Protein-Drug Interaction Studies for Development of Drugs Against *Plasmodium falciparum*

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Abstract: The study of protein-drug interaction is of pivotal importance to understand the structural features essential for ligand affinity. The explosion of information about protein structures has paved the way to develop structure-based virtual screening approaches. Parasitic protein kinases have been pointed out as potential targets for antiparasitic development. The identification of protein kinases in the *Plasmodium falciparum* genome has opened the possibility to test new families of inhibitors as potential antimalarial drugs. In addition, other key enzymes which play roles in biosynthetic pathways, such as enoyl reductase and chorismate synthase, can be valuable targets for drug development. This review is focused on these protein targets that may help to materialize new generations of antimalarial drugs.

Key Words: *Plasmodium falciparum*, antimalarial drugs, structure-based virtual screening, protein kinases, enoyl reductase, chorismate synthase.

INTRODUCTION

The progress of structural genomics projects has greatly contributed to the growth in the number of macromolecular structures that established the basis for virtual screening approaches in drug discovery and development [1-3]. Structure-based virtual screening initiatives bring decades of accumulated experience in structural studies focused on the analysis of protein-ligand interactions together with chemoinformatics and bioinformatics methodologies for the analysis of drug-likeness. This association allows a synergetic relationship that benefits the process of drug discovery [4-16].

The central problem in the analysis of protein-drug complexes is the relationship between the three-dimensional structure of the complex protein-drug and activity. Data originated from the analysis of hundreds of binary complexes indicate that the affinity between a drug and its protein target is determined by intermolecular hydrogen bonds and ionic interactions, as well as by the shape and charge complementarity of the contact surfaces of both partners [17-19]. Fully understanding of the structural basis for inhibition of protein targets relies heavily on detailed three-dimensional information about protein-ligand interactions, which play a central role in the early stages of virtual screening process. The identification of an enzyme's active site, and the key-residues involved in substrate binding, are remarkable achievements that guide the efforts to identify new potential inhibitors.

Most of the structural information about protein-ligand complexes is deposited at Protein Data Bank (PDB) [20-24]. This remarkable relational database is one of the most accessed biological databases, and works as a repository for three-dimensional structures of proteins, DNA, RNA, and also complexes involving these biological macromolecules. Currently, PDB has about 60,000 macromolecular structures, each receiving an alphanumeric code. These codes are composed of a series four of letters and numbers which are written in a form that can be easily processed and retrieved by a computer. Besides the atomic coordinates for each structure available at the PDB, there is also information about ligand, if present, primary references related to the deposited structure, and details about the technique used to solve the structure. Over 85 % of this structural information was obtained applying X-ray crystallography diffraction techniques. Other techniques that contribute to increase the arsenal of existing methodologies to address protein-ligand structures are mainly Nuclear Magnetic Resonance (NMR) and Electron Microscopy (EM). Nevertheless, the vast majority of the structural information about protein-ligand complexes was obtained applying biocrystallography methodologies.

The origin and development of PDB is the result of decades of research focused on three-dimensional structures of biological macromolecules. One interesting aspect of this database is that we may correlate this structural information to experimentally determined ligand-binding affinities, which opens the possibility to bring both sets of information together and derive a general model to predict affinity from the

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structures. In addition, specific databases dedicated to structures of binary complexes have been created, such as PDBBind [25, 26], PLD [27], AffinDB [28], PDBCal[29] and BindDB [30]. Therefore, binding-affinity in a given conformation can be calculated and correlated with experimental determined information. This structural information is the foundation for structure-based virtual screening projects. Methodologies employed in these projects usually include molecular docking algorithms to predict conformations of protein-ligand complexes, followed by a process to evaluate ligand-binding affinity.

