INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

Volume: 2: Issue-4: Oct - Dec -2011 IJABPT *ISSN 0976-4550*

ELUCIDATING PYRAZINAMIDE RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* BY MOLECULAR DOCKING

A. Nusrath Unissa, S. Sudha and N. Selvakumar

Department of Bacteriology, National Institute for Research in Tuberculosis (NIRT)
Chetput, Chennai 600 031, Tamil Nadu, India.
E-mail:nusrathunissa@gmail.com

ABSTRACT: Pyrazinamide, PZA - an important drug in the anti-tuberculosis therapy, activated by an enzyme Pyrazinamidase, PZase. The basis of PZA resistance in *Mycobacterium tuberculosis* is owing to mutation in *pncA* gene coding for PZase. The identification of the structural or functional defects in the mutant enzymes leading to resistance still remains an area to be explored. In the light of which, in the present study, the Wild-type and five mutant models Asp8Gly, Lys96Thr, Ser104Arg, Cys138Ser and Cys138Tyr were docked with PZA and its derivatives. Docking results predicts the compounds-10 and 4 were the good derivatives of PZA to bind with mutants of PZase. These models represent the first *in-silico* evidence for the binding interaction of PZase with PZA derivatives and analogues. The models may provide useful insights for designing new anti-TB agents in order to overcome the resistance developed with PZA.

Key Words: *Mycobacterium tuberculosis,* PZase, PZA resistance, Mutants, Derivatives, Docking.

INTRODUCTION

Tuberculosis (TB) is a major cause of mortality worldwide. The emergence of multi-drug resistant (MDR-TB) and extensively drug resistant (XDR-TB) has further worsened the situation. Drug resistance in TB is essentially a potential threat to the TB control programmes.

PZA remains as one of the most active compounds used to treat and prevent TB worldwide. The use of PZA (Pyrazine-2-carboxamide -IUPAC name), as an effective anti-TB drug began in 1980. It is an important sterilizing drug, acting as a principle component in the current six-month short course chemotherapy. PZA plays a unique role in shortening the therapy from a period of 9 to 12 months down to 6 months, because PZA kills a population of semi dormant tubercle bacilli, residing in an acidic environment, which cannot be killed by other TB drugs [1].

PZA is a pro-drug that requires cellular activation by PZase protein to its active form (**Figure 1**) before exerting its toxic effect on the bacillus. In *Mtb* the susceptibility to PZA correlates with the presence of a single enzyme (PZase) with nicotinamidase and pyrazinamidase

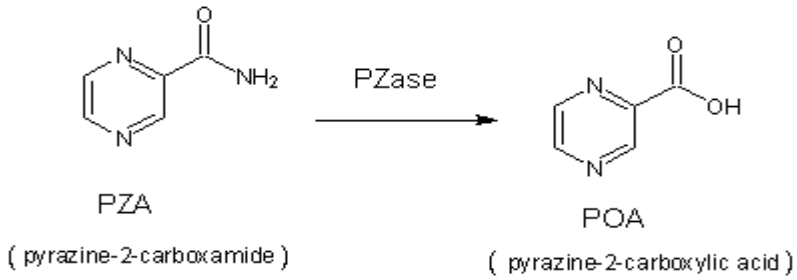

Figure 1. PZase mediates the activation of PZA

activities. Strains of *Mtb* that are resistant to PZA are often defective in PZase activity [2]. PZA-resistant (PZA^r) *Mtb* clinical isolates are usually defective for PZase activity, and there is very good correlation between PZA resistance and loss of this enzyme. Scorpio and Zhang in 1996 had identified the PZase gene (*pncA*) from *M. tuberculosis* and had shown that *pncA* mutations are a major mechanism of PZA resistance [3]. The identified *pncA* mutations are largely missense mutations causing amino acid substitutions, and in some cases nucleotide insertions or deletions and nonsense mutations in the *pncA* structural gene or in the putative promoter region of *pncA* [4]. The uniqueness in the mutations of *pncA* gene is its diversity and scattering along the whole gene though there does appear to be some degree of clustering at three regions of PncA protein (3 to 17, 61 to 85, and 132 to 142). These regions are likely to contain catalytic sites for the PZase enzyme [5]. PZA as a prodrug needs to be activated by the bacterial nicotinamidase-PZase into pyrazonic acid (POA), the active moiety of the drug [6].

