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Towards the Selective Inhibition of Glycolytic Pathway Enzymes: A Computational Analysis of Plasmodium falciparum and Human Triosephosphate Isomerase Ligand Interactions.

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Peer Review
This work has undergone a double-blind review by a minimum of two faculty members from institutions of higher learning from around the world. The faculty reviewers have expertise in disciplines closely related to those represented by this work. If possible, the work was also reviewed by undergraduates in collaboration with the faculty reviewers.

Abstract
Plasmodium falciparum is main causative agent of malaria, a disease that affects half of world's population. The parasite's dependence on glycolysis makes this pathway a suitable target for drug development. Understanding the structure-function dynamics of glycolytic enzymes in different species has an important impact on the development of selective drug analogues. Parasitic resistance and drug side effects of current antimalarial drugs have complicated and increased the cost of curing malaria.

Molecular docking was used to explore structural motifs responsible for the interactions between triose phosphate isomerase (TIM) from *Plasmodium falciparum* (PFTIM) and human (HTIM) tissues. Fourteen antimalarial drugs were examined. The binding affinities and domains identified serve as a basis for modeling novel analogues.

For all drugs modeled, PFTIM complexes displayed stronger binding affinities compared to HTIM. A dissociation constant (K_I) of 40.2 mM was obtained for the interaction between primaquine and HTIM and 1.22 mM with PFTIM. This represents a 33-fold increase in selective binding to PFTM compared to HTIM. Mefloquine shows a 24-fold increase, and one new test ligand showed 25-fold increase in binding.

The dimer interface and other pocket close to the active site are the main pockets observed by the docking studies. Key residues at the dimer interphase (Y48, D49, V46, S45, E65, S211) form a tight pocket with favorable polar contacts. 75% of PFTIM ligand complexes preferred the dimer interphase suggesting a potential site for non-competitive inhibition. These data suggest that TIM is a candidate for development of antimalarial drugs.

Keywords
malaria, glycolytic enzymes, AutoDock 4.2, dissociation constant, triosephosphate isomerase, binding affinity

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INTRODUCTION

Parasitic organisms, such as plasmodium with a fully compartmentalized glycolytic pathway, are responsible for the world scourge (malaria) prevalent in tropical and subtropical regions of the world (Kehr et al., 2010). Malaria if untreated can lead to very debilitating conditions such as coma, brain damage, loss of muscle function and death (Bloland, 2001). Global mapping data estimates that about 3.2 billion people are at risk of contracting malaria every year (Guerra et al., 2006).

The plasmodium parasite's sole dependence on glycolysis for energy needs makes the pathway enzymes potential targets for antimalarial chemotherapies. (Sameer et al., 1997; Kim et al., 2005; Verlinde et al., 2001). The decrease in efficiency of current antimalarial agents in many affected regions of the world due to toxic side effects, parasitic resistance and mutation has significantly increased the cost and complexity of curing malaria (Peterson et al., 2011; Bray et al., 2003; Briolant et al., 2010). The limited number of antimalarial drugs, and parasitic resistance to almost every available chemical therapy continues to spur the search for novel, cheaper and better analogues (Plowe et al., 2007; Tony et al., 1997). The molecular mechanism of how some antimalarial drugs exert their antimalarial activity is not completely understood.

The differences in the way triosephosphate isomerase (TIM), in human cells (HTIM) and plasmodium cells (PFTIM) interact with 14 common antimalarial agents were explored. We postulate that this can serve as basis for developing novel analogues. The increased application of Computer Aided Drug Design (CADD) methods in pharmaceutical industry and academia is a direct result of increase in computer speed, and the reliability of simulation theories and docking software (Schneider et al., 2002; Seeliger et al., 2010).

TIM is a key dimeric enzyme that speeds up the final investment phase of glycolysis (Scheme 1).

