Dynamic conformational states of apo, ATP and cabozantinib bound TAM kinases to differentiate active-inactive kinetic models

Gatta K. R. S. Naresh and Lalitha Guruprasad

School of Chemistry, University of Hyderabad, Hyderabad, 500046, India.
Communicated by Ramaswamy H. Sarma

1. Introduction

The receptor tyrosine kinases (RTKs) are single-pass membrane spanning proteins, recognized by specific extracellular ligands to cause receptor dimerization followed by kinase activation and intracellular autophosphorylation (Endicott et al., 2012). This leads to a cascade of downstream signalling process that allow the molecular pathways to communicate within intracellular components and integrate with the nucleus to regulate the normal physiological events in cell survival, proliferation, growth and death. The upregulation and overexpression of the RTKs lead to abnormal intracellular signalling resulting in disease. The kinase domain in RTKs is a drug target for various diseases ranging from immunogenic, autoimmune, diabetes, cardiac and cancer. Most kinase inhibitors have been designed and validated for cancer treatment (Manning et al., 2002). The Tyro3, Axl and Mer (TAM) RTKs are a family of kinases, extracellular ligands such as growth arresting-specific 6 protein (GAS-6) and protein S (Pros1) activate TAM kinases (Grebuer et al., 2013). GAS-6 has specific binding affinities for each of the three TAM RTKs, whereas Pros1 has specific affinity to bind Tyro3 and Mer (Johnson et al., 1996). TAM RTKs activate specific intracellular kinases to regulate cell growth, survival and apoptosis (Cowan-Jacob et al., 2005). TAM RTKs are overexpressed in acute myeloid leukaemia, breast, colorectal, lung, ovarian cancers and glioblastoma. These three TAM kinase members share a high degree of sequence and structural homology in their kinase domains (Naresh & Guruprasad, 2021).

Cabozantinib is a small molecule inhibitor that is targeted towards multiple kinases such as Axl, c-Met, VEGFR2, RET, KIT and FLT3 (Grüllich, 2014). Cabozantinib was approved by U.S. Food & Drug Administration (FDA) for advanced renal cell carcinoma, hepatocellular carcinoma and medullary thyroid cancer. In September 2021, FDA has approved cabozantinib also for differentiated thyroid cancer that has progressed following prior VEGFR-targeted therapy. Cabozantinib is reported to bind TAM kinases with high affinity at nanomolar concentrations (Gajiwala et al., 2017; Herum et al., 2017; Lacy et al., 2018; Myers et al., 2019; Pantano et al., 2016; Qin et al., 2019; Robinson, 2013; Skora et al., 2013; Sultan et al., 2017; Turner & Blythe, 2019).

The kinase activity of RTK is triggered by its binding to ATP and Mg$^{2+}$ that results in the transfer of γ-phosphate group to tyrosine containing protein target. The ATP binding cleft is located between the N- and C-terminal lobes, and at the hinge region connecting the two lobes (Kornev et al., 2006). The structurally important regions required for the activity of a protein tyrosine kinase include, the P-loop located between β1 and β2 strands, catalytic helix (α-helix) in the N-terminal lobe comprising the essential amino acid (Glu585, Axl amino acid numbering) with its side-chain fluctuating between the active and inactive states of kinase. The distinction between the active and inactive states is also based upon the α-helical...
movement towards or away from the ATP binding site. The presence of catalytically important Lys567 (close to P-loop)–Glu585 (α-helix) is an essential ionic interaction in the active Axl kinase from the crystal structure in protein data bank (PDB). The disordered activation loop (689–724) in the C-terminal lobe has altered conformational states that are variable among the kinase structures reported so far. An ionic interaction between the side-chains of Asp581 (α-helix) and Lys695 (activation loop) is important in the kinase structure and allosteric. The synchronous fluctuations in the P-loop, α-helix and activation loop leads to spatial alteration in the shape of the enzyme active site pocket and distinct structural features such as the inward/outward rotation of α-helix and expansion of the activation loop. The Lys567–Glu585 salt bridge is the indication for the active state of TAM RTKs. A kinase domain has two kinds of active sites; regulatory substrate site and catalytic active site that become available during allosteric competitive inhibitor binding pathways in the cellular signal transduction process. Structure analyses revealed the presence of two non-contiguous structural motifs termed regulatory (R) and catalytic (C) spines (Hu et al., 2015; Mohanty et al., 2016; Robinson, 2013) that are required for stabilizing the protein in the active state.

Since the Axl kinase domain is crystallized in both active and inactive forms, we performed classical long range molecular dynamics (MD) simulations of active and inactive states of TAM kinases to obtain key insights into the spatial dynamics and to understand the cellular mechanistic pathways of inhibitor, cabozantinib binding to kinases that will prevent internalization and expansion of the activation loop. The Lys567–Glu585 salt bridge is the indication for the active state of TAM RTKs. A kinase domain has two kinds of active sites; regulatory substrate site and catalytic active site that become available during allosteric competitive inhibitor binding pathways in the cellular signal transduction process. Structure analyses revealed the presence of two non-contiguous structural motifs termed regulatory (R) and catalytic (C) spines (Hu et al., 2015; Mohanty et al., 2016; Robinson, 2013) that are required for stabilizing the protein in the active state.

Since the Axl kinase domain is crystallized in both active and inactive forms, we performed classical long range molecular dynamics (MD) simulations of active and inactive states of TAM kinases to obtain key insights into the spatial dynamics and to understand the cellular mechanistic pathways of inhibitor, cabozantinib binding to kinases that will prevent internal signaling by up-regulation or overexpression of kinases. In this manuscript, the highly unstable conformational transition states including R and C-spines in the kinase domains are reported by studying the apo, ATP and cabozantinib bound TAM RTKs each for 1 μs MD simulations using AMBER 18.14 suite of programs.

2. Materials and methods
2.1. Structures of apo, active and inactive TAM RTK kinase domains
The three-dimensional crystal structures of Axl (PDB id: 5U6B) (Gajiwala et al., 2017) A and B chains exist as inactive and active states, respectively. The missing residues in the activation loop were built using ‘Model/Refine Loops’ in ‘Structure Editing’ tool in UCSF Chimera 1.12. (Yang et al., 2012) The active and inactive homology model structures of Mer and Tyro3 were built based on the crystal structures of 5U6B, B and A chains, respectively, using MODELLER (Webb & Sali, 2014) as described previously (Naresh & Guruprasad, 2021).

2.2. Molecular docking of ATP and cabozantinib
The inhibitor cabozantinib was docked into the ATP binding pocket of the active and inactive conformers of TAM kinases and ATP was docked into the ATP binding pocket of the active state of TAM kinases using AutoDock (Morris et al., 2009). The docking pose with lowest binding energy and maximum docking poses was utilized for further MD simulations to decipher the molecular basis for interactions between protein and ligand.