The decrease in PZase activity observed in the mutant proteins correlates well with the structural modifications as was evident from a study [7] that has clearly shown the structure-function relationships of PncA protein (PZase). In another report [8] comprehensive enzymatic characterization of PZase was done along with the generation of nine different mutants. It suggested that the Asp8, Lys96 and Cys138 were key residues for catalysis, and Asp49, His51, His57 and His71 were essential for metal ion binding. Very recently, crystal structure of PZase was elucidated [9]. Also; another study suggests that considerable amount of PZA resistance is determined by other factors than PZAse activity [10]. Both of the reports [7, 8] demanded the need for further structural studies. In our earlier reports [11, 12], we developed three dimensional (3D) models of PZase (wild and five mutants) with Asp8Gly (D8G), Lys96Thr (K96T), Ser104Arg (S104R), Cys138Tyr (C138Y), and Cys138Ser (C138S) and explored its interactions with PZA. This study is an extension of the previous reports, was aimed to determine the profile of interactions of PZase models with PZA derivatives.

MATERIALS AND METHODS

Proteins

The modeled mutants of PZase (WT and the

five mutants) were obtained from our earlier published data [11, 12] for molecular docking.

Ligands

The ligands chosen in this study were the derivatives of PZA (**Figure 2**). The Simplified Molecule Input Line Entry System (SMILES) formulae for some of the PZA derivatives obtained from the NCBI-database were converted to chemical structure using Chem sketch software Version (V)-10 (**Table 1**). Some of derivatives obtained from National Center for Biotechnology Information (NCBI) Pubchem-database whose characteristics and the chemical formulae are provided in **Table 2**. The Chem 3D Pro 12.0- trial version was used for chemical structure generation for other derivatives obtained from the literature. The structures of the derivatives were converted into Protein Data Bank (PDB) files using DS. The labeling of the derivatives/compounds is as per the order given in **Table 1.**

Docking software

The GOLD protocol is an implementation of the genetic algorithm wherein the receptor is held rigid while the ligand is allowed to flex during the refinement process**.** The docking of flexible ligand and a protein with flexible hydroxyl groups makes it a good choice when the binding pocket contains amino acids that form H bonds with the ligand. GOLD uses a scoring function that is based on favorable conformations found in Cambridge Structural Database and on empirical results on weak chemical interactions.