Molecular docking is a computer simulation procedure to predict the conformation of a receptor-ligand complex, where the receptor is usually a protein or a nucleic acid molecule (DNA or RNA) and the ligand is either a small molecule or another protein. It can also be defined as a simulation process where a ligand position is estimated in a predicted or predefined binding site. There are several docking programs, such as DOCK [31], AUTODOCK [32, 33], GOLD [34, 35], FLEXX [36, 37], ZDOCK [38], M-ZDOCK [39], MS-DOCK [40], Surflex [41], MCDOCK [42], and others. Each docking application is mainly based on a specific search algorithm, such as Incremental Construction (IC) [35, 36], Genetic Algorithm (GA) [32, 43], and Monte Carlo (MC) [42]. The docking program can search for the best fit between two or more molecules taking into account several parameters, obtained from receptor and ligand input coordinates, such as geometrical complementarity, regarding atomic van der Waals radius and charge, receptor or ligand structure flexibility, or considering interatomic interactions, such as hydrogen bonds and hydrophobic contacts. As the result, docking applications return the predicted orientations (poses) of a ligand in the target's binding site. Usually the posing process returns several possible conformations. Scoring functions, which are able to evaluate intermolecular binding affinity or binding free energy, are employed in order to optimize and rank the results, obtaining the best orientation after the docking procedure. This approach to the study of ligand-binding affinity started with the pioneering work of Böhm [44-49].

The major source of structural data for virtual screening with small-molecules to be docked against a protein target are the several available small molecule databases, such as ZINC [50], PubChem [51], and DrugBank [52], which together surpass one million of deposited structures of potential ligands. The procedure of virtual screening through docking has become crucial when it is necessary to test a database of thousands (or even millions) of compounds against one or more targets in a short period of time. This search would be impossible to be reproduced experimentally at a so small economic and time cost. The main aspects of these methodologies have been recently reviewed [53-63].

The present review article aims to give an overview of some protein targets identified in *Plasmodium falciparum* genome. We will discuss structural features of protein targets in complex with small molecules currently employed to better understand protein-drug interactions, and present few well-established protein targets in complex with ligands. This structural information is at the core of all structure-based virtual screening projects that may help to develop new generations of drugs against parasite-mediated diseases.

MOLECULAR TARGETS FOR PLASMODIUM FALCIPARUM

Malaria, caused by *Plasmodium* spp.,is one of the most important public health problems worldwide [64]. The most effective measures for combating malaria rely on prevention of transmission and the use of antiparasitic drugs, but the increasing numbers in cases of drug-resistance *Plasmodium* strongly indicate the urgent need to develop new antiparasite drugs [65].

A search in the PDB using the word "Plasmodium falciparum" returns 256 structures, of which 149 are ligand-bound complexes. Analysis of these structures brings information about several potential protein targets, such as protein kinases 5 and 7 (PfPK5 and PfPK7), which are promising targets for antimalarial drug design [66]. Structural comparison of human CDKs and PfPKs indicates that folding and the mechanism of inactivation of these kinases are well-conserved. Furthermore, the identification of a family of kinases in *P. falciparum* with a high degree of sequence conservation to the mammalian CDKs has opened the possibility to transfer all information obtained in decades of studies with CDK inhibitors, previously identified as potential anti-cancer drugs [17-18]. In addition to PKs, we will also describe in the present review two other protein targets for *P. falciparum*, chorismate synthase and enoyl reductase, which participate in important biosynthetic routes in the parasite.

PROTEIN KINASES (PKs)

In humans the cell cycle progression is tightly controlled by the activity of cyclin-dependent kinases (CDKs) [67]. CDKs are inactive as monomers, and activation requires binding to cyclins, a diverse family of proteins whose levels oscillate during the cell cycle, and phosphorylation by CDK-activating kinase (CAK) on a specific threonine residue [68]. CAK is also known as CDK7. Eleven CDKs have been identified in the human genome, most of them involved in cell cycle. Besides their role in cell cycle regulation, several CDKs participate in other physiological processes, such as neuronal function (CDK5, CDK11), apoptosis (CDK1, CDK5), and transcription (CDK2, CDK7, CDK8, CDK9, CDK11), which are also affected by pharmacological inhibitors of CDKs [69-70]. Several protein kinases similar to CDKs have been identified in *P. falciparum* [71]. PfPKs can be inhibited by mammalian CDK inhibitors (CDI) *in vitro*, which demonstrates that structural features required for inhibition are conserved in the PfPKs [71-72].

