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Original Article

Pharmacophore based High Throughput Virtual Screening towards the Discovery of Novel BLK (B-lymphocyte kinase)-tyrosine Kinase Inhibitors

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ABSTRACT

Aim: LK (B-lymphocyte kinase) is a protein from the family SRC (Proto-oncogene tyrosine-protein kinase), an important cell signaling molecule that influences cellular response. The BLK tyrosine-protein kinase has been a potential target for cancer therapy. As a result, this could be an initial step toward the development of novel inhibitors to fight cancer. **Materials and Methods:** A homology model of human BLK tyrosine kinase was constructed using Phyre2. Active site prediction was done for the model using the CASTp server. High Throughput virtual screening was performed with the help of a ligand-based pharmacophore model of FDA (Food and Drug Administration) approved SRC tyrosine family kinase inhibitors using the PharmaGist and ZINCPharmer servers. The 250 novel compounds obtained were docked by a Python script-based method with Autodock Vina. To ensure drug safety, ADME/Tox (Absorption, Distribution, Metabolism, Elimination, Toxicity) analysis was performed for the molecules with the lowest binding energy. Six compounds that passed ADME/Tox analysis were again utilized to perform molecular docking with Autodock4. The active residues were then identified using PLIP [protein ligand interaction profiler]. **Results and Conclusion:** Six compounds passed ADME/Tox analysis. Based on the molecular docking analysis, the compound ZINC57306994 showed an increased binding affinity with the target BLK tyrosine kinase. The compound ZINC57306994 may serve as a lead molecule that could be developed into a potent BLK tyrosine kinase inhibitor.

Keywords: Pharmacophore modeling, Molecular docking, BLK tyrosine kinase, Cancer.

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INTRODUCTION

Cancer is a serious disease caused by cells dividing uncontrollably and spreading into surrounding tissues and distant organs. If the tumor has progressed to the stage where it cannot be treated, it eventually leads to the death of the affected patient. Cancer is caused due to changes in our genetic material, DNA. The most common risk factors for cancer include aging, tobacco chewing, exposure to radiation, chemicals, some bacteria and viruses, family history of cancer, certain hormones, alcohol, an unhealthy diet, lack of physical activity, and obesity. An estimated 19.3 million new cancer cases were found worldwide, and almost 10.0 million cancer deaths occurred in 2020.[1] Female breast cancer is the most diagnosed cancer in 2020. About 2.3 million cases were estimated. One way to overcome cancer is through the identification of drugs against potential protein targets. Tyrosine kinase are a family of enzymes that catalyze phosphorylation reactions by using ATP and are involved in cell proliferation, differentiation, migration, metabolism, and programmed cell death. Recent studies have revealed that tyrosine kinase is involved in the pathophysiology of cancer.[2,3] BLK is a non-receptor tyrosine kinase belonging to the SRC kinase family.[4] The SRC family kinase consists of proteins SRC, YES1, HCK, FYN, FGR, LCK, LYN, and BLK that are present in humans, all of which are characterized by the presence of an SH3 and SH2 domain N-terminal to the catalytic kinase domain. BLK is functionally involved in B-cell signaling and B-cell development and is ectopically involved in hematological and multiple non-hematological malignancies, including breast, kidney, and lung cancer, indicating that BLK could be a new potential target for the therapy of cancer.

MATERIALS AND METHODS

Collection of data

PubChem contains data on a wide variety of chemical entities, including small molecules, lipids, carbohydrates, amino acids, and nucleic acids.[5] The chemical structures of the FDA-approved drugs, Dasatinib, Ponatinib and Saracatinib were downloaded from PubChem.[6-9] Uniprot is a knowledge base where over 120

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million proteins from all branches of life were collected, with their sequences and annotations. The amino acid sequence of various protein tyrosine kinase from the SRC kinase family was taken and its amino acid sequence was retrieved from Uniprot.[10] The multiple sequence alignment was performed using the fasta sequence that we obtained from Uniprot.[11] The Protein Data Bank (PDB) contains more than 134,000 structures of biomolecules that have been identified by crystallography, NMR spectroscopy, and 3D electron microscopy.[12] Here we used the PDB web server to download protein structures in PDB format. Open Babel software which was used to convert different file formats used in this study.[13] The ZINC database is a collection of chemical compounds that are commercially available and specially prepared for virtual screening.[14] The ligands downloaded from here were used in the virtual screening process. An extendable application called chimera allows for the interactive viewing and analyzing of molecular structures and associated data.[15]

