times 10^{-4}$	12.29
Olverembatinib	Activated	—	—	—	—
	Inactivated	—	—	$1.84 \times 10^{-5} \pm 1.04 \times 10^{-5}$	—

conformational analyses were performed. While global descriptors such as solvent-accessible surface area (SASA) and RMSF provide valuable information on the overall stability and flexibility of the protein, they do not directly quantify the energetic contributions responsible for ligand stabilization or identify the residues involved in ligand recognition.

The binding energetics of the HOLO systems were therefore evaluated using the Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) approach, including both the decomposition of the total binding free energy into its individual energetic components and a per-residue energy decomposition analysis to identify the principal interaction hotspots within the binding site.

To investigate the conformational behavior of the protein independently of ligand binding, the free energy landscape (FEL) analyses were performed for both the APO and HOLO systems. These analyses enabled the identification of the dominant collective motions, the conformational states preferentially sampled by each system, and ligand-induced changes in the underlying conformational energy landscape. Together, the MM/GBSA and FEL analyses provide complementary energetic and conformational information, allowing a comprehensive characterization of protein–ligand recognition and of the effect of ligand binding on the dynamic behavior of cKIT.

2.2.1. Comparative analysis of active (hyper-phosphorylated) cKIT* vs inactive (hypo-phosphorylated) cKIT

We started comparing the hyper- (active) and hypo- (inactive) phosphorylated forms to characterize the different dynamics induced by the phosphorylation. In this case, the existing models contain only two phosphorylation in the active conformation, the Y568 and Y570.

The simulation models differ in structure and residue count. The 1PKG (active kinase) model includes residues N567–G927, omitting I698–M757. The 1 T45 (inactive kinase) model spans Y547–I935 but lacks T694–S753. Typically, the models employed omit the region spanning residues around 690 to 760. This omission is attributable to the fact that this segment represents a highly flexible loop, for which it is not possible to obtain a precise electron density map as a result of its inherent instability.

The RMSF profiles (Fig. 2) reveal broadly similar fluctuation patterns for the N-terminal region of cKIT in both the active and inactive conformations. The most pronounced difference is observed around residue F600, which displays increased flexibility in the active state. Additional differences are evident within residues 800–850, corresponding to the activation loop, a region that undergoes substantial conformational rearrangement between the two functional states. Consistent with these structural differences, the activation loop exhibits slightly lower flexibility in the active conformation than in the inactive one. Fig. S1 presents the SASA analysis for each residue of the protein in both its active and inactive conformations. Significantly, residues Y568 and Y570 (circled in red in Fig. S1), which undergo phosphorylation in the active state, are fully solvent-accessible whereas they are only partially exposed in the inactive conformation. Conversely, residues D572 and T574 remain shielded from solvent exposure when the protein is inactive. In the active conformation, a buried cluster is observed between L637 and L645. Residues 631–647 form the kinase's α C-helix, a key segment for its movement during activation and deactivation [54]. While this region is usually buried in the inactive state, M638 and Y646 show partial solvent exposure per SASA analysis, possibly due to outward helix displacement. cKIT activation depends on the α C-helix's conformation; an inward α C-helix promotes the Glu-Lys salt bridge and aligns the catalytic site for ATP binding [28]. Structural and computational studies confirm that inward rotation stabilizes the regulatory spine and active kinase conformation [55]. In contrast, the autoinhibited state features an outward α C-helix that disrupts these interactions, keeping cKIT inactive [28]. Additionally, the segment between K786–A794 includes the HRD motif and residues that stabilize the geometry of the active site. The regulation of catalytic activity is, indeed, strongly dependent upon the precise spatial orientation of conserved structural

components, specifically the DFG motif, the C-helix, and the HRD motif located within the catalytic loop. Within the active conformational states of c-KIT, the His-Arg-Asp (HRD) triad serves as a fundamental structural and electrostatic framework that facilitates substrate coordination and ensures the stability of the catalytically functional activation loop in its DFG-in orientation. Specifically, the histidine and arginine residues of the HRD motif establish a hydrogen-bonding network that interconnects the catalytic loop with the activation segment, whereas the aspartate residue operates as the requisite catalytic base for phosphotransfer reactions. Perturbation of this network impairs the stability of the active activation loop conformation, thereby promoting a transition toward inactive states. This mechanism highlights the significance of the HRD motif as an allosteric nexus that harmonizes activation loop dynamics with the catalytic apparatus of the kinase domain.

Elevated mobility distinguishes the 810–830 region in the inactive state, with residues 813, 814 and 815 not exposed to the solvent in the inactive form. On the other hand, only residue 814 remains buried in the active conformation, while residues 813 and 815 are exposed to the solvent. Both states feature two clusters of buried residues, although their composition varies slightly: residues 831–841 and 846–866 in the inactive state, and 834–841 and 846–866 in the active conformation.

A superposition of the cKIT in the active and inactive conformations is reported in Fig. 3, while Fig. 4 shows the architecture of cKIT with all its constituent regions.

2.2.2. cKIT with Avapritinib

After the characterization of these two conformers, we moved to the analysis of cKIT interacting with Avapritinib, starting from the active cKIT* form. The Avapritinib crystal structure reveals a dimer consisting of chain A and chain B; however, our calculations utilize only chain A to minimize computational expenses. The Root Mean Square Fluctuation (RMSF) was calculated to assess the dynamic behavior of the protein in both the presence and absence of the ligand. The aim was to determine the extent to which the ligand modulates the flexibility and mobility of individual residues.

Avapritinib binding mainly affects the residue 600, as illustrated in Fig. 5, Panel A, resulting in decreased flexibility when the ligand is present. Significant differences in flexibility are noted within the region defined by residues 817–825 (Fig. 5, Panel B), where ligand binding results in decreased conformational mobility. As previously noted, this region aligns with the activation loop, indicating its potential role in ligand binding.

SASA analysis (Fig. S2) reveals some differences between the bound and unbound form of the protein. More specifically, in the APO state, F600 and K602 within the P-loop region are solvent-exposed, whereas G601 and V603 remain buried. Avapritinib binding alters this configuration by also exposing G601 and V603 to the solvent.

In the region corresponding to the α C helix (H630–L647), a cluster of primarily buried residues (L637–L645) is observed in the APO form; however, Avapritinib binding leads to M638 becoming solvent-exposed.

Avapritinib binding also leads to an increase in the cluster of buried residues in the D768–A777 region. In this region, Y774 was exposed to the solvent in the protein's APO form, but became buried upon binding to Avapritinib.

Furthermore, within the activation loop, V833 are rendered buried upon ligand binding. Conversely, Avapritinib binding induces solvent exposure of D908.

The MM/GBSA calculations predicted a favorable binding free energy of $\Delta G_{\text{bind}} = -63.1 \pm 0.05$ kcal/mol between cKIT and Avapritinib. As shown in Fig. 6, Panel A, the stabilization of the complex was primarily driven by favorable van der Waals interactions and electrostatic contributions, which were partially counterbalanced by unfavorable polar desolvation effects. The consistency of the calculated energetic contributions across independent replicas further supports the robustness of the predicted energetic profile.

However, the global binding free energy does not provide

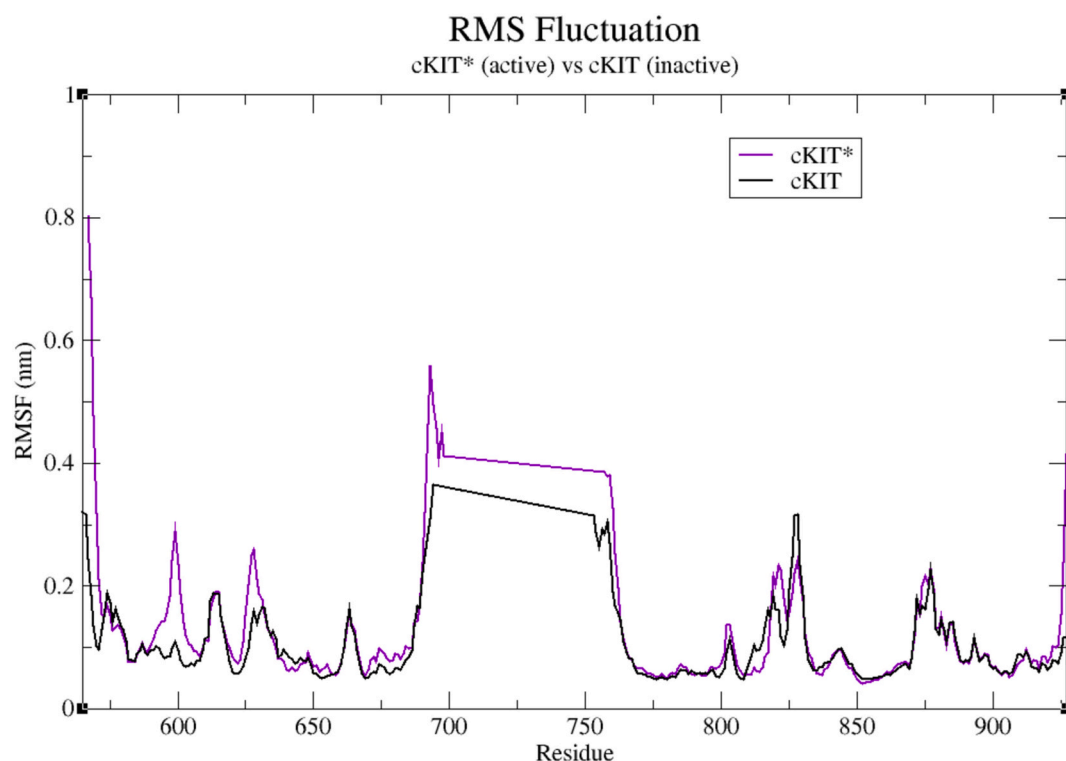


Fig. 2. RMSF comparison between hyper- (active) and hypo- (inactive) phosphorylated forms of cKIT. The active and inactive conformations are depicted in violet and black, respectively.

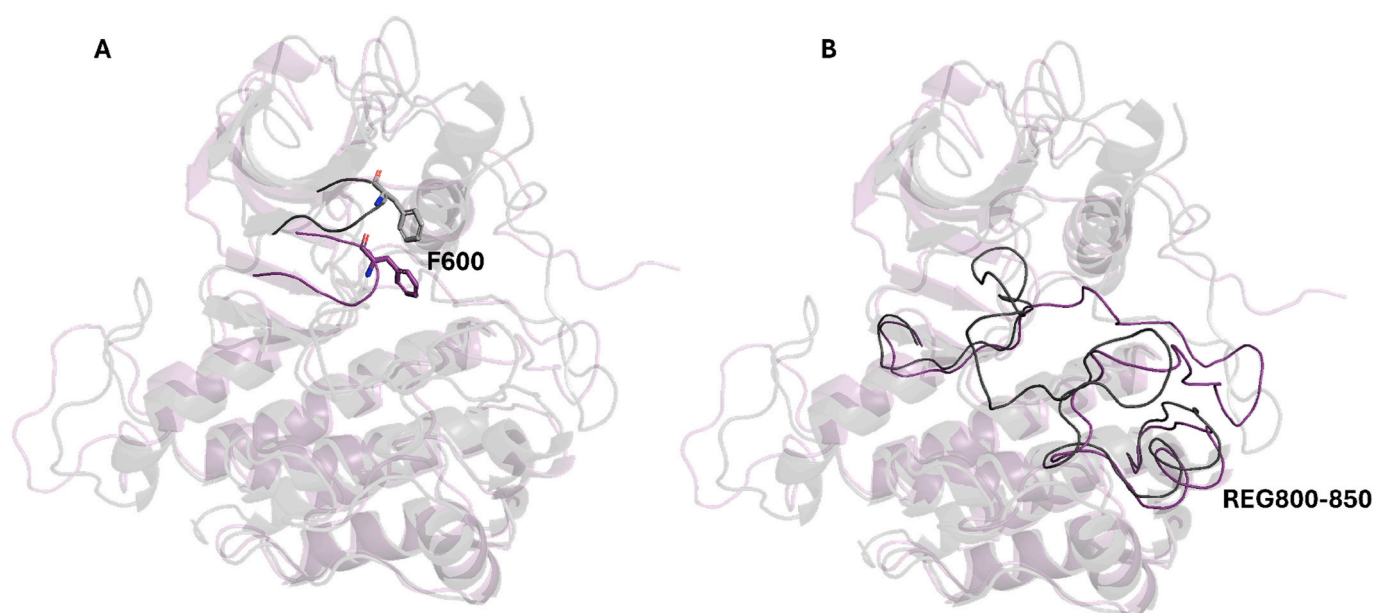


Fig. 3. Superposition of cKIT in its active (violet) and inactive (dark grey) conformations. A) F600 along with TYR568 and TYR570 are shown in stick. The phosphorylation is reported as pY. B) region between 598 and 633 is reported. The cluster of residues reported as not exposed to the solvent in which E605 is found is reported in sticks for both models. C) Region between 800 and 850 is highlighted. D) Region between 870 and 900 is highlighted.

information about the individual residues responsible for ligand stabilization. Therefore, a per-residue energy decomposition analysis was performed to identify the specific amino acids contributing to ligand binding. Residues showing a favorable energetic contribution lower than -1 kcal/mol were selected as energetic hotspots, as these residues represent the major contributors to ligand stabilization within the binding site. The identified hotspot residues were ranked according to their ΔG contribution and visualized through residue-wise energy

decomposition analysis (Fig. 6, Panel B).

According to the per-residue energy decomposition, V603 emerges as the primary molecular determinant for ligand recognition, situated within the core binding pocket of cKIT (Fig. 6, Panel B). A secondary significant energetic contribution arises from hydrophobic associations with L595. Furthermore, residues C673 and Y672 located in the hinge region facilitate critical interactions with Avapritinib. The structural organization of this region is essential for stabilizing the complex and

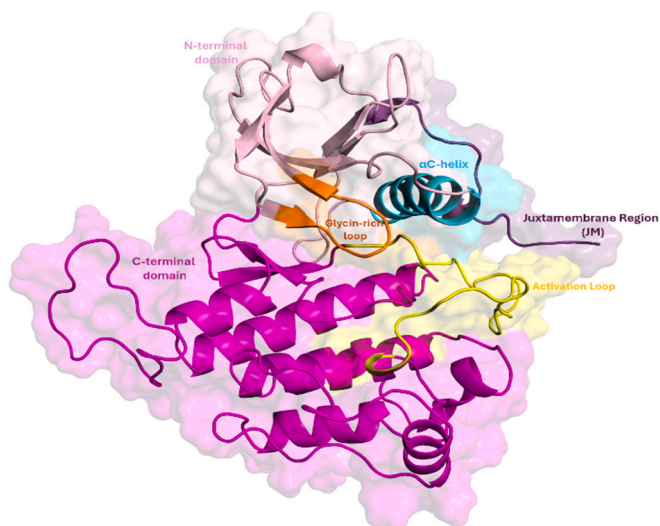


Fig. 4. c-KIT architecture. PDB ID (1PKG). The N-terminal domain, the C-terminal domain, the Gly rich loop, the α C-helix, the activation loop and the JM region are depicted in light pink, magenta, orange, cyan, yellow and purple, respectively.

accounts for the high degree of binding complementarity observed. Fig. 6, Panel C shows the 3D localization of the aforementioned residues.

The free energy landscapes (FELs) obtained from the first two principal components are shown in Fig. 7, Panel A, and reveal that both the apo and ligand-bound systems populate multiple metastable conformational states. In all replicas, one or more well-defined low-energy basins are observed, indicating the existence of preferred conformations sampled during the simulations.

The apo state exhibits heterogeneous conformational landscapes across the three independent replicas. Although each simulation identifies distinct low-energy basins, the overall topology of the FELs suggests that the protein is able to explore multiple conformational substates, with differences in the extent of sampling among replicas.

Similarly, the ligand-bound simulations display multiple energetic minima. However, the sampled conformational space appears generally more localized around a limited number of low-energy basins, suggesting that ligand binding favors specific conformational substates while preserving a certain degree of structural flexibility.

2.2.3. cKIT with Olverembatinib and Labuxtinib

After the analysis of Avapritinib, we moved to the other compounds, firstly analyzing Olverembatinib and Labuxtinib with the inactive form of cKIT. The RMSF results are reported in Fig. 8 for Olverembatinib and Fig. 9 for Labuxtinib, while the MM/GBSA calculations results for both compounds are reported in Fig. 10.

The primary differences observed in the RMSF analysis of cKIT in the presence and in the absence of Olverembatinib are mainly localized within regions 585–650 (Fig. 8, Panel A). Notably, ligand binding appears to increase overall residue fluctuation, with distinct peaks evident in this region. From the SASA analysis (Fig. S3, Panel A), we could detect that Olverembatinib binding alters the JM region by exposing G565 and P573 to the solvent. In the P-loop region, ligand presence leads to solvent exposure of G596 and F600. Upon ligand binding, residues H630 and T632 in the α C helix become fully solvent-exposed, whereas they are only partially exposed in the absence of the ligand; conversely, A636 becomes partially solvent exposed upon ligand interaction. The S639–L647 segment forms a buried cluster, Y646 remaining partially exposed in the APO protein. Additionally, ligand binding renders E640 solvent exposed. The DFG peptide accessibility is also affected, as D810 is partially exposed in the bound state but buried when unbound. In the activation loop, ligand binding results in solvent exposure of R815; no

significant changes are observed within the C-terminal lobe.

The MM/GBSA calculations predicted a favorable binding free energy of $\Delta G_{\text{bind}} = -66.6 \pm 1.04$ kcal/mol, suggesting that the complex remains energetically stable throughout the trajectories. As evidenced by the energetic profile, the stabilization of the system is primarily driven by a substantial van der Waals contribution, reflecting a high degree of steric complementarity between Olverembatinib and the inactive conformation of the cKIT binding pocket (Fig. 10, Panel A). This stabilization is further reinforced by favorable electrostatic interactions, which represent specific polar contacts and hydrogen bonds established with the surrounding residues. While van der Waals forces describe the overall shape complementarity, the electrostatic component provides directional interactions that are critical for orienting and anchoring the ligand within its binding pose.

