

## Screening potential inhibitors of lactate dehydrogenase from *Plasmodium knowlesi* via virtual screening approaches

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**Abstract.** Lactate dehydrogenase from *Plasmodium knowlesi* (*Pk*-LDH) has been suggested as a potential therapeutic target for the development of drugs against malaria disease. This paper reported the screening of compounds which have potentials to be developed as drugs specific for malaria caused by *P. knowlesi* via *in silico* screening. Due to the unavailability of *Pk*-LDH crystal structure, a protein model was built based on the crystal structure of the closest similar protein, lactate dehydrogenase from *Plasmodium falciparum* (*Pf*-LDH) with 91% sequence identity between the two enzymes. The model was developed using MODELLER program and verified in Structural Analysis and Verification Server. Primary and secondary structure features were determined and based on Globplot, two disordered regions were predicted at amino acid numbers 85-95 and 269-281. Meanwhile, results of PPCpred server predicted that *Pk*-LDH is crystallisable with predicted crystallisation propensity of 0.766. Verification of the model was performed with the ERRAT quality factor of 92.2% while Verify 3D gave the percentage of 85.76%. Ligand-based drug design was performed using Ultra-Fast Shape Recognition with Atom Types (USFRAT) with scores for compounds most resemble oxamate ranged from 0.832-0.914. Meanwhile, the results from structure-based screening using Autodock4 and Cygwin gave minimum binding energies ranged from -3.59 to -0.07. Taken together, this study has successfully generated a verified model structure of *Pk*-LDH and yielded a list of compounds that have potentials to be developed as antimalarial drugs.

### INTRODUCTION

The World Health Organisation (WHO) estimated that 214 million malaria cases were reported in 2015, with approximately 438 000 deaths occurred globally. Currently, a number of Southeast Asean countries are facing a new malaria threat, as a large group of human infections caused by *P. knowlesi*, an emerging zoonotic malaria parasite, has been identified in this region (Ministry of Health Malaysia, 2013; Singh *et al.*, 2004). In Malaysia specifically, 38% of malaria cases reported is due to *P. knowlesi* infection, which triggers a concern among the local community.

The causative agent of malaria is a parasitic protozoa from the genus *Plasmodium*, which is spread through the bites of infected *Anopheles* mosquitoes (Ministry of Health Malaysia, 2013). It was long known that malaria in human is typically caused by *P. falciparum*, *P. malariae*, *P. vivax* or *P. ovale*, but research findings recently revealed that the host of a rare form of malaria, caused by *P. knowlesi*, has shifted from macaques to human (Cox, 2010). A large cluster of human infections of *P. knowlesi* was first observed in Sarawak, Malaysia in 2004, and additional cases have been reported throughout Southeast Asia since then (Sabbatani *et al.*, 2010). Severe

malaria caused by *P. knowlesi* is life-threatening due to vital organs dysfunction and further complications such as enlargement of the spleen, which is mainly due to its asexual erythrocytic cycle that completes within 24 hours, accompanied by a fever that typically occurs at the same frequency (Carter and Mendis, 2002; Chin *et al.*, 1965; Cox-Singh *et al.*, 2008).

Although many antimalarial drugs have been the treatments of choice for both uncomplicated and severe malaria caused by *P. knowlesi*, respectively, mammalian toxicity and parasite resistance to the drugs complicate these efforts (Ashley *et al.*, 2014). Unfortunately, highly effective vaccines, critical in targeting malaria for global eradication, have not yet been developed. Furthermore, in contrast to *P. malariae*, which multiplies every three days in the blood and does not cause severe infections, *P. knowlesi* multiplies daily and often results in patient's death (Antinori *et al.*, 2013). These challenges further emphasise the importance of developing new antimalarial drugs that will circumvent the parasites' resistance and have little or no toxic effects in mammalian hosts.

The glycolytic pathway in *Plasmodium* spp. is an attractive target for new antimalarial drugs because during its intra-erythrocytic phase, the parasite depends solely on glycolysis for its survival in mammalian hosts. At this stage of its complex life cycle, the parasite lacks a functional tricarboxylic acid cycle, and increased glycolysis is responsible for its persistent pathogenesis. In particular, higher glucose consumption rates and lactate levels have been observed in infected erythrocytes than in uninfected cells (Harris *et al.*, 2013). The importance of the pathway was further demonstrated when inhibitory treatment with glucose analogues depleted the parasites' ATP levels (Slavic *et al.*, 2010). Together, these results highlight the advantages of considering the glycolytic pathway as a target during antimalarial drug design and discovery.

*Pf*-LDH is essential for the anaerobic lifestyle of *Plasmodium*, thus serves as a potential drug target. The enzyme is an

oxidoreductase [EC 1.1.1.27] and as the last enzyme of the glycolytic pathway, it plays a key role in energy metabolism of malaria parasites as it converts pyruvate to lactate while regenerating NAD<sup>+</sup> for continued use in glycolysis. The enzyme present bountifully in malaria parasites and was reported to be biochemically, immunologically and structurally different from mammalian and bacterial LDHs (Singh *et al.*, 2012). The plasmodial LDH has also been shown to be a potential target for chemotherapy and the *P. falciparum* LDH has recently been used for anti-malarial screening by docking studies (de Souza *et al.*, 2014; Penna-Coutinho *et al.*, 2011). On the other hand, in the quest to elucidate the functional role of LDH from *P. knowlesi*, attempts to express and purify the bacterially-expressed enzyme have been pursued (Singh *et al.*, 2012).

