Effect of mutations on the structure of Mycobacterium tuberculosis KatG protein and interactions with Isoniazid - An in-silico study

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Abstract
Fight against tuberculosis (TB) is still ongoing in India. The problem of drug resistance in Mycobacterium tuberculosis (M.tb) has obligated the need of studying key enzymes involved in drug interaction. One of the known M.tb enzyme targets for the primary drug isoniazid is catalase-peroxidase (KatG).

In this study, we induced mutations (observed in clinical samples) at specific locations around the catalytic binding site of KatG protein and elucidated the impact of these mutations on the protein structural stability, secondary structure deformation and binding interaction with the drug ligand. It is clear from this study that mutations near the heme region have impacted the structure of the protein and the binding pattern with the drug. It can be hypothesized that this change leads to inhibition of KatG protein catalase and free radical generation activity. This could impact the KatG-Isoniazid interaction and overall make the bacteria resistant to the drug.

Keywords: Catalase-Peroxidase Enzyme, KatG, Secondary Structure, Structure Stability, Drug Resistance.

Introduction
Tuberculosis (TB) is one of the leading causes of death and the second known infectious disease of the respiratory tract after COVID-19 with a remarkably high mortality rate worldwide. Even though, there was a reduction in TB cases observed during the COVID-19 waves in India, the National Tuberculosis Elimination Programme (NTEP) noted down that the percentage of active TB cases was high. In 2021, there were 19,33,381 TB cases as compared to 16,28,161 in the year 2020. In 2021, 21,35,830 drug-sensitive TB cases were diagnosed and 30,509 (95%) were under different treatment strategies. Both public and private sectors had around 83% and 82% treatment success rates.

However, in 2021, 48,232 multidrug-resistant tuberculosis/rifampicin-resistant patients were diagnosed, making TB treatment difficult even today. Regulating the spread of TB, especially drug-resistant TB became an alarming issue in 2020. The number of TB infections was higher than infections caused by HIV/AIDS. Mortality rates due to COVID-19/TB in 2020 were also higher than HIV/AIDS. Therefore, elucidation of the mycobacterial drug resistance properties has become crucial.

Isonicotinic acid hydrazide/Isoniazid is a prodrug used as a first-line drug against TB and is known to reduce the overall bacterial load in the initial phase of therapy. It was first reported in 1952. An isoniazid monotherapy (duration: 9 months) is used for treating latent infections. However, it has been reported that when isoniazid is used alone, mutations in katG, inhA, kasA and ahpC genes make M.tb resistant to isoniazid.

The catalase-peroxidase enzyme (KatG) is encoded by the katG gene. It is a bifunctional hemoprotein with a heme prosthetic group. This enzyme due to its catalase and peroxidase activities plays a role in protecting M.tb from ROS damage. However, it also has detrimental effects when isoniazid is present. In presence of the drug, KatG activates the isoniazid by oxidizing it leading to the formation of isonicotinoyl radicals, which further form an isoniazid-NAD adduct with NAD+/NADH. This adduct inhibits the action of other genes inhA, kasA and ahpC and thus inhibits mycolic acid synthesis in M.tb rendering it susceptible to isoniazid. Therefore, it is important to study the enzyme in detail, as it will further aid in understanding the mechanism used by the TB bacillus to survive and persist in the host. Most importantly the study will also help to develop an improved isoniazid therapy.

Evaluation of clinical samples revealed multiple mutations associated with KatG protein that displays differential levels of resistance to isoniazid. Studies have proved that S315T mutation in the katG gene has contributed significantly to isoniazid resistance. Brossier F. et al. have shown the presence of mutations G118D, G121D, L121P and S315T leading to high isoniazid resistance and impaired catalase and peroxidase activities, reducing free radical production and isonicotinoyl-NAD formation. KatG protein requires residues close to the heme region for its activity and adjacent residues at the heme-binding region consist of a proximal and distal pocket.

Residue 336 near the proximal pocket, residues 118 and 121 at the entrance to the distal pocket and residue 141 at the distal pocket are considered for this study. The putative binding site regions of the isoniazid ligand in the KatG protein were correlated to 3WXO, the KatG protein from Synechococcus elongatus and were used for further experimentation.
In general, changes in the amino acid residues influence the structure and function of the protein. The present study focuses on determining the overall impact of G118D, G121D, L121P and L336S mutations of KatG protein observed among clinical samples and highlights the overall structural stability of the protein in terms of secondary structural changes. Further, the study also intends to explore the interaction of isoniazid with putative binding sites suggested by literature and to decipher the impact and mutations on the interaction.