CH_2—O—PO_3^{2-} | C=O | CH_2OH ⇌ (TIM) CH_2—O—PO_3^{2-} | H—C—OH | CH_2OH

Dihydroxyacetone phosphate (DHAP) — glyceraldehyde-3-phosphate G3P

Scheme 1: Isomerization of DHAP and G3P by TIM

HTIM and PFTIM share an amino acid sequence identity of 42 %. Despite the 58 % difference in sequence identity, both molecules have similar structural folds. A root mean square deviation (RMSD) of 0.825 Å is obtained when both enzymes are structurally aligned (Figure 1). HTIM and PFTIM do, however, have some amino acid residues located in key binding motifs with different side chain polarities (Figure 1). Position 96 that is close to TIM active site residues (K12, H95 and E165) in many TIM sequences is usually occupied by serine residue (Ser), but it is replaced by Phenylalanine (Phe) in PFTIM sequence. (Parthasarathy et al., 2002). One goal of this study is to determine whether such differences and other unique structural motifs are critical for the selectivity of interactions between HTIM and PFTIM enzyme receptors.

The molecular modeling software AutoDock 4.2 was used to screen the interactions of some antimalarial agents and as a tool to fine-tune analogues. The docking was also used to map different binding pockets and determine residues involved in complexes formed by HTIM and PFTIM. Furthermore, the binding affinities and dissociation constants of antimalarial agents and analogues are compared to determine what ligand features enhance selective affinity to TIM.

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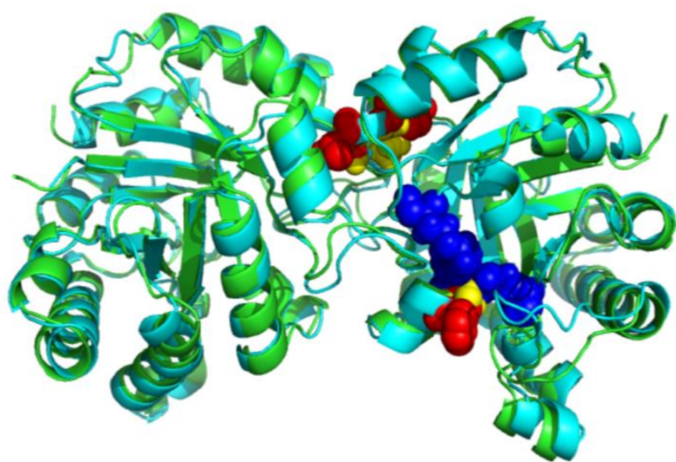

Figure 1: HTIM (green) and PFTIM (cyan) structures aligned. Key variant residues highlighted. HTIM-Yellow (A46, I48, S96) and PFTIM red (V46, Y48, F96). Active site residues (K12, H95 and E165) in Blue.

MOLECULAR SYSTEMS AND COMPUTATIONAL METHODS

The X-ray crystal structures of HTIM and PFTIM have been determined to atomic resolution and were downloaded from the RCSB Protein Data Bank (Berman et al., 2000), with accession codes 4POC (Roland et al., 2015) and 2VFI respectively (Gayathri et al, 2009). The molecular modeling software pymol (Delano, 2002) and chimera (Petterson et al., 2004) were used to refine the TIM three-dimensional structures prior to docking and create ligand protein complex figures.

AutoDock 4.2 has been successfully used to predict the binding affinities and conformations between a number of ligands and molecular targets (Morris et al., 2009). In this study, antimalarial drugs were docked to HTIM and PFTIM (Table 1). The three-dimensional structures of the ligands were obtained from ligand databases DrugBank (Law et al., 2014) and Ligand explorer site (Moreland et al., 2005; Berman et al., 2000). The AutoDockTools suite was used to prepare the ligand and receptor structures, add appropriate Gasteiger charges, identify and modify ligand rotatable bonds (Morris et al., 2009).