2.3. Molecular dynamics simulations
All MD simulations were achieved using AMBER (Gotz et al., 2012) version 18.14 for the apo, ATP bound active and cabozantinib bound active and inactive states of TAM kinases. The best docking pose of each complex was utilized as input for MD simulations. The force fields for the entire systems were generated with Antechamber using am1bcc method (Colovos & Yeates, 1993; Wang et al., 2006). All input parameter files for MD simulations were generated after adding hydrogen atoms in tLeap module in AMBER tools (Anandakrishnan et al., 2012; Lindorff-Larsen et al., 2010). Sodium and chloride ions were added to the systems to neutralize the charge, each molecular system was solvated within a 10 Å size box. The final ionic concentration for the systems was set to 100 mM. The Amberff99sb-ildn force field was used for entire model system with TIP3P water model for AMBER molecular parameters (Mark & Nilsson, 2001; Meagher et al., 2015). All MD simulations were run at 300 K temperature and 1 atm pressure with Monte Carlo barostat (Salomon-Ferrer et al., 2013). Energy minimization was carried out by using steepest descent method for 40,000 cycles to overcome short range null contacts among the molecular system in solvent (Darden et al., 1993). Long range electrostatic interactions were considered with Particle Mesh Ewald algorithm (Jorgensen et al., 1983) with cut-off range 9 Å and order 4. All model systems were equilibrated for 5 ns before the production run, and the coordinates in the production run were saved after every 5 ps (McGibbon et al., 2015; Salomon-Ferrer et al., 2013). The MD simulations of each molecular system were carried out for 1 μs, accounting for a total of 12 μs simulations time.

2.4. Data analysis
The MD trajectory data analysis was carried out using cpptraj and pytraj in Amber tools 18 (Hornak et al., 2006). The average structures after MD simulations, root mean square deviation (RMSD), root mean square fluctuation (RMSF) and principal component analysis (PCA) were derived from the trajectory analysis. For the sake of data space minimization during post MD analysis, the Markov state model (MSM) analysis was carried out on 40 K frames out of 200 K frames and the PCA was carried out on the data from 1 K frames generated from each molecular system. To build the MSM, datasets of close accessible kinetic metastable states associated with protein conformational ensemble obtained from large scale simulations are required. These states can be defined in pyEMMA Python library (Scherer et al., 2015). To generate the MSMs 40 K conformations were sampled. All twelve MD simulations datasets were transformed in terms of protein Cα backbone dihedrals, Cα backbone atomic positions and distances from their trajectories. All MD simulations trajectories were analyzed for 1000 ns (200 K frame data) by sampling the MSM predictions (Harrigan et al., 2017). This identified kinetically metastable transitions among cluster k-means lag time (250 degrees of
freedom) of protein conformations (Perez-Hernandez et al., 2013). The extrapolation of the real time data into pictorial and graphic vectorized data points was achieved with maplotlib and numpy data frames into 2D plotting space. The state distributions of kinetic metastable data points were featureized and cluster analysis was applied using time lagged independent component analysis (tICA) (Noo & Clementi, 2015; Pedregosa et al., 2011; Perez & Granger, 2007).

3. Results and discussion

The amino acid sequence alignment of Tyro3, Axl and Mer kinases is shown in supplementary material (Figure S1A), the final modeled (after MD simulations) structures of the active and inactive kinases display significant conformational alterations in the P-loop, α-helix and activation loop as shown in the Figure 1A. The three-dimensional structures of active and inactive forms of Axl kinase domain were taken from the crystal structure (SU68) (Gajiwala et al., 2017) B and A chains, respectively. The homology models of active and inactive forms of Mer and Tyro3 kinase domains were constructed and validated (Naresh & Guruprasad, 2021). The models of TAM kinase domains constructed were compared with model structures generated using Phyre2 (Kelley et al., 2015) and AlphaFold (Varadi et al., 2022) by structure superposition. As shown in supplementary material (Figure S1B), the structures superpose with low RMSD.

From the docking of cabozantinib into TAM kinases, it was observed that it binds to the ATP binding pocket mediated via several non-bonding interactions. The hinge region residues Phe622, Met623 (Axl kinase domain) interact with dimethoxy quinoline ring nitrogen of cabozantinib. The para-fluoro phenyl interacts with Phe691 aromatic ring (DFG motif in Axl) and Asp690 forms hydrogen bond with amide nitrogen located between the cyclopropyl and phenyl rings of the inhibitor. The cofactor ATP binds the active site of TAM kinases, intermolecular hydrogen bonding interactions are observed with Pro621 (hinge region) and Asp627 (hinge region), Asn677 (catalytic loop region) in the Axl kinase domain. The protein–ligand interaction plots are shown in supplementary material (Figure S1C). The structures of apo, active TAM kinases complexed with ATP, active and inactive TAM kinases complexed with cabozantinib were subjected to 1 μs MD simulations each, using AMBER18. Throughout MD simulations all molecular system appeared to be stable as observed from temperature versus time and total energy versus time plots shown in supplementary material (Figure S1D and E).

3.1. Active–inactive kinetic state models of TAM RTKs

From the long range MD simulations of TAM RTKs the kinetic state models are defined according to the internal structural dynamical features such as P-loop (544–549 amino acid residues), α-helix (576–591) and activation loop (689–724) from the trajectories of the MD simulations data. The active/inactive conformers of TAM kinases are clearly distinguished. In the active state, the side-chain of Glu585 on α-helix is rotated inwards towards the substrate binding site to make salt bridge interaction with Lys567 in the case of ATP bound Axl RTKs. The side-chain of Asp690 from the DFG motif also projects towards the active site. The outward orientation of Glu585 side-chain away from the substrate to dissociate the ionic interaction with Lys567 (P-loop), and rotation of Asp690 side-chain inwards into kinase active site is indicative of an inactive state of kinase (Gajiwala et al., 2017). In the inactive state, the α-helix undergoes outward rotation, followed by the activation loop inward folding to minimize the drug binding active site that can be seen from Figure 1A. These are the key structural features implicated in the regulation of protein kinase activity and influence the effective binding of inhibitors. The binding of cabozantinib influenced various states of active/inactive models in Tyro3, Axl and Mer kinase domains. The active kinetic models are indicated by the ionic interaction between Lys567 and Glu585, inward rotation and activation loop extended to further maximize inhibitor binding site. In the ATP bound active TAM kinase structures, this ionic interaction is retained throughout the MD simulations (Figure 1B) indicating that ATP bound TAM kinases retain the active state.