It was believed that drug-resistant malaria parasites emerged through mutations in the active sites of drug targets or from biochemical changes in the drug receptors. The lactate dehydrogenase enzyme from *P. falciparum* (*Pf*-LDH) has been considered as a potential molecular drug target. Chloroquine, one of the drugs used to treat malaria, interacts specifically with *Pf*-LDH in the NADH binding pocket, occupying a position similar to that of the adenyl ring cofactor and hence acting as a competitive inhibitor for this critical glycolytic enzyme (Read *et al.*, 1999). However, current drug research conducted on the glycolysis of malarial parasites of humans focuses mainly on *P. falciparum*, while a limited number of studies on other *Plasmodium* species, especially *P. knowlesi*, have been conducted. Our preliminary sequence analysis revealed a high level of identity (91%) between *Pf*-LDH and *Pk*-LDH, which suggest a probability for the latter enzyme to also be an ideal drug target, specifically to cure malaria caused by *P. knowlesi*.

The main aim of our research is to identify new drugs for the treatment of *knowlesi* malaria. Hence, in this paper, a series of virtual screening strategies are reported, with the aim of finding novel drug-like inhibitors. A ligand-based drug design experiment was conducted by screening

similar compounds which are mimics of known inhibitor of *Pf*-LDH; oxamate. Subsequently, a series of docking and scoring experiments were conducted, following the generation of a model of *Pk*-LDH structure, based on the crystal structure of *Pf*-LDH.

## MATERIALS AND METHODS

### **Amino acid sequences of lactate dehydrogenase from *P. knowlesi* (*Pk*-LDH)**

The amino acid sequence of *Pk*-LDH was retrieved from protein database of National Centre for Biotechnology Information (Accession No: AEL88505). The protein is made up 316 amino acids and used for sequence similarity analysis.

### **Physico-chemical characteristics of *Pk*-LDH**

Primary structure prediction was determined using ExPasy's Prot Param tool (Gasteiger *et al.*, 2005). By using default parameters, the system calculates the physico-chemical characteristics of the protein, such as the number of amino acids, molecular weight, theoretical isoelectric point (pI), extinction coefficient, total number of positive and negative residues, and grand average hydropathicity (GRAVY) (Geourjon and Deleage, 1995).

### **Analysis of secondary structure of *Pk*-LDH**

Self-Optimise Prediction Method with Alignment (SOPMA) programme, which is available at <https://npsa-prabi.ibcp.fr> was used to predict the secondary structure properties of *Pk*-LDH including  $\alpha$ -helix, Pi helix, Extended strand, Bend region,  $3_{10}$  helix, Beta bridge, Beta turns, Random coil, Ambiguous state and other states.

### **Prediction of disease-causing region**

Prediction of the disease region was performed by using Globplot 2.3. Globplot is a common gateway interface (CGI) web-based server available at <http://globplot.embl.de>, and is used for exploring disorder and globular segments (GlobDoms). The

server sent the *Pk*-LDH sequence by default to the public SMART queue that also predicts Pfam domains. This website searches for order/globularity or disorder tendency in *Pk*-LDH protein based on the running sum of the propensity for an amino acid to be in ordered or disordered state, by searching domain database and known disorder in proteins. In addition, PPCpred analysis available at <http://biomine-ws.ece.ualberta.ca> was also performed to predict protein crystallisation, purification, and production propensity.

### **Selection of template and sequence alignment analysis**

PSI (Protein-Specific Iterative) BLAST was performed to find out the best protein that may serve as a template for protein modelling studies. The programme finds the distant relatives of a protein by creating all closely-related proteins, and these proteins were subsequently combined into a general "profile" sequence. The profile was run as a query against the protein database, and a bigger set of proteins was identified. Another profile was constructed using this group, and the process was repeated. Three replication of PSI BLAST search was performed, prior to template selection. In this work, the PDB structures of *Pf*-LDH selected were the crystal structures of *Pf*-LDH mutant W107fA (4PLZ), *Pf*-LDH in complex with NAD<sup>+</sup> and 4-hydroxy-1,2,5-oxadiazole-3-carboxylic acid (1T24), *Pf*-LDH in complex with DNA aptamer (3ZH2) and *Pf*-LDH in complex with chloroquine (1CEQ) (Boucher *et al.*, 2014; Cameron *et al.*, 2004; Cheung *et al.*, 2013; Read *et al.*, 1999). The selected PDB structures and sequences of similar proteins from other species were aligned with the query sequence by using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), where the percentage of similarity of these proteins with *Pk*-LDH were obtained.

