IDENTIFICATION OF ETHR INHIBITOR TARGETING *MYCOBACTERIUM TUBERCULOSIS*: AN INSIGHT FROM MOLECULAR DOCKING STUDY

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ABSTRACT

Objective: *Mycobacterium tuberculosis* (MTB) is a pathogenic bacterium of the Mycobacteriaceae family that causes TB. EthR is a transcriptional regulator which is involved in the repression of the monooxygenase EthA which is responsible for the formation of the active metabolite of Eth. Inhibitors of the EthR DNA binding protein induce a conformational change in this repressor, thus preventing its binding to DNA operator, consequently resulting in increased transcription of EthA and bioactivation of Eth.

Methods: In this study, we used first-line and second-line drugs and their analogues to validate the binding affinity of EthR DNA binding protein of MTB. Molegro Virtual Docker (MVD) is utilized for virtual screening and validation of MolDoc, Rerank, and hydrogen bond parameters of ETH, isoniazid (INH), clofazimine (CLF), and its modified derivatives to the EthR DNA binding protein of MTB. The modified molecules; ETH4, INZ2, CLF3, and CLF4 show more binding affinities than that of native compounds ETH, INH, and CLF to the EthR DNA binding protein of MTB. The top scoring compound was docked by auto dock vina in PyRx to get the best conformer pose for intermolecular interactions.

Results: CLF4 had the best lowest MolDock score -176.29kcal/mol and H-bonding energy -6.89kcal/mol in the MVD virtual screening. The best conformer pose generated by PyRx was shown -7.1 binding affinity and ligand generated hydrogen bond interactions with THR130 and LYS68, respectively, which stabilized the ligand in the active site of EthR protein.

Conclusion: We concluded that CLF4 has shown better inhibitory efficacy than other compounds towards EthR protein. However, these results need to be further substantiated through *in vitro* and *in vivo* experimental studies.

Keywords: *Mycobacterium tuberculosis*, Molecular docking, EthR, Ethambutol, Isoniazid, Clofazimine, Ethionamide.

INTRODUCTION

Tuberculosis (TB) is one of the top ten causes of global mortality, with an estimated 1.5 million deaths due to TB in 2018, globally [1]. *Mycobacterium TB* (MTB) infection in humans does not usually lead to active disease. The vast majority of infections are clinically latent, with just 10% developing active TB. In humans, latent TB is defined as the absence of clinical illness in a TB-positive individual. A latently infected person is likely to harbor the organism for life and is at risk of reactivation TB. Animal models have been used to study the immune responses that lead to the management of acute infections and the likely formation of latency [2].

The most common cause of mortality from TB is caused by a single infectious pathogen, MTB. The only vaccine currently available for TB is Bacillus Calmette-Guérin, provides little protection from TB beyond infancy as currently utilized, and new vaccination strategies are greatly needed. Anti-tubercular chemotherapy is effective in treating the infection with drug-susceptible strains of MTB, but innovative approaches in treating TB disease are required to meet the growing threat posed by drug-resistant MTB [3]. The initially prescribed regimen for the treatment of TB usually consists of four drugs: Isoniazid (INH), rifampin, pyrazinamide, and ethambutol (ETH) [4]. Of the >10 million new cases reported, 4.6% are resistant to both the key first-line drugs rifampicin and INH and are classified as multidrug-resistant (MDR). About 8.5% of MDR-TB cases were extensively drug-resistant (XDR), where resistance to two of the key second-line drugs is also present. Only 55% of the MDR-TB and 30% of the XDR-TB cases are treated successfully [5].

Current TB treatments involve several prodrugs that must be metabolically modified inside the mycobacterial cell to gain an active form. One of these is Eth, an antimicrobial employed as the second-line medication for MDR TB therapy [6]. It is a transcriptional repressor that increases MTB resistance to Eth [7]. It is bioactivated inside the mycobacteria by the flavin-containing monooxygenase EthA into an Eth-NAD adduct [6]. This active Eth-NAD complex inhibits the synthesis of mycolic acids by targeting InhA [7,8]. EthR belongs to the tetR/CamR family of a transcriptional repressor that negatively regulates the expression of the EthA enzyme. It plays a key role in the control of the Eth activity as it represses the expression of EthA, then reduces the effectiveness of Eth. Conversely, abolishing EthR DNA-binding function with small molecules has been shown to improve Eth potency [9].

