

# Diagnosis and Management of Tuberculosis

## Chapter 2

### New Insights to Resistance of a Novel Drug Bedaquiline using *in-vitro* Mutants of ATP Synthase in *Mycobacterium Tuberculosis*

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#### Abstract

Bedaquiline (BDQ) is the new first-in-class anti-tuberculosis (TB) compound belonging to the class of diarylquinolone with activity against drug-sensitive and drug-resistant *Mycobacterium tuberculosis* (*M. tuberculosis*). This novel drug has the immense potential to shorten TB treatment duration and has been advocated for multi-drug resistant (MDR)-TB treatment. Therefore, BDQ resistance can be considered as a major public health problem and molecular investigation of the same is the utmost need of the hour. The target based concept of resistance to BDQ is caused by mutation in c-ring of adenosine tri phosphate (ATP) synthase, a critical enzyme in the synthesis of ATP in *M. tuberculosis* coded by *atpE* gene. BDQ inhibits the proton pump of mycobacterial ATP synthase. To understand the molecular basis of BDQ resistance using mutants (MTs) as the emergence of strains resistant to BDQ may pose a potential threat to the TB control program, we undertook a initiative to study seven *in vitro* mutants of *AtpE* viz., Asp28Gly, Asp28Ala, Asp28Pro, Asp28Val, Glu61-Asp, Ala63Pro and Ile66Met (D28G, D28A, D28P D28V, E61D, A63P, and I66M) which involves in resistance against BDQ. Molecular modeling and docking was performed to understand the interacting behaviour of mutant (MT) enzymes with BDQ in comparison to wild-type (WT). These results indicate that the substitutions in *AtpE* except D28G showed high affinity towards the drug in comparison to the WT. This could be due to the favourable interactions in mutants compared to WT.

It can be inferred from this concise analysis that the mutants (D28A, D28P D28V, E61D, A63P, and I66M) due to high affinity binds with the BDQ tightly leading to slow or no release of the BDQ which eventually results in high level BDQ resistance, compared with D28G that could lead to low-level resistance.

**key words:** Mycobacterium tuberculosis; BDQ resistance; ATP synthase; mutants

## 1. Introduction

Despite being a controllable, preventable and curable disease, tuberculosis (TB) still remain as a major public health problem in many parts of the world. The increase in multi-drug resistant (MDR-TB) is defined as resistance to the two most effective first line TB drugs: rifampicin (RIF) and isoniazid (INH) has intensified the magnitude of the situation. Extensively drug-resistant TB (XDR-TB) is emerging as an even more ominous threat, that is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs (capreomycin, kanamycin, and amikacin), in addition to INH and RIF. Drug resistance in TB is essentially a potential threat to the TB control programs. The most recent drug-resistance surveillance data issued by the World health organization (WHO) estimates that an average of roughly 9 % of MDR-TB cases are XDR-TB [1].

### 1.2. Properties of Bedaquiline (BDQ)

Bedaquiline (BDQ) is also known as TMC207 or R207910, is a drug belonging to the class of diarylquinoline and found to be effective against both drug susceptible and drug resistance TB [2]. It is a very promising and relatively new candidate drug for treatment of TB and mycobacterial infections [2]. The activity spectrum of TMC207 includes the mycobacterial species that are pathogenic to humans, such as *Mycobacterium tuberculosis* (*M. tuberculosis*), but also atypical pathogenic species, such as *M. avium complex*, *M. kansasii*, and the fast growers *M. fortuitum* and *M. abscessus* [2]. It is found to be active within macrophages, and an important agent in shortening the duration of anti- TB treatment [3].

### 1.3. BDQ in MDR treatment

Preclinical studies have shown the efficacy of BDQ in terms of reduction in bacterial load and treatment duration. Based on the positive influence of BDQ for treatment of MDR-TB [4], in 2012, food and drug administration (FDA) approved BDQ for treatment of MDR-TB and XDR-TB, followed this many clinical trials were undertaken and was also evaluated in a multi-centric study for treatment of MDR-TB and XDR-TB [5]. Phase II clinical studies have established the safety, tolerability and earlier sputum conversion time in patients with MDR-TB [6]. It is currently in phase III clinical trials for patients with MDR-TB. Recently, a study from France based on large proportion of patients showed that BDQ-containing regimens achieved favourable outcomes and prolonged treatment was overall well tolerated in their cohort [7].