The ligands were then blindly docked into the rigid HTIM and PFTIM enzyme receptor using AutoDock 4.2. A grid-based method was used to enhance the quick evaluation of the binding energy of conformations of the complexes formed. The grid boxes were centered using coordinates of a virtual center of mass atom for PFTIM and HTIM. The grid box had dimensions of 120 Å x 120 Å x 120 Å, large enough to allow for sampling of binding domains or pockets on the entire protein surface by the probe ligand atoms. The Lamarckian genetic algorithm (Morris, 1998) was used in configuration space search and determination binding energy of the complexes. The interaction affinity and selectivity of the docked complexes with the enzymes was evaluated using relative binding energies (Equation 1) and dissociation constants (K_i, Equation 2). The energetics of the binding process was estimated using pair-wise terms based on the semiempirical force field expression described in Equation 1 (Huey, 2006). The first term evaluates the 6/12 Lennard Jones potential (dispersion and repulsion interactions). The second term is a

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directional hydrogen bond based potential term (Goodford, 1985). Screened Coulomb potentials where a distance dependent dielectric constant is used to model solvent effects describe the third term (Mehler, 1991). The loss of conformational entropy due to binding is calculated in the fourth and fifth terms. The last term describes the solvation and desolvation effects as the protein or ligand atoms are fully hydrated or buried in the complex (Wesson, 1192). The docked complexes were then analyzed using a combination of AutoDockTools, chimera and pymol molecular visualization software.

$$\Delta G_{bind} = \Delta G_{vdw} + \Delta G_{hbond} + \Delta G_{elect} + \Delta G_{conf} + \Delta G_{tor} + \Delta G_{solva} \quad (1)$$

$$K_i = e^{\frac{\Delta G}{RT}} \quad (2)$$

RESULTS

Binding Energies

In these simulations, 2×10^6 energy evaluations between each antimalarial drug and HTIM/PFTIM enzymes were screened to identify binding domains, important amino acid residues, binding energies and dissociation constants. For each docked system simulated, one hundred high affinity complexes were analyzed for binding domains and contact residues. All antimalarial drugs studied were successfully docked to the enzymes. The pairwise intramolecular, intermolecular and desolvation energies of the interaction between the ligands and enzymes was calculated using Equation 2. The binding energy difference between the ligand bound and unbound state was used to determine strength of interaction. The complexes with more negative binding energy were selected for further analysis. Strong binding energies ranging from -4 to -12 kcal/mol were observed for HTIM and PFTIM interactions (Figure 2). Current antimalarial drugs like atovaquone, doxycycline, chloroquine, pentamidine, primaquine and mefloquine displayed a stronger affinity for PFTIM compared to HTIM.

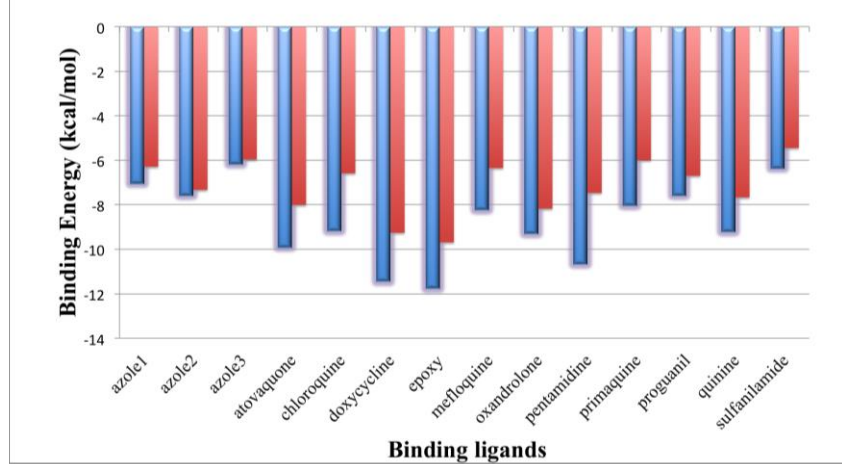

Figure 2: Relative binding energies between antimalarial drugs with HTIM (red) and PFTIM (blue). The estimated binding energies are computed using a combination of molecular mechanics forcefields and empirical parameterizations to estimate enthalpic and entropic contributions of interactions (equation 1).

