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IN SILICO STRUCTURAL DATA ANALYSIS TO THE IMPACT OF LEU27PRO AND ASP49GLY MUTATIONS ON PZA RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

This study reports on the structural and functional basis of pyrazinamide (PZA) resistance conferred by the mutations Leu27Pro and Asp49Gly in *pncA* gene as sequenced from a PZA resistant *Mycobacterium tuberculosis* strain. Molecular modelling studies of Wild Type (WT) and Mutant Types (MT1, MT2) of Pyrazinamidase (PZase) showed the mutation at Leu27Pro and Asp49Gly do not impact on the conformation of the protein. However, comparing all the docking score, energy and interaction pattern with wild type Pzase, the M1 Pzase has no significance influence of mutant residues Leu27Pro which is reside in the N-terminal helices α3. But in case of M2 (Asp49Gly) mutant Pzase, the Asp49Gly is the prime residues for their active functionality by forming coordination interaction with Fe^{2+} is lost due to the mutation, which subsequently effects in their binding energy and docking score of -25.533 kcal/mol and -5.093 respectively. Moreover, the orientation of the PZA molecules was shifted of about 2.06 Å with respect to C3 atom and moved apart from its interaction with Ala102 instead it forms a hydrogen bond between D8 OD1 and N3 by contact distance of 2.667. Though, other interaction was retained, the less efficiency nature of coordination interaction and proper interaction pattern of PZA with Pzase makes M2 mutant shown efficacy less interaction nature and which indirectly lead for the resistant against PZA.

KEYWORDS: **Mycobacterium tuberculosis, Pyrazinamide, Pyrazinamidase, Nicotinamidase, DNA sequencing**

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INTRODUCTION

Pyrazinamide (PZA) is an important first-line tuberculosis (TB) drug. Along with isoniazid (INH), rifampicin (RMP), and ethambutol (EMB), PZA is part of the currently used short course treatment regimen, also called DOTS (for directly observed therapy, short course) recommended by the World Health Organization[1]. PZA plays a unique role in achieving this shortened therapy, because PZA is believed to kill a population of semi dormant tubercle bacilli residing in an acidic environment (e.g., as in active inflammation sites with low pH)in vivo that may not be affected by other TB drugs. Despite its role in shortening the TB therapy, PZA has no apparent activity against tubercle bacilli under normal pH conditions; the activity is only present at acidic pH[2].PZA, an analog of nicotinamide, is not active against *M. tuberculosis* under normal culture conditions, but it is active in acid medium (pH 5.5) and in host macrophages. The mode of action of PZA is not understood. It is thought that the bacterial enzyme pyrazinamidase (PZase) is required to convert PZA to pyrazinoic acid (POA), which is toxic to *M. tuberculosis*. Resistance to PZA develops readily, and in a fashion analogous to INH resistance, PZA-resistant *M.tuberculosis* strains lose both PZase and nicotinamidase activities. These two enzyme activities are due to a single enzyme that acts on both nicotinamide and PZA. Loss of PZase correlates with resistance to PZA, and negative PZase tests for clinical isolates of *M. tuberculosis* are indicative of PZA resistance[3].The *pncA* gene encodes pyraziniamidase (PZase), and mutations in *pncA* are associated with resistance to PZA or loss of PZase activity. PZA acts by targeting the fatty acid synthase/synthetase enzyme, and is responsible for the killing of persistent tubercle bacilli in the initial intensive phase of chemotherapy. It is a prodrug that is converted to its active form namely, pyrazinoic acid (POA) by the catalytic action of PZase enzyme, encoded by the *pncA* gene in *M. tuberculosis.* Interestingly, PZA is active only at low pH since acidic environment favours accumulation of POA in the cytoplasm due to an ineffective efflux pump, thereby leading to improper efflux out of the amidase from the cell to the exterior[4]. Although a large number of mutations have been described, no mutational hotspots have been identified so far[5].This can be explained by the fact that mutations occur along the entire length of the *pncA* gene[6]. However, studies show that mutations that confirm the PZA resistance occur mainly in the putative promoter region of the *pncA* gene. 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*[7]. The uniqueness of 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).The crystal structure of the *M. tuberculosis pncA* protein has been determined, showing significant differences in the substrate binding cavity when compared to the pyrazinamidases from *Pyrococcus horikoshii* and *Acinetobacter baumanii*. In *M. tuberculosis*, this region was found to hold a Fe^{2+} ion coordinated by one aspartate and three histidines, the most crucial structural element in this loop appears to be the specific positioning of residue His57 which is directly involved in the coordination of the Fe^{2+} ion. The overall architecture of the pyrazinamidase of *M. tuberculosis* is similar to that reported for the other pyrazinamidases of *A. baumanii* and *P. horikoshii*[8].In the *pncA* model, the putative catalytic centre would be located in a pocket formed by one α-helix (αE), four β-strands (β1, β2, β3 and β4) consisting of β1 (Asp-8 and Phe-13), β2 (Asp-49), β3 (Lys-96), β4 (Ala-134 and Thr-135) and αE (Cys-138). In this pocket, the conserved active cysteine residue Cys-138 is located close to the conserved residues: Asp-8, Trp-68, Lys-96, Ser-104, Ala-134 and Thr-135. In the *pncA* model, the side chains of the two residues Asp-8 and Lys-96 are found to point towards Cys-138 of the active-site. The modification of the amino acid residues Asp-8, Lys-96 and Ser-104 in the mutants D8G, K96T and S104R resulted in enzymes showing specific activities drastically impaired (%0.004 unit mg), thus suggesting that these residues are essential for the *pncA* activity. The amino acids found at positions 8, 13, 61, 69, 96, 103, 104 and 146 are functionally and or structurally important in *pncA*[9]. Hence, in this study we attempt to utilize this empirical structural data to analyze to the impact of Leu27Pro and Asp49Gly mutation on PZA resistance.