## 1.4. Mechanism of action of BDQ

BDQ exhibits a novel mechanism of action which efficiently inhibits the adenosine 5-triphosphate (ATP) synthase of mycobacteria such as *M. tuberculosis* [2]. It targets subunit c of the ATP synthase of *M. tuberculosis*, leading to inhibition of the activity of proton pump in the enzyme [8]. The structure of ATP synthase was described in terms of two sectors, a membranous  $F_0$  ( $ab_2c_{10-15}$ ) and a membrane-extrinsic  $F_1$  ( $\alpha_3\beta_3\gamma\delta\epsilon$ ) [9]. Binding of BDQ at the level of the proton-binding site to the oligomeric and proteolipic subunit c in  $F_0$  domain of ATP synthase blocks rotation of discs of ATP synthase, which culminates in the inhibition of ATP synthesis and eventually to the death of mycobacteria [10,11].

## 1.5. Mechanism of resistance of BDQ

Target based resistance was found to occur in resistant isolates with mutations in subunit c at positions 28 (Asp→Gly/Ala/Val/Pro), 61 (Glu→Asp), 63 (Ala→Pro), and 66 (Ile→Met) based on the *M. tuberculosis* amino acid numbering system [12,13]. The assumption was supported by the fact that the mycobacterial species naturally resistant to BDQ, i.e., *M. xenopi*, *M. novacastrense*, and *M. shimoidei*, display a Met at position 63 in subunit c in place of a conserved Ala in the species susceptible to the drug [12,13].

Recent elucidation of crystal structure of ATP synthase from *M. Phlei* (4VIF, 4VIG) [14], enables the mechanism of action and resistance of BDQ in *M. tuberculosis* in a more precise manner compared to earlier studies [12,15]. In the light of the above in this study, to understand the molecular basis of BDQ resistance, seven mutants (MT) proteins of ATP synthase with important resistant mutations at codon position 28 such as Asp→Gly/Ala/ /Pro/Val (D28G, D28A, D28P and D28V) and the remaining three mutants at codon 61, 63 and 66 with Asp, Pro, Met in place of Glu, Ala and Ile (E61D, A63P, and I66M) were modeled and docked with BDQ using *in silico* approaches.

## 2. Materials and Methods

### 2.1. Proteins: Template selection and model building

In the present study, ATP synthase was modeled using MODELLER 9.14 [16], because its crystal structure is so far not deduced. ATP synthase is coded by *atpE* gene; the target *AtpE* protein (Rv1305) sequence of *M. tuberculosis* obtained from the Tuberculist database was submitted to BLASTp [17] program and searched against protein data bank (PDB). The WT protein from *M. phlei* (PDB code-4V1F) [14] was considered as template protein displaying maximum identity with the WT protein. In addition, the minimum inhibitory concentration (MIC) reported for *M. phlei* is very low (0.05 mg/ml) and basically identical to that reported for *M. tuberculosis* (0.06 mg/ml) [18]. In the PDB file of template 4V1F, heteroatoms such

as water and other chains were removed; retaining chain A and command line options were provided for model building in MODELLER9v14. The software aligns the FASTA sequence of template and target, following which it takes up the PDB template file and generates the model based on the concept of sequence predict structure. Sequence alignment between WT and template protein was performed with the command line options, and a series of commands were provided for model building using the software MODELLER9v14. Residues at positions 28, 61, 63, and 66 of WT AtpE protein were substituted for generating seven different MT proteins, following the above procedure. The same set of protocol was followed for chain B generation for the WT and seven mutants.

## **2.2. Chain combination**

In this study, two (A and B) chains were modeled separately following the above procedure; chains were then combined using Amber software (Version- 12) [19] to enable docking efficiently, since, ATP synthase is a complex oligomeric protein comprises of 11 AtpE subunits, for proper binding of BDQ at least two chains are required.

## **2.3. Model evaluation**

Validation of the models was done by ramachandran plot [20]. Further the deviation between the WT and the template 4V1F upon structural superimposition was determined using PDBeFOLD [21].

## **2.4. Ligand**

The ligand (BDQ) chosen in this study was obtained from PDB structure of 4V1F [14].

