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The 3D Modeling and Characterization of Wild Type and Mutated *Plasmodium falciparum* Cytochrome b: A Computational Approach

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Abstract.- Multi-drug resistant malaria parasite promotes the search of new analogues against mutated drug targets. *Plasmodium falciparum* cytochrome b (*Pf*Cytochrome b) was a promising drug target but resistance linked to the mutation has been reported. Different combinations of point mutations have been observed in atovaquone-resistant strains of *Plasmodium falciparum (P. falciparum)*. The crystal structure of wild type and mutant *Pf*Cytochrome b has not been reported till present. In current study, the analyses and characterization of mutated primary sequences and generation of 3D structure for wild type and mutated *Pf*Cytochrome b taken into account. The spotlight of this study is to generate 3D structural model of the *Pf*Cytochrome b by utilizing *in silico* methods. The template based protein modeling servers were considered for 3D structural modeling and finally selected structures were validated by utilizing computational tools. Docking studies revealed that conformational changes in active site are potentially associated with resistance development due to point mutations. These findings are useful for further structure based investigations to design new drugs against mutated protein.

Key words: Anti-malarial drug target, *Pf*Cytochrome b, 3D-Structural modeling, Atovaquone.

INTRODUCTION

Malaria is an infectious disease with high rate of morbidity and mortality in underdeveloped and developing countries. An estimation of 207 million cases and 627000 deaths were reported worldwide in a year (World Malaria Report, 2013). Despite of global efforts complete eradication could not achieved mainly due to development of drug resistant strains. *Plasmodium falciparum (P. falciparum*) is the most virulent human parasite. Many proteins of *P. falciparum* have been targeted by antimalarial drugs including dihydrofolate reductase–thymidylate synthase, dihydropteroate synthase, adenosine triphosphatase, cytochrome b etc. Monotherapeutic approach for practice of a drug as first line treatment develops selection pressure on strains.

Development of resistance against applied antimalarial drugs in endemic areas stimulates the search for new drugs and need of change in treatment policy. Artemisinin-based monotherapies recommended to be withdrawn by the World health Assembly in 2007 and most recently artemisinin-based combination therapy (ACTs) has been advised as first-line treatment for malaria caused by *P. falciparum*. Effectiveness of ACTs is also compromised as resistance against artemisinin has been reported in endemic countries but it can be effective due to efficacy of partner drug. In case of resistance against both components of ACTs, non-artemisinin-based combination such as atovaquone-proguanil (AP) can be recommended (World Malaria Report, 2012).

Atovaquone-proguanil is a combination of two drugs, distributed with commercial name Malarone. Malarone validated as effective drug against multi-drug resistant *P. falciparum* malaria (Srivastava *et al.,* 1999; Vaidya and Mather, 2000). It has minor side effects and a high cure rate (Looareesuwan *et al.,* 1999). Atovaquone (hydroxyl-naphthoquinone) and proguanil (isopropylbiguanide) are potent inhibitor of *P.*

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falciparum cytochrome bc1 (Cyt bc1) complex (Kessl *et al.,* 2003, 2005; Korsinczky *et al.,* 2000; Painter *et al.,* 2007) and dihydrofolate reductase (Kessl *et al.,* 2005; Srivastava *et al.,* 1997; Vaidya *et al.,* 1993) respectively. Atovaquone also reported efficacious for human immuno-deficiency virus, Pneumocystis, and AIDS. It has strong blood schizonticidal action, efficient against the sexual (Fleck *et al.,* 1996) and pre-erythrocytic stages (Korsinczky *et al.,* 2000) of the malaria parasite. In *P. falciparum* atovaquone targets Cytochrome b. Cytochrome b plays a key role as respiratory chain enzyme present in mitochondrial membrane. Inhibition of *Pf*Cytochrome b results into the collapse of mitochondrial membrane potential for caring electron transport chain in *P. falciparum* (Painter *et al.,* 2007; Srivastava *et al.,* 1999). *Pf*Cytochrome b is a promising drug target as its quinone oxidation (Q_o) site is most likely to be blocked by competitive inhibitors (Fisher *et al.*, 2012).

