ORIGINAL ARTICLE

In Silico Identification of Novel Tuberculosis Drug Targets in Mycobacterium tuberculosis P450 Enzymes by Interaction Study with Azole Drugs

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ABSTRACT

Introduction: Tuberculosis (TB) is one of the utmost serious infectious diseases worldwide. The emergence of multi-drug resistance demands the development of better or new putative drug targets for tuberculosis. Recent studies suggest *Mycobacterium tuberculosis* cytochrome P450 enzymes as promising drug targets and azole drugs as potential inhibitors. **Methods:** Various computational tools, like Expasy Protparam, Swiss model, RaptorX and Phyre2 were used to analyze 12 *Mycobacterium tuberculosis* P450 enzymes and determine their three-dimensional structure. The structural validation was done through a Ramachandran plot using RAMPAGE server. The docking of P450 enzymes with azole drugs was done with autodock ver 4.2.6. **Results:** Based on sub-cellular localization prediction using CELLO tool, P450 enzymes CYP123A1, CYP132A1, CYP135A1, CYP136A1, CYP140A1, and CYP143A1 were predicted to be in the cytoplasm. Through structure assessment by Ramachandran plot, the best homology modelled proteins were docked with azole drugs like clotrimazole, croconazole, econazole, fluconazole, itraconazole, itraconazole, ketaconazole and micronazole by using autodock. By docking method it is identified that ketaconazole drug has a high affinity towards most of the mycobacterium P450 enzymes followed by the itrconazole drug. CYP123A1 enzyme is preferable as a drug target due to high binding affinity towards ketoconazole followed by CYP135A1, CYP140A1 enzymes. **Conclusion:** This study would help in identifying putative novel drug targets in *Mycobacterium tuberculosis*, which can lead to promising candidates for the optimization and development of novel anti-mycobacterial agents.

Keywords: Tuberculosis, Azoles, Ketoconazole, Drug Resistance, Cytochrome P-450 Enzyme System

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INTRODUCTION

Tuberculosis (Tb) is one of the infectious bacterial diseases worldwide caused by bacterium *Mycobacterium tuberculosis* (Mtb). In recent years, this is a major health problem and it has re - emerged in many industrialized developing countries (1). Tb being a greater global problem than it was at the beginning of the twentieth century although we are already entering the new millennium era. Tb mainly attacks the lung but also can attack any part of our body system. In most healthy people, their own immune system would be able to destroy this infection. Tb became the world's leading cause of death from bacterial infection, with 9.6 million cases and 1.5 million deaths in 2014 (2). Although tuberculosis is more prevalent in the developing world, it affects virtually all over the world (3, 4). Recently, the Mtb strain H37Rv genome has been well studied and fully characterized to understand this strain, which helps us to act to prevent this disease and to treat this disease. Mtb, is far less well known than that of *Escherichia coli*, potentially because Tb was more or less effectively controlled throughout the world (5). In recent years, this human pathogen, Mtb made a dramatic resurgence. Like many organisms, the entire Mtb genome sequence revealed the existence of cytochrome P450 (P450) enzymes unexpectedly with a high number and many parallel studies indicated that P450-inhibiting azole drugs had a potent anti-tuberculosis activity (6). The clarification of the P450 catalyzed reaction would enable a better understanding of Mtb physiology and the design of selective inhibitors. (7).

A P450 superfamily is a large group of hemoprotein monooxygenases found both in prokaryotes and eukaryotes. These enzymes play a major role in the oxidative metabolism of a wide range of exogenous and endogenous substrates. More than 230 different P450 genes and pseudogenes have currently been identified (8). P450 common structural and functional domain architecture is well known. Apart from P450 itself, these

systems can include several fundamentally different protein components or domains with different functions. (9).

Computational genome analysis of Mtb revealed that 14α-demethylase plays an essential role in sterol biosynthesis. (10). Azoles compounds block sterol biosynthesis by inhibiting fungal sterol 14α-demethylase which explains their antifungal activity (11). Azole drug inhibits a number of P450s and has a number of targets in Mycobacteria, thus providing another new antibiotic approach against Mtb. (12). Recently, both in vitro and ex vivo experiments, the anti-tuberculosis potential of azole drugs against latent or persistent tuberculosis was also demonstrated. The results suggest that these drugs hold the potential to enhance the efficacy of currently prescribed anti-tubercular drugs (13). The frequency of infections with Mtb resistant to anti-tuberculous drugs is increasing globally. Due to this increase, it becomes a major threat to tuberculosis treatment and control program (14). Recently, azole drugs have proven to be more effective than rifampicin in controlling persistent bacilli in mice. (15). Therefore, new drug approaches are therefore desperately needed to combat the increasing prevalence of tuberculosis, in particular the multi-drug - resistant form (MDR - TB), and to shorten the period of tuberculosis chemotherapy (16). The Mtb genome encodes 20 cytochrome P450 enzymes, of which 8 Mtb P450s are experimentally solved and and and these structures are available in both ligands - free and with substrates in the Protein Data Bank (PDB). In this study, remaining 12 Mtb P450s whose structures are unavailable were characterized through computational approach and interaction between different azole drugs were studied through molecular docking approach.