Homology modeling and structural validation of protein

Analysis of conserved sequences throughout the protein SRC tyrosine kinase family of proteins, SRC, YES1, LCK, HCK, and BLK was done using Clustal Omega.[16] It gives the alignment between two or more sequences by using seeded guide trees and the HMM profile technique. The BLK tyrosine kinase protein was homology modeled using the PHYRE2 server.[17] The amino acid sequence of BLK was subjected to PHYRE2 to recognize the most acceptable crystal structure in the database as a template for modeling BLK. Sequence identity, resolution, domain coverage, and cognate ligand binding give the best template selection. And the structure validation was done by using the servers QMEAN4, ProSA, PROCHECK and verify3D.[18-20]

Active site prediction of protein model

Active site residues of the modeled BLK tyrosine kinase were found using the CASTp server.[21] The CASTp server recognizes the surface of all the pockets and their interior cavities throughout the channels in the structure of a protein and gives detailed information about all the atoms taking part in the formation. The CASTp server also measures the exact area and volume of the pocket. The structure of PDB ID 2C0I was retrieved from the protein data bank and the active residues were identified by using the PLIP [protein ligand interaction profiler] server. The Plip server detects and visualizes the target and ligand interactions. PLIP identifies the halogen bonds, water bridges, salt bridges, hydrogen bonds, hydrophobic contacts, pi-stacking, and pi-cation interactions between the target and ligand.[22]

Molecular docking of FDA-approved BLK inhibitors

Molecular docking was done using Autodock 4.2.6 software.[23] It is used to fit a ligand into a 3D structure binding site and undergoes two steps. The first is the search for conformational space for binding, and the second is binding by releasing free binding energy. At first, the macromolecule modeled BLK tyrosine kinase was checked for missing amino acid residues, and the missing residues were added with the help of the misc option. Polar hydrogen and partial Kollman charges were assigned. The torque bonds of the inhibitors were selected and defined. A Lamarckian genetic algorithm was used to explore flexible inhibitors. Grid maps of the interaction energy between atoms and proteins present in inhibitors have been pre-calculated using the Autogrid program. The three-dimensional grid box with a 60-grid size (x, y, z) with a spacing of 0.500 and a grid center (x, y, z) (-0.06844, 65.4316, 20.9191) was created. Active site residues of the docked complex of FDA drugs and modeled BLK tyrosine kinase were found using the PLIP server.

Pharmacophore modeling

Ligand-based pharmacophore modeling using FDA-approved drugs Dasatinib, Ponatinib, and Saracatinib was done using the PharmaGist server.[24] It is an ensemble of steric and electronic features that are important to recognize the optimal supramolecular interactions with the protein and trigger its biological response. By this definition, the interactions between the proteins and the bioactive molecules can be represented through a 3D arrangement. The interaction types include hydrogen bonds, metal interactions, charged interactions, and hydrophobic and aromatic contacts. High-throughput virtual screening of BLK tyrosine kinase inhibitors was done using the ZincPharmer server.[25] It gives us a database of conformations that has been calculated from the zinc database of purchasable compounds. The filters used were as follows: Molecular weights ranging from 450 to 550 and rotatable bonds ranging from 6 to 9 were used to identify 250 zinc-purchasable compounds.

Virtual screening

Here, virtual screening was employed to help with lead optimization and hit detection. A python script-based approach using Autodock vina was used to do virtual screening on 250 active ligands taken from the top hits of the zinc purchasable database on the target.[26] The macromolecule was prepared the same as in Autodock 4.2.6. The three-dimensional grid box with a 60-grid size (x, y, z) with a spacing of 0.500 and a grid center (x, y, z) (-0.06844, 65.4316, 20.9191) was created, and virtual screening was performed.

ADMET/Tox prediction

The top six ligands with the lowest binding energy from 250 hits were taken and ADMET was analyzed. Drug ADME prediction and drug-likeness prediction were done using the Swiss ADME server.[27] In the discovery and development of a drug, chemical absorption, distribution, metabolism, excretion, and toxicity (ADMET) play an important role. For drug likeliness, Lipinski's Rule of Five was considered because a molecule will not be orally

active if it violates two of the four Lipinski's Rule of Five.[28] The drug toxicity analysis was done using OSIRIS Property Explorer and the Pro-Tox II.[29]

Molecular docking of ADME passed novel drugs

Molecular docking analysis of modeled BLK tyrosine kinase against the drugs that passed ADME was done. The six ligands that passed the ADME test, were docked uniformly by using Autodock4. The macromolecule was prepared and grid preparations were done as previously. And the binding free energy and inhibition constant of the macromolecule-ligand complexes were obtained.