MATERIALS AND METHODS

Patients attending the Tuberculosis Clinics (Puducherry, India) with signs and symptoms of pulmonary tuberculosis were examined. The patients included both males and females, irrespective of age. Based on the provisional clinical diagnosis of tuberculosis, the relevant investigations, including chest X-ray, staining, and culture by BACTEC and L-J of pulmonary samples, were performed. In the present study, a total number of 60 definite cases of multidrug resistant suspects (confirmed clinically and by lab testing) were chosen. The sputum samples collected from the pulmonary tuberculosis patients, hospitalized in Government Hospital for Chest Disease at Puducherry was used for this study.

Pyrazinamidase Assay (PZase Assay)

The PZase assay was performed by the method described in the Clinical Microbiology Procedure Handbook[10]. Briefly, 6.5g of Dubos broth base, 0.1g of PZA, 2.0g of sodium pyruvate and 15.0gof agar were dissolved in 1L of distilled water and heated to dissolve the components. The solution was dispensed in 5ml amounts into screw-cap tubes and stored at 2 to 8°C until use after solidification of the agar with the tubes in an upright position. A heavy loopful of growth from an actively growing subculture was inoculated. After incubation at 37°C for 4 or 7 days, 1ml of freshly prepared 1% ferrous ammonium sulphate was added to each tube. A pink band in the agar indicated a positive test.

Mycobacterium DNA Extraction

One loopful of culture was homogenized in 100 µl of sterile distilled water. The entire homogenized samples

were treated with 50 µl of lysozyme (10 mg/mL) at 37 °C for overnight incubation. 70 µl of 14% SDS and 6 µl of Proteinase K (10 mg/mL) was added and incubated at65 °C for 15 minutes. 10 µl of 5 M NaCl and 80 µl of CTAB/NaCl were added and were incubated at65 °C for 10 minutes. 800 µl of Phenol: Chloroform: Iso amyl alcohol (25:24:1) mixture was added and centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and 600 µlof Isopropanol was added to precipitate the DNA and incubated overnight at −20 °C and centrifuged at12, 000 rpm in 4°C for 10 minutes. The pellet was washed with 70% ethanol to remove any remaining solutes. The pellet was air-dried and was dissolved in 20 µl of 1× TE buffer[11].

PCR Amplification of pncA

The isolated template DNA was amplified using *pncA1* primers 5'GGCGTCATGGA CCCTATATC3' and *pncA*2: 5'CAACAGTTCATCCCGGTTC3[12]in an authorized thermal cycler (Eppendorf Gradient Cycler). The PCR cycling parameters were 95°C for 5 minutes; followed by 40 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute; and a final extension of 74°Cfor 10 minutes which yielded a 670bp product. For each PCR run, negative control and positive control were included in the study. The PCR results were considered valid only when the negative control was negative without amplicon and the positive control yielded specific band. The amplified PCR product was withdrawn from thermal cycler and run on a 2% Agarose gel in TAE buffer. The Ethidium bromide stained gels were observed in a UV Trans -illuminator and photographed using a Geldoc.