The C-spine and R-spine dictate the positions of ATP and substrate in the kinase domain. These spines play a key role in the catalysis of kinases while binding with ATP. We have mapped the location of R-spine and C-spine on the structures of TAM kinases based on the structures of C-Src (Robinson, 2013). The R-spine consists of four non-consecutive hydrophobic amino acid residues aligned vertically from N-terminal lobe towards the C-terminal lobe through the activation loop (Kim et al., 2017). These hydrophobic residues in Axl kinase domain are Leu600 (βx-strand); Met589 (α-helix); Phe691 (DFG motif); His670 (catalytic loop) and an additional residue Asp731 from the C-terminal lobe (Figure 2A–C). The C-spine consists of eight non-consecutive hydrophobic amino acid residues aligned vertically from N-terminal lobe towards the C-terminal lobe through the hinge region. These hydrophobic residues in Axl kinase are, Val550 (β2-strand), Ala565 (β3-strand), Phe622, Leu628 (hinge region), Met679, Leu580 (catalytic loop), Met739, Ile742 (αx-helix from C-terminal lobe) as shown in supplementary material (Figure S1F) and supporting videos 0-Axl-apo, 1-Axl-Cabo-active and 2-Axl-Cabo-inactive.

The RMSD plots are shown in Figure 3A–F. The RMSD plots of protein Ca atoms (Figure 3A), indicate that the structures converged at about 100ns of MD simulations and the RMSD values lie within a narrow range from 2 to 4.5 Å. The ATP bound TAM kinase domains have lowest RMSD among all the systems studied. The TAM active state kinases form stable complexes when bound to cabozantinib. The apo Tyro3 and Axl have higher RMSD values among all systems studied. The RMSD analysis of specified regions in kinases are the key components to describe the distribution among inactive and active states. The R-spine of Tyro3 and Axl have well differentiated active states based on the lower RMSD (~2.8 Å) while the Mer active state has higher RMSD (~4.5 Å). All the inactive states of TAM kinases have an RMSD of 4 Å in the R-spine. The C-spine RMSD is higher in the case of apo Axl kinase but the ATP bound Mer and Tyro3 have lower
C-spine RMSD (~2.8 Å). The RMSD of N-terminal P-loop are nearly similar in all the molecular systems studied. The RMSD of α-helix region is distinguished among all TAM kinases studied and lie within a range of 1.5–3.0 Å. The active and apo states of Tyro3, the inactive and apo states of Axl and the apo Mer kinases have higher and nearly similar RMSD values of the α-helix among all the kinase states. The RMSD is lowest in the inactive Tyro3, Axl active, active and inactive Mer complexes. The RMSD of the activation loop is quite opposite to the α-helix region. The active state Tyro3, active and apo states of Axl and apo state of Mer kinase have lower and nearly similar RMSD values among all the systems. The activation loop in the apo and inactive Tyro3, inactive Axl, active and inactive Mer has highly dynamical conformation as can be seen from the higher RMSD values. Among all the systems studied, the inactive Axl activation loop is highly variable. The RMSD of R- and C-spines in the ATP complexes of TAM kinases is lower than 3 Å. The P-loop and α-helix have lower RMSD (1.5 Å) and the RMSD of the activation loop is in between 1.5 and 3 Å. The cofactor ATP stabilized TAM kinases with the adenine group coordinated at the hinge region of the kinase domain. The results from the
RMSD are in correspondence with the RMSF plots (Figure 4A–C). In the hinge region, cabozantinib bound Tyro3 inactive is distinct from the active and apo Tyro3 kinase domains. The side chains of hinge region amino acid residues (607–611) have greater fluctuations. In the Axl kinase domain, the apo form has a distinct conformation in the hinge region compared to the inactive and active cabozantinib bound states. The side chains of hinge region amino acid residues (622–625) have greater fluctuations. Whereas in the Mer kinase domain, all the three states (apo, cabozantinib bound active and inactive forms) have nearly similar conformations in the hinge region (666–669). From the supplementary material Figure S1G, it can be seen that the hinge region is most stable in the Mer kinase domain when complexed with cabozantinib. From the analyses of the RMSD and RMSF plots, we believe that TAM kinase domains have unique hidden dynamic states that can be distinguished from further analyses of MD trajectories.

3.2. Tam kinase-cofactor complex activation pathway

The kinetic states appear due to the stereo-spatial arrangement of certain residues in specific α-helices, β-sheets and loop regions in the kinase domain. These kinetic states provide key insights into the activation of protein kinase in the presence of ATP and inhibitor bound to the active site. The apo, ATP bound and active/inactive Axl-cabozantinib molecular
systems consist of well-defined kinetic state models during the MD simulations. The precise representation of the local spatial pattern in the active and inactive states of a kinase domain can be accessed via the R-spine and C-spine. The R-spine controls substrate molecule in the active site ($\alpha$-helix and activation loop). The C-spine regulates catalysis by allowing the ATP binding site at hinge region. The inactive kinase state should be converted into active state with help of substrate binding at activation loop through the influence of R-spine hydrophobic residues which connect the dynamical movement of catalytic loop in $\alpha$F-helix. The coordination between R-spine and C-spine evolve a dynamical conformation for the transfer of

Figure 3. RMSD plots of apo, ATP bound TAM RTKs and cabozantinib bound active and inactive TAM RTKs from 1 $\mu$s MD simulations. (A) Tyro3, Axl, Mer Cx atoms in the kinase domain (B) Activation loop (C) Regulatory spine (D) Catalytic spine (E) P-loop (F) $\alpha$-helix.
\( \gamma \)-phosphate from ATP to the substrate protein (Kim et al., 2017; Kornev et al., 2006; Mohanty et al., 2016; Myers et al., 2019; Robinson, 2013).

The R-spine is continuous and linear in the case of normal metabolic kinase activity. The hydrophobic surface in the R-spine is vertically aligned (Leu-Met-Phe-His) in the apo form of all TAM kinases as can be seen from the Figure 2(A–C). In the Axl active state, the R-spine is broken in the case of inhibitor bound form due to the expansion of activation loop that results in the extended space between \( \alpha \)-helix Met589 and DFG motif-Phe691 at ATP binding site. The inactive Axl bound to cabozantinib has an intact R-spine due to the expansion of space between \( \alpha \)-helix Met589 and \( \beta_4 \)-strand Leu600 as a result of the outward rotation of...
Figure 3. Continued.
Figure 4. RMSF plots of TAM kinase domain from 1 μs MD simulations. Kinase domains are numbered as per their primary structure. Axl indexing 539–553 (β1–β3 turn in the N-terminal domain-P-loop); 579–591 (α-helix in the C-terminal domain); 689–724 (activation loop). (A) Tyro3; (B) Axl; (C) Mer.
Figure 5. Distance plots between side-chains of Lys P-loop-Asp α-helix pairs in apo, ATP and TAM-cabozantinib bound active and inactive kinase domain from 1 μs MD simulations. Axl (K567–E585); Tyro3 (K552–E570); Mer (K612–E630); (A) ATP bound; (B) Active inhibitor bound; (C) Inactive inhibitor bound; (D) Apo state.
α-helix. The R-spine is retained in a similar way in the cabozantinib bound Tyro3 kinase domain in the active and inactive states. In the case of the cabozantinib bound active state Mer RTK, the R-spine fragmentation occurs between the P-loop, α-helix and activation loop, whereas in the cabozantinib bound inactive Mer, the R-spine is retained. In the active site in Axl and Mer kinases, inhibitor occupies the shallow depth in between the α-helix–activation loop, resulting in the broken R-spine. In the case of inactive states, the inhibitor binds at the hinge region of TAM kinases and therefore retaining the R-spine.