## **2.5. Molecular docking**

The GOLD protocol is based on the principle of genetic algorithm, with the rigid receptor and the flexible ligand during the refinement process, details of which have been described elsewhere [22]. Docking was performed between BDQ and seven MT proteins of AtpE (D28G, D28A, D28P, D28V, E61D, A63P, and I66M) in comparison to WT with the help of software GOLD (Version- 4.0.1). The input atom files for both the proteins and the ligand were created. The ligand and the models were added with hydrogen atoms before docking. The cavity atom file containing the atom number of binding residues (Gly58, Ala62, Phe65, Ile66 and Ala69) was prepared for BDQ. The binding residues were selected on comparison between binding regions of BDQ with crystal structures of 4V1F. 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. Ide-

ally, 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.

## 2.6. BIOVIA software

Biovia -2015 was used for visualization purpose of modelled proteins, docking data and to determine the interactions between the ligand and proteins [23].

## 3. Results and Discussion

BDQ or TMC207 is a new anti-TB drug belonging to the class of diarylquinoline, which selectively inhibits the mycobacterial energy metabolism *i.e.* ATP synthesis. BDQ is found to be effective against all states of *M. tuberculosis* like active, dormant, replicating, non-replicating, intracellular and extracellular. Although the contribution of BDQ against the treatment of drug resistant TB seems significant to the TB control program, however, some *in vitro* studies [12,13] have shown the emergence of resistant strains to BDQ. In a report [12], *in vitro* resistant mutants of BDQ from *M. tuberculosis* and diverse atypical mycobacteria were isolated. Six distinct mutations, Asp28→Gly, Asp28→Ala, Leu59→Val, Glu61→Asp, Ala63→Pro, and Ile66→Met have been identified in the subunit c forming a C ring in the ATP synthase, in order to map the amino acid residues involved in the binding of BDQ [12,13].

The catalytic core of the membrane-embedded rotor ring of the sodium ion-translocating ATP synthase contains  $\alpha\beta\beta\gamma$  subunits arranged in a hexagon of alternating  $\alpha$  and  $\beta$  subunits with helices of  $\gamma$  in the center. ATP synthesis and hydrolysis reactions occur at three catalytic sites [9]. In the present study, ATP synthase was modeled because its crystal structure of *M. tuberculosis* is not so far deduced due to the complexity involved in crystallizing the protein. The template chosen was the crystal structure of 4V1F from *M. Phlei* at 1.7 Angstrom (Å) resolution that showed 90% identity with the target *M. tuberculosis* protein (**Figure 1**). Moreover, 4V1F was in complexed with BDQ, which enabled the docking process easier [14].

In this study, seven MT models of AtpE were built based on the WT sequence of AtpE protein (Rv1305) through substitution at position 28 with four mutants: Gly, Ala, Pro, Val in place of Asp, the remaining three mutants at codon 61, 63 and 66 with Asp, Pro, Met in place of Glu, Ala and Ile, respectively. (**Figure 2**). They were validated by ramachandran plot (RM) which showed 100% and above 97% of residues in the favoured regions for WT and seven mutants, respectively (**Figure 3 and Table 1**). In addition to evaluation by RM plot, they were also validated by structural superimposition (**Figure 4**) which showed a root mean square deviation (RMSD) of 1.2 Å between 4V1F and WT suggesting a reliable model.

NCBI Blast:Protein Sequence (81 letters)

Chain A, Crystal Structure Of A Mycobacterial Atp Synthase Rotor Ring In Complex With Bedaquiline

Sequence ID: [4V1F\\_A](#) Length: 86 Number of Matches: 1  
[e 8 more title\(s\)](#)

Range 1: 3 to 83 [GenPept](#) [Graphics](#)

[Next Match](#) [Previous Match](#)

