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IKKβ inhibitors identification part I: Homology model assisted structure based virtual screening

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ABSTRACT

Control of NF-κB release through the inhibition of IKKβ has been identified as a potential target for the treatment of inflammatory and autoimmune diseases. We have employed structure based virtual screening scheme to identify lead like molecule from ChemDiv database. Homology models of IKKβ enzyme were developed based on the crystal structures of four kinases. The efficiency of the homology model has been validated at different levels. Docking of known inhibitors library revealed the possible binding mode of inhibitors. Besides, the docking sequence analyses results indicate the responsibility of Glu172 in selectivity. Structure based virtual screening of ChemDiv database has yielded 277 hits. Top scoring 75 compounds were selected and purchased for the IKKβ enzyme inhibition test. From the combined approach of virtual screening followed by biological screening, we have identified six novel compounds that can work against IKKβ, in which 1 compound had highest inhibition rate 82.09% at 10 μM and IC_{50} 1.76 μM and 5 compounds had 25.35–48.80% inhibition.

1. Introduction

NF-κB regulates gene expression in immunity, stress responses, inflammation and the inhibition of apoptosis.[1] A key role of the transcription factor is to induce and coordinate the expression of broad spectrum of proinflammatory genes, including cytokines, chemokines, interferons, MHC proteins, growth factors and cell adhesion molecules.[2] The IkB inhibitory proteins sequester NF-kB in the cytoplasm by masking its Rel homology domain. The IKKβ is a serine-threonine protein kinase, which is critically involved in the activation of the nuclear factor kappa B (NF-kB) in response to various stimuli. The transcription of both the TNF-α and IL-1β is dependent on the transcriptional activator nuclear factor (NF)-κB, as the expression of other proteins that play important roles in immunologic and inflammatory processes.[3] IKKβ plays a major role in activation of NF-κB via classical (canonical) pathway, and activation of the classical pathway that can promote the production of TNF-α. IL-1, intercellular adhesion molecule (ICAM)-1, and cycloxygenase (COX)-2.[4]

The IKK complex comprises of two catalytic subunits, such as, IKKα and IKKβ and a regulatory subunit IKKγ. Although both the catalytic subunits can catalyze the phosphorylation of IκBα, the IKKβ subunit seems to have the dominant role in the canonical pathway in cells and IKKα has a crucial role in mediating p52 activation through the 'non-canonical' pathway.[5] The IKKα can form an alternative complex (without IKKβ and IKKγ), and the function of which is required for the development of lymphoid organ and the maturation of B cell.[6]

Activated NF-kB then translocated into nucleus, where it can mediate proinflammatory gene expression and ultimately turns into awful swelling, redness, pain, heat, and loss of function of an organ. Activation of the most forms of NF-κB, especially the most common form—the p50–RelA dimer—depends on phosphorylation-induced Ubiquitination of the IκB proteins. This sequential modification depends on two protein complexes: the IκB kinase (IKK) complex and the $E3^{IκB}$ ubiquitin ligase complex.[7] Inhibitors of this enzyme represent a promising target for the development of novel agents to treat rheumatoid arthritis and other inflammatory diseases.[8] The identification of isoform selective IKK inhibitors can provide pharmacological reagents to further address the differential role of these kinases in health and disease.[9]

Virtual screening is a complementary approach to HTS that allows the discovery of novel lead-like molecules from large libraries of diverse compounds by using information about the structure of

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the protein binding cavity or known ligands. We have developed homology model of IKKβ catalytic domain and an extensive enrichment study has been conducted to examine whether the developed homology model is capable enough for structure based virtual screening. Finally, the results of the validations show that the model is reasonably good in the present scenario of non-availability of structural information about IKKβ. Further, we have extended this screening process to the structure-based design. There has been no crystal structure information reported on IKKβ yet and therefore, in the absence of an experimentally determined structure, a homology model can be used instead. Importance of hinge region, Glu97 and Cys99 hydrogen bonding has already been explained by other researchers, who working on the IKK inhibitor design and synthesis.[4,9,10] Our docking simulation study can be correlated with the previous study and exhibits the hinge region hydrogen bonding with ligand.[11] We have successfully employed the homology model in the structure based VS scheme to search ChemDiv database that contains 26,953 compounds. The biological screening reveals one potential hit with IC_{50} 1.76 μM, and five compounds having moderate inhibition rate. Therefore, these compounds are entering into lead optimization process in our research group.