In the kinase active state, the R-spine is broken in Axl and Mer RTKs, whereas the C-spine is retained in the active state of Axl and Mer RTKs with no breakage in the hinge region. The R and C-spines are coordinated in such a way that if the R-spine is broken, the C-spine is retained and vice-versa. The active Axl and Mer RTKs have broken R-spine but the C-spine is intact, but in the rest of the molecular systems the R-spine is intact and the C-spine is broken. It is like a lever pulling mechanism in the presence of higher concentration of inhibitor bound at regulatory site of kinase. The spine coordinated mechanism is important to ensure that the kinase is regulated from inactive state to active state mode in the presence of higher concentrations of substrate or high concentration of ATP in the cellular cytosol. Therefore, the cabozantinib binding in the active state kinase influences at specified locations of the R-spine residues rather than C-spine. This can lead the C-spine to initiate catalytic activity towards passive mechanism to alert the body immune system with help of chemokines. Whereas, in the inactive kinase state, the inhibitor binding to the regulatory active site or hinge region, R-spine activates either the dynamical movement of catalytic loop or C-spine to initiate the catalysis process with help of cofactor ATP. As a consequence, both the spines are well coordinated in the case of inhibitor bound to both active and inactive states to trigger apoptosis in malignant cells.

3.3. Confirmation of the existence of active states in ATP and active/inactive states in cabozantinib bound TAM kinases

A kinase domain can switch from active to inactive states and vice versa due to either inhibitor binding, or influence of the R-spine and C-spine during MD simulations at longer time-scales. We observed noticeable changes in the spatial conformational states with inhibitor binding at the active site of the TAM RTKs. However, the specified regions of spatial orientations are not directly observable from the conventional RMSD plots. The inhibitor bound Axl kinase activation takes place in the transition from active to inactive kinetic models. Therefore, these states coexist with broken R-spine in the active and inactive metastable states at the specified timescales of MD simulations. In addition, the RMSD of Axl differentiates due to the coexistence of active-inactive states throughout 1 μs time-scales (Figure 3A). The RMSD of specific loops in Axl is observed at higher square fluctuations occurring at the loop connecting β4–β5 strands (Glu609-Pro614), α-helix and activation loop. It is evident from the RMSF plots that cabozantinib drug binding influences the inactive state of Axl and Mer kinase more than their active states (Figure 4). When cabozantinib binds the kinetically metastable states of TAM RTKs, it arrests the mechanism of kinase activity by inhibiting the up-regulation of its enzymatic activity. R-spine is broken in inhibitor bound active state of Axl and Mer but it is intact in apo and cofactor (ATP) bound kinase state. The active kinetic states of TAM kinase bound ATP at hinge regions shows Lys–Glu salt bridge distance retained within 4 Å range throughout 1 μs MD simulations. This indicates that the ATP bound active TAM kinases retain their active state throughout the MD simulations. While only the cabozantinib bound active state of Axl has the salt bridge distance between P-loop and α-helix, the Mer and Tyro3 kinases have longer distances (>7.5 Å) due to the core expansion of activation loop region. In the case of inactive states of Axl, Mer and Tyro3 kinases these distances drastically
This signifies that the Axl and Mer RTK kinetic models have well distinguished proportions of active and inactive state, while Tyro3 has similar ratios of active and inactive intermediate states (average 12.5 Å salt bridge distance between (P-loop) Lys and Glu (α-helix) in Tyro3 active and inactive). The salt bridge distance between α-helix Asp/Glu and Lys in activation loop of ATP bound states in Axl and Mer (<5 Å) and Tyro3 (>5 Å) indicates highly dynamical structure than among all active and inactive states. These salt bridge distance analyses clearly differentiate cofactor (ATP) and inhibitor (cabozantinib) bound kinase domains at active site and active/inactive states, respectively (Figure 5A–D). The inhibitor bound active/inactive kinase states are highly dynamical in nature than cofactor bound kinase states, therefore the inhibitor bound kinase might trigger apoptotic signaling pathways leading to inhibition. Based upon individual RMSD plots of the R-spine and activation loop (Figure 3B, C), it can be seen that cabozantinib binding influences the activation loop and hydrophobic spine in individual kinetic states. A specific spatial conformational variation in RTKs occurs only in the activation loop and R-spine. The active and inactive forms of apo and active ATP bound conformers of TAM kinases appeared to have intact R-spine. This could lead to the normal signal transduction process while the regular ligands (GAS-6 and Pros1) bind to the extracellular regions of TAM RTKs.

The analysis of salt bridge distance between the activation loop and α-helix reveals the hidden conformers among the apo, ATP and inhibitor bound TAM kinases. The salt bridge interaction in the apo kinase is retained within a reasonable distance between the α-helix and activation loop residues Asp581–Lys695 (3.99 Å, Axl) or Glu626–Lys739 (3.86 Å, Mer) or Glu566–Arg680 (3.44 Å, Tyro3). The salt bridge distance between Asp/Glu (α-helix) - Lys/Arg (activation loop) in cabozantinib bound active states increases in Axl and Mer RTKs due to the expanded core in the inhibitor binding site in RTKs (Tyro3: 3.16 Å; Axl: 13.92 Å; Mer: 10.28 Å), but in the inactive states of TAM RTKs salt bridge distance between α-helix and activation loop is lower for Axl RTK (Tyro3: 6.82 Å; Axl: 2.83 Å; Mer: 8.83 Å) supplementary material (Figure S2A–D). These salt bridge distances provide support to the stationary state distribution in
apo TAM RTKs. These salt bridge distances in the ATP bound TAM kinases is observed to be greater than 5 Å (Tyro3: 7 Å; Axl: 10.0 Å; Mer: 12.0 Å).

The salt bridge is retained in the apo form, active states of Tyro3 and inactive states of Axl. The salt bridge distance analysis provides a clear evidence that the kinases coexist in active and inactive state models while binding with inhibitor at the active site. The large distance across the regulatory site of kinase active states occurred due to a β-sheet formation in the activation loop and inward rotation of α-helix. This causes the extended nature of regulatory active site between α-helix and activation loop. The inactive state
models have \(\alpha\)-helix outward rotation and activation loop undergoes shift to helical structure to minimize the active space across \(\alpha\)-helix and C-lobe in the RTKs. These results provide further support to R-spine analysis. But most of the active states in Axl and Mer forms have broken R-spine between \(\alpha\)-helix and activation loop therefore the distance between these domains is extended and the salt bridge interaction is disturbed due to the increased distances between Asp/Glu (\(\alpha\)-helix) and Lys (activation loop). In the inactive state of TAM RTKs, the R-spine is reinstated due to the bound inhibitor at the hinge region of kinase and expansion of space between P-loop and \(\alpha\)-helix. In overview, in the cabozantinib bound TAM kinases, the salt bridge distance is higher in active states than inactive states, as the distance of salt bridges Asp/Glu (\(\alpha\)-helix) and Lys/Arg (activation loop) in the active states are above 10 Å, the inactive states have below 10 Å (Figure S2D).