The scoring function is force field based and

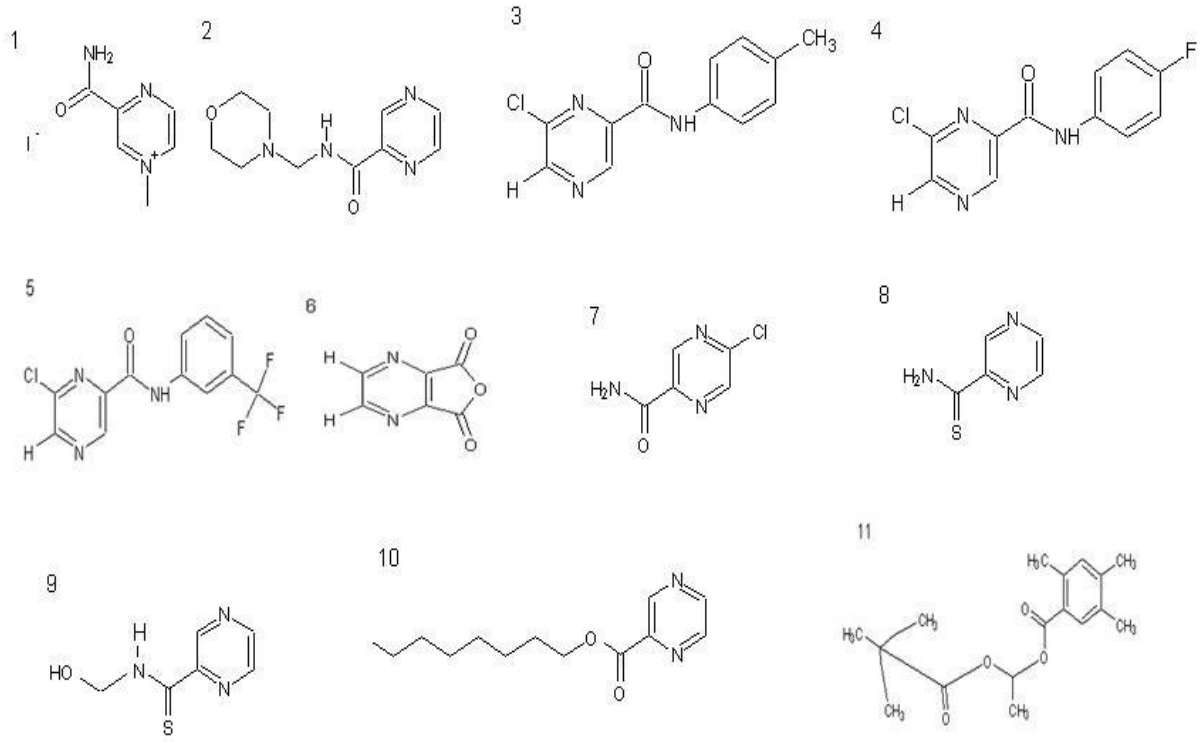

Figure 2. Chemical Structure of PZA derivatives (1-11)

Table 1. Name of Compounds

S.no.	IUPAC	SMILES formulae
1.	4-methylpyrazin-4-ium-2-carboxamide iodide	C[N+]1=CC(=NC=C1)C(=O)N.[I-]
2.	(morpholin-4-ylmethyl) pyrazine-2-carboxamide	C1COCCN1CNC(=O)C2=NC=CN=C2
3.	6-chloro-*N*-(4-methylphenyl)pyrazine-2-carboxamide	O=C(NC1CCC(C)CC1)C2CNCC(CL)N2
4.	6-chloro-*N*-(4-fluorophenyl) pyrazine-2-carboxamide	O=C(NC1CCC(F)CC1)C2CNCC(CL)N2
5.	6-chloro-*N*-[3-(trifluoromethyl)phenyl] pyrazine-2-carboxamide	O=C(NC1CC(CCC1)C(F)(F)F)C2C NCC (CL)N2
6.	pyrazine-2,3-dicarboxylic acid anhydride	O=C1OC(=O)C2=NC=CN=C12
7.	5-chloropyrazinamide	C1=C(N=CC(=N1)Cl)C(=O)N
8.	pyrazine thiocarboxamide	C1=CN=C(C=N1)C(=S)N
9.	N-hydroxymethyl pyrazine thiocarboxamide	C1=CN=C(C=N1)C(=S)NCO
10.	pyrazinoic acid n-octyl ester	CCCCCCCCOC(=O)C1=NC=CN=C1
11.	pyrazinoic acid pivaloyloxymethyl ester	Not determined

includes three terms (a H bonding term, a 4–8 intermolecular dispersion potential, and a 6–12 intramolecular potential for the internal energy of the ligand) [13].

Docking protocol

Docking was performed for 11 derivatives of PZA with the generated proteins models (WT and the five mutants) of PZase (D8G, K96T, S104R, C138Y and C138S) with the help of software GOLD (Evaluation V- 4.0.1). The input atom files for both the proteins and the ligands were created. The ligands and the models were added with H atoms using GOLDMINE before docking. The cavity atom file containing the atom number of binding residues such as Lys96, Asp49, Asp8, Cys138, Trp68, Phe13, Ala134, and Thr135 (**Figure 3**) was prepared for PZA and its derivatives. Dockings were performed under 'Standard default settings' mode, number of islands was 5, population size of 100, number of operations was 100,000, a niche size of 2, and a selection pressure of 1.1.

Ten docking poses were obtained for each ligand. Poses with highest GOLD score were used for further analysis. The docked poses of the ligands were visualized using Hermes software. The scoring function of GOLD provides a way to rank placements of ligands relative to one another. Ideally, the score should correspond directly to the binding affinity of the ligand for the protein, so that the best scoring ligand pose are the best binders.