2. Results and discussion

2.1. IKKβ structure and ATP interaction

The structure based virtual screening is the most efficient way for identifying the small molecule inhibitors from a large drug-like database. Till date there has been no experimental structure reported for the IKKβ, which remained as a big obstacle in the structure based virtual screening. Hence, we have developed a homology model based on four different kinases as given in Table 1. The IKKβ model (Fig. 1) exhibits a well defined β strand rich N-lobe and α helices rich C-lobe. The structural analysis indicates that the glycine-rich loop (G-Loop) and hinge region are more or less similar with other kinases. G-loop forms a portion of the ATP-binding site and connecting lope of N and C lobe is hinge region, which can interact with adenine portion of ATP. The IKKβ model represents activation loop (A-Loop) with 177 and 181 phosphoserine,[12] which is responsible for the IKKβ activation. However, the A-loop state is poorly understood. As it has been observed in other kinases, the IKKβ model also exhibits hydrophobic core pocket where, the adenine moiety of ATP is stabilized (Fig. S1). From docking of the ATP molecule, it is understood that the putative interaction (Fig. 2), adenine interacts with Glu97(N) and Cys99(O), ribose sugar forms an interaction with Asp103 (sidechain O) and Glu149(O), phosphate region interacts with G-Loop region Thr23(N), 2 hydrogen bonding with oxygen of Gly24, 1 with Gly27 nitrogen and 1 with Asn28 oxygen. A total of 11 hydrogen bonds can be formed by the ATP.

2.2. Homology model selection

Initially, three protein models are generated, in which the model **1** is considered to be the best, based on the least 'discrete optimized potential energy' (DOPE) score of −26222, and this score is found to be very close to other models. Energy minimization with tripos field, turnouts the relaxed model having −587.601 kcal/mol. Interestingly, the model **1** shows an interac-

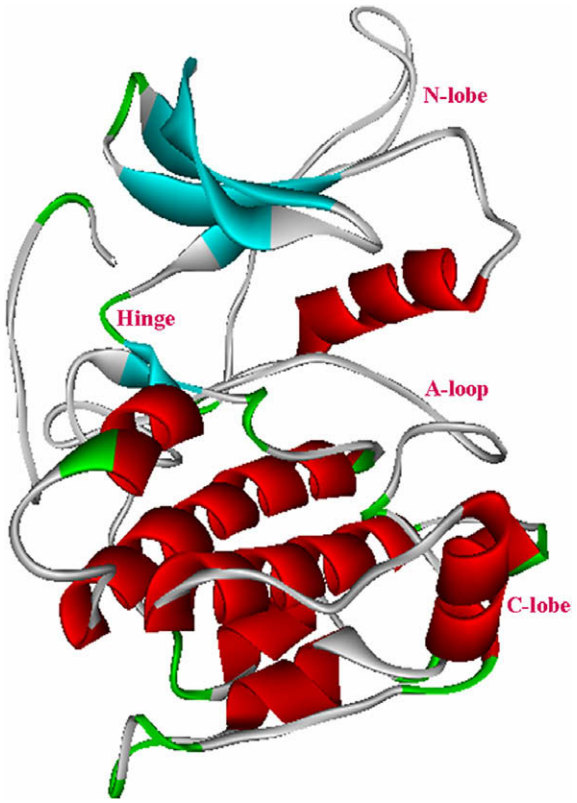


Figure 1. IKKβ putative model exhibiting well defined N and C terminal lobe, hinge region, G-loop and A-Loop.

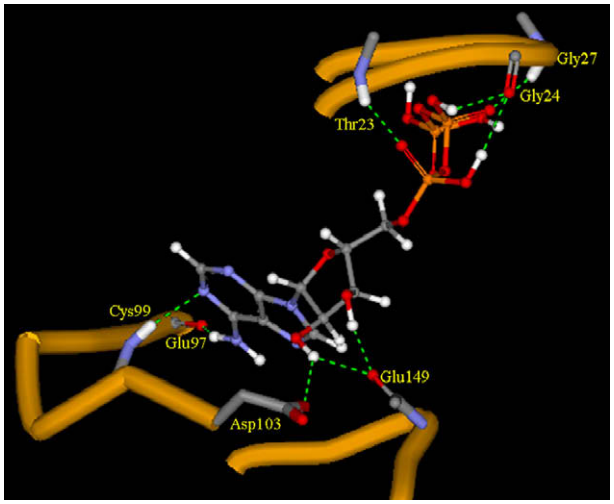


Figure 2. Plausible binding mode of ATP with IKKβ. Adenine portion of ATP forms 2 hbond with hinge region and sugar moiety forms 3 bonds with Asp103 and Glu149, phosphate region interacts with g-loop.