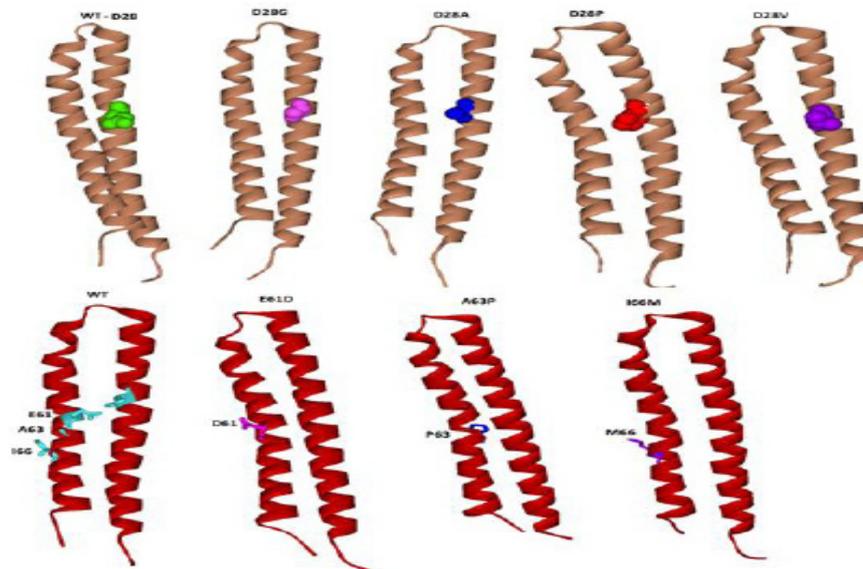
**Related Information**

[Structure](#) - 3D structure displays

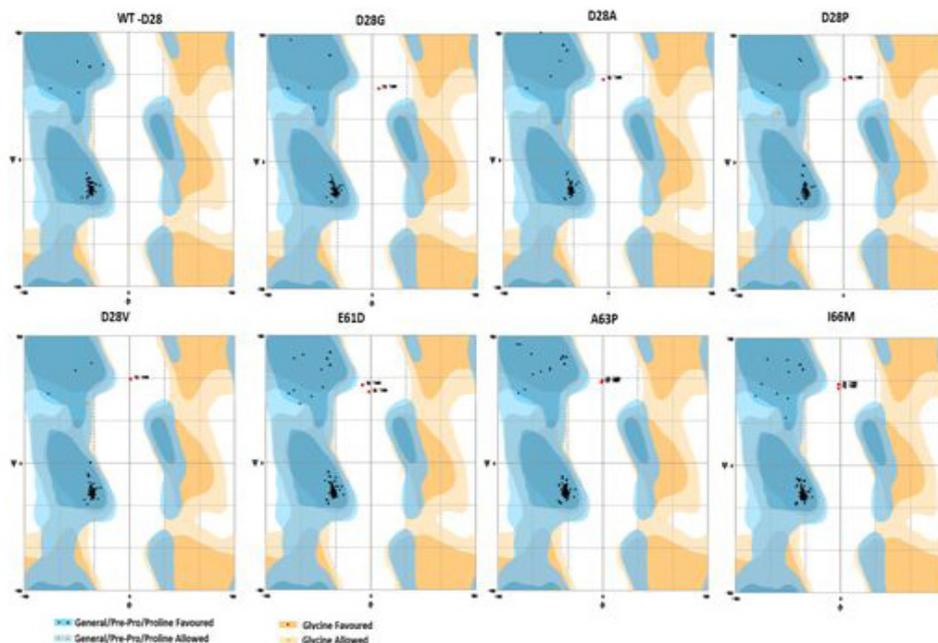
[Identical Proteins](#) - Identical proteins to 4V1F\_A

Score	Expect	Method	Identities	Positives	Gaps
95.1 bits(235)	3e-27	Compositional matrix adjust.	73/81(90%)	76/81(93%)	2/81(2%)
Query 1	MDPT--IAAGALIGGGLIMAGGAIGAGIGDGVAGNALISGVARQPEAQGRLLTPFFITVG				58
	+DP I AGALIGGGLIM GGAIGAGIGDG+AGNALISG+ARQPEAQGRLLTPFFITVG				
Sbjct 3	LDPNALITAGALIGGGLIMGGGAIGAGIGDGIAGNALISGIARQPEAQGRLLTPFFITVG				62
Query 59	LVEAAYFINLAFMALVFATP				79
	LVEAAYFINLAFMALVFATP				
Sbjct 63	LVEAAYFINLAFMALVFATP				83

**Figure 1:** pBLAST results showing maximum identity (90%) between the template 4V1F of ATP synthase from *M. Phlei* and the target WT protein sequence - Rv1305 of ATP synthase of *M. tuberculosis*.



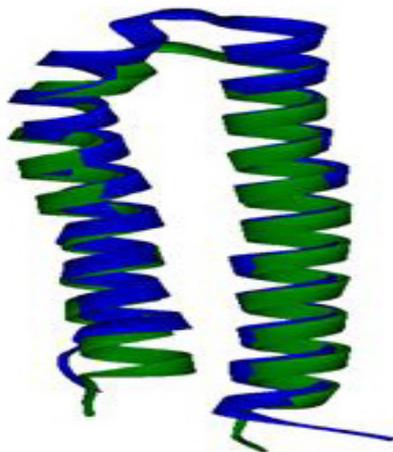
**Figure 2:** Three-dimensional models of AtpE showing WT and mutated residues at respective codon positions in *M. tuberculosis*.