Per-residue MM/GBSA decomposition identified K623 (Fig. 10, Panel B) as the residue providing the largest favorable contribution to ligand binding, indicating that it represents one of the principal energetic anchors of the complex. Such a marked contribution suggests that K623 establishes persistent interactions with the ligand throughout the simulations, likely through a combination of electrostatic contacts and hydrogen-bonding interactions, which contribute to maintaining a stable binding pose. The prominent energetic contribution of K623 is particularly noteworthy, as this highly conserved catalytic lysine is essential for ATP recognition and kinase activation [27]. Persistent interactions with this residue may therefore contribute to the effective stabilization of the ligand within the ATP-binding pocket while simultaneously interfering with the molecular interactions required for catalytic activity. Structurally, residues C809 to R815 belong to the activation loop (A-loop) of the kinase domain, a regulatory element that plays a central role in controlling the transition between inactive and active conformations of cKIT [56]. Ligand interactions with this region are therefore particularly relevant, as stabilization of specific A-loop conformations can directly influence kinase activity. The favorable energetic contributions observed for these residues indicate that the ligand establishes extensive contacts with the activation loop, potentially restricting its conformational flexibility and contributing to the stabilization of the bound state observed during the molecular dynamics simulations. The energetic importance of these residues is also consistent with their location within the ATP-binding cleft. Together with K623, which lies within the N-lobe close to the ATP-binding region, the C809–R815 segment forms part of the structural environment surrounding the ligand. Consequently, the favorable MM/GBSA contributions reflect both the high geometric complementarity of the ligand within the binding pocket and the establishment of multiple stabilizing non-covalent interactions distributed across functionally important regions of the kinase domain.

The free energy landscape (FEL) analysis (Fig. 7, Panel B) reveals that cKIT, when sampled in its inactive state, explores a variety of metastable conformational states, a behavior similar to that observed in the active conformation. In particular, replica 2 exhibits a notably heterogeneous landscape, characterized by a broader exploration of the conformational space. Conversely, replicas 1 and 3 display more restricted landscapes with a limited number of well-defined low-energy basins. In the presence of Olverembatinib, the protein appears to undergo a stabilizing effect, as evidenced by the consistent population of only two localized energy basins across the trajectories. This observation suggests that the ligand promotes the occupancy of specific structural substates, thereby enhancing the overall stability of the complex.

RMSF analysis of cKIT in complex with Labuxtinib shows an increased flexibility in the region delimited by the residues 610–616 (Fig. 9, Panel A), while in the C-terminal lobe a decreased flexibility is observed in the region delimited by residues 800–850. More in depth, residues in the region 800–820 exhibit reduced flexibility in the protein-ligand complex compared to the unbound protein. This decreased mobility is likely attributable to their location within the binding site; interaction with the ligand may restrict their movement, or they may

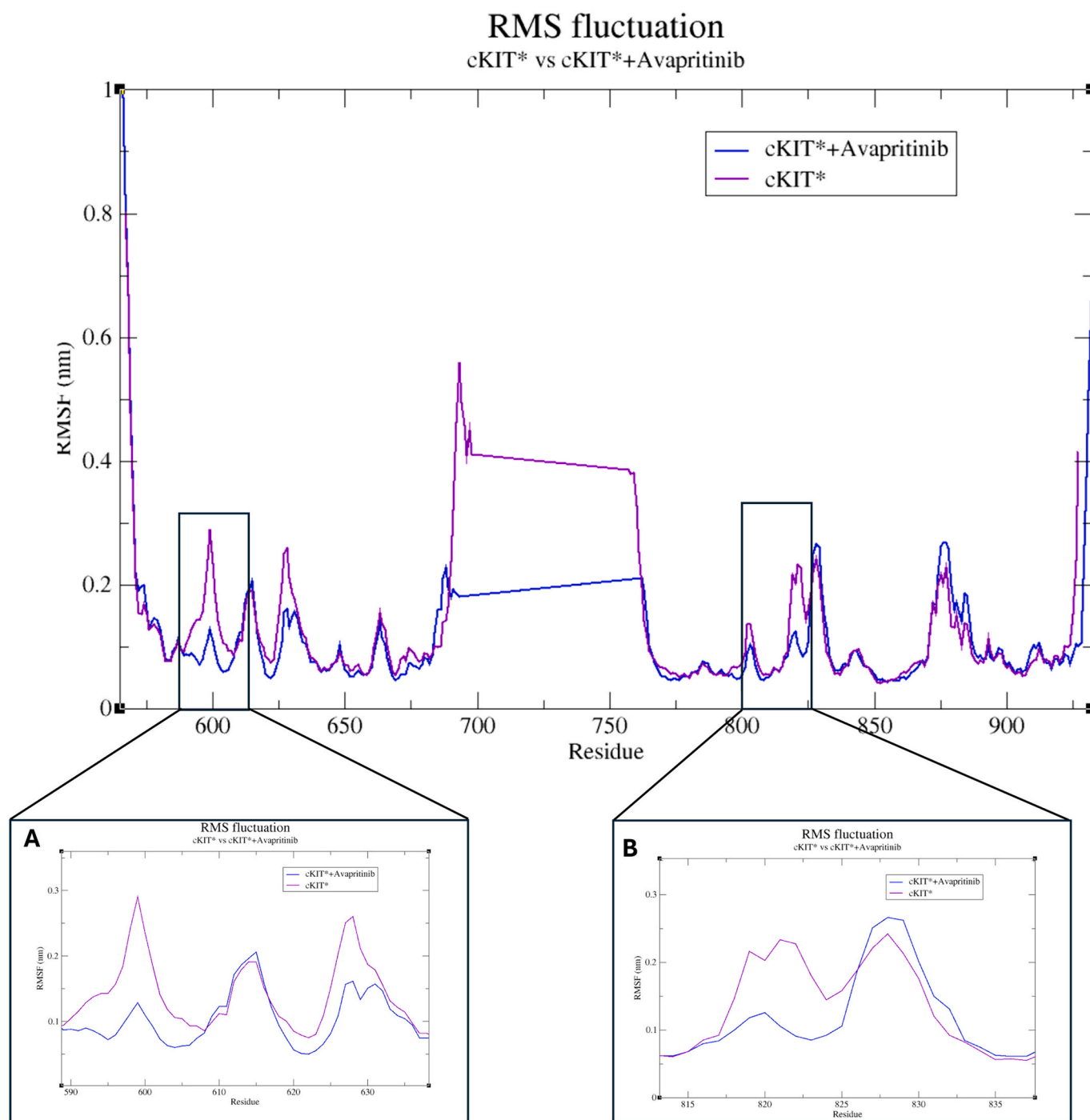


Fig. 5. RMSF analysis of cKIT* not complexed (violet) and complexed (blue) with Avapritinib.

participate directly in ligand binding, thereby limiting their flexibility.

The SASA analysis of cKIT in complex with Labuxtinib (Fig. S3, Panel B) indicates no significant differences between the APO and HOLO forms in the N-terminal domain. Notably, an important variation is observed in the region containing residues Y672-G676, which constitutes part of the hinge region frequently involved in ligand binding [57]. Within this segment, Y675 becomes partially solvent-exposed upon ligand engagement. The region defined by residues V846-G866 forms a structure that is largely protected from solvent exposure in the APO form. However, the presence of Labuxtinib alters its conformation, resulting in increased solvent accessibility for residues Y846, T847, and Q849. Additionally, in the C-terminal lobe, the I928 residue is only partially exposed to the solvent in the presence of the ligand.

As observed for Olverembatinib, the stabilization of the system is primarily driven by a substantial van der Waals contribution also in cKIT-Labuxtinib complex (Fig. 10, Panel D). Electrostatic interactions are less favorable if compared with those of Olverembatinib. However, the total ΔG_{bind} of -61.7 ± 0.62 suggests the occurrence of favorable binding interactions.

The energetic per-residue hotspots identified by the MM/GBSA decomposition are distributed across several functionally important regions of the kinase domain, including the N-terminal lobe (W556 and V603), the ATP-binding cleft and gatekeeper region (T670 and Y672), and the activation loop (C809 and D810), together with residues from the C-terminal lobe such as L799 (Fig. 10, Panel E). This distribution indicates that ligand stabilization does not rely on a single dominant

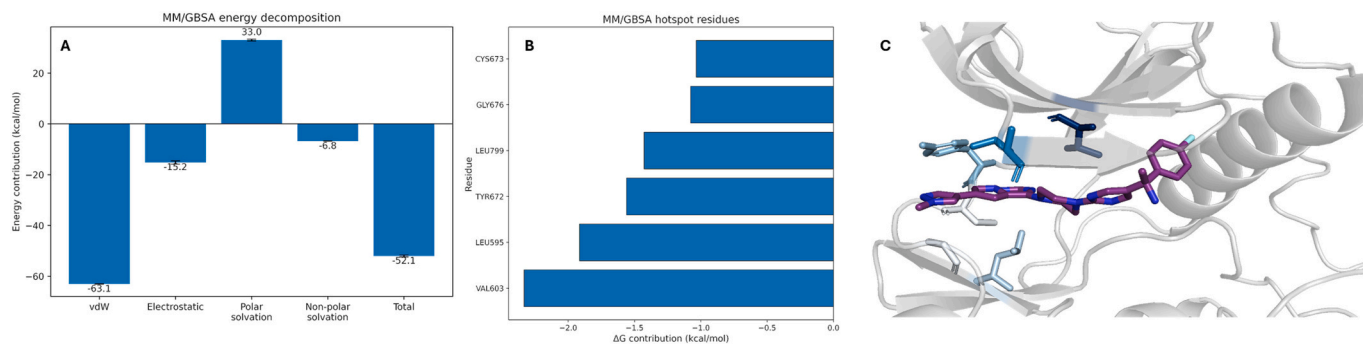


Fig. 6. MM/GBSA analysis of the cKIT-Avapritinib complex. (A) Binding free energy decomposition into the main energetic components, including van der Waals (vdW), electrostatic polar solvation, non-polar solvation, and the resulting total binding free energy (ΔG_{TOTAL}). (B) Per-residue MM/GBSA decomposition highlighting the hotspot residues with individual energetic contributions lower than -1 kcal/mol, identified as the principal contributors to ligand stabilization. (C) Three-dimensional representation of the protein–ligand binding site showing the spatial distribution of the energetic hotspot residues identified in panel B. Residues are colored according to their energetic contribution using a blue gradient, where darker shades indicate more favorable (more negative) contributions to ligand binding, whereas lighter shades correspond to weaker stabilizing effects.

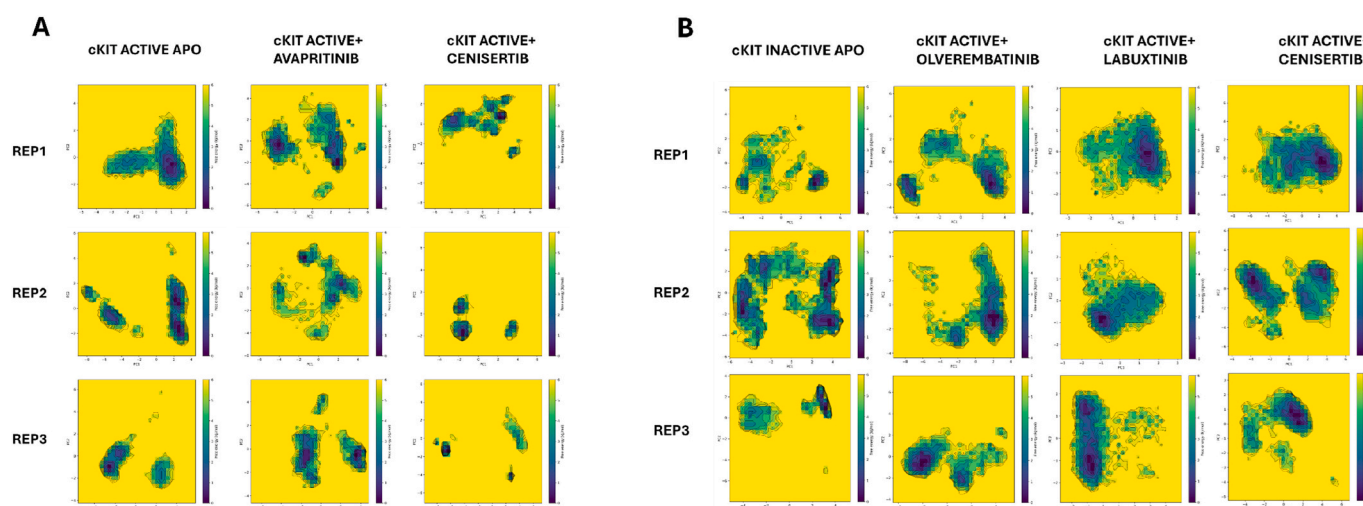


Fig. 7. Free Energy Landscape (FEL) for apo and holo systems.

interaction but instead arises from a cooperative network of hydrophobic and electrostatic contacts spanning the entire binding pocket. Such a binding mode is consistent with the favorable van der Waals and electrostatic contributions observed in the global MM/GBSA analysis and supports the predicted stability of the protein–ligand complex throughout the molecular dynamics simulations.

The free energy landscapes (Fig. 7, Panel B) obtained from the three independent replicas revealed a highly consistent conformational behavior. In two replicas, the HOLO complex was characterized by a single, well-defined low-energy basin, indicating that the system predominantly sampled one energetically favorable conformational state throughout the simulations. Such a landscape is generally associated with a structurally stable complex undergoing only limited large-scale conformational rearrangements. Although the third replica displayed two low-energy basins, these remained confined within a restricted region of the conformational space, suggesting transitions between closely related substates rather than sampling of structurally distinct conformations. The presence of an additional minimum therefore likely reflects the intrinsic dynamic nature of the protein rather than a loss of complex stability.

2.2.4. cKIT with Cenisertib

The investigation was then expanded to Cenisertib in conjunction with both forms of the protein. Cenisertib, being a Type I inhibitor as

demonstrated by X-Ray crystallography (see below), can bind both active and inactive conformations of cKIT.

Fig. 11 shows the RMSF analysis for Cenisertib in complex with the active cKIT*. Notably, differences in residue fluctuations are most prominent within residues 600–622 (Fig. 11, Panel A), where ligand binding appears to decrease the F600 fluctuations, while increasing the mobility of residues 612, 613, and 614. Conversely, three distinct peaks are observed between residues 800–840 (Fig. 11, Panel B); Cenisertib binding reduces fluctuations at positions such as 804 and 820–823, while increased flexibility is observed in the activation loop from the residues 825 to 835. Lastly, increased fluctuations are also evident in the C-terminal region encompassing residues 870–890 (Fig. 11, Panel C).

In the active state (Fig. S4), SASA analysis reveals that residues 564–569 of the JM region are exposed or partially exposed to the solvent. T574, is completely exposed to the solvent in the APO form, while is only partially exposed upon Cenisertib binding. Within the P-loop region, F600 is not exposed in the APO form, but it is partially exposed in presence of the ligand, as well as V603, which becomes exposed only upon ligand binding. Additionally, ligand binding in the APO form disrupts the cluster of buried residues from N652 to T661, inducing the partial exposure of L657 upon ligand binding. Cenisertib binding induces also a conformational change in the DFG peptide, notably causing G812 to become buried, indicative of ligand interaction, while in the C-lobe, D908 becomes solvent-exposed.

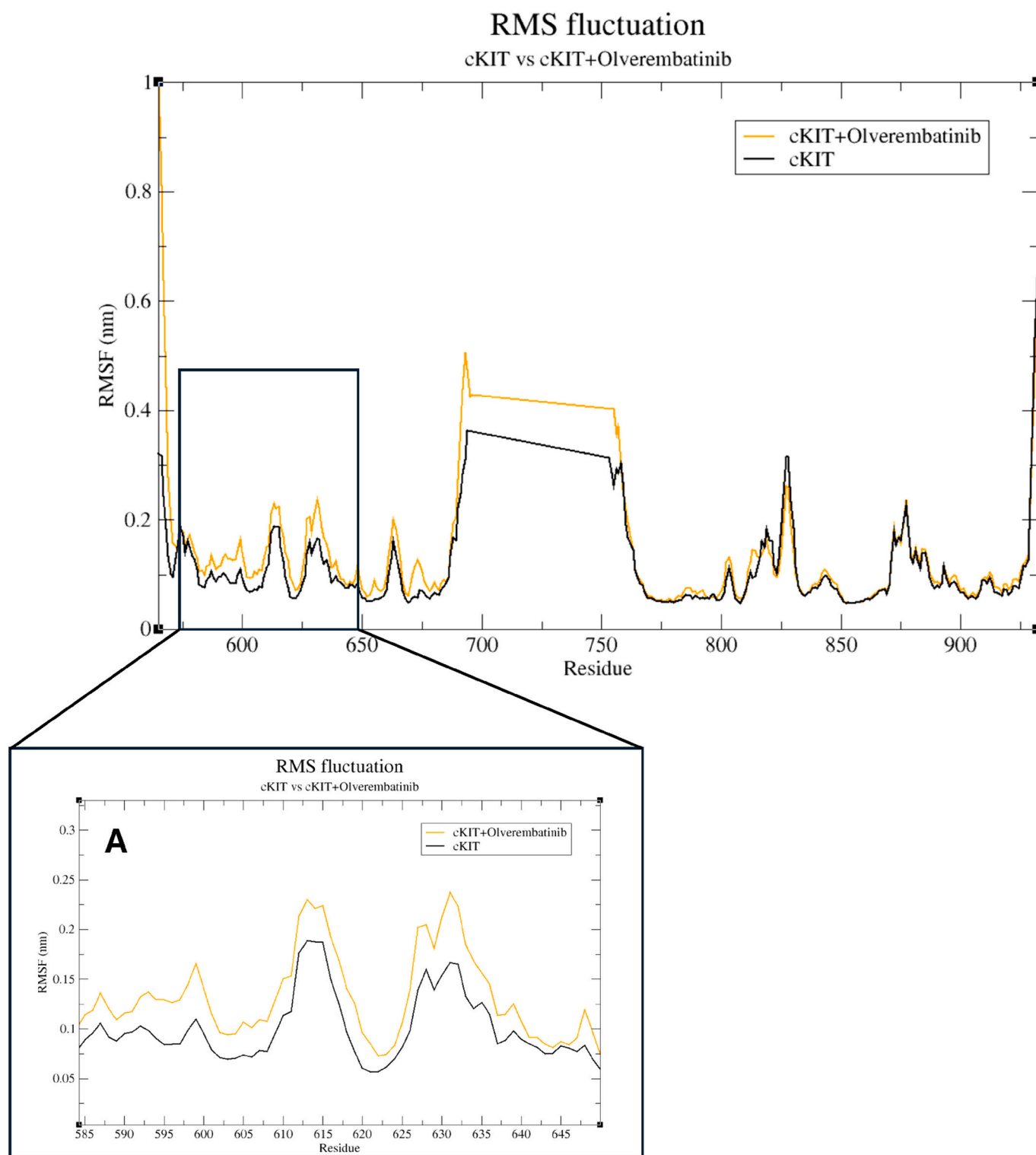


Fig. 8. RMSF analysis of cKIT not complexed (violet) and complexed (orange) with Olverembatinib.