### **Modeling of *Pk*-LDH Structures**

Three dimensional (3D) constructions of proteins and their assemblies were performed using MODELLER programme, by satisfaction of spatial restrains. The programme was used for homology or

comparative protein structure modelling. *Pk*-LDH sequences, together with the sequence template served as the input for the programme. The output, which was the 3D model of *Pk*-LDH was obtained, consisting all non-hydrogen atoms and the main chains.

#### **Structure refinement of *Pk*-LDH model**

ModRefiner is an algorithm for atomic-level, high-resolution protein structure refinement, which may begin from either C-alpha trace, main-chain model or full-atomic model (Dong Xu and Yang Zhang, 2011). The PDB file of *Pk*-LDH model was loaded into the programme to obtain the hydrogen bonds, backbone topology and side-chain positioning of the model that is close to their native states. Besides that, it generates improvement in terms of physical quality of local structures. There were two steps of energy minimisation at atomic-level in refining protein structure from C $\alpha$  traces; the main chain structures are constructed from initial C $\alpha$  and later, the side-chain rotamers were refined together with the backbone atoms by using composite physics and knowledge-based force field (Hasan *et al.*, 2015).

#### **Verification and validation of the *Pk*-LDH model structure**

*Pk*-LDH model structure was verified and validated using Structure Analysis and Verification Server (SAVES). SAVES is a metaserver that runs six programmes for checking and validating protein structures during and after model refinement. The six programmes include PROCHECK (Laskowski *et al.*, 1996), ERRAT (Colovos and Yeates, 1993), WHAT\_CHECK (Lüthy, Bowie & Eisenberg, 1992), VERIFY 3D (Bowie *et al.*, 1991; Lüthy *et al.*, 1992), PROVE and Ramachandran Plot. However, only three programmes were available at the time this study was conducted, which were ERRAT, Verify 3D and PROCHECK, thus only the verification from these programmes were considered for structure verification.

#### **Ligand-based drug design**

An initial ligand-based pharmacophore screening was carried out by utilising

oxamate, the ligand in complex with the crystal structure of *Pf*-LDH (PDB ID: 4PLZ). The programme UFSRAT (Ultra-Fast Recognition with Atom Types), which is available at <http://opus.bch.ed.ac.uk/ufsrat/> was used to search the virtual databases for molecules with different types of similarity to the known ligands. The chosen database used in this study was EDULISS (Edinburgh University Ligand Selection System) 2 Unique database that stores 4, 012, 677 small molecules. The search took into account the overall shape similarities of all atoms, hydrophobic, hydrogen bond acceptors and hydrogen bond donors. The predicted similarity of the molecules was based on the geometric distribution descriptors of the query molecule, which is calculated along with the candidate's. Subsequently, a scoring function was employed to develop a single numerical data ( $0 < \text{score} < =1$ ).

#### **Structure-based drug design**

The structure-based drug design requires the understanding of the 3D structure of *Pk*-LDH model obtained from the MODELLER programme. This approach allows compounds from UFSRAT to be docked into the catalytic site of *Pk*-LDH model by using automated computational procedures. The first step is the modification of protein structures, by removal of water molecules from the cavity, stabilises the charges, fills in the missing residues and side chains generation according to the default parameters. Following modification, the *Pk*-LDH model structure is considered as biologically active and stable. Subsequently, the predicted active sites for *Pk*-LDH were identified. The presence of water molecules and heteroatoms were removed prior to docking. After that, the ligands obtained from UFSRAT databases were sketched using Chems sketch and the file format was changed to PDB format using OpenBabelGUI software. The final step was docking the selected compounds into the catalytic site of *Pk*-LDH using Autodock4 and Cygwin. The minimum binding energy of the compounds and *Pk*-LDH with the best dock poses was selected.

## RESULTS

### Physico-chemical characteristics of *Pk*-LDH and the secondary structure

The parameters computed by ProtParam analysis were important in showing the protein function and stability. Results of the primary structure analysis are as shown in Table 1. Secondary structure features were predicted by SOPMA. Secondary structure analysis was useful for analysing the interaction between hydrogen bond donor and hydrogen bond acceptor residues. The results of secondary structure analysis of *Pk*-LDH are presented in Table 2.

### Prediction of disease-causing region

Fig. 1 shows the results of prediction by Globplot, exhibiting the disease-causing regions of *Pk*-LDH. Two disordered regions were predicted at acid amino numbers 85-95 and 269-281, as indicated in blue colour. Results of PPCpred server predicted that *Pk*-LDH is crystallisable with predicted crystallisation propensity of 0.766. Results of individual steps in the crystallisation process are as follows; probability that production of protein material fails is 0.368, probability that purification fails is 0.207, probability that crystallisation fails is 0.116 and probability that target will yield diffraction-quality crystals is 0.531.

### Comparison of LDH from *P. knowlesi* and other human malaria parasites

The amino sequences of *Pk*-LDH and similar proteins were aligned using protein BLAST and Clustal Omega. Clustal Omega aligned more rapidly and yielded accurate sequences. Results of BLAST and Clustal Omega revealed the highest similarity between *Pf*-LDH and *Pk*-LDH with the score of 91%. Hence, *Pf*-LDH crystal structure was chosen as the template for modelling of *Pk*-LDH.