MATERIALS AND METHODS

Sequence retrieval

The 12 sequences belonging to Mtb P450 protein were retrieved from the Cytochrome P450 Homepage (17). These sequences were cross-checked with the Uniprot database (18) and retrieved with respective Uniprot IDs. For further analysis, all 12 Mtb P450 sequences have been downloaded in a FASTA format.

Physicochemical characterization

The physicochemical parameters of Cytochrome P450 protein were evaluated by using the Expasy ProtParam tool (19). The computed parameters include molecular weight, theoretical PI, extinction coefficient, instability index, aliphatic index and also Grand average of hydropathicity (GRAVY).

Subcellular Localization and Transmembrane Prediction
Subcellular localization was predicted using CELLO tool (20) and the signal peptide and transmembrane region were predicted using signal (21), HMMTOP (22) and TMHMM (23).

Homology Modelling

Since the cytochrome P450s crystal structures were unavailable in PDB (24), they were modelled through a homology modelling approach. The homology modeling was carried out to construct a three - dimensional (3D) structure based on the similar protein of the known PDB structure. The protein sequences were submitted to different homology modeling servers like Swiss-model (25), RaptorX (26), and Phyre2 (27) respectively. These tools were used to generate automated, three - dimensional protein structures based on a comparative method. The modeled protein was evaluated by Ramachandran using the RAMPAGE server. (28). The best models were selected based on the Ramachandran plot. The selected best models were energy minimized using YASARA server (29).

Ligand retrieval

The ligand structures of croconazole, clotrimazole, econazole, fluconazole, miconazole, itranazole, voriconazole and ketoconazole were obtained from the Pubchem database. (30). The selected 3D structure of the ligands was retrieved from the PubChem Compound database in SDF format followed by conversion in the PDB format and optimized using UCSF chimera (31).

Molecular docking

Screening of eight azole drugs namely croconazole, clotrimazole, econazole, fluconazole, miconazole, itranazole, voriconazole, ketoconazole was docked using Auto dock vina (32). Based on their binding energy from a screening of azole drug with the proteins, the drug targets were prioritized. The lower the energy, the better their binding.

RESULTS

Physicochemical parameters include molecular weight, aliphatic index, instability index, theoretical value and GRAVY. In molecular weight, six proteins weighed more than 50kDa while other six proteins which weighed below were CYP123A1, CYP138A1, CYP139A1, CYP140A1, CYP141A1 and CYP143A1 (Table I). In the instability index, the result showed that two proteins are stable while the other ten proteins are unstable. Two stable proteins were CYP123A1 and CYP128A1 (Table I). In GRAVY, out of twelve proteins, only two proteins, CYP137A1 and CYP135B1 had positive value while the other ten were negative (Table I). By using CELLO web-tool, those six proteins were found inside the membrane and another six proteins were found in cytoplasmic. It has been predicted that CYP123A1, CYP132A1, CYP135A1, CYP136A1, CYP140A1 and CYP143A1 are in the cytoplasm and that other P450 protein is in the membrane. Furthermore, in signal peptide prediction, all 12 proteins showed no signal peptide inside the proteins. In predicting transmembrane helices using HMMTOP, one transmembrane helix was predicted. (Table II).

