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In Silico Pharmacology

In Silico Discovery of Fructosamine-3-Kinase Inhibitors Targeting the Nrf2-Mediated Antioxidant Response in Cancer Cells: A Molecular Dynamics Approach

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Abstract

Fructosamine-3-kinase is an enzyme responsible for deglycating the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which regulates the expression of numerous genes involved in the cellular defence against oxidative stress. To identify inhibitors against FN3K, which may disrupt antioxidant-homeostasis in cancer cells, noncovalent docking was performed for a set of 83 ATP-mimicking kinase inhibitors, followed by flexible side-chain docking to re-confirm the trend in binding energies. Covalent docking was also performed for a set of 36 known covalent kinase inhibitors with the active site Cys150 residue. The recently reported crystal structure of Human FN3K [PDB:8UE1] was employed for docking. The pharmacokinetic and toxicological properties of the top noncovalent and covalent inhibitors were subsequently studied. Kinase inhibitors with appropriate ADMET properties that are suitable for human administration and have favourable binding energies were subjected to molecular dynamics simulations to study the stability of their protein-ligand complexes. On the basis of 110 ns MD simulations, it was concluded that trilaciclib, tucatinib and olmutinib can be considered candidate inhibitors for HsFN3K inhibition. Trilaciclib and tucatinib also showed favourable thermodynamic binding free energies of -23.37 ± 3.03 and -18.36 ± 1.83 kcal/mol respectively, as calculated via MM/PBSA method. Olmutinib displayed very stable binding as a complex with FN3K with no dramatic shifts in protein structure or having any impact on its folding. The solvation and hydration effects seen on the ATP-binding site in response to ligand-binding were also extensively studied which corresponds to efficient ligand entry. These drugs are expected to have favourable in-vitro and in-vivo activity inhibiting HsFN3K on the basis of our results.

Keywords

Molecular dynamics, Molecular docking, FN3K-Nrf2 Axis, Protein deglycation, Oxidative stress, Cancer

Introduction

Cancer remains a significant public health concern, with increasing cases and complications worldwide [1]. Common treatments for cancer such as radiotherapy, immunotherapy, and chemotherapy are effective and have led to a significant increase in survival rates, however, they can often be ineffective against more aggressive forms of cancer. Additionally, drug resistance across chemotherapy, and other targeted therapies, is the most fundamental challenge in cancer treatment, and is linked to the aggressive progression of tumours [2]. Oxidative stress is one of the main drivers of DNA damage and can lead to oncogenic mutations. It refers to the production of reactive oxygen species (ROS) having free, unpaired electrons that are capable of scavenging or attacking other stable molecules within the cell, including DNA, proteins, and lipids disrupting their chemical integrity [3]. Various biomarkers, including small molecules such as 8-Hydroxy-2-deoxyguanosine (8-OHdG) and antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase), are used to study oxidative stress in the context of cancer pathophysiology [4].

Nrf2 (nuclear factor erythroid 2-related factor 2) is a transcription factor which activates an antioxidant response element (ARE) which bears genes involved in glutathione (GSH) production, redox balance, xenobiotic detoxification, and cellular anabolism [5]. Keap1 is involved in the formation of an E3 ligase complex that ubiquitinates Nrf2, leading to its proteasomal degradation by the 26S proteasome [6]. Under oxidative stress, Nrf2 is released by Keap1 through the formation of disulfide bonds via Keap1's key Cys residues [7]. Keap1 mutations occur in approximately 20% of lung adenocarcinomas and 25-30% of lung squamous cell carcinomas, which leads to a constantly active Nrf2 phenotype, improving the cancer ROS defence mechanism [8]. In the nucleus, Nrf2 heterodimerises with small Maf proteins (sMAFs), subsequently binding to AREs and upregulating phase I and II biotransformation enzymes [9].

Fructosamine-3-kinases (FN3Ks) are a group of deglycation enzymes responsible for removing ribose and fructose sugars from lysine residues on protein surfaces. These sugars are added via non-enzymatic glycation (Maillard reaction) on lysine residues [10]. Schiff bases are formed by the addition of glucose to these amine groups. The early glycation products can subsequently undergo Amadori rearrangements to form more stable ketoamines or Amadori products [11]. However, these glycated protein moieties can form advanced glycation end-products (AGEs) by forming cross-links between many proteins, altering their structure, and function, and ultimately having an aggregation effect [11]. In contrast to AGEs which are thought to be irreversible and accumulate over the life-span of a protein, deglycation mechanisms involving FN3K are present which reverse early glycation events [12]. FN3K phosphorylates the ketose structure by hydrolysing ATP, followed by rearrangement of this structure, releasing 3-deoxyglucosone (3DG) (if the sugar moiety is fructose) and a free amine group with a lysine residue [13].

Thus, inhibition of FN3K leads to prolonged Nrf2 glycation, reducing its activity and ability to dimerise and cause subsequent protein aggregation in cancer cells. In-vitro studies on cancer cell lines (T47D) treated with brusatol, an Nrf2 blocker, and oxaliplatin (platinum-based FN3K inhibitor) have shown that Nrf2 related antioxidant proteins are downregulated in breast cancer cells [14]. Additionally, with respect to human safety, the characteristic phenotype of FN3K-knockout mice, which display increased protein glycation despite reduced FN3K levels, suggests that FN3K inhibitors are likely to be well tolerated by the body [15]. Further combination studies, involving administration of FN3K inhibitors can increase chemosensitivity by reducing the antioxidant ability of cancer cells.

The crystal structure of human FN3K (HsFN3K) was discovered and reported in 2024 [12]. Earlier in-silico studies involving the identification of potent FN3K inhibitors have relied primarily on homology modelling with *Arabidopsis thaliana*'s FN3K (AsFN3K) as a template [16]. Although HsFN3K (PDB:8UE1) has a resolution value of 2.85 Å, it is the only available crystal structure for the human ortholog of FN3K. Our work relies on the crystal HsFN3K structure to accurately identify small-molecule kinase inhibitors via computational methods; to do so, mostly ATP-mimicking molecules have been studied for their ability to bind to the ATP-binding site in FN3K. Since FN3K is a kinase, these compounds bearing heterocyclic ATP-mimicking scaffolds (pyrimidines, purines, pyridines, quinazolines) are pharmacologically pre-validated to complement the conserved ATP-binding pocket of the kinase fold [17].

In recent years, computational methods relying on protein structures for drug discovery and studying ligand-binding interactions have significantly advanced. It has been clearly established that protein flexibility, and dynamic changes in protein structures are crucial for function [18]. Even before conformational changes are induced by ligand-binding events, the active site needs to be flexible to allow for binding of these ligands in some cases [19]. Thus, our study also employed flexible side-chain docking before MD simulations to further validate the trend in binding energies as seen by rigid side-chain docking. The selective receptor flexibility method implemented in Autodock4, allows designated active-site residues to adopt alternative rotameric states during docking for prediction of induced-fit binding poses [20].

For kinase inhibitors bearing electrophilic warheads capable of forming irreversible covalent bonds with active site Cys/Lys/Arg residues, specialised covalent docking protocols have been developed. For cysteine dependent covalent docking, the thioether adduct between the target inhibitor and cysteine is modelled, and subsequently docking is performed. Further, the integration of AI-predicted protein structures with physics-based MD simulations and free-energy calculation methods such as MM/PBSA have bridged the gap between structural prediction and studying the dynamic conformational behaviour of these protein-ligand complexes in solution [21].

Materials and Methods

Small molecule kinase inhibitor database

A total of 119 known kinase inhibitors were chosen to evaluate their secondary effects as FN3K inhibitors. All drugs and their IUPAC names are presented in Tables S1 and S2. The three-dimensional structures of these compounds were extracted from the PubChem Database as SDF files. Among these 119 drugs, 83 are known to noncovalently act as kinase inhibitors, while 36 are known covalent kinase inhibitors with electrophilic warheads. The 36 covalent kinase inhibitors were extracted from the DrugBank open database [22]. Amongst the 83 noncovalent inhibitors, 67 are FDA-approved [23], and only 16 are non-FDA approved. Additionally, 15 of these 83 drugs are carbohydrate kinase inhibitors such as hexokinase and PI3K inhibitors [23].

Protein preparation

The crystal structure of HsFN3K was taken from the Protein Data Bank (PDB:8UE1). This structure has a resolution of 2.85 Å and was identified via X-ray diffraction. HsFN3K is a 35 kDa monomer with 309 residues [10], however, this PDB structure has 8 missing residues (A:129-136), which are part of a loop extension (A:116-138) along with a missing C-terminal Lys (K309) residue. The loop and K309 were built using the PyMOL Builder tool [24], followed by loop modelling to satisfy spatial restraints with the ModLoop online tool [25, 26].

The protein was prepared for docking by adding Kollman charges and hydrogens using the AutoDock software (ADT) MGLTools 1.5.7.

Hydrogen atoms were added at physiological pH (7.4) and energy minimisation was performed for all 83 noncovalent ligands by using the Universal Force Field (UFF) with a conjugate gradient algorithm for 200 steps via PyRx [27].

All other cofactors and non-protein elements were also removed. This loop, which is native only to HsFN3K, was excluded from all MD simulation analyses, excluding RMSF and secondary structure composition, because the loop is disordered, even absent in AsFN3K, unresolved in the PDB crystal structure, and dispensable for kinase activity [12]. Further, our docking analysis showed none of these loop residues being involved in ligand-binding interactions, the loop was built only for the sake of continuity in the polypeptide chain during the MD simulation.

Molecular docking preparation

To identify the ATP-binding active site of FN3K, ATP (PubChem CID:5957) was blind-docked to the protein, and it was found that in 8 poses, ATP was bound in a relatively similar position with no significant change in the RMSD.

Thus, the site of binding was defined as the volume occupied by ATP in the binding site. Additionally, in-vitro studies have identified the key ATP-binding residues through mutagenesis [12], which are in alignment with our own predicted active site.

The 3D grid box dimensions were X: 25.3751, Y: 20.7225, and Z: 18.0386 Å, and the coordinates were X: -19.0821, Y: 16.5640, and Z: -23.1739 to suit the ATP-binding site of FN3K. The final rigid docking run for the 83 ligands with the built-loop protein structure was run via PyRx software.

For flexible docking, out of all the residues involved in binding interactions, 8 were selected as flexible based on their direct involvement in ligand contacts and availability of rotatable side chains. These residues included Phe252, Glu55, Cys150, Phe153, Asp217, Trp219, Asn222, and Asp234. Similar to the rigid docking, the box size was kept as 25.50 x 21.00 x 18.75 Å with a grid spacing of 0.375 Å. A Lamarckian Genetic Algorithm (LGA) was used for a total of 50 runs for each ligand.

A total of 36 covalent kinase inhibitors were covalently docked against an active site cysteine residue present within FN3K. Cys150, located within the ATP-binding cleft of HsFN3K, was selected as the nucleophilic target residue for covalent bond formation with these warheads. Covalent docking was performed for these drugs following the flexible side-chain protocol [28]. In this method, the thioether adduct between each ligand and Cys150 was first generated and aligned into the receptor by superimposing it into the receptor's existing Cys150. The covalent ligand-Cys150 adduct was then treated as a flexible residue, with the backbone C α atom held fixed, while the side chain and attached ligand were allowed to rotate freely. AutoGrid4 was used to generate affinity grid maps on a 126 × 126 × 126 grid with 0.375 Å spacing, centered on the Cys150 S γ atom. Docking was performed with 50 Lamarckian genetic algorithm (LGA) runs, each with 10 million energy evaluations.

The RMSD tolerance was kept to 2.0 Å for flexible and covalent docking, both were performed using Autodock 4.2.6 and MGLTools 1.5.7.

The protein-ligand conformations were subsequently studied via the Discovery Studio 2025 Client and PyMOL, the former was also used to generate 2D protein-ligand interaction images.

ADMET analysis

All drugs are required to have adequate pharmacokinetic properties to reach their target site of action. Even if a drug strongly binds with a protein, it may not have a physiological effect if its ADME (absorption, distribution, metabolism, excretion) and toxicological properties do not support bioavailability and show toxicity.

Physicochemical properties (molecular weight, no. of rotatable bonds, H-bond acceptors/donors, topological polar surface area etc.), drug-likeness (Lipinski, Veber [29, 30]), log P, log S, and medicinal chemistry properties were studied to provide a perspective on the ability of the drug to be metabolised within the body, in addition to its binding chemistry.

The SwissADME server [31] was used to evaluate the ADME properties of the top 15 drugs on the basis of their binding energies.

The examination of the toxicity effects of any drug is necessary before it is deemed a potential lead compound. In this study, the ProTox 3.0 webserver was used to evaluate the toxicological properties of the top drug candidates. Specifically, the hepatotoxicity, carcinogenicity, mutagenicity, cytotoxicity, and LD50 (lethal dose 50) of the drugs were studied. All these properties coexist with toxicological risk.