Table 1
Templates used for IKKβ catalytic domain homology modeling

Protein	Accession	Identity (%)	Resolution (Å)	Crystal state	Reference
Human calmodulin-dependent protein kinase	2JC6	31	2.30	Co-crystal	16
Catalytic and ubiqutin-associated domains, Human	2HAK	32	2.60	—	18
Human ZIP Kinase	2J90	32	2.00	Co-crystal	16
Protein kinase A fivefold mutant model of Rho-kinase, Bovine	2GNF	29	2.28	Co-crystal	17

Table 2
The data represents the predicted binding energies measured by f_scoring function of the compounds docked at ATP binding pockets of three different models

Compound	Model 1	Model 2	Model 3
1	–	–	–
2	−27.101 kJ/mol	–	–
3	–	–	–
4	−12.991 kJ/mol	–	–
5	−26.577 kJ/mol	–	−20.022 kJ/mol
6	−24.260 kJ/mol	–	−26.504 kJ/mol
7	−21.243 kJ/mol	–	–
8	–	–	–

Some compounds shown to interact with the non ATP binding sites were not considered.

tion with five ligands in the ATP binding pocket without any docking constraints (Table 2). The model **2** does not show any interaction in ATP binding pocket, whereas the model **3** has an interaction with two ligands at ATP binding pocket. Further, the model **1** was subjected to Profunc analysis to access the stereochemical quality of the model. Ramachandran plot (Fig. S2) has reported that 94.8% of residues are found in the most favoured and additional allowed regions, 4.0% residues reported in generously allowed region and 1.2% of residues in disallowed region. Obviously, all the functionally important and theoretically identified residues responsible for selectivity located at the most favoured region of plot.

2.3. Key residues

Almost all the kinases have a ATP binding pocket, wherein most of the inhibitors can interact to inhibit the kinase activity. Sequence alignment describes the sequence conservation especially at the ATP binding pocket (Fig. 3). The docking study of various known compounds conferred an immense idea about the ATP binding pocket and the interaction mode. Sequence alignment of IKK isoforms, other kinase proteins with IKKβ and docking studies have revealed that in ATP binding pocket (i) the residues are variably found in IKKβ (ii) residues can frequently interacting with ligands and (iii) the residues are held responsible for IKKβ selectivity. Comparisons with the other known kinases sequence propose that the Glu100, Asp103, Lys106, Tyr107 and Glu172 are unique residues in IKK. Moreover the docking simulations suggest Glu97, Cys99, Arg20, Leu21, Thr23, Gly24, Lys44, Asp103, Lys147, Glu149, Asn150, Ala170, Lys171 and Glu172 has frequent interaction with known inhibitors. While, Glu97 and Cys99 forms a hinge loop and tend to form a three (acceptor–donor–acceptor) hydrogen bonds. Besides, sequence comparison of IKKα and IKKβ represents Glu100, Tyr107, and Glu172 residues are exclusively found in IKKβ. The overall fashion of structure based investigation directs that the Glu172 recurrently interacts with known ligands, whereas Glu100 twisted apart from interaction region and Tyr107 far from binding pocket. Therefore, this approach theoretically can conclude that the Glu172 is held responsible for the IKKβ isoform selectivity.