RESULTS AND DISCUSSION

Homology modeling and structural validation of protein

By performing conserved sequence analysis, it was found that the HCK kinase protein has the highest sequence similarity to our protein of interest, BLK tyrosine kinase. The homology model was constructed from the phyre2 server using template PDB ID 2C0I (HCK tyrosine kinase) with a sequence identity of 69% with BLK. Structure validation of the model was done by using many servers like ProSA-web, PROCHECK, QMEAN4, and verify3D, and the results are shown in (Table 1). The Ramachandran plot and its statistics for the model BLK tyrosine kinase were obtained using the PROCHECK server.[30] Ramachandran plot statistics implied that 87.60% of residues are present in the favored region. The ProSA - webserver was used to find the Z-score. It indicates the quality of overall protein model. Here we obtained a Z-score value of -9.4 for our homology model. A high Z score indicates high quality. Ramachandran plot and Z-score plot shown in (Figure 1). QMEAN4 [qualitative model energy analysis] is the composite scoring function that describes the geometrical aspects of protein structures. Here we got a QMEAN4 score of 0.73 for our model. Furthermore, the model was subjected to the Verify3D algorithm for a more thorough evaluation, which measures protein model compatibility with its amino acid sequence. It was found that the residue had an average 3d-1d score>= 0.2 and gave a value of 99.10%.

Active site prediction of protein model

The active site residues of template PDB ID 2C0I were found on the PLIP server. The template residues are Leu-247, Val-255, Ala-267, Phe-281, Met-288, Ile-310, Leu-367, Leu-367, Ala-377, and Leu-381. Their distances are 3.81 Å, 3.82 Å, 3.97 Å, 3.68 Å, 3.95 Å, 3.58 Å,3.70 Å,3.98 Å, 3.88 Å, 3.83 Å respectively. An active pocket of the BLK model was identified using the CASTp server it gives pockets of the binding sites for the ligand. We finalized pocket 2, which has a volume of (723.546). And this pocket had active site residues matching the template PDB ID 2COI.