Table I: Physio-chemical characterization of Mycobacterium tuberculosis P450 enzymes

S.NO	Cytochrome P450	Molecular weight, M_w (Da)	Theoretical PI	Extinction coefficient (M^{-1} cm^{-1})	Instability Index		Aliphatic index	*Grand average of hydropathicity (GRAVY)*
					computed	classification		
1	CYP123A1	45421.45	5.34	38975	35.54	Stable	92.74	-0.234
2	CYP128A1	53313.14	9.33	42775	36.79	Stable	91.25	-0.105
3	CYP132A1	52229.07	8.93	74285	44.87	Unstable	87.22	-0.228
4	CYP135A1	50010.80	9.83	55920	52.52	Unstable	95.41	-0.129
5	CYP135B1	50687.53	9.93	38055	46.08	Unstable	96.00	0.011
6	CYP136A1	56227.67	6.57	67695	41.95	Unstable	81.65	-0.310
7	CYP137A1	52265.60	10.06	35535	50.68	Unstable	101.47	0.019
8	CYP138A1	49260.62	10.01	51910	58.70	Unstable	88.28	-0.235
9	CYP139A1	48641.65	9.88	74495	47.62	Unstable	93.37	-0.175
10	CYP140A1	48871.93	6.58	42190	47.72	Unstable	101.07	-0.168
11	CYP141A1	43730.88	5.06	21345	52.67	Unstable	92.88	-0.047
12	CYP143A1	43541.38	6.33	46535	56.26	Unstable	96.82	-0.075

Table II: Prediction of subcellular localization by CELLO, signal peptide prediction by SignalP, transmembrane region by HMMTOP and TMHMM

S.No	Cytochrome P450	CELLO	Signal peptide	HMMTOP	TMHMM
1	CYP123A1	Cytoplasmic	NO	0	0
2	CYP128A1	Membrane	NO	0	0
3	CYP132A1	Cytoplasmic	NO	0	0
4	CYP135A1	Cytoplasmic	NO	0	0
5	CYP135B1	Membrane	NO	1	0
6	CYP136A1	Cytoplasmic	NO	0	0
7	CYP137A1	Membrane	NO	1	0
8	CYP138A1	Membrane	NO	1	0
9	CYP139A1	Membrane	NO	1	0
10	CYP140A1	Cytoplasmic	NO	1	0
11	CYP141A1	Membrane	NO	0	0
12	CYP143A1	Cytoplasmic	NO	1	0

For the cytochrome P450 whose crystal structures were unavailable for 12 Mtb P450s were homology modelled using three different servers. All the energy minimized models were assessed by Rampage server. Rampage server used a Ramachandran plot image to give a better validation and understanding about the number of residues of the proteins (Table III). In this study, docking was done by using Autodock server with eight different ligands of azole drugs (Figure 1). The best homology modeled proteins were docked with azole drugs like clotrimazole, croconazole, econazole, fluconazole, itraconazole, ketoconazole, micronazole and voriconazole (Table IV). Azole drugs like croconazole, econazole, fluconazole and miconazole have a low binding affinity towards Mtb P450 (Figure 2). Three P450 proteins which could not be docked with any of azole drugs due to large protein size were CYP136A1, CYP137A1 and CYP144A1.

DISCUSSION

In this study, 12 Mtb P450s whose three-dimensional structures are unavailable were studied. As a primary analysis for identifying the suitable drug target, the ProtParam tool was used to check their physicochemical parameters. The determination of the molecular weight of proteins is essential for its biochemical characterization and is ideal for the development of drug targets due to the ease of crystallization. Based on the results (Table I), the six Mtb P450 was found to be preferable for drug target which had less than 50kDa. Theoretical PI showed that seven proteins had a value higher than seven while another five proteins had a value below seven (Table I). Depending on its amino acid composition and pH, a protein may have a net negative or net positive charge. The molecule can be electrically neutral at certain pH values of solutions if negative and positive charges are balanced. In the instability index study, CYP123A1 and CYP128A1 proteins had an index below 40. If the index was greater than 40, it is considered as not stable (33). The coefficient of extinction shows how much light a protein absorbs at a certain wavelength. For a protein which is a spectrophotometer when purifying it, it is useful to have an estimate of this coefficient. The aliphatic index is used to calculate the relative volume of aliphatic side chains and a positive indicator of thermostability of globular protein. This method is only available with the presence of amino acid. The aliphatic index is directly associated with the protein mole fraction of alanine, isoleucine, leucine and valine

Table III: Homology modelling of Mycobacterium tuberculosis P450 enzymes by Phyre2, RaptorX, Swiss model servers. Best homology models selected were highlighted in bold