DNA Sequencing

The amplified PCR product *pncA* gene from clinical isolate strains were run on 2% Agarose gel and the PCR product purified using PCR purification kit (Invitrogen). DNA Sequencing of amplicons was carried out using an ABI prism 3110 automated DNA sequencer. Cycle sequencing of the amplified products was carried out using ABI Prism Big Dye terminator kit (Applied Biosystems, USA) following manufacturer's instruction. The sequences were analyzed by CLC protein work bench version 5 sequence alignment software. The sequences generated were compared with the wild type sequence (Genbank accession no X68081) by using multiple sequence alignment software to identify the presence of mutation or polymorphism.

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Tertiary structure prediction

The three dimensional structure of mutant (M1: L27P and M2: D49G) pyrazinamidase (Pzase) *pncA* from *M. tuberculosis* were modelled by Modeller 9v8 program using the wild type pyrazinamidase (PDB ID: 3PL1)[9] as the template. The alignment of both template and target was done using the align2D script in the Modeller 9V8[13, 14]. Then the aligned sequence is used for model generation using the model_ligand.py script in the Modeller9V8[13, 14]. Here, the coordinate information of the template Fe^{2+} was also used for the mutant Pzase model generation. From the ten models best model was chosen based on their Discrete Optimized Protein Energy (DOPE) and Modeller Objective Function (MOF) scores. The best model was further validated using structural analysis and verification server (SAVES), which includes various tools like Ramachandran plot[15], ERRAT[16]and VERIFY_3D[17].

Docking of PZA at the active site of Pzase

The pyrazinamide is the first line drug used in the treatment of tuberculosis was retrieved from the PubChem database as 3D conformer (http://pubchem.ncbi.nlm.nih. gov/search/search.cgi) and prepared using LigPrep module of Maestro 9.1 software by applying the OPLS 2005 force filed. Possible ionization states at pH 7.0 ± 2.0 were used to generate their specified chiralities. In case of Pzase the wild type were retrieved from the PDB database (ID: 3PL1) and the modelled mutant were prepared by protein preparation wizard of the Maestro 9.1 software (Schrodinger Software Suite 2010)[18]. The bond order and hydrogen were assigned to coordinate the protein molecules. The hydrogen bond of the protein molecule was also assigned based on the sample water orientations and optimized using exhaustive sampling methods. Further, the protein energy minimization was carried out by applying OPLS2005 force field. Further the receptor grid was generated for wild and mutant type Pzase by selecting the active site residues D8, F13, T61, P69, K96, Y103 and C138 as centroid with a 15 Å radius to dock the PZA molecule. Then, PZA was docked into the prepared proteins using the standard precision (SP) protocol of Maestro 9.1 with default parameter setting.

RESULTS AND DISCUSSION

The samples for this study were collected mainly on the basis of MDR suspect criteria of Revised National Tuberculosis Control Programme. All 60 MDR suspects sputum samples were processed for the Ziehl Nielsen straining and Auromine O phenol staining.Overall, 60 pooled Multi Drug Resistant suspects sputum specimens were tested by in house PCR assay to target *pncA* gene by using PCR mediated direct DNA sequencing.The results of the sequence analysis of *pncA* from 31 PZA-resistant isolates are presented in Table 1. The nature of the *pncA* mutations includes substitutions of amino acids (31 of 60 totals MDR isolates), insertions causing nonsense peptides (3 of 60 isolates). The distribution of *pncA* mutations is dispersed along the gene. The reason for treatment failure in the 29 PZA sensitive cases could be due to some other mechanisms. The susceptible clinical *M.tuberculosis* isolates were found to have identical wild-type sequence, while 75–95% of PZA resistant isolates had a point mutation (missense / nonsense or insertion / deletion) and these were spread over the entire length of the *pncA* gene[19, 20, 21]. It is generally considered that mutations leading to PZA resistance are scattered along the *pncA* gene[12, 22].Among the PZA resistant isolates, 31 (53%) exhibited 18 different changes in *pncA* nucleotide sequence (Table 1). The mutations in *pncA* included thirteen nucleotide substitutions causing amino acid change in 28 isolates, mutations caused by insertions in three isolates. However, it was observed that every isolate that presented a *pncA* alteration lacked PZase activity. Prior studies have shown that common mutations in the *pncA* are located in three regions, 3–17, 61–85 and 132–142[23]. In this study it is found that