Material and Methods

Selection of wild-type KatG and preparation of mutant KatG: The KatG protein selected for the study was a crystal structure of M.tb catalase-peroxidase (PDB ID: 1SJ2) with a resolution of 2.41 Å retrieved from the RCSB Protein Data Bank. Substitution mutations were induced in KatG using the Dunbrack rotamer library embedded in Chimera software.14,15

Predicting the impact of mutations on KatG function: The impact of clinical mutations (G118D, G121D, L121P and L336S) on the biological function of the protein was evaluated using Protein Variation Effect Analyzer (PROVEAN) tool and changes imposed by the mutations on its structure and function were analyzed using Missense 3D online server. For analysis, both the KatG protein sequence P9WIE5 (Uniprot Id) and protein structure 1SJ2 were used.

Protein-ligand interaction study: The putative binding sites suggested for Synechococcus elongatus catalase (KatG) protein (PDB ID: 3WXO) were mapped on KatG of M.tb using pairwise sequence alignment [data not shown] and 3 putative binding sites of 1SJ2 were identified. Molecular docking studies were performed to determine the binding affinities of isoniazid [Pubchem Id: 3767] and KatG using Autodock 4.2 MGL tools. After selecting the best pose for docking, molecular docking was also performed on the mutant KatG model and isoniazid.

Analysis of protein dynamics using molecular dynamics (MD) simulations: The wild-type protein was prepared using the protein preparation wizard in maestro and refined to optimize any overlapping atoms observed in the crystallized protein structure. Water beyond 5 Å in heterogeneous atoms was eliminated and bond order and hydrogenation of the structure were performed. For the study, we worked on chain A of the KatG protein. Further, a system was built for the protein to study MD. An orthorhombic box was used to solvate the protein. After orienting the protein in the box, it was subjected to MD simulation studies with a sampling interval of 100ns and an NPT ensemble. The same processing steps were followed for the mutant KatG protein and evaluation of binding free energy and interaction analysis was done using the Desmond41 package.

To understand the interaction analysis between KatG and isoniazid, the Simulation Interactions Diagram tool in maestro was used for analyzing the Desmond trajectory files generated. Data on protein root mean square deviation (RMSD), root-mean-square fluctuation (RMSF) for proteins and secondary structure analysis were obtained.

Results and Discussion

Selection of KatG protein and preparation of mutants: KatG is a hemoprotein that exhibits catalase-peroxidase activity and helps in the generation of free radicals. Brossier et al4 reported that point mutations L336P, G118D, G121D and L141S highlighted a decrease in catalase and free radical generation properties. This study focused on determining the effect of these mutations on protein stability and further evaluated the interaction pattern of KatG with isoniazid. Prodrugs like isoniazid require KatG for activation which helps them target the mycolic acid synthesis pathway and overall makes the M.tb susceptible to lysis. In this study, 1SJ2 was the protein structure selected from PDB for our analysis. Mutations were induced and the regions of mutations on KatG are highlighted in figure 1.

Figure 1: Figure highlighting the mutated regions (pink) in a KatG WT protein structure.
Predicting the impact of mutations on KatG function:
The impact of mutations on KatG biological function is predicted using the PROVEAN tool and the impact on structure and function analyzed by missense 3D is represented in table 1. For PROVEAN analysis, a default threshold of -2.5 is considered neutral and values below the threshold are deleterious. The analysis showed that all the mutations were deleterious with a score below -5.0. The Missense 3D analysis of KatG protein showed the damaging effect at the structural level caused by L336P, G118D and G121D mutations except for L141S.

Protein-ligand interaction studies: The putative binding sites of KatG-Isoniazid interaction suggested for Synechococcus elongatus were used as a reference for *M. tb* protein. The binding pockets and binding energy showing the interaction between the KatG-Isoniazid are highlighted in table 2. Binding site 1 showed lower binding energy demonstrating stable interaction than the other 2 putative binding sites. Binding site 1 was considered for further studies to analyze the impact of KatG mutants. The interaction of KatG mutant and isoniazid showed a change in binding energy to -4.00 Kcal/Mol. KatG protein-isoniazid ligand interaction profiling showed hydrophobic interactions and hydrogen bonds being formed between the ligand and the protein. The interacting residues, the distance between the atoms of ligand and protein and the donor-acceptor status for hydrogen bond are highlighted in table 3 and 4 and diagrammatically in figure 2.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>PROVEAN analysis</th>
<th>Missense 3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>G118D</td>
<td>ROVEAN score -6.861, Deleterious, Buried Gly replaced, Buried charge introduced</td>
<td>Damaging</td>
</tr>
<tr>
<td>G121D</td>
<td>ROVEAN score -6.847, Deleterious, Triggers disallowed phi/psi alert. The phi/psi angles are in the favored region for wild-type residue but the outlier region for mutant residue. Buried Gly replaced replaces glycine originally located in a bending curvature</td>
<td>Damaging</td>
</tr>
<tr>
<td>L141S</td>
<td>ROVEAN score -5.884, Deleterious, Nil</td>
<td>No structural damage detected</td>
</tr>
<tr>
<td>L336P</td>
<td>ROVEAN score -6.831, Deleterious, Introduces a buried proline.</td>
<td>Damaging</td>
</tr>
</tbody>
</table>