The RMSF analysis of the inactive conformation of cKIT in complex with Cenisertib indicates no significant differences between the two models, as illustrated in Fig. 12. Ligand binding does not notably affect fluctuation patterns, especially within key regions such as residues 600 to 620. Conversely, the fluctuation profile reveals notable differences within the 800–830 region (Fig. 12, Panel A), where ligand binding affects the residues by reducing the fluctuations. Ligand binding appears to influence the dynamics of residues 825–830, and although

fluctuations are elevated, their extent remains less pronounced than observed in the APO form.

With respect to SASA (Fig. S5), the segment encompassing residues N555 to 560 in the JM is totally buried. I571 is buried upon ligand binding, while is partially exposed to the solvent in the APO form. Conversely, E583 is only shielded from solvent exposure in the presence of Cenisertib. Ligand binding also affects F600 in the P-loop region, rendering it partially solvent-exposed, while G610 becomes buried.

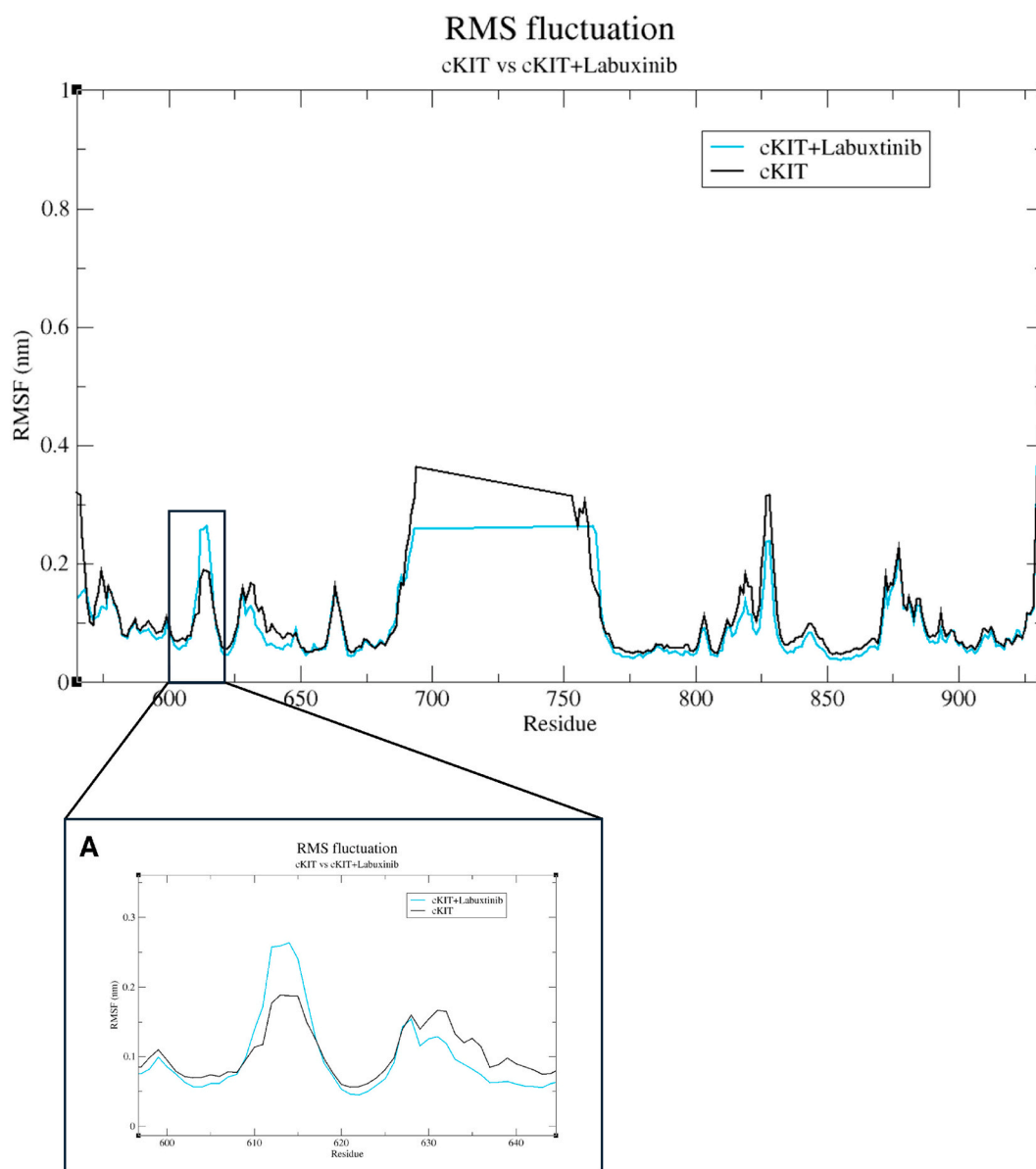


Fig. 9. RMSF analysis of cKIT not complexed (violet) and complexed (cyan) with Labuxtinib.

Most α C-helix residues are buried in both APO and HOLO conformations; however, M638 transitions to a buried state exclusively upon ligand binding, indicating potential involvement in ligand interactions. L631 increases its exposure to the solvent, moving from the HOLO to the APO form. No other important differences in SASA analysis were detected.

The MM/GBSA analysis (Fig. 13) revealed that the overall binding free energy was remarkably similar for the active and inactive conformations of cKIT, suggesting that the ligand is able to establish a comparably stable bound state in both kinase conformations. Although the energetic balance leading to complex stabilization differed slightly between the two systems, the resulting binding free energy remained essentially unchanged, indicating that the ligand can efficiently accommodate both conformational states of the kinase. A comparison of the individual energetic components showed that the van der Waals contribution was nearly identical in the active and inactive forms (Fig. 13, Panel A and Panel D). This observation indicates that the overall steric complementarity between the ligand and the binding pocket is largely preserved irrespective of the kinase conformation. The ligand therefore maintains an extensive network of short-range

hydrophobic contacts in both systems, suggesting that the architecture of the binding cavity remains sufficiently conserved to support a similar packing efficiency. In contrast, the inactive conformation displayed a more favorable electrostatic contribution, indicating subtle differences in the orientation of polar or charged residues surrounding the ligand. Such variations are expected considering the structural rearrangements accompanying the transition between active and inactive kinase states, which may alter hydrogen-bond geometries or electrostatic interactions without substantially modifying the overall topology of the binding pocket. Interestingly, the stronger electrostatic stabilization observed in the inactive conformation did not translate into a more favorable overall binding free energy. This finding indicates that the energetic gain provided by electrostatic interactions is largely compensated by other energetic terms, particularly the polar solvation contribution, resulting in an almost identical ΔG_{bind} for the two conformational states. Consequently, ligand recognition appears to be governed by a robust energetic balance rather than by the dominance of a single interaction type.

Per-residue MM/GBSA decomposition (Fig. 13, Panel B and Panel E) further supports this interpretation. The energetic profiles of the active and inactive complexes were highly similar, with most hotspot residues

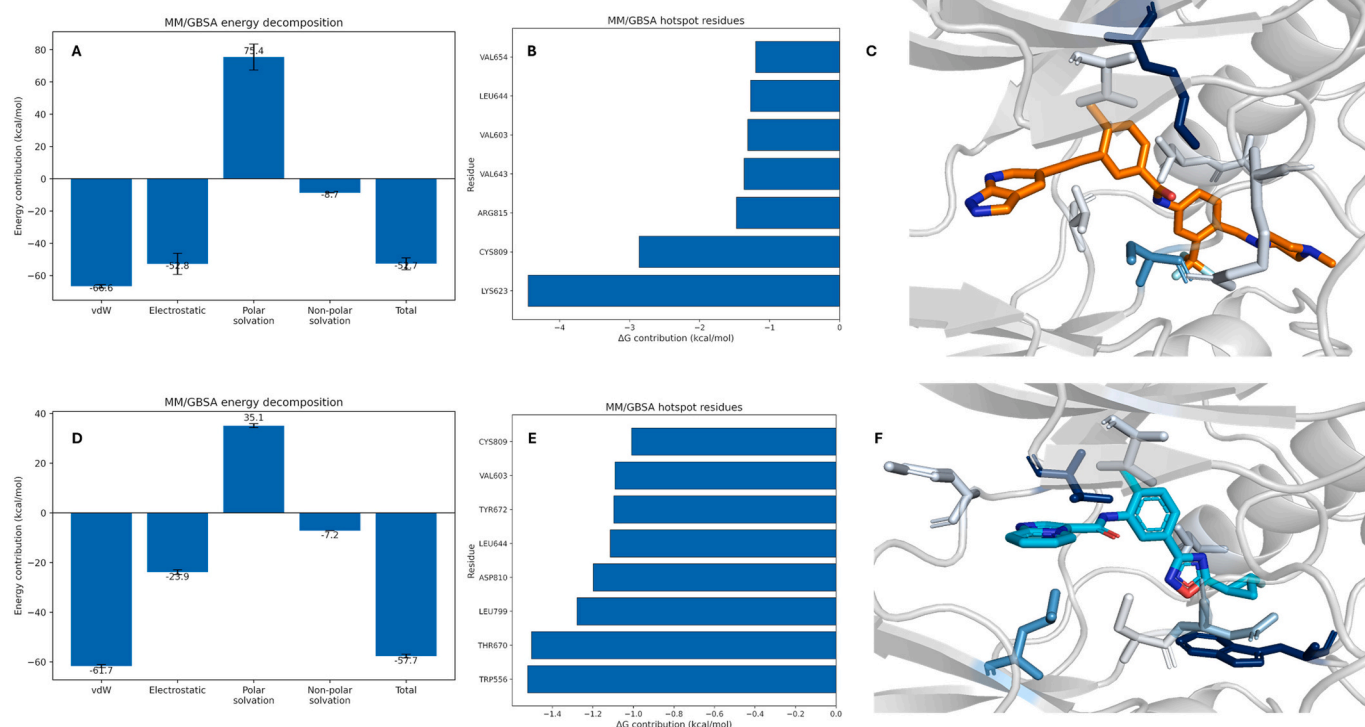


Fig. 10. MM/GBSA analysis of cKIT-Olverembatinib (A–C) and cKIT-Labuxtimib complexes (D–F). Energetic characterization of the Olverembatinib complex, displaying the total binding free energy decomposition into its fundamental components (A), identification of per-residue energetic hotspots with contributions below -1 kcal/mol (B), and the spatial mapping of these residues within the binding pocket (C). Residues in the 3D representation are colored via a blue gradient, with darker intensities representing stronger stabilizing contributions. (D–F) Corresponding energetic and structural analyses for the cKIT-Labuxtimib system illustrating the principal molecular determinants of ligand recognition.

contributing comparably to ligand stabilization in both systems. The principal difference involved F811, which emerged as the dominant energetic contributor in the inactive conformation, whereas C673 provided the largest contribution in the active state. This shift likely reflects local rearrangements of the ligand within the binding pocket, allowing different residues to establish the most favorable contacts while preserving the overall interaction network. Importantly, the remaining hotspot residues were largely conserved between the two conformations. Many of these residues are predominantly hydrophobic and constitute the structural framework of the ATP-binding pocket, explaining the nearly identical van der Waals contribution observed in the global MM/GBSA analysis. Their persistence across both conformations indicates that ligand stabilization is primarily driven by a conserved hydrophobic interaction network, whereas conformational changes mainly redistribute local electrostatic interactions without altering the overall binding strength.

FEL analysis (Fig. 7, Panel A and Panel B) of both active and inactive conformation of cKIT in complex with Cenisertib reveals a good stability of the complex, with few observed minima. In particular, the cKIT active-Cenisertib complex explores a relatively restricted conformational space, with the vast majority of sampled conformations clustered within a well-defined low-energy basin. The presence of a dominant free energy minimum indicates that, following the initial equilibration, the complex preferentially populates a single energetically favorable conformational state rather than continuously exploring multiple structurally distinct conformations. This behavior suggests that ligand binding effectively confines the conformational ensemble of the protein, limiting large-scale structural rearrangements while preserving the integrity of the binding mode throughout the simulation.

In the inactive conformation, the free energy landscape revealed a broader exploration of the conformational space compared with the active state. Nevertheless, the sampled conformations remained

confined to one or, at most, two well-defined low-energy basins across the independent replicas. This observation indicates that, despite a greater degree of conformational freedom, the protein–ligand complex still preferentially populates a limited number of energetically favorable states rather than continuously exploring multiple unrelated conformations. The observation of a secondary basin in replica 2, for example, most likely reflects transitions between closely related conformational substates separated by relatively low free energy barriers, rather than the existence of alternative binding modes or partial dissociation events. This interpretation is further supported by the MM/GBSA analysis, which yielded binding free energies comparable to those obtained for the active conformation, indicating that these substates correspond to energetically equivalent bound configurations.

2.3. LiP-MS analysis

As validation of MD simulations, a Limited Proteolysis-Mass Spectrometry (LiP-MS) approach was employed to identify the binding site and the conformational changes induced on both active and inactive cKIT forms by the binding of selected inhibitors. This methodology is based on the evidence that in the protein–ligand complex, the binding site is less accessible to protease hydrolysis than when the protein is in its native free-state. The conformational differences occurring upon the formation of the protein–inhibitor complex can be monitored by using proteases as conformational probes. More recently, a limited proteolysis protocol combining a first short hydrolysis step with an unspecific protease, i.e. Proteinase K, followed by an extensive trypsinization has been developed and applied to the identification of drug's target proteins at the level of a whole proteome [58]. We applied a similar procedure to probe the binding region of the 4 cKIT inhibitors, Cenisertib, Olverembatinib, Labuxtinib and Avapritinib. The procedure was applied in parallel on the protein in the presence and in the absence (control) of

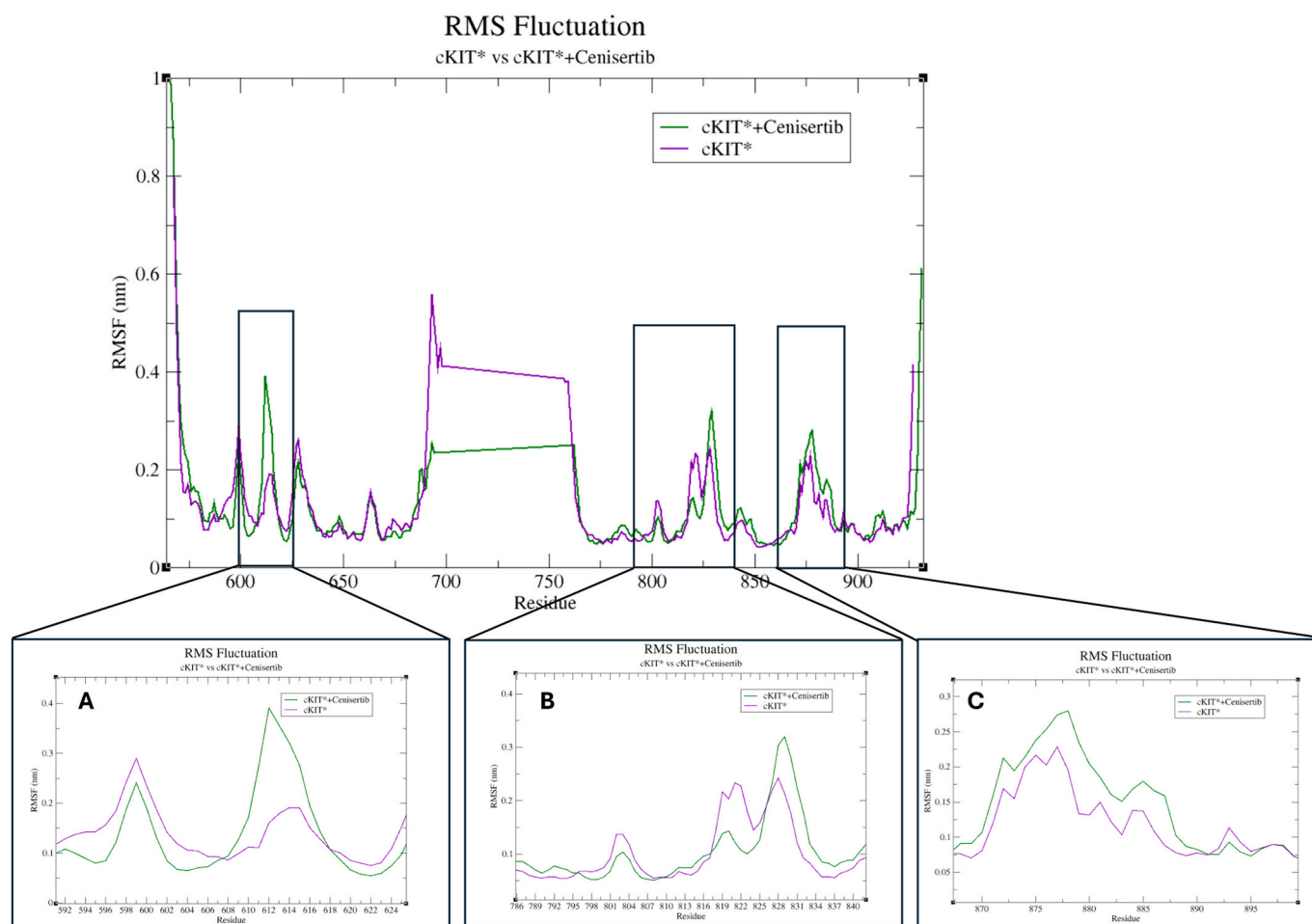


Fig. 11. RMSF analysis of cKIT* not complexed (violet) and complexed (green) with Ceniseritib.

each potential inhibitor. A relative quantification of tryptic and semi-tryptic peptides released in both conditions allowed the identification of all regions sensitive to the occurrence of binding (“hot regions”), which include the region(s) directly involved in the interaction with the ligand (short range effects) but also those regions far away from the binding pocket, whose conformations change as consequence of protein flexibility (long range effects). In particular, tryptic and semi-tryptic peptides released upon the proteolysis from the “hot regions” are expected to be up- and down-regulated, respectively, in the complexed protein in comparison to the control (Fig. 14).