one third of the mutations are distributed in the 132–142 region and 30.7% of mutations from 12 isolates are located in the 61–85 region. The regions 3–17 accounts for mutations in 6 (15%) of the resistant isolates. Overall, these three regions alone contribute to drug resistant mutations in 79.5% of the isolates. These three regions are important in the formation of the active site of the enzyme[23]. Twenty nine clinical isolates carried the wild-type *pncA* sequence and retained PZase activity. This finding supports the hypothesis that other mechanisms may be involved in PZA resistance - possibly alteration in Pyrazinamide uptake, increased POA active efflux[22] or mutations leading to the modification or amplification of an unknown POA target[21].

Table 1
pncA nucleotide and amino acid changes in PZase–negative M.tuberculosis isolates

S.No.	Amino acid changes	No of isolates	Percentage (%)
1.	Asp 63 Gly	1	3.23
2.	Ser 185 Ala	1	3.23
3.	Val 7 Ala	2	6.46
4.	Leu 27 Pro4	3	9.69
5.	Asp 12 Ala	1	3.23
6.	Ile 5 Ser	3	9.69
7.	Val 139 Ala	3	9.69
8.	Glu141Pro	1	3.23
9.	Leu27Pro	2	6.46
10.	Ala171Pro	2	6.46
11.	Val180Gly	1	3.23
12.	Asp129Gly	1	3.23
13.	Asp49Gly	2	6.46
14	Val44Asp	1	3.23
15	Asp 16 B	4	12.92
16	T 142 Ala	1	3.23
17	Val139 Ala	1	3.23
18	Ser 66 Val	1	3.23

The structure of wild and mutant type of Pzase

The crystallographic structure of Pzase is made of a six-stranded parallel beta sheet with helices packed on either side to form a/b single domain (Fig 1A). Here, there are totally eight alpha helices α1-α2 and six beta sheet β1-β6. The active resides D8 residues in the loop connecting β1 to α1, F13 in α1, T61 and P69 resides in the loop connecting α3-α4, β3 and α7 bears the K96 and C138 active site residues and Y103 resides in the loop connecting β3-α5. The metal binding residues H51, H57, H71 and D49 makes coordination interaction with Fe^{2+}. As combination of both active and metal binding site constitutes the binding cavity which includes C138-D8-K96 motif involved in cysteine-based catalytic mechanism of substrates. The mutant structure M1 has the mutation at the position of L27P (Fig 1B) whereas M2 has at the position of D49G (Fig1C) been clearly mentioned in the structure with sphere representation. Moreover, there is no structural difference were observed in the modelled structure of mutant type of Pzase. Also the validation analysis, which showed that all models had good quality factors and were reliable (Table 2) for further studies.

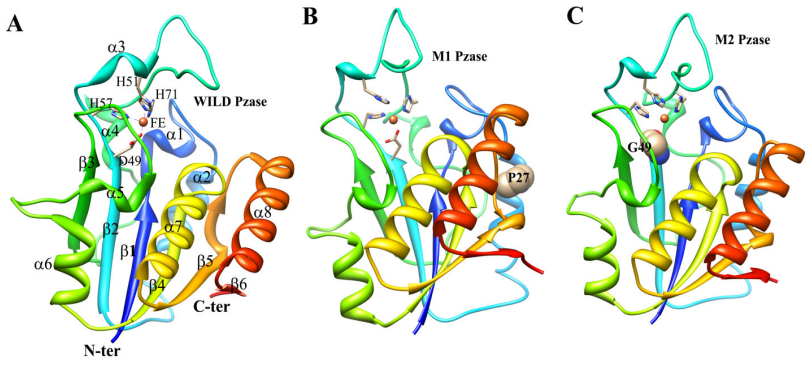

Figure 1
The three dimensional structure of wild and mutant M1 and M2Pzase protein. A) The wild type Pzase protein shown with helices α1-α8 and sheets β1-β6. The FE^{2+} coordinate interaction with H51, H57, H71 and D49 were shown. B & C) shown same representation of M1 (L27P) and M2 (D49G) mutation respectively, where the mutant residues were shown in sphere representation.