### 3.4. Post-MD data analysis of TAM RTK kinase domain

The preliminary MD simulations data acquired from AMBER trajectories was analyzed to ensure that kinetically active and inactive states were investigated with the help of PCA. PCA analysis was carried out on 1 K conformer samples of trajectories out of 40 K for clear visualization of data points from kinetic transition states in the active to inactive kinases (Supporting Figure S3A–C). The histogram showed that the random distribution of kinase state trajectories data was extrapolated as training and test sets of individual components validated with shuffle-split cross-validation in PCA plot. All apo and inhibitor bound forms of TAM RTKs have random distribution of states that are very unique in nature from the respective scatter plots of kinase trajectory analysis. This is a preliminary analysis to propose the hidden dynamic states existing in longer timescale MD simulations and trajectory data of kinases.

The metastable kinetic models were built based upon advanced trajectory data analysis using python based scripts. All TAM trajectories data was sampled into vectorized and clustering was done using Keras-state algorithm for MSM model generation (Harrigan et al., 2017; Prinz et al., 2011; Schwantes & Pande, 2013). The MSM data of TAM kinases was bootstrapped from 1 μs of trajectory data to generate Hidden Markov models (HMM) to reveal the unfolding and refolding of the activation loop from active state to inactive states. The metastable trajectories are well converged as shown by VAMP score. Discrete clustering of protein backbone state distribution featurization was performed to show distinct kinetic stable states in all TAM kinases. All HMM states are key intermediate conformers to describe the kinase inhibitory activity when bound to cabozantinib. As per the analysis of metastable kinetic state forms, higher numbers of active state models are present in Axl and Mer than the number of kinetic transitions states of inactive forms. However, the Tyro3 has approximately similar numbers of kinetic state models in their respective active and inactive states which are included in state distributions (Table 1).

| Kinases types | Axl | Mer | Tyro3 |
|---------------|-----|-----|-------|
| **Kinetic metastable states** | G/\(kT\) (kcal per HMM state) | G/\(kT\) (kcal per HMM state) | G/\(kT\) (kcal per HMM state) |
| **Active** | 1 | 0.080859 | 2.515052 | 0.072653 | 2.622066 | 0.000000 | inf |
| | 2 | 0.000000 | 2.347855 | 0.095574 | 2.10675 | 0.155743 |
| | 3 | 0.328240 | 1.114010 | 0.271887 | 1.302367 | 0.094495 | 2.359208 |
| | 4 | 0.206730 | 1.576340 | 0.286168 | 1.251176 | 0.343637 | 1.068168 |
| | 5 | 0.384171 | 0.956668 | 0.273718 | 1.295657 | 0.351193 | 1.046420 |
| **Transition states** | \(S_1-S_5\) | \(S_1-S_5\) | \(S_1-S_5\) |
| | \(S_1-S_5\) | \(S_1-S_5\) | \(S_1-S_5\) |
| **Apo** | G/\(kT\) | G/\(kT\) | G/\(kT\) | | | |
| | 1 | 0.008222 | 4.800930 | 0.081258 | 2.510125 | 0.078556 | 2.543949 |
| | 2 | 0.052043 | 2.955685 | 0.239732 | 1.428232 | 0.083054 | 2.488261 |
| | 3 | 0.080056 | 2.525032 | 0.131020 | 2.032406 | 0.139106 | 1.972522 |
| | 4 | 0.165858 | 1.796625 | 0.228406 | 1.476629 | 0.293660 | 1.225334 |
| | 5 | 0.693821 | 0.365541 | 0.319583 | 1.140738 | 0.405625 | 0.902326 |
| **Transition states** | \(S_1-S_5\) | \(S_1-S_5\) | \(S_1-S_5\) |

**Table 1.** Tyro3, Axl and Mer kinetic transition states analysis with specified free energy of nine HMM states. Bold indicates metastable kinetic equilibrium transitions states. Italic indicates metastable kinetic non-equilibrium transitions states.
changes in the kinase domain is due to the conversion of active to inactive states through kinetic transition metastable equilibrium states.

In the inhibitor bound form of TAM kinases, greater state distribution models coexist in the active forms than in the inactive forms. The drug bound to kinase active state influences the kinetic signaling pathways more rather than the inactive state (Roskoski, 2015; Shukla et al., 2014; Sultan et al., 2018; Taylor & Kornev, 2011). Therefore, the active state kinase bound to inhibitor is more susceptible to arrest the dysregulated kinase activity (shown by the broken R-spine) in all kinetic HMM states. These observations provide key insights to describe that the kinase activity can be arrested through active state models of inhibitor bound RTK, where R-spine breaks in between activation loop and α-helix in the active states (Bowman & Pande, 2010; Parsons & Parsons, 2004; Robinson, 2013). The hydrophobic surface R-spine is retained in the apo form of all the three TAM kinases. The R-spine is retained in the cabozantinib bound Tyro3 RTK in the active and inactive states, due to the increased distance between P-loop and α-helix. This retaining of R-spine in Tyro3 RTKs indirectly influences the number of active and inactive state distribution in equal proportions. In the inactive Axl and Mer RTKs, intact R-spine is observed due to increase in the distance between the P-loop and α-helix, whereas in the active Axl and Mer RTKs, R-spine fragmentation occurs between α-helix and activation loop, due to the lower distance between P-loop and α-helix. These observations are shown in Figure 2(A–C). The discrete clustering of MSM estimation and validation was done with reversible estimation equilibrium transition probabilities. The discrete kinetic state models were further validated by analysis of hidden Markov kinetic models. The implied relaxation timescales are extracted to validate the HMM in order to ensure the conditional transition probabilities among 250 microstates. Therefore, the implied timescale analyses indicated that the kinetic state distribution occurred within time intervals of a few nanoseconds range among 1 μs MD simulations timescale; supporting material (Figure S6A–C).

The Mer active states have longer MD kinetic relaxation timescales among the active MSM kinetic forms of TAM RTKs. The inactive Axl kinetic state models have higher relaxation timescales within short range of time intervals. The critical observation from all TAM apo and inhibitor bound active and inactive kinetic states implied from timescale plots, with 4.5 ns timescale separation as the average implied relaxation timescale among all. The Tyro3 apo has more relaxation time intervals than the rest of kinase systems; supporting material (Figure S6C). The kinetic relaxation time intervals revealed that the inhibitor bound TAM RTKs showed kinetic metastable state transitions due to various periodic time laps even though all TAM RTKs bound with same inhibitor (cabozantinib); supporting material (Figure S6D).