2.3.1. Conformational comparison of active (cKIT*) vs inactive cKIT

As previously done for MD, the LiP-MS method was employed to preliminarily probe the conformational perturbations occurring in the hyperphosphorylated state of the active cKIT*, in comparison with the hypo-phosphorylated inactive form. Active (cKIT*) and inactive cKIT recombinant intracellular domains were first digested with Proteinase K for one minute and then hydrolysed overnight with trypsin in denaturing conditions.

The obtained peptide mixtures were then analyzed by LC-MS/MS system and tryptic and semi-tryptic peptides identified and quantified through Proteome Discoverer software. Five replicates were performed for each condition, with two injections of each sample. Perseus was employed for statistical analysis (see M&M).

The obtained MS and MSMS data were first analyzed with the aim to identify and relatively quantify the phosphorylation sites on both active and inactive cKIT forms. Given the enormous heterogeneity of the phosphorylated species, we decided to focus on sites showing a

phosphorylation level over 25% of all the forms present (Fig. 15, Panel A, Supplementary Table S1), assuming they would have the greatest impact on the conformational states of the kinase. In addition, it is noteworthy that the sites showing phosphorylation greater than 25% are those already reported in the literature [36,37,59].

As expected, a greater number of phosphosites were identified in the active form of cKIT in comparison to the inactive form. As expected, the vast majority of these phosphosites have been reported to be implicated in the process of activation of the kinase (see Supplementary Table S1).

Then we moved to the analysis of the distribution of hydrolysis sites identified with the LiP-MS approach for the characterization of the conformational differences occurring between the active and inactive cKIT forms. Differently from the canonical LiP-MS approach focused on the identification of disappearing hydrolysis sites upon the interaction with a ligand, in this case we monitored both the increasing and decreasing semi-tryptic peptides amount, in order to monitor both positive (appearing) and negative (disappearing) variations.

Utilizing this approach and applying the stringent parameters for data analysis reported in M&M session, we were able to identify 206 statistically significant peptides, allowing a sequence coverage of 94%. This achievement is of paramount importance since it suggests that the protocol developed allowed monitoring conformational variations overall the protein. From the list of peptides, we filtered the ones that respected LiP-MS criteria for the identification of conformational changing regions. The results are summarized in Supplementary Table S2 and graphically reported in Fig. 15, Panel B.

In particular, the sites resulted higher or lower exposed in the active cKIT* intracellular domain in comparison to its inactive form are

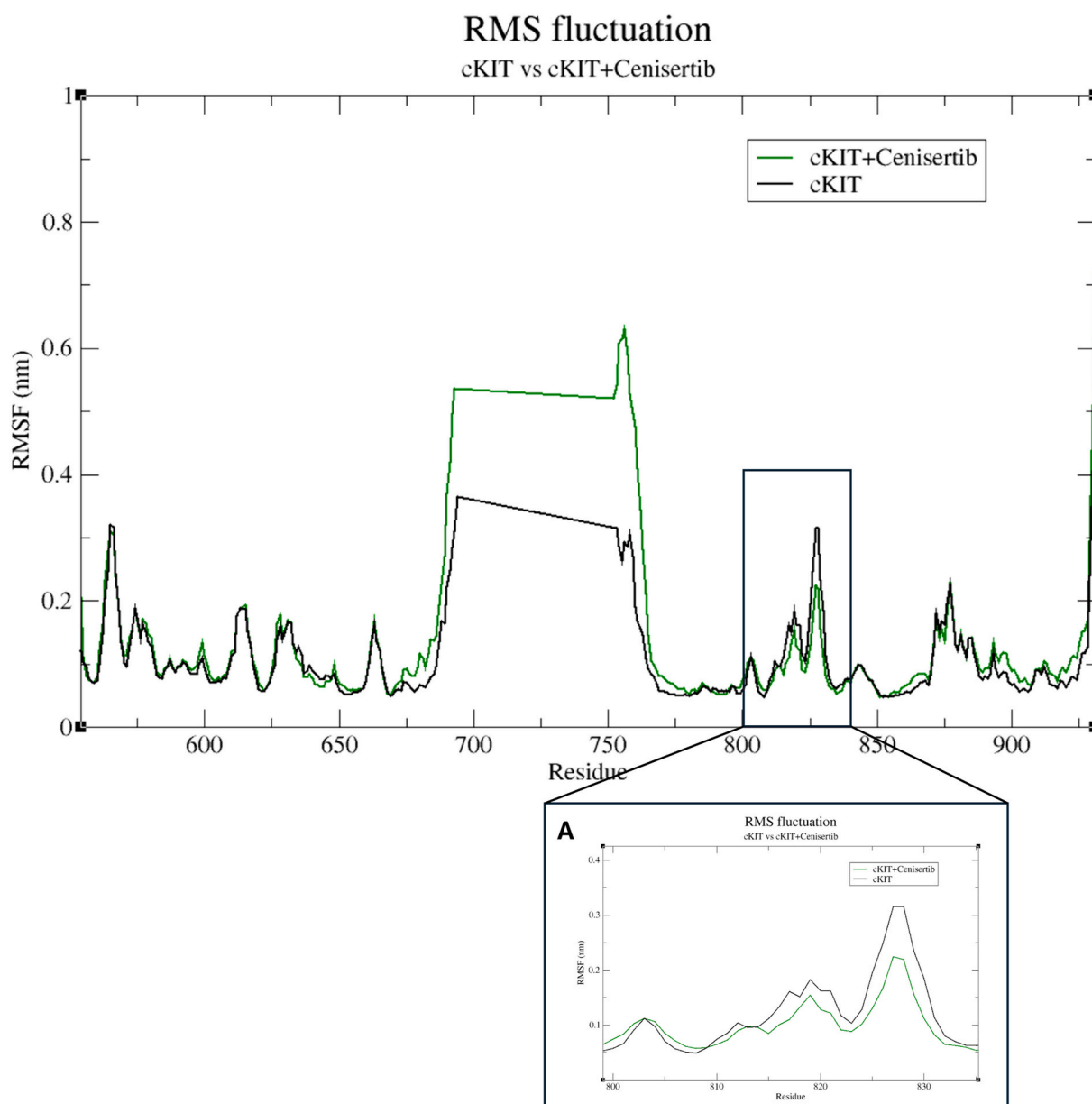


Fig. 12. RMSF analysis of cKIT not complexed (violet) and complexed (green) with Cenisertib.

reported in dark and light black, respectively. The residues reported to be involved in ATP binding are highlighted in blue. The residues represented in an intermediate shade don't have a unique behavior, since they were found contemporarily among the buried and exposed residues.

As can be seen, the highest part of sites changing their exposure encompasses residues C691-S729. These latter fall in a huge loop, slightly down-stream the ATP binding pocket, characterized by a great disorder and flexibility, as highlighted by the high values of the FCs measured for the complementary tryptic/semityryptic peptides. Moreover, this feature is confirmed by the identification of two residues (C961, S962), both in up- and down-regulated semi-tryptic peptides. In fact, it can be hypothesized that the flexibility of this loop is so high, to continuously and quickly change its conformation, leading C961 and S962 residues sometimes to be exposed and sometimes to be hidden.

Notably, some of the hidden LiP residues (E605, A621 and E633) in cKIT* are located within the ATP binding site: this finding suggests that the ATP binding pocket might be less accessible in the active form for the presence of the cofactor or for a conformational change leading to the

partial closure of the cavity. Finally, some residues at the N- (such as Y570, D572, T574) and at the C- terminus (S943) were univocally identified as more exposed in the active form. The higher solvent exposition of Y570 is also confirmed by the SASA results, in which V569 (SASA active: $0.63 \pm 1.72 \times 10^{-1}$ M; SASA inactive: $0.50 \pm 1.40 \times 10^{-1}$ M) and I571 (SASA active: $0.44 \pm 2.63 \times 10^{-2}$ M; SASA inactive: $0.23 \pm 2.32 \times 10^{-2}$ M) are more exposed in the active form compared to the inactive one.

The LiP sites identified by comparing the active and the inactive forms of cKIT represent the hallmarks of conformational changes occurring upon the phosphorylation of specific tyrosine residues in the kinase activation process. Therefore, this aspect has been taken into account in the interpretation of LiP-MS data referring to the effects of the inhibitors.

2.3.2. LiP-MS analysis of complexes of cKIT and Avapritinib

Upon the characterization of the conformational differences between the active (cKIT*) and the inactive forms of cKIT, deriving from their different phosphorylation states, the focus shifted to the study of the

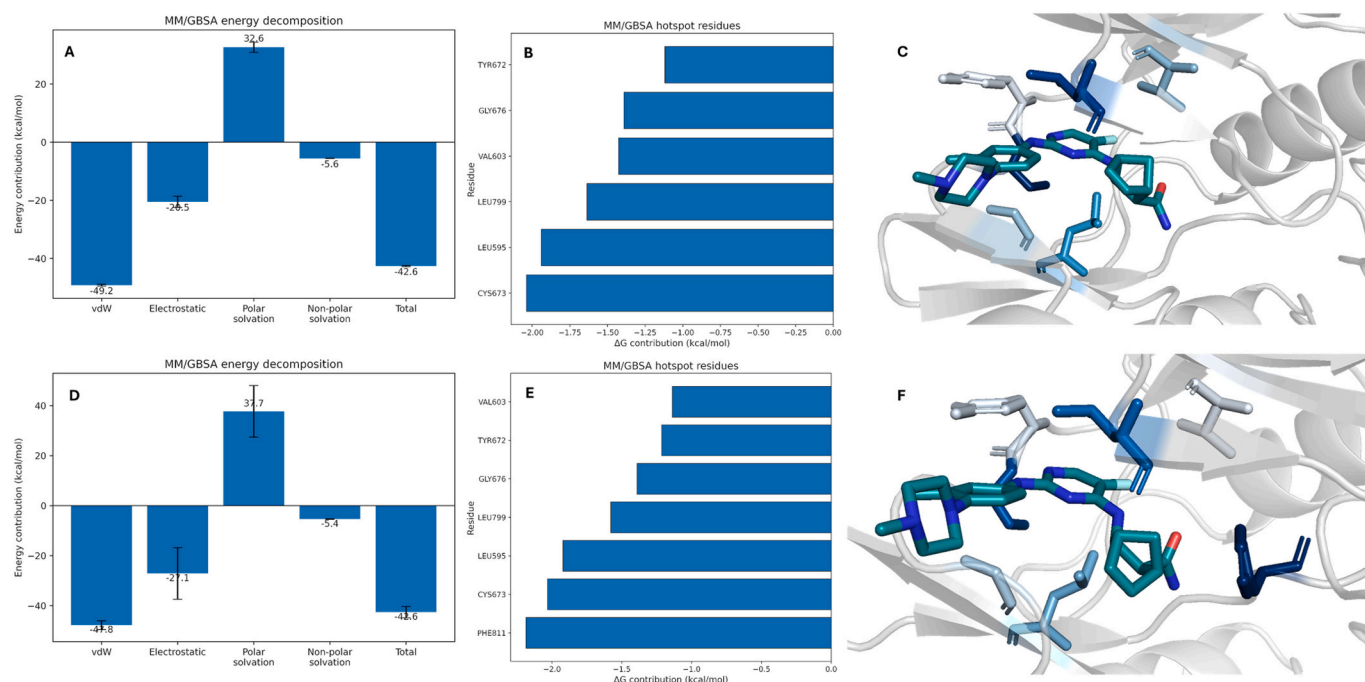


Fig. 13. MM/GBSA analysis of cKIT-Cenisertib in the active (A–C) and inactive (D–F) conformation. Energetic characterization of the cKIT displaying the total binding free energy decomposition into its fundamental components (A, D), the identification of per-residue energetic hotspots with contributions below -1 kcal/mol (B, E), and the spatial mapping of these residues within the binding pocket (C, F). Residues in the 3D representation are colored via a blue gradient, with darker intensities representing stronger stabilizing contributions.

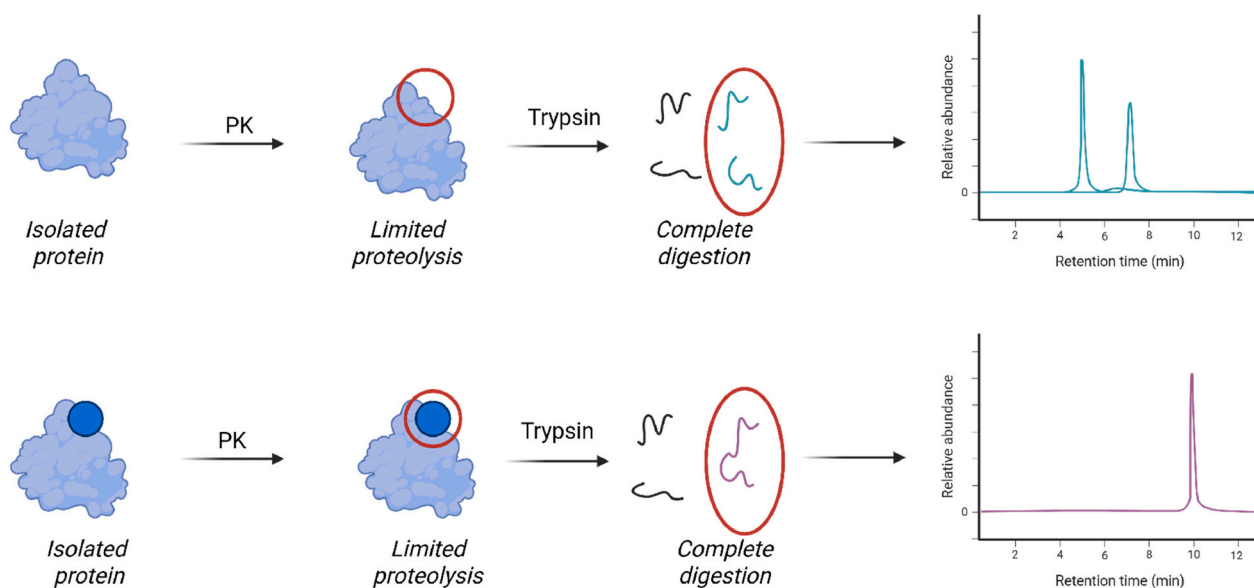


Fig. 14. Schematic representation of LiP-MS approach. The isolated protein and the complex protein-ligand are hydrolysed under controlled conditions and then a complete trypsination is performed. The differences lie in the relative abundances of the semi- and tryptic peptides in the regions affected by the binding with the small molecule.

binding mode of 4 cKIT inhibitors, Avapritinib, Olverembatinib, Labuxtinib and Cenisertib, monitored for both kinase conformations.

cKIT was incubated with each molecule for 30 min at r.t. and, afterwards, we proceeded with a limited proteolysis experiment in parallel on complex and the free protein for each condition (active and inactive). We expected that, in such instances, the binding of the small molecule to the protein results in the occlusion of the binding site, thereby preventing its exposure to enzyme-mediated hydrolysis. Therefore, in this application, the up-regulated tryptic and the down-regulated semi-

tryptic peptides quantified in the complexed protein in comparison with the free one and released from the same region were exclusively monitored.

We started analyzing the complex with Avapritinib, a well-known cKIT inhibitor. The crystal structure of the Avapritinib-cKIT* complex has been resolved with $1.7 \text{ \AA}$ resolution [47], making this experiment a validation of LiP-MS approach.

According to LiP-MS results, many sites (see Supplementary Table S3) were hidden upon the incubation with the ligand, as reported in

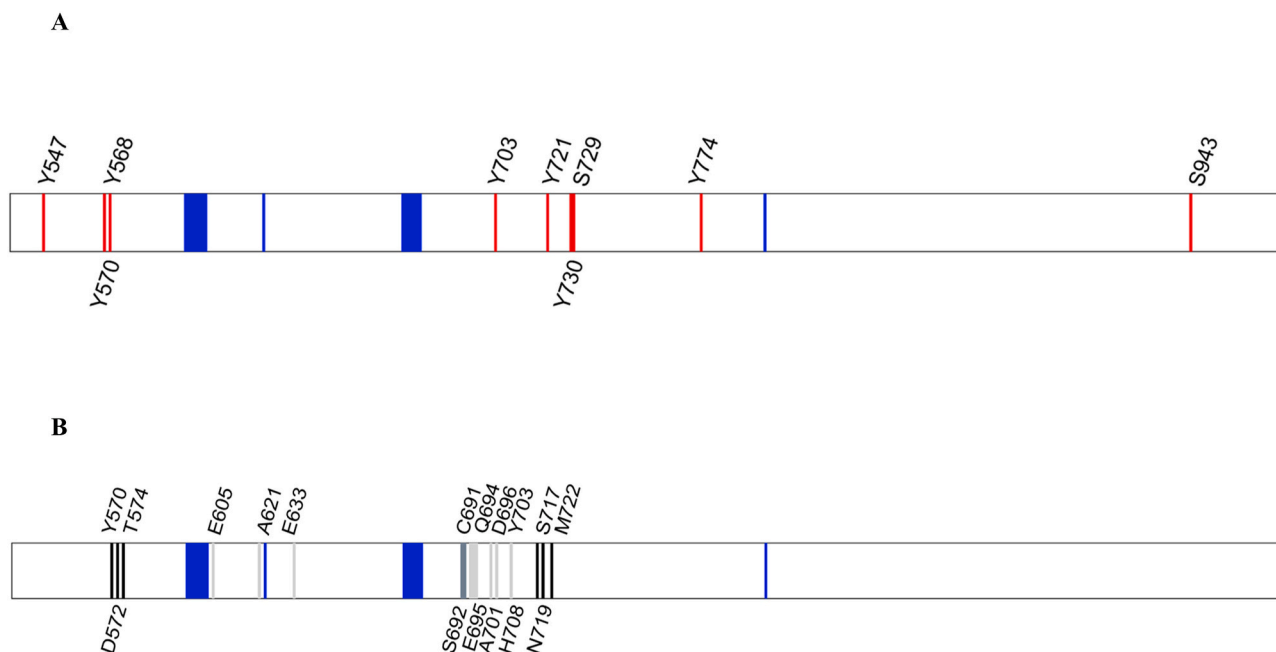


Fig. 15. A) Graphical representation of the intracellular domain of c-KIT. In blue the ATP binding sites reported in Uniprot (P10721). In red the identified phosphosites are reported. B) Intracellular domain of c-KIT. In dark and light black the most or less exposed residues are represented, respectively, in the active form vs the inactive one. In slate-grey, the residues that are both exposed and buried are reported. In blue, the ATP binding sites of Uniprot are reported.