Resistance development in plasmodium strains against atovaquone compromised its efficacy. The mechanism of resistance is most likely to involve point mutations around putative binding site. Different studies reported different set of mutation(s) associated with resistance development and have the capacity to change the catalytic turnover (Korsinczky *et al.,* 2000; Schwöbel *et al.,* 2003; Fisher *et al.*, 2012). Most of the mutations, related to resistance development occurred in highly conserved region of eukaryotic Cytochrome b (Korsinczky, *et al.,* 2000). In *P. falciparum*, M133I/V, V140T, L144S, L181V, F267V, Y268S/C, L271F, K272R, P275T, G280D, L283I and V284K are reported to be associated with atovaquone resistance (Table I). Understanding of conformational changes in mutated active site provides an insight for designing effective agents against *Pf*Cytochrome b. These mutations may produce slight variations in hydrophobicity and drug binding affinity. The underline mechanism can be explored by visualization of mutated binding site.

Analysis and characterization of primary sequence before and after mutation delicate residues provide a useful insight about probable functional changes of proteins. In current study, various *in silico* tools have been utilized for prediction of functional region, conserved residues, physico-chemical properties and post-translational modifications to explore the changes at primary sequence level. In previous structural studies, crystal structure of cytochrome b from different model organisms like chicken (PDB ID: 1BCC), bovin (PDB ID: 1BE3) and yeast (PDB ID: 3CX5) have been utilized to understand the binding pattern of ligands due to unavailability of crystal structure of *Pf*Cytochrome b. In this study, a wild type and four reported combinations of mutations in *Pf*Cytochrome b are modeled (3D) and validated to understand the conformational changes conferring resistance against effectual drugs.

Table I.- List of mutations reported in *Pf*Cytochrome b

Sr. No	Mutation	Reported by
1	M133V	(Nam *et al.*, 2011; Bopp *et al.*, 2013; Schwöbel *et al.*, 2003; Korsinczky *et al.*, 2000; Ekala *et al.*, 2007)
2	F267V	(Nam *et al.*, 2011; Bopp *et al.*, 2013)
3	L144S	(Nam *et al.*, 2011; Bopp *et al.*, 2013)
4	M133I	(Nam *et al.*, 2011)
5	V140T	(Nam *et al.*, 2011)
6	L181V	(Nam *et al.*, 2011; Ekala *et al.*, 2007)
7	L271F	(Schwöbel *et al.*, 2003; Ekala *et al.*, 2007)
8	V284K	(Ekala *et al.*, 2007; Korsinczky *et al.*, 2000)
9	L283I	(Ekala *et al.*, 2007; Korsinczky *et al.*, 2000)
10	G280D	(Ekala *et al.*, 2007; Korsinczky *et al.*, 2000)
11	K272R	(Ekala *et al.*, 2007; Korsinczky *et al.*, 2000)
12	P275T	(Ekala *et al.*, 2007; Korsinczky *et al.*, 2000)
13	Y268S	(Korsinczky *et al.*, 2000; Vallieres *et al.*, 2012; Fisher *et al.*, 2012)
14	Y268C	(Vallieres *et al.*, 2012)

MATERIALS AND METHODS

Primary sequence selection

The primary sequence selection search of *P. falciparum* Cytochrome b retrieved two sequences (Accession No Q02768 (CYB_PLAFA) and Q7HP03 (Q7HP03_PLAF7)) from UniProt database of protein sequences (http://www.uniprot.org/). The retrieved sequences were aligned by Clustal Omega

(Sievers *et al.,* 2011) for selection of one sequence for 3D modeling experiments.

Modification and characterization of sequence

Point mutations associated with resistance development were grouped into four sets and introduced in selected wild type sequence manually. The wild type and four modified/mutated sequences of *Pf*Cytochrome b were characterized *in silico* by Expasy-ProtParam (Gasteiger *et al.,* 2005). ConSurf (http://consurf.tau.ac.il/) was utilized for the identification of functional regions and conserved residues of *Pf*Cytochrome b. Expasy-ProtScale (Gasteiger *et al.*, 2005)) was utilized to investigate the probability of change in hydrophobicity index in mutated sequences. CYS_REC tool (http://linux1.softberry.com/) was utilized to investigate the presence of Cys-Cys linkage in *Pf*Cytochrome b sequence. Post translational acetylation prediction analysis was also performed by using PAIL (Li *et al.,* 2006) tool to explore its functional impact in wild type and mutated *Pf*Cytochrome b.