Inhibiting EthR with small molecules/drug analogues increases the sensitivity of the MTB Eth both *in vitro* and *in vivo*, thus EthR can be a potential new drug target to fight TB [10]. The current study has been undertaken to evaluate the binding affinity of existing drugs ETH [11], INH [12], clofazimine (CLF) [13], and its different functional derivatives against EthR of MTB using molecular docking [14].

METHODS

The present study involved retrieval of the 3d structure of the target protein and ligands from Protein Data Bank [15] and PubChem database [16], respectively. The proposed ligands and their modified structures were designed in Hyperchem8 [17]. Virtual screening experiments were performed by Molegro Virtual Docker (MVD2019.7.0.0) [18] and analyzed its data by Molegro Data

modeler [19]. The top-scoring ligand was docked using AutoDock vina in PyRx [20].

Target protein

The three-dimensional structure of EthR ligand complex DNA binding protein of MTB (PDB ID: 6R1P) was retrieved from Protein Data Bank. Structural details of protein were retrieved from the PDBsum server which includes topology of the secondary structure, promotif documentation (total residues 193, 10 helices, 20 helix-helix interactions, 2beta turns, and 1 gamma turn). Before docking the water molecules, unwanted hetero atoms and other ligand compounds were removed by MVD.

Drug candidate's structural information

The structures of ETH, INH, and CLF and its derivatives were drawn in Hyperchem 8 package; they were pre-optimized using the molecular mechanics force field (MM+, AMBER) procedure. To obtain the conformers with the lowest energy, the semi-empirical method AM-1 was applied to the molecular structures. To avoid the local stability, each molecular structure was optimized several times with different starting points using the Polak-Rebiere algorithm, until the root-mean-square gradient is equal to 0.01 kcal $Å^{-1}$ mol^{-1} [21]. The four modified structures of ETH; ETH1, ETH2, ETH3, and ETH4 were modified at carbon-11 by adding alkyl halide, alkane, primary amine, and polar carbonyl groups, respectively. The four modified structures of INH in which INZ1 and INZ4 were modified at carbon-7 by adding alkyl halide and polar carbonyl groups, respectively; INZ2 and INZ3 were modified at carbon-5 by adding alkane and primary amine groups, respectively. The four modified structures of CLF; CLF1, CLF2, CLF3, and CLF4 were modified at carbon-3 by adding alkyl halide, alkane, primary amine, and polar carbonyl groups, respectively. Then the energy minimizations of all ligand structures were done using Hyperchem 8. The first step was by calculating a single point that was used to determine the total molecular energy of the structure, the second step to employ the energy minimization algorithms that locate the stable structure was using geometric optimization calculation (MM+, and AMBER force field). Then these 12 modified structures along with native ETH, INH, and CLF were utilized for molecular docking calculations.

Active site of protein

The drug-binding cavities in EthR protein of MTB for ETH, INH, and CLF are not well characterized. The amino acid residues responsible for cavity formation in EthR protein were detected through the MVD cavity detection algorithm. The program generally identified five different cavities. MVD identified three separate cavities in EthR protein with a volume of 80.38, 36.86, and 13.82, respectively. The high volume of the cavity was used for molecular docking.

Preparation of target protein and ligand

Protein

The EthR ligand complex DNA binding protein of MTB (PDB ID: 6R1P) was selected for molecular docking studies using MVD. Using the utilities provided in MVD all necessary valency checks, H atom addition, and protein preparation (protonation) were done and repaired and rebuilt. For precise docking, it is important that the imported structures must be prepared accurately, that is the atom connectivity and bond orders are correct, and partial atomic charges are assigned. PDB files often have a poor or missing assignment of explicit hydrogen's, and the PDB file format cannot accommodate bond order information. Then the repair and rebuilt protein were saved in *.Mol format. The final structure was visualized and analyzed with SPDBV 4.1 [22].