Among the protein kinases identified in the *Plasmodium falciparum* genome, three present three-dimensional information, two of them (PfPK5 and PfPK7) have their structures elucidated by X-ray diffraction crystallography (PDB access codes: 1OB3 and 2PML, respectively) [66,71] and PfPK6 has been modeled in complex with roscovitine and olomoucine [65]. PfPK7 has been recently deposited in the PDB, and the article describing its structure has not been published yet, and therefore it will be not discussed in this review. Structures of PfPK5 and PfPK6 are very similar to human CDK2. In sequence comparison with members of human CDK family, they present identity around 60 %.

Analysis of the crystallographic structure of PfPK5 and the homology model of PfPK6 indicated that they are bilobal, typical for most of protein kinases as can be seen in Fig. (**1**). PfPK5 and 6 present a smaller N-terminal domain consisting of a sheet of five antiparallel β-stands and a single large α-helix. The larger C-terminal domain consists primarily of α-helices. It contains a pseudo-4-helical bundle, a small β-ribbon, and two additional α-helices. The ATP-binding pocket is found in the cleft between the two lobes. The adenine base can be easily positioned in a hydrophobic pocket between the β-sheet of the small domain. The ATP phosphates are held in position by ionic and hydrogen-bonding interactions with several residues, including Lys33, Asp143, and the backbone amides of the glycine-rich loop (residues 10-17). It was observed that ATP binding to CDK2 appears to induce a slight closure of the cleft by a 2.1^{o} hinge movement around an axis parallel to the longitudinal axis of the ATP molecule [66], which is also expected for PfPK5.

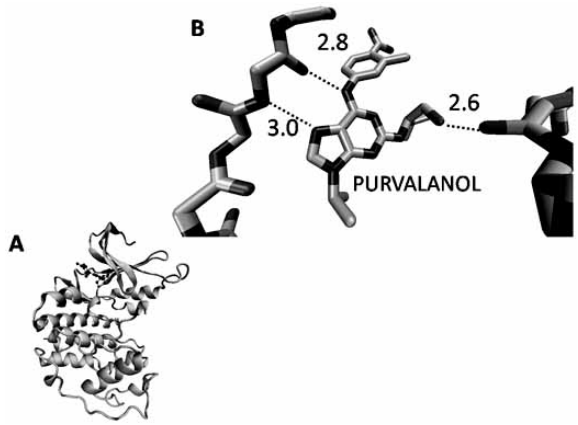

Fig. (1). A. Crystallographic structure of PfPK5. B. ATP-binding site showing intermolecular hydrogen bonds with purvalanol (distances in Å) (PDB access code: 1V0P).

In all structures of human CDK2, except for CDK2-cyclin A complex, electron density is weak and poorly defined in two regions in the structure, spanning residues 36-47 which links the N-terminal domain and "PSTAIRE" (in human CDK2) and PSTTIRE in PfPK5 or cyclin recognition helix and residues 150-164 (148-162 in PfPK5) of the "T loop" containing the activating phosphorylation site. All inhibitors and ATP bind in the deep cleft between the two domains.