The MSMs of the members from same class of protein kinase complexes (TAM kinases bound to cabozantinib) is expressed as different relaxation timescale intervals obtained from the MD simulations. The free energy and stationary state distribution of apo Axl is higher than Tyro3 and Mer.

![Figure 7. (A) Schematic view of three kinetic equilibrium metastable states among HMM states involved allosteric activation and deactivation from active to inactive states in RTKs. (I) active state; (II & III) transition like states; (IV) inactive state.](image-url)
From Table 1, we infer that there are unique kinetic Markov state models existing among them. These are classified as ‘kinetic non-equilibrium transition state models’ (Tyro3 apo, Mer active and Axl inactive). This is further discussed in kinetic transition analysis. The lowest free energy and equal stationary distribution exist in stable kinetic model states of TAM kinases (Axl-active, Mer-inactive).

The kinetic transition state between Axl active and Mer inactive has higher free energy and approximately equal stationary distribution values (Tyro3 active/inactive) and is classified as ‘kinetic equilibrium transition state models’. As per the state distribution difference between active-inactive states of Axl inactive HMM has half (1/2) of the stationary distribution of Axl active (more active state distribution). The inactive Mer has $\frac{3}{4}$ of the state distribution of active Mer RTK. The Tyro3 has equal contribution in active and inactive stationary distributions among kinetic HMM states. The surface free energy of Axl has same energy values in the active and inactive states ($\sim 4.0 \text{ kcal/kT per 5 states-Axl}$) but Tyro3 and Mer have 0.5 and 1.2 kcal, respectively per five MSM states energy difference between the active and inactive hidden Markov states; supporting material (Figure S7A–C). Each hidden MSM state contains five metastable kinetic conformers from sampling of 40 K conformers to study the MSM validation.

### 3.5. Kinetic transition state analysis

The estimated five state kinetic metastable models were designed based upon active space distribution of HMMs in TAM RTK kinase domains. All the five metastable state transitions occurred based upon kinetic transition energy (Weinan & Vanden-Eijnden, 2010; Salvalaglio et al., 2014; Metzner et al., 2009; Table 1). The apo Axl has higher transition energy (4.8 kcal), inactive Mer (3.4 kcal) and inactive Tyro3 (3.5 kcal). Out of the nine kinetic states, six kinetic transition states are represented as metastable kinetic equilibrium transition states as these kinetic transitions occurred in $S_4$–$S_5$ states. The metastable kinetic non-equilibrium transitions exist in various types of kinetic metastable states (Tyro3 apo: $S_3$–$S_5$; Mer-active: $S_2$–$S_4$; Axl inactive: $S_2$–$S_3$) from the nine metastable transition states (Figure 6A–C). All non-equilibrium kinetic transitions occur with a very low transition energy (2–2.6 kcal). These hidden states are classified based upon kinetic transition energy and state transitions. All the metastable kinetic equilibrium transitions occurred with a high energy (2.3–4.8 kcal); supporting material (Figure S8A–F). As per the individual TAM RTK, the Axl apo kinase has higher kinetic transition energy among all TAM RTKs in the apo and inhibitor bound active and inactive forms. The next higher kinetic transition energy exists for Mer and Tyro3 inactive forms. It is evident that all inhibitor bound RTKs exhibit different kinetic metastable states in the overexpressed RTKs during the protein function. According to approximate difference in transition probability of active to inactive metastable kinetic states in Tyro3, Axl and Mer RTKs, for Tyro3, 1st MSM state has higher transition probability difference (50%), for Axl and Mer RTKs, 2nd MSM states have higher transition probability difference; Supporting material (Figure S8G–I).
The transition of kinase active state to inactive state can be explained based upon kinetic metastable states of these specified MSM conformer analysis (Husic & Pande, 2018). From Figure 7, it can be seen that the stationary state distributions in Axl active are doubled when compared to Tyro3 active, and Mer inactive states has only 4/3 proportion. Therefore, Axl active RTK has more active stationary states. The relative transition state probability is explained on the basis of salient feature analysis in hidden Markov kinetic states. These could be key intermediate structures among subfamily of TAM RTK kinase domains. However, these protein kinases driven from active to inactive states expressed significant structural changes upon binding with inhibitor. The active state of Axl kinase consists of activation loop that transits from β-sheet to α-helical structure in the inactive state (Figure 7A, B). Mer RTK shows high structural changes in the activation loop which converts from loop (active) to helical (inactive) in their respective state transitions (2-2 transition probability), while Tyro3 does not have any significant change in the MSM kinetic states (Figure 8A, B).

### 3.6. Mechanistic strategy of TAM RTKs activation when complexed with cabozantinib

The dynamical movement of the R and C-spine residues is a result of the coordinated alterations in the kinase structural domain during the cellular signal transduction process. The selective kinase inhibitor (cabozantinib) arrests the activity of these overexpressed kinase domains via the dynamic movement of both these spines and distancing the space between α-helix and activation loop (Supporting material Figure S2). This can be supported from the results of distance plots shown in the active states of Tyro3, Axl and Mer that have undergone large expansion of protein core between α-helix-activation loop in regulatory active site; supporting material (Figure S2). Therefore, the kinase activation is carried out by the active state modes. The five metastable states from
Chapman-Kolmogorov test described transition probability from 40 K frames of dynamic kinetic metastable states for each of the protein complex trajectories, was obtained from AMBER MD data with 95% confidence level; supporting material (Figure S7A–C). Combining all these transition probabilities with transition states and assigning five sampled metastable states could provide good insights and predict long lived transition states in the MD simulations trajectories with Perron-cluster cluster analysis (PCCA++) clustering algorithm (Harrigan et al., 2017; Perez-Hernandez et al., 2013; Scherer et al., 2015; Schwantes & Pande, 2013).