### Generation of *Pk*-LDH protein model

The model of *Pk*-LDH (Fig. 2) was built based on BLAST results, whereby there were four protein structures selected (PDB ID: 1T24, 3ZH2, 1CEQ and 4PLZ) as the templates. These structures were the structures of

Table 1. The primary structure analysis of *Pk*-LDH

Parameter	Value
Number of amino acids	316
Molecular weight	34192.04
Theoretical pI	6.96
Ext. coefficient	17670
Abs 0.1% (=1 g/l) 0.517, assuming all pairs of Cys residues form cystines	
Ext. coefficient	17420
Abs 0.1% (=1 g/l) 0.509, assuming all Cys residues are reduced	
Instability index:	31.77
This classifies the protein as stable	
Grand average of hydropathicity (GRAVY)	0.138
Aliphatic index:	108.83

Table 2. The secondary structure analysis of the sequence of *Pk*-LDH

Secondary structure	Percentage (%)
Alpha helix (Hh)	37.66
Random coi (Cc)	32.59
Extended strand (Ee)	21.84
Beta turn (Tt)	7.91
310 helix (Gg)	0.00
Pi helix (Ii)	0.00
Beta bridge (Bb)	0.00
Bend region (Ss)	0.00

*Pf*-LDH crystal structures with different types of ligands' derivatives. The structures were chosen based on their high similarity to *Pk*-LDH, as well as the availability of the crystal structures.

### Predicted model refinement, verification and validation

Refinement of the model was performed through Modrefiner to get an improved quality of the model. The model was then verified by a programme known as Structure Analysis and Verification Server (SAVES), by which this programme tested the model on six different platforms. However, only three platforms were considered in this study. The results from ERRAT shows a quality factor of 92.2%, while Verify 3D shows that 85.76% of the residues have an average 3D-

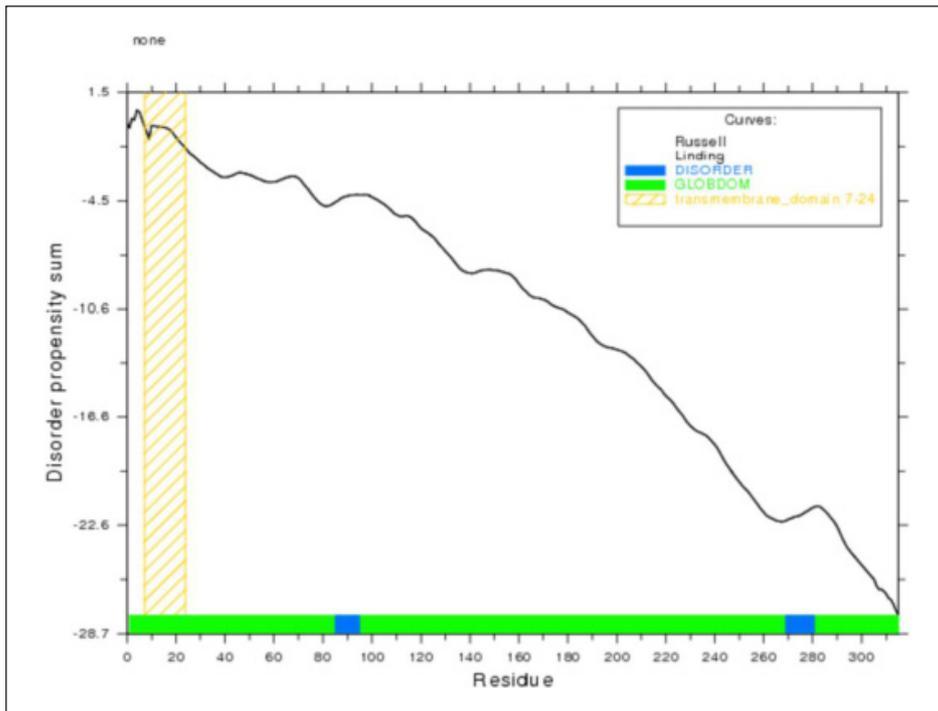


Figure 1. Protein disordered regions in *Pk*-LDH predicted by Globplot. Two disordered regions were predicted at the acid amino numbers 85-95, 269-281, as indicated in blue colour.

