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In silico modeling of *Plasmodium falciparum* chloroquine resistance transporter protein and biochemical studies suggest its key contribution to chloroquine resistance

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ABSTRACT

Chloroquine (CQ) has been used for decades as the primary chemotherapeutic drug for the treatment of malaria. The emergence of drug resistance in *Plasmodium falciparum* has been considered to be because of the excessive use of antimalarial drugs worldwide. Moreover, the intense distribution and prevalence of chloroquine-resistant strains in endemic regions has aided the incidence of more complications to malaria treatment and control. Due to the lack of literature that portrays evident molecular mechanisms of drug resistance, it has been difficult to understand the drug resistance conferred by *Plasmodium* species. Intensive research on CQ drug resistance has identified the association of *P. falciparum* chloroquine resistance transporter protein (PfCRT), which belongs to the drug/metabolite transporter and EamA-like superfamily. Additionally, it has shown that K76 T mutation in PfCRT protein has mainly attributed to CQ resistance than other mutations. This study deals with the development of an *in silico* model of the PfCRT protein and its interaction with the CQ ligand molecule as well as the biochemical and biophysical characterization of the transmembrane domain 1 (TMD 1) peptide of the PfCRT protein. The physiochemical analysis of the PfCRT protein identified basic differences between the wild and mutant forms of the protein, as well as identifying the high hydrophobic nature of the mutant-type protein. The tertiary structure of the PfCRT protein was predicted and interaction with CQ revealed different active pocket binding regions in both the wild and mutant form of PfCRT proteins. The CQ^{2+} molecule interacts with TMD 10 of the wild-type PfCRT protein, whereas it interacts with TMD 1 of the mutant-type protein. Studies on the TMD 1 peptide revealed the insertion of the peptide in the micelles adopting stable alpha-helical structure. Binding studies with the CQ molecule detected high binding affinity toward the mutant-type TMD 1 peptide rather than the wild-type, thus confirming that the TMD 1 peptide is involved in substrate selectivity. Our findings help to characterize the structure of the PfCRT protein and the role played by the TMD 1 region in CQ resistance using *in silico* and biochemical approaches. Molecular docking and ligand binding studies confirm that TMD 1 is involved in substrate selectivity and aids in CQ efflux, thereby contributing to the parasite's CQ drug resistance mechanism.

Abbreviations: PfCRT, *P. falciparum* chloroquine resistance transporter protein; CQ, chloroquine; TMD, transmembrane domain; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol; DLS, dynamic light scattering; CD, circular dichroism; ITC, isothermal titration calorimetry

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1. Introduction

Chloroquine (CQ) was synthesized in 1934 (Wellems and Plowe, 2001) and introduced for the treatment of infectious malaria disease close to the end of the World War II (Coatney, 1963). CQ, 4-aminoquinoline, is a diprotic weak base with a half-life of 6–14 days (Dzekunov et al., 2000), and is readily absorbed in the gastrointestinal tract. CQ is effective against the erythrocytic stages of the parasite and accumulates in the digestive vacuole of the parasite. The parasite digests the hemoglobin into component peptides and heme in the parasite's digestive vacuole. Heme is a toxic substance to the parasite and hence converted into inert hemozoin pigment via a polymerization pathway (Zhang et al., 1999). CQ interrupts the detoxification pathway and increases the level of the toxic monomeric heme, leading to its accumulation, which in turn kills the parasite (Martin and Kirk, 2004).

In the 1950s, the National Malaria Control and Eradication Programme was launched to treat and eradicate malaria throughout the world (Antony and Parija, 2016; Farooq and Mahajan, 2004; Klein, 2013). However, CQ resistance was first detected in Columbia and along the Thailand-Cambodia border in the late 1950s (Payne, 1987). The resistant parasite steadily spread across the globe and reversed the efforts achieved through the malaria control program. The mechanism of CQ drug resistance has not been clearly understood, with theories supporting reduced CQ accumulation in the digestive vacuole, which includes some of the following reasons: (1) increased efflux of CQ at the cytoplasmic membrane of the parasite, (2) decreased influx of CQ at the digestive vacuolar membrane of the parasite, potentially due to the changes in vacuolar pH, (3) reduced access of CQ to the heme molecule, and (4) increased detoxification of CQ-heme complexes via the glutathione-mediated pathway (Fidock et al., 2000; Lakshmanan et al., 2005; Wellems and Plowe, 2001).

A genetic crosslink study between CQ-sensitive strain (HB3, Honduras) and CQ-resistant strain (Dd2, Indo-China) revealed a 36-kb segment gene on chromosome 7, which was involved in CQ resistance in *P. falciparum* (Wellems et al., 1991). Further studies revealed a strong association between CQ resistance and polymorphisms in the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene in both laboratory and field isolates (Baro et al., 2013; Durand et al., 2001; Ecker et al., 2012; Fidock et al., 2000). PfCRT is a transmembrane protein, encoding 424 amino acid residues with a molecular mass of 48.6 kDa and has 13 exons (Fidock et al., 2000). It has ten putative transmembrane domains (TMDs) spanning around the digestive vacuole of the parasite (Martin and Kirk, 2004). The PfCRT protein belongs to the drug/metabolite transporter superfamily, EamA-like transporter family, and CRT-like transporter family.