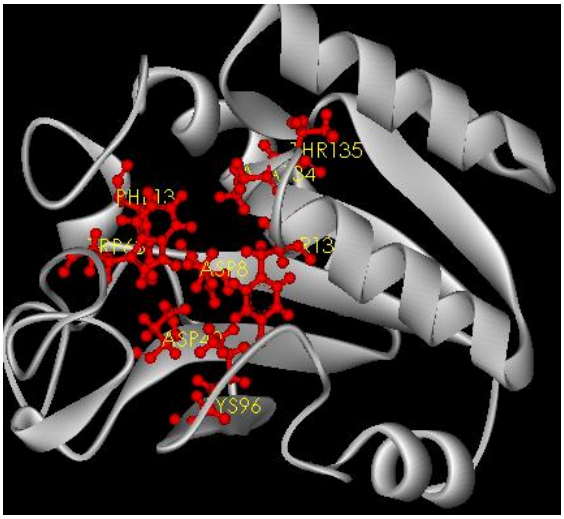

Figure 3. Structure of PZase showing the nine active site residues coloured in red and displayed in ball and stick model. The active site residues in PZase are Lys96, Asp49, Asp8, Cys138, Trp68, Phe13, Ala134, and Thr135.

Table 2. Characteristics of PZA derivatives

Properties	PZA derivatives				
	1	**7**	**8**	**9**	**10**
Compound ID	171646	181450	2797467	3001381	465427
Molecular Weight [g/mol]	265.05169	157.55776	139.1783	169.20428	236.3101
Molecular formula	$C_6H_8IN_3O$	$C_5H_4ClN_3O$	$C_5H_5N_3S$	$C_6H_7N_3OS$	$C_{13}H_{20}N_2O_2$
XLogP	0	-0.5	0	-0.6	3.3
H-Bond Donor	1	1	1	2	0
H-Bond Acceptor	3	3	2	3	4
Rotatable Bond Count	1	1	1	2	9
Tautomer count	2	2	2	2	-
Exact Mass	264.971205	157.004289	139.020418	169.030983	236.152478
MonoIsotopic Mass	264.971205	157.004289	139.020418	169.030983	236.152478
Topological Polar Surface Area	59.9	68.9	51.8	58	52.1
Heavy Atom Count	11	10	9	11	17
Formal Charge	0	0	0	0	0
Complexity	137	141	115	142	210
Isotope Atom Count	0	0	0	0	0
Defined Atom StereoCenter Count	0	0	0	0	0
Undefined Atom StereoCenter Count	0	0	0	0	0
Defined Bond StereoCenter Count	0	0	0	0	0
Undefined Bond StereoCenter Count	0	0	0	0	0
Covalently-Bonded Unit Count	2	1	1	1	1

RESULTS

An *in-silico* study based on the structure of receptor (PZase) was performed with analogues of PZA in this study with the purpose of contributing to the rational design of tuberculostatic leads. The models (WT and mutants of PZase) were used to evaluate a training set containing 11 compounds by a process of docking performed using GOLD. Few of the compounds (1, 7, 8, 9, 10) were selected at random from NCBI-Pubchem database, others from the available literature. Out of the 11 compounds tested for binding activity, four of them are analogues of PZA (8, 9, 10, and 11) were compounds with antimycobacterial activity.

Compounds with highest and lowest affinities

In accordance to the general conception, that high score is indicative of high affinity

between the protein and ligand molecule, the top GOLD score (-55.04 kcal/mol) obtained between the WT and compound-10 (pyrazinoic acid n-octyl ester) suggests high affinity. The score was -51.96, -49.88 and -57.79 kcal/mol for compound-10 with the mutants D8G, K96T and S104R respectively. The score for the compound-4 (6-chloro-*N*-(4-fluorophenyl) pyrazine-2-carboxamide) was found to be -55.63 and -54.90 kcal/mol for mutants C138Y and C138S respectively (**Table 3 and Figure 4a&b**). One of the compound-6 (pyrazine-2,3-dicarboxylic acid anhydride) was interestingly found to have a remarkable decrease (-24.64 kcal/mol) lower than that of parental PZA itself (-34.98 kcal/mol) in degree of binding affinity with WT PZase. The compound also imparts decrease in affinity with all PZase mutants (D8G, K96T, S104R, C138Y and C138S).