Ligand

The molecular docking technique was adopted to know the binding activity of the ligand to the protein and obtain the best binding scores. The selected ligands and their modified structures were built using Hyperchem 8 software and imported to MVD workspace in *.mol

format. Before import, the ligands underwent a series of steps that generate variation and optimization of the structure.

Molecular docking procedure

The validated protein is utilized to test the ability of ETH, INH, and CLF drugs and their modified structures to bind the MTB EthR protein. (MVD2019.7.0.0) program was used for the validation of molecular docking. The docking scoring function MolDock score energy, E_{score} is defined by (1), where E_{inter} is the ligand-receptor interaction energy and E_{intra} is the internal energy of the ligand. According to (2) equation, E_{inter} is calculated.

$$E_{score} = E_{inter} + E_{intra} \tag{1}$$

$$E_{inter} = \sum_{i=ligand} \sum_{j=protein} \left[E_{PLP(rij)} + 332.0 \frac{q_i q_j}{4r_{ij}^2} \right] \tag{2}$$

According to (3) equation E_{intra} is calculates the energy involves in the pair of atoms of the ligand and torsional energy where h is the torsional angle of the bond. E_{clash} assigns a penalty of 1000 kcal/mol if the distance between two heavy atoms similar than 2.0Å.

$$E_{intra} = \sum_{i=legand} \sum_{j=protein} [E_{PLP}(r_{ij})] \\ + \sum_{flexibleband} A[1 - \cos(m\theta - \theta_\circ)] + E_{dash} \tag{3}$$

The MolDock scoring function was also set with a grid resolution of 0.30 A. It was set at a maximum iteration of 1500 with a simplex evolution size of 50 and a minimum of 10 runs were performed for each compound with threshold energy of 100. In addition, the simplex evolution was set for 300 steps with a neighbor distance factor of 1.00. The best pose of each compound was chosen for subsequent ligand-protein interaction energy analysis [23,24]. Hydrogen bond interaction and its binding energy were observed between the amino acid residues in the target site with the functional group of the modified molecules. The top-scoring ligands were screened and validated its conformer poses by AutoDock vina in PyRx. The best pose was taken into consideration for further analysis.

ADMET and drug likeness properties

Along with native compounds, the modified molecules were further used to estimate pharmacokinetic properties, drug-likeness, and toxicity using the pkCSM [25], SwissADME [26], and molinspiration tools [27].

RESULTS

Target protein conformation

The EthR ligand complex DNA binding protein of MTB (PDB ID: 6R1P) with resolution 1.80Å was retrieved from PDB database. The 3d, secondary structural analysis, and Ramachandran plot are shown in Fig. 1. The cavities (active sites) of the protein EthR were obtained through MVD. The protein was loaded into PyRx (vina) 0.8. Identical chains have been removed and polar hydrogens were added, further the same was converted to pdbqt format, which includes Kollman charges.

Determination of ligand structures

The structural information of native ligands; ETH, INH, and CLF are shown in Supplementary Table 1. The 12 modified structures of Eth, INH, and CLF (addition of functional groups such as alkyl halide, alkane, primary amine, and carbonyl) were used to find out their potential binding with the target protein EthR and its structural flexibility after single point energy and after geometrical optimization energy is shown in Supplementary Fig. 2. In the present study, the top-scoring ligand CLF4 is considered the best ligand. The 3D structure of the CLF4 was drawn and geometrically optimized its energy by Hyperchem8.0. Molecular docking was carried out by AutoDockVina. The CLF4 ligand

least confirmation E=1632.86 was selected and converted to pdbqt format for further analysis.

Docking results and validation
The EthR protein active sites and their volume detected by MVD detection algorithm Fig. 2. The interactions (MOlDock score, re-rank score, the total interaction energy between the pose and the target molecule, and hydrogen bond energy) of ETH, INH, CLF, and its analogs with EthR protein are shown in Supplementary Table 2.

Molecular docking techniques aim to predict the best matching binding mode of a ligand to a macromolecular partner (here just proteins are considered). It consists of the generation of several possible conformations/orientations, that is, poses, of the ligand within the protein binding site [28]. In the studies reported here, MVD and PyRx were used for virtual screening and molecular docking, respectively.