It has been observed in several structures of CDK2, and also in the structures of PfPK5 complexed with inhibitors, the participation of a molecular fork (Fig. (**2**)). This fork is composed by a C=O group of Glu88 (Glu81 in the CDK2 sequence) and the N-H and C=O group of Leu82 (Leu83 for CDK2), which allows the formation of intermolecular hydrogen bonds between the kinases (CDK2 and PfPK5) and the inhibitors. This molecular fork, composed of two hydrogen bond acceptors (C=O) and one hydrogen bond donor (N-H), allows a wide range of different molecules to dock on to the ATP binding pocket, such as: olomoucine, isopentenyladenine, and roscovitine [18, 73], staurosporine [74], purvalanols [75], indirubins [76], and hymenialdisine [77]. Fig. (**3**) shows these CDK inhibitors. All these inhibitors have pairs of hydrogen bond partners that show complementarity to the molecular fork on CDK2 and PfPK5, most of them involving at least two hydrogen bonds with the molecular fork.

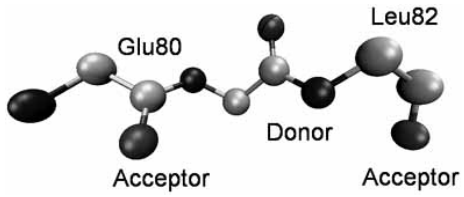

Fig. (2). Molecular fork present in protein kinases and CDKs.

Analysis of the binary complexes involving PfPKs and inhibitors [66] indicates that the relative orientation of the inhibitors in the ATP-binding pocket of PfPKs locates one hydrogen bond donor close to C=O in Glu81 and/or Leu82, and an acceptor close to N-H in Leu82. Such simple paradigm is conserved in all PfPK5-inhibitor and CDK2-inhibitor complex structures solved so far.

Homology-modeling studies were able to generate reliable structures for PfPK6 in complex with canonical CDK inhibitors, such as roscovitine and olomoucine [65]. Superpositions of the CDK2-ATP onto CDK1, CDK5 and PfPK6 structures complexed with roscovitine and olomoucine indicate that the two ring systems of roscovitine with ATP and olomoucine with ATP overlap approximately in the same plane [17,18] however, with different orientations. As observed in the crystallographic structures of CDK2-roscovitine and CDK2-olomoucine, the region of CDK1, CDK5 and PfPK6 occupied by the phenyl rings of roscovitine and olomoucine are pointing away from the ATP-binding pocket, and partially exposed to solvent in the both complexes. For the complexes CDK2-roscovitine and CDK2-olomoucine, the contact areas between inhibitor and CDK2 are 320 and 269 Å^2, respectively. This analysis was used for comparison of the CDK1, CDK5 and PfPK6 complexed with the same ligands. The active site of PfPK6 is structurally similar to the CDKs. Two intermolecular hydrogen bonds between PfPK6 and inhibitors, involving the residue Leu95, were observed for the binary complexes. The contact areas for the complexes of PfPK6 with roscovitine and PfPK6 with olomoucine are 356 and 297 Å^2, respectively, compatible with the values observed to CDK2-roscovitine and CDK2-olomoucine complexes. However, the IC_{50} is higher for PfPK6 than for CDK2. The structural basis for this higher IC_{50} relies on the presence of two tyrosine residues in the entry of the ATP-binding pocket observed in the complex PfPK6-roscovitine and PfPK6-olomoucine. This pair of tyrosines is not observed in the CDK2 complexes.

It has been suggested that these tyrosines offer further hindrance to the docking of roscovitine and olomoucine to the ATP-binding pocket of PfPK6, justifying the higher IC_{50} observed for the inhibition of PfPK6 by roscovitine and olomoucine (30 and 180 lM, respectively) [78], when compared with the inhibition of CDK2 (0.7 and 7 lM, respectively) [78]. The values of contact area to CDK1 and CDK5