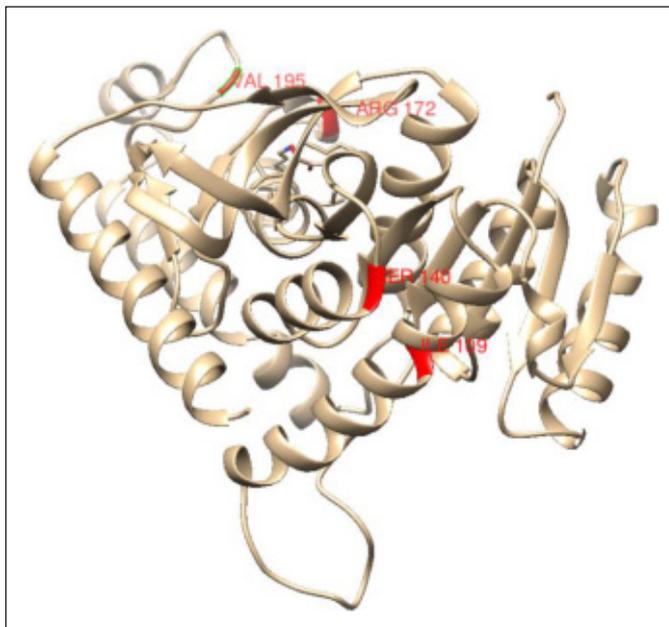


Figure 2. The model of *Pk*-LDH based on the crystal structure of *Pf*-LDH. The amino acid residues predicted to play significant roles in the active site are as labelled in red.

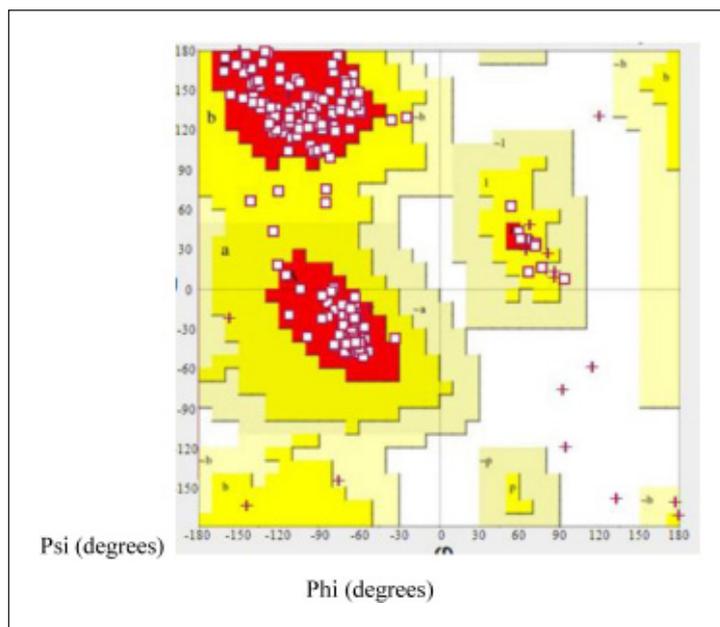


Figure 3. Ramachandran Plot analysis of *Pk*-LDH whereby white indicate disallowed region, light yellow for generously allowed region, yellow for allowed regions and red regions for favoured regions.

1D score of  $\geq 0.2$ . The passing mark for this programme is at least 80% of the amino acids scores  $\geq 0.2$  in the 3D/1D profile. PROCHECK calculated the accuracy and stereo chemical features of the model of *Pk*-LDH by using Ramachandran Plot Analysis, as shown in Fig. 3 (Laskowski *et al.*, 1993; Thillainayagam *et al.*, 2014).

### Identification of Small Molecule Analogues

A search was carried out by using known inhibitor of LDH (oxamate) as the query molecule, to search for similar candidates from a large multi-conformer library comprising of 4 012 677 molecules from EDULISS 2 Unique database. In this programme, the similarity score of zero indicates the least similar compounds, whereas a score near to one suggests a closer resemblance. The top six compounds most resemble oxamate show similarity scores ranging from 0.914–0.832 (Table 3).

These compounds satisfied the Lipinski's rule of five with no violations. Topological parameters of these compounds such as

number of atoms, molecular weight, number of hydrogen donors and number of hydrogen acceptors were calculated for all five molecules that satisfy the Lipinski rules. It is found that all six molecules have shown bonds in the range of two to four. The mentioned parameters of rule of five are listed in the Table 3. Compound 1 to 5 had satisfied the parameters with  $mlogp$  between -1.69 to 0.17 and drug likeness between -1.33 to -0.67. All compounds were calculated for toxicity using Toxicity Estimation Software (TEST) and the Quantitative Structure Activity Relationships (QSAR) results are shown in Table 4.

Generally, the predicted values obtained from different test endpoint analyses show that the smaller the value, the compound is more toxic, while larger value indicates that the compound is less toxic (Ruiz, *et al.*, 2012). Based on the toxicity prediction values in Table 4, compounds 3, 4 and 6 are non-toxicant based on developmental toxicity. It is noteworthy to note that all compounds are negative for mutagenicity.