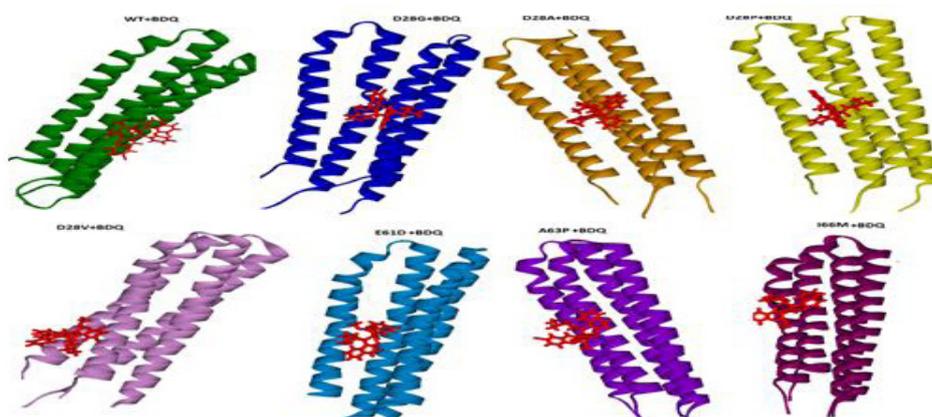
**Figure 3:** Ramachandran Plot for WT and MT models of AtpE from *M. tuberculosis*

**Table 1:** RM plot analysis of WT and MT models of AtpE from *M. tuberculosis*

Evaluation of residues	WT	D28A	D28G	D28P	D28V	E61D	A63P	I66M
Number of residues in favoured region (~98.0% expected)	79 (100)	77 (97.5)	78 (98.7)	77 (97.5)	77 (97.5)	78 (98.7)	78 (98.7)	78 (98.7)
Number of residues in allowed region (~2.0% expected)	0	1	0	1	1(1.3)	0	0	0
Number of residues in outlier region	0	1	1	1	1	1(1.3)	1(1.3)	1(1.3)

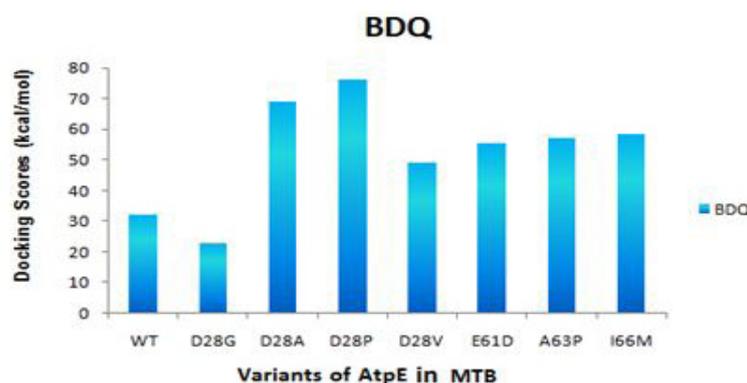
**Figure 4:** Validation of modeling through superimposition of template 4V1F (green) with WT protein of AtpE from *M. tuberculosis* (dark blue).

The mutant models such as D28G, D28A, D28P D28V, E61D, A63P, and I66M were created and docked with BDQ along with WT, the docked BDQ and AtpEs complex are illustrated in **Figure 5**.

**Figure 5:** Docking of BDQ (red) with WT and mutants of AtpE.

The docking of BDQ with AtpEs resulted in ten poses. Of the ten poses produced, the best ligand pose was selected based on top GOLD score. Among the seven mutants, the high score of - 76.04 kcal/mol was obtained for the MT-D28P, followed by D28A, I66M, A63P, E61D and D28V compared to the WT and the D28G-MT showed relatively low score of -22.86 kcal/mol compared to the WT. The binding energy between WT and BDQ was found to be -32.1 kcal/mol (**Figure 6**). Thus, the docking results suggests that in comparison to the WT, binding affinity of D28P with BDQ was shown to be more, followed by others. In contrast,

D28G, displayed low binding affinity compared to the WT protein.