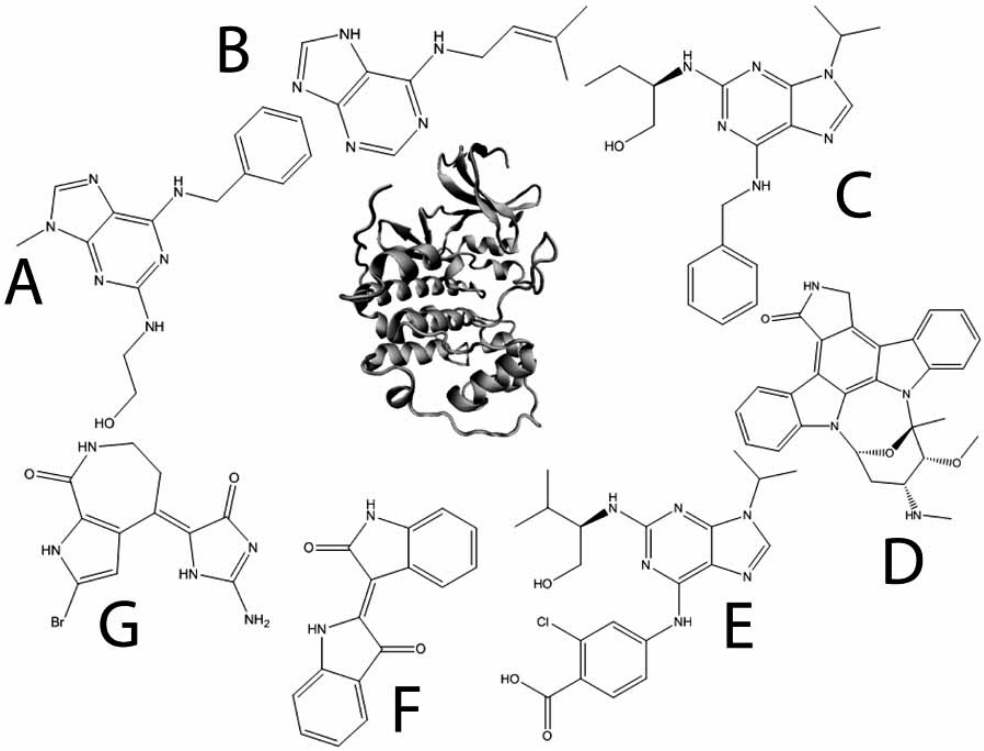

Fig. (3). Molecular structures of CDK inhibitors. A. olomoucine, B. isopentenyladenine, C. roscovitine, D. staurosporine, E. purvalanols, F. indirubins , G. hymenialdisine [77].

are in agreement with the IC_{50} values. Analysis of the charge distribution of the binding pockets indicates the presence of charge and shape complementary between CDK1 complexed with roscovitine, CDK5 complexed with roscovitine and PfPK6 complexed with roscovitine and olomoucine. Analysis of the structural models of PfPK6 in complex with roscovitine and olomoucine strongly indicates that roscovitine and olomoucine are more specific for CDK1, CDK2 and CDK5 than for PfPK6. A significant difference was observed in the PfPK6 models in complex with roscovitine and olomoucine. These models present a pair of tyrosines, which make a barrier for the ligand. Fig. (**4**) shows the ATP-binding pocket of PfPK6. The presence of Tyr13 and Tyr96 in the entrance of the ATP-binding pocket reduces the volume available for ligand binding in the PfPK6 active site. The presence of this pair of tyrosines is the structural basis for the high values of IC_{50} for PfPK6 when compared to CDK1, CDK2 and CDK5. These structural models are available for download (http://www.biocristalografia.df.ibilce.unesp.br/tools/hmdb/index.php) (access codes: 1PFPK6 for PfPK6-roscovitine and 2PFPK6 for PfPK6-Olomoucine), which allow structure-based virtual screening approaches using this kinase as target.