Fig. 16. Panel A for the active form. From a preliminary and general overview, the proteolytic profile of cKIT* appears much richer than the inactive form (Supplementary Table S3).

Looking at the details of sites' distribution, it immediately appears that the clustering of most of the identified sites occurs in correspondence of the loop already discussed in the previous paragraph, which is

extremely subject to rapid and continuous conformational movements. Therefore, in light of this peculiar characteristic, we can speculate that the variations recorded at the level of this loop might derive from long range effects which strongly impact the most flexible regions such as this loop as well as the free N- and C- terminus ends of the recombinant protein.

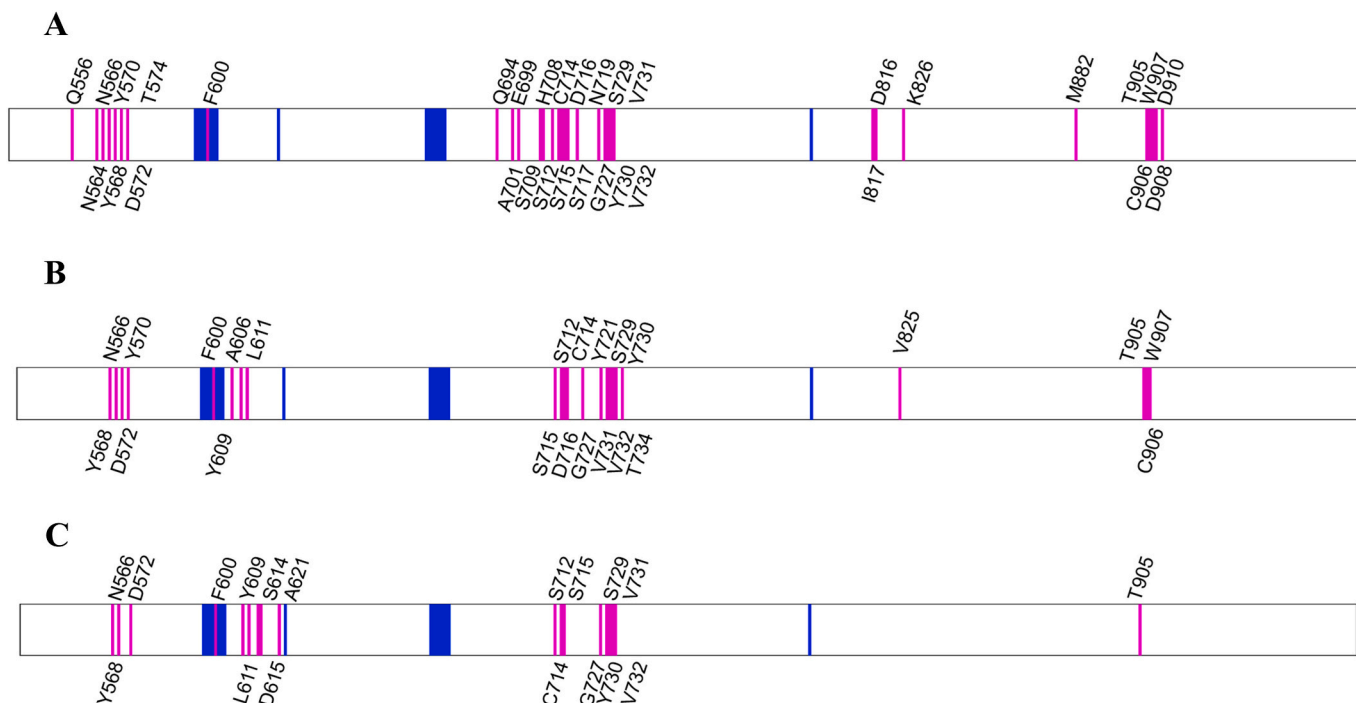


Fig. 16. A) LiP-MS results of the interaction between Avapritinib and c-KIT. Intracellular domain of c-KIT. In pink the residues buried after the interaction with Avapritinib in the active form of the protein are reported. In blue, the ATP binding sites of Uniprot are reported. B) LiP-MS results of the interaction between Olverembatinib and c-KIT. Intracellular domain of c-KIT. In pink the residues buried after the interaction with Olverembatinib in the inactive form of the protein are reported. In blue, the ATP binding sites of Uniprot are reported. C) LiP-MS results of the interaction between Labuxtinib and c-KIT. Intracellular domain of c-KIT. In pink the residues buried after the interaction with Labuxtinib in both forms of the protein are reported. In blue, the ATP binding sites of Uniprot are reported.

The most significant result concerns the identification of F600 as a hidden residue in cKIT* upon the interaction with the inhibitor. This amino acid is reported to participate in the ATP binding. This finding suggests that Avapritinib interacts with cKIT* in correspondence of the ATP binding site, according to the crystal structure and with the behavior expected for a I type inhibitor. Moreover, we also found buried the residues D816, I817 and Y826, again in agreement with the molecular dynamic results. In fact, the RMSF analysis showed a decreased flexibility between the residue 816 and 825.

Once validated the method with Avapritinib, we moved to the characterization of the binding with the other three compounds.

2.3.3. LiP-MS analysis of complexes of cKIT and Olverembatinib and Labuxtinib

The incubation of cKIT with Olverembatinib gave a reverse result in respect to the previous molecule (Fig. 16, Panel B and Supplementary Table S4). Indeed, the proteolytic pattern obtained for the inactive form was thicker of LiP sites than the one shown by cKIT* (Supplementary Table S4), suggesting a preferential binding with the inactive kinase.

Conversely, it is noteworthy that the impressive number of proteolytic sites identified in the inactive form, most of them specifically buried close to the ATP binding pocket (from F600 to L611). In the SASA analysis, G601 (SASA complex: $0.01 \pm 6.44 \times 10^{-3}$ M; SASA inactive: $0.04 \pm 1.29 \times 10^{-2}$ M) and K602 (SASA complex: $0.29 \pm 1.05 \times 10^{-2}$ M; SASA inactive: $0.37 \pm 1.28 \times 10^{-2}$ M) also are more buried when the protein interacts with the small molecule, according to LiP-MS results. These results agree with the expected behavior of a Type II compound as Olverembatinib is, which binds in the orthosteric site of the inactive kinase.

Very similar results were obtained analyzing the interaction of the kinase with Labuxtinib (Fig. 16, Panel C and Supplementary Table S5). As in the case of Olverembatinib, we identified many more proteolytic sites disappearing upon the interaction of Labuxtinib with the inactive form compared to the active one (Supplementary Table S5). Once again, the effects on the active form are concentrated at the level of unstructured regions (middle loop, N- and C-terminus), further confirming the considerations previously reported for Olverembatinib. Moreover, even in the presence of this inhibitor, on the inactive form, the most crowded region (neglecting the effects on the usual loop and on the amino and carboxy-terminal ends) occurs between F600 and A621, suggesting that

Labuxtinib, similarly to Olverembatinib, is interspersed in the ATP binding pocket. Overall, our data support the hypothesis that both these molecules are type II orthosteric inhibitors of cKIT, interacting with its inactive form at the level of the ATP binding site.

It is surprising that the LiP approach highlights the masking of F600 in the presence of all three orthosteric inhibitors, suggesting a partial common positioning of the three molecules in the binding site. However, in the presence of the two type II inhibitors, Labuxtinib and Olverembatinib, the number of residues that resulted in being hidden in the binding pocket was largely greater than that described for Avapritinib. This finding could derive from the fact that in the inactive form the binding pocket that normally hosts ATP is free and therefore larger than in the cKIT*, and the binding effects in the presence of orthosteric inhibitors are then more evident.

2.3.4. LiP-MS analysis of complexes of cKIT and Cenisertib

Finally, we analyzed the interaction between the two forms of cKIT and Cenisertib. In this case, the situation is a bit different compared to the other three compounds. In the LiP-MS results (Fig. 17 and Supplementary Table S6), we found enriched proteolytic patterns in both forms, according to the SPR analysis. However, except for the numerous sites present at the level of the usual loop, the two proteolytic patterns appear different. In particular, the distribution of hydrolysis sites at the N- and C-terminus found in cKIT* is compliant with the ones described in the other complexes. Also, in the inactive cKIT we found many sites at the ends of the kinase although its N-terminus appears less changeable than in cKIT* and the variations at C-terminus occur in a more extreme region, between N958 and S966. To these differences, a further, even more significant is added: in the inactive form, in the presence of the inhibitor, many sites within the ATP binding site between Y609 and A621 disappear, a phenomenon which is not evident in cKIT*. Among these hidden sites, we didn't identify the F600. Overall, these findings may suggest that Cenisertib might bind cKIT/cKIT* in different ways in comparison to the other three inhibitors. Apparently, neglecting the possible non-specific interactions involving the usual suspect regions, it behaves as a type II inhibitor, occupying the ATP binding pocket in the inactive form.

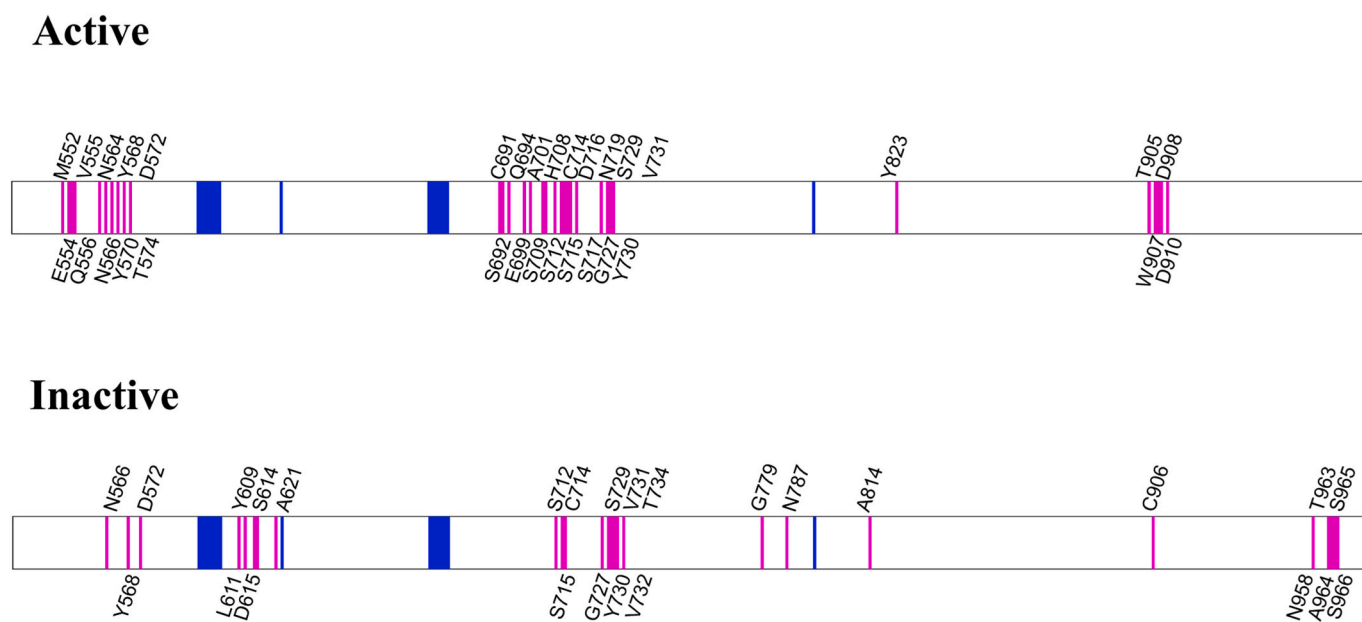


Fig. 17. LiP-MS results of the interaction between Cenisertib and c-KIT. Intracellular domain of c-KIT. In pink the residues buried after the interaction with Cenisertib in both forms of the protein are reported. In blue, the ATP binding sites of Uniprot are reported.

2.4. X-Ray crystallography

To characterize further details and confirm the binding mode of the compounds, we performed X-Ray crystallography for cKIT in interaction with Avapritinib, Labuxtinib and Cenisertib.

This approach allowed us to obtain an atomic resolution of the interaction between the kinase and each small molecule, further elucidating the binding mode.

2.4.1. Avapritinib

The Avapritinib crystal structure in complex with cKIT was solved in 2023 by A. Teube et al. [47]. In their work, they described a peculiar binding mode of Avapritinib, which acts as an ATP-competitive type 1.5 inhibitor, binding the active DFG-in conformation of KIT/PDGFR α while engaging in the hinge region. Notably, its binding induces the formation of a previously uncharacterized accessory sub-pocket (α -pocket) located between the α C-helix and the Gly-rich loop, which is not accessible in canonical APO or active structures. This pocket accommodates the fluorobenzene moiety and represents a key structural determinant underpinning the high potency and selectivity of the inhibitor. Additionally, a water-mediated hydrogen bond interaction is observed between the inhibitor and the gatekeeper residue T670, further

contributing to binding stabilization.

In addition to A. Teube et al., we also determined the crystal structure of the cKIT–Avapritinib complex, modelling a structure encompassing amino acid residues 567–931 in chain A and 579–931 in chain B. Residues 694–753 are missing from both chain A and B due to a deletion of Δ S688–D765 and replacement by a smaller peptide EFVPYKVAPED-LYKDFLT. In chain A, all amino acids in the smaller peptide are visible in the electron density, whereas in chain B nine amino acids of the peptide are so poorly visible in the electron density that they have not been modelled. The naming of the amino acids in the peptide is identical to the deposited structures (PDB ID: 6GQJ and 6GKQ). In addition to amino acids, two ligands (285), eleven chlorine (CL) and one bromine (BR) have been modelled as well as two glycerol (GOL) molecules and 374 water molecules. Since the crystal was obtained from a mixture of chlorine, bromine and iodine, the halogen most matching the electron density has been modelled. The binding site of the ligands in molecule A and B is more or less identical apart from the cysteine modification of residue Cys 788 (CSO) in chain B and the extra ligand interactions in chain A with Asn 567 and Tyr 568 from the traceable N-terminus.

As shown in Fig. 18, Panel A, Avapritinib occupies the ATP-binding site, with the induced α -pocket clearly visible and accommodating the fluorobenzene moiety of the ligand. Access to this sub-pocket is enabled

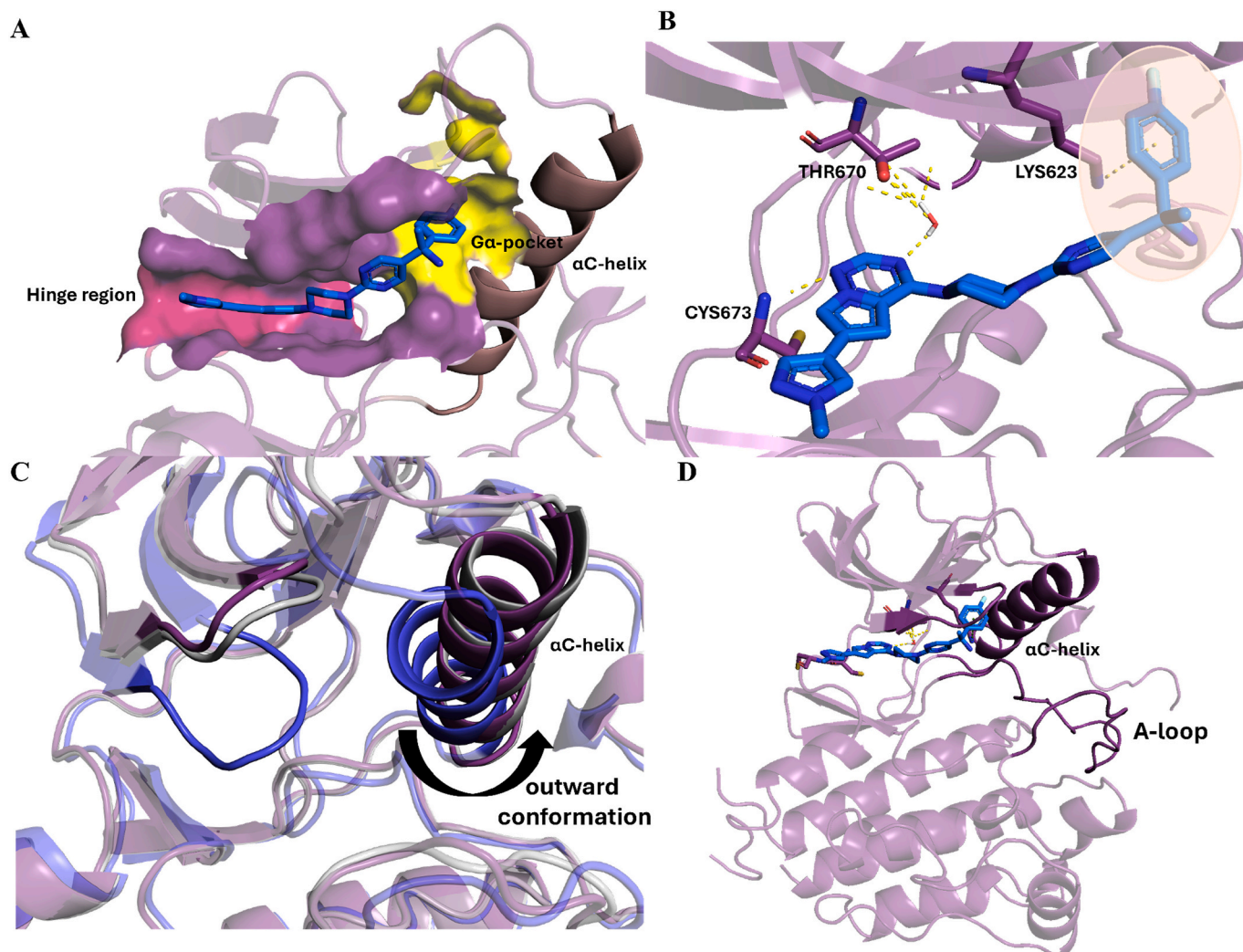


Fig. 18. Binding mode of Avapritinib as determined by our internal crystal structure: A) The fluorobenzene moiety is positioned within an accessory sub-pocket first identified by A. Teuber et al. B) Principal interactions observed between cKIT and Avapritinib. C) Comparison of α C-helix conformation among Avapritinib-bound models and the standard active conformation of cKIT (PDB ID 1PKG). Our model, the model of A. Teuber et al. (PDB ID 8PQ9), and 1PKG are depicted in violet, light grey, and blue, respectively. D) Overall structural representation of our model.

by the outward displacement of the α C-helix. The structural superimposition of 8PQA, our crystal structure, and 1PKG (Fig. 18, Panel C) reveals a conserved α C-helix conformation in both 8PQA and our model, whereas in 1PKG the helix is oriented toward the interior of the ATP-binding site. Consistently, the glycine-rich loop adopts an upward-displaced conformation in Avapritinib-bound structures, allowing access to the G α -pocket, while it remains closed in 1PKG. The activation loop adopts an extended active-like conformation consistent with a DFG-in state.