Table 3. Docking Score

S. no.	PZA and its derivatives	PZase and its variants					
		WT	**D8G**	**K96T**	**S104R**	**C138Y**	**C138S**
	PZA	34.98	25.96	29.91	32.31	33.22	33.01
1	4-methylpyrazin-4-ium-2-carboxamide iodide	34.80	34.12	31.03	32.01	34.66	34.15
2	(morpholin-4-ylmethyl)pyrazine-2-carboxamide	53.68	48.90	44.13	49.49	49.97	50.27
3	6-chloro-*N*-(4-methylphenyl)pyrazine-2-carboxamide	45.08	44.87	47.41	45.84	50.08	49.46
4	6-chloro-*N*-(4-fluorophenyl)pyrazine-2-carboxamide	52.87	38.71	44.97	40.86	**55.63** ✓	**54.90** ✓
5	6-chloro-*N*-[3-(trifluoromethyl)phenyl] pyrazine-2-carboxamide	47.64	42.81	42.26	45.14	51.25	50.97
6	pyrazine-2,3-dicarboxylic acid anhydride	**24.64***	**23.89***	**20.78***	**27.23***	**26.16***	**26.41***
7	5-chloropyrazinamide	35.75	31.23	30.03	31.14	33.78	33.57
8	pyrazine thiocarboxamide	39.37	38.77	36.08	37.49	36.97	37.30
9	N-hydroxymethyl pyrazine thiocarboxamide	42.52	37.36	38.70	40.49	42.49	42.76
10	pyrazinoic acid n-octyl ester	**55.04** ✓	**51.96** ✓	**49.88** ✓	**57.79** ✓	52.54	52.12
11	pyrazinoic acid pivaloyloxymethyl ester	48.81	44.95	41.87	48.21	52.97	51.73

Tick mark ✓ indicates highest score. Asterick * indicates lowest score.

Overall affinity of PZase and its mutants with the Compounds

The WT PZase affinity towards compounds-1, 7, and 8 found to have values in range of thirty (30s') whereas compounds-3, 5, 9, and 11 have values in the range of forty (40s'), compounds-2, 4, 10 with values in range fifty (50s'). (1) For the mutant-D8G, compounds-1, 4, 7 and 9 displayed values in 30s' series whereas compounds-2, 3, 5, 6, 11 showed values in 40s' series and a compound-10 showed value in the range of