The guided differential evolution technique was used to execute ten separate docking runs for each ligand, with each docking run-producing one solution (pose). The Moldock scoring function employed by MVD is

taken from Gehlhaar et Alscoring's functions, which were then expanded by Yang *et al.* Using a more sophisticated scoring mechanism, the ten solutions acquired from the ten separate docking runs were re-ranked to improve docking accuracy even more [29]. The grid resolution of 0.30 Å was likewise chosen for the MolDock scoring algorithm. It was set to a maximum of 1500 iterations with a simplex evolution size of 50 and a minimum of 10 runs for each compound with threshold energy of 100. Furthermore, the simplex evolution was adjusted to 300 steps with a neighbor distance value of 1.00. Each compound's optimum posture was chosen for future ligand-protein interaction energy analysis.

Among all docked compounds, to scoring ligand the CLF modified ligand CLF4 showed better binding inhibitory effect with the target protein. According to the average MolDock score and better H-bonding energy, we have selected the best ligand CLF4 (Fig. 3) for further analysis. In the virtual screening by MVD, CLF4 revealed the best lowest MolDock score -176.29kcal/mol and H-bonding energy -6.89kcal/mol.

Furthermore, CLF4 was docked with EthR protein by PyRx to get the best conformer pose and observe the intermolecular interactions between the protein-ligand complexes. Fig. 4 shown the two-dimensional representation of the interaction formed by EthR protein with CLF4 compound. The best top five conformers pose and its binding affinity is shown in Table 1.

The ligand was formed hydrogen bond interactions with residues THR130 and LYS68, respectively, stabilizing the ligand at the active site. The other various intermolecular interactions are illustrated in Table 2.
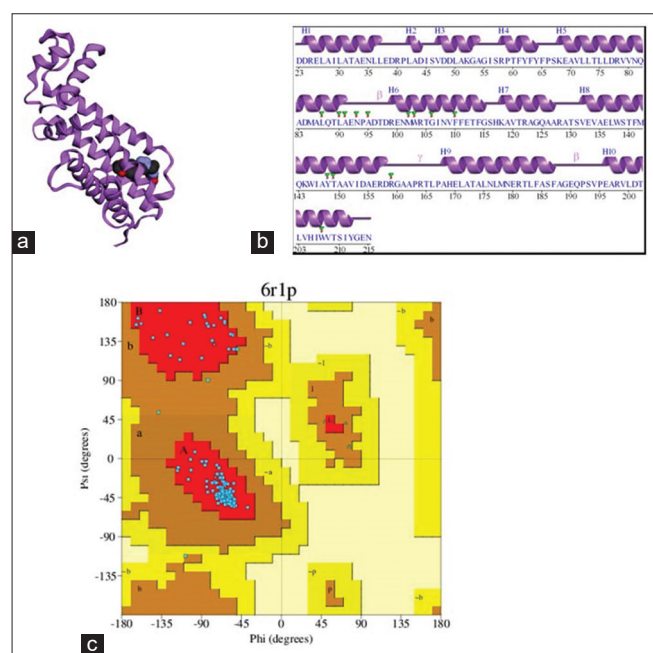
Table 1: The best 5 conformer poses of EthR protein with CLF4 compound

Conformers	Binding affinity	Rmsd/ub	Rmsd/lb
1	-7.1	0	0
2	-6.9	15.05	11.71
3	-6.8	36.73	33.55
4	-6.7	14.03	11.19
5	-6.6	29.18	25.24

Table 2: Various intermolecular interactions noticed between the protein and the target

Name of the bond	Interacting residues
Conventional hydrogen bond	THR130, LYS68
Pi-anion	GLU69
Pi-sigma	LUE42

Fig. 1: Structural information of EthR (a) 3d structure of EthR, (b) secondary structure of 3pty mapped obtained using PDBsum, (c) Ramachandran plot
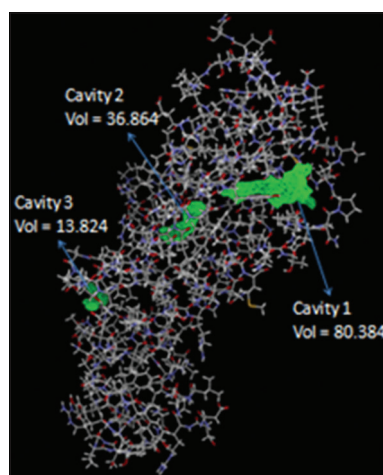