Molecular dynamics simulations

The compounds were subjected to molecular dynamics simulations of 110 ns each on the basis of their ADMET properties and binding energies. The MD simulations were performed using the Gromacs-2024.5 packages with the CHARMM36 force field [32, 33]. The topologies of all ligands were prepared by first adding hydrogen atoms using Avogadro2 software and then using the CGenFF server to generate topologies [34, 35]. The protein-ligand complexes were solvated using the CHARMM-modified TIP3P water model in triclinic boxes separately. Since the system was inherently neutral, no counterions were added. Energy minimisation via the steepest descent minimisation algorithm of 50000 steps was performed to remove any steric clashes. All systems were then equilibrated for NVT and NPT for 500 ps each. NVT equilibration was performed at 300 K using V-Rescale thermostat, and NPT equilibration was carried out at 1.0 atm using Parrinello-Rahman barostat [36, 37]. The 110 ns MD simulation was carried out and a total of 11000 frames were saved at an interval of 10 ps.

Each protein-ligand simulation was carried out having three independent MD replicates, further, the protein and ligand RMSD was computed and compared for each simulation. RMSD for all protein-ligand simulation triplicates can be found in Fig. S1, S2, S3, and S4. The trajectory files were subjected to periodic boundary corrections before analysis using standard Gromacs tools. The RMSD was calculated for the backbone, whereas, radius of gyration (Rg) and SASA were calculated for whole protein and RMSF for C α .

MM/PBSA free energy calculations

The Molecular Mechanics/Poisson Boltzmann Surface Area method was used to perform free energy calculations on 100 frames extracted from 70-110 ns of the entire trajectory. This method can be used to calculate the free energy associated with various intermolecular interactions, as well as the per-residue free energy decomposition as carried out in our study [38]. The free energy of binding of a protein-ligand complex can be estimated as:

$$\Delta G_{binding} = \langle G_{complex} \rangle - \langle G_{receptor} \rangle - \langle G_{ligand} \rangle$$

where G represents the free energy of the system, thus, $\Delta G_{binding}$ refers to the free energy available within the system to allow binding of the ligand to the protein. Each free energy term on the right side can be calculated as:

$$\langle G_x \rangle = \langle E_{MM} \rangle + \langle G_{solvation} \rangle - \langle TS \rangle$$

$\langle E_{MM} \rangle$ refers to the gas phase molecular mechanical energy, $\langle G_{solvation} \rangle$ is the solvation free energy, and $\langle TS \rangle$ accounts for entropy. The molecular mechanical energy in the gas phase can further be defined as a sum of ΔE_{bonded} (internal energy) and $\Delta E_{nonbonded}$ corresponding to van der Waals and electrostatic contributions.

$$\Delta E_{MM} = \Delta E_{bonded} + \Delta E_{nonbonded} = \Delta E_{bonded} + (\Delta E_{vdW} + \Delta E_{ele})$$

$\langle G_{solvation} \rangle$ has two components, the polar ΔG_{polar} and the non-polar contribution $\Delta G_{nonpolar}$ [39]:

$$\Delta G_{solvation} = \Delta G_{polar} + \Delta G_{nonpolar}$$

The electrostatic contribution to the solvation free energy was computed using the Poisson–Boltzmann (PB) model with CHARMM-optimized atomic radii (PBRadii = 7), an ionic strength of 0.15 M to mimic physiological salt concentration, and a grid fill ratio of 4.0. The nonpolar solvation term was estimated from the solvent-accessible surface area (SASA), these two terms are usually assumed to be directly proportional. The standard numerical values of γ are 0.00542 kcal/(mol·Å²), and β is 0.92 kcal/mol for the Poisson-Boltzmann method.

$$\Delta G_{nonpolar} = \gamma \times SASA + \beta$$

Results and Discussion

Pharmacokinetics and Toxicology of drugs

Ideal drug candidates for further research must possess optimal ADME and toxicological properties. The SwissADME webserver was used to analyse the pharmacokinetic properties of our hit compounds, including

drug-likeness, the partition coefficient, solubility, GI absorption, blood-brain barrier permeability, cytochrome P450 isoform inhibition, and several other parameters. The toxicity effects of these drugs, primarily their hepatotoxicity, carcinogenicity, mutagenicity, cytotoxicity and LD50 were assessed using the ProTox 3.0 webserver. Among the top scoring drugs based on covalent and noncovalent docking, only 3 drugs in total were able to pass the ADME profiling and toxicity assessment. The ADME properties of the noncovalent and covalent inhibitors are shown in Tables S3 and S4 respectively.

Drug-likeness is an important parameter studied in the selection of compounds during the early phases of drug discovery. It can be defined as the sum of the physiochemical properties that are characteristic of chemical drugs. Several drug-likeness rules have been proposed, including Lipinski's rule of five (which refers to drugs with MW less than 500 g/mol, less than 5 HBD, less than 10 HBA and $M \log P$ less than 4.15), and Veber et al. (those drugs with less than 10 rotatable bonds, and a topological polar surface area less than $140 \text{ \AA}^2$) which were used in this study [29, 30]. Amongst the noncovalent inhibitors, compounds **1**, **4**, **6**, **8**, and **12** violate at least one of Lipinski's rules, and are expected to have low bioavailability through the oral route, whereas only compound **6** violates Veber's rules. There are a total of 7 drugs out of 15 covalent drugs having binding energies < -7.0 kcal/mol that violate no drug-likeness rules, these include compounds **3**, **7**, **8**, **11**, **12**, **13**, and **14**.

Aqueous solubility is represented by the term $\log S$, where S is the concentration of the drug in mol/L, and a $\log S$ value ranging from -1.0 to -5.0 is expected to reflect the optimal hydrophilicity for aqueous dissolution, and hydrophobicity for passage through the cell membrane [40]. Table S3 shows that eight drugs exhibit solubility outside the acceptable range: compounds **1**, **2**, **4**, **5**, **6**, **8**, **10**, **15** having $\log S$ values < -5.0 . The drugs part of this study display a low potential to cross the blood-brain barrier, in fact, only compound **10** has shown this ability. The same trend is seen in Table S4 for the covalent inhibitors, only 3 drugs exhibit solubility outside the determined range, i.e., compounds **5**, **11**, and **12**. The majority of drugs also exhibit high theoretical gastrointestinal absorptivity.

Most of the noncovalent drugs showed no PAINS (Pan Assay Interference Compounds) alerts, as shown in Table S3, except, drugs **3** and **18** having a single alert each. PAIN compounds are those that show nonspecific biological activity, instead of highly specific drug-target interactions [41].

The cytochrome P450 (CYP) superfamily is involved in metabolic biotransformation in the liver, which primarily catalyses phase I reactions. The inhibition of CYP3A4 by kinase inhibitors, specifically tyrosine kinase inhibitors (TKIs) has been widely studied, and several kinase inhibitors are known to demonstrate time-dependent or quasi-irreversible inhibition of CYP3A4 [42, 43]. Thus, it is not surprising that some of the drugs listed in Tables S3 and S4 are represented as CYP inhibitors.

Skin permeability is quantified using $\log K_p$ (skin permeation coefficient), which refers to the rate of permeation of a chemical through the outermost layer of the epidermal skin [44]. $\log K_p$ is inversely proportional to the permeability of a molecule. All drugs were found to have $\log K_p$ measurements within the acceptable range (-1.0 to -10.0). The toxicity predictions for the 10 noncovalent and 7 covalent drugs that did not have any Lipinski or Veber violations were assessed and are reported in Table 1. The hepatotoxicity, carcinogenicity, mutagenicity, and cytotoxicity of these drugs were determined using the ProTox 3.0 server.

The toxicity prediction is given with a probability of accuracy (0.0 – 1.0). On the basis of these results, tucatinib, larotrectinib, pacritinib, and trilaciclib are indicated to have nontoxic effects. A probability of approximately 0.50 represents uncertainty in their accuracy. Trilaciclib and larotrectinib have no toxic effects with a probability of inactivity ≥ 0.50 . Tucatinib and pacritinib are predicted to be slightly carcinogenic, with a probability of activity < 0.55 . The prediction of the LD50 in a rat model revealed that these four compounds have lethal dose 50 values ranging from 2000 to 3160 mg/kg, and are expected to have nontoxic effects. Amongst covalent drugs, the majority of them were predicted to show no toxic effects except for abivertinib, canertinib, and poziotinib. However, these toxicity predictions are not conclusive and are only used to provide insights into the potential toxic behaviour of these compounds along with quantification for their accuracy and magnitude of toxicity (LD50).

The ADMET analysis revealed that compounds tucatinib and trilaciclib have no Lipinski or Veber drug-like violations, additionally, their toxicity effects are either absent or negligible. Thus, tucatinib and trilaciclib can potentially be orally administered drug candidates. Olmutinib, on the basis of its high covalent docking score and predicted non-toxic effects is also recommended to be a favourable target. Consequently, compounds

tucatinib, trilaciclib, and olmutinib were chosen for MD simulations to study the stability of their binding with HsFN3K.

Table 1 Toxicity predictions of noncovalent and covalent drugs with appropriate ADME properties

N	Name	Hepatotoxicity	Carcinogenicity	Mutagenicity	Cytotoxicity	LD50 (mg/kg)
2	Tucatinib	Inactive - 0.59	Active - 0.53	Inactive - 0.52	Inactive - 0.73	3160
3	Capmatinib	Active - 0.55	Inactive - 0.53	Inactive - 0.60	Inactive - 0.73	300
5	Dactolisib	Inactive - 0.57	Inactive - 0.70	Inactive - 0.54	Inactive - 0.82	1000
7	Larotrectinib	Inactive - 0.53	Inactive - 0.50	Inactive - 0.52	Inactive - 0.52	2000
9	Pirtobrutinib	Active - 0.60	Inactive - 0.55	Inactive - 0.60	Inactive - 0.78	550
10	Pacritinib	Inactive - 0.78	Active - 0.52	Inactive - 0.63	Inactive - 0.60	3160
11	Trilaciclib	Inactive - 0.70	Inactive - 0.57	Inactive - 0.59	Inactive - 0.64	2000
13	Lorlatinib	Inactive - 0.53	Inactive - 0.55	Active - 0.54	Inactive - 0.61	710
14	Pazopanib	Inactive - 0.65	Active - 0.66	Inactive - 0.70	Active - 0.50	2000
15	Regorafenib	Active - 0.82	Inactive - 0.50	Inactive - 0.79	Active - 0.77	800
3	Olmotinib	Inactive - 0.66	Inactive - 0.53	Inactive - 0.53	Inactive - 0.62	870
7	Evobrutinib	Inactive - 0.66	Inactive - 0.50	Inactive - 0.61	Inactive - 0.67	1000
8	Abivertinib	Inactive - 0.57	Inactive - 0.52	Active - 0.52	Inactive - 0.53	800
11	Nazartinib	Inactive - 0.68	Inactive - 0.62	Inactive - 0.55	Inactive - 0.52	1000
12	Branebrutinib	Inactive - 0.56	Inactive - 0.55	Inactive - 0.50	Inactive - 0.57	3029
13	Canertinib	Active - 0.51	Inactive - 0.56	Inactive - 0.55	Inactive - 0.59	1500
14	Poziotinib	Active - 0.52	Inactive - 0.50	Inactive - 0.53	Inactive - 0.60	2935

Molecular docking analysis

First, for the noncovalent docking, ATP was blind-docked to the entire HsFN3K protein to identify the most likely site of binding, and to define a binding site for our ligands. The binding site was seen to be composed mostly of polar, hydrophilic amino acids, with a few non-polar ones. ATP is a highly polar molecule, having four negative charges at physiological pH that require hydrophilic residues to stabilise it via electrostatic interactions and hydrogen bonding [45].

As shown in Fig. 1a, ATP interacts with a total of nine residues, forming strong H-bonds with a distance of <3.50 Å with Asn287 and Cys150, along with weaker H-bonds formed by interactions with His288 and Asp234. These residues stabilise the alpha, beta, and gamma phosphate groups, along with the partial positive charges on the carbon and hydrogen atoms formed due to inductive effects. The purine ring is stabilised and bound by hydrophobic interactions with residues Ala236, Glu55, Lys41, and Met51.

Molecular docking was subsequently performed for the 83 ligands at this ATP-binding cleft with the HsFN3K protein. The top 15 compounds on the basis of their binding affinities and chemical structures can be found in Table 2. The binding affinities of all 83 compounds calculated by rigid noncovalent docking with HsFN3K (PDB:8UE1) are available in Table S1.

The top 15 compounds presented highly favourable docking scores with all of them having binding energies not greater than -9.4 kcal/mol. These findings indicate that these compounds have potential inhibitory activity as they strongly bind with the protein of interest. Conformational studies of these docked protein-ligand complexes were subsequently carried out to study the amino-acids that bind the ligand and the types of noncovalent interactions involved in the process.