Dissociation Constants

The dissociation constants (K_i) computed with equation 2, describes how tightly bound the ligands are to HTIM and PFTIM. In addition to binding energies, this thermodynamic constant also has dimensions of concentration and describes the solution equilibrium between the enzyme receptors and the ligands (small organic molecules). K_i are also commonly used to rank the order strength of receptor ligand interactions. Nanomolar and submicromolar ranges are common for many drugs. The smaller the dissociation constant (nM or μM) the more tightly bound the ligand is. It is also the ligand concentration at which the active site, allosteric or other binding domains of a receptor like TIM is half filled. A number of the ligands screened showed significant differences in dissociation constants between HTIM and PFTIM (Figure 3). Primaquine binds PFTIM with a K_i of 1.22 μM and 40.22 μM for HTIM. This suggests that the binding between primaquine and PFTIM is about 33 times stronger than the interaction with HTIM. Similarly, mefloquine also shows an increased affinity to PFTIM by a factor of 24, while sulfanilamide an antibacterial drug with one benzene ring shows an increased affinity of a factor of 5. The dissociation constants suggest that PFTIM can potentially be a suitable target for selective inhibition by primaquine, mefloquine, chloroquine, and pentamidine.

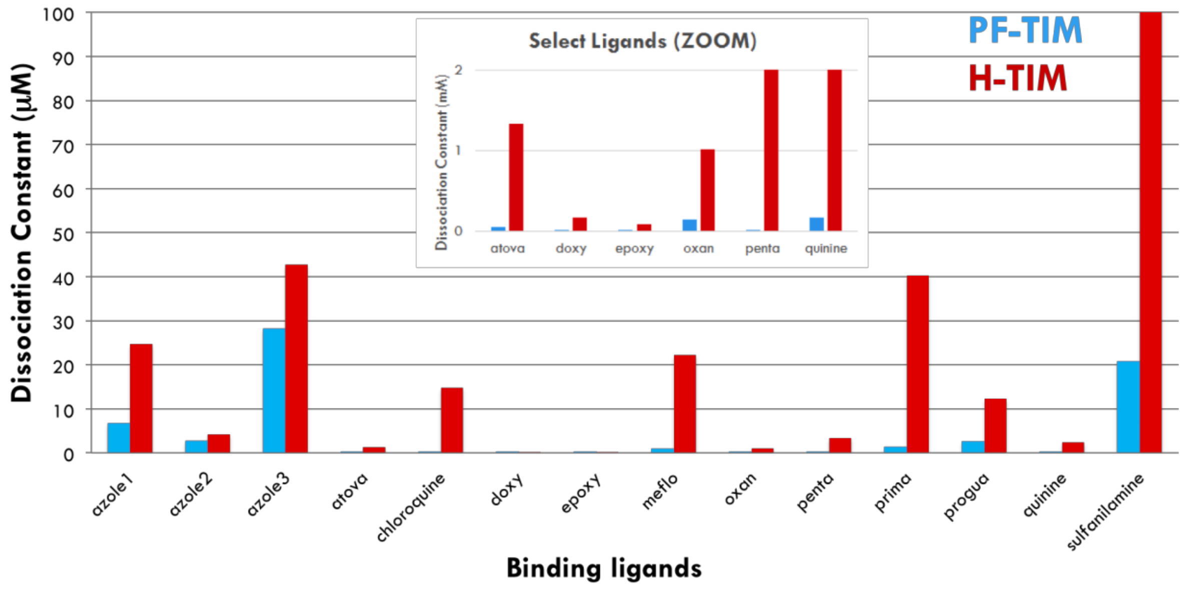

Figure 3: Dissociation constant between ligands with HTIM (red) and PFTIM (blue). The insert scales the K_i of ligands not visible on larger bar chart. K_i is computed from the estimated binding free energy of complex using equation 2.

Many ligands have been used to curb the effects of malaria, targeting different receptors in the plasmodium parasite with different degrees of success (Table 1) (Harinasuta et al., 1985; Huy et al., 2002; Tan et al., 2011; Derbyshire et al., 2012). Their efficacy is however, hindered by parasitic resistance and side effects. The indication from the micromolar dissociation constant in interactions with the glycolytic enzymes HTIM and PFTIM as potential molecular targets to fight malaria is interesting.