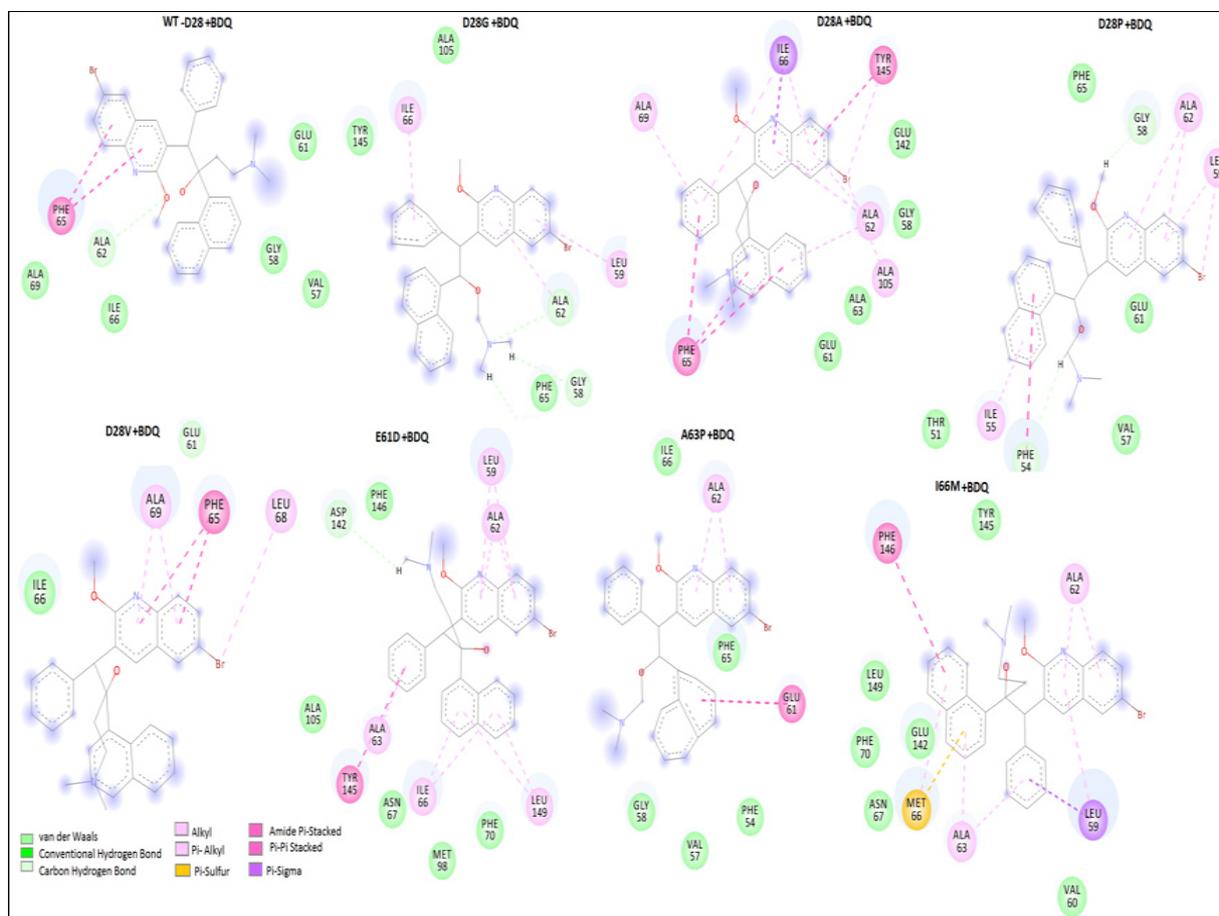


**Figure 6:** Docking score of BDQ with WT and mutants of AtpE

The interaction profile of WT and MT-AtpEs with BDQ at its binding site are illustrated in **Figure 7**. In general, the inhibitor and enzyme make a pattern of complementary hydrogen (H) bonds between their respective backbone atoms. In case of WT-AtpE complexed with BDQ, single carbon H bond was seen followed by two Pi-pi stacked interactions with Phe65, and other residues in van der Waals contact distance as shown in **Figure 7**. In case of MT-D28G, three carbon H bonds were observed between BDQ and residues Glu61, Phe62 and Phe65, respectively, followed by weak Pi-alkyl interactions. In contrast to WT in MT-D28A, several interactions principally of alkyl, Pi-alkyl, Pi-Pi stacked were found and Pi-sigma between the drug and Phe66 was also found. In MT-D28P complexed with the drug molecule, two carbon H bonds were formed with residues Phe54 and Gly58, followed by alkyl, Pi-alkyl Pi-pi stacked interactions. Surprisingly, in MT-D28V complexed with BDQ no H bonds were found. Notably, bromine atom of the BDQ was involved in alkyl interaction with the residue Leu68. In addition, alkyl, Pi-alkyl and two amide Pi-stacked types of interactions were observed. Interestingly, bromine atom of the BDQ was involved in alkyl interaction with the residue Leu68 (**Figure 7**). In case of MT- E61D, a carbon H bond with Asp142 was formed and Pi-alkyl, Pi-pi stacked interactions were found as shown in **Figure 7**. In MT- A63P complexed with BDQ, an amide-Pi stacked and Pi alkyl interactions were found with Glu61 and Ala62 residues. In MT- I66M with BDQ, interestingly, sulphur based interactions with Met66 itself was found. Then, a pi-Sigma with Leu59 and Pi-Pi T-shaped with Phe146 were also found (**Figure 7**).

The reason for the high score in all mutants (D28A, D28P D28V, E61D, A63P, and I66M except D28G) with BDQ could be attributed to the presence of favourable interactions that lacks in WT. This could in turn be due to the substitution of Ala/Pro/Val in the MT proteins in place of Asp in WT at position 28. In these mutants (D28A, D28P D28V), Ala contains one extra methyl group, Pro contains a hetero cyclic group and Val contains two methyl groups instead of Asp which is an acidic amino acid, might have induced more structural changes in the protein's side chain, which was obvious in changes in the pattern of interactions (many alkyl, Pi-alkyl, Pi-Pi stacked and Pi-sigma in D28A; two carbon H bonds, alkyl, Pi-alkyl, Pi-pi stacked interactions in D28P and alkyl, Pi-alkyl and two amide Pi-stacked in D28V)