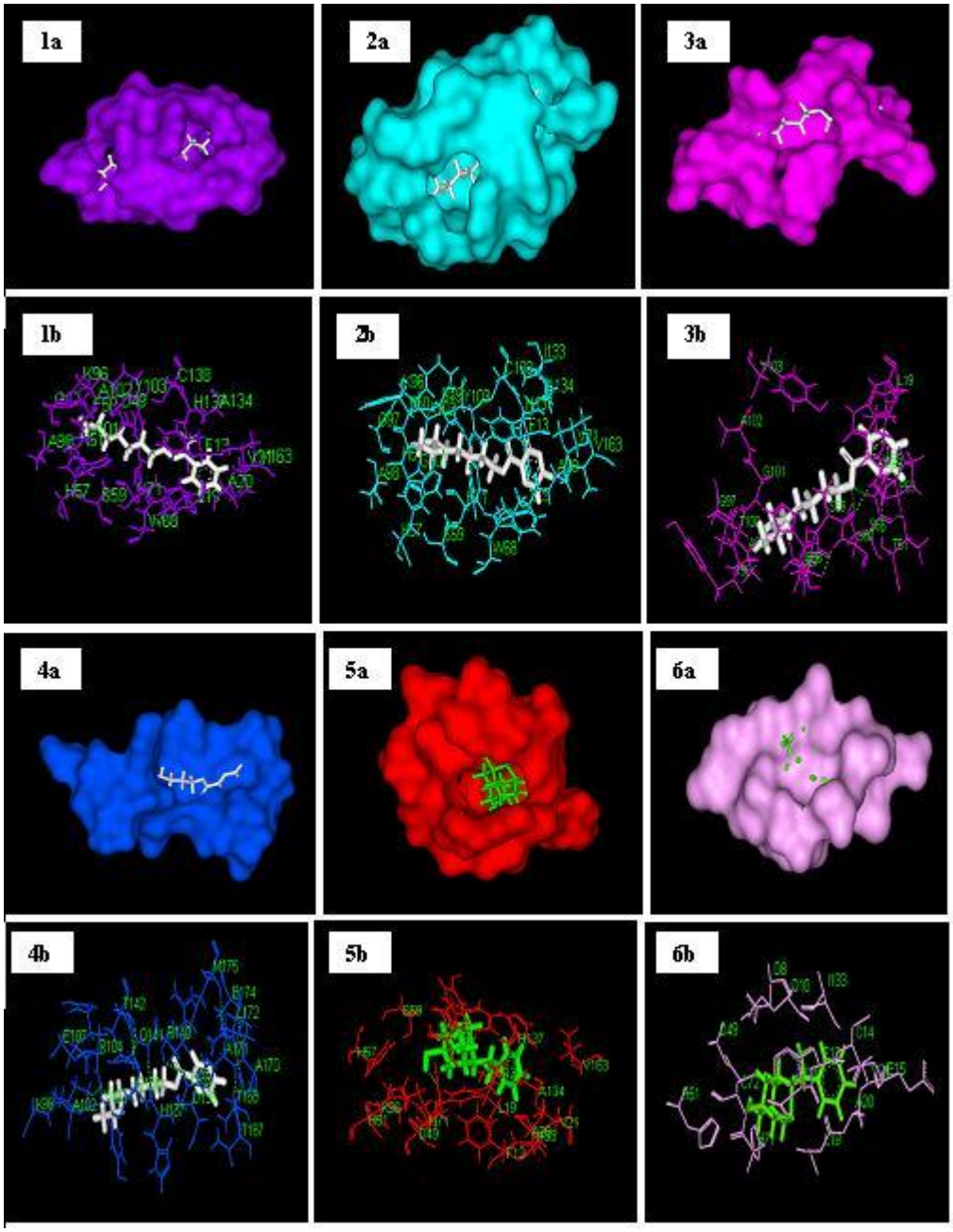

Figure 4a. Surface representation of docked PZA derivatives (white and green colored) 4b. H bond display in dotted green lines with PZase and its mutant. 1a &b-WT and compound-10. 2a &b- D8G and compound-10. 3a&b-K96T and compound 10 4a&b:S104R and compound -10. 5a&b: C138Yand compound-4. 6a & b: C138S and compound-4.

50s'. (2) The mutant K96T discharge values in the range of 30s' with the Compounds-1, 7-9 found to, followed by compounds-2-5, 10, 11 displayed in 40s series. Surprisingly, no compound showed the affinity in the range of fifty compared to other mutants for this mutant. (3) In case of mutant-S104R, compounds-1, 7, 8 found to discharge affinity in the range of 30s' and compounds-2-5, 9-11 displayed in 40s', with compound-10 in the range of fifty. The pattern of results obtained for the mutants D8G, K96T, S104R were found to be more or less similar. (4) The mutant-C138Y showed scores in 30s for compounds-1, 7, 8 and that for the compounds- 2, 9 were in 40s and for compounds-3-5, 10, 11 were in 50s. (5) The affinity of the mutant C138S with compounds-1, 7, 8 was in the 30s while the affinity of compounds-3 and 9 were in 40s and compounds -2, 4-7 in 50s series. Thus, the C138Y and C138S mutants showed more or less similar pattern of results compared to D8G, K96T and S104R mutants. The scores indicate that the compound-10 showed highest affinity with the WT and also with the mutants D8G, K96T, S104R whereas compound-4 displayed highest affinity with the mutants C138Y and C138S. Of interest, compound-6 showed significant lower values in degree of binding affinity with WT PZase and all its mutants (Table 3).

Hydrogen bond profile at the ligand binding sites

It is well known that Hydrogen (H) bonds play an important role in the maintenance of stability, structural integrity and function of biological molecules, especially for enzyme catalysis. Three H bonds formation was observed at the ligand binding regions in the WT with compound-10 whereas for the mutant S104R, 13-H bonds were found. This seems to be very important because none of the other mutants (D8G, K96T, C138Y and C138S) showed the involvement of the Cys138 residue in H bond formation, which in turn signifies the residue 138 is not perturb in this mutant. Further the mutants C138Y and C138S forms 1 and 8 H bonds with compound-4 respectively. The other mutants D8G and K96T forms 3 and 7 bonds, of notice the mutant K96T showed the involvement of two H bonds with the compound-10 (**Table 4**).