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Table 1. Antimalarial Drugs and Ligand structures used in docking studies and associated side effects, target receptors and mutations responsible for resistance. (Bloland, 2001; Nixon, 2013; Hyde, 2007).

Ligand Name	Target receptor	Side Effects	Drug Resistance
Pyrimethamine	Dihydrofolate reductase (DHFR) Thymidylate synthetase (TS)	Nausea, stomach cramps, vomiting, headache, itching,	Resistance is due to point mutations e.g. C59R, I164L
Proguanil	Dihydrofolate reductase (DHFR) Thymidylate synthetase (TS)	Agranulocytosis, megaloblastic and aplastic anemia, thrombocytopenia	Resistance involve point mutations at S108N, N51I
Quinine	P-glycoprotein homologue 1 (Pgh1)	Ringing in the ears, vomiting, diarrhea, nausea, blurred vision, cold sweats, convulsions, drowsiness	Resistance due to mutation in *pfmdr-1* and *pfcrt* genes and corresponding protein target
Chloroquine	Chloroquine resistance transporter (CRT)	Fever, chills, headache, flu-like symptoms	Drug resistance due to mutation (K76T) in *Pfmdr 1* and *Pfcrt* which encode CRT receptors
Primaquine	Unknown	Nausea, stomach pain, vomiting, loss of appetite, abdominal cramps, heartburn	Resistance and compliance issues
Atovaquone	Target the mammalian serotonin receptor Cytochrome b	Maculopapular rash, nausea, diarrhoea and headache	Drug resistance is given by mutation at Y268S/N/C
Mefloquine	P-glycoprotein homologue 1 (Pgh1) Multi drug resistance 1 (MDR1)	Headache, nausea, vomiting, hair loss, anorexia,	Resistance is due to point mutations e.g. C59R, I164L
Sulfanilamide	Dihydropteroate synthetase	Itching, anemia and swollen tongues	Resistance is due to point mutations e.g. E540K, A437G

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Binding Site and Complexes

Complexes between primaquine, mefloquine, sulfanilamide and TIM were selected for further analysis**.** Two main binding domains were observed out of 100 complexes analyzed for each screened ligand**.** For interactions involving PFTIM, the ligands preferred a binding site at the dimer interface of the enzyme. There was a more even distribution between dimer interphase, active site proximity and other sites in the binding with HTIM (Figures 4 and 5). The favorable interactions observed at the dimer interface are mainly due to polar contacts with ligand atoms (Table 2).

Table 2. Percentage of complexes formed in dimer interface and other binding domains on HTIM and PFTIM.

	HTIM %		**PFTIM %**	
Ligand	Dimer	Other	Dimer	Other
Sulfanilamide	55	45	73	27
Primaquine	34	64	72	28
Mefloquine	60	40	68	32

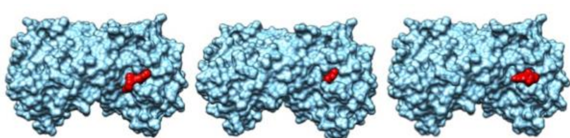

Figure 4: Surface diagram showing binding modes of primaquine (left), sulfanilamide (middle) and mefloquine (right) in interactions with HTIM. Ligand in red color.

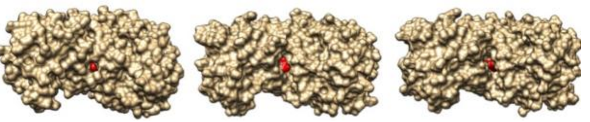

Figure 5: Surface diagram showing the dimer interphase binding modes of primaquine (left), sulfanilamide (middle) and mefloquine (right) in interactions with PFTIM. The ligand is buried in red.

DISCUSSION

AutoDock 4.2 was used as a preliminary tool to identify binding affinities and binding domains between a number of antimalarial therapeutic agents and the glycolytic enzyme TIM from human and *Plasmodium falciparum* cells. The major goal was to computationally identify ligands with the potential to selectively interact and hence inhibit glycolytic enzymes of plasmodium cells as opposed to human cells. This is to determine whether glycolytic enzymes are suitable candidates and to continue the search for ligands that can replace current antimalarial agent whose efficiency is reduced by parasitic resistance.