We found trilaciclib interacted with FN3K with a binding affinity of -9.6 kcal/mol. As shown in Fig. 1b, trilaciclib was bound to the ATP-cleft primarily by hydrophobic interactions (π -sigma, π -anion, Alkyl, and π -alkyl interactions) and a few H-bonds. Ser26 formed a strong H-bond at a bond length of 3.08 Å, and Pro235, Met51, Glu55, and Gly152 were also involved in forming weak polar contacts (bond length >3.50 Å). Trp219, His288, Phe252, and Ala236 are involved in hydrophobic interactions via π -sigma, Alkyl and π -alkyl interactions. Asp217 showed π -anion interactions with a resonating Pyridine ring. Hydrophobic interactions play a key role in stabilising the closed-ring structures of the ligands.

Tucatinib exhibited a binding affinity of -10.2 kcal/mol when docked with HsFN3K. As displayed in Fig. 1c, this complex was stabilised by three strong H-bonds, two formed by Ser26, and one formed with an imine group by Asp217. The hydrophobic interactions included stacked π - π interactions formed by Trp219, π -sigma interactions between alkyl groups interacting with His288 and His291, and lastly π -anion and π -sulfur bonds formed by Asp234, and Met51 respectively. π - π stacking interactions are non-covalent attractive forces that occur between aromatic ring systems of the ligand and aromatic amino acid residues (such as Tyr, His, Phe, and Trp) at the protein binding site, the geometric parameters for perfect π - π stacking interactions are 4.5 to 5.5 Å for distance and the angle between normal vectors of two rings should be in range of 0 to 90 degrees [46]. These π - π interactions and stacking are essential for dispersion interactions, whereas cation- π contacts produce further electrostatic contributions [47]. Stacking interactions contribute greatly towards stability of the complex through van der Waals and non-polar solvation energy components as they stabilise the aromatic ring systems of the ligand within the binding pocket.

Docking analysis revealed that tucatinib formed a greater number of strong H-bonds than trilaciclib did, while both of them were strongly stabilised by hydrophobic interactions to assist in their binding and to accommodate their bulky nonpolar rings. The binding of trilaciclib and tucatinib with FN3K is shown in Fig. 2a and 2b respectively.

For comparison, Sorafenib, a known FN3K kinase inhibitor with in-vitro validation was taken as a reference compound and all analysis was performed in a similar manner for it [16]. As shown in Fig. 1d, sorafenib is stabilised by two strong H-bonds with Met51 and Asp217 both formed with imine groups (2.62 and 2.46 Å respectively). Further weak polar contacts were formed via Cys150, Pro235, and His288. The stacking/hydrophobic interactions included His288, Phe252, Phe292, Lys41, and Asp234 forming π - π stacked, π - π T-shaped, π -alkyl, π -cation and π -anion bonds respectively. Sorafenib was docked with a binding energy of -9.3 kcal/mol, lower than that of both tucatinib and trilaciclib.

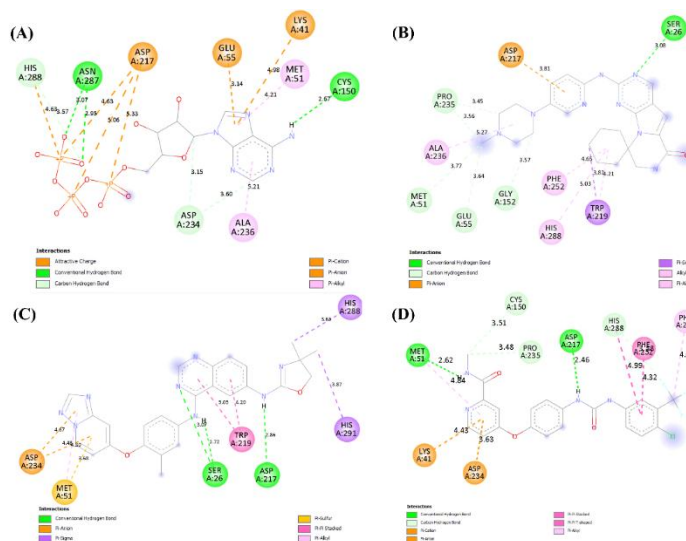
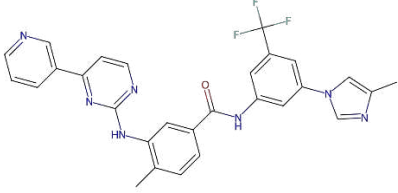
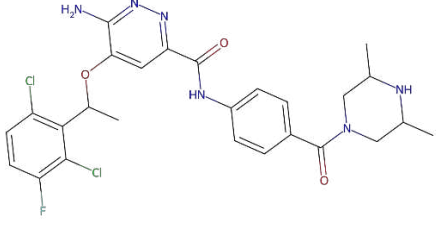
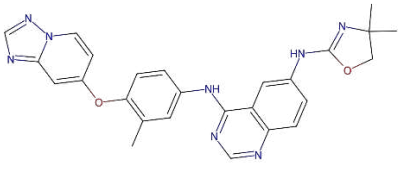
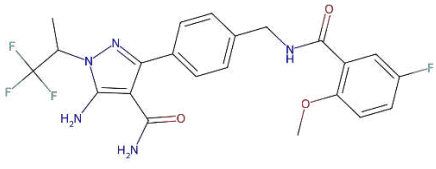
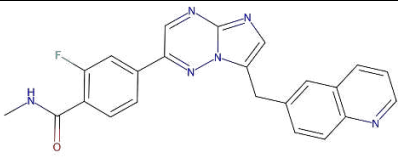
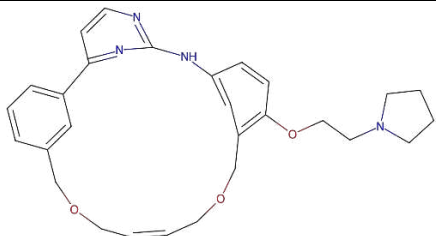
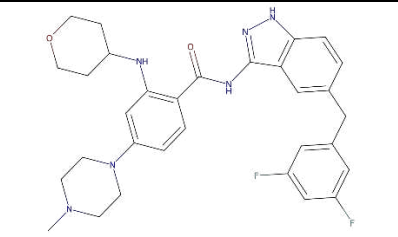
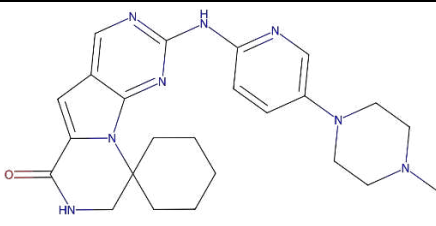
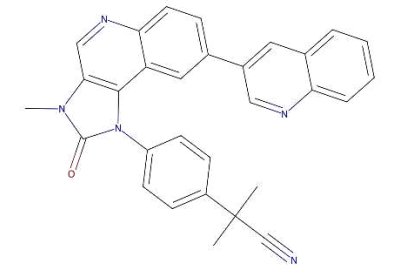
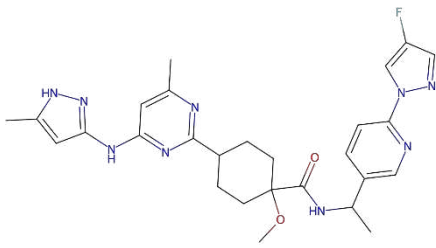
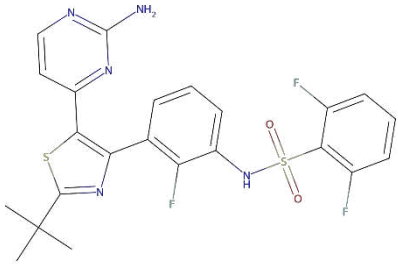
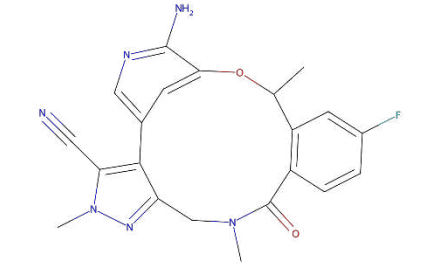
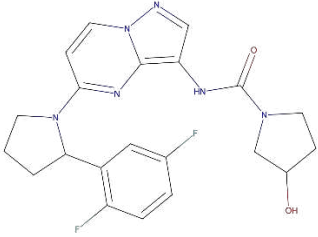
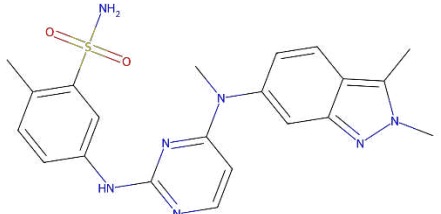
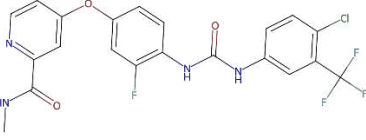


Fig. 1 2D interaction diagrams displaying binding of ligand with ATP-binding cleft of FN3K (A) FN3K-ATP, (B) FN3K-trilaciclib, (C) FN3K-tucatinib, (D) FN3K-sorafenib

Table 2 2D Structures and binding energies (kcal/mol) of top 15 compounds and Acalabrutinib

Chemical Structure	BE	Chemical Structure	BE
 Nilotinib	-10.3	 Ensartinib	-9.7
 Tucatinib	-10.2	 Pirtobrutinib	-9.6
 Capmatinib	-10.0	 Pacritinib	-9.6
 Entrectinib	-9.9	 Trilaciclib	-9.6
 Dactolisib	-9.8	 Pralsetinib	-9.5
 Dabrafenib	-9.8	 Lorlatinib	-9.5

 Larotrectinib	-9.8	 Pazopanib	-9.4
 Regorafenib			-9.4

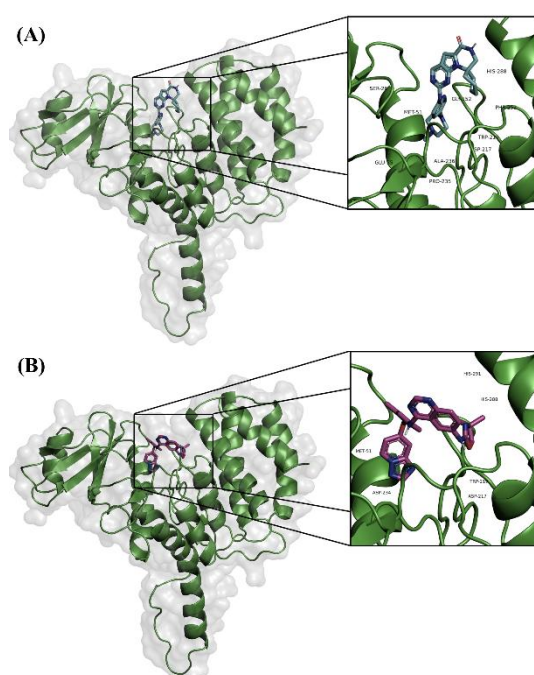


Fig. 2 3D images of (A) FN3K-trilaciclib, (B) FN3K-tucatinib docked complexes at the ATP-binding cleft

Protein structures are known to be highly dynamic in nature, and their flexibility is most apparent during ligand-binding events. Enzymes do not necessarily have rigid, static structures, it has been explained through the “Induced-fit” model that the active site can conform to the ligand’s structure to facilitate binding [48]. Thus, we performed flexible side-chain docking for the top drugs- trilaciclib, tucatinib, and sorafenib for comparison to study whether the favourable binding energies as seen through rigid docking still remain consistent when the active site is made flexible and allowed to rotate. The residues Phe252, Glu55, Cys150, Phe153, Asp217, Trp219, Asn222, and Asp234 were kept flexible to generate different conformations during docking.

The results of flexible side-chain docking showed that trilaciclib, tucatinib, and sorafenib were docked with a binding energy of -13.16, -14.46, and -11.92 kcal/mol respectively. The same order of favourability as seen through rigid docking was maintained even in docking where protein flexibility was accounted for (tucatinib>trilaciclib>sorafenib). There was an increase in the number of polar interactions formed by these ligands in each case, trilaciclib transitioned from a pose forming only one H-bond to forming 3 H-bonds with Asn222, Asp217, and Asp234 as shown in Fig. 3a. Fig. 3b showed that sorafenib continued to form the same number of H-bonds however the Met51 H-bond was displaced by Trp219. Similarly, the H-bond formed by Ser26 with tucatinib was displaced by Trp219 during flexible docking. Although tucatinib didn’t show any

change in the number of H-bonds, it formed two new salt-bridges with Asp234 and Asp217 as seen in Fig. 3c. Thus, Trp219 can be considered to be of significance for H-bond formation across multiple ligand scaffolds.