At the molecular level (Fig. 18, Panel B), a water-mediated hydrogen bond involving the N11 atom of the pyrrolotriazine scaffold is observed. In addition, cation- π interactions between the fluorobenzene moiety and the catalytic K623 contribute to ligand stabilization. The hinge region is also engaged, with the pyrrolotriazine nitrogen forming a hydrogen bond with the carbonyl group of C673.

The structure was crystallized as a dimer. Overall, chains A and B

display a highly similar structural organization, and no substantial chain-dependent differences were identified in the overall protein conformation. Thus, the two chains do not appear to represent clearly distinct global conformational states within the crystal structure. However, residues K693, D762 and T932 are resolved in chain A but not in chain B. This may reflect local differences in structural definition between the two copies present in the asymmetric unit, potentially arising from local conformational heterogeneity, differences in the crystallographic environment, or differences in the quality of the experimental electron density. Accordingly, chain A was selected as the starting model for the molecular dynamics simulations because it provided the more complete experimentally resolved structure. The selection of chain A was therefore based on the completeness of the available experimental coordinates.

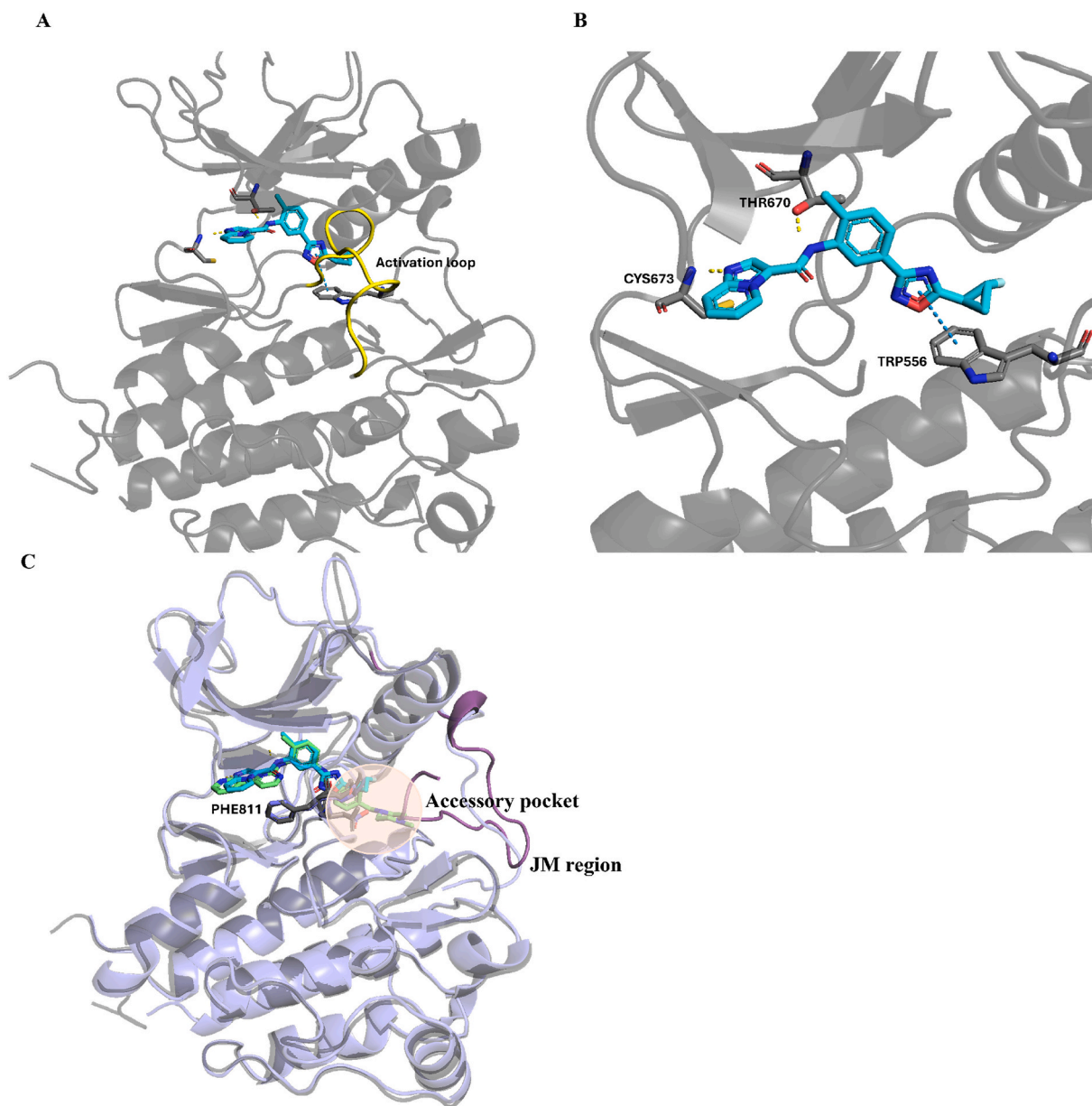


Fig. 19. A) structure of cKIT complexed with Labuxtinib, where the ligand and activation loop are indicated in cyan and yellow, respectively. B) binding mode of Labuxtinib. C) Superimposition of cKIT bound to Imatinib (PDB ID 1T46) and our model, shown in light blue and grey, respectively. PHE811 is rendered as sticks in both structures to emphasize the DFG-out conformation. The JM region in the current model is highlighted in violet. Imatinib and Labuxtinib are represented in green and cyan, respectively.

2.4.2. Labuxtinib

Labuxtinib is a tyrosine kinase inhibitor for which no crystal structure in complex with kinases has previously been reported. Here, we present the first crystal structure of cKIT in complex with Labuxtinib and provide a detailed characterization of its binding mode. The structure of cKIT in complex with Labuxtinib was determined to 2.0 Å. The structure is composed of one protein chain. Chain A includes amino acid residues 553–931. Residues 695–762 are missing due to a deletion of ΔS688–D765 and replacement by the smaller peptide EFVPYKVAPEDLYKDFLT. Other residues not visible in the electron density map were removed from the model. In addition to amino acids, 2 glycerol and 65 water molecules were built into the structure.

Our structural data indicate that Labuxtinib acts as a type II inhibitor, binding to and stabilizing the inactive conformation of cKIT, although it does not fully occupy the accessory pocket typically engaged by canonical type II inhibitors. The Labuxtinib–cKIT complex is shown in Fig. 19, Panel A and Panel B, where the activation loop adopts a closed conformation consistent with the inactive state. At the molecular level, Labuxtinib establishes key interactions with residues C673, T670, and W556, the latter already predicted by the MM/GBSA analysis.

To further rationalize the distinct binding mode of Labuxtinib, a structural comparison with cKIT bound to the prototypical type II inhibitor Imatinib (Fig. 19, Panel C) was performed. The superposition reveals a largely conserved global fold, with strong alignment of the central β-sheet and surrounding α-helices. However, clear differences emerge within the binding site: notably, the fluoroisopropyl group of Labuxtinib occupies only a limited portion of the accessory pocket compared to Imatinib, highlighting its atypical engagement of this region.

Importantly, our structure also resolves the JM region starting from residue E553, extending beyond what is observed in most reported cKIT structures, which typically begin at G565. This improved coverage enables a more comprehensive analysis of this regulatory segment. In our model, the N-terminal portion of the JM region is oriented toward the binding site, partially occupying the accessory pocket. Structural superposition indicates that this region overlaps with the space occupied by the methylpiperazine moiety of the ligand in the 1 T46 structure. These observations suggest that the distinct binding mode of Labuxtinib, likely related to its smaller size, permits a different positioning of the JM

region, potentially reflecting ligand-induced conformational adaptation.

2.4.3. Cenisertib

For the first time, we have elucidated the cKIT crystal structure in complex with Cenisertib.

The recombinant construct encompasses the region 564–931, carrying a deletion ΔS688–D765 and replaced by the shorter peptide EFV-PYKVAPEDLYKDFLT. The naming of the amino acids in the peptide is identical to the deposited structures (PDB IDs: 6GQJ and 6GKQ). In addition, one bromine ion (BR), one polyethylene glycol (PEG) fragment, and 24 water molecules have been modelled. Since the crystal was obtained from a mixture of chloride, bromide and iodide salts, the halogen anion most matching the electron density has been modelled.

The model indicates that Cenisertib binds to the active cKIT* conformation, as expected by a Type I inhibitor which occupies the ATP-binding site without accessing additional subpockets. As shown in Fig. 20, Panel A and, more in detail, Panel B, the αC-helix assumes an inward orientation, stabilized by the conserved LYS-GLU salt bridge (K623–E640), a hallmark of catalytically competent kinases [60]. Additionally, the A-loop is fully open.

Cenisertib forms multiple interactions within the cKIT binding site. The *N*-methylpiperazine group interacts with a water molecule adjacent to the binding site; this water molecule is not structural and likely not essential for Cenisertib activity. Consistent with typical type I inhibitors, Cenisertib establishes hydrogen bond interactions with C673. Its binding is further stabilized by the carboxamide moiety's triple interactions with D810, R796, and N797.

In Fig. 21, Panel A, a structural superposition between cKIT in complex with a type I inhibitor (PDB ID: 7KHK) and our model is shown. Overall, the two structures display a high degree of similarity. The DFG-in conformation allows both ligands to establish interaction with D810 (Fig. 21, Panel B).

Structurally, our model resolves several regions that are not defined in 7KHK. In particular, residues G827, N828, and A829 within the activation loop and P627, S628, A629, and H630 located adjacent to the αC-helix, are clearly modelled in our structure (highlighted in pale cyan), whereas they are missing in 7KHK and therefore represented as dashed segments.

Additionally, residues R586, N587, and R589 are highlighted in

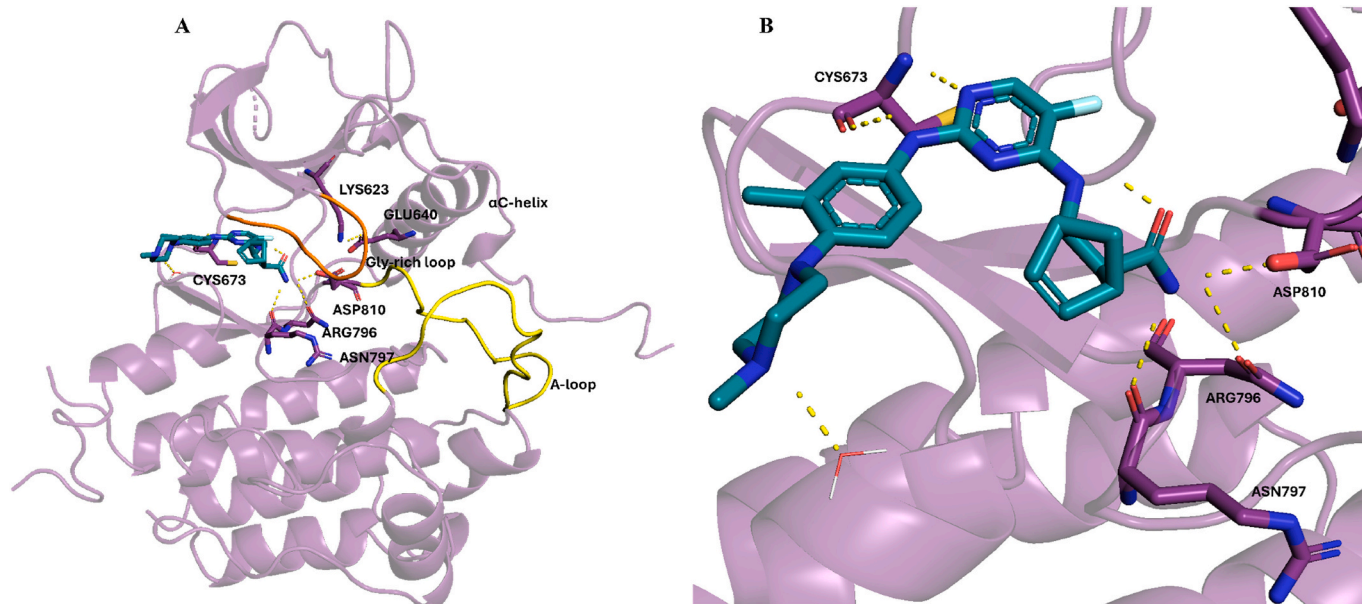


Fig. 20. Binding mode of Cenisertib as determined by our model: A) Structure of cKIT in complex with Cenisertib. The activation loop is depicted in yellow, while the glycine-rich loop is shown in orange. Key interacting residues and the ligand are illustrated in stick representation. B) Detailed binding mode of Cenisertib, with interactions indicated by yellow dashed lines.

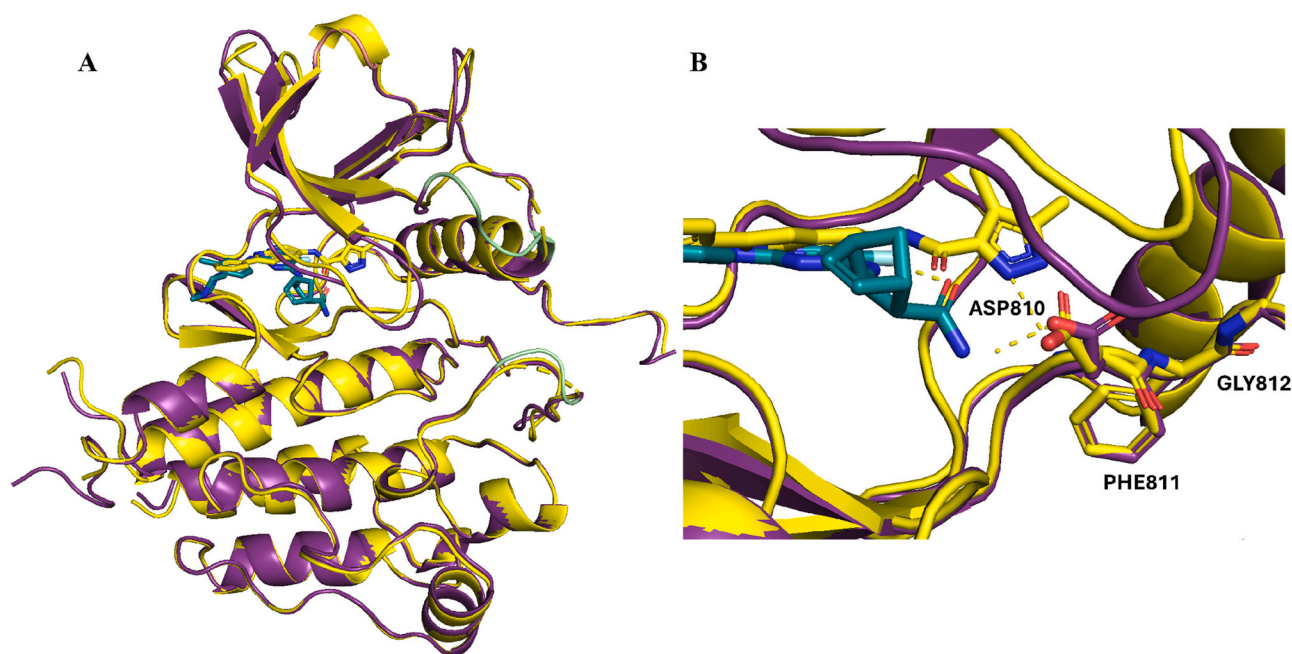


Fig. 21. Superposition of our model and 7KHK. A) Full kinase architecture. Residues GLY827, ASN828, and ALA829 within the activation loop and PRO627, SER628, ALA629, and HIS630 located adjacent to the α C-helix are represented in pale cyan, while the portion corresponding to Arg586, Asn587, and Arg589 is represented in salmon. B) DFG-in conformation establishing contacts with ligands.

salmon to emphasize a secondary structure difference: while this segment adopts a β -sheet conformation in 7KHK, it remains unstructured in our model and instead forms a flexible loop.