Table 3. Physicochemical and pharmacophore properties and the similarity scores (Sqc) for compounds obtained from UFSRAT

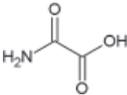
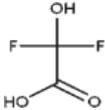
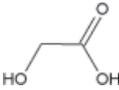
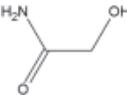
Compound	2D structure	Similarity score (Sqc)	Molecular weight	Number of HBA/HBD	MolLogP	Drug-likeness
1		0.914	89.01	3/3	-1.30	-1.02
2		0.885	114.01	3/3	0.17	-0.67
3		0.883	76.02	3/2	-1.08	-0.83
4		0.878	90.00	4/2	-0.54	-0.97
5		0.837	88.02	3/1	-0.49	-1.20
6		0.832	75.03	2/3	-1.61	-1.19

Table 4. Estimation of toxicity values for all compound using Quantitative Structure Activity Relationships (Hierarchical method)

Toxicity endpoints	Predicted value					
	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5	Compound 6
Oral LD <sub>50</sub> (mg/kg)	4260.70	203.69	2597.90	592.91	5662.36	2797.88
96 hour <i>Fathead minnow</i> LC50 (mg/L)	520.93	683.18	1904.50	2114.14	1532.74	1455.73
48 hour <i>Daphnia magna</i> LC50 (mg/L)	95.66	1287.31	1878.14	20.53	227.28	255.04
48 hour <i>Tetrahymena pyriformis</i> IGC50 (mg/L)	182.84	NA	808.39	20.53	1825.96	9929.65
Bioaccumulation factor	0.27	0.56	0.54	0.24	0.70	0.55
Developmental toxicity	Toxicant	Toxicant	Non-toxicant	Non-toxicant	Toxicant	Non-toxicant
Mutagenicity	Negative	Negative	Negative	Negative	Negative	Negative

### Docking studies

Prior to docking of the selected compounds, the docking procedure was validated using pose selection method, where in this method a compound with known conformation and orientation from a co-crystal structure was re-docked into the target's active site. A docking procedure was considered successful to be performed when the program is able to return poses below a preselected Root Mean Square Deviation (RMSD) value, from the known conformation (usually 1.5 or 2 Å depending on ligand size). Pose selection is then followed by scoring and ranking to determine which of the available scoring functions most accurately rank the poses with respect to their RMSD values (Hevener *et al.*, 2009).

*Pf*-LDH (PDB ID: 4PLZ) crystal structure was selected to verify the docking procedure. We selected the commonly used RMSD of

2 Å as our threshold for determining the success or failure of docking. This validation is considered to be successful because the scored poses with RMSD is equal to 2 Å. Furthermore, structure analysis showed that there were four Hbond between OXM and *Pf*-LDH, two with Arg 157, one with Asn 126 and one with His181, as shown in Fig. 4(a). The docking procedure was validated by retrieving the OXM from the crystal structure of *Pf*-LDH and docked into the catalytic site (Arg 157) of *Pf*-LDH. The AutoDock program has successfully reproduced similar binding pattern of OXM to the respective dehydrogenase as shown in Fig. 4(b). The position is almost the same as in the crystal structure of *Pf*-LDH with three Hbond is formed at Glu 244, Ala 241 and Leu 153, respectively.

The minimum binding energy (kcal/mol) for docking of OXM in *Pf*-LDH crystal structure is -3.46 kcal/mol, while the

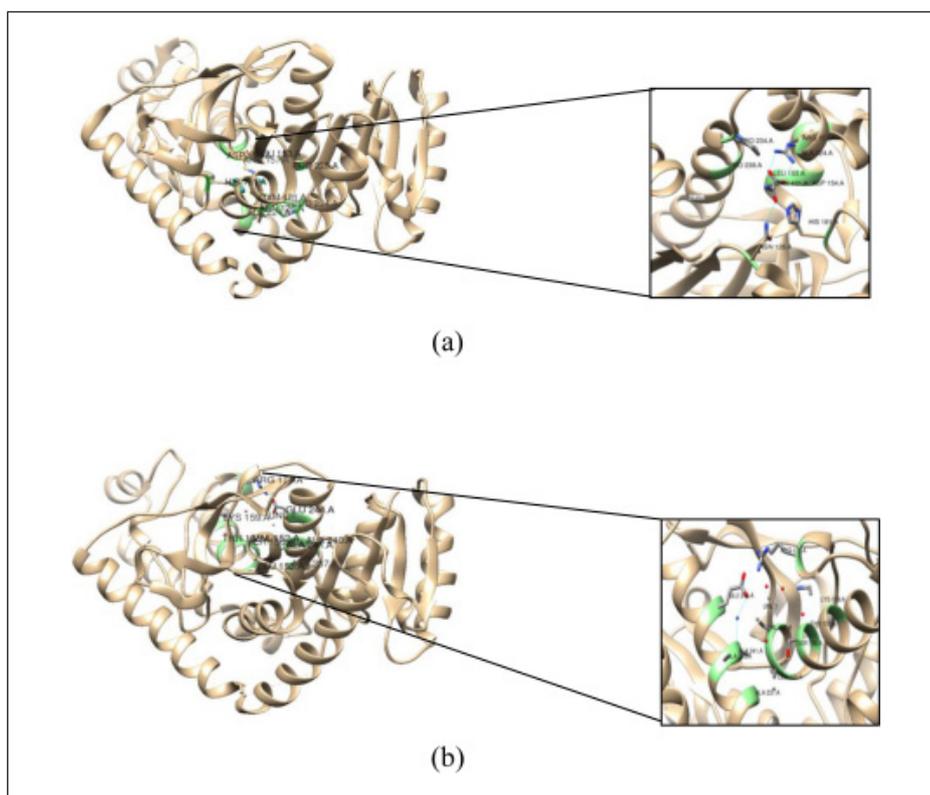


Figure 4. (a) Crystal structure of *Pf*-LDH-Oxamate complex obtained from PDB (ID:4PLZ) with four Hbond formed; two with Arg 157, one Hbond with Asn 126 and one Hbond with His181. (b) *Pf*-LDH-Oxamate complex through molecular docking analysis at Arg 157 active site, three Hbond formed with one Hbond at Glu 244, Ala 241 and Leu 153 each.