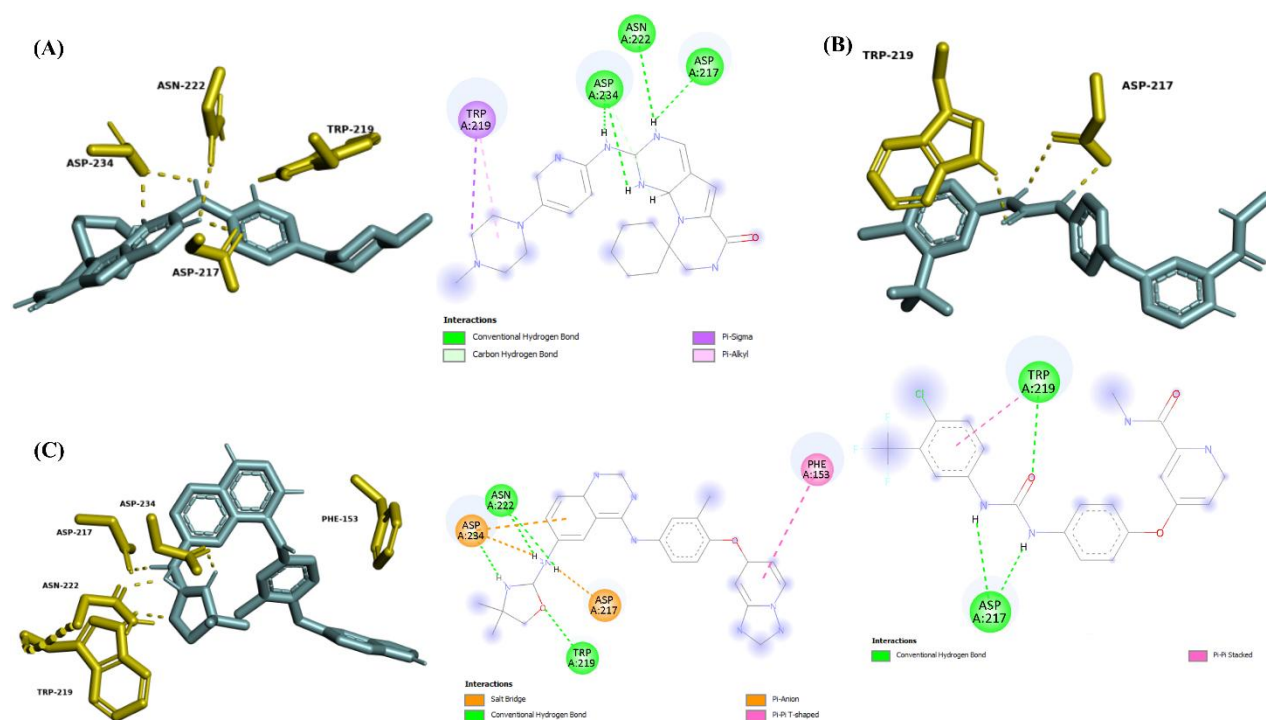


Fig. 3 Flexible side-chain docking of (A) FN3K-trilaciclib, (B) FN3K-sorafenib, and (C) FN3K-trilaciclib at the ATP-binding active site

Covalent docking analysis

Covalent kinase inhibitors (CKI) unlike noncovalent reversible drugs modify a proximal nucleophilic residue on the protein via a reaction with an electrophile to form a covalent protein-ligand complex [49]. In our study, we have taken the active site Cys150 residue, the nucleophilic thiol group is chosen to form an adduct with the ligand which is subsequently superimposed to the protein's own Cys150 and different conformations are explored through flexible side-chain docking keeping only the backbone C α atom fixed. The most common electrophilic warhead present on these covalent inhibitors is the acrylamide group which can act as a Michael acceptor forming a thioether product with the cysteine. This Michael reaction involves the addition of an enolate (Michael donor) to the β -carbon of an alkene conjugated to an electron withdrawing group (Michael acceptor). In this case, the cysteine thiol (Michael donor) is added to the β -carbon of the drug.

The covalent docking binding energies for all 36 inhibitors have been shown in Table S2, upon analysis, Olmutinib was identified to have the most favourable binding energy (-8.91 kcal/mol) while also having acceptable ADMET properties.

The acrylamide group in olmutinib has been highlighted in Fig. 4a. The thiol group of this residue undergoes a Michael addition with the β -carbon of the acrylamide forming a covalent complex as shown in Fig. 4b and the docked pose is shown in Fig. 4c. Cys150 is an active site residue present at the ATP-binding cleft, it has shown to form both weak and strong H-bonds during rigid noncovalent docking. The conformations assumed by this complex are also favourable, i.e., they lead to no steric clashes with other parts of the protein structure which is evident by the low binding energy of this docked pose.

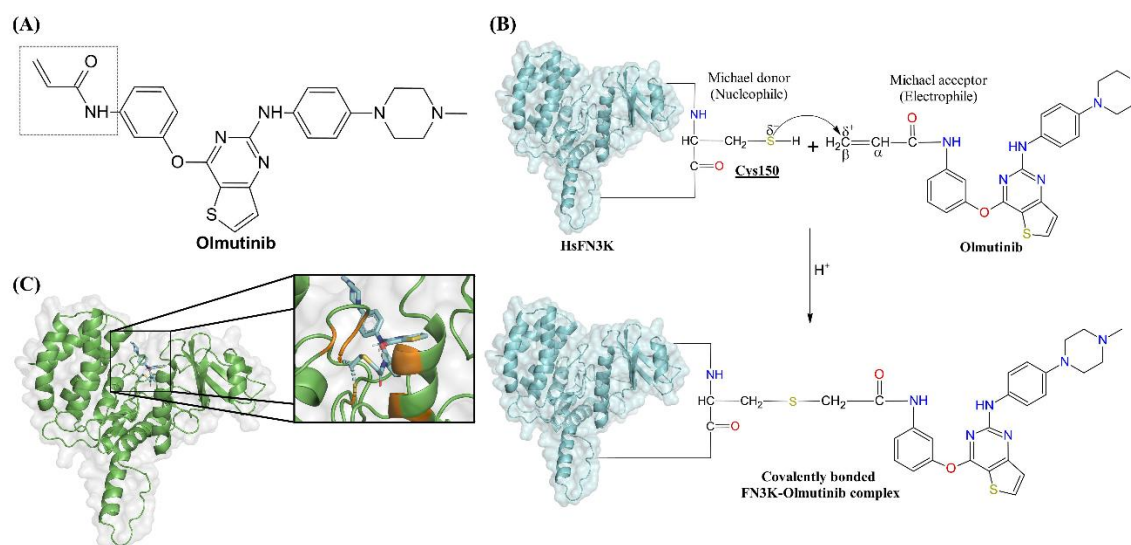


Fig. 4 Covalent docking of Olmutinib with HsFN3K, (A) electrophilic warhead (acrylamide) in Olmutinib, (B) Michael addition of Cys150(FN3K) with acrylamide of Olmutinib, (C) docked Olmutinib-FN3K complex

Molecular dynamics simulations

The lowest energy rigid docked conformations of the ligands were used to study the stability of these complexes by simulating them in an aqueous environment. Various parameters such as RMSD, RMSF, Rg, and H-bonds were used to study the dynamic nature of HsFN3K and its protein-ligand complexes. The disordered loop which was built in FN3K was excluded from all MD analyses except for the protein residue RMSF, and secondary structure composition by specifying and excluding the missing loop residues in an index file. Each protein-ligand simulation was run in triplicate; each replicate was initiated with different random velocity seeds drawn from a Maxwell-Boltzmann distribution at 300 K. The ligand and protein RMSD were calculated for each simulation and have been compared. These have been shown for all 4 simulations in Fig. S1-S4, all systems displayed RMSD values less than 0.3 nm on average. The maximum inter-replicate protein RMSD difference for the equilibrated 65-110 ns regions were 0.027, 0.045, 0.047, and 0.038 nm for trilaciclib, tucatinib, sorafenib, and olmutinib MD simulations respectively, confirming reproducibility across independent runs. Further analysis has been carried out using the first representative simulation trajectory for each system. To establish the convergence and to show that the systems have stabilised, block averaging for RMSD and Rg was performed for 65-110 ns as shown in Fig. S5. There were no major directional trends in RMSD for all systems, with only a minor downward and upward trend in FN3K-apo and FN3K-sorafenib complexes respectively, the hit drug complexes remained highly stable. The Rg values also remained highly consistent throughout all 3 blocks.

One of the main observations in identifying potent FN3K inhibitors is that the ATP-binding cleft comprises mostly of hydrophilic amino acids to accommodate charged ATP; therefore, in an unbound state, to stabilise the protein, the active site is solvated by water molecules. A stable FN3K inhibitor would need to have optimal polarity to displace the water molecules and overcome this solvation free energy barrier, while also being hydrophobic enough to remain drug-like and have the ability to cross the lipid bilayer without active transport. To further evaluate this, we computed if there were any bridging water molecules that interact with both the ligand and some active site residue. The waters forming H-bonds with the ligands and binding site residues (Ser26, Cys151, Cys150, Gly152, Trp219, Asn222, Pro235, Ala236, Ile248, Phe252, His288, Phe292, Tyr296) were evaluated, the waters appearing in both sets, i.e., Hydrogen bonding with both the ligand and binding site were classified as water bridges. This analysis was performed at 50 and 60 ns frames to study any dynamic changes in this water-mediated ligand binding and the total number of water bridges at the representative frames and residues involved are shown in Table 3.

Trilaciclib showed a decrease in water bridges formed at 60 ns, while tucatinib remained consistent at 4 water bridges, sorafenib instead showed an increase going from 50 to 60 ns. Gly152 and Trp219 were the most consistent bridging residues involved across all three ligand scaffolds, highlighting their importance as key binding residues. The water bridges formed between ligands and residues have been visualised in Fig. 5a.

Table 3 Water bridges formed between ligand and binding site residues

System	Frame	Water Bridges	Bridging Residues
Trilaciclib-FN3K	50 ns	4	His288, Trp219, Asn222, Gly152
Trilaciclib-FN3K	60 ns	2	Trp219, Phe292
Tucatinib-FN3K	50 ns	4	Pro235, Ala236, Gly152, His288, Asn222
Tucatinib-FN3K	60 ns	4	Gly152, Trp219, Pro235
Sorafenib-FN3K	50 ns	1	Gly152
Sorafenib-FN3K	60 ns	2	Gly152

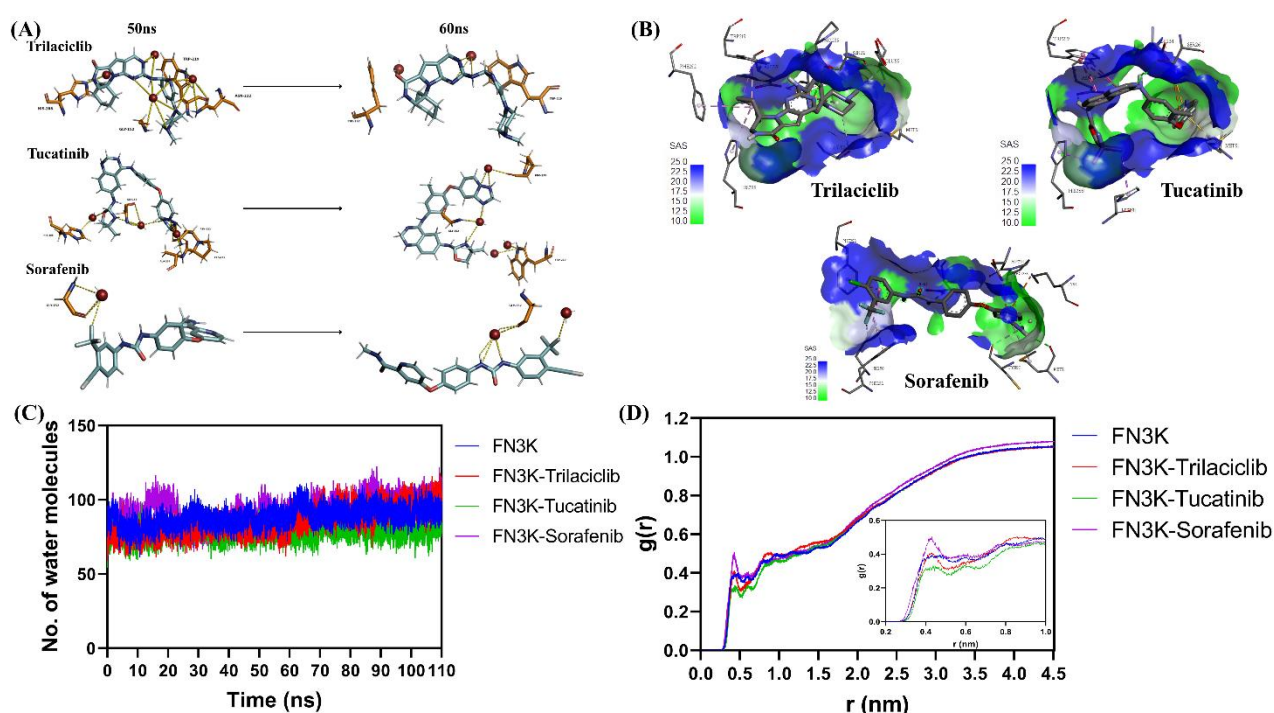


Fig. 2 (A) Water bridges formed between ligands and protein residues at 50 and 60 ns frames (*H*-bond criteria: Donor-Acceptor distance <3.5 Å, Donor-H...Acceptor angle >130°; (B) Solvent accessible surface (Å²) of FN3K-trilaciclib, FN3K-tucatinib and FN3K-sorafenib; (C) No. of water molecules present at active site; (D) Radial distribution function for the probability of finding water molecules around active site residue, first solvation shell has been highlighted

The solvent accessible surface area for the ligands has been shown in Fig. 5b for trilaciclib, tucatinib, and sorafenib respectively, where a greater blue portion correlates to a higher degree of solvent availability in that region. The no. of water molecules around 4 Å of the same 13 binding site residues was also calculated represented in Fig. 5c, in the apo state the active site had 88.4±6.5 water molecules on average. The protein-ligand complexes, FN3K-trilaciclib, FN3K-tucatinib, and FN3K-sorafenib had 85.4±9.6, 77.5±5.2, and 92.3±9.0 water molecules at the active site respectively throughout the 110 ns simulation. Ligand placement within the active site is expected to displace water molecules and reduce the total number of water molecules on average, this trend is observed for trilaciclib and tucatinib, however, an increase is observed with sorafenib. One possible explanation could be that due to the presence of multiple halogens and carbon-halogen (C-X) bonds the ligand is hydrated to a greater extent on average.