Table II.- Resistance-associated mutations in *Pf*Cytochrome b: To build four mutated structures.

Sr. No	Model Code	Mutations	Reference
1	M-wild	None	Uniprot ID: Q02768
2	M-I	M133V, V284K, L283I, G280D, K272R, P275T	(Ekala *et al.*, 2007; Korsinczky *et al.*, 2000)
3	M-II	M133I, V140T, L181V	(Nam *et al.*, 2011)
4	M-III	Y268S	(Fisher *et al.*, 2012; Korsinczky *et al.*, 2000)
5	M-IV	M133V, F267V, L144S	(Boop, 2013)

*Homology modeling of Pf*Cytochrome b

As experimental crystal structure of *Pf*Cytochrome b is not available, homology based molecular modeling, can provide reasonably accurate and precise structural model for structure based studies. In current study, three homology based modeling servers ModWeb (Eswar *et al.,* 2003), I-TASEER (Zhang, 2008) and SWISS-MODEL (Kiefer *et al.,* 2009) were utilized to generate a wild type (M-wild) and four mutated (M-I, M-II, M-III, M-IV) structures of *Pf*Cytochrome b (Table II).

Template selection

In homology based modeling, the quality of 3D model directly depends upon template selection. I-TASEER and ModWeb select the template by automated mode while SWISS-MODEL can also generate 3D structure of protein by "user specified" template. HMMER (Finn *et al.,* 2011) is aLinux based program to search homologous sequences against a specified sequence. Selection of template was performed by using HMMER. Multiple sequence alignment (MSA) of cytochrome b from different species such as *P.falciparum* (Uniprot ID: Q02768), *Bos taurus* (Uniprot ID: P00157), *Gallus gallus* (Uniprot ID: P18946) and *Saccharomyces cerevisiae* (Uniprot ID: P00163) was generated by Clustal Omega (Sievers *et al.,* 2011). This MSA file worked as input for HMMER to search a suitable template.

Model generation and validation

A wild type and four multiple mutated models of *Pf*Cytochrome b were generated by utilizing I-TASEER, ModWeb and SWISS-MODEL servers by taking 2ibz chain C, 2a06 chain P and 1bcc chain C as template respectively. PROCHECK (Laskowski *et al.,* 1993) and ProSA-web (Wiederstein and Sippl, 2007) were brought into account to validate the generated models. Ramachandran plots were also generated for selection of most appropriate modeled structures. To check the quality of the generated structures PDBsum Generate (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) analysis were applied.

Docking studies

Docking analyses were performed to generate binding complexes of atovaquone with newly modeled (M-wildM-I, M-II, M-III and M-IV) *Pf*Cytochrome b structures. ChemSketch and Discovery Studio were utilized to prepare ligand (atovaquone) and receptors respectively. Molecular Operating Environment (MOE) software package

was utilized to run docking analyses. Ligand interaction tool of MOE and Chimera was utilized to investigate and visualize the generated complexes.

RESULTS AND DISCUSSION

The primary sequence for *Pf*Cytochrome b 3D7 isolate was retrieved from Uniprot database with accession No. Q7HP03 found with low evidence level. The only primary sequence for *Pf*Cytochrome b with good evidence level was Q02768. To resolve the conflict of selection, both Q7HP03 and Q02768 were subjected to sequence alignment by Clustal Omega and revealed that both sequences have 100% identical sequence. Finally, Q02768 was selected as primary sequence (376 amino acids) for construction of homology based structural model of *Pf*Cytochrome b.

Atovaquone-resistant isolates of *P. falciparum* have been investigated to understand the mechanism of resistance. Various studies discussed and provided evidences for the probable association between point mutation(s) in *Pf*Cytochrome b and development of resistance. Different sets of point mutations have been reported by different groups (Table I). In current work, all associated mutations were divided into four groups to explore the mutation-linked changes in conformation of *Pf*Cytochrome b active site. Mutations were introduced in selected wild type sequence for the construction of four mutated structures named as M-I, M-II, M-III and M-IV (Table II).