SNO	Cytochrome P450	Number of residues in favoured region	Number of residues in allowed region	Number of residues in outlier region
1	CYP123A1	Phyre: (~98.0% expected): 375 (93.8%)	Phyre: (~2.0% expected): 19 (4.8%)	Phyre: 6 (1.5%)
		RaptorX: (~98.0% expected): 382(95.5%)	**RaptorX: (~2.0% expected): 14 (3.5%)**	**RaptorX: 4 (1.0%)**
		Swiss model: (~98.0% expected): 360 (92.8%)	Swiss model: (~2.0% expected): 21 (5.4%)	Swiss model: 7 (1.8%)
2		Phyre: (~98.0% expected): 355 (91.5%)	Phyre: (~2.0% expected): 22 (5.7%)	Phyre: 11 (2.8%)
	CYP128A1	RaptorX: (~98.0% expected): 374(93.0%)	RaptorX: (~2.0% expected): 19 (4.7%)	RaptorX: 9 (2.2%)
		Swiss model: (~98.0% expected): 356 (93.2%)	**Swiss model: (~2.0% expected): 16 (4.2%)**	**Swiss model: 10 (2.6%)**
3		Phyre: (~98.0% expected): 416 (91.8%)	Phyre: (2.0% expected): 22 (4.9%)	Phyre: 15 (3.3%)
	CYP132A1	RaptorX: (~98.0% expected): 424(92.4%)	RaptorX: (~2.0% expected): 21 (4.6%)	RaptorX: 14 (3.1%)
		Swiss model: (~98.0% expected): 419 (92.7%)	**Swiss model: (~2.0% expected) : 25 (5.5%)**	**Swiss model: 8 (1.8%)**
4		Phyre: (~98.0% expected): 392 (91.8%)	Phyre: (~2.0% expected): 27 (6.3%)	Phyre: 8 (1.9%)
	CYP135A1	RaptorX: (~98.0% expected): 415(92.8%)	RaptorX: (~2.0% expected): 25(5.6%)	RaptorX: 7(1.6%)
		Swiss model: (~98.0% expected): 407 (94.0%)	**Swiss model: (~2.0% expected): 16 (3.7%)**	**Swiss model: 10 (2.3%)**
5		Phyre: (~98.0% expected): 391 (90.7%)	Phyre: (~2.0% expected): 26 (6.0%)	Phyre: 14 (3.2%)
	CYP135B1	RaptorX: (~98.0% expected): 428(91.1%)	RaptorX: (~2.0% expected): 28 (6.0%)	RaptorX: 14 (3.0%)
		Swiss model: (~98.0% expected): 407 (92.7%)	**Swiss model: (~2.0% expected): 26 (5.9%)**	**Swiss model: 6 (1.4%)**
6		Phyre: (~98.0% expected): 399 (91.7%)	Phyre: (~2.0% expected): 26 (6.0%)	Phyre: 10 (2.3%)
	CYP136A1	**RaptorX: (~98.0% expected): 457(93.3%)**	**RaptorX: (~2.0% expected): 21 (4.3%)**	**RaptorX: 12(2.4%)**
		Swiss model: (~98.0% expected): 404 (93.1%)	Swiss model: (~2.0% expected): 25 (5.8%)	Swiss model: 5 (1.2%)
7	CYP137A1	Phyre: (~98.0% expected): 406 (92.5%)	Phyre: (~2.0% expected): 21 (4.8%)	Phyre: 12 (2.7%)
		RaptorX: (~98.0% expected): 445(93.9%)	**RaptorX: (~2.0% expected): 18(3.8%)**	**RaptorX: 11 (2.3%)**
		Swiss model: (~98.0% expected): 391 (91.1%)	Swiss model: (~2.0% expected): 25 (5.8%)	Swiss model: 13 (3.0%)
8	CYP138A1	**Phyre: (~98.0% expected): 390 (92.2%)**	**Phyre: (~2.0% expected): 20 (4.7%)**	**Phyre: 13 (3.1%)**
		RaptorX: (~98.0% expected): 402(91.6%)	RaptorX: (~2.0% expected): 26(5.9%)	RaptorX: 11(2.5%)
		Swiss model: (~98.0% expected): 385 (90.6%)	Swiss model: (~2.0% expected): 29 (6.8%)	Swiss model: 11 (2.6%)
9	CYP139A1	Phyre: (~98.0% expected): 385 (93.7%)	Phyre: (~2.0% expected): 21 (5.1%)	Phyre: 5 (1.2%)
		RaptorX: (~98.0% expected): 407(93.8%)	**RaptorX: (~2.0% expected): 17(3.9%)**	**RaptorX: 10 (2.3%)**
		Swiss model: (~98.0% expected) : 381 (89.9%)	Swiss model: (~2.0% expected): 33 (7.8%)	Swiss model: 10 (2.4%)
10	CYP140A1	Phyre: (~98.0% expected): 378 (89.6%)	Phyre: (~2.0% expected): 26 (6.2%)	Phyre: 18 (4.3%)
		RaptorX: (~98.0% expected): 407 (93.3%)	**RaptorX: (~2.0% expected): 19(4.4%)**	**RaptorX: 10 (2.3%)**
		Swiss model: (~98.0% expected): 368 (92.2%)	Swiss model: (~2.0% expected): 25 (6.3%)	Swiss model: 6 (1.5%)
11	CYP141A1	Phyre: (~98.0% expected): 379 (95.9%)	Phyre: (~2.0% expected): 9 (2.3%)	Phyre: 7 (1.8%)
		RaptorX: (~98.0% expected): 382(96.0%)	**RaptorX: (~2.0% expected): 11 (2.8%)**	**RaptorX: 5 (1.3%)**
		Swiss model: (~98.0% expected): 366 (93.6%)	Swiss model: (~2.0% expected): 21 (5.4%)	Swiss model: 4 (1.0%)
12	CYP143A1	Phyre: (~98.0% expected): 346 (88.7%)	Phyre: (~2.0% expected): 26 (6.7%)	Phyre: 18 (4.6%)
		RaptorX: (~98.0% expected): 370(94.6%)	**RaptorX: (~2.0% expected): 11 (2.8%)**	**RaptorX: 10 (2.6%)**
		Swiss model: (~98.0% expected): 363 (93.8%)	Swiss model: (~2.0% expected): 16 (4.1%)	Swiss model: 8 (2.1%)