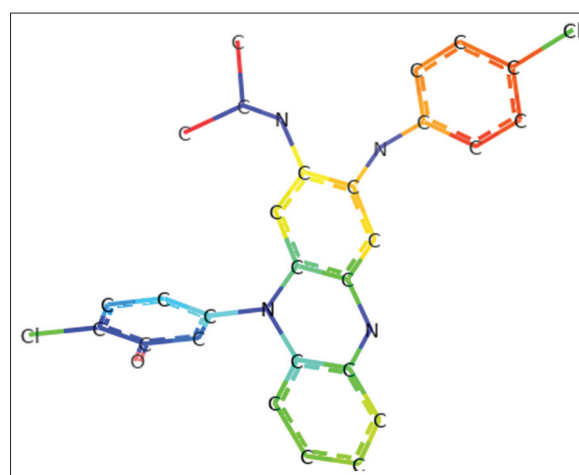**Fig. 2: Active sites of protein EthR**

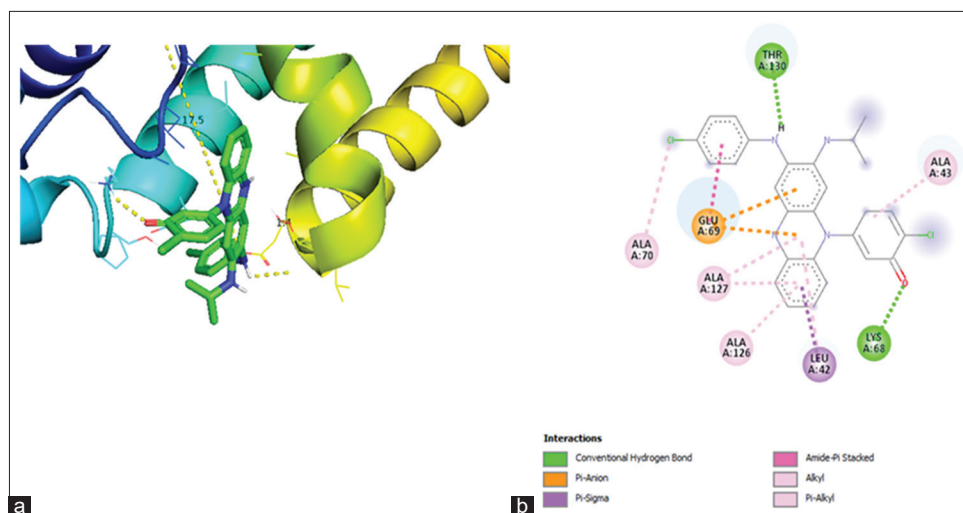**Fig. 3: Structure of Clofazimine 4 ligand**

Fig. 4: (a) The dock pose of the protein-ligand complex (b) two-dimensional representation of the interaction formed by EthR protein with clofazimine 4 ligand

Table 3: Drug-likeness properties of CLF4 ligand

Property	Model Name	CLF4	Unit
Absorption	Water solubility	-4.42	log mol/L
	Caco2 permeability	0.45	log Papp in 10–6 cm/s
	Intestinal absorption (Human)	93.52	%Absorbed
	Skin Permeability	-2.73	log Kp
Distribution	VDss (Human)	-0.44	log l/kg
	Fraction unbound (Human)	0.031	Fu
	BBB permeability	0.071	log BB
	CNS permeability	-1.273	logPS
Metabolism	CYP2D6 substrate	No	Yes/No
	CYP3A4 substrate	Yes	
	CYP1A2 inhibitor	No	
	CYP2C19 inhibitor	No	
	CYP2C9 inhibitor	No	
	CYP2D6 inhibitor	No	
	CYP3A4 inhibitor	No	
Excretion	Total clearance	0.069	log ml/min/kg
	Renal OCT2 substrate	No	Yes/No
Toxicity	AMES toxicity	Yes	
	Max. tolerated dose (Human)	0.219	log mg/kg/day
	hERG I inhibitor	No	Yes/No
	Oral rat acute toxicity (LD50)	2.154	Mol/kg
	Oral rat chronic toxicity (LOAEL)	1.798	log mg/kg_bw/day
	Hepatotoxicity	Yes	Yes/No
	Skin sensitization	No	
	T.pyriformis toxicity	0.28	log µg/L
	Minnow toxicity	-3.26	log µg/L
Drug likeness	Lipinski	Yes	Yes/No
Bioactivity Score	GPCR ligand	0.08	
	Ion channel modulator	-0.07	
	Kinase inhibitor	0.14	
	Nuclear receptor ligand	-0.40	
	Protease inhibitor	0.01	
	Enzyme inhibitor	0.03	