The RTKs are involved in signal transduction process in which dysregulated kinase is inhibited such that the cells initiate programmed cell death with the help other proteases belonging to the caspase enzyme (Kim et al., 2017). The regulated and dysregulated kinases can be distinguished with help of R-spine (Leu-600 311β4-sheet P-loop; Met-589 α-helix; Phe-691 DFG-activation loop; His-670 catalytic loop) closed and open conformers of apo, ATP and inhibitor bound TAM RTKs, respectively, due to significant conformational changes. A regulated kinase has closed and continuous R-spine in both active apo and ATP bound form in RTKs. The hydrophobic surface is in a closed manner and continuous in apo and ATP bound form of active Tyro3, Axl and Mer RTKs. This space has expanded in the case of active states of normal physiological kinase mechanism. This is achieved based upon activation loop refolded into β-sheet and π-stacking with β-sheet structure of the catalytic loop in active state model (observed in Axl). But inhibitor bound dysregulated kinase experiences large conformational deformations in their regular structures due to the influence in certain parts of RTKs with overwhelmed hidden dynamic states to trigger kinase domain equilibration between...
active and inactive states. Indeed, the drug (cabozantinib) bound at RTKs active site, triggers the activation loop folding into either β-sheet (Axl active-state) or α-helix (Mer inactive-state) (Figure 7A). These kinetic metastable states have transition from active to inactive states through intermediate structure (transition-state) and vice-versa (Metzner et al., 2009; Noe & Clementi, 2015; Weinan & Vanden-Eijnden, 2010). The dynamic states would proceed through mechanistic pathways to initiate signaling process as expanding or compressing of the activation loop, outward/inward rotation of α-helix and extended movements in the P-loop. It can be seen that the active site cavity is enhanced in the presence of inhibitor bound active state that has broken R-spine obtained by moving apart the Glu residue on α-helix and Phe residue in DFG motif associated with the activation loop. The uncertainty of migrated residues could be withheld in a particular state of kinase domain vertically from N-lobe towards C-lobe. The R-spine is broken in the active state only in situ with all four residues moving away of the broken hydrophobic surface between α-helix bound Met-589 and DFG motif bound Phe-691 due to the extend space of activation loop and inward rotation of α-helix. Inactive state model kinase consists β4-strand bound Leu-600 and α-helix bound Met-589 in situ R-spine intact in a continuous manner due to the outward rotation of α-helix and the activation loop has recoiled into α-helix where DFG motif and α-helix moves away from the P-loop of β-sheet (Figure 7B).

From the PCA, it is revealed that all TAM kinase domains have random distribution of states. The analysis of metastable kinetic states revealed higher numbers of active state models in Axl and Mer kinase domain than the number of kinetic transition states of inactive forms, however Tyro3 kinase domain has similar numbers of coexisted metastable state transitions among the active and inactive forms. The MSMs (Husic & Pande, 2018) of the TAM kinases is expressed as different relaxation timescale intervals. The Tyro3 apo, Axl inactive and Mer active have higher relaxation timescales. The Tyro3 has equal contribution in active and inactive states, (K567 P-loop - S2–S5 and Mer-active S2–S5) due to different kinetic transitions occurring among them (Table 1). The rest of the six HMM states undergo S2–S5 kinetic transitions among five state model system mentioned as ‘equilibrium kinetic transition states’ (Figure 8, supplementary material, Figures S10, S11). The activation loop undergoes β-sheet formation in the case of active Axl and α-helix formation in the case Mer inactive state during S2–S5 kinetic transitions. In the case of Tyro3 active and inactive states, the activation loop remains in a random loop conformation. The TAM receptor tyrosine kinase bound with ATP as active state mode to facilitate phosphorylation of substrate (tyrosine amino acid). But the inhibitor (cabozantinib) bound to TAM RTKs active and inactive mode states. This result describes that the effective inhibitor bound the active receptor tyrosine kinase to arrest the substrate binding state of kinase domain to effective block overexpressed TAM RTK’s. The inactive state kinase bound inhibitor could arrest the change of protein conformations in transduction signal process to initiate the effective apoptotic signals to nullify the any malignant protein bound inhibitor state by immune cells. Therefore, the kinase bound specify states are very crucial to understand the RTKs involved in various types of cancers. In summary, we observed salient changes in the spatial conformational states due to inhibitor binding to the active site during MD simulations in various regions of Tyro3, Axl and Mer kinases. From these research findings, the kinetic active and inactive state mechanisms could explain how cabozantinib arrests the overexpressed TAM RTKs in malignant cells, a key step to inhibit the kinase signaling pathway in cellular signaling process.

4. Conclusions

From 1 μs MD simulations each of apo, ATP, cabozantinib bound active and inactive TAM kinases, metastable active and inactive conformational states are revealed. The α-helix region is highly distinguished and its conformational flexibility is complementary to the activation loop. The dynamical movement of the overall R and C-spines consisting hydrophobic residues coordinated in kinase internal domain initiate cellular signal transduction process. The R-spine is intact and vertically aligned in ATP bound TAM kinases that continue to remain in the active conformation. However, it is broken in cabozantinib bound active and inactive TAM kinases due to the expansion of protein core arising from fluctuations in P-loop, α-helix and activation loop. The selective TAM kinase inhibitor (cabozantinib) arrests the overexpression of kinase domains via blockage of dynamical movement of both these spines by undergoing a fragmentation of hydrophobic surface at binding site between α-helix and activation-loop (shown in Figure 2).

The RTKs bind inhibitor in two different conformations, as active and inactive states, (K567 P-loop-α-helix E-584 inward/outward rotations in the case of Axl). The kinase activation is in the active state mode, as the distance plots show the active states of Tyro3, Axl and Mer have large core expansion between α-helix-activation loop in regulatory active site (Figure 5). The cabozantinib binding stabilized the hidden Markov state structures of active and inactive Axl, whereas the hidden Markov state conformations from the three Mer structures are closely associated with each other. From PCA, it is revealed that all TAM RTK kinase domains have random distribution of states. The analysis of metastable kinetic state forms revealed higher numbers of active state models in Axl and Mer RTKs than the number of kinetic transition states of inactive forms, however Tyro3 RTK has similar numbers of coexisted metastable state transitions among the active and inactive forms. The MSMS of the TAM kinases is expressed as different relaxation timescale intervals. Three HMM states are classified as non-equilibrium kinetic transition states (Tyro3-apo S2–S5, Axl-inactive S2–S5 and Mer-active S2–S5) due to different kinetic transitions among the nine kinetic metastable states (Table 1). The possible kinetic transitions occur via equilibrium kinetic transitions from Axl active to Mer inactive through Tyro3 active transition state (S2–S5). In the presence of inhibitor, kinase domain proceeds as inactive state to block transduction of cellular mechanistic signal pathways in cancer therapy. The one μs MD simulations each of apo, ATP and cabozantinib inhibitor bound active and inactive TAM kinases
describes the abnormal activation and overexpression of RTKs resulting in several forms of cancer and inhibition.

Disclosure statement
The authors declare no competing interests.

Funding
NGKRS thanks University of Hyderabad for UGC Non-NET fellowship. The authors thanks DST-PURSE and UGC UPE2 for funding and CMSD for computational facilities.

ORCID
Gatta K. R. S. Naresh http://orcid.org/0000-0001-5194-0148
Lalitha Guruprasad http://orcid.org/0000-0003-1878-6446

Data availability statement
All 12 µs MD structures and trajectory data, Markov models are available upon reasonable request to corresponding author (lalitha.guruprasad@uohyd.ac.in). The rest of the all data held with manuscript supporting information including videos.

Author contributions
Naresh GKRS carried out the research work, methodologies and wrote the manuscript. Lalitha Guruprasad conceived the idea, supervised and wrote the manuscript.