ENOYL ACYL CARRIER PROTEIN REDUCTASE (ENR)

Fatty acids are a well-established source of metabolic energy, which makes members of their biosynthetic pathway valuable protein targets for anti-parasites drugs. A number of recent reviews summarize the progress in this field [79-84]. Multifunctional single polypeptides involved in fatty acid synthesis have been characterized in higher eukaryotes and yeast, these polypeptides referred to as type I fatty acid synthases (FAS-I). The FAS-I system utilizes acetyl CoA for iterative 2-carbon elongation of fatty acids. On the other hand, in most prokaryotes and plants these enzymatic steps are performed employing separated enzymes, called type II fatty acid synthases (FAS-II) [85-88]. FAS-II is composed of the following enzymes: β-ketoacyl-ACP synthase III, β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydratase, and the final step is catalyzed by a single NADH-dependent enoyl-ACP reductase that converts *trans*-2-enoyl-ACP to acyl-ACP [89]. This enzyme belongs to the short chain dehydrogenase/reductase (SDR) family of enzymes. This metabolic pathway is absent in humans, which makes enzymes of

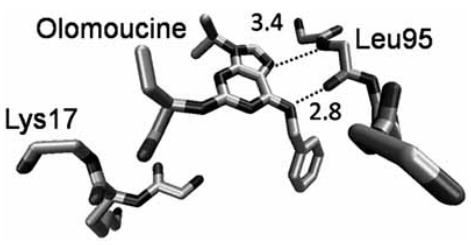

Fig. (4). ATP-binding pocket for PfPK6 showing olomoucine and two intermolecular hydrogen bonds (distances in Å).

this route interesting targets for antimicrobial drug development [84].

Recently, triclosan was found to inhibit *P. falciparum* growth [90]. Triclosan is a well-known antibacterial agent (Fig. (**5**)), and structural studies identified ENR as the protein target for triclosan in *P. falciparum* [89, 90]. Furthermore, the elucidation of the three-dimensional structure of ENR from *P. falciparun* (PfENR) opened the possibility of structure-based virtual screening studies [91].

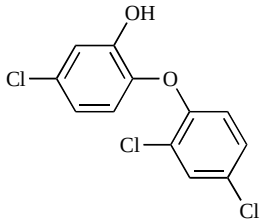

Fig. (5). Molecular structure of triclosan.

The analysis of structures of ENR and the equivalent protein in *Mycobacterium tuberculosis* (MtInhA) [92-94] indicates that the main characteristic of this family is a polypeptide backbone topology in which each subunit consists of a single domain with a central core that contains a Rossmann fold supporting an NADH binding site. The structure displays a α/β folding consisting of a central β-sheet composed of parallel strands and flanked by α-helices. Fig. (**6**) shows the PfENR structure. Analysis of the complex involving PfENR and triclosan indicates an intricate map of intermolecular interactions. Fig. (**7**) shows the active site triclosan and PfENR.

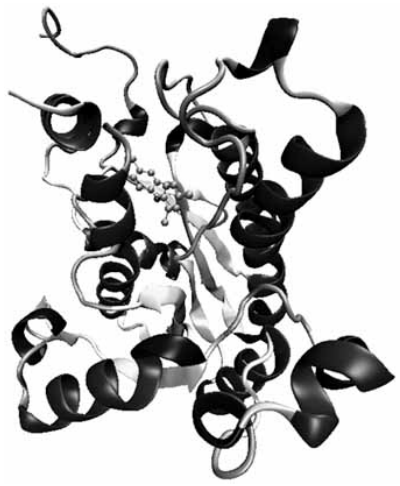

Fig. (6). Crystallographic structure of PfENR (PDB access code: 2O2Y).