ConSurf used to predict valuable information about functional regions, conserved residues and overall architecture of protein by predicting exposed and buried residues. ConSurf reported that M133I/V, V140T, L144S mutations are present in a highly conserved region ranging from T121 to L144. Another conserved region started from P260 to L281 contains six mutation sites like F267V, Y268S/C, L271F, K272R, P275T and G280D (Fig. 1). As shown, most of the conserved region residues are predicted to be buried while few of them are exposed in functional conformation of *Pf*Cytochrome b and potentially critical for protein's function. Hydrophobicity is characteristic feature of integral proteins such as *Pf*Cytochrome b. In current study, hydrophobicity index for all five sequences presented graphically by Expasy-ProtScale tool. M-II shows little variation in a peak around mutated residues (Fig. 2). Moreover, there is no potential for Cys-Cys bonding in *Pf*Cytochrome b as analyzed by CYS_REC (Table III).

Table III.- CYS_REC output: prediction for Cys-Cys linkage.

Sr. No	Residue	Theoretical Status	Score
1	Cys18	18 is not SS-bounded	Score= -17.0
2	Cys73	73 is not SS-bounded	Score= -20.6
3	Cys156	156 is not SS-bounded	Score= -19.7
4	Cys182	182 is not SS-bounded	Score= -53.0
5	Cys324	324 is not SS-bounded	Score= -39.9
6	Cys334	334 is not SS-bounded	Score= -27.6
7	Cys354	354 is not SS-bounded	Score= -44.4

Comparative analyses of wild type and mutated sequences provided useful information about change in functional potential of a protein. Expasy-ProtParam tool, utilized for initial characterization, estimated the molecular weight, isoelectric point, instability index, number of negative and positively charged residues and GRAVY (Grand Average Hydropathicity) for wild type and mutated primary sequences (Table IV). The estimated value of instability index for wild type is 44.09 while for mutated it is ranges from 41.65-42.92. It shows slight decrease in the *in vitro* instability of mutated *Pf*Cytochrome b as compare to wild type.

Post-translational modifications play vital role in functional switch of proteins (Wajahat *et al.,* 2006). Integral proteins such as Cytochrome b is suspected to be modified by addition of acetyl-group on side chain amino group of Lys residue (Ryan *et al.,* 2010). Mutation on such potentially modified residue can mimic the functional alteration of protein. PAIL is an *in silico* tool for the prediction of posttranslational acetylation sites. Analysis of *Pf*Cytochrome b by using PAIL predicted that four residues Lys167, Lys207, Lys272 and L277 are potentially modifiable site (Table V). Among these, Lys272 is the most important modifiable site as mutation (K272R) has been reported at this position (Ekala *et al.*, 2007; Korsinczky *et al.*, 2000). Absence of modification as a result of mutation is likely to play its role in development of resistance.

Fig. 1. ConSurf results for *Pf*Cytochrome b. e, An exposed residue according to the neural-network algorithm; b, A buried residue according to the neural-network algorithm; f, A predicted functional residue (highly conserved and exposed); s, A predicted structural residue (highly conserved and buried); X, Insufficient data - the calculation for this site was performed on less than 10% of the sequences.

In the absence of crystallographic structure for *Pf*Cytochrome b, homology based protein modeling used to generate 3D model for wild type and mutated structures. ModWeb, I-TASEER and SWISS-MODEL are automated homology based protein modeling servers utilized to generate 3D structures. ModWeb is a reliable protein modeling server as it utilizes MODELLER (protein modeling software) to build 3D models. I-TASSER was - selected for 3D model generation of *Pf*Cytochrome b as it is significant for generating structure with complete range of residues in target protein sequence. ModWeb and I-TASEER selected template by automated search while SWISS_MODEL is an automated homology based protein modeling server that gives free access to generate user-specified template based structure.