Table 2
The validation report of wild and mutant Pzase

Validation methods	Wild Pzase	M1 Pzase	M2 Pzase
Ramachandran Plot Assessment			
Residues in favored region (%)	92.3	91.3	92.9
Residues in allowed region (%)	6.6	7.1	5.4
Residues in outlier region (%)	1.1	1.6	1.6
ERRAT			
Overall quality factor	92.045	87.079	94.381
Verify3D			
The percentage of the residues had an averaged 3D-1D score >=0.2	98.92	100	100

Interaction analysis of PZA with wild type Pzase

The substrate pyrazinamide (PZA) forms stable interaction with wild type Pzase active site with the glide docking energy of -35.498 kcal/mol (Table 3). The hydrophobic residues A102 forms backbone hydrogen bond with N_3 of PZA with a contact distance of 2.744 Å. Also, the W68, H137 and F13 forms hydrophobic cavity and initiates π-π and T-shaped π interaction with PZA. Accordingly the aromatic ring of PZA forms π-π interaction with ring of W68 and H137 with a contact distance of 4.9 and 5.7 Å respectively (Fig 2A). The PZA ring also forms T-shaped interaction with F13 by a distance of 4.2 Å. Along with that, PZA O1 forms metal coordination interaction with FE^{2+}. Moreover, the coordination interaction formed between Fe^{2+} and H51, H57, H71 and D49 also maintained. Other active site residues such as hydrophobic nature (L19, C138 and Y103), polar (H137 and H71) and charged (K96 and D49) residues were also contributed for the VdW interaction with PZA and stabilized their interaction. All these interactions were discussed here are well correlated with already proven interacting complex published already.

Interaction analysis of PZA with mutant Pzase

As similar to wild type Pzase interaction with PZA, M1 mutant of Pzase also forms backbone hydrogen bond between A102 with N_3 atom of PZA by contact distance of 2.894Å with total glide docking energy of -35.714 kcal/mol (Table 2). Similarly, M1 mutant Pzase forms hydrophobic packet constituted by W68, H137 and F13 and forms π-π and T-shaped π interaction with PZA (Fig 2B). Other residues hydrophobic in nature (L19, C138, Y103, V163 and A134), polar (H71) and charged (K96 and D49) also forms strong interaction with PZA as similar to the wild type interaction pattern. In addition, the coordinates interaction formed by Fe^{2+} and PZA also reside in this mutant complex. Hence, comparing all the docking score, energy and interaction pattern with wild type Pzase, the M1 Pzase has no significance influence of mutant residues L27P which is reside in the N-terminal helices α3. But in case of M2 (D49G) mutant Pzase, the D49 is the prime residues for their active functionality by forming coordination interaction with Fe^{2+} is lost due to the mutation, which subsequently effects in their binding energy and docking score of -25.533 kcal/mol and -5.093 respectively (Table 2). Moreover, the orientation of the PZA molecules was shifted of about 2.06 Å with respect to C_3 atom and moved apart from its interaction with A102 instead it forms a hydrogen bond between D8 OD1 and N_3 by contact distance of 2.667. Further, the hydrophobic pocket formulated by F13 and H137 interaction was lost. Only, the T-shaped interaction was observed between W68 and aromatic ring of PZA (3.3 Å). Other interaction, hydrophobic (A102, C138, F13, L19 and W68), polar (H71, H57 and H51) and charged (K96 and D8) retains their interaction with PZA (Fig 2C). Though, other interaction was retained, the less efficiency nature of coordination interaction and proper interaction pattern of PZA with Pzase makes M2 mutant shown efficacy less interaction nature and which indirectly lead for the resistant against PZA.

Table 3
Interaction score, energy and interacting residues of wild and mutant Pzase with PZA.