SHIKIMATE PATHWAY ENZYMES

Shikimate pathway is a metabolic route that catalyzes the conversion of erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) to chorismate. A search in the KEGG database [95] indicates that E4P is also an intermediate in the

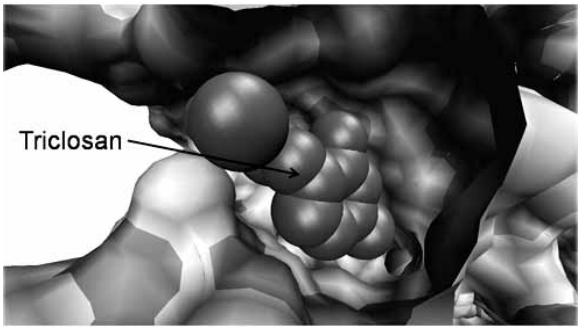

Fig. (7). Contact surfaces for complex PfENR-triclosan (PDB access code: 2O2Y).

Calvin cycle and in pentose phosphate pathway, and PEP is present in glycolysis / gluconeogenesis, citrate cycle, aminophosphonate metabolism, pyruvate metabolism, carbon fixation, reductive carboxylate cycle (CO_2 fixation), biosynthesis of phenylpropanoids, phosphotransferase system.

Chorismate is the branch point in the biosynthesis of many aromatic molecules. Therefore, it was named chorismate, which means, in Greek, separation, split, or divorce. The chorismate is the common precursor for the biosynthesis of a wide range of primary and secondary metabolites, including aromatic amino acids (phenylalanine, tyrosine and tryptophan), folate, naphthoquinones, menaquinones and mycobactins. Identification of the shikimate pathway presence in apicomplexan parasites is of great interest since the metabolic pathway is absent in mammals but is apparently essential for survival of the parasites [96-98]. The shikimate pathway is composed of the following enzymes: 3-deoxy-darabino-heptulosonate-7-phosphate synthase (DAHPS), 3-dehydroquinate synthase (DHQS), 3-dehydroquinate dehydratase (DHQD), shikimate-5-dehydrogenase (SDH), shikimate kinase (SK), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and chorismate synthase (CS) [98]. All these enzymes have been submitted to structural studies in the last few years [99-111], generating enough structural information to guide virtual screening initiatives focused on bacterial proteins.

Analysis of the *P. falciparum* genome indicated the presence of chorismate synthase (PfCS), the last step in the shikimate pathway. Nevertheless, no structural information is available for this enzyme. Chorismate synthase has been validated as a target for antimalarial drug discovery [112-114]. Sequence comparison of PfCS and bacterial chorismate synthases indicates high identity among these sequences. This identity is enough to guide molecular modeling initiatives, as has been previously applied to model other enzyme of shikimate pathway, such as shikimate kinase [115] and EPSP synthase [99] from *M. tuberculosis*.

FINAL REMARKS

The genomic information of protozoan parasites unveils a new world, increasing our understanding about parasite metabolism and how it works in host. This useful information became a hallmark of molecular biology, as the identification of genes and which proteins are expressed allows the identi-

fication of new molecular targets. In addition, elucidation of three-dimensional structures of validated protein targets makes the virtual screening projects possible. As examples, the analysis of the interaction of canonical CDK inhibitors with protein kinase identified in the *P. falciparum* opened the possibility to test CDK inhibitors [116]. In addition, validation of ENR and CS as targets for antimalarial drugs creates a scenario of multiple targets to be evaluated. Virtual screening projects focused on these enzymes, as well as in other available targets, are underway, which may help in the development new generation of antimalarial drugs.

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ABBREVIATIONS

CDK	=	Cyclin-dependent kinase
CS	=	Chorismate synthase
DAHPS	=	3-deoxy-darabino-heptulosonate-7-phosphate synthase
DHQD	=	3-dehydroquinate dehydratase
DHQS	=	3-dehydroquinate synthase
E4P	=	Erythrose 4-phosphate
ENR	=	Enoyl acyl carrier protein reductase
EPSP	=	5-enolpyruvylshikimate 3-phosphate synthase
PEP	=	Phosphoenolpyruvate
Pf	=	*Plasmodium falciparum*
PK	=	Protein Kinase
SDH	=	Shikimate-5-dehydrogenase
SK	=	Shikimate kinase

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