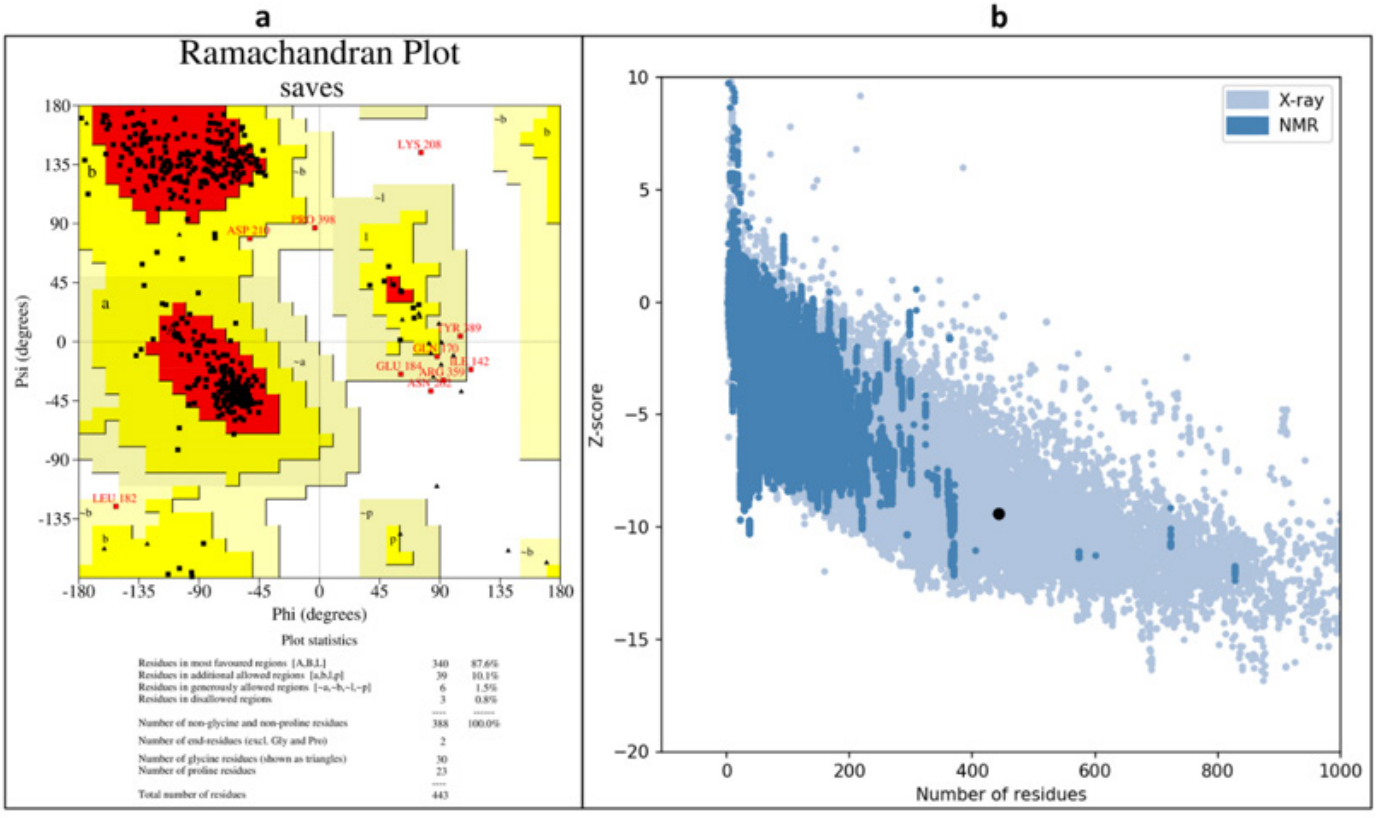

Figure 1: Structure validation results of homology model by (a) Ramachandran plot obtained from PROCHECK, (b) Z-score graph obtained from PROSA.

Table 1: Structure validation results of homology model performed with various servers.

Sl. No	Server	Parameters	Values
1	PROSA	Z-SCORE	-9.4
2	PROCHECK	Residues in most favored region	87.60%
3	Qmean4	Q-mean score	0.73
4	VERIFY 3D	The residues that have averaged 3d-1d score>=0.2	99.10%

Table 2: Molecular docking analysis results of known FDA approved drugs used for pharmacophore model with modelled BLK tyrosine kinase.

Sl. No	Drugs	MOLECULAR FORMULA	Binding energy (kcal/mol)	Inhibition constant (nM)
1	Dasatinib	$C_{22}H_{26}C_lN_7O_2S$	-9.38	132.62
2	Ponatinib	$C_{29}H_{27}F_3N_6O$	-10.22	32.33
3	Saracatinib	$C_{27}H_{32}C_lN_5O_5$	-8.59	507.81

Table 3: Active site residues of modelled BLK tyrosine kinase interacting with FDA approved inhibitors.

Sl. No	Drugs	Hydrogen-bond	Residual hydrophobic interactions
1	Dasatinib	Gly-253, Val-255, Lys-269, Asp-378, Phe-379	Lys-269, Leu-271, Phe-281, Met-288, Lys-269, Val-297, Leu-299, Ile-310, Phe-379. Leu-381
2	Ponatinib	Val-255	Val-255, Lys-269, Leu-271, Phe-281, Met-288, Ile-310, Leu-381
3	Saracatinib	Gly-253, Val-255	Gln-251, Phe-252, Val-255, Leu-381. Ile-385

Table 4: ADME prediction results of novel shortlisted compounds based on best binding energy and other physiochemical properties.

Physiochemical Properties	ZINC57306994	ZINC33263215	ZINC02709773	ZINC11696138	ZINC19166011	ZINC12637348
Molecular weight	476.55	481.54	484.57	489.59	474.96	500.61
Num. rotatable bonds	6	9	6	6	6	7
H-bond acceptors	6	6	6	5	6	5
H-bond donors	1	2	1	1	1	1
TPSA[1](A°[2])	111.56	132.53	111.02	105.57	111.56	102.33
Lipophilicity (LogP)	3.01	3.21	3.27	2.85	3.04	3.21
Metabolism						
CYP1A2*	No	No	No	No	Yes	No
CYP2C19*	Yes	Yes	No	Yes	Yes	Yes
CYP2C9*	Yes	Yes	No	Yes	Yes	Yes
CYP2D6*	No	Yes	No	No	Yes	Yes
CYP3A4*	Yes	Yes	Yes	Yes	Yes	Yes
Excretion						
P-Gp[2] substrate	Yes	Yes	No	Yes	No	No

Abbreviations [1]Total polar surface area, [2]P-glycoprotein, * Cytochrome P450 inhibitors