The data involving rigid enzyme targets reveal that primaquine prefers an allosteric binding site on the PFTIM. The dimer interphase of PFTIM has a collection of polar and hydrophobic amino acid residues (S45, V46, Y48, Q64, N65, F96) facing each other that may be an important binding domain for the ligands studied (Figure 6 and 7). For example, 75 % of the high affinity complexes analyzed in PFTIM occur at the dimer interphase (Table 2). The complexes with HTIM were evenly distributed between the dimer interphase and a site proximal to the active site. This is despite the fact that the dimer interphase in both species have amino acids with similar physico-chemical properties and polarities. One possible reason for the difference in complex distribution with HTIM is the residue substitutions at position A46, I48, S96. The importance of these amino acid residue substitutions to the observed differences in ligand affinities was however, not explored further in this study using point

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mutation studies. The polar contacts, which include hydrogen bonding and van der Waals interactions on both sides of the ligands, create a pocket that provides more favorable binding with PFTIM as opposed to HTIM (Figures 2, 6 and 7). The stronger affinity of these known ligands towards PFTIM as opposed to HTIM is mainly due to electrostatic forces. For example, PFTM is dimeric with an overall charge of -8e as opposed to -6e for HTIM. This difference in charge suggests that the amine-based ligands will show some selectivity. The importance of electrostatic interaction in ligand binding selectivity has been well described in a number of studies (Astorga et al., 2012). For example quinolone-based ligands do not compete for the cofactor of binding site of alcohol dehydrogenase like glyceraldehyde phosphate dehydrogenase (GAPDH) due to unfavorable intrinsic electrostatics and hydrogen bonding (Waingeh et al., 2013). Similarly, the discrimination between K^+ and Na^+ by the narrowing potassium channel KscA is due to the intrinsic electrostatics and coordination between ions and carbonyl groups (Noskov et al., 2004). In general a single molecular determinant cannot usually explain the high affinity or selectivity in functional protein complexes (Astorga et al, 2012). The ligands that show strongest affinity and improved selective binding to PFTIM over HTIM (Figure 8) possess key features. These ligands have a good combination of lipophilicity and polarity. The hydrophobic character is introduced by the presence of 2 or more phenyl rings and short alkyl chain. The lipophilic groups are flanked by hydrogen donors and acceptors creating a dipolar small organic molecule with ionizable features (Table 1). This is the case for ligands with high K_i selectivity factors like chloroquine and primaquine. The K_i selectivity factor for Proguanil a mostly polar molecule is small (Figure 8). Azole-based ligands with lots of hydrophobic moieties or sulfanilamide fewer hydrophobic moieties (single phenyl ring) have smaller K_i selectivity factor.

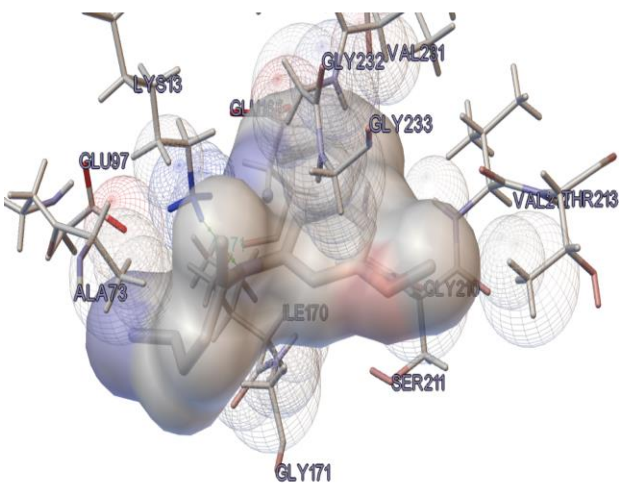

Figure 6: HTIM primaquine interaction region showing contact residues responsible for polar contacts. Contact residues show in ball and stick and the ligand in surface representation.