Table 1
Table depicting the impact of mutations on KatG function and structure using PROVEAN and Missense 3D database.

<table>
<thead>
<tr>
<th>Putative binding site</th>
<th>Residues</th>
<th>Binding affinities of WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding site 1</td>
<td>Pro136, Asp137, Leu205, Leu227, Asn231, Pro232, Ser315</td>
<td>-5.44 Kcal/Mol</td>
</tr>
<tr>
<td>Binding site 2</td>
<td>Trp90, Trp91, Lys143, Lys301, Gly307, Thr308</td>
<td>-3.30 Kcal/Mol</td>
</tr>
<tr>
<td>Binding site 3</td>
<td>Ala278, Lys310, Asp311, Pro347, Arg373</td>
<td>-2.67 Kcal/Mol</td>
</tr>
</tbody>
</table>

Figure 2: Diagrammatic representation of hydrophobic interactions and hydrogen-bonding pattern between the (A) KatG WT and Isoniazid [orange ring] and (B) KatG MT and Isoniazid [orange ring].
## Table 3
Table depicting the hydrophobic interactions and hydrogen-bonding pattern between the KatG WT and Isoniazid.

<table>
<thead>
<tr>
<th>Hydrophobic interactions</th>
<th>Hydrogen bonding pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Index</strong></td>
<td><strong>Residue</strong></td>
</tr>
<tr>
<td>1</td>
<td>228A</td>
</tr>
<tr>
<td>2</td>
<td>108A</td>
</tr>
<tr>
<td>3</td>
<td>137A</td>
</tr>
<tr>
<td>4</td>
<td>229A</td>
</tr>
<tr>
<td>5</td>
<td>230A</td>
</tr>
</tbody>
</table>

## Table 4
Table depicting the hydrophobic interactions and hydrogen-bonding pattern between the KatG MT and Isoniazid.

<table>
<thead>
<tr>
<th>Hydrophobic interactions</th>
<th>Hydrogen bonding pattern</th>
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<tbody>
<tr>
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<td>3</td>
<td>229A</td>
</tr>
<tr>
<td>4</td>
<td>230A</td>
</tr>
</tbody>
</table>

### Analysis of protein dynamics using MD simulations:

The dynamic behavior of protein in terms of RMSD and RMSF was analyzed for the KatG Wild Type (KatG WT) and KatG Mutant Type (KatG MT) using Desmond molecular simulation software. The RMSD graphs of the WT and MT were designed and compared after inducing mutations near the distal and proximal pockets of KatG MT. Figure 3 highlights the variation patterns observed for 100 ns simulations. In these graphs, the stability pattern of the WT and MT protein backbone is shown. Throughout the trajectory, there was an overall stable pattern observed in the WT protein whereas in the case of MT, the protein equilibrated after 60 ns at 5.6 Å.

The RMSF measures the deviation of a particle on an average, from a particular position over time. Therefore, with the help of RMSF graphs, we can analyze the portions in the protein structure that fluctuate the most/least from their mean structure. In both the WT and MT KatG with/without the isoniazid, the terminal regions showed a peak as they tend to fluctuate the most at the N terminal. The area of the proteins that fluctuates the most, is depicted by peaks in other regions of the proteins also (Figure 4). Overall, the WT protein structure was stable except with high peaks observed at positions: alanine 531 (4.10 Å), serine 530 (3.67 Å), alanine 532 (3.44 Å) and glycine 358 (2.95 Å) that depicted large fluctuations.
Figure 3: RMSD graphs for the backbone of WT, MT (without the ligand) and WT, MT (with the ligand). The plot of RMSD for (A) wild type (B) mutant for 100 ns of simulation (C) KatG WT with Isoniazid and (D) KatG MT with Isoniazid [The orange line represents the ligand RMSD]

Figure 4: RMSF graphs for the backbone of WT, MT (without the ligand) and WT, MT (with the ligand). The plot of RMSF for (A) wild type (B) mutant for 100 ns of simulation (C) wild type with Isoniazid and (D) mutant type with Isoniazid.