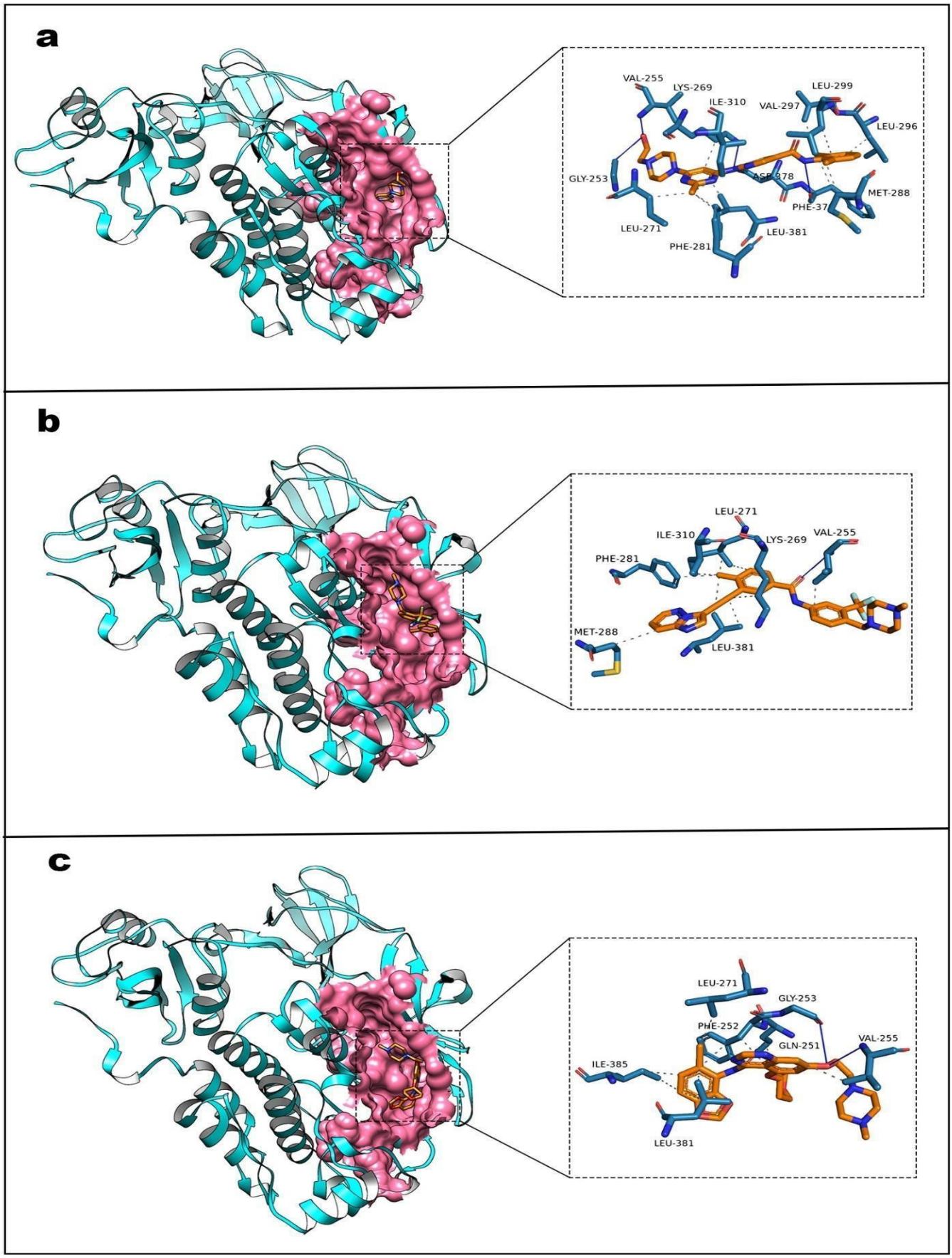

Figure 2: Active site residues of a Docked complex of modeled BLK tyrosine kinase and FDA approved drugs (a) Dasatinib, (b) Ponatinib, (c) Saracatinib.