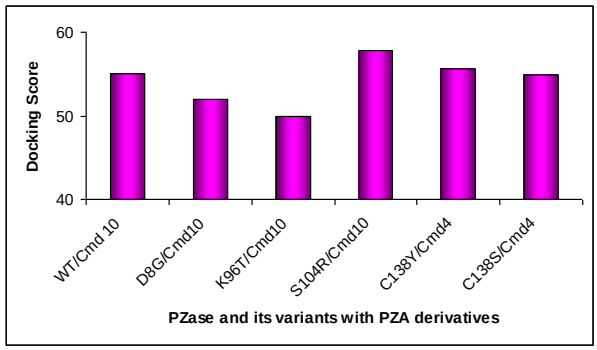

Figure 5. PZase and its mutants displaying highest affinity with PZA derivatives, Cmd = compound.

DISCUSSION

Genetic and molecular analysis of drug resistance in *Mtb* suggests that the bacilli usually acquire resistance either by alteration of the drug target through mutation or by titration of the drug through overproduction of the target. In this study, based on the concept of drug target alteration through mutation, five mutants of PZase were used for evaluation of chemically versatile analogues/derivatives of PZA. From this primary *in-silico* screening

Table 4. Details of H bond formation at the ligand binding site

PZase models	No. of Hb	Hb donor	Hb acceptor	Bond distance
D8G + Cmd10				
	3	HIS51:HN	CYS72:O	2.04
		LYS96:HZ2	ALA102:O	2.45
		LYS96:HZ3	ASP49:OD1	2.39
K96T+ Cmd10				
	7	GLY60:HN	HIS57:O	1.99
		THR61:HN	TRP68:O	1.84
		THR61:HG1	SER66:O	1.74
		SER66:HG	SER59:O	2.09
		SER66:HG	UNK1:O3	1.15
		SER67:HN	UNK1:O3	1.59
		TRP68:HN	SER59:O	2.04
S104R + Cmd10				
	13	LYS96:HZ2	TYR103:O	2.32
		LYS96:HZ3	ASP8:OD1	2.38
		LYS96:HZ3	ASP8:OD2	2.29
		ARG140:HN	THR135:O	2.41
		GLN141:HN	HIS137:O	2.06
		THR142:HN	CYS138:O	1.92
		THR142:HG1	CYS138:O	2.40
		GLU144:HN	ARG140:O	1.93
		ALA170:HN	ASP166:O	2.05
		ALA171:HN	THR167:O	1.94
		LEU172:HN	THR168:O	1.92
		GLU174:HN	ALA170:O	1.92
		MET175:HN	ALA171:O	1.92
C138Y + Cmd4				
	1	UNK1:H19	HIS71:NE2	2.39
C138S + Cmd4				
	8	PHE13:N	GLN10:O	3.04
		CYS14:N	ASN11:O	3.03
		GLY17:N	CYS14:O	2.89
		LEU19:N	ASP12:O	2.90
		VAL21:N	PHE13:O	2.66
		GLY24:N	VAL21:O	2.91
		ASP49:N	GLN10:OE1	2.83
		HIS51:N	CYS72:O	2.91
		HIS71:ND1	PRO69:O	2.81
		CYS72:N	ASP12:OD2	2.63

UNK1 = PZA derivative; Hb = Hydrogen bond

method, inter-differences between the binding affinities amongst these structurally diverse molecules were determined for the prediction of better leads which can be used as fundamental raw material for structure based drug designing.

Eleven molecular descriptors of PZA with various types of physicochemical, steric, geometrical, and electronic properties have been tested for its ability to bind with WT and mutants of PZase. However, in spite of its chemically complex nature, compound-10 displayed the highest score with three mutants and WT, also compound-4 with two mutants C138Y and C138S. Compared to other compounds and PZA itself, these two compounds (4, 10) displayed higher values (**Figure 5**). Another interesting insight comes from the analysis of ligand-6 in exhibiting lowest binding affinity (lower than that of parental PZA) with WT PZase and its mutants (D8G, K96T, S104R, C138Y and C138S).