Similarly, in the MT, peaks were observed at positions: alanine 531 (6.28 Å), alanine 532 (5.51 Å), glycine 358 (4.34 Å), serine 530 (4.11 Å) and lysine 213 (4.07 Å) that also depicted large fluctuations.

Secondary structure analysis of WT and MT KatG: On analyzing the secondary structure elements (SSE) (Figure 5) contributing to the overall stability of the KatG protein, it was observed that all the protein structures maintained an SSE value of above 75% and were composed for helical structures rather than strands and loop regions with minor differences. The KatG protein consists of 700 amino acids. The KatG MT showed a reduction in SSE percentage at residue 310 and residue 570 for beta sheets. Therefore, this indicates that there were secondary structural changes after inducing mutations. Similarly, secondary structural changes were observed between the KatG WT-Isoniazid complex and KatG MT-Isoniazid complex obtained after docking. Helices at residue 310 reduced in the KatG MT-Isoniazid structure when compared with the KatG WT-Isoniazid structure. At residue 570, loop regions were converted to sheets leading to changes in KatG protein conformation.

Protein and ligand contact studies of WT and MT KatG docked with isoniazid: As shown in the KatG-Isoniazid interaction diagram (Figure 6), a change in the binding pattern was observed in the KatG WT bound to isoniazid when compared to KatG MT bound to isoniazid. There were more water bridges observed during the trajectory in the MT. The mutations induced have led to conformational changes in the protein which has changed the interaction patterns as suggested by the Missense 3D prediction.
Figure 5: Changes in protein secondary structure at different residues in (A) WT, (B) MT (without Isoniazid) and (C) WT, (D) MT (with Isoniazid).

Figure 6: Ligand-protein interaction pattern observed in (A) KatG WT, (B) KatG MT (with Isoniazid).

Figure 7: Protein-ligand bonding pattern observed in (A) KatG WT, (B) KatG MT (with Isoniazid).

Figure 7 highlights the bonds formed between the KatG WT and isoniazid during the entire run time. KatG MT bound to isoniazid showed a hydrophobic interaction maintained at 26% during the time compared to the 17% hydrophobic interaction maintained at residue 232 in the WT protein.

There were more water bridges formed after the protein was mutated. In the MT, the water bridges were maintained at residue 314, 65.25% of the simulation time. Ionic bond interactions were lost after mutating the protein.

In general, binding of the ligand involves breaking hydrogen bonding with the help of water molecules and further leading to the formation of new hydrogen bonds\textsuperscript{17}. At residue 108, 80.5% of the simulation time, the H-bond interaction was maintained and more water bridges were observed. This
change was observed because the KatG MT has a high affinity for isoniazid and thus forms a bond.

These results show that mutations at sites in the distal and proximal pockets of the heme active site are instrumental in changing the protein folding pattern, affecting the secondary structure of the protein as well as changing the binding pattern of a ligand. The mutations simulated in this study may have affected the charge transfer pathways or had an overall impact on the loop-forming regions that are known to support hydrogen-bonding networks that participate in isoniazid binding.

Conclusion
Isoniazid is one of the most important primary anti-TB drugs. Free radical generation and catalase activity are significant in the adduct formation step which is a crucial step in the KatG-isoniazid interaction.

This study determined the effect of distal and proximal pocket mutations on the structure and function of the KatG protein. The sequence and structural analysis of the mutant KatG showed a deleterious biological effect and structurally damaging impact. Putative pockets showed variation in binding energy between KatG and isoniazid for suggested binding sites. The interaction in terms of binding energy was higher, indicating temporary adduct formation by KatG. Mutations induced in the KatG protein affected the catalase and free radical generation activity leading to an overall reduction in the protein function. Mutations also led to changes in the secondary structure, the overall folding pattern of KatG and binding patterns with the isoniazid in a dynamic environment.

Acknowledgement
The authors would like to thank St Xavier’s College (Autonomous), Mumbai for providing the computational facility for this in silico project. Special thanks go to DBT-Builder for the grant in procuring the molecular dynamic simulation facility used extensively for this study. We are grateful to Dr. Elvis Martis and Ms. Afreen Khan from Bombay College of Pharmacy, Mumbai for sharing their expertise in computational analysis.

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