and consequently reflected in high score. In case of E61D, A63P, and I66M, Asp contains an acidic group, Pro contains a hetero cyclic group and Met contains functional side chain of methyl group and sulphur atom in place of Glu61, Ala63 and Ile66, might have induced more structural changes in the protein's side chain, which was obvious in changes in the pattern of interaction.



**Figure 7:** Differences in network of interactions between WT and mutants of AtpE from *M. tuberculosis* with BDQ

A carbon H bond, Pi-alkyl, Pi-pi stacked interactions in E61D and amide-Pi stacked and Pi alkyl interactions in A63P; interactions such as sulphur based with Met66 itself was found. A pi-Sigma with Leu59 and Pi-Pi T-shaped with Phe146 were found in MT- I66M. These types of interactions in all mutants consequently reflected in high score than the WT. In contrast in case MT-D28G, as Gly does not contains a functional group or side chain, resulted in lower score (only Pi-alkyl) than the WT (two Pi-pi stacked interactions). Thus, based on these findings it can be assumed that the mutants -D28A, D28P, D28V, I66M, A63P, and E61D could lead to high level resistance compared to MT-D28G that may lead to low level resistance. Therefore, in this pilot study, a primary effort was taken to understand the effect of binding affinity of WT and MT proteins of AtpE with BDQ, which showed more affinity towards the mutants compared to the WT. Therefore, it can be suggested that the mutants displayed more affinity with BDQ, because of the substitution that induces structural changes and BDQ binds very tightly leading to the slow or no release of the drug to mediate its inhibitory activity, thereby leads to BDQ resistance.

## 4. Conclusions

The findings in this concise report have provided some useful insights towards understanding the basis of *in vitro*-BDQ resistance in *M. tuberculosis*. Although, the effect of docking could be better explained after performing molecular dynamics for understanding their function precisely, yet, the information provided over here can be useful to understand the impact of such substitution and consequent changes in binding ability. However, further structural studies are needed to get a deeper understanding of the mechanism of ATP resistance to BDQ that will aid in development of inhibitors that are selective against ATP synthase which can circumvent the problem of BDQ resistance. In addition, to understand more about the binding aspects of BDQ with other non-target based resistance *i.e.*, efflux-based resistance to BDQ which was identified in paired isolates from patients treated with BDQ, as well as in mice, showing cross-resistance to clofazimine [24]. Thus, structural studies related to efflux-based resistance mutants leading to cause of BDQ resistance are also needed.

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