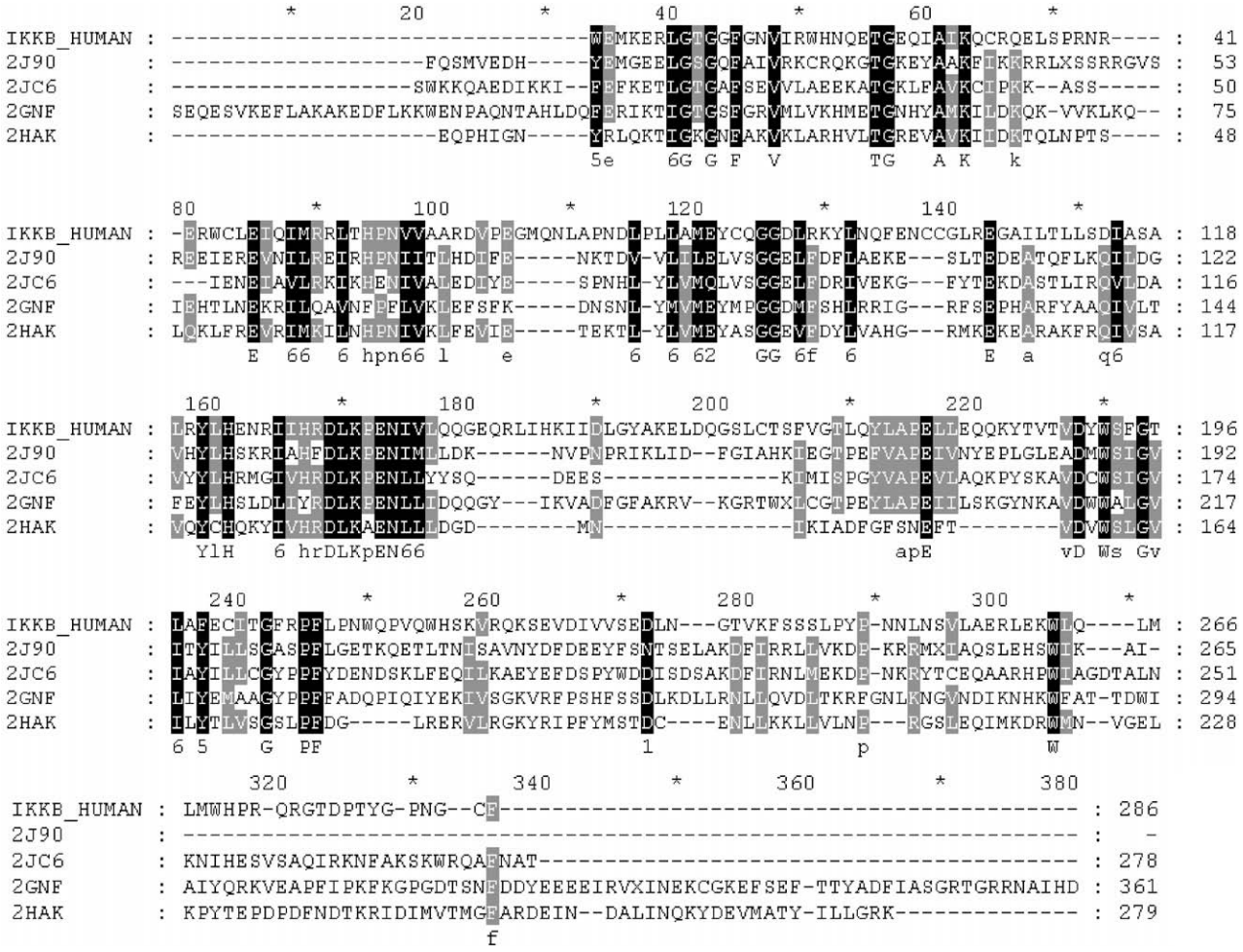


Figure 3. IKKβ (human) sequence aligned with four different template sequences. 2J90, Human ZIP kinase; 2JC6, Human calmodulin-dependent protein kinase; 2GNF, Protein kinase A fivefold mutant model of Rho-kinase, Bovine; 2HAK, Catalytic and ubiqutin-associated domains, Human.

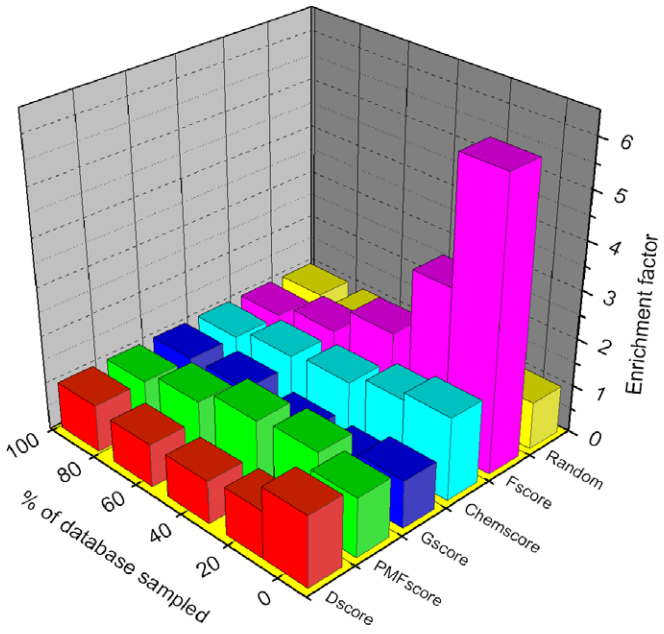


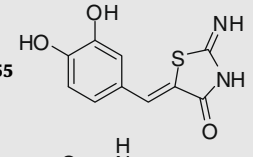
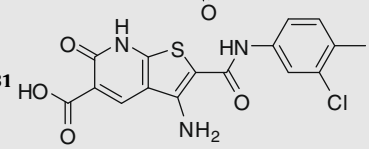
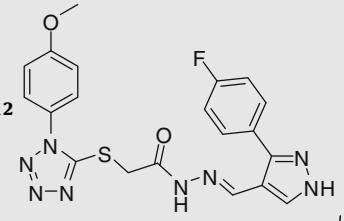
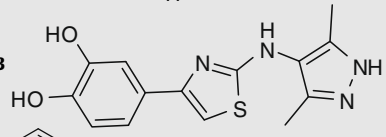
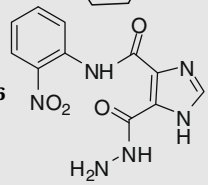
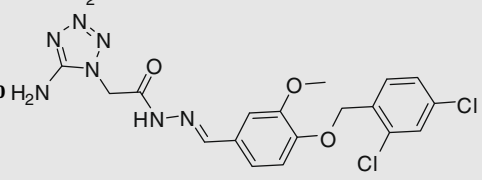
Figure 4. Enrichment factor calculated for five different scoring functions at 10%, 25%, 50%, 75% and 100% database sampling. f_scoring function has 6.25 increased fold to report active compounds as hits than other scoring functions.

Totally, 187 known compounds are subjected to docking simulation; of them 80% of compounds are succeeded. All the compounds docked successfully were able to make two hinge region hydrogen bonding interaction at the ATP binding pocket. On the other hand, some of the compounds are failed to dock, and the probable reasons are allosteric region binding, structural rigidity or little tendency of model to report a false negative. However we are interested in identifying ATP competitive inhibitors from the large database. Overall docking result has suggested that, the model is found to be highly suitable for the structure based virtual screening, while there is a no crystal available to this target.

2.4. Enrichment calculations

Enrichment factor study can be considered as a powerful computational filter for reducing the size of a chemical library that will be further experimentally tested. In structure based screening method, analyzing all the docking interactions is time consuming and does not signify any point. Therefore, we always consider the top level data sets based on its score. Among the various scoring functions tested, only f_scoring function was able to identify the more number of active compounds (hits), at initial level of database sampling than any other scoring functions (Fig. 4). This scoring function has 6.25-fold increased chance to identify the active compounds from decoys at initial 10% of database screening. Although PMF_score is found to have a close correlation to f_score

Table 3
Hit compounds and its inhibition rate of IKKβ enzyme (measured by TR-FRET method)

Compound ID	f_Score	% of inhibition @ 10 μM	IC_{50} in μM
M4891-3155 	−7.747	82.09	1.76
M4296-0831 	−16.545	48.80	ND[a]
M8012-3312 	−10.405	35.40	ND
M7790-1103 	−16.775	33.23	ND
M2295-0236 	−15.929	25.40	ND
M8008-5430 	−17.438	25.35	ND

[a] ND, not determined.

scoring, the top level database sampling does not support the PMF_score. The enrichment calculation has suggested that the f_scoring function is fairly suitable for structure based virtual screening approach of IKKβ inhibitors.

2.5. Virtual screening of ChemDiv database

Structure based virtual screening of ChemDiv database, consist of 26,953 compounds returns 277 compounds as a hits. These compounds had f_score ranges from −30.771 to 5.013 kJ/mol. Finally, 75 compounds were selected for the biological screening. In the compound selection prime criteria was set to f_score ranking, however, based on the compounds structure diversity and commercial availability some of the highly ranking compounds were not included for in vitro assay. The enzyme inhibition assay reveals six compounds posses >25% inhibition at 10 μM concentration (Table 3), in which the compound **M4891-3155** has 82.09% inhibition and IC_{50} = 1.76 μM and the compound **M4296-0831** has 48.80% inhibition (IC_{50} value is not measured) (Fig. 5). From the successful implication of the structure based virtual screening (SBVS) and in vitro analysis, we have identified six inhibitors, in which one compound has high inhibition rate. But, that compound has already been patented[13,14] by two countries. Hence, we have tried to identify the similar types of compounds from the commercial database and also, initiated the process of optimizing the other compounds in our lab.

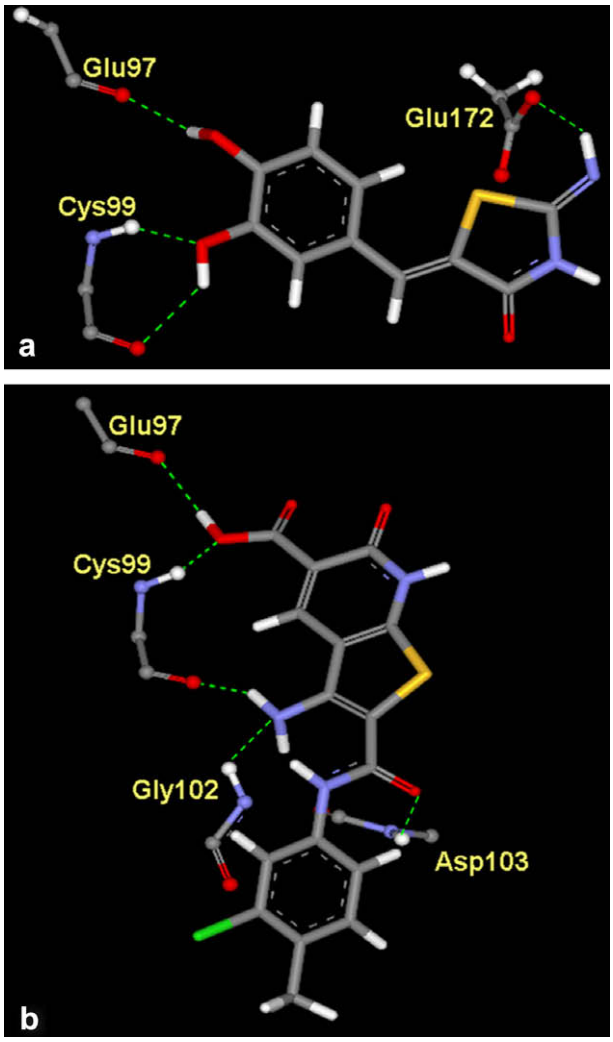


Figure 5. Pictorial representation of the two active compounds and its corresponding hydrogen bonding interaction with active site residues. Hydrogen bonding represented in green dotted lines, residues are marked with ball & stick and compounds in stick format. (a) compound M4891-3155 and (b) M4296-0831.

3. Conclusion

Control of NF-κB release through IkB kinase β inhibition represents the potential target for the modulation of immune function; and hence, the development of drugs for the treatment of auto-immune diseases is necessitated. In this present work, we have identified six novel lead molecules that can inhibit IKKβ action, by means of applying the virtual screening protocol. This consists of homology modeling and docking simulations, followed by enzyme inhibition assay. The virtual screening scheme helps to identify six compounds, which can inhibit the IKKβ enzyme. Thus, the model is not serendipity; it could be a powerful filter to screen IKKβ inhibitors from large database, as there is no crystal structure reported yet. The six lead-like molecules reveal structure diversity and one compound can inhibit with IC_{50} 1.76 μM value in a significant manner. Also, a detailed kinase activity profiling; selectivity profiling and lead optimization processes are under way.

4. Experimental

4.1. Homology model building

Inhibitor kappa B kinase protein catalytic domain (Fig. 6) sequence was collected from Swiss-Prot protein database (ID: O14920).[12] Sequence similarity search of IKKβ catalytic domain against protein databank sequences was performed using NCBI BLAST server.[15] Four potential protein kinase catalytic domains structures were identified as homologs from PDB[16–18] (Table 1). The automated sequence alignment and analysis of template and target has been carried out by CLUSTAL X 1.81[19] program and GENEDOC 2.6 program[20] used to analyse the alignment (Fig. 3). Discovery studio 2.0 (http://accelrys.com/) has inbuilt MODELER[21] package was used to model a IKKβ protein sequence. Four different kinase (template) sequences aligned with IKKβ sequence and this alignment was supplied along with the 3D coordinates of templates as an input to the program. Modeler implements comparative protein structure modeling by satisfaction of spatial restraints.[21,22] Initially three models generated were subjected to energy minimization process by using the Powell method[11] for about 1000 iterations, with tripos force filed in SYBYL 7.3[23] package running on LINUX server, to get the relaxed conformation. To assess the quality of the minimized models Profunc PROCHECK[24–26] analysis has been ensued.

4.2. Criteria for model selection

Ultimate utility of the homology model is implicating in receptor based virtual screening the large commercial database. Identifying suitable model is very important criteria in structure based virtual screening, which can potentially drop down the false positives rates. Here, we considered two aspects in choosing the model, one is 'energy minima' and another is optimum binding site to accommodate the IKKβ inhibitors. We conducted a docking simulation with **8** highly active ligands reported for IKKβ (Fig. 7) that were collected

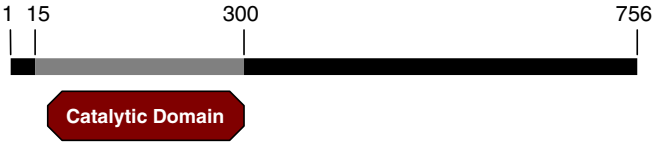


Figure 6. Catalytic domain region in IKKβ sequence.

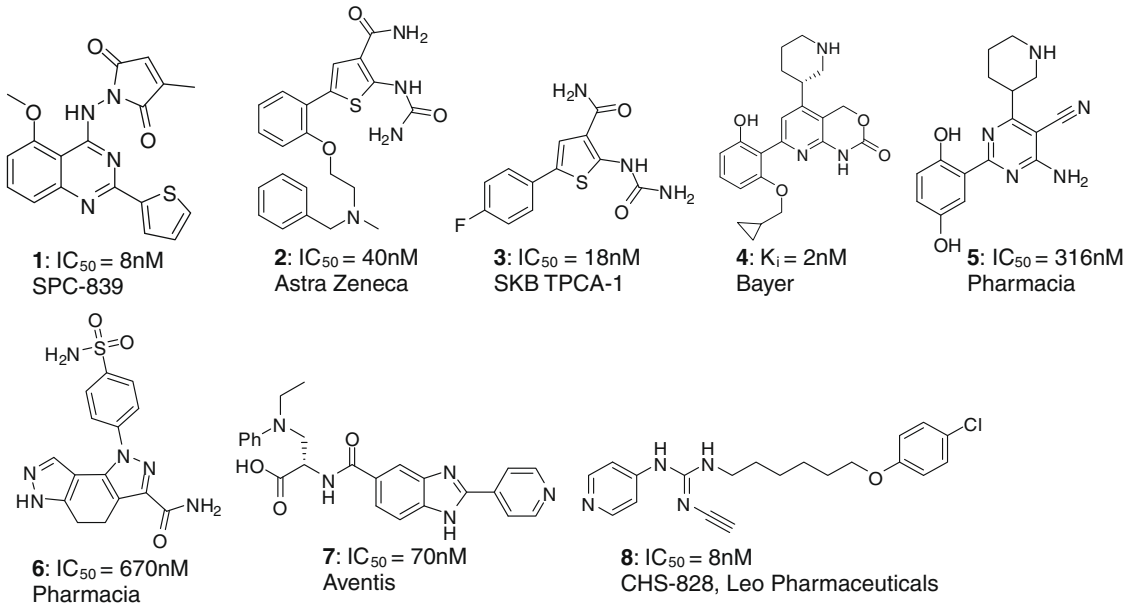


Figure 7. IKKβ selective inhibitors used in the docking study.

from the review article.[27] All the **8** compounds were docked with three models to infer the model, which can interact with more number of active compounds at ATP binding site. Besides the energy criteria and docking analysis, we have subjected the models for Profunc analysis to access the stereochemical quality of the model.

4.3. Functionally important residues

In a protein, every residue is not equally important. Some are essential for the proper function and structure stability, which are known as conserved residues. But, others can be replaced by means of course of evolution. Identification of functionally important residues can provide a clear insight into the structural aspects of IKKβ. Both sequence and structural based approaches were applied in identification of functionally important residues. Multiple sequence alignment (MSA) is the widely used method to asses the sequence conversation across the sequences. The IKKβ catalytic domain sequence aligned with GSK, Aurora and CDK2 catalytic domain kinase sequences, which are well studied and the structural information is known. This alignment explains the sequence level similarities and differences of IKKβ in comparison with other kinases (Fig. S3). Sequence alignment between the IKKα and IKKβ of human, bovine and mouse revealed the residues that are responsible for selectivity among the isoforms (Fig. S4).

Docking simulations can help to identify the residues involved in the interactions with ligand at 3-D space of binding pocket. The 187 known IKKβ inhibitors with structures were collected from Integrity[28] database and the compounds were converted in 3D format using CONCORD program.[29] The ATP binding residues and the surrounding residues within the distance of 6.5 Å were defined as an active site and three pharmacophore constraints defined at hinge region, CYS99 donor as essential, GLU97 and CYS99 acceptors as optional. Apparently, the docking study helped us to understand the docking manner of the various active compounds apparently (Fig. 8).

4.4. Scoring function identification

In order to identify the best scoring function in virtual screening of IKKβ inhibitors, we have conducted exhaustive enrichment study. In this study we used different scoring functions available in FlexX module. Totally 40(7.40%) active compounds that have IC_{50} value ranges from 8 nM to 1500 nM were collected from the literature[2,5,7,9,10,30–37] and randomly chose 500 (92.60%) ChemDiv[38] GPCR focused library compounds that were used as decoys. FlexX-Pharm was used to define pharmacophore constraints, hinge region Cys99 donor mentioned as an optional constraint. The main advantage of this optional constraint is to direct the compounds into ATP binding pocket, without forcing the interaction. Among 540 compounds, one inactive compound has failed to dock, and CScore[39] subset module applied to extract the ligands with different scoring functions ranking. According to each scoring function ranking, 10%, 25%, 50%, 75% and 100% database was sampled; and the numbers of true hits were accounted in each sampling level.

Enrichment factor is represented as

$$EF = \left(N_{active(\%)} \times N_{all}\right) / \left(N_{(\%)} \times N_{active}\right)$$

$N_{active(\%)}$ is the percentage of actives found in x%, N_{all} is the number of compounds used in the test, $N_{(\%)}$ is the x% of the compounds used in the calculation of EF (%), N_{active} is the number of all actives used in the calculation of enrichment factor.[40] Random theoretical data used as a comparison scale to weigh the scoring efficiency of different scoring function in the supplied receptor. This approach was also helpful in investigating the utility of the homology models capability in differentiating active and inactive from a mixture of compounds.

4.5. Virtual screening

Structure based virtual screening of ChemDiv database containing 26,953 compounds, conducted with homology model. The main reason for choosing this database is, attributed to the diversity of new structures with validated quality, compounds were pre filtered with drug-likeness properties, without any duplicate records and can be supplied in a short period of time by suppliers. Residues Leu21, Thr23, Gly24, Glu97, Cys99, Asp103, Lys147, Glu149, Asn150, Glu172 and 6.5 Å surrounding residues are defined as an active site. This completely covers the ATP binding pocket, which can facilitate the virtual screening of the ATP competitive inhibitor. As it has been observed from the previous docked IKKβ known compounds, we can infer the binding mode and the frequently interacting residues. Sequence analysis has