(34). The GRAVY value of the protein was calculated by adding the amino acid residue hydropathy values and dividing by the number of residues in the sequence. The high positive result showed greater hydrophobicity. Based on the physicochemical analysis the preferable drug target was CYP123A1 which was predicted to be low molecular weight and stable in instability index prediction. It is worthwhile to compute an amino acid sequence's physicochemical characteristic like isoelectric point (theoretical pI), molecular weight (Mw), theretical PI, extinction co-efficient, instability index, alphatic index and GRAVY as these data indicate the approximate area of a 2D-gel where a protein of interest can be detected and purified. Understanding a protein's physicochemical properties also supports the development of drugs and quality control. Subcellular localization was done to analyze where protein resides inside the cell. The subcellular localization of proteins is closely correlated to its biological function (35). Signal peptides are responsible for targeting proteins to

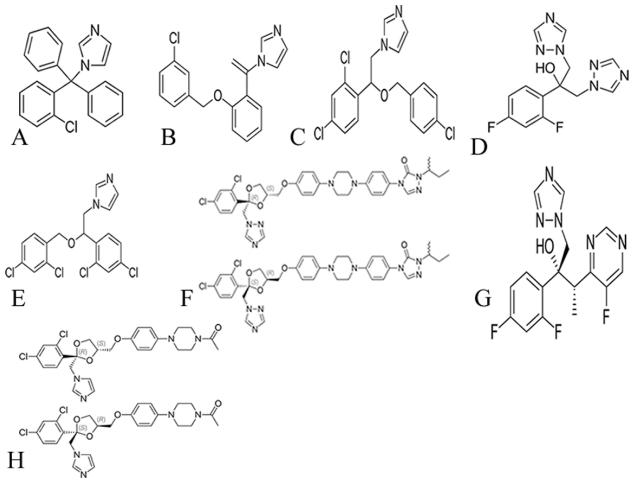

Figure 1: 2D strucure of selected azole drugs. (A). clotrimazole (B). croconazole (C). econazole (D). fluconazole (E). miconazole (F). itraconazole (G).voriconazole (F).ketaconazole

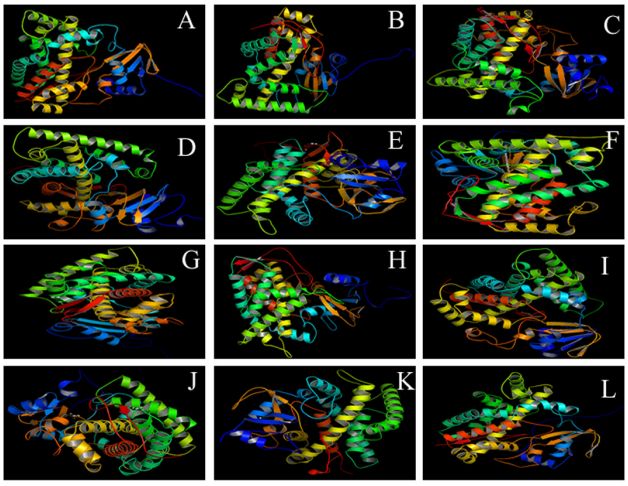

Figure 2: Homology models of selected Mycobacterium tuberculosis P450s. (A).CYP123A1 (B).CYP128A1 (C).CYP132A1 (D).CYP135A1 (E).CYP135B1 (F).CYP136A1 (G).CYP137A1 (H). CYP138A1 (I).CYP139A1 (J).CYP140A1 (K).CYP141A1 (L).CYP143A1