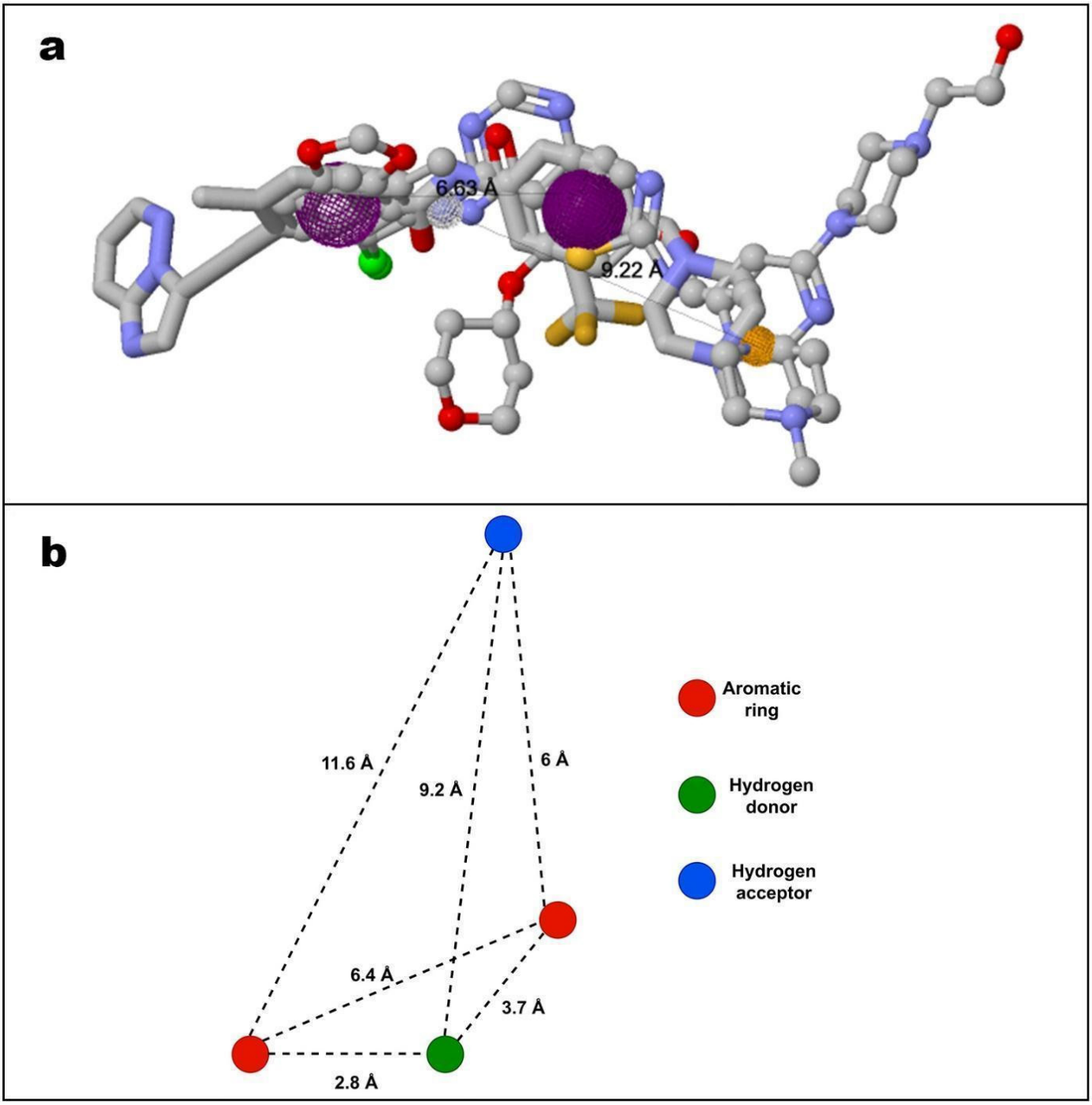

Figure 3: (a) Pharmacophore model of FDA-approved SRC tyrosine kinase inhibitors, (b) Four-point model of pharmacophore with the distances between molecular descriptors.

Molecular docking analysis of FDA-approved BLK inhibitors

Molecular docking analysis was done by using Autodock4 for BLK tyrosine kinase and FDA-approved SRC tyrosine kinase inhibitors, the binding energies and inhibition constants are listed in (Table 2). The binding energies of Dasatinib, Ponatinib, and Saracatinib were -9.38 kcal/mol, -10.22 kcal/mol, and -8.59 kcal/mol, respectively. The inhibition constant wavelengths are -132.62nm, 32.33nm, and -507.81nm, respectively. The active site residues of all docked complexes were noted by PLIP, and the results are shown in (Table 3) and (Figure 2).

Pharmacophore modeling

Ligand-based Pharmacophore modeling of FDA-approved drugs was done using the PharmaGist server. The drugs in complex