3. Discussion

In the present study, we combined complementary biophysical, structural, and computational approaches to describe in detail the binding modes of four cKIT inhibitors and to dissect how phosphorylation-dependent conformational states of cKIT influence it. By integrating SPR kinetics, enzymatic assay, MD simulations, SASA analyses, MM/GBSA, Free Energy Landscape, LiP-MS profiling, and X-Ray crystallography, we provide a comprehensive, state-resolved view of cKIT regulation and inhibition by describing each interaction and the consequent effects with different levels of detail, taking into account also of conformational long- and short-range rearrangements. Each of these techniques has its limitations and its advantages. These limitations can be overcome through the complementarity of the other approaches, enabling a detailed structural analysis of the protein and its complexes, also taking into account the effects of post-translational modifications, i.e. phosphorylation. Computational methods, such as MD and SASA, are extremely useful in predicting possible regions and modes of interaction. In this context, MM/GBSA binding free energy calculations and free energy landscape (FEL) analysis provide complementary insights by quantifying the energetic determinants of ligand recognition and describing the conformational states preferentially sampled during the simulations. Together, these approaches strengthen the interpretation of the MD results, providing a more comprehensive description of both the thermodynamic and conformational stability of the investigated complexes. However, these approaches should be complemented by additional computational analyses and, ultimately, validated through experimental evidence. To this aim, high-resolution crystallography and LiP-MS approaches for conformational analysis in solution have been

employed. Crystallography relies on the high resolution of ordered regions, but lacks information about more flexible regions, such as connecting loops and unstructured regions. The latter, conversely, are well described by LiP-MS, as this approach is applied to proteins in their native state in solution. This method provides insight into dynamic conformational changes, although it lacks the resolution of crystallography.

Avapritinib consistently behaves as a Type I inhibitor with preference for the active state of cKIT. SPR measurements show comparable affinities for both states, but with fast kinetics and short residence times typical of ATP-competitive inhibitors. LiP-MS identifies protection of key residues within the ATP-binding site, most notably F600, together with elements of the activation loop. MD simulations agree with this binding mode by showing reducing flexibility in the activation loop upon binding, alongside localized changes in solvent accessibility. Crystallography data confirm that Avapritinib occupies the ATP-binding site while stabilizing the DFG-in conformation and inducing the formation of an accessory sub-pocket. The combination of MM/GBSA and FEL analyses provided complementary evidence that ligand binding is both energetically favorable and conformationally stable throughout the simulations, demonstrating that Avapritinib locks cKIT in a catalytically competent conformation thus preventing ATP access, and also propagates long-range conformational effects to distal regions of the kinase. These findings are consistent with those previously reported by Teuber et al. [47], who first described Avapritinib as a 1.5 inhibitor. In contrast, Olverembatinib displays a clear preference for the inactive conformation of cKIT. SPR data indicated no interaction with the active state, while a weak binding is detectable in the inactive form. LiP-MS reveals extensive protection of residues spanning the ATP-binding pocket, particularly in the F600-A621 region, indicating an engagement of the orthosteric site. MD and SASA analyses are in line with these findings by showing increased burial of pocket residues and enhanced flexibility in surrounding regions, consistent with ligand-induced stabilization of the

DFG-out conformation. Furthermore, both MM/GBSA analysis and FEL demonstrated the high stability of the ligand in complex with the inactive form of cKIT. The breadth of protected residues suggests a more extensive occupation of the binding cavity compared to Type I inhibitors, as expected for a Type II binding mode. Even if crystallographic data are not available for the interaction between Olverembatinib and cKIT, it has been resolved the structure of Olverembatinib with LRRK2 (PDB: 8TZE), demonstrating its binding as a type II inhibitor. Together, these published results and our own work suggest Olverembatinib as a type II inhibitor.

Olverembatinib has been approved in China for the treatment of chronic myeloid leukemia (CML) as a BCR-ABL1 inhibitor [61]. However, accumulating evidence suggests that, unlike imatinib, it can also inhibit cKIT, including in patients who are resistant to other BCR-ABL1 inhibitors [48]. These findings indicate that Olverembatinib has a broader kinase inhibition profile, potentially increasing its therapeutic versatility.

Labuxtimib exhibits a comparable but even more significant stabilizing profile, characterizing it as the most potent inhibitor evaluated. These findings are substantiated by SPR measurements, which demonstrate nanomolar binding affinity and remarkably slow dissociation rates, reflecting the prolonged residence time typical of high-occupancy inhibitors. Furthermore, LiP-MS data reveal extensive structural protection across the ATP-binding cleft of the inactive kinase conformation, specifically focused on the F600-A621 segment. Consistently, molecular dynamics simulations indicate a reduction in local flexibility for the involved residues. According to the per-residue decomposition analysis, amino acids within this region - particularly V603 - emerge as critical molecular determinants, providing substantial energetic contributions to ligand stabilization. Crystallographic data further confirm that Labuxtinib stabilizes the inactive conformations of cKIT, although with a partially atypical engagement of the accessory pocket. Compared to Olverembatinib, Labuxtinib appears to achieve tighter binding through a combination of deep pocket insertion. This may explain the higher affinity recorded in the SPR data compared to Olverembatinib. While some previous studies have reported on the potency of this inhibitor for cKIT [62,63], no study has yet examined its binding mode in detail.

Cenisertib exhibited a more complex behavior. SPR data indicate measurable affinity for both active and inactive forms, with a moderate preference for the inactive one. However, LiP-MS reveals markedly different protection patterns between the two states. In the inactive conformation, Cenisertib induces protection within the ATP-binding pocket, consistent with a Type II-like binding mode. In contrast, in the active cKIT, the protection of these residues is less pronounced. MD simulations mirror this dual behavior: in the inactive form, ligand binding stabilizes ATP-pocket region and reduces local fluctuations, whereas in the active form it induces more distributed dynamic changes, particularly in the activation loop and C-terminal. Crystallographic data indicate that Cenisertib can adopt a Type I-like binding mode in the active cKIT, occupying the ATP pocket without engaging additional sub-pockets. Altogether, these findings suggest that Cenisertib is capable of adapting its binding mode to the conformational state of cKIT, combining features of both Type I and Type II inhibition. The robustness of the predicted interaction is further substantiated by integrated energetic analyses, which yielded remarkably similar binding free energies for both kinase conformations. This suggests that Cenisertib can effectively accommodate the inactive state of the protein, a finding that remains in high agreement with experimental evidence derived from SPR measurements. Cenisertib has not previously been clearly reported as a cKIT inhibitor, and this is the first study to examine this interaction in detail. However, it is described as an ATP-competitive kinase inhibitor

for the Aurora kinase [64], which confirms the binding mode identified in this paper. Across all compounds, a recurring feature is the involvement of highly flexible regions, particularly the loop downstream the ATP-binding site and the terminal segments. LiP-MS consistently identifies these regions as highly responsive to both phosphorylation and ligand binding, consistently with MD simulations which also highlights their elevated mobility. These segments appear to function as conformational amplifiers, transmitting local perturbations at the binding site into global structural rearrangement. Notably, phosphorylation itself induces substantial reorganization of these regions, leading to increased solvent exposure of regulatory residues and partial occlusion of the ATP-binding pocket in the active form.

Overall, this work demonstrates that a detailed understanding of kinase inhibition requires an integrative strategy that combines kinetic measurements, structural approaches, and molecular simulations. While each technique has inherent limitations when considered in isolation, their convergence here provides a robust and internally consistent framework for assigning inhibitor binding modes and understanding state-dependent kinase regulation. Applied to cKIT, this approach clearly discriminates Type I from Type II inhibition mechanisms and reveals how phosphorylation modulates both local pocket architecture and long-range dynamics. More broadly, this integrated methodology is readily transferable to other kinases and offers a powerful platform for rational drug discovery and the development of next-generation, state-selective kinase inhibitors.

4. Materials and methods

4.1. Enzymatic assay and SPR

The recombinant human cKIT enzyme was obtained from Carna Biosciences (Japan). The following reagents were purchased from Sigma Aldrich (USA): Hepes, Tris, EGTA, MgCl₂, dithiothreitol (DTT), Tween 20, ATP, staurosporine, and EDTA. The Ulight TK peptide substrate, europium labeled anti phospho PT66 antibody, and detection buffer were supplied by Revvity (USA).

The kinase reaction buffer consisted of 40 mM Hepes/Tris (pH 7.4), 0.8 mM EGTA/Tris, 8 mM MgCl₂, 4.5 mM DTT, and 0.008% Tween 20. All components were prepared fresh on the day of the experiment. Kinase reactions were performed in an assay buffer by combining the test compound, reference inhibitor (staurosporine), or DMSO (vehicle control) with 0.1 ng of cKIT enzyme. Reactions were initiated by the addition of 100 nM Ulight TK peptide substrate and 50 μM ATP. The mixture was incubated for 15 min at room temperature to allow phosphorylation of the peptide substrate. For basal (maximum inhibition) control measurements, 0.1 μM staurosporine was included in the reaction mixture. Enzymatic activity was terminated by adding 13 mM EDTA to each reaction well. After a 5 min incubation, the europium labeled anti phospho-PT66 antibody was added according to the manufacturer's recommended dilution. Plates were incubated for an additional 60 min at room temperature to allow antibody binding and TR FRET complex formation. Time-resolved fluorescence resonance energy transfer (TR FRET) signals were measured on a Pherastar microplate reader (BMG). The excitation wavelength was set at 337 nm, and emission was recorded at 620 nm (donor) and 665 nm (acceptor). The TR FRET signal was calculated as the ratio of fluorescence at 665 nm to 620 nm. Enzyme activity in the presence of test compounds was expressed as a percentage of the DMSO control. Percent inhibition was calculated from the TR FRET ratios. Staurosporine was included as a reference inhibitor in every experiment and tested across multiple concentrations to generate a concentration-response curve. The half maximal inhibitory

concentration (IC_{50}) was determined by nonlinear regression analysis.

Recombinant biotinylated intracellular domains of human cKIT, used for SPR measurements, were purchased from Carna Biosciences (Cat# 08-456-23 N and 08-456-21 N). The study was conducted on a BIAcore 8 K+ system (GE Healthcare Life Sciences, Uppsala, Sweden).

cKIT constructs were immobilized on a streptavidin-coated Serie S SA sensor chip to reach a level of approximately 5500 resonance units (RU) using the immobilization buffer HBS-EP+ (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% Surfactant P20) supplemented with 1 mM TCEP. On each channel, flow cell (FC) 1 was prepared as the reference surface and FC2 as the active surface.

For direct interaction studies, compounds were tested in HBS-EP+ buffer containing 1 mM TCEP and 3% DMSO, with an association time of 120 s, dissociation time of 120 s, and a flow rate of 50 μ L/min.

Binding and data analysis were performed using double referencing: subtraction of the reference surface (FC1) followed by subtraction of the zero concentration. Sensorgrams were fitted with a 1:1 binding model equation or steady-state affinity model. For each condition, at least two independent experiments were performed.

4.2. Molecular modelling

The cKIT structures in complex with Avapritinib and Cenisertib (active form), as well as the inactive cKIT in complex with Labuxtinib, were obtained from internal XRAY models developed at Domp . The APO cKIT structures representing both active and inactive conformations were retrieved from the Protein Data Bank. Specifically, the 1 T45 model was utilized for simulating the APO cKIT in its inactive conformation, whereas the 1PKG model was employed to represent the APO cKIT in its active conformation. As this model was complexed with ATP and no other APO models were available, the ATP molecule was removed. All 3D structures were optimized with the Protein Preparation Wizard (Schr dinger v2025.3), by adding hydrogen, refining loops, completing side chains, and removing ions and water.

For structures crystallographically resolved as homodimeric assemblies—exemplified by 1PKG and our Xray model of the cKIT-Avapritinib system—chain A was exclusively selected for all production trajectories reported herein.

Regarding the cKIT-Avapritinib complex, this selection was dictated by the superior structural integrity of chain A. Specifically, critical residues including K693, D762, and T932 remained unresolved in the B chain, whereas chain A offered a more comprehensive experimental mapping of the protein scaffold.

In the context of the 1PKG X-ray model, chain A was prioritized for MD analysis based on rigorous structural and methodological criteria. A comparative structural inspection revealed that both protomers exhibit high conformational similarity, maintaining an equivalent architecture of the catalytic site. Since the dimeric state observed in the crystal lattice typically originates from packing effects rather than the physiological activation of the receptor, simulating a single protomer sufficiently captures the intrinsic dynamics of the kinase domain. Chain A was specifically chosen due to its greater completeness, as chain B lacks the segment 816–830 within the catalytic region. Consequently, chain A provides a more robust model of the fully resolved catalytic pocket, ensuring that all molecular determinants relevant to ligand recognition and site-specific fluctuations were preserved throughout the simulations.

The inactive conformation of cKIT in complex with Olverembatinib was obtained by carrying out a Ligand Docking by using GLIDE. In order to maintain the bias created by the presence of the ligand in the inactive conformation, the docking experiments were carried out by using the 1

T46 model. In this crystal structure, cKIT is bound to its established inhibitor, Imatinib. Initiating the analysis with an experimentally determined conformation of the protein-inhibitor complex was considered appropriate. Accordingly, the ligand and protein were separated, after which the protein was optimized and subsequently used to generate the grid for molecular docking simulations. Model optimization was performed utilizing the Protein Preparation Wizard tool implemented in Maestro us (Schr dinger) and the ligands were prepared by means of LigPrep tool (Schr dinger v2025.3). Hydrogens were added, salts were removed, and ionization states were calculated using Epik at pH 7.4.

Molecular Docking was carried out by means of the Glide (version 2025.2) using the SP (standard precision) algorithm and running 10 poses per ligand.

The grid box was built by using the model co-crystallized ligand as centroid. The best poses were selected by combining visual inspection and Glide Gscore.

The inactive cKIT–Cenisertib complex model was built starting from the 3D structure of cKIT (PDB ID: 6KLA), which represents cKIT in the inactive conformation bound to a type I inhibitor, a binding mode consistent with that of Cenisertib. Since the docking calculations did not produce satisfactory poses with acceptable docking scores, the complex was instead generated by superimposing the in-house experimentally X-ray derived model of Cenisertib and manually positioning the ligand within the binding site of the 6KLA structure. A local energy minimization was then performed using the Maestro GUI to remove possible steric clashes between atoms. Finally, the resulting model was subjected to molecular dynamics simulations as described below.

4.3. Molecular dynamics simulation (MDs)

One microsecond of molecular dynamic simulations was conducted using GROMACS package v.2023.3 by employing AMBER ff99SB force field. Ligand parameters were generated with Antechamber software, and conversion to GROMACS format was accomplished using the AnteChamber PYthon Parser InterfacE (ACPYPE) tool. The protein–ligand complex.

The system was immersed in a box with dimensions of $7.78 \times 7.28 \times 7.42$ nm, ensuring a minimum solute–box distance of 1.0 nm and solvated by employing TIP3P explicit solvent model, subsequently neutralized by adding Na^+ and Cl^- ions and brought to a physiological salt concentration of 0.1 M.

The system was subjected to energy minimization prior to equilibration using the steepest descent algorithm implemented in GROMACS. The minimization was performed for up to 50,000 steps or until convergence was reached with a maximum force threshold of $1000 \text{ kJ mol}^{-1} \text{ nm}^{-1}$. A Verlet cutoff scheme was employed to calculate non-bonded interactions, with a neighbor list updated every step. Short-range electrostatic and van der Waals interactions were truncated at 1.2 nm. Electrostatic interactions at long range were treated using the Particle Mesh Ewald (PME) method. Van der Waals interactions were also cut off at 1.2 nm with a force-switching function applied between 1.0 and 1.2 nm to ensure a smooth decay of the potential. Periodic boundary conditions were applied in all three spatial directions (xyz). Long-range dispersion corrections were not applied. The system was equilibrated following energy minimization through a two-step equilibration protocol consisting of constant volume (NVT) and constant pressure (NPT) ensembles. The NVT equilibration was performed for 100 ps using a leap-frog integrator with a 2 fs time step, applying position restraints on both the protein and ligand. Temperature was maintained at 300 K using the V-rescale thermostat, with separate

coupling groups defined for the protein and for water and ions. All bond lengths involving hydrogen atoms were constrained using the LINCS algorithm, and long-range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method with a real-space cutoff of 1.2 nm. Van der Waals interactions were calculated using a cutoff scheme with a force-switching function between 1.0 and 1.2 nm.

Subsequently, the system was further equilibrated for 100 ps under isothermal-isobaric (NPT) conditions using the Berendsen barostat to maintain a pressure of 1 bar with isotropic pressure coupling and a coupling constant of 2.0 ps. Temperature was again kept at 300 K using the V-rescale thermostat with the same coupling scheme as in the NVT phase. Position restraints on the protein and ligand were maintained during this stage to allow solvent relaxation while preserving the structural integrity of the complex. Electrostatic and van der Waals interactions were treated identically to the NVT equilibration using PME and a 1.2 nm cutoff, respectively. The system was finally prepared for production molecular dynamics simulations after achieving stable temperature and pressure convergence.

To improve the robustness and statistical reliability of the molecular dynamics results, three independent replicas were performed for each simulated system. Each replica was initiated from the same equilibrated structure but with different initial atomic velocities generated by assigning a different random seed (*gen_seed*) during the velocity generation step, following the standard GROMACS protocol. This approach enables independent sampling of the conformational space while maintaining identical simulation conditions across all replicas. Performing multiple independent simulations reduces the likelihood that the observed behavior arises from stochastic fluctuations or insufficient sampling, improves the reproducibility of the results, and allows statistical analyses of the calculated structural, energetic, and thermodynamic descriptors.

4.4. Root-mean square fluctuation (RMSF)

The root mean square fluctuation (RMSF) on the backbone was calculated to evaluate the flexibility of individual residues throughout the production trajectory. The RMSF was computed using the *gmxf* utility in GROMACS, after aligning all trajectory frames to the reference structure in order to remove overall rotational and translational motions. The calculation provides the time-averaged atomic positional deviations according to Formula 3. The resulting RMSF values were plotted as a function of residue number to identify flexible regions and structural stability within the protein. All analyses and plots were generated using GROMACS analysis tools and visualized with Grace and PyMOL.