Interaction with PZA	Glide docking score	Glide Energy (kcal/mol)	Hydrogen bond		Other interaction residues
			Bond	Distance (Å)	
Wild type Pzase	-7.319	-35.498	A102-OD2::N3-ZA	2.744	F13, L19, D49, H51, H57, W68, H71, K96, H137, A102, Y103, A134, C138
M1mutant Pzase	-7.262	-35.714	A102-OD2::N3-ZA	2.894	F13, L19, D49, H51, H57, W68, H71, K96, H137, A102, Y103, A134, C138
M2 mutant Pzase	-5.093	-25.533	D8-OD1::N3-PZA	2.667	D8, F13, L19, G49, H51, H57, W68, H71, K96, C138

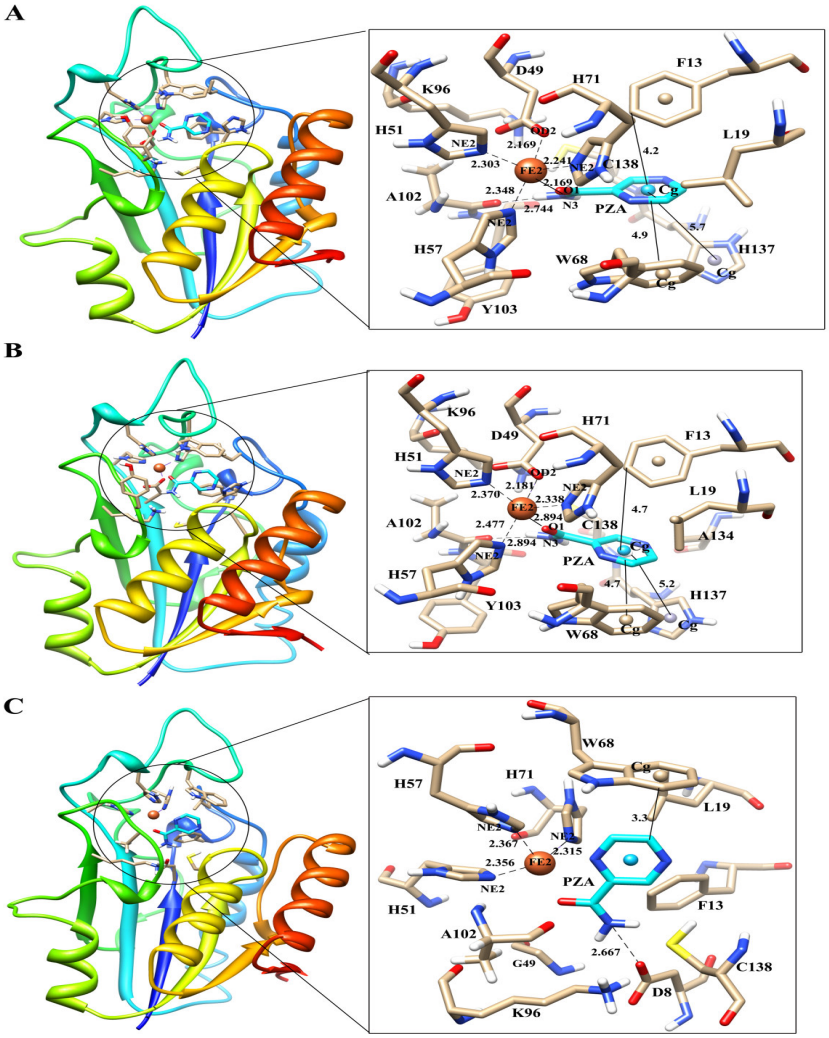

Figure 2

The interaction of PZA with wild and Mutant (M1 and M2) Pzase. A) The interaction network of PZA with wild type Pzase B) interaction of PZA with M1 mutant Pzase and C) interaction mode of PZA with M2 mutant Pzase. Here, the PZA were shown in cyan colour with hetero atom representation whereas the residues were shown in tan colour. Hydrogen bonds are denoted as dotted dashed line whereas the special types of interaction are shown in line representation.

CONCLUSION

Molecular modelling and docking analysis showed that Mutant Types has a higher inhibitory constant than Wild Type in terms of PZA binding, which indicates that the drug affinity is highly affected in the Mutant Types due to structural changes. The molecular dynamics simulation and gyration analysis together infer the change in flexibility at the active site cavity of Mutant Types. Hence, this study gives insight on the impact of mutations on the activity of this protein which can be attributed to the drug resistance observed. This study also presents a need for the discovery of potential newer drug molecules against mutant types.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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