Drug-likeness and ADMET analysis

In our present study, we compared the modified compound's drug-likeness and ADMET properties with native compounds. The weaker binding will ultimately have a rapid dissociation rate. In this study, the modified compounds showed the lower binding energy which means strong binding with EthR protein, suggesting that these analogues could be used as inhibitors of the EthR protein to combat TB. Table 3 shows the pharmacokinetics, bioavailability, and drug-likeness properties of the CLF4 compound.

DISCUSSION

Developing drugs to decrease the symptoms of MTB infection are the main task now worldwide. Nowadays first-line and second-line drugs were used to treat TB in many countries. The new research was adopted to find the best binding inhibitory drugs and drug repurposing medicines for the possible treatment. In our study, we targeted the EthR protein of the MTB for identifying modified first-line (ETH, INH) and second-line (CLF) drugs to get the better inhibitory effect on EthR protein to combat TB, using *in silico* analysis. According to our results, CLF4 shows better binding efficacy on EthR protein which shows the best lowest MolDock score -176.29kcal/mol and H-bonding energy -6.89kcal/mol.

CONCLUSION

This study encompasses the designing of novel inhibitor molecules against EthR protein with a focus on the binding interactions of ETH, INH, CLF, and their modified derivatives to EthR of MTB. The 12 modified ligands were designed and computed for their binding affinity with the target protein EthR. The ETH modified molecule ETH4, the INH modified molecule INZ2, and the CLF modified molecules of CLF3 and CLF4 have shown better binding efficacy than that of the original native compounds. Furthermore, concluded that CLF4 revealed harmonious binding affinity on EthR protein to combat TB. However, *in vitro* and *in vivo* experimental studies are warranted to prove the above results.

AUTHORS' CONTRIBUTIONS

SCP and PKP conceptualized, designed interpreted data, and edited manuscript. All authors have approved the manuscript in the current form.

CONFLICT OF INTERESTS

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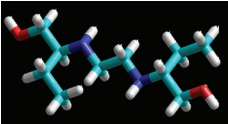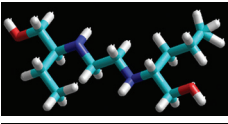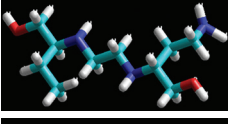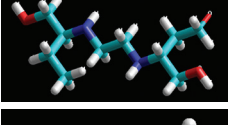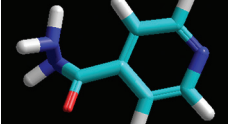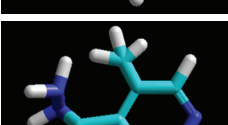
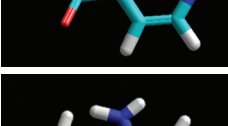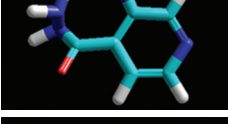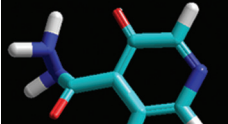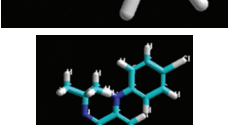1. Wong YJ, Noordin NM, Keshavjee S, Lee SW. Impact of latent tuberculosis infection on health and wellbeing: A systematic review and meta-analysis. Eur Respir Rev 2021;30:1-11.
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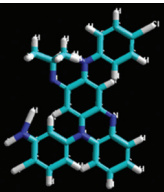
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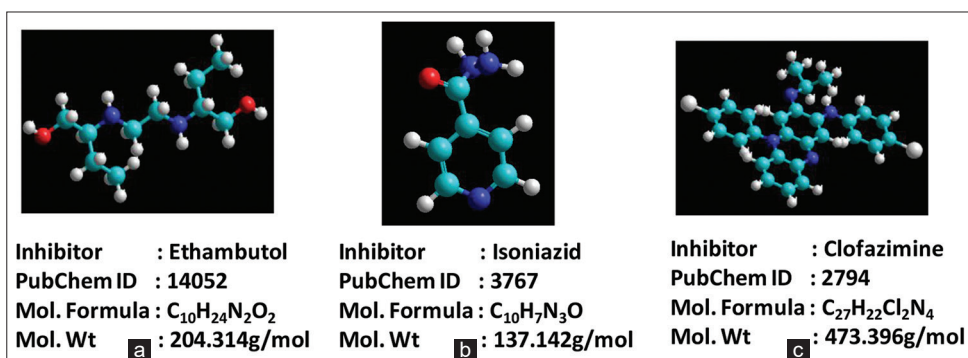
Supplementary Fig.1. Structural information about EthR inhibitors (a). Ethambutol, (b). Isoniazid, (c). Clofazimine.clofazimine 4 ligand