Lastly, radial distribution functions $g(r)$ which measure the probability of finding water molecules at distance r from the centre of mass (COM) of hydrophilic active site residues (Ser26, Cys150, Cys151, Asn222, His288,

Tyr296) were computed and shown in Fig. 5d. It was observed that sorafenib had the highest $g(r)$ at the first solvation shell consistent with its high-water molecule count, we suspect that it is an outcome of the presence of several highly polar C-X bonds as part of its structure. Excessive solvation can also hinder ligand binding by shielding binding sites, increasing entropy, and competing with ligands for binding positions which is already a hindrance for a hydrated ATP-binding cleft [39]. Tucatinib which displayed the highest binding energy during docking has the smallest peak at 0.4 nm, indicating that it binds tighter than other ligands, shielding the active site from solvent. Trilaciclib displayed nearly similar behaviour as the apo simulation in the first solvation shell.

We also studied the stability of these FN3K-inhibitor complexes as shown in Fig. 6 to identify any ligand-unbinding events that may have occurred due to unfavourable binding [50]. Snapshots taken at 70, 90, and 110 ns towards the end of simulation depict that all three systems remained bound in a stable manner with no loss of binding.

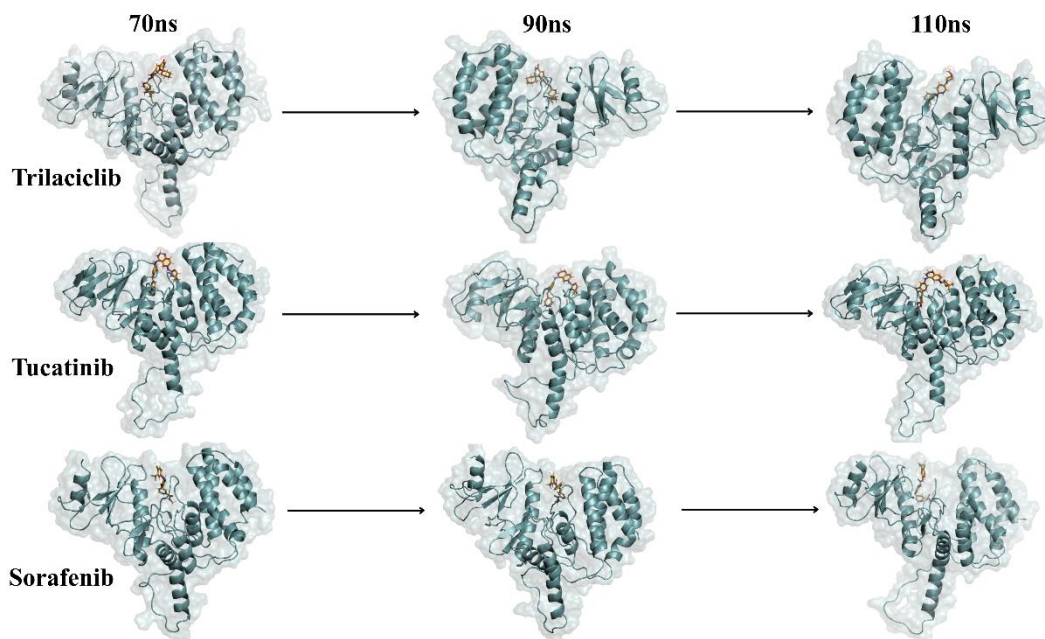


Fig. 6 Structural snapshots of FN3K-inhibitor complexes at 70, 90, and 110 ns of MD simulation. The snapshots illustrate the conformational dynamics and stability of these complexes over the simulation trajectory.

Analysis of RMSD and RMSF

The root mean squared deviation (RMSD) was calculated with respect to the pre-simulation, energy minimised structure of the complex. The RMSD data comparing the complex systems with the apo state is presented in Fig. 7a. The RMSD of FN3K alone shows some variation until approximately 70 ns, after which it plateaus and then gradually decreases. The FN3K-trilaciclib and FN3K-tucatinib complexes also fluctuated until 70 ns, after which they showed some variance, but no major changes or peaks. The average RMSD values of the FN3K, FN3K-trilaciclib, FN3K-tucatinib and FN3K-sorafenib complexes were 0.190 ± 0.0328 , 0.205 ± 0.0227 , 0.197 ± 0.0281 , and 0.234 ± 0.0295 nm, respectively. The RMSD of all the systems remained well below 0.30 nm, with acceptable standard deviations for RMSD. The ligand RMSD fitted to the ligand itself was also computed as shown in Fig. 7b, all three ligands exhibited values well less than 0.3 nm on average, specifically 0.165 ± 0.0297 , 0.175 ± 0.0228 , and 0.190 ± 0.0367 nm for trilaciclib, tucatinib and sorafenib respectively. The stability of the protein residues in the presence and absence of ligands was also evaluated by calculating the root mean square fluctuations (RMSFs). The RMSF values of all 309 FN3K residues in complexed and uncomplexed states are shown in Fig. 7c. This RMSF also includes the disordered loop (A:129-136), and its high fluctuation in RMSF is another justification for its exclusion in MD analysis. The average RMSFs of the FN3K, FN3K-trilaciclib, FN3K-tucatinib, and FN3K-sorafenib complexes were 0.138 ± 0.0885 , 0.141 ± 0.1013 , 0.145 ± 0.1153 , and 0.110 ± 0.0701 nm, respectively. The trilaciclib and tucatinib complexes had slightly higher RMSF values, however, they remained well below 0.2 nm for all the systems, indicating stability.

The RMSD and RMSF analysis revealed that the protein alone and when bound with ligands, was stable in an aqueous environment. The RMSF for all the atoms of the three ligands was also calculated as shown in Fig. 7d. Both candidate drugs seem to exhibit a similar pattern of fluctuation, with the 69 atoms in trilaciclib having a RMSF of 0.131 ± 0.0586 nm and the 70 atoms in tucatinib having a RMSF of 0.126 ± 0.0546 nm. The RMSF of trilaciclib is only marginally greater than that of tucatinib. Sorafenib displayed much higher RMSF as compared to the other drugs equivalent to 0.170 ± 0.0669 nm.

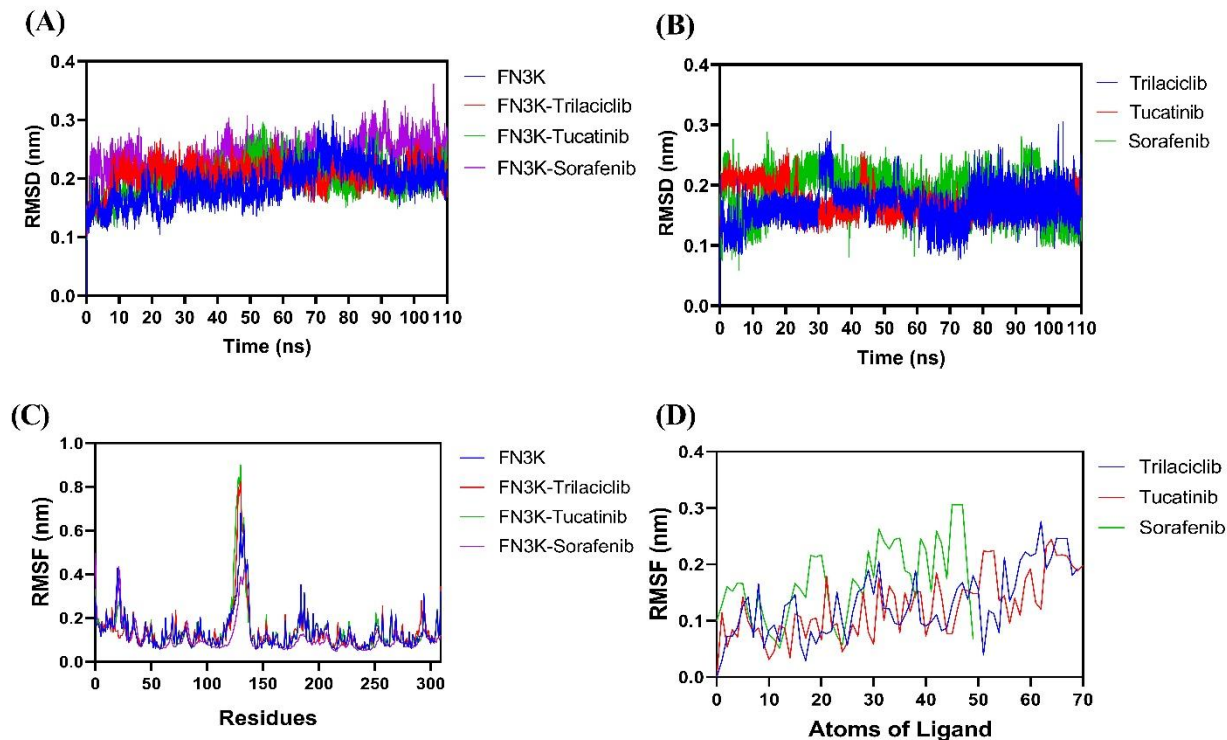


Fig. 7 (A) RMSD of protein, (B) RMSD of ligand and, (C) RMSF of FN3K, FN3K-trilaciclib, FN3K-tucatinib, and FN3K-sorafenib complexes, (D) RMSF of trilaciclib, tucatinib and sorafenib bound to FN3K

Radius of gyration, solvent accessible surface area and energy analysis

The radius of gyration refers to the compactness of a protein, or its globularity. The folding rate of a protein decreases with increasing size, and FN3K is a relatively small globular protein that is expected to have a stable Rg throughout the simulation [39]. The Rg values of FN3K and its complexes are depicted in Fig. 8a. The Rg of FN3K alone remained relatively stable throughout the 110 ns simulation, whereas the values of the FN3K-trilaciclib, and FN3K-tucatinib complexes showed some variation, but became stable after approximately 75 ns. The average Rg values of FN3K, FN3K-trilaciclib, FN3K-tucatinib, and FN3K-sorafenib complexes were 2.086 ± 0.0101 , 2.107 ± 0.0155 , 2.096 ± 0.0122 , and 2.102 ± 0.0151 nm respectively. Thus, on average, the protein showed no significant change in its folding or compactness in any of the systems.

The solvent accessible surface area (SASA) of proteins is a decisive factor when studying protein folding and stability, and is defined as the surface characterised around a protein by the hypothetical center of a solvent sphere with the van der Waals contact surface of the molecule [51]. In an aqueous environment, water molecules are used to calculate the SASA. The SASA of all the systems was very stable, with no major fluctuations as shown in Fig. 8b. The average SASA of FN3K, FN3K-trilaciclib, FN3K-tucatinib, and FN3K-sorafenib were calculated to be 157.941 ± 2.5343 , 158.902 ± 2.6258 , 158.739 ± 2.3670 , and 158.659 ± 3.1185 nm², respectively.

The total and potential energies of all four systems were also calculated and are presented in Fig. 8c. Both of these parameters remained stable with no significant difference from one another, collectively validating the stability of the complexes during the MD simulation.