Template selection was done by searching

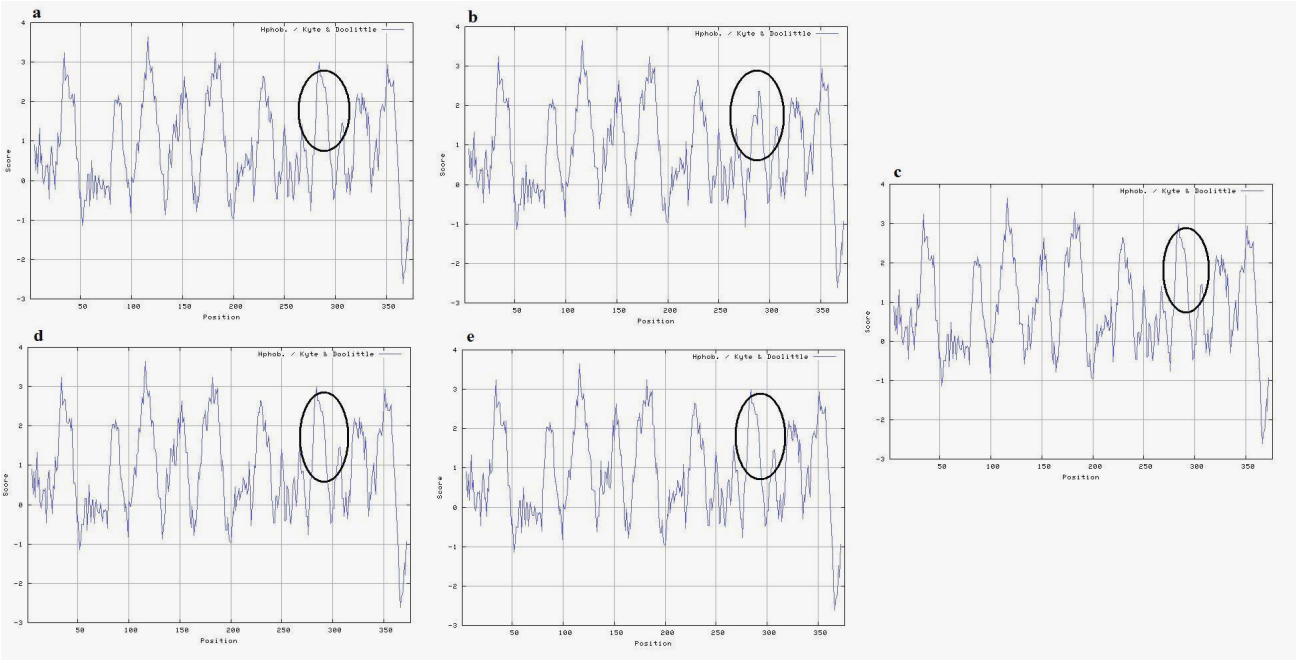

Fig. 2. Expasy-ProtScale output for hydropathicity. a, for wild type sequence; **b,** for M-I sequence; **c,** for M-II sequence; **d**, for M-III sequence; **e**, for M-IV sequence.
*Encircle peak for M-I shows considerable difference as compare to others due to mutation at consereved residues.

Table IV.- ProtPram results: Predicted physico-chemical properties of wild type and mutated sequence of *Pf*Cytochrome b.

Sequence code	Molecular weight	Theoretical pI	Total number of negatively charged residues	Total number of positively charged residues	Instability index	GRAVY*
M-Wild	43376.7	8.84	11	17	44.09	0.857
M-I	43463.7	8.84	12	18	41.76	0.834
M-II	43346.6	8.84	11	17	42.92	0.852
M-III	43300.6	8.85	11	17	42.59	0.858
M-IV	43270.5	8.84	11	17	41.65	0.855

*Grand Average of Hydropathicity.

Table V.- PAIL results: prediction for post-translational acetylation.