minimum binding energy of OXM in *Pk*-LDH model structure is -2.95 kcal/mol. Docking of OXM on *Pf*-LDH was set as the positive control for this study. Table 5 tabulates the results from docking studies for the top six compounds similar to oxamate, which were obtained from USFRAT. The compounds were ranked based on the minimum binding energy when docked into *Pk*-LDH model structure. Compound 5 exhibits the lowest binding energy with -3.59 kcal/mol, while compound 3 with -0.07 kcal/mol exhibits the highest binding energy, as compared to the positive control through docking analysis. This may suggest potential binding competition between the compounds with the substrate during inhibition analysis.

However, compound 4 is most likely to be used in *Pk*-LDH inhibition analysis based on its binding affinity value (-2.59 kcal/mol) that is also close to OXM, the dock poses with 3 Hbond formed at Lys 160 and Asn 175 (Fig. 5), negative for mutagenicity, non-toxicant in developmental toxicity and requires the highest concentration in one of the toxicity test end point.

## DISCUSSION

As the crystal structure of *Pk*-LDH is currently unavailable, the structure of the enzyme has been modelled by using *Pf*-LDH as the template. This was conducted based on high percentage of sequence similarity between the two proteins, the availability of *Pf*-LDH crystal structure in complex with the inhibitors and morphological similarity. It is noteworthy to note that a sequence similarity analysis between *Pk*-LDH and human LDH was also conducted, and resulted with low percentage (data not shown), which suggest that both enzymes are structurally different. This further strengthens the possibility for *Pk*-LDH to be a good drug target, with no or minimal interference with the corresponding enzyme in human. On top of that, crystal structures of both *P. falciparum* and human LDH show two key differences which are the positioning of the NADH factor and the secondary structure of a loop region that closes down on the active site during catalysis

Table 5. The minimum binding energy obtained from docking studies on *Pk*-LDH model, ranked from the lowest to the highest values

Compound No.	Minimum Binding Energy (kcal/mol)
Oxamate	-2.95
Compound 5	-3.59
Compound 1	-2.76
Compound 4	-2.59
Compound 2	-2.57
Compound 6	-2.47
Compound 3	-0.07

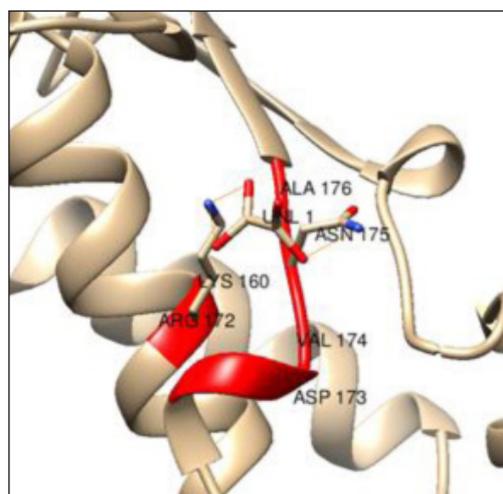


Figure 5. Compound 4 docking analysis shows three Hbond formed with two Hbond at Lys 160 and one Hbond at Asn 175.

(Cameron *et al.*, 2004). Besides that, kinetic differences between human and *Pf*-LDH are so great that the observed LDH activity can be used as an indication of *in vivo* parasitaemia (Roth *et al.*, 1982).

In order to obtain a well-validated model structure, details on the physico-chemical characteristics were obtained from the ExPasy ProtParam tool. This protein analysis tool explores the physico-chemical properties of *Pk*-LDH based on its sequences, and revealed the instability index of 31.77, which indicates that this protein is stable *in vitro*. This index is a measurement of the stability of protein in the test tube (Gasteiger *et al.*, 2005). Meanwhile, secondary structure analysis by SOPMA predicted that the

percentage of  $\alpha$ -helix in this protein is 37.66%, while random coils is 32.59%, indicating high conservation and stability of the model (Geourjon and Deleage, 1995).

The prediction of protein disordered region was conducted to shed more lights on the protein's function. Short linear peptides motifs often located in disordered regions, which are normally important for protein function. The use of protein disorder prediction server is to assist the experimental solution of protein structure and will allow one to implement a more targeted method to the experimental studies (Atkins *et al.*, 2015; Linding *et al.*, 2003). For *Pk*-LDH, Globplot showed two disordered regions, in which this intrinsically-disordered protein regions may exist as unstructured and may also become structured when bound with other molecules (Dunker *et al.*, 2008). Meanwhile, PPCpred webserver is frequently used to forecast whether a protein is capable to be expressed, purified and crystallised. This server integrates a disorder prediction within the calculations. Both the Globplot and PPCpred results predicted that *Pk*-LDH is crystallisable.