References
Anandakrishnan, R., Aguilar, B., & Onufriev, A. V. (2012). Hþþ 3.0: Automating pK prediction and the preparation of biomolecular structures for atomistic molecular modeling and simulations. Nucleic Acids Research, 40(Web Server issue), W537–541. https://doi.org/10.1093/nar/gks375

Bowman, G. R., & Pande, V. S. (2010). Protein folded states are kinetic hubs. Proceedings of the National Academy of Sciences of the United States of America, 107(24), 10890–10895. https://doi.org/10.1073/pnas.1003962107

Colovos, C., & Yeates, T. O. (1993). Verification of protein structures: Patterns of nonbonded atomic interactions. Protein Science: a Publication of the Protein Society, 2(9), 1511–1519. https://doi.org/10.1002/pro.5560020916

Cowan-Jacob, S. W., Fendrich, G., Manley, P. W., Jahnke, W., Fabbro, D., Liebetanz, J., & Meyer, T. (2005). The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. Structure (London, England : 1993), 13(6), 861–871. https://doi.org/10.1016/j.str.2005.03.012

Darden, T., York, D., & Pedersen, L. (1993). Particle mesh Ewald: An N·log(N) method for Ewald sums in large systems. The Journal of Chemical Physics, 98(12), 10089–10092. https://doi.org/10.1063/1.464397

Endicott, J. A., Noble, M. E., & Johnson, L. N. (2012). The structural basis for control of eukaryotic protein kinases. Annual Review of Biochemistry, (81), 587–613. https://doi.org/10.1146/annurev-biochem-052410-090317

Gajiwala, K. S., Grodsky, N., Bolanos, B., Feng, J., Ferre, R., Timofeevski, S., Xu, M., Murray, B. W., Johnson, T. W., & Stewart, A. (2017). The Axl kinase domain in complex with a macromolecular inhibitor offers first structural insights into an active TAM receptor kinase. The Journal of Biological Chemistry, 292(38), 15705–15716. https://doi.org/10.1074/jbc.M116.771485

Gott, A. W., Williamson, M. J., Xu, D., Poole, D., Le Grand, S., & Walker, R. C. (2012). Routine microsecond molecular dynamics simulations with AMBER on GPUs. 1. Generalized Born. Journal of Chemical Theory and Computation, 8(5), 1542–1555. https://doi.org/10.1021/ct2000909

Gruen, E. K., Smith-Pearson, P., Wang, J., & Pendergast, A. M. (2013). Role of ABL family kinases in cancer: from leukemia to solid tumours. Nature Reviews. Cancer, 13(8), 559–571. https://doi.org/10.1038/nrc3563

Grüllich, C. (2014). Cabozantinib: A MET, RET, and VEGFR2 tyrosine kinase inhibitor. In U. M. Martens (Ed.), Small molecules in oncology (pp. 207–214). Springer.

Harrigan, M. P., Sultan, M. M., Hernandez, C. X., Husic, B. E., Eastman, P., Schwantes, C. R., Beauchamp, K. A., McGibbon, R. T., & Pande, V. S. (2017). MSMBuilder: Statistical models for biomolecular dynamics. Biophysical Journal, 112(1), 10–15. https://doi.org/10.1016/j.bpj.2016.10.042

Herum, K., Lunde, I., McCulloch, A., & Christensen, G. (2017). The soft-and hard-heartedness of cardiac fibroblasts: Mechatronaduction signaling pathways in fibrosis of the heart. Journal of Clinical Medicine, 6(5), 33. https://doi.org/10.3390/jcm6050035

Hovek, V., Abel, R., Okur, A., Stockbine, B., Roitberg, A., & Simmerling, C. (2006). Comparison of multiple Amber force fields and development of improved protein backbone parameters. Proteins, 65(3), 712–725. https://doi.org/10.1002/prot.21123

Hu, J., Ahuja, L. G., Meharena, H. S., Kannan, N., Kornev, A. P., Taylor, S. S., & Shaw, A. S. (2015). Kinase regulation by hydrophobic spine assembly in cancer. Molecular and Cellular Biology, 35(1), 264–276. https://doi.org/10.1128/MCB.00943-14

Husic, B. E., & Pande, V. S. (2018). Markov state models: From an art to a science. Journal of the American Chemical Society, 140(7), 2386–2396. https://doi.org/10.1021/jacs.7b12191

Johnson, L. N., Noble, M., & Owen, D. J. (1996). Active and inactive protein kinases: Structural basis for regulation. Cell, 85(2), 149–158. https://doi.org/10.1016/s0092-8674(00)81092-2

Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., & Klein, M. L. (1983). Comparison of simple potential functions for simulating liquid water. The Journal of Chemical Physics, 79(2), 926–935. https://doi.org/10.1063/1.445869

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. Nature Protocols, 10(6), 845–858. https://doi.org/10.1038/nprot.2015.053

Kim, J., Ahuja, L. G., Chao, F. A., Xia, Y., McClendon, C. L., Kornev, A. P., Taylor, S. S., & Veglia, G. (2017). A dynamic hydrophobic core orches- trates allosterity in protein kinases. Science Advances, 3(4), e1600663. https://doi.org/10.1126/sciadv.1600663

Kornev, A. P., Haste, N. M., Taylor, S. S., & Eyck, L. F. (2006). Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. Proceedings of the National Academy of Sciences of the United States of America, 103(47), 17783–17788. https://doi.org/10.1073/pnas.0607651013

Lacy, S., Nielsen, J., Yang, B., Miles, D., Nguyen, L., & Hutsmacher, M. (2018). Population exposure-response analysis of cabozantinib efficacy and safety endpoints in patients with renal cell carcinoma. Cancer Chemotherapy and Pharmacology, 81(6), 1061–1070. https://doi.org/10.1007/s00280-018-3579-7

Lindoff-Larsen, K., Piana, S., Palmo, K., Maragakis, P., Klepeis, J. L., Dror, R. O., & Shaw, D. E. (2010). Improved side-chain torsion potentials for the Amber ff99SB protein force field. Proteins, 78(8), 1950–1958. https://doi.org/10.1002/prot.22711

Manning, G., Whyte, D. B., Martinez, R., Hunter, T., & Sudarsanam, S. (2002). The protein kinase complement of the human genome. Science (New York, N.Y.), 298(5600), 1912–1934. https://doi.org/10.1126/science.1075762

Mark, P., & Nilsson, L. (2001). Structure and dynamics of the TIP3P, SPC, and SPC/E water models at 298 K. The Journal of Physical Chemistry A, 105(43), 9954–9960. https://doi.org/10.1021/jp003020w

McGibbon, R. T., Beauchamp, K. A., Harrigan, M. P., Klein, C., Swails, J. M., Hernandez, C. X., Schwantes, C. R., Wang, L. P., Lane, T. J., & Pande, V. S. (2015). MDTraj: A modern open library for the analysis of