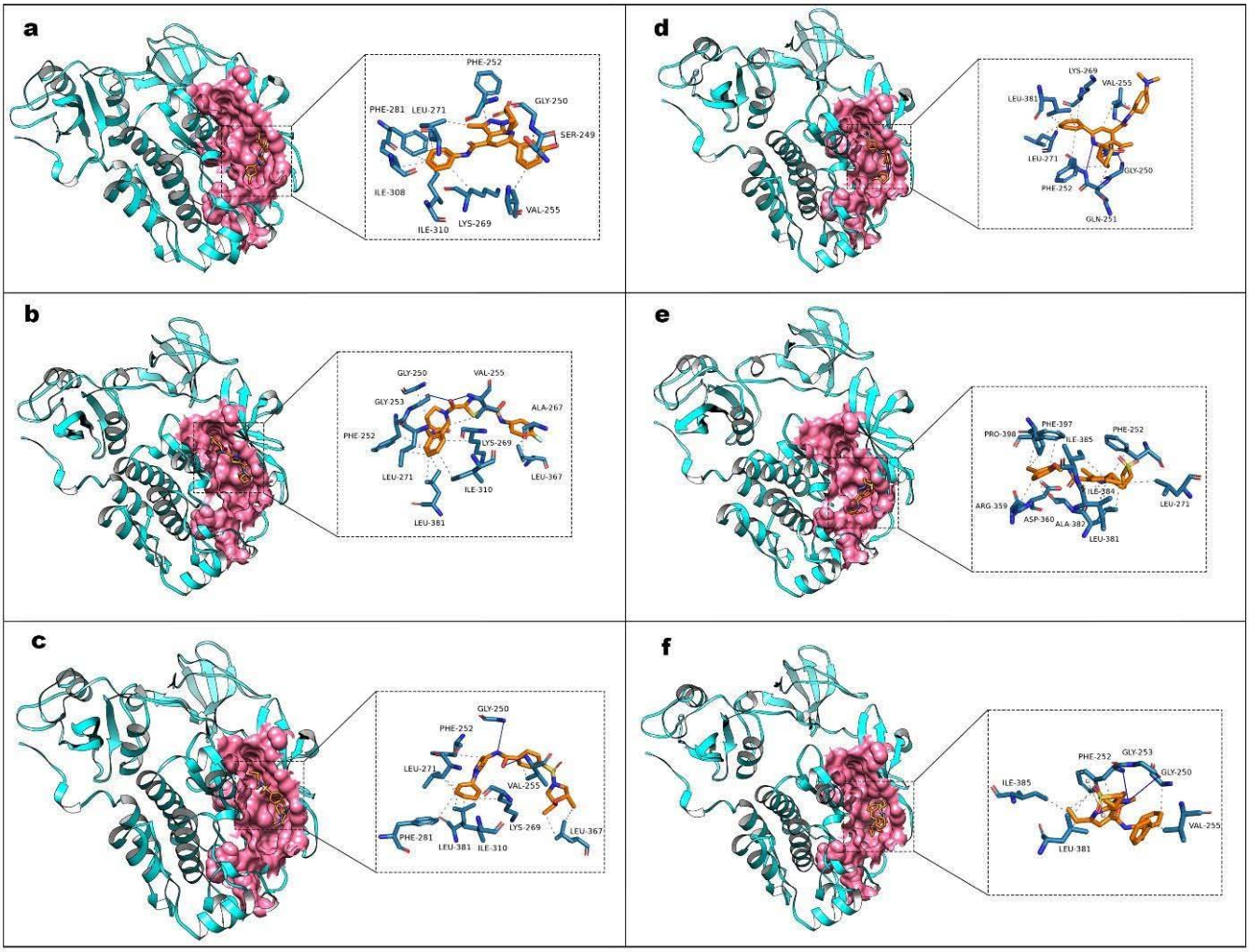

Figure 4: Active site residues of Docked complex of modelled BLK tyrosine kinase and its Novel inhibitors (a) ZINC57306994, (b) ZINC33263215, (c) ZINC02709773, (d) ZINC11696138, (e) ZINC19166011, (f) ZINC12637348.

Table 5: Toxicity prediction results of novel shortlisted compounds.

Toxicity	ZINC57306994	ZINC33263215	ZINC02709773	ZINC11696138	ZINC19166011	ZINC12637348
Toxicity Class	4	4	5	4	4	4
Hepatotoxicity	Inactive	Inactive	Active	Inactive	Active	Inactive
Carcinogenicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Immunotoxicity	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Mutagenicity	Inactive	Inactive	Active	Active	Inactive	Inactive
Cytotoxicity	Inactive	Inactive	Inactive	Active	Inactive	Inactive
Irritant	No	No	No	No	No	No

with the protein model generated a total of four pharmacophore features and their molecular descriptors two aromatic rings, one hydrogen donor, and one hydrogen acceptor. The Pharmacophore model having a Jmol score of 22.405 was selected and it's shown in (Figure 3). ZINCPharmer server was used for High-Throughput Virtual screening of BLK tyrosine kinase inhibitors. Although it can automatically extract a pharmacophore from the interactions the user further increases the applicability and specificity of the results by editing query properties or by applying filters to get high-quality hits. Each hit shows a unique orientation and conformation to the query. Finally, the best 250 hits of Zinc purchasable compounds were selected.

Virtual screening

Virtual screening of the top 250 hits was obtained using the Autodock Vina software. AutoDock Vina is a program for

Table 6: Molecular docking analysis results of ADMET passed drugs with BLK.

Sl. No	Compound name	Molecular formula	Binding energy (kcal/mol)	Inhibition constant (nM)
1	ZINC57306994	$C_{25}H_{24}N_4O_4S$	-11.23	5.89
2	ZINC33263215	$C_{24}H_{24}FN_5O_3S$	-11.17	6.47
3	ZINC02709773	$C_{24}H_{28}N_4O_5S$	-10.98	8.88
4	ZINC11696138	$C_{26}H_{27}N_5O_3S$	-10.92	9.88
5	ZINC19166011	$C_{22}H_{23}ClN_4O_4S$	-9.39	130.39
6	ZINC12637348	$C_{28}H_{28}N_4O_3S$	-8.8	414.3

Table 7: Active pocket residues of shortlisted compounds against modelled BLK tyrosine kinase.