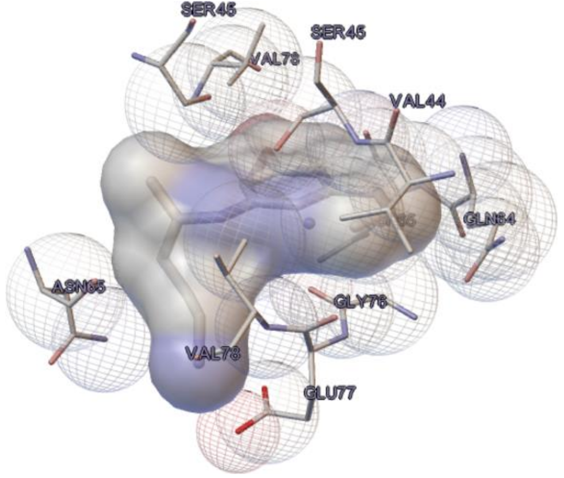

Figure 7: PFTIM primaquine interaction region showing contact residues responsible for polar contacts. Contact residues show in ball and stick and the ligand in surface representation.

Table 3: Structure and binding energy of fine-tuned ligand analogues. The binding energies are color coded (PFTIM is blue and HTIM is red).

A	B	C
-5.72 kcal/mol -6.27 kcal/mol	-6.66 kcal/mol -7.27 kcal/mol	-8.11 kcal/mol -7.77 kcal/mol
D	**E**	**F**
-7.42 kcal/mol -7.45 kcal/mol	-7.69 kcal/mol -5.79 kcal/mol	-10.09 kcal/mol -8.91 kcal/mol

Six new ligands (A, B, C, D, E and F) were designed and docked to HTIM and PFTIM (Table 3). The chemical modifications involved the increase of phenyl rings, substitution of fluorine for chlorine, and a change in position of polar fluorine atoms. The K_i data indicates that only compound E amongst the analogues improves the selective affinity for PFTIM over HTIM. The enhancement by a factor of 25 is better or on par with some current antimalarial agents (Figure 8). The enhanced selectivity factor for compound E is still however, lower than that of chloroquine and primaquine.

CONCLUSIONS

The data suggest that TIM is a potential candidate for development of antimalarial drugs. Some antimalarial agents likely interact with PFTIM and HTIM using different binding domains. The dimer interface and other pocket close to the active site are the main pockets observed by the docking studies. Key residues at the dimer interphase (Y48, D49, V46, S45, E65, S211) form a tight pocket with favorable polar contacts. 75% of PFTIM ligand complexes preferred the dimer interphase suggesting a potential site for non-competitive inhibition. Ligand analogues can therefore be fine-tuned to take advantage of differences in binding domains and residues between PFTIM and HTIM.

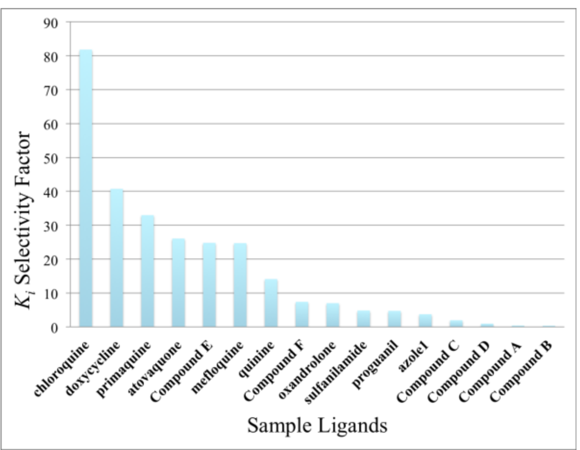

Figure 8: K_i selectivity factor for ligand binding with HTIM and PFTIM.

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In addition, the ligands do not seem to be competing for the active site of TIM but interact at proximal sites. Work is currently underway to determine how the flexibility and dynamic motions of both ligand and enzyme will affect the interactions using molecular dynamics simulations.

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