Sr. No	Peptide	Position	Score	Threshold
1	VSDPTI**K**RFFVLH	167	0.54	0.2
2	GYDTAL**K**IPFYPN	207	0.27	0.2
3	PFYAML**K**TVPSKP	272	0.77	0.2
4	LKTVPS**K**PAGLVI	277	0.90	0.2

homolog sequence with the help of HMMER. P18946 (Uniprot ID) was retrieved as most suitable template sequence that is associated with Mitochondrial Cytochrome Bc1 Complex of *Gallus gallus* (PDB ID: 1BCC) at resolution of 2.7 Å obtained from PDBsum and utilized as a template protein showing 44% identity with the target protein. SWISS-MODEL generated 3D models for M-wild, M-I, M-III and M-IV by using chain C of 1BCC as template while M-II was generated against chain C of 3l70 as template.

All three servers generated 3D models for *Pf*Cytochrome b named, M-wild, M-I, M-II, M-III and M-IV. Newly modeled structures were

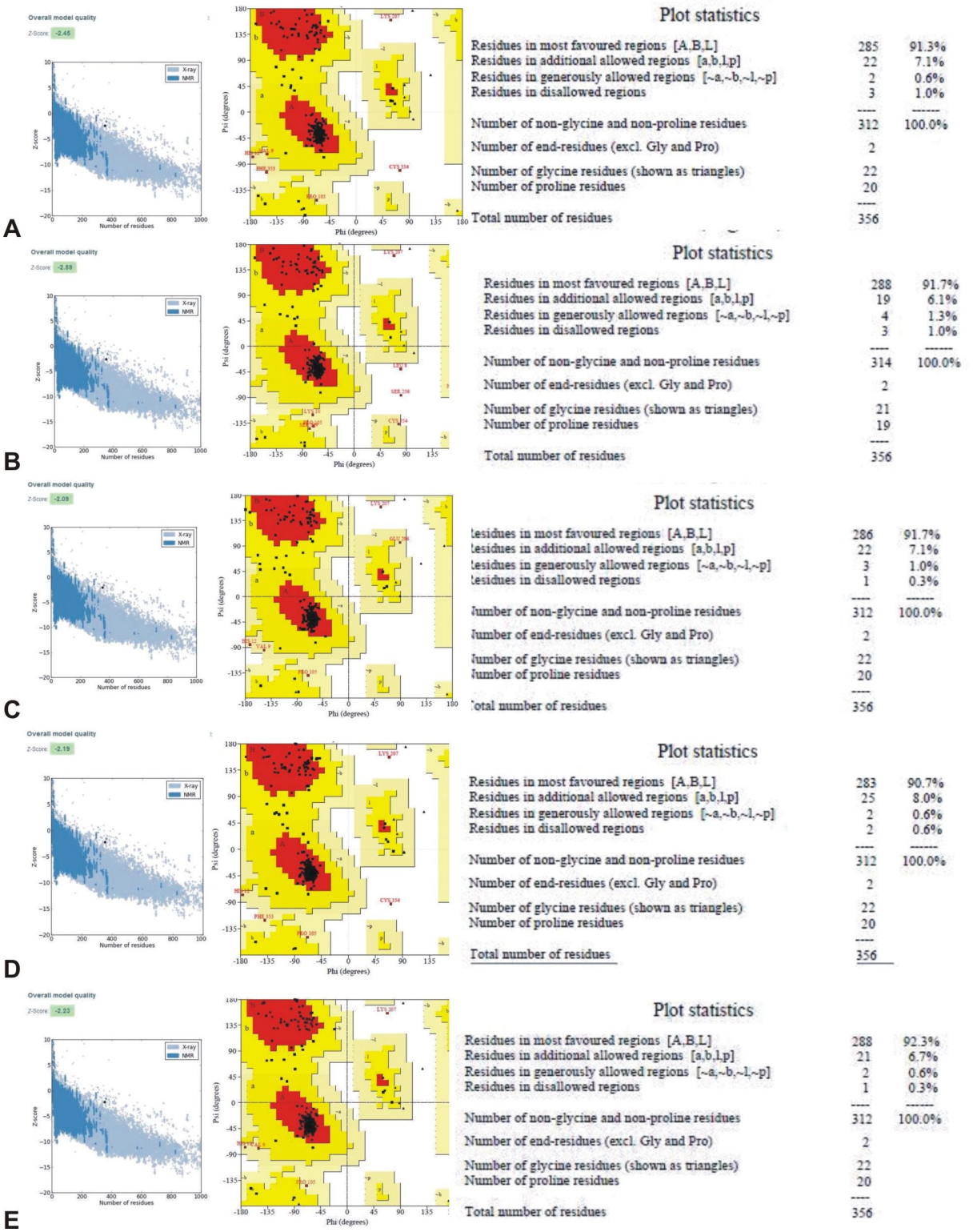

Fig. 3. ProSA-web and Ramachandran Plot for M-wild (A), M-I (B), M-II (C), M-III (D) and M-IV (E), *Pf*Cytochrome b structure.