Sl. No	Compound name	Hydrogen-bond	Residual hydrophobic interactions
1	ZINC57306994	Ser-249	Gly-250, **Phe-252**, **Val-255**, **Lys-269**, **Leu-271**, **Phe-281**, Ile-308, **Ile-310**
2	ZINC33263215	Gly-250, Gly-253, **Val-255**	Gly-250, **Phe-252**, **Val-255**, Ala-267, **Lys-269**, **Leu-271**, **Ile-310**, Leu-367, **Leu-381**
3	ZINC02709773	Gly-250, **Val-255**	**Phe-252**, **Val-255**, Lys-**269**, **Leu-271**, **Leu-381**, **Ile-310**, Leu-367, **Leu-381**
4	ZINC11696138	Gly-250, Phe-252, **Val-255**	**Gln-251**, **Phe-252**, **Val-25**5, **Lys-269**, **Leu-271**, **Leu-381**
5	ZINC19166011	Gln-251, Phe252	**Phe-252**, **Leu-271**, Arg-359, Asp-360, **Leu-381**, Ala-382, Ile-384, **Ile-385**, Phe-397, Pro-398
6	ZINC12637348	Phe-252, **Gly-253**	Gly-250, **Phe-252**, **Val-255**, **Leu-381**, **Ile-385**

*Bold Amino acid residues coincide with the active residues of FDA approved BLK inhibitors

virtual screening. It uses a sophisticated gradient optimization method in its local optimization procedure. From among the top 250 hits obtained, we chose the best 50 ligands that have free binding energies of 14 to -8 kcal/mol and were further utilized for ADMET analysis.

ADMET/Tox prediction

ADME properties were predicted using SwissADME server, all the parameters predicted are shown in (Table 4). First, drug likeliness was seen where the Lipinski's Rule of Five Violations was analyzed, and compound 6 had one Lipinski's Rule violation of having a molecular weight greater than 500g/mol. Then the properties like skin permeation value, polar surface area, gastrointestinal absorption, and blood-brain barrier permeability were obtained, and all six compounds have high gastrointestinal absorption, but there is no blood-brain barrier permeability. Then P-gp(P-glycoprotein) substrates and cytochrome P450 inhibitors were obtained, and it was found that all compounds were P-gp substrates. P-gp involved in the efflux function. It extrudes toxins and xenobiotics out of the cell. The measurement of toxicity is an important step towards the selection of a better compound. Electronic toxicity measurement shows more accuracy and accessibility, which is why it is widely used and can show information about any natural or synthetic compound. By using the Pro-Tox II server and OSIRIS Property Explorer software toxicity prediction was performed, all the parameters predicted are shown in (Table 5). At first, Toxicity Class and irritant effect were predicted and all the compounds didn't have any irritant effect. Finally, toxicity prediction was performed using the Pro-Tox II server include hepatotoxicity, i.e., liver toxicity; carcinogenicity, i.e., the ability to cause cancer; mutagenicity, i.e., the ability to induce mutation, and cytotoxicity, i.e., cell toxicity. All six compounds do not show any carcinogenicity or immunotoxicity.

Molecular docking analysis of ADME passed novel drugs

Among all the 250 hits, 6 ligands that passed the ADME were taken for molecular docking analysis. It was done using Autodock4. (Table 6) gives information about the molecular docking analysis of ADMET-passed drugs. Here we note the binding energy in kcal/mol (ranges from -11.23 to -8.8) and inhibition constant in nM. Among all the six ligands, ZINC57306994 has the best binding energy of -11.23 kcal/mol and an inhibition constant of 5.89 nM. (Table 7) and (Figure 4) gives the active site residues obtained from the PLIP server.

CONCLUSION

The study design is based on a bioinformatics approach that has successfully established a novel drug, ZINC57306994, as a potential lead molecule against BLK protein tyrosine kinase using

homology modeling, ligand-based pharmacophore modeling, high-throughput virtual screening, ADME/Tox prediction, and molecular docking. The compound ZINC57306994 was found to have the least binding energy of -11.23 kcal/mol with an inhibition constant of 5.89 nM (*in-silico*). This compound may act as potent inhibitor against BLK protein tyrosine kinase. Further studies including *in-vitro* and *in-vivo* have to be carried out for checking the efficacy of the novel compound. This study is an attempt to screen and shortlist candidate molecule as a potent BLK inhibitor.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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