INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print); 2222-5234 (Online)
Website: https://www.innspub.net
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Vol. 27, Issue: 5, p. 182-197, 2025

RESEARCH PAPER OPEN ACCESS

Targeting proteolytic enzymes in the hemoglobin degradation pathway to inhibit *Plasmodium falciparum*: An *in silico* approach

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Key words: Malaria, *In silico*, Hemoglobin degradation pathway, Proteases, Drug repurposing

DOI: https://dx.doi.org/10.12692/ijb/27.5.182-197 Published: November 18, 2025

ABSTRACT

Malaria caused by *Plasmodium falciparum* continues to be a major health burden, and the parasite depends on hemoglobin digestion for its survival inside human red blood cells. The enzymes operating in this pathway plasmepsins, falcipains, aminopeptidases and serine proteases play specific roles in the stepwise breakdown of hemoglobin, and therefore serve as important points for drug intervention. In this work, twelve proteases with experimentally solved structures were prepared using standard protein-refinement procedures, binding sites were identified, and a library of 326 compounds was docked using Glide-SP. The screened molecules included approved antimalarial drugs, repurposable candidates and selected marine natural products with antimalarial and antiprotozoal activity. Plasmepsin IV produced the strongest binding scores, with WEHI-842 and saquinavir showing tight fitting in the active site. HIV-protease inhibitors and diamidines also showed good interactions with several proteases, which is consistent with earlier reports of their antimalarial effects. Cysteine proteases and metallo-aminopeptidases gave moderate binding for a few compounds like pentamidine, aliskiren and carvedilol. Some large natural molecules also showed notable pocket fitting, suggesting scope for future exploration. The results indicate that structure-based screening can help narrow down promising molecules before lab testing and can support repurposing ideas when the mechanism of binding overlaