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Identification of novel inhibitors against *Mycobacterium tuberculosis* L-alanine dehydrogenase (MTB-AlaDH) through structure-based virtual screening

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ABSTRACT

Mycobacterium tuberculosis (MTB) the etiological agent of tuberculosis (TB) survives in the human host for decades evading the immune system in a latent or persistent state. The Rv2780 (ald) gene that codes for L-alanine dehydrogenase (L-AlaDH) enzyme catalyzes reversible oxidative deamination of L-alanine to pyruvate and is overexpressed under hypoxic and nutrient starvation conditions in MTB. At present, as there is no suitable drug available to treat dormant tuberculosis; it is essential to identify drug candidates that could potentially treat dormant TB. Availability of crystal structure of MTB L-AlaDH bound with co-factor NAD^+ facilitated us to employ structure-based virtual screening approach to obtain new hits from a commercial library of Asinex database using energy-optimized pharmacophore modeling. The resulting pharmacophore consisted of three hydrogen bond donor sites (D) and two hydrogen bond acceptor sites (A). The database compounds with a fitness score more than 1.0 were further subjected to Glide high-throughput virtual screening and docking. Thus, we report the identification of best five hits based on structure-based design and their in vitro enzymatic inhibition studies revealed IC_{50} values in the range of 35–80 μM.

1. Introduction

Tuberculosis (TB) is the leading cause of bacterial infectious disease mortality, and the World Health Organization (WHO) estimates that at least one-third of the world's population could be infected with latent form of *Mycobacterium tuberculosis* (MTB), the etiological agent of TB [1]. Due to the emergence of multidrug-resistant tuberculosis (MDR-TB), high incidence of HIV/TB co-infection and lack of new anti-tubercular drugs, WHO has declared TB a global health emergency. Currently available anti-tubercular drugs were found to be ineffective to treat dormant MTB; thus there is an urgent need for a new anti-tubercular drug to cure dormant TB.

Dormancy has been associated with non-replicating or very slow growth of MTB that resides in granulomas, a heterogeneous assembly of macrophages in the lungs of infected individuals. It is generally assumed that the microenvironment inside the granulomas is characterized by hypoxia, nutrient starvation, and reactive oxygen and nitrogen species [2]. Comparison of gene expression profiles and proteome analyses of active versus non-replicating bacteria have identified a number of up-regulated genes in persistent MTB [3]. One of these genes, Rv2780 was found to be overexpressed under hypoxic [3] and nutrient starvation [4] regimes that encode L-alanine dehydrogenase (L-AlaDH). Increased levels of this enzyme has been linked to the generation of alanine for peptidoglycan biosynthesis [3] and the maintenance of the NAD^+ pool under conditions when the terminal electron acceptor oxygen become limiting [5]. NAD(H)-dependent L-AlaDH catalyze the oxidative deamination of L-alanine to pyruvate and ammonia (catabolic reaction) or, in the reverse direction, the reductive amination of pyruvate to L-alanine (biosynthetic reaction). A recent analysis involving microarray and other data had identified this enzyme to be among the top three drug targets, especially against persistence [6]. Since no inhibitors are reported till date for MTB L-AlaDH, in the present work we took an effort to design some novel inhibitors for MTB L-AlaDH by energy-based pharmacophore (e-pharmacophore) modeling and virtual screening and thus we report herein the first set of novel diverse inhibitors.

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2. Materials and methods

2.1. Computational details

All computations were carried out on an Intel Core 2 Duo E7400 2.80 GHz capacity processor with memory of 2 GB RAM running with the RHEL 5.2 operating system. PHASE 3.3 implemented in the Maestro 9.3 software package (Schrodinger, LLC) was used to generate e-pharmacophore [7]. Glide energy grids were generated for each of the prepared protein complexes. The binding site was defined by a rectangular box surrounding the X-ray ligand. Ligand was refined using the "Refine" option in Glide, and the option to output Glide XP descriptor information was chosen (Glide v5.7, Schrodinger, LLC, New York, NY). For the refinement and docking calculations, the default settings as available in the software package were used.

2.2. Protein target

The crystal structure of the protein L-AlaDH from MTB (PDB code: 2VHW) was retrieved from protein data bank (PDB) and was utilized for molecular modeling [8]. Hydrogen atoms, bond orders and formal charges were added using the protein preparation wizard of the Maestro software package [7]. From the atomic coordinates, water molecules were removed, thus the resulting structure was energy minimized. The interactions of the ligand (co-factor) with the protein residues in the active site were visualized using ligand interaction diagram in Schrodinger suite version 9.3 [9].

2.3. Protein and ligand preparation

The protein file was prepared using protein preparation wizard and impact energy minimization was performed using 500 cycles of steepest descent (SD) and 5000 cycles of conjugate gradient (CG) methods. The optimized potential for liquid simulations (OPLS) 2005 force field was attained using Schrodinger suite version 9.3 [7]. The active site of the protein was defined and grid files were generated using receptor grid generation panel.

The reference ligand NAD^+ structure was downloaded from PDB and minimized using impact energy minimization with 100 cycles of SD and 500 cycles of CG. The three dimensional structure of the compounds were retrieved from Asinex database and were employed for the virtual screening. The database compounds were energy minimized and used as a single file using LigPrep (LigPrep v2.2, Schrodinger, LLC, New York, NY) module of Schrodinger suite.

2.4. Glide XP (extra-precision) docking

The generated grid file from the prepared receptor was used for the Glide XP docking calculations. The minimized conjugate gradient output of the ligand NAD^+ was used. The "Write XP descriptor information" option and "Compute RMSD" option was enabled and the settings were kept default for the rest of the parameters. The XP Glide scoring function was used to order the best ranked compounds and the specific interactions like π–cation and π–π stacking were analyzed using XP visualizer in Glide module. The input RMSD of the ligand was also ascertained.

2.5. e-Pharmacophore generation

The pharmacophore hypothesis was created for the ligand NAD^+ by using the Xpdes file of the Glide XP output in the docking post processing tool of the scripts module. Default settings were used for refinement and scoring. Starting with the refined crystal ligand, pharmacophore sites were automatically generated with Phase (Phase, v3.0, Schrodinger, LLC, New York, NY) [10,11] using the default set of six chemical features: hydrogen bond acceptor (A), hydrogen bond donor (D), hydrophobic (H), negative ionizable (N), positive ionizable (P), and aromatic ring (R). Hydrogen bond acceptor sites were represented as vectors along the hydrogen bond axis in accordance with the hybridization of the acceptor atom. Hydrogen bond donors were represented as projected points, located at the corresponding hydrogen bond acceptor positions in the binding site. Projected points allowed the possibility for structurally dissimilar active compounds to form hydrogen bonds at the same location, regardless of their point of origin and directionality. The ligand was docked with Glide XP and the pose was refined. The Glide XP scoring terms were computed, and the energies were mapped onto atoms. The pharmacophore sites were generated, and the Glide XP energies from the atoms that comprised each pharmacophore sites were summed up. These sites were then ranked based on the individual energies, and the most favourable sites were selected for the pharmacophore hypothesis [12]. This pharmacophore model was then used as query for virtual screening.

2.6. e-Pharmacophore-based database screening and docking studies

Asinex database containing 500,000 unique structure records were used in this study [13]. For the e-pharmacophore approach, explicit matching was required for the most energetically favourable site (scoring better than $-1.0\,kcal\,mol^{-1}$). For filtering the database molecules, a minimum of 4 sites were required to match for hypotheses with 5 or more sites [14]. The above criterion was followed in the present work to screen the Asinex database. In order of their Fitness score, database hits were ranked to measure how well the aligned ligand conformer matched the hypothesis based on RMSD, site matching, vector alignments and volume terms [15]. Database ligands after e-pharmacophore filter were docked into the binding sites of the protein with Glide 5.7 (Glide, version 5.7, Schrodinger, LLC, New York, NY, 2011), utilizing the high-throughput virtual screening (HTVS) scoring function to estimate protein–ligand binding affinities. Ligands filtered from HTVS were subjected to Glide SP (standard precision) docking. The center of the Glide grid was defined by the position of the co-crystallized ligand. Default settings were used for both the grid generation and docking. Post docking minimization was implemented to optimize the ligand geometries. Compounds with best docking and Glide scores were then subjected to Glide XP (extra precision) docking.

2.7. Cloning and purification of L-AlaDH

Gene encoding MTB L-AlaDH was amplified from H37Rv genomic DNA by using the forward primer (5′CCCAAGCTTATGCGCGTCGGTATTCCGACCGAG3′) and the reverse primer (5′CCCAAGCTTTCAGGCCAGCACGCTGGCGGGCTCGGT3′) flanked with Hind-III site. The amplified PCR products were digested with Hind-III and cloned at Hind-III restriction enzyme site of the pET-28a (Novagen) expression vector to generate the pET-28a-ADH construct. Further, this clone was transformed into C41(DE3) cells (Lucigen). Transformants were grown in LB broth at 37 °C with constant aeration in the presence of 50 μg/mL kanamycin. Exponentially growing cultures (A600 of ~0.6) were induced with 0.2 mM IPTG and further grown for 12–16 h at 18 °C. Cells were then harvested and lyzed by sonication in lysis buffer. The cell lysate containing His6-fusion proteins were mixed with equilibrated Ni-NTA affinity resins and the tagged proteins were eluted with buffer containing 500 mM imidazole.

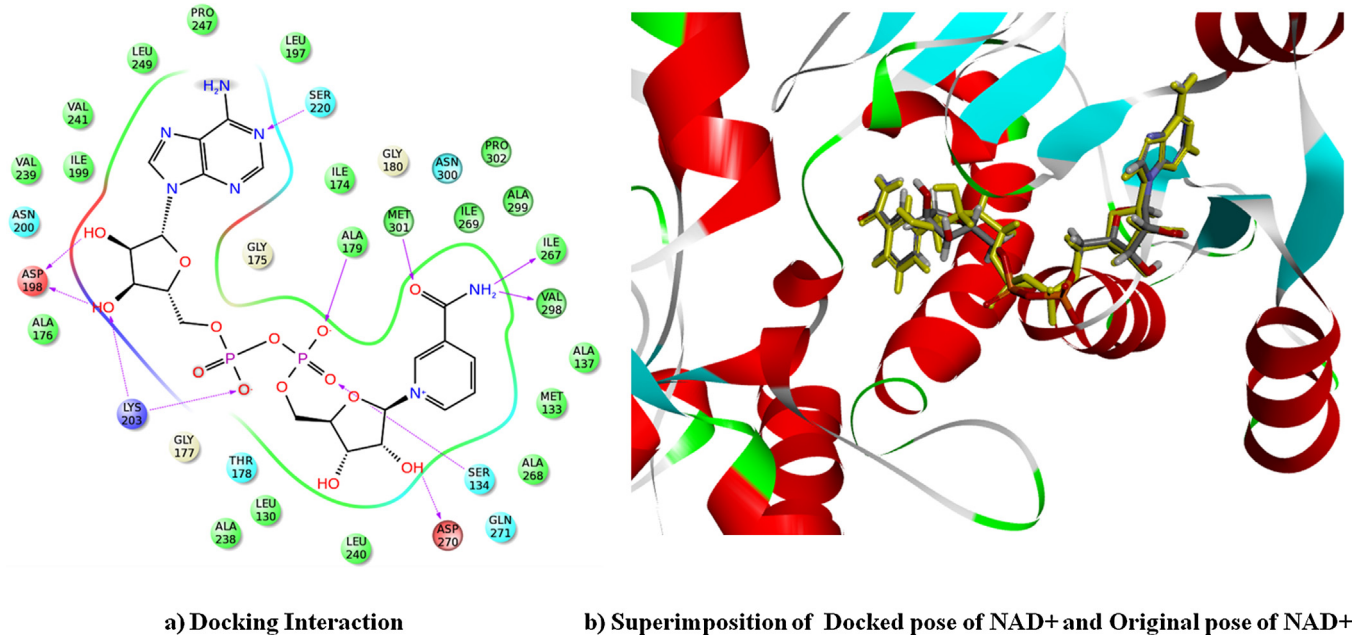

Fig. 1. (a) XP docking showing interactions of NAD^+ with the active site residues of MTB L-AlaDH protein. (b) Superimposition of docked pose of the NAD^+ to the original pose of the NAD^+.

2.8. In vitro MTB L-AlaDH enzyme inhibition assay

To each well of a 96-well plate, the reaction mixture consisted of 125 mM glycine/KOH (pH 10.2), 100 mM L-alanine, 1.25 mM NAD^+ and 6.026 pM of MTB-AlaDH in a final volume of 200 μL diluted in 125 mM glycine/KOH (pH 10.2). Compounds were then added to plates. Reaction was initiated with the addition of 10 μL of ADH diluted in buffer. Enzymatic activity was measured by the rate of production of NADH that accompanied the conversion of alanine to pyruvate by oxidative deamination [16]. The reaction components, except MTB-AlaDH, were mixed in the well and the background reaction was measured; MTB-AlaDH was then added and the reaction kinetics was monitored. Similar protocol was followed for testing the designed inhibitors in various concentrations. All measurements were performed at 340 nm with heat-controlled Perkin Elmer Victor V3 spectrophotometer.

3. Results and discussion

Information on the common properties of the binding groups is essential for resolving the type of inhibitor binding to the target protein. Major aim of drug design is the identification and development of new ligands with high affinity of binding towards protein receptor. A very useful model for achieving this goal was reported to be pharmacophore modeling [17]. In the present study, the co-factor (NAD^+) bound to the protein was re-docked with the MTB L-AlaDH and the output file was used to identify a pharmacophore hypothesis to be further utilized for screening database compounds of Asinex. Thus identified compounds were evaluated for in vitro enzymatic inhibition studies.

3.1. e-Pharmacophore of NAD^+

The crystal structure of the protein L-AlaDH from MTB (PDB code: 2VHW) was used for structure-based pharmacophore modeling. Till date as no inhibitors were reported for L-AlaDH, in this study we attempted to utilize the co-factor NAD^+ bound with the protein along with pyruvate as substrate for the generation of an e-pharmacophore hypothesis. The asymmetric unit of AlaDH consisted of six chains [8] and the subunit of L-AlaDH was built up of two distinct domains, the substrate-binding domain and the NAD-binding domain, connected by two α-helices. The reference ligand NAD^+ was re-docked with the active site residues of the MTB L-AlaDH protein to validate the active site cavity. The ligand exhibited a highest Glide score of $-11.42\ kcal\ mol^{-1}$ and was found in the vicinity of important amino acids like Asp270, Val298, Thr178, Ser220, Lys203, Asp198, Met301, Ile267, Ser134, Leu240 and Ala179. Re-docking results (Fig. 1) showed that the compound exhibited similar interactions as that of the original crystal structure and showed a RMSD of 0.897 Å (Fig. 1b). The result of this docking was used to ascertain the pharmacophoric features of MTB L-AlaDH. The binding mode of the NAD^+ ligand to the MTB L-AlaDH protein was defined by the interaction pattern of its pharmacophore.

Pharmacophore hypotheses were developed by mapping Glide XP (Glide, version 5.7, Schrodinger, LLC, New York, NY, 2011) energetic terms onto pharmacophore sites which was calculated based on the structural and energetic information between the protein and the ligand using Phase module (Phase, v3.3, Schrodinger, LLC, New York, NY). The initial number of pharmacophore sites was set up to 10 for the crystal structure. The docking result (Xpdes) was then imported to find the structure-based pharmacophoric features, which would help in finding the best featured functional groups. The results of the e-pharmacophore generation of the reference ligand used to identify the best pharmacophoric features are given in Table 1. The e-pharmacophore features consisted of

Table 1
Scores of hypothesis in e-pharmacophore.

Rank	Feature label	Score	X	Y	Z	Type
1	D16	−0.8	−52.328	61.8632	14.889	D
2	D17	−0.8	−52.172	64.0717	15.797	D
3	D19	−0.75	−52.468	60.6048	1.3518	D
4	A2	−0.7	−46.361	66.2155	14.8008	A
5	A12	−0.7	−48.258	56.4722	−1.1937	A
6	D22	−0.7	−46.29	57.6156	−0.2416	D
7	D23	−0.7	−47.154	58.2248	1.1599	D

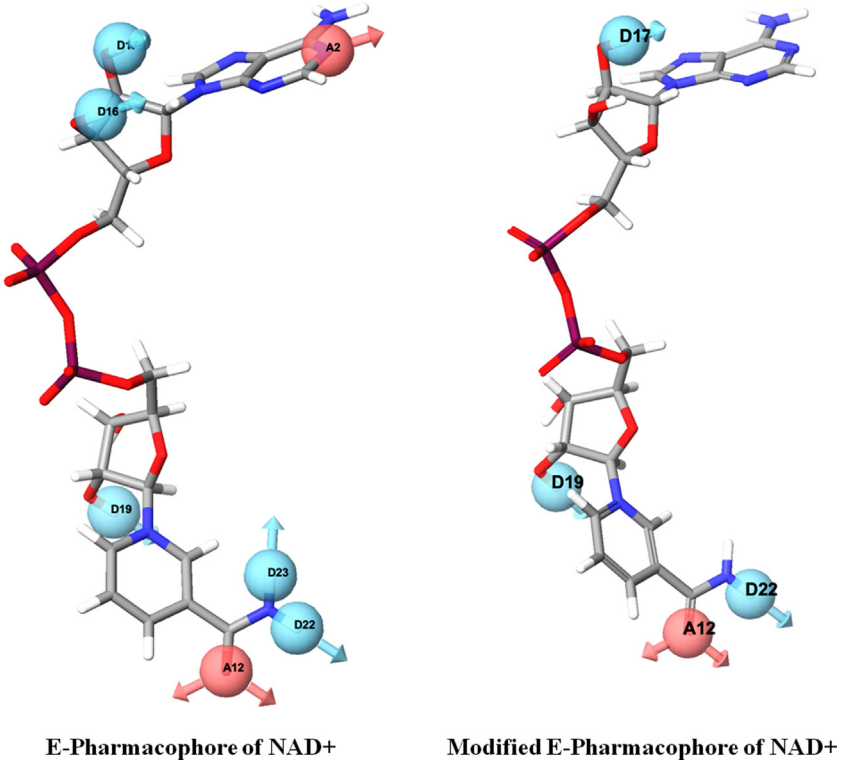

Fig. 2. (a) The energy based pharmacophoric features of NAD^+. (b) Modified energy based pharmacophoric features of NAD^+.

five hydrogen donor moieties and two hydrogen acceptor groups (Fig. 2a). The energy contribution for binding of ligand to the protein was the key to derive pharmacophoric features in structure-based design [18]. Among the seven pharmacophore sites, i.e. four hydrogen bond donors (D16, D17, D19, D22, D23), and two hydrogen bond acceptors (A2 and A12) generated by the structure-based pharmacophore for the MTB L-AlaDH inhibition, we selected five sites as D17, D19, D22 A2 and A12 using e-pharmacophore method script on the basis of their energy scores and pharmacophoric feature distances. The D16 and D17 showed a 2.39 Å distance while between D22 and D23 was only 1.75 Å distance, and hence found to be very close to each features and with same energy score. Hence in the final hypothesis we considered one of the representatives from each site (Fig. 2b). Hence other than ranking for the selection of the vital functional groups, the Glide XP docking results were also considered in support of the e-pharmacophore generated.

Also the important sites obtained in e-pharmacophore model such as D16, D17, D19, D22, D23 were found to correspond to the important amino acid residues such as Lys203, Asp198, Asp270, Ile267, and Val298 respectively, while A2 and A12 corresponded to Ser220 and Met301 residues respectively. The five point e-pharmacophore hypothesis was then utilized for the virtual screening of commercial database (Asinex) following a protocol as summarized in Fig. 3. The option implying "find matches" to the hypothesis in Maestro was employed for this step. Thousand hits with pharmacophoric features similar to the known ligand were then obtained.

3.2. Virtual screening of pharmacophore screened database compounds

The basic goal of any virtual screening protocol would be to reduce enormous virtual chemical space of small organic molecules to a manageable number of compounds that could inhibit the protein with a higher chance so as to identify a candidate drug [19]. The virtual screening workflow option was performed with the e-pharmacophore filtered ligands. The virtual screening options for HTVS (High Throughput Virtual Screening), SP (Standard Precision) and Glide XP (extra precision) docking were all checked to be executed. A fit value was a measure of how well the ligand fits the e-pharmacophore model. In this study, the hits retrieved by the e-pharmacophore model with a fit value above 1.0 were carried forward for HTVS. Top hits from HTVS resulting in a score of ≥ -6.0 kcal mol^{-1} were subjected to another round of docking by Glide SP. The survivors of these preliminary screening steps were taken up to Glide XP docking. The results of the virtually screened ligands using Glide XP docking helped to find precisely the hydrogen-bond interactions, electrostatic interaction, hydrophobic enclosure, and π–π stacking interactions.

3.3. Molecular docking studies

In order to understand how these ligands bind to the enzyme, hits molecules obtained from Glide SP were further evaluated with Glide XP and GOLD 4.1.2 (genetic algorithm based ligand docking) [20] programmes to confirm their potency. A total of 100 compounds selected from the SP were subjected to Glide XP and GOLD docking. The results showed scores ranging from −7.471 to −10.523 kcal mol^{-1}. In addition to the docking scores, final short listing of possible lead compounds were based on visual inspection of the important amino acid residues involved in binding that included hydrogen bonding with Lys203, Asp198, Ser220, Ser134, Asp270, Leu240 and Ala238. Thus we selected top five compounds from the Glide XP docking study with the best Glide scores (−7.47199 to −10.523) and GOLD scores (50.5–72.57), suggesting strong protein–ligand interactions. The chemical structures of these lead compounds (**Lead 1** to **Lead 5**) are illustrated in Fig. 4.

All these top five hits showed good docking score and interaction with important amino acids such as Lys203, Asp198, Ser220, Ser134, Asp270, Leu240 and Ala238. Fig. 5 presents the binding

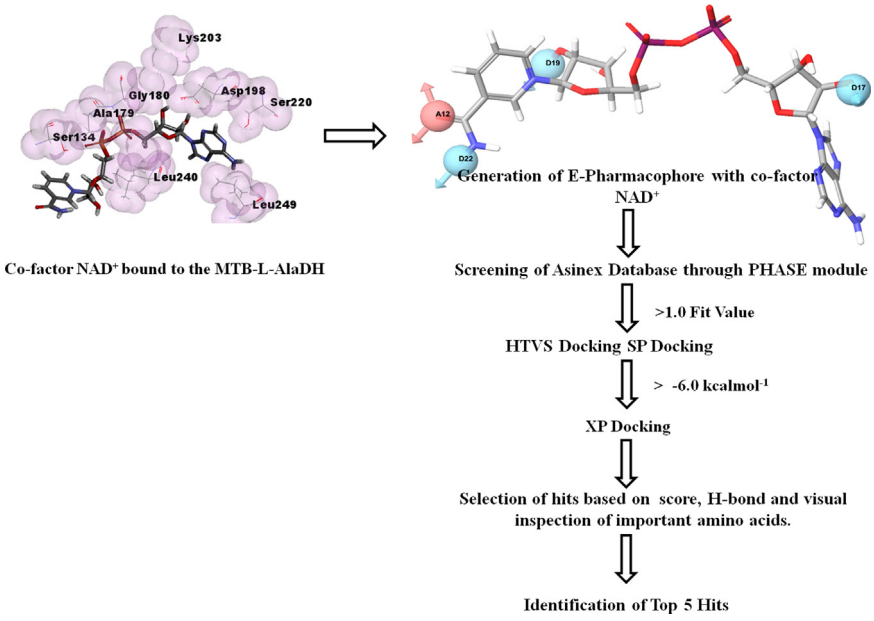

Fig. 3. Virtual screening workflow.

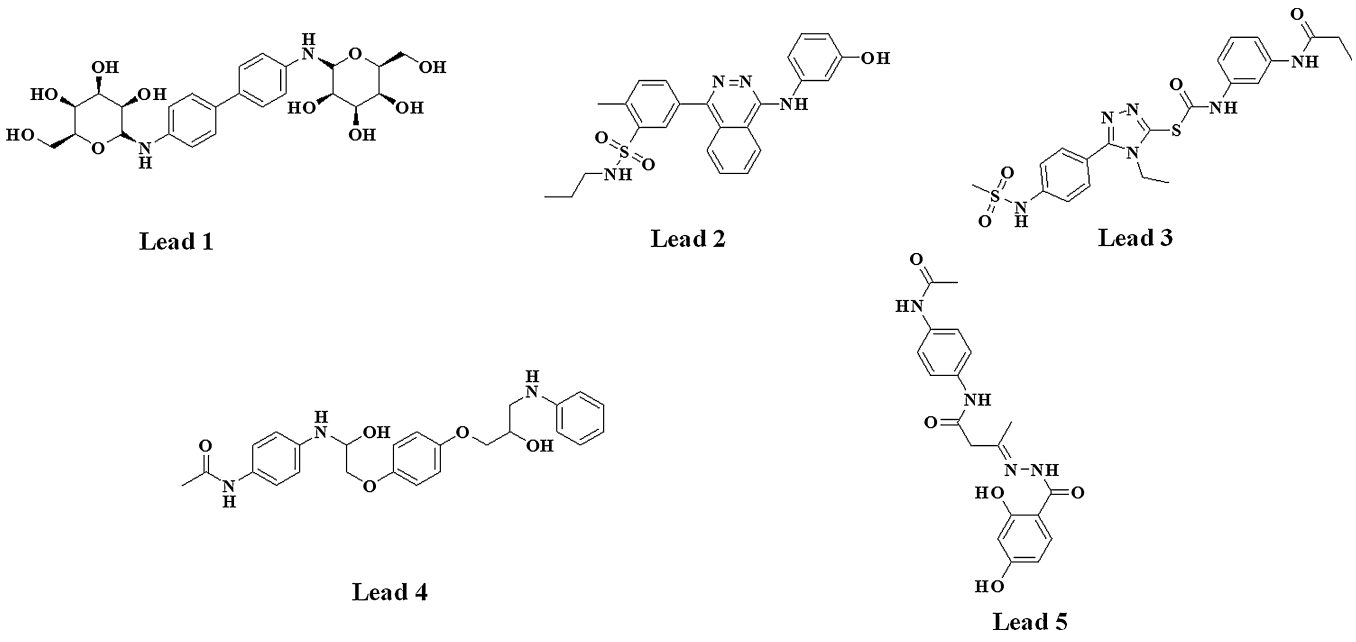

Fig. 4. 2D structures of top five hits after virtual screening.

modes of all five lead molecules and their interacting pattern in the active site of the protein using ligand interaction diagram in Schrodinger suite version 9.3. These compounds were well fit in the active site cavity of the protein. The binding patterns of top five hits in 3D are represented in Fig. 6. The docking score, H-bond and important interactions of these hits can be seen in Table 2. Further to prove the design concept, the final hits were procured from Asinex database, and subjected to biological assay.

Table 2
The docking score, fitness and hydrogen bond interaction of best fit ligands.

Ligand	Fitness	Docking score	Glide score	H-bond	GOLD score	Interaction
Lead 1	1.63319	−9.880	−9.880	5	54.44	Asp198, Ser220, 2Lys203, Asn200
Lead 2	1.3423	−7.01956	−7.92956	7	50.5	Leu137, Ser220, Asp198, Leu240, Gly180, Ala238
Lead 3	1.17877	−7.50913	−7.50913	5	58.43	Asp270, Ala178, Asp198, Leu240, Ser220
Lead 4	1.34514	−10.523	−10.523	9	58.9	Asp198, Ser220, Lys203, 2Ser134, Arg15, Ala299, Gly177, Leu240
Lead 5	1.2349	−7.47199	−7.47199	7	72.57	Asp198, Thr178, Ala238, Ala299, Lys75, Arg15

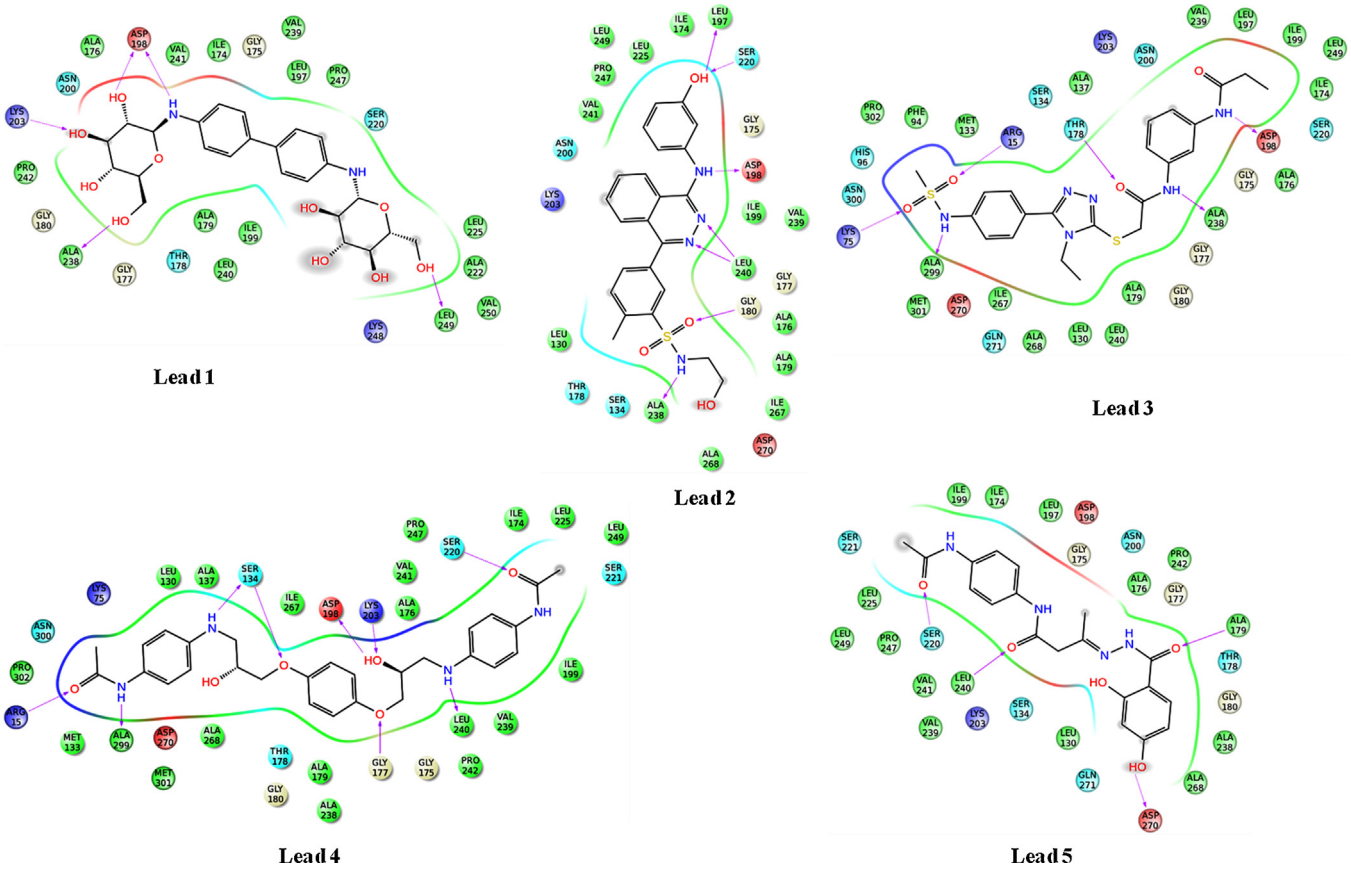

Fig. 5. Protein–ligand contacts of MTB L-AlaDH with the top five hit obtained using virtual screening. Ligand-interactions diagram representing protein–ligand of the top five hits in two-dimension with the protein.

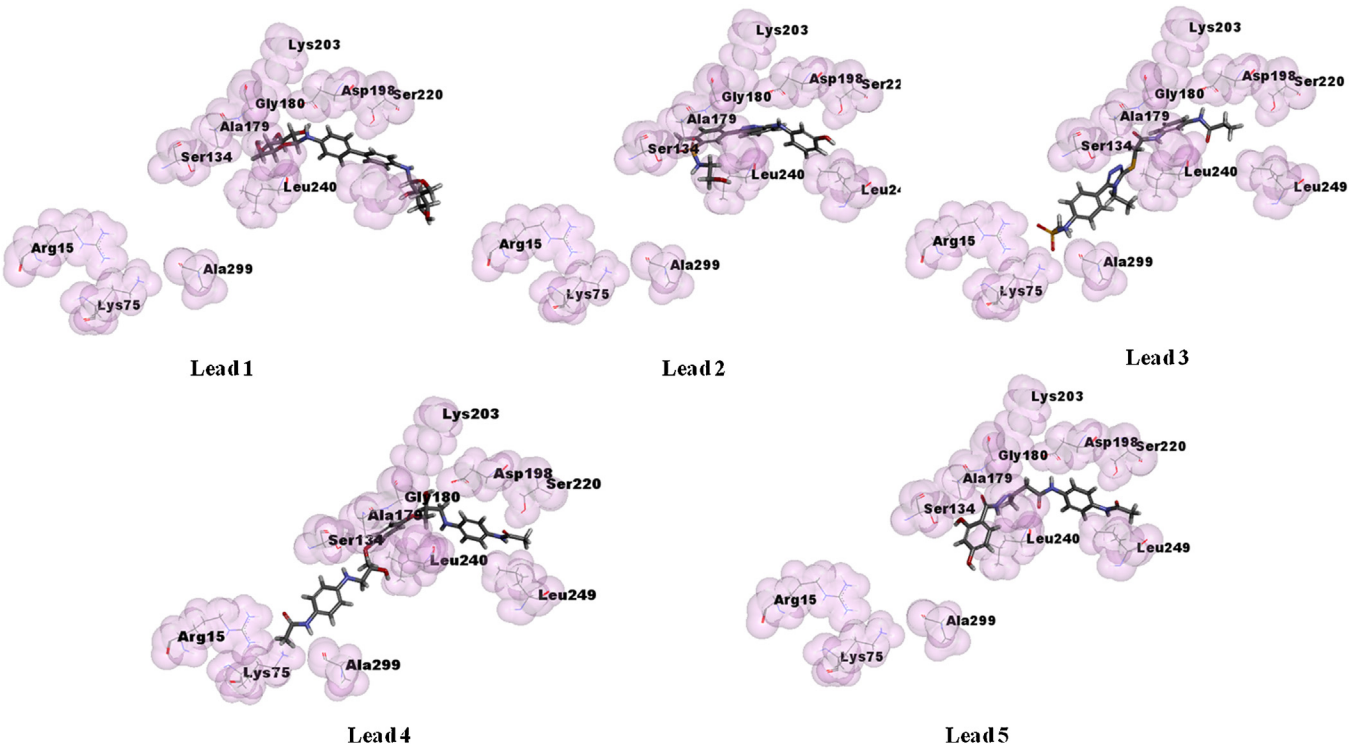

Fig. 6. Binding pattern of top five hits in the MTB L-AlaDH active site.

Table 3
Activity table showing the IC_{50} value of all the five top hits obtained through virtual screening.

Leads	IC_{50} (μM)
Lead 1	35.54 ± 0.0033
Lead 2	80.37 ± 0.010
Lead 3	51.529 ± 0.0048
Lead 4	36.84 ± 0.030
Lead 5	73.84 ± 0.0232

3.4. *In vitro enzyme inhibition of MTB-L-AlaDH protein*

The top hits were screened initially at 100 μM concentration and later at 50 and 25 μM against MTB-L-AlaDH using an assay based on the spectrophotometric determination in a 96-well plate using Perkin Elmer Victor V3 spectrophotometer. Photometric determination of L-AlaDH activity was accomplished by measuring the rate of production of NADH that accompanied the conversion of alanine into pyruvate in the oxidative deamination. The five hit candidates exhibited IC_{50} values in the range of 35–80 μM, and the IC_{50} of two most active compounds **Lead 1** and **Lead 4** were found to be 35.54 and 36.84 μM, respectively. These compounds were the ones that showed high docking score and number of H-bond interaction (Table 2) which thus correlated with our in vitro enzymatic inhibition studies. The IC_{50} values are presented in Table 3. These compounds could be further developed into drug candidates by medicinal chemistry approach.

4. Conclusion

The main objective of the present study was to identify the first set of inhibitors for MTB-L-AlaDH enzyme by using structure-based modeling. Structure-based virtual screening and pharmacophore modeling have emerged as corresponding methods to high-throughput screening of large chemical databases. The availability of the crystal structure bound with co-factor of MTB-L-AlaDH was explored using pharmacophore models based on interaction energy and docking to yield diverse leads. Structure-based pharmacophore and virtual screening of Asinex database retrieved five top hit compounds with good docking score and interaction pattern. In vitro enzymatic inhibition studies of these five ligands yielded two compounds (**Lead 1** and **Lead 4**) with good IC_{50} of 35.54 and 36.84 μM, respectively. Thus, the overall analysis of results suggest that the structure-based pharmacophore modeling provided useful information required for proper understanding of the important structural and binding features for designing novel MTB-L-AlaDH inhibitors.

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