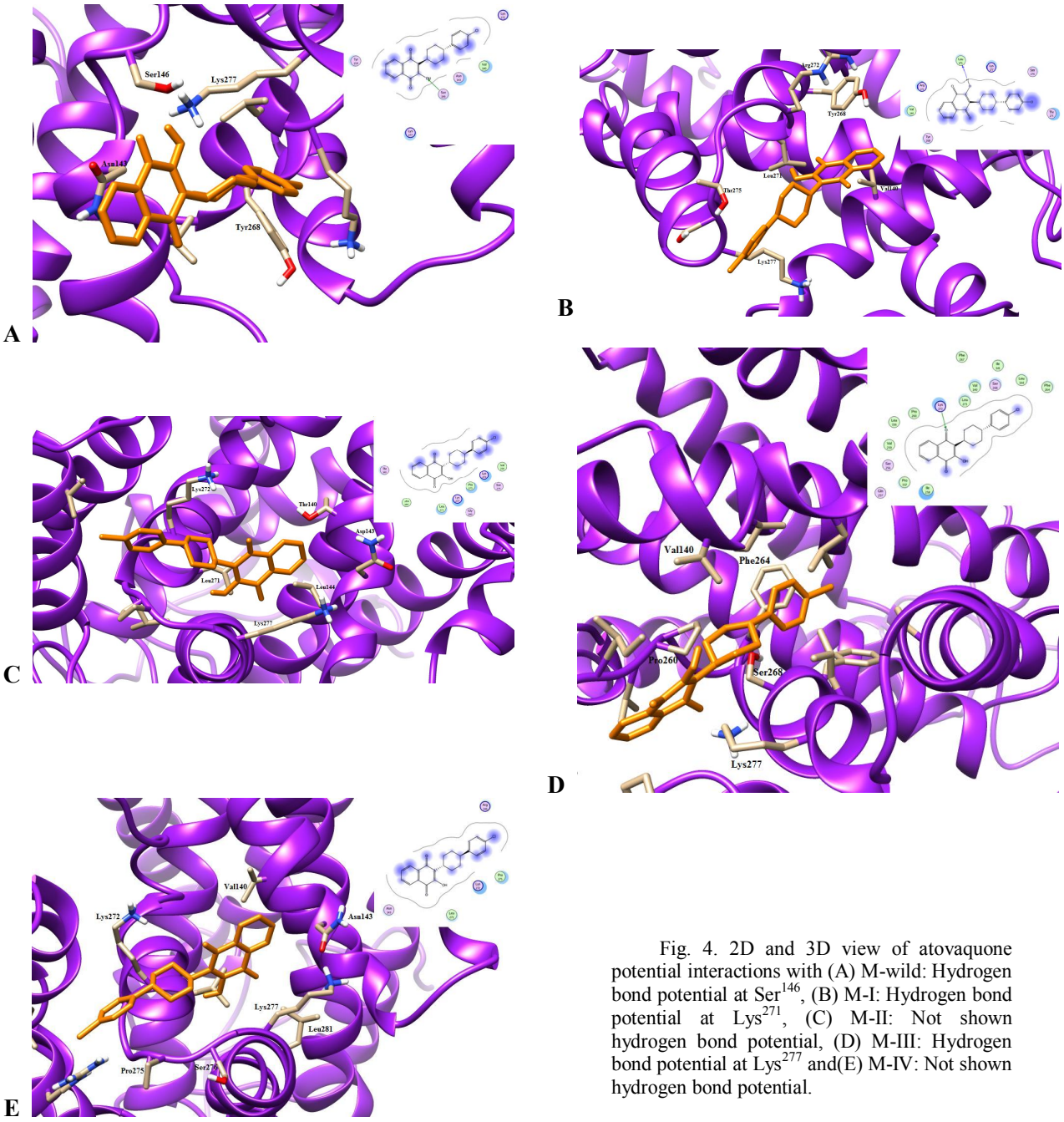

Fig. 4. 2D and 3D view of atovaquone potential interactions with (A) M-wild: Hydrogen bond potential at Ser^{146}, (B) M-I: Hydrogen bond potential at Lys^{271}, (C) M-II: Not shown hydrogen bond potential, (D) M-III: Hydrogen bond potential at Lys^{277} and(E) M-IV: Not shown hydrogen bond potential.

subjected to validation through PROCHECK and ProSA-web (Fig. 3A-E). Selection of most accurate model was done by calculating Z-score for overall quality of model by ProSA-web and Ramachandran plot by PROCHECK (Table VI). 3D models generated by ModWeb qualified as most accurate and selected for further characterization and visualization of mutated active site of *Pf*Cytochrome b.

Docking analyses performed by using MOE dock tool to generate binding complexes of atovaquone with newly modeled wild type and mutated *Pf*Cytochrome b structures. Top ranked complexes with minimum free binding energy

Table VI.- Z score calculated by ProSA-web and residue percentage present in most favored region calculated by Ramachandran plot generation.

Model code	Modeling server used					
	I-TASEER		ModWeb		SWISS MODEL	
	Z score by ProSA-web	Ramachandran residue percentage in most favored region	Z score by ProSA-web	Ramachandran residue percentage in most favored region	Z score by ProSA-web	Ramachandran residue percentage in most favored region
M-wild	-1.65	90.07	-2.45	91.3	-2.25	81.9
M-I	-1.71	89.5	-2.59	91.7	-2.31	82.3
M-II	-1.71	91.3	-2.09	91.7	-2.05	88.1
M-III	-1.52	89.5	-2.19	90.7	-2.25	82.2
M-IV	-1.73	90.4	-2.23	92.3	-2.19	81.9

selected to visualize the binding interactions between atovaquone and active site for each model (Fig. 4A-E). Results of docking analysis were in good consensus with previous findings as maximum binding affinity showed by wild type structure (M-wild) while minimum binding affinity shown by M-I. M-I contains six mutations as M133V, V284K, L283I, G280D, K272R and P275T. Among these six mutations K272R and P275T cause a considerable addition of side chain atoms in active site environment (Fig. 4B) that can disrupt the binding of drug (Ekala *et al.