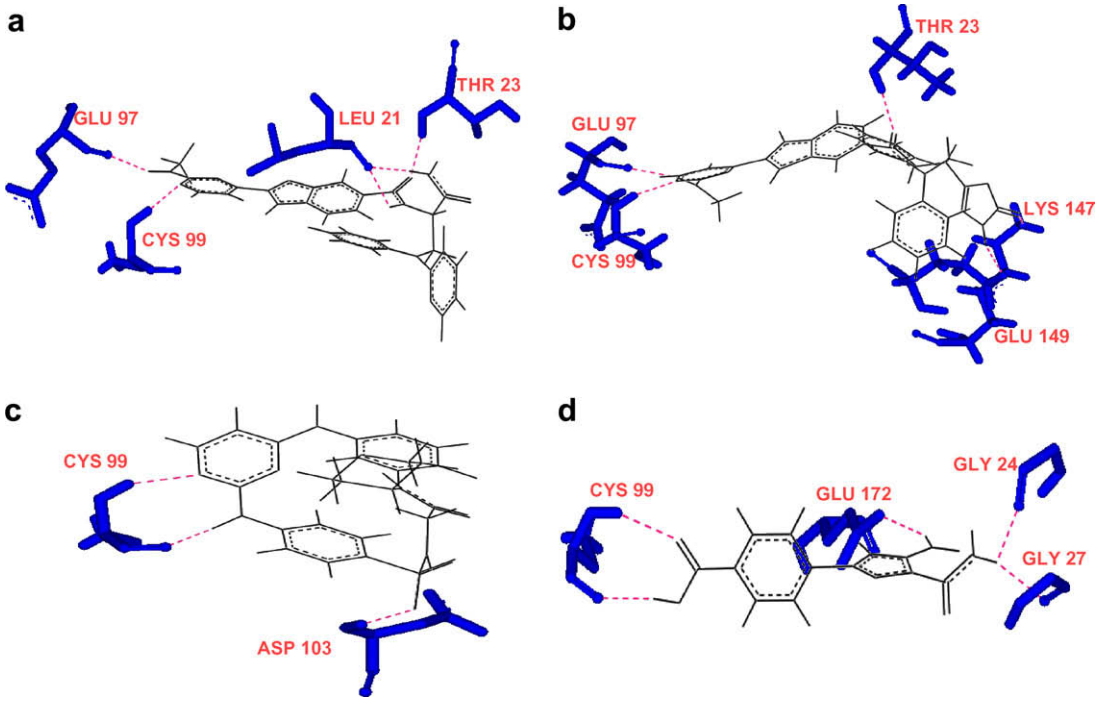


Figure 8. (a–d) depicts interaction manners of various known IKK inhibitors with homology model.

suggested that the Glu172 may be responsible for the selectivity. Because of that reason pharmacophoric constraint has been applied to Glu172, in structure based virtual screening.

Totally, five pharmacophoric constraints were defined using FlexX-Pharm module, hinge region acceptor–donor–acceptor defined as an essential constraints, Asp103 backbone nitrogen and Glu172 sidechain carboxylic oxygen defined as an optional constraints in which, at least one optional constraint is essential. As per the constraints definition, the successfully docked compound should have three hydrogen bonding interaction with hinge region and one hydrogen bonding with either Asp103 or Glu172. According to the docking scheme, the compounds which are capable of forming a minimum 4 hydrogen bond and energetically stable at ATP binding pocket are considered as hits.

4.6. IKKβ enzyme inhibition assay (IKKβ-TR-FRET)

IKKβ-TR-FRET reactions for the search of the IKKβ inhibitors were carried out based upon the suggestions of IMAP-TR-FRET system (MDS Analytical Technologies, Sunnyvale, CA, USA). IKKβ kinase reactions were performed in reaction buffer (10 mM Tris–HCl, pH 7.2, 10 mM $MgCl_2$, 0.05% NaN_3), contained with 1 mM DTT and 0.01% Tween-20 (Sigma–Aldrich Co., St. Louis, MO, USA) to help stabilize the enzyme. The reactions were done at room temperature for 2 h in white standard 384 plates (3572, Corning Life Sciences, Lowell, MA, USA), using 0.5 μg/ml IKKβ (Millipore Co., Billerica, MA, USA), 1 μM I_KBα-derived substrate (5FAM-GRHDSGLDSMK-NH2; R7574, MDS Analytical Technologies), and 3 μM ATP (Sigma–Aldrich Co.) unless otherwise noted. The total reaction volumes were 20 μl and 10 μM compounds were preincubated with IKKβ enzyme for 10 min before substrate and ATP were added. For TR-FRET reaction, 60 μl of detection mixture (1:600 dilution of IMAP binding reagent and 1:400 dilution of Terbium donor supplied by MDS Analytical Technologies) was added 15 h before reading the plate. The energy transfer signal was measured in a multilabel counter with a TR-FRET option (Victor II, PerkinElmer Oy, Turku, Finland). The counter setting was 340 nm excitation, 100 μs delay, and dual-emission collection for 200 μs at 495 and 520 nm. The energy transfer signal data were used to calculate percentage inhibition and IC_{50} values.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.041.

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