The generation of *Pk*-LDH 3D model was performed by using MODELLER programme, which calculates a model containing all non-hydrogen atoms. Subsequently, the model was refined to place the majority of residues in satisfactory core regions and further validated using a structure validation tool, Verified 3D. This tool matches the 3D structure and its specific sequence to decide the accurateness of the 3D structure, whereby a high score is expected between the two (Bowie *et al.*, 1991). ERRAT is another programme that was utilised in this study, in which its purpose is to verify protein structures normally obtained from crystallography method. The programme plotted the error values as a function of the position of a sliding 9-residue window. The error function is based on the statistics of non-bonded atom-atom interactions in the reported structure compared to a database of reliable high-resolution structures (Colovos and Yeates, 1993). ERRAT interpreted the quality of *Pk*-LDH model with 92.2% score, while the rejection limit is 95% (Hasan *et al.*, 2015).

Based on these results, it can be concluded that the refined and validated *Pk*-LDH can be utilised for the subsequent docking analysis.

For the subsequent docking, the binding pocket or the catalytic site for *Pk*-LDH was determined based on the residues corresponding to the ones involved in the active site of *Pf*-LDH crystal structure. In this case, the substrate in complex with the enzyme was used as an initial point for positioning these ligands' derivatives. Meanwhile, the UFSRAT algorithm was used to screen for candidates similar to OXM, whereby the overall shape similarities are not the only consideration, as it also examines the types and molecular topology of the existing atoms. Compounds which are mimics of OXM, a known inhibitor of *Pf*-LDH were ranked based on their similarity scores compared to the query molecule, OXM.

As shown in Table 3, the top six compounds that most resemble OXM show similarity scores ranged from 0.914–0.832, which exhibits high similarity to the query molecule. It is also noteworthy to note that the majority of compounds obtained from UFSRAT adhere to the Lipinski's rule of five criteria (Lipinski *et al.*, 1997; Taylor *et al.*, 2008) which is essential in drug design study when a pharmacologically-active lead structure is optimised for improved activity and selectivity. These selected compounds are already commercially available compounds that can be purchased from various suppliers.

Docking calculations can be affected by different protonation states, such as producing different binding poses (Warren *et al.*, 2005). Docking data from study of antimalarial activity of bisquinoline and monoquinoline against chloroquine-resistant parasites suggest that the protonated forms of both compounds present the most stable energetic conformations at the binding site of NADH inside *Pf*-LDH (Aguiar *et al.*, 2012). Hence, in this study, we selected the protonated compounds for docking analysis.

Following the validation of *Pk*-LDH model, docking and scoring functions were performed by utilising the mimics of OXM which were obtained from UFSRAT screening. The scoring function generates

scores based on mathematical methods that calculate the strength of the non-covalent interactions between the two molecules, the enzyme and the ligand. In this study, the minimum binding energies of the top six compounds ranged from -0.07 to -3.59 (Table 5). Binding energy quantifies the binding strength of a ligand to a protein. The lower the energy suggested tighter binding and more stable complex. Compounds with lower energy binding values have higher potential to compete with the substrate in the active site of the protein. Hence, compounds with the lowest binding energy and the closest value to OXM are considered to be the best results in this study.

Other studies that have been conducted in the search for antimalarial activity of potential inhibitors of *Pf*-LDH by docking methods showed that there were three compounds, analogues of NADH, with the best binding energies (itraconazole, atorvastatin and posaconazole) (Penna-Coutinho *et al.*, 2011). However, this study used different type of query molecule and utilised a different software. *In silico* search for inhibitors of *Pf*-LDH by docking showed that sequentially ZINC27313038, ZINC13759138, ZINC13759183, ZINC13759202, ZINC59648667 and ZINC11159075 have the most binding capacity with *Pf*-LDH, as compared with gossypol with the highest binding energies of -11.0, -10.3, -10.3, -10.1, -10.1 and -10.0kcal/mol respectively (Saddala *et al.*, 2014). All of these studies have shown one in thing in common, where the results indicate that selection of compounds through docking studies is an appropriate measure for antimalarial drug development. Furthermore, this technique is economical, the compounds are commercially-available and some of them are already approved for human used.

## CONCLUSION

This study has successfully generated a verified 3D model structure of *Pf*-LDH. Making use of both the ligand-based and structure-based screening approaches, the most similar compounds to the query

molecule, oxamate, obtained from the former method, were docked into the predicted catalytic site of *Pf*-LDH model, by using the latter method. The top six compounds obtained from the docking study demonstrated minimum binding affinities and compound 4 have potentials to be further validated through *in vivo* and *in vitro* studies, to verify the *in silico* evaluation, so that their biological activities can be determined. However, more extensive studies using a variety of docking software packages instead of a single docking software package, might be valuable in the near future.

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