*, 2007). M-II and M-IV are triple mutant structure including M133I, V140T, L181V and M133V, F267V and L144S respectively. Both sets of triple mutation have been found in atovaquone resistant strains and associated with resistance development (Nam *et al.*, 2011; Fisher *et al.*, 2012; Korsinczky *et al.*, 2000). In M-II, V140T mutation replace a hydrophobic residue with a polar amino acid (Fig. 4C) that potentially important to lower the binding affinity. M-III contains a single mutation (Y268S) that has been reported to alter the catalytic turnover of *Pf*Cytochrome b (Fisher *et al.*, 2012; Korsinczky *et al.*, 2000). Ser268 added hydroxyl group into the binding pocket that is directed towards central cyclohexane moiety of atovaquone (Fig. 4D) and suspected to alter the optimum conformation of drug to bind in active site of *Pf*Cytochrome b.

CONCLUSIONS

*Pf*Cytochrome b is one of the most considered antimalarial drug targets. The development of resistance due to the point mutations and the unavailability of the crystal structure of the *Pf*Cytochrome b are two main reasons to explore this protein. The current study focused on the development of homology based 3D structures of wild type and mutated *Pf*Cytochrome b by in silico means. Docking studies were also performed to investigate the conformational changes in putative binding sites of *Pf*Cytochrome b. This study will eventually be helpful to design structure based antimalarial drugs to cure malaria. Furthermore, this study will also assist to understand the variations in binding energies due to point mutations.

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