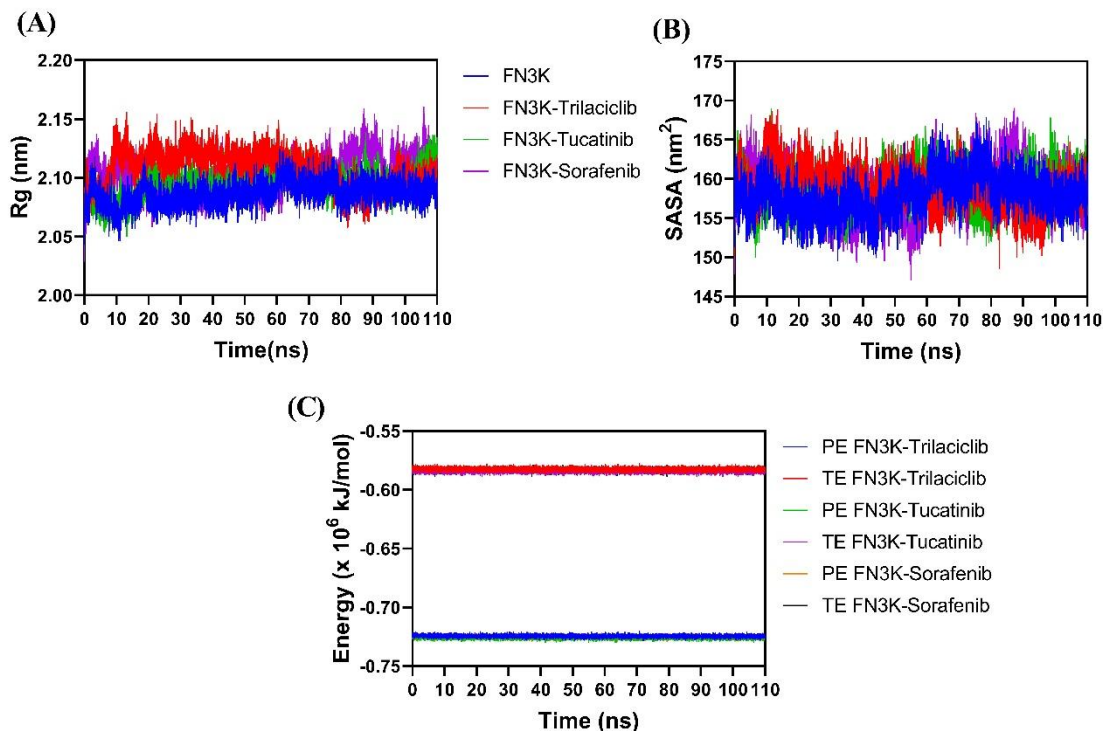


Fig. 8 (A) Radius of gyration, (B) solvent accessible surface area (SASA) in nm², (C) total and potential energies of FN3K, FN3K-trilaciclib, FN3K-tucatinib, and FN3K-sorafenib complexes

Principal component analysis (PCA)

PCA is used to reduce the dimensionality of high-dimensional datasets to reveal the most important motions in proteins during the MD simulation. It greatly reduces the dimensionality of their configurational space, facilitating further analysis of the motions and states acquired by the protein [52, 53]. PCA is performed by reducing the complexity of the data set without losing important information, which is characterised by the eigenvectors [54]. PCA allowed us to view the flexibility and conformational space occupied by FN3K alone, and in its complexed states. The projection of the top two eigenvectors in the bound and unbound states is presented in Fig. 9a. Relative to the apo state, when bound to trilaciclib, FN3K occupied less conformational space, indicating that it exhibited lower structural flexibility. When bound to tucatinib, FN3K showed no major change in its conformation, with similar flexibility as the apo state and a similar condition was seen in the FN3K-sorafenib complex.

The free energy landscapes (FEL) of FN3K alone and its complexed states were also plotted. The top two principal components (PC1 and PC2) are used to plot this FEL, and the probability distributions of these collective variables are used to find the minimum energy (blue/green) and unstable (red) conformational states of the protein. FELs are used to identify any conformational changes and changes in protein folding in the apo FN3K state, versus the complexed states. FEL mapping elucidates ligand-induced changes in protein conformational stability and accessibility of alternative states.

Consistent with the 2D eigenvector projection in Fig. 9a, FN3K in its apo state occupied a large conformational space denoted by the blue area with low free energy in Fig. 9b, whereas FN3K when bound to trilaciclib exhibited less structural flexibility, as there is only a smaller blue region in Fig. 9c which can be interpreted as the protein exhibiting a more constrained structure. Compared with trilaciclib, the FN3K complex with tucatinib resulted in greater structural variation. Multiple blue regions can be seen in Fig. 9d along PC1, indicating that FN3K can possibly exhibit several conformations, with the largest blue region being the most stable. The FEL of sorafenib shown in Fig. 9e is similar to FN3K's apo state, with one large energy basin not having multiple low energy conformations. The minimum energy structures of FN3K were extracted and have been depicted in Fig.

10a-10d relative to the energy basins present within the free energy landscapes. These structures represent the most energetically stable forms of the protein.

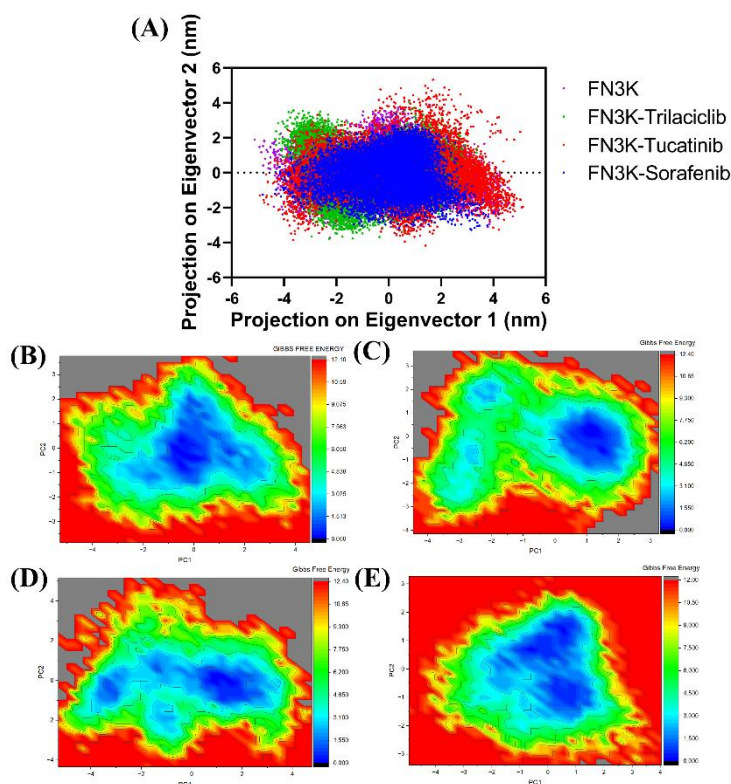


Fig. 9 (A) 2D Eigenvector projection plot for FN3K and its complexes, 2D free energy landscape for (B) FN3K-apo, (C) FN3K-trilaciclib, (D) FN3K-tucatinib, (E) FN3K-sorafenib

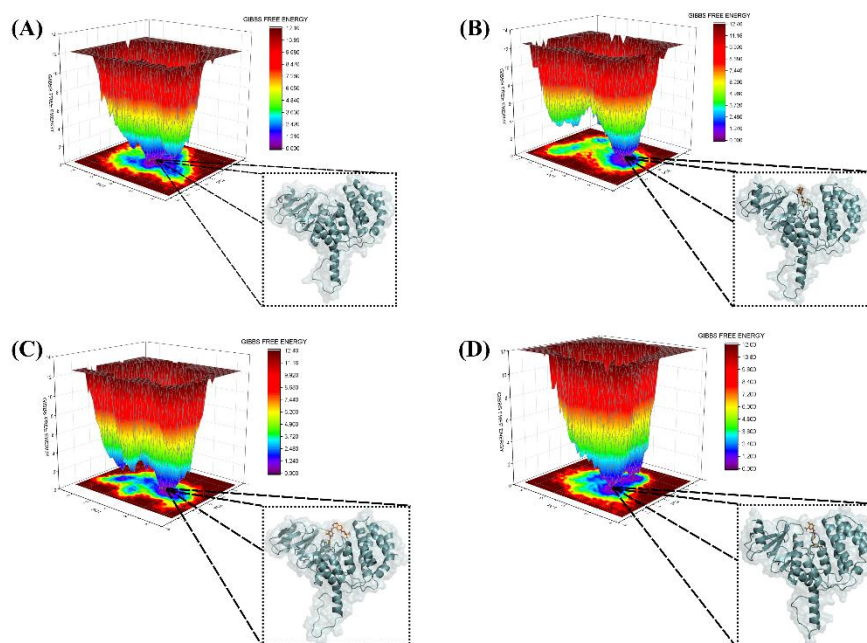


Fig. 10 Representative energy minima structures corresponding to lowest energy basins in free energy landscapes, (A) FN3K-apo, (B) FN3K-trilaciclib, (C) FN3K-tucatinib, (D) FN3K-sorafenib

Secondary structure and Hydrogen-bonds analysis

The changes in different secondary structure motifs were studied by comparing the complex and un-complexed states of FN3K. The secondary structure composition of FN3K is shown in Fig. 11a. The loops, bends, turns, polyproline helices, 3(10)-helices, beta-strands and bridges, and alpha-helices accounted for 19.60, 10.17, 7.23, 1.63, 2.00, 14.30, 1.34, and 43.73%, respectively, of FN3K in its unbound state. There was no significant change in the protein motif composition upon complexing with ligands.

Hydrogen-bonds are important noncovalent interactions needed for the binding of a ligand to its active site, and the number of hydrogen-bonds formed between the ligands and FN3K throughout the simulation is presented in Fig. 11b [55]. All ligands formed H-bonds with FN3K during the simulation, however, since these drugs are mostly hydrophobic with few polar functional groups, the number of H-bonds formed showed some variation. The primary mode of binding for these ligands was through hydrophobic interactions, as shown by docking analysis. To further dissect the residues and binding moieties of the ligands involved in H-bond formation, the occupancy or number of frames in which a H-bond was present throughout the simulation was calculated and shown in Table 4. A H-bond was defined as having a donor-acceptor distance <3.5 Å, and an acceptor...H-donor angle $>130^\circ$. Gly152, despite being a non-polar amino acid, was involved in the most prominent H-bond formation through its backbone imine (-NH-), it was a major contributor for all three complex systems.

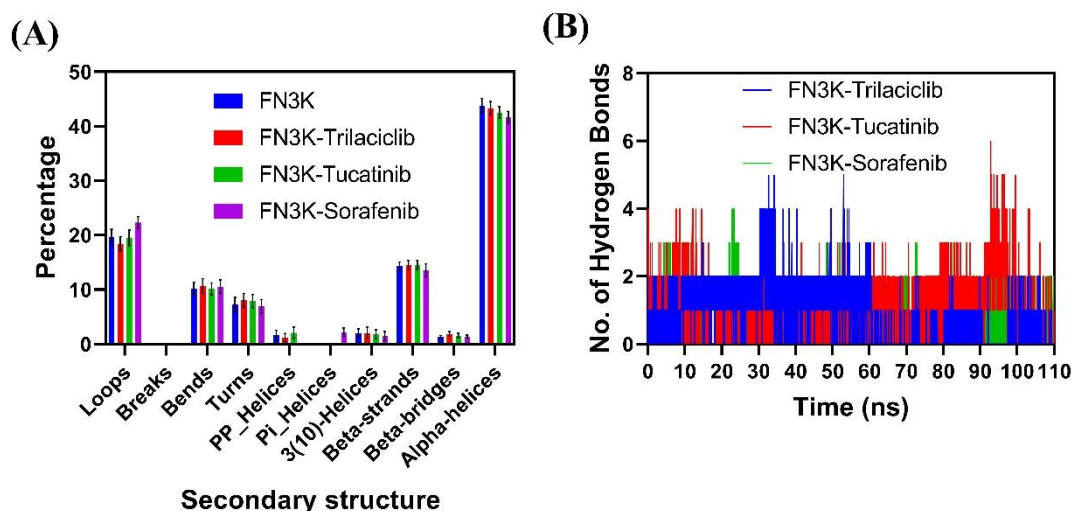


Fig. 11 (A) Secondary structure composition of FN3K, FN3K-trilaciclib, FN3K-tucatinib, and FN3K-sorafenib complexes, (B) H-bond profile for the interaction of trilaciclib, tucatinib and sorafenib with FN3K

Notably, Gly152 maintained the highest occupancy for trilaciclib (66.03%) with decent occupancy with tucatinib (16.40%), while sorafenib showed markedly reduced Gly152 engagement (7.59%), reflecting its weaker anchoring at the backbone hinge of the ATP-binding cleft. Asn222 (56.68%) was another strong contributor for Trilaciclib. Tucatinib additionally recruited Pro235 (38.10%) as its greatest contributor along with Asp217 (14.24%) as major donors. Sorafenib interestingly also recruited Gln47 for H-bonding which was absent among the other two complexes.