Genetic mutation in the *Pfcrt* gene, resulting in amino acid substitutions in the encoded protein, plays a major role in determining CQ resistance. The K76 T mutation in the PfCRT protein is the primary determinant for CQ resistance and susceptibility in *P. falciparum* (Fidock et al., 2000). The positively charged lysine residue at position 76 in TMD 1 is replaced by the neutrally charged threonine residue, that facilitates the transport of deprotonated CQ out of the digestive vacuole by an active efflux mechanism (Martin and Kirk, 2004). This mutation, located toward the C-terminal end of TMD 1, is predicted to be involved in substrate recognition and selectivity (Martin and Kirk, 2004). However, there is not much structural information available to understand the key role these point mutations, that aids in the binding and transport of CQ. Thus, we intend to provide more information for a better understanding of the role played by the PfCRT protein by predicting its 3D structure and binding motifs of wild- and mutant-type of protein, apart from docking with the CQ^{2+} ligand molecule. Additionally, we further aimed to characterize the potent role of K76T mutation in TMD 1 through biochemical studies of the wild- and mutant-type TMD 1 of the PfCRT protein.

2. Materials and methods

2.1. Sequence retrieval and analysis

The primary sequence of the wild-type PfCRT protein from the *P. falciparum* 3D7 strain (CQ-sensitive) was retrieved from the UniProt database (UniProt Consortium, 2015) with the accession number Q8IBZ9. The mutant-type PfCRT protein sequence from the *P. falciparum* Dd2 strain (CQ-resistant) was retrieved from the NCBI Protein database with the accession number AAF26926. The physiochemical properties of the protein were analyzed using the ProtParam tool (Gasteiger et al., 2005), that computes various parameters such as molecular weight, theoretical pI, GRAVY index, etc. The InterProScan tool helps to classify the protein into families and predict domains by scanning against InterPro's signatures (Jones et al., 2014). The TMpred server, based on the statistical analysis of the TMbase, was used to predict the membrane-spanning regions and its orientation of the protein (Hofmann and Stoffel, 1993). The signal peptide and surface accessibility of the protein were predicted using tools such as the SignalP version 4.1 server (Petersen et al., 2011) and the NetSurfP version 1.1 (Petersen et al., 2009).

2.2. Secondary and tertiary structure prediction

The secondary structure of the wild- and mutant-type PfCRT protein was predicted using the PSIPRED version 3.3 server (Jones, 1999). The full-length sequences of both the wild- and mutant-type PfCRT protein were modeled using the Iterative Threading Assembly Refinement (I-TASSER) server (Roy et al., 2010), the Phyre2 server (Kelley et al., 2015), and the Robetta server's structure prediction program (Kim et al., 2004). The I-TASSER and Robetta programs constructed five models for both the wild- and mutant-type PfCRT protein, while the Phyre2 method constructed two models based on basic and intensive searches. Each of these generated models was then subjected to validation using the Volume Area Dihedral Angle Reporter (VADAR) server (Willard et al., 2003), as well as Structural Analysis and Verification Server (SAVES) version 4, with tools including PROCHECK and VERIFY_3D (Laskowski et al., 1993; Eisenberg et al., 1997).

2.3. Prediction of binding sites and docking with ligand

The Robetta server's generated model was used to predict the binding region in the wild- and mutant-type PfCRT protein using the Computed Atlas of Surface Topography of proteins (CASTp) server (Dundas et al., 2006). To further understand the activity of CQ resistance in PfCRT protein, molecular docking protocols were followed to study the molecular basis for the interacting mechanism and the affinity of CQ^{2+} with the wild- and mutant-type three-dimensional structure of PfCRT. The structure of the CQ ligand compound was drawn using Marvin Bean software version 5.1.1 available in the ChemAxon website (ChemAxon, 2015). The LibDock docking tool implemented within Discovery Studio version 3.1 was used for performing interaction analysis of CQ with wild- and mutant-type PfCRT protein. For defining the active site, residues of TMD 1 (amino acid ranging from 59 to 79) were taken and then proceeded with the docking protocols of LibDock.

2.4. Peptide synthesis

Peptides were synthesized corresponding to TMD 1 of wild- and mutant-type PfCRT protein and purchased from GenPro Biotech (New Delhi, India). All the peptides were purified by HPLC with > 95% purity, which was confirmed by mass spectrometry. Table 1 describes the amino acid sequences of the peptides used in the study. Lysine residues were added to the C- and N-termini of the amino acid sequences because of the hydrophobic nature of the peptides, thereby increasing