Docking many ligands to the same protein followed by scoring them for their relative strength of interaction has been proposed as a procedure to identify candidates for drug development. In connection to which, in the present study, that the ligand-10 followed by compound-4, whose score was higher than other compounds, can be used as possible leads in the future for treatment of TB. Compound-6 whose score was lower than that of parental PZA, suggests that cannot be considered as lead for the TB drug development process. This has led to assumed that the rationale behind higher affinity lies in the structural complexity as well as the sterical demands imparted by these ligands relating to binding affinity. Thus, the finding has provided some insights towards understanding the basis for rationalization of PZA resistance in naturally occurring PZase mutant strains of *Mtb*. However further studies are needed to validate the binding aspects of PZase with PZA derivatives.

CONCLUSION

In this study, the models of PZase in association with PZA and its derivatives were determined, which may provide a basic structural framework for developing rational drug designing, also, can be useful for QSAR studies. The docking results has aided in predicting the best form of PZA to bind with mutants of PZase. The findings suggest that redesign of the PZA molecule to improve drug binding may be a viable approach to overcome resistance in PZase.

ACKNOWLEDGEMENTS

Dr. A. Nusrath Unissa received financial support from the Indian Council of Medical Research as Senior Research Fellow.

REFERENCES

1. Somoskovi A, Parsons LM, Salfinger M. The molecular basis of resistance to isoniazid, rifampin and pyrazinamide in *Mycobacterium tuberculosis*.Respir Res 2001; 2: 164-168.
2. Ramaswamy S and Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber. Lung. Dis.1998;79: 3-29.
3. Scorpio A and Zhang Y. Mutations in pncA, a gene encoding Pyrazinamidase/ nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat. Med. 1996; 2: 662-667.
4. Sreevatsan S, Pan X, Zhang Y, Kreiswirth BN and Musser JM. Mutations associated with pyrazinamide resistance in pncA of *Mycobacterium tuberculosis* complex organisms. Antimicrob Agents Chemother. 1997;41:636-640.

5. Lemaitre N, Sougakoff W, Truffot-pernot C and Jarlier V. Characterization of new Mutations in Pyrazinamide-Resistant Strains of *Mycobacterium tuberculosis* and Identification of Conserved Regions Important for the Catalytic Activity of the Pyrazinamidase PncA. Antimicrob Agents Chemother. 1999; 43:1761-1763.
6. Zhang Y, Scorpio A, Nikaido H and Sun Z. Role of Acid pH and Deficient Efflux of Pyrazionic Acid in Unique Susceptibility of *Mycobacterium tuberculosis* to yrazinamide. J. Bacteriol. 1999;181:2044-2049.
7. Lemaitre N, Callebaut I, Frenois F, Jarlier V and Sougakoff W. Study of the structure-activity relationships for the pyrazinamidase (PncA) from *Mycobacterium tuberculosis.* Biochem. J. 2001; 353:453-458.
8. Zhang H, Characterization of nicotinamidase/pyrazinamidase in *Mycobacterium tuberculosis.* FEBS J. 2008;275:753-762.
9. Petrella S, Gelus-Zietal N, Maudry A, Laurans C, Boudjelloul R, Sougakoff W Crystal Structure of the Pyrazinamidase of *Mycobacterium tuberculosis*: Insights into Natural and Acquired Resistance to Pyrazinamide PLoS One. 2011; 6 (1):e15785.
10. Quiliano M, Gutierrez AH, Gilman RH, López C, Evangelista W, Sotelo J, Sheen P, Mirko and Zimic M. Structure-Activity relationship in mutated pyrazinamidases from *Mycobacterium tuberculosis*. Bioinformation. 2011; 6(9): 335–339.
11. Nusrath Unissa A, Sameer Hassan, Selvakumar N and Narayanan PR. In silico studies on modeling of wild type and mutants of pyrazinamidase from *Mycobacterium tuberculosis* and docking with pyrazinamide. Int J App Bioeng. 2007; 1: 48-50.
12. Nusrath Unissa A, Sameer Hassan and Selvakumar N. Insight to pyrazinamide resistance in *Mycobacterium tuberculosis* by molecular docking. Bioinformation. 2009; 4: 24-29.
13. Jones G, Willett P and Glen RC. Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. J. Mol. Biol. 1995; 245: 43-53.