the endoplasmic reticulum for further transport via the secretory pathway (36). Predicting the signal peptide and transmembrane helices is an important step in understanding the membrane protein structure topology and from table II it is inferred that all the proteins had less than only one transmembrane protein and no signal peptide was predicted in all the proteins. Out of 20 Mtb P450, 8 crystal structures of CYP51B1 (PDB ID: 1EA1), CYP121A1 (1N40), CYP124A1 (2WM4), CYP125A1 (2X5W), CYP126A1 (5LI6), CYP130A1 (2UUQ), CYP142A1 (2XKR), and CYP144A1 (5HDI) available in PDB were not considered for study. Homology modeling is a method for creating three - dimensional protein structure models using experimentally determined family structures as templates. Three types of servers were used for homology modeling, Swiss model, Phyre2 and RaptorX. The best homology models selected were energy minimized by the steepest descent method using the YASARA server. The best models among three servers were selected based on Ramachandran plot with the majority of the residue clustered together or closely in the favored region with a very few in outliers' region (Table III). The docking procedure is to predict the correct poses of ligand at the protein's binding site and to score them within a reasonable time frame based on the strength of the interaction. (37). By this docking method, it was identified that ketoconazole drug was the best ligand because of a high affinity toward most of the Mtb P450 proteins (CYP123A1, CYP128A1, CYP135A1, CYP139A1, CYP140A1, CYP141A1) followed by itraconazole three proteins (CYP135B1, CYP138A1, CYP143A1) and one for clotrimazole (CYP132A1) (Figure 2). In summary, based on physico-chemical characterization, sub-cellular prediction and docking study, CYP123A1 enzyme was identified as a preferable drug target with a good binding affinity towards

Table IV: The interaction between Azole drugs with Cytochrome P450 of Mycobacterium tuberculosis. The azole drug which have high binding affinity are shown in bold

NO	Cytochrome P450	Azole Drugs							
		Clotrimazole	Croconazole	Econazole	Fluconazole	Miconazole	Itraconazole	Voriconazole	Ketaconazole
1	CYP123A1	-5.970	-6.160	-6.350	-5.430	-7.450	-7.420	-6.070	**-7.940**
2	CYP128A1	-7.550	-7.530	-7.500	-5.570	-7.480	-7.450	-6.150	**-8.350**
3	CYP132A1	**-7.750**	-7.260	-6.560	-5.580	-7.130	-7.240	-6.800	-7.540
4	CYP135A1	-6.120	-6.620	-7.980	-5.210	-5.670	-7.840	-5.410	**-8.030**
5	CYP135B1	-7.340	-7.230	-6.860	-5.400	-7.060	**-8.220**	-5.820	-6.990
6	CYP136A1	-	-	--	-	-	-	-	-
7	CYP137A1	-	-	--	--	--	--	--	-
8	CYP138A1	-7.590	-7.700	-6.250	-6.300	-7.500	**-10.480**	-6.260	-8.140
9	CYP139A1	-7.820	-7.430	-7.320	-6.100	-8.410	-8.550	-6.480	**-9.110**
10	CYP140A1	-8.070	-7.030	-6.950	-6.220	-7.100	-7.580	-6.890	**-8.360**
11	CYP141A1	-6.640	-8.500	-7.400	-6.440	-7.740	-8.500	-7.570	**-9.480**
12	CYP143A1	-6.090	-6.920	-6.6100	-5.160	-6.700	**-9.080**	-5.170	-8.960

ketoconazole followed by CYP135A1, CYP140A1 enzymes.

CONCLUSION

Tb is one of the major bacterial diseases that affect the lungs. There is an urgent requirement for identifying new drug target. Through bioinformatics approach, 12 Mtb P450 enzymes were modeled and docked with azole drugs. From this study, CYP123A1 enzyme with ketoconazole azole drug was predicted to be the suitable drug target and drug. However, further in vivo studies are needed to confirm this study.

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