Table 4 H-bond occupancy analysis during MD simulations for the FN3K-ligand systems

Complex	Donor	Acceptor	Occupancy (%)
FN3K-Trilaciclib	Gly152	N8	66.03
	Asn222	N7	56.68
	N2	His291	8.1
FN3K-Tucatinib	N2	His288	5.4
	N6	Pro235	38.1
	Gly152	N5	16.4
	N6	Asp217	14.24
FN3K-Sorafenib	N6	His291	7.6
	Gln47	O2	17.08
	N2	Gly152	7.59
	N2	Asn290	5.17
	N2	Phe153	2.09

The energy minima structures from the free energy landscapes in Fig. 10 were extracted for the complexed and un-complexed states to plot the Ramachandran plots using the Ramplot webserver [56]. The Ramachandran plots presented in Fig. 12 show that nearly all the residues of FN3K were in the allowed regions in all three systems. The percentages of residues with Φ and Ψ angles in favoured, allowed, and disallowed regions, as calculated from the Ramachandran plots are represented in Table 5. A protein structure with $\geq 90\%$ of its residues in the most favoured regions is considered stable and accurate [57]. The findings show that there is only a marginal difference in the backbone conformations of the protein with FN3K-sorafenib having the least percentage of favourable dihedral angles.

Table 5 % of residues having favoured, allowed, and disallowed Φ and Ψ angles for the protein backbone in complexed and un-complexed noncovalent FN3K systems

Complex	Favoured (%)	Allowed (%)	Disallowed (%)
FN3K apo	94.585	5.054	0.361
FN3K-Trilaciclib	94.465	5.166	0.369
FN3K-Tucatinib	92.78	6.137	1.083
FN3K-Sorafenib	92.164	7.09	0.746

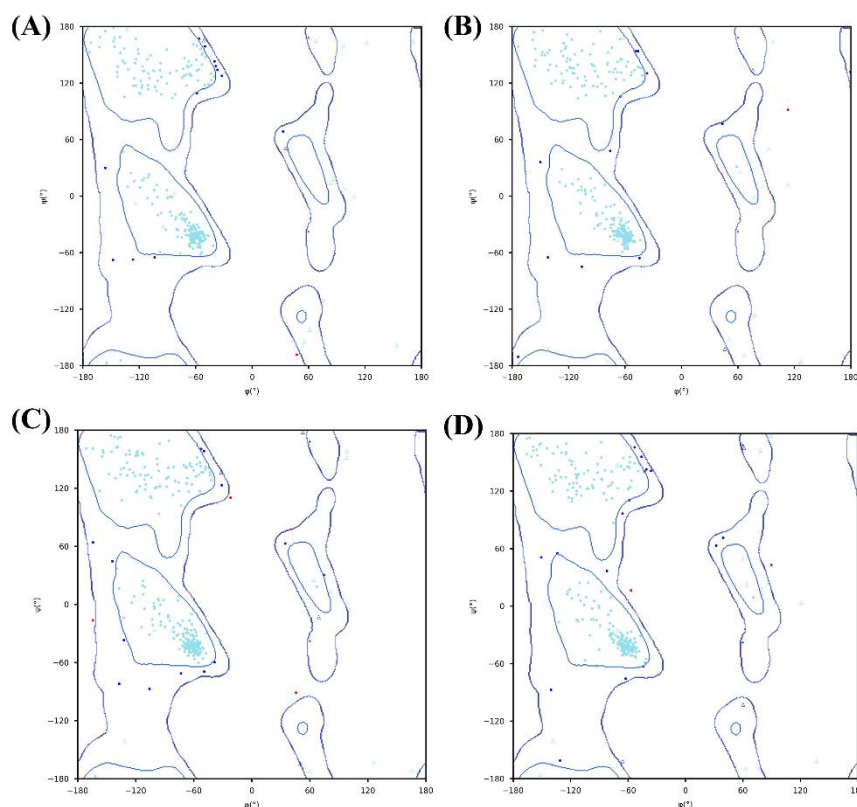


Fig. 12 Ramachandran plots for energy minima structures (A) FN3K, (B) FN3K-trilaciclib, (C) FN3K-tucatinib, (D) FN3K-sorafenib

Covalent complex MD simulations

The covalent FN3K-Olmutinib complex built by forming a thioether adduct between the drug's acrylamide group and active site Cys150 was also simulated in an aqueous environment to study stability of the complex and dynamic changes in protein structure. The MD simulation analysis shows that the covalent binding of Olmutinib to FN3K stabilises the protein structure and restricts its conformational dynamics.

The FN3K-Olmutinib protein showed similar fluctuation on average (0.201 ± 0.0184 nm) in comparison to the apo simulation (0.190 ± 0.0328 nm) as seen in Fig. 13a, olmutinib's RMSD (0.121 ± 0.0217 nm) was also much lower as compared to all other noncovalent ligands with the values being consistently well less than 0.2 nm as shown in Fig. 13b. This behaviour is to be expected as due to the covalently bound nature of the ligand, it is expected to remain burrowed within the ATP-binding cleft while being anchored to the nucleophilic cysteine residue. Fig. 13c shows that the protein RMSF for this covalent complex was also much lower than that for the apo simulation with a value of only 0.102 ± 0.0608 nm. The ligand RMSF (0.079 ± 0.0303 nm) also displayed a significant decrease as compared to the solely noncovalently interacting ligands (trilaciclib, tucatinib, and sorafenib) which is depicted in Fig. 13d. These parameters suggest that Olmutinib remains stably bound to the protein structure while also stabilising the backbone and side-chains of FN3K itself. The no. of H-bonds formed by Olmutinib were quite low and constantly fluctuating as shown in Fig. 13e, however, since the ligand is covalently bound to the protein itself, H-bonds are not solely responsible for ligand-binding. The C-S bond length is known to be of 1.82 Å on average, we further studied the variation in bond length throughout the simulation which may indicate unstable or uncomfortable fitting of the ligand [58]. Fig. 13f shows that there were some fluctuations however there were no major directional shifts in bond length throughout the simulation, with an average length of 0.183 ± 0.0037 nm. The FN3K-Olmutinib complex exhibited a marginally higher Rg (2.106 ± 0.0092 nm) compared to apo FN3K (2.086 ± 0.0101 nm), this can be attributed to the covalent attachment of Olmutinib to the surface-exposed Cys150.

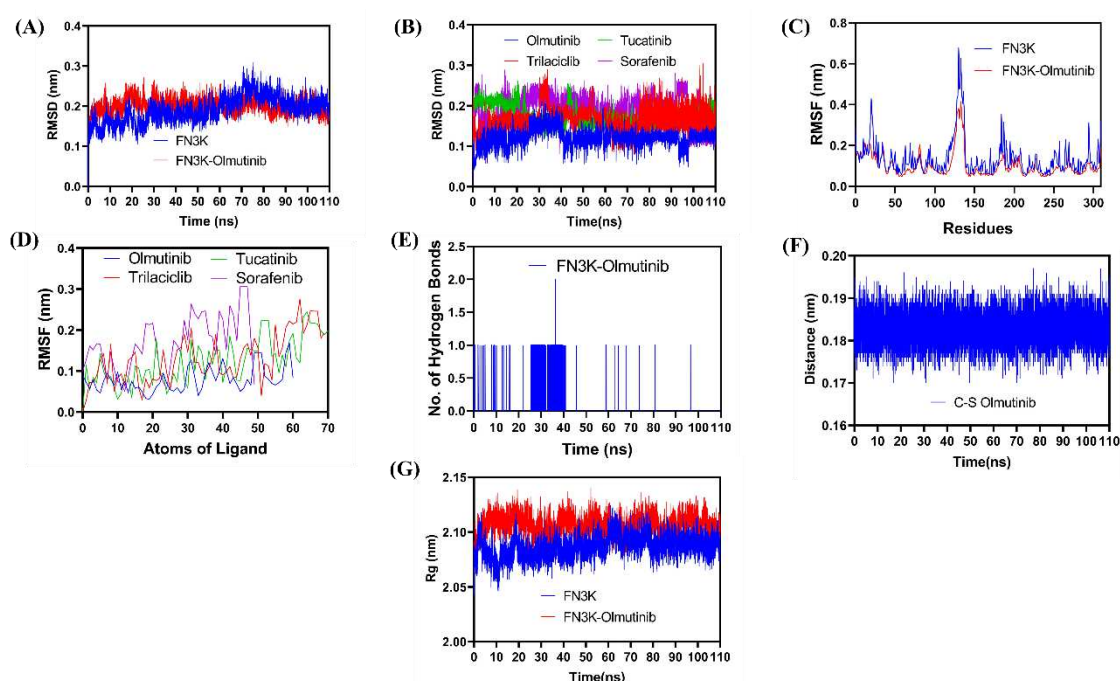


Fig. 13 (A) Protein RMSD (nm) for apo and covalent complex, (B) Ligand RMSD (nm) comparing Olmutinib with noncovalent inhibitors, (C) Protein RMSF (nm) for apo and covalent complex, (D) Ligand RMSF (nm) comparing Olmutinib with noncovalent inhibitors, (E) No. of Hydrogen bonds formed between FN3K residues and Olmutinib, (F) Time series for C-S Bond length (nm) of thioether adduct formed between Olmutinib and Cys150, (G) Radius of gyration (nm) for apo and covalent complex

There were no significant changes in the protein motifs as shown in Fig. 14a. However, the free energy landscapes in Fig. 14b and 14c show that there is a single dominant energy minimum at the centre of PC1 and PC2. The covalent bond anchors the ligand, preventing conformational transitions that noncovalent inhibitors might undergo, and the circular, compact basin indicates the complex doesn't drift along any particular collective motion. The Ramachandran plot measuring the freedom of backbone dihedral angles in Fig. 14d showed that 93.993, 5.300, and 0.707% of residues were in favoured, allowed and disallowed regions respectively indicating presence of majorly permissible dihedral rotations. There were also no major effects on the extent of hydration at the active site as the RDF in Fig. 14e shows that the first solvation shell showed $g(r)$ very similar to the apo complex, and there was no significant shift in trend for the number of water molecules (85 ± 5.3) present at the ATP-binding cleft as depicted in Fig. 14f.

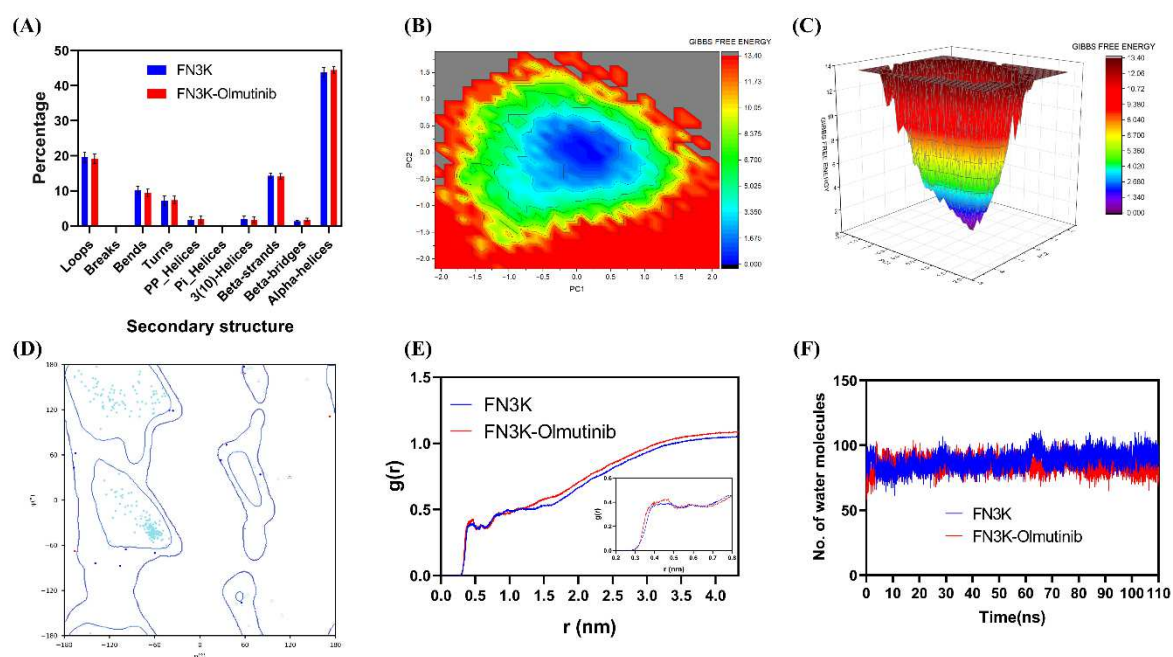


Fig. 14 (A) Secondary structure composition of FN3K and FN3K-Olmutinib complex, Free energy landscapes for FN3K-Olmutinib complex (B) 2D, (C) 3D, (D) Ramachandran plot for energy minima structure of FN3K-Olmutinib, (E) Radial distribution function $g(r)$ for the probability of finding water molecules at COM of hydrophilic active site residues (Ser26, Cys151, Asn222, His288, Tyr296), first solvation shell has been highlighted (F) No. of water molecules within the active site

MM/PBSA free energy calculations

The overall binding energetics of the ligands with the protein were studied using MM-PBSA calculations to determine the contribution of each intermolecular interaction in stabilising the complex, and the total binding free energy ΔE_{BE} of the system. The free energy was calculated corresponding only to enthalpic contributions,

The charmm_radii set was used for the Poisson Boltzmann radii, as all our systems were prepared via the CHARMM36 force field.