Suplementary Table 1: List of 12 modified ligand structures.

Ligands	Addition of functional group	position	structure	After single point energy	After geometry optimization energy
ETH1	ALKYL HALIDE C-CL	Atom no 11 carbon		684366.14 kcal/mol	7.45 kcal/mol
ETH2	ALKANE C-CH3	Atom no 11 carbon		684477.3 kcal/mol	9.2 kcal/mol
ETH3	PARIMARY AMINE C=NH2	Atom no 11 carbon		684402.3 kcal/mol	11.41 kcal/mol
ETH4	CARBONYL C=O	Atom no 11 carbon		684366.14 kcal/mol	7.45 kcal/mol
INZ1	ALKYL HALIDE C-CL	Atom no 7 carbon		142.808 kcal/mol	12.5 kcal/mol
INZ2	ALKANE C-CH3	Atom no 5 carbon		650.01 kcal/mol	19.02 kcal/mol
INZ3	PRIMARY AMINE C-NH2	Atom no 5 carbon		191.81 kcal/mol	18.31 kcal/mol
INZ4	CARBONYL GROUP C=O	Atom no 7 carbon		142.808 kcal/mol	12.53 kcal/mol
CLF1	ALKYL HALIDE C-CL	Atom no 3 carbon		1852.37 kcal/mol	36.35 kcal/mol
CLF2	ALKANE C-CH3	Atom no 3 Carbon		2379.32 kcal/mol	113.12 kcal/mol

Suplementary Table 1: *(Continued)*

Ligands	Addition of functional group	position	structure	After single point energy	After geometry optimization energy
CLF3	PARIMARY AMINE C=NH2	Atom no 3 Carbon		2187.96 kcal/mol	41.34 kcal/mol
CLF4	CARBONYL C=O	Atom no 3 carbon		1852.37 kcal/mol	36.35 kcal/mol

Supplementary Table 2: MolDock score, rerank score, interaction, torsions and hbond energy of the docked compounds.

Ligand	MolDock Score	Rerank Score	Interaction energy	HBond
Ethambutol	-105.14	-87.82	-113.57	-5.92
Isoniazid	-68.55	-62.77	-87.94	-5.44
Clofazimine	-93.95	312.607	-134.68	-2.35
ETH1	-109.05	-88.51	-120.29	-4.96
ETH2	-109.87	-76.13	-120.28	-5.66
ETH3	-115.27	-88.67	-121.51	-4.96
ETH4	-110.18	-78.76	-114.97	-5.87
INZ1	-71.53	-62.58	-89.82	-0.10
INZ2	-68.47	-55.85	-87.75	-6.77
INZ3	-72.19	-62.35	-89.22	-0.52
INZ4	-73.87	-69.11	-92.18	-0.0009
CLF1	-82.07	428.82	-112.03	-0.53
CLF2	-92.23	235.19	-132.53	-2.34
CLF3	-73.64	488.66	-94.20	-4.27
CLF4	-176.29	476.65	-112.23	-6.89