4.5. Solvent accessible surface area

The solvent accessible surface area (SASA) of the protein was calculated to evaluate changes in solvent exposure during the molecular dynamics simulation. The analysis was carried out using the *gmxf* tool in GROMACS, applying the Lee and Richards algorithm with a probe radius of 0.14 nm, corresponding to the approximate radius of a water molecule. The SASA values were computed for the entire protein over the equilibrated and production trajectories, providing insights into the overall compactness and conformational stability of the system. The time evolution of the total SASA and its average values were analyzed to monitor potential structural rearrangements and solvent accessibility variations. The normalized SASA was obtained as specified above.

4.6. Binding free energy calculations

Binding free energy calculations were performed using the Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) approach implemented in *gmxmmpbsa* (v1.6.5). The Amber topologies required for the calculations were generated using the *ff99SB* force field available in AmberTools, which provide the topology form as required by *gmxmmpbsa*, for the protein and the General Amber Force Field (GAFF) for the ligand, consistent with the ligand parametrization performed with Antechamber. The Generalized Born implicit solvent model *igb* = 5 (Onufriev-Bashford-Case, OBC II) was employed with an internal dielectric constant of 1.0, an external dielectric constant of 78.5, and an ionic strength of 0.1 M. The non-polar solvation contribution was estimated using the solvent-accessible surface area (SASA) model with a surface tension coefficient of 0.0072 kcal mol⁻¹ Å⁻² and a solvent probe radius of 1.4 Å.

MM/GBSA calculations were performed on snapshots extracted from the equilibrated portion of each trajectory. Frames from 1 to 10,000 were analyzed by sampling one frame every 10 trajectory frames, yielding 1000 conformations for each independent simulation. The binding free energy was calculated according to Formula 3, where the molecular mechanics contribution comprises van der Waals and electrostatic interactions, whereas the solvation term includes polar (Generalized Born) and non-polar contributions. Entropic contributions were not included in the calculations (quasi-harmonic, interaction entropy, and C2 entropy were disabled), as the objective was to compare the relative energetic stabilization of closely related conformational states rather than to determine absolute binding free energies.

Per-residue free energy decomposition was carried out using the *idecomp* = 1 protocol, which assigns 1–4 interactions to the internal energy term, and energetic contributions were calculated for all protein residues. To improve the statistical robustness of the analysis, MM/GBSA calculations were independently performed for each of the three molecular dynamics replicas, and residue-wise binding free energies were averaged over the three simulations before comparative analysis.

4.7. Principal component analysis and free energy landscape analysis

Principal component analysis (PCA) was performed on the molecular dynamics trajectories to characterize the dominant collective motions of the protein. The covariance matrix was calculated using the backbone atoms after least-square fitting of the trajectories, and the first two principal components (PC1 and PC2) were extracted using the *gmxf* *covar* and *gmxf* *anaeig* tools implemented in GROMACS. The conformational free energy landscape (FEL) was subsequently constructed by projecting the trajectories onto the first two principal components. The probability distribution of the conformational states was converted into relative free energy values according to the Boltzmann relationship (Formula 4).

The FEL was generated using the *gmxf* *sham* tool in GROMACS, and the resulting free energy maps were used to compare the conformational stability and the accessible energy states of the apo and ligand-bound protein systems.

The depth and distribution of the energy minima were used as indicators of the conformational stability and heterogeneity of the different protein states.

4.8. Limited proteolysis protocol

The Limited Proteolysis – Mass Spectrometry protocol was performed as described by Monaco et al. [65]. Briefly, the recombinant

active and the inactive intracellular domain of cKIT (Carna Biosciences, Japan) were incubated with each inhibitor in 50 mM Tris-HCl, 150 mM NaCl at pH 7.5 for 30 min in a thermomixer at room temperature with stirring. Limited proteolysis was performed with the proteinase K at a weight ratio of 1:100 for 1 min at 37 °C. The reaction was stopped boiling at 95 °C for 5 min. All samples and controls were incubated with dithiothreitol (DTT) 12 mM in 50 mM Tris-HCl, 150 mM NaCl at pH 7.5 for 30 min at 37 °C followed by iodoacetamide (IAM) 40 mM in 50 mM Tris-HCl, 150 mM NaCl at pH 7.5 in the dark for 30 min. The same procedure was applied to isolated proteins, employed as controls of corresponding experiments. For each experiment, 5 replicates were produced independently. The reactions were stopped by rapid desalting using a ZipTip C18 system. The resulting mixtures were dried using a SpeedVac system and then processed by extensive hydrolysis by using trypsin proteomic grade at a weight ratio of 1:50 at 37 °C overnight and then stopped by the addition of 20% TFA in a volume ratio of 1:10. Samples were dried using a SpeedVac system.

4.9. Mass spectrometry analysis and peptide identification and quantification

The peptide mixtures were resuspended in 16 µL of 0.2% formic acid (HCOOH) LC-MS grade, and 2 µL of these were injected and analyzed by the mass spectrometer in technical duplicate. The instrument used was an Orbitrap Exploris 240 instrument (Thermo Fisher Scientific, Massachusetts (USA)) equipped with a Vanquish nanoUHPLC system and Nanospray Flex ion source. Samples were fractionated on a C18 capillary reverse phase column (150 mm, 75 µm, 2 µm 100 Å) at a flow rate of 250 nL/min. A linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid and 2% acetonitrile in LC-MS grade water) was used from 2% to 90% in 27 min. The MS/MS method used a Data-Dependent Acquisition (DDA) mode consisting of a full scan from 375 to 1200 *m/z* range followed by fragmentation of the top 30 ions (MS/MS scan) selected by intensity and charge state (+2, +3 and multi-charges) with a dynamic exclusion time of 40s.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [66] partner repository with the dataset identifier PXD074531.

Protein identification and quantification were performed with Proteome Discoverer Software (v.2.5.0.400) using the raw files and the specific FASTA database of the intracellular domain of cKIT. Identification and quantification were performed with an FDR cut-off of 5% and using carbamidomethyl (Cys) as the fixed modification, while Gln- > pyro-Glu (N-term Gln), oxidation (Met) and phosphorylation (S/T/Y) were used as the variable modifications. The Perseus software (v.2.1.3.0) was used for the statistical analysis. In particular, only the peptides with a LFQ value intensity differing from zero in at least 7 replicates out of 10, in at least one group, were considered. The remaining missing values were imputed from normal distribution. Due to the substantial number of peptides for each condition, it was decided to implement rigorous filters for the fold change threshold (FC): specifically, only peptides upregulated with an FC greater than 2 and those downregulated with an FC less than 0.5 were considered. Furthermore, a Student-*t*-test was applied to our replicates, and only peptides with FDR of less than 0.05 were deemed to be statistically significant [65,67,68]. For the graphical representation of the intracellular domain of cKIT, we decided to use the numeration reported in the full-length protein, to better compare the results obtained from the other approaches.

4.10. Quantification of phosphorylated peptides

The LC-MSMS data collected for the isolated active cKIT (cKIT*) were also analyzed with Proteome Discover (v.2.5.0.400) to identify and relatively quantify the phosphorylated residues. In particular, only those peptides with the probability of phosphorylation overcoming the 75% in a specific site were considered. A second filter was applied, considering phosphopeptides exclusively identified on the base of MSMS spectra ("high" confidence) in at least 70% of the 10 replicates.

For the relative quantification of each phosphosite, the Formula 5, adapted from Iacobucci et al. [69] was employed. Only the phosphorylation sites overcoming 25% of relative phosphorylation were taken in account.

4.11. Protein expression and purification

The plasmid encoding KIT was co-transformed with PTPN1 into BL21 (DE3) using standard procedures. One of the colonies obtained after transformation was used to make a glycerol stock. The glycerol stock was used to inoculate an LB preculture, and on the following day the preculture was used to inoculate 4 × 1 L LB cultures. The cultures were incubated at 37 °C until OD600 reached 0.8. Protein expression was induced by addition of 1 mM IPTG at 18 °C overnight. On the next day the cells were harvested by centrifugation at 6000 *xg* for 10 min at 4 °C.

The cell pellet was resuspended in IMAC buffer A (40 mM HEPES pH 8.0, 300 mM NaCl, 10% glycerol and 1 mM TCEP) supplemented with Pierce protease inhibitor mini tablets (EDTA-free), DNase, and was sonicated on ice for 15 min using 5 s on/5 s off pulses. The lysate was clarified using centrifugation at 30000 *xg* for 1 h at 4 °C. Clarified lysate was loaded onto a 10 mL HisTrap FF column (Cytiva) equilibrated in IMAC buffer A supplemented with 20 mM imidazole. On the next day, the resin was washed stepwise with 20 mM and 50 mM imidazole. Protein elution was performed with a linear gradient of IMAC buffer B up to 250 mM imidazole. The fractions from the IMAC purification were analyzed on SDS-PAGE (Fig. S6, Panel A).

The main peak containing the protein of interest was pooled and cleaved with TEV protease at a 1:20 (w/w) TEV to protein ratio. On the next day the sample was collected and loaded onto a 10 mL HisTrap FF IMAC column, equilibrated in IMAC buffer A with 20 mM imidazole. The column was washed with 50 mM imidazole and protein elution was performed with a linear gradient of IMAC buffer B up to 50% (250 mM imidazole). Samples collected during this purification step were analyzed on SDS-PAGE (Fig. S6, Panel B).

TEV-cleaved protein that eluted in the flow-through and in the wash was combined, concentrated and centrifuged at 17000 *xg* for 10 min prior to injection onto a size-exclusion chromatography column (SEC) (HiLoad 16/60 Superdex S75 prep grade, Cytiva) equilibrated in SEC buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1 mM TCEP). Collected fractions were analyzed on SDS-PAGE (Fig. S6, Panel C). Major peak fractions containing the protein of interest were pooled (fractions 1C8 1D8) and concentrated to 7.1 mg/mL in 150 µL (final yield of 1 mg). KIT was flash frozen in liquid nitrogen and stored at -80 °C.

4.12. c-KIT-Avapritinib X-ray

Co-crystals of 5.3 mg/mL KIT in buffer (200 mM NaCl, 2 mM TCEP and 20 mM Tris pH 8.0) appeared overnight at 20 °C with 2 mM of compound 8 (L00011285SIM) added or 2 mM of compound 61 (L00011250) added (using a compound stock solution at 100 mM dissolve in DMSO). After 2 h of incubation on ice, 100 nl complex of KIT

and compound 8 was mixed with 100 nL reservoir (0.1 M MES/Imidazole pH 6.5, 30 mM sodium chloride, 30 mM sodium bromide, 30 mM sodium iodide, 18% (v/v) glycerol and 9% (w/v) PEG 4000). After the same incubation time 100 nL complex of KIT with compound 61 was mixed with 100 nL reservoir (0.1 M PCTP1 pH 6.4, 30 mM sodium chloride, 30 mM sodium bromide, 30 mM sodium iodide, 16% (v/v) glycerol and 8% (w/v) PEG 4000). The sitting drop was equilibrated over 40 μ L reservoir. The crystallization thus is similar as described by Kettle et al. [70].

After growing for a few days the KIT with compound 8, co-crystal was transferred to: 24% (v/v) glycerol, 12% (w/v) PEG 4000, 0.1 M MES/Imidazole pH 6.5, 20 mM Tris/HCl pH 8, 30 mM sodium chloride, 30 mM sodium bromide and 30 mM sodium iodide as well as 2 mM compound 8 and flash-frozen in liquid nitrogen, mounted in a nylon loop. In the case of the KIT with compound 61 co-crystal, it was used as a control in a soaking experiment. In brief 1.5 μ L of the composition of the reservoir supplemented by 2% DMSO and 20 mM Tris/HCl pH 8.0 was added to the crystals. The crystals were incubated overnight and next day flash-frozen in cryosolution containing: 2% DMSO, 24% (v/v) glycerol, 12% (w/v) PEG 4000, 0.1 M PCTP pH 6.4, 20 mM Tris/HCl pH 8, 30 mM sodium chloride, 30 mM sodium bromide and 30 mM sodium iodide.

The datasets of KIT in complex with compound 8 and compound 61 were collected at the beam line i03, Diamond Light Source, Didcot, UK at 100 K and $\lambda = 0.9763$ Å. 3600 images were collected with an oscillation range of 0.1° per image using an EIGER2 XE 16 M detector. Data were processed using the xia2 dials pipeline [71], which includes the software XDS(3) and further using Aimless [72]. The structure was refined using Refmac5 [73] and model building was carried out in Coot [74]. After initial refinement with ligand removed, clear density for compound 8 was found in both molecule A and molecule B in the asymmetric unit. Ligand coordinates and a restraints file for compound 8 were generated using the smiles string and Grade2 [75] and used in Refmac5. The final model has been refined to Rmodel/Rfree of 0.165/0.190.

4.13. c-KIT Labuxtinib X-RAY

Initially, apo crystals of KIT were soaked for 18 h in a precipitant solution supplemented with 1 mM of compound. Afterwards, crystals were transferred to a fresh droplet of cryo-solution before they were flash-cooled in liquid nitrogen. The best datasets of KIT in complex with compound 65 were collected at the BioMAX beamline, MAX IV, Lund, Sweden at 100 K and $\lambda = 0.9760$ Å. 3600 images were collected with an oscillation range of 0.1° per image using a Dectris EIGER 16 M detector. The data were processed with the EDNA pipeline. The structure was refined using Refmac [73,74] model building was carried out in Coot [74]. After initial refinement, clear density for compound 65 was observed. Ligand coordinates and a restraints file for compound 65 were generated using the SMILES string and the program Grade2 [75].

150 nL of KIT solution at 5.3 mg/mL containing 2 mM of compound 66 L00004862SIM was mixed with 120 nL reservoir solution (10% PEG 4000, 20% glycerol, 0.1 M bicine/Trizma base pH 8.5, 20 mM 1,6-hexanediol, 20 mM 1-butanol, 20 mM (RS)-1,2-propanediol, 20 mM 2-propanol, 20 mM 1,4-butanediol, 20 mM 1,3-propanediol. To facilitate crystal nucleation, 30 nL of seed stock (0.1 M MES/Imidazole pH 6.5, 30 mM sodium chloride, 30 mM sodium bromide, 30 mM sodium iodide, 14% (v/v) glycerol and 7% (w/v) PEG 4000) from KIT compound 66 co-crystals was added to the drops. The crystallization plate was incubated at 20 °C. Crystals were briefly dipped in solution with the same composition as the reservoir solution but supplemented with 10% glycerol and subsequently flash frozen in liquid nitrogen. Complete data were collected to 2.45 Å resolution at the BioMAX beamline, MAX IV Laboratory, Lund, Sweden at 100 K and $\lambda = 0.9763$ Å. 3600 images were collected with an oscillation range of 0.1° per image. The beamline is equipped with a Dectris EIGER 16 M detector. Data were processed using autoPROC pipeline [70]. The structure was refined using Refmac5 and model building was carried out in Coot [74]. After initial refinement with ligand removed, clear difference electron density for compound 66 was found. Ligand coordinates and a restraints file for compound 66 were generated using the SMILES string and the program Grade2 [75] and used in Refmac5. The final model has been refined to Rmodel/Rfree of 0.187/0.256.

The Table S7 summarizes the different experiments performed for the inhibitors.

Formula 1. Fractional SASA.

$$fractional.SASA = \frac{SASA_{observed}}{SASA_{max}}$$

Formula 2. RMSF calculation, where $x_i(t)$ represents the position of atom i at time t , and $\langle x_i \rangle$ is its average position over the trajectory.

$$RMSF(i) = \sqrt{\langle (x_i(t) - \langle x_i \rangle)^2 \rangle_t}$$

Formula 3. Equation used to calculate the binding free energy (ΔG_{bind}) in the MM/PBSA analysis, where the total binding free energy is obtained as the sum of the van der Waals interaction energy (ΔE_{vdW}), electrostatic interaction energy (ΔE_{ele}), polar solvation free energy (ΔG_{polar}), and nonpolar solvation free energy ($\Delta G_{nonpolar}$).

$$\Delta G_{bind} = \Delta E_{vdW} + \Delta E_{ele} + \Delta G_{polar} + \Delta G_{nonpolar}$$

Formula 4. Boltzmann relationship. R is the gas constant, T is the simulation temperature (300K), and $P(x,y)$ represents the probability of each conformational state.

$$G(x,y) = -RT \ln P(x,y)$$

Formula 5. Percentage of phosphorylation for each phosphosite. Y_x/Z_x corresponds to the specific X tyrosine or Z serine under investigation.

$$\% \text{phosphorilation} = \frac{\sum \text{LFQ intensity}_{\text{peptideY}_x/\text{S}_z \text{ phosphorylated}}}{\sum \text{LFQ intensity}_{\text{peptideY}_x/\text{S}_z \text{ phosphorylated}} + \sum \text{LFQ intensity}_{\text{peptideY}_x/\text{S}_z \text{ not modified}}}$$

4.14. c-KIT Cenisertib X-ray

Co-crystallization of KIT in the buffer (200 mM NaCl, 3 mM TCEP and 20 mM Tris pH 8.0) was set up using sitting drop vapor diffusion.

CRedit authorship contribution statement

Irene Cipollone: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Carmen Gratter:** Writing – original

draft, Software, Methodology, Investigation, Formal analysis. **Carminé Talarico**: Investigation. **Andrea Rosario Beccari**: Conceptualization. **Dedna Zoubri**: Methodology, Investigation, Formal analysis. **Alexandre Baron**: Methodology, Investigation. **Vanessa Porkolab**: Writing – original draft, Investigation. **Daniela Iaconis**: Resources, Conceptualization. **Maria Monti**: Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

We have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2026.154141>.

Data availability

The Molecular Dynamics simulations were deposited to the Zenodo repository with the DOI <https://doi.org/10.5281/zenodo.20180996>. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD074531.

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