The MM/PBSA calculations are reported in Table 6. As evident, van der Waals forces play the greatest role in stabilising the interactions of the ligands with FN3K, followed by the electrostatic energy. There is also a small contribution from the non-polar energy, which is typically estimated using the surface area (SA) method to account for hydrophobic interactions. However, as expected, the polar solvation energy greatly hindered binding, having a net positive ΔE_{PSE} value. The binding free energy of trilaciclib was more favourable than that of tucatinib, with values of -23.37 ± 3.03 and -18.36 ± 1.83 kcal/mol, respectively. Sorafenib (-15.09 ± 2.93 kcal/mol) had the least negative binding free energy as compared to the two hit drugs indicating that they may bind even stronger than sorafenib, a known FN3K inhibitor.

MM/PBSA was further applied to identify the free energy contributions of residues within a 4 Å radius from the ligands. The list of all contributing residues is presented in Table 7. Ala236, Asn222, Cys151, His288, Ile248, Phe252, Phe292, Ser26, and Trp219 are common residues involved in the binding of both trilaciclib and tucatinib. Thus, these residues can be identified as key contributors to the binding of small molecules at the ATP-binding cleft in HsFN3K. Additionally, Lys41 seemed to have a significantly greater contribution of -5.721 kcal/mol than the other interacting amino acids. Asn222 and Lys41 have been reported to be indispensable for the kinase activity of HsFN3K, as N222A and K41A mutant forms of HsFN3K have had abolished activity [12]. Our study provides further insight into the significance of these two residues in ligand-binding events. The much lower electrostatic energy of tucatinib is further highlighted by the significant contributions of Lys41.

Table 6 Free energy associated with various intermolecular interactions and total binding free energy for the interaction of trilaciclib and tucatinib with FN3K

Energy Parameter	Trilaciclib (kcal/mol)	Tucatinib (kcal/mol)	Sorafenib (kcal/mol)
ΔE_{vdW}	-27.83 $\pm$ 1.21	-41.92 $\pm$ 0.59	-34.16 $\pm$ 0.87
ΔE_{ele}	-11.18 $\pm$ 2.62	-21.05 $\pm$ 1.59	-11.11 $\pm$ 1.47
ΔE_{PSE}	19.79 $\pm$ 0.57	49.68 $\pm$ 0.52	34.45 $\pm$ 2.26
ΔE_{NP}	-4.15 $\pm$ 0.04	-5.07 $\pm$ 0.05	-4.28 $\pm$ 0.22
ΔE_{BE}	-23.37 $\pm$ 3.03	-18.36 $\pm$ 1.83	-15.09 $\pm$ 2.93

ΔE_{vdW} van der Waals energy, ΔE_{ele} electrostatic energy, ΔE_{PSE} polar solvation energy, ΔE_{NP} non-polar energy, ΔE_{BE} binding energy

Table 7 Average free energy contributions (kcal/mol) of residues within 4 Å of ligand bound to FN3K

Residues	Trilaciclib				Tucatinib			
	E_{vdW}	E_{ele}	E_{PSE}	E_{total}	E_{vdW}	E_{ele}	E_{PSE}	E_{total}
SER:26	-0.0121	-0.0328	0.0198	-0.0243	-0.01455	-0.01713	-0.00663	-0.03881
LYS:41	—	—	—	—	-0.28109	-6.88396	1.44396	-5.72139
MET:51	—	—	—	—	-1.09426	-0.06693	0.78396	-0.37772
PHE:52	—	—	—	—	-0.28604	-0.06782	0.192871	-0.1602
CYS:150	—	—	—	—	-1.54584	-0.4099	1.88505	-0.07149
CYS:151	-0.6942	-0.8137	0.2956	-1.2133	-0.78337	0.337624	-0.13911	-0.58475
GLY:152	-0.47	-1.7067	0.9964	-1.1799	—	—	—	—
PHE:153	—	—	—	—	-3.02683	-0.29832	0.852574	-2.47228
TRP:219	-2.0899	-0.462	1.0401	-1.5115	-0.23485	0.013168	0.044158	-0.17703
ASN:222	-0.5513	-0.8546	0.2817	-1.1235	-0.07644	0.102574	-0.26069	-0.23495
PRO:235	-0.2197	0.0426	0.1132	-0.0639	—	—	—	—
ALA:236	-0.2085	-0.0153	0.0725	-0.1511	-0.33356	-0.14119	0.112376	-0.36198
ILE:248	-0.0686	-0.0024	-0.0762	-0.1475	-0.02485	0.032376	-0.07614	-0.06842
PHE:252	-0.778	-0.0635	0.13	-0.7118	-0.06	0.003069	-0.00911	-0.06436
ASN:287	—	—	—	—	-0.64465	0.860495	-0.4502	-0.23485
HIS:288	-1.0143	-0.1505	0.4043	-0.7611	-0.9199	-0.11832	0.44495	-0.59376
HIS:291	—	—	—	—	-1.72861	0.130891	0.51	-1.08842
PHE:292	-1.332	-0.223	0.3893	-1.1657	-1.15743	-0.09515	0.311287	-0.94139
TYR:296	-0.0797	0.0153	0.0168	-0.0475	—	—	—	—

E_{vdW} van der Waals energy, E_{ele} electrostatic energy, E_{PSE} polar solvation energy, E_{total} total energy

Conclusion

Fructosamine-3-kinase has been widely identified as a novel therapeutic target for increasing the efficiency of existing cancer treatments. Our study aimed to identify and employ FDA-approved, ATP-mimicking molecules as noncovalent, and drugs bearing electrophilic warheads as covalent FN3K inhibitors by relying on computational tools such as MD simulations to evaluate the binding stability of these inhibitors. Based on our results, trilaciclib, tucatinib, and olmutinib are proposed as potential inhibitors of FN3K. Both trilaciclib and tucatinib displayed significantly better binding energies and complex stability as compared to sorafenib, a known FN3K inhibitor with in-vitro validation. Trilaciclib is a CDK4/6 inhibitor normally administered prior to chemotherapy [59], whereas tucatinib is a tyrosine kinase inhibitor used to treat HER2-positive breast cancer by targeting ErbB2/HER2 [23]. Olmutinib is an orally active epidermal growth factor receptor (EGFR) inhibitor used in treatment of T790M mutation positive non-small cell lung cancer [60].

HsFN3K has a solvent-exposed, shallow ATP-binding cleft that is targeted for inhibitor binding, additionally, since HsFN3K is an intra-cytosolic protein [61], drugs need to be able to cross the lipophilic plasma membrane. As a result, potent FN3K inhibitors need to be hydrophobic enough to pass through the lipid bilayer, while also being hydrophilic enough to interact with a hydrated, ATP-binding active site. This hydration ability has also been extensively studied by focussing on parameters such as radial distribution functions, formation of water bridges and water molecule count at this binding site.

Unlike prior homology model based studies [16], our study relied on the 3D HsFN3K crystal structure to perform molecular docking and MD simulations to assess the stability of HsFN3K-ligand binding, and whether any major conformational or structural changes occur within the protein due to these binding-events. The MD results strengthen our claim, and the experimentally determined HsFN3K structure yields significantly more stable MD behaviour and allows a finer dissection of ligand-induced effects. However, the low-resolution value (2.85 Å) of HsFN3K (PDB: 8UE1) remains to be a limitation of our study; better resolved structures, when available, may further refine the docking poses and binding energy estimates reported herein. Further in-vitro and in-vivo studies are also needed to assess the efficiency of these noncovalent and covalent drugs against HsFN3K.

Declarations

Ethical approval

This declaration is not applicable.

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Data availability

All relevant data are within the manuscript and the supplementary information files.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Author Contributions

M.A.A. performed the in-silico analysis. M.A.A. and N.P. wrote the main manuscript text. Both authors reviewed the manuscript.

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Figures

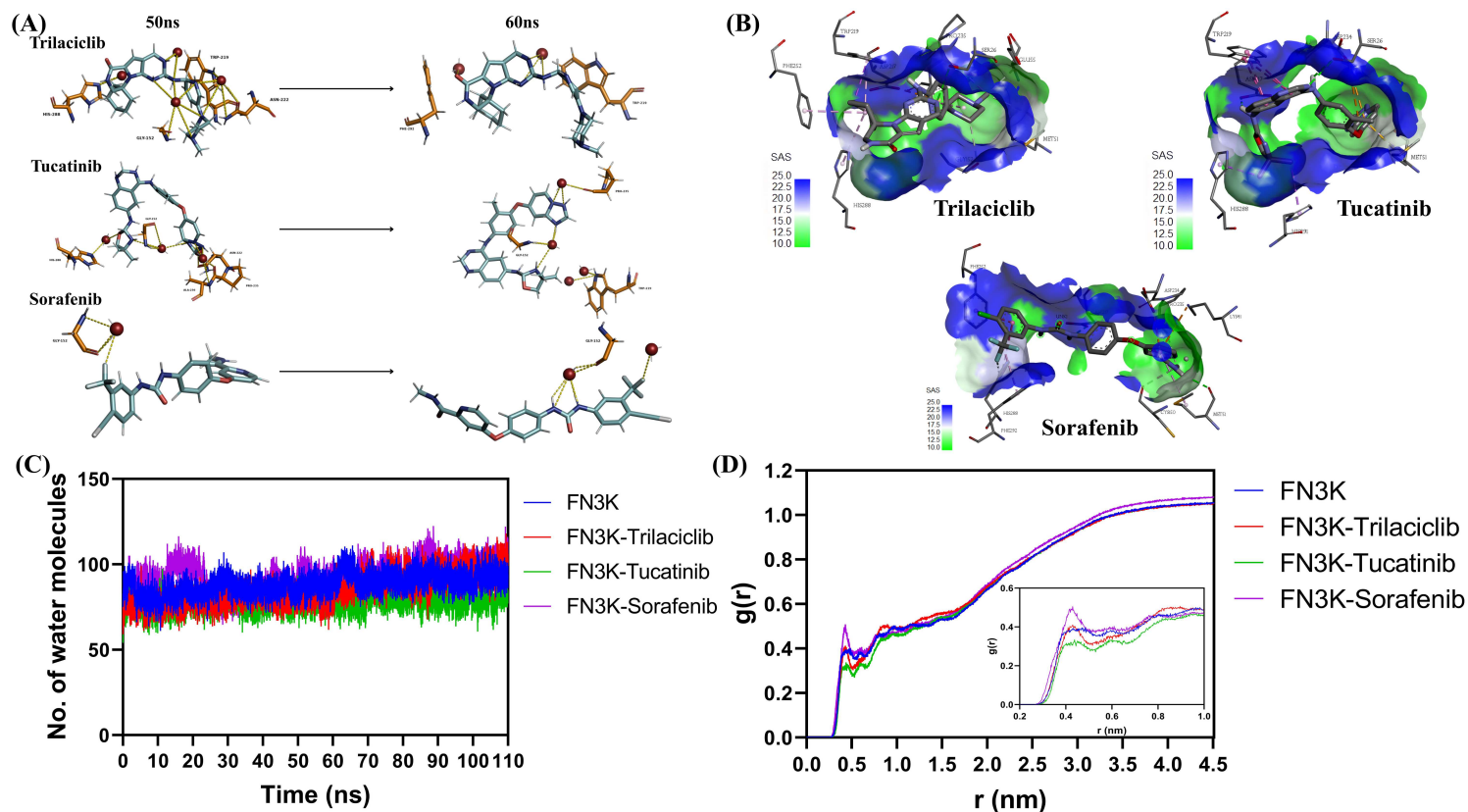


Figure 5

(A) Water bridges formed between ligands and protein residues at 50 and 60ns frames (H-bond criteria: Donor-Acceptor distance < 3.5 Å, Donor-H...Acceptor angle $> 130^\circ$); (B) Solvent accessible surface ($\text{\AA}^2$) of FN3K-trilaciclib, FN3K-tucatinib and FN3K-sorafenib; (C) No. of water molecules present at active site; (D) Radial distribution function for the probability of finding water molecules around active site residue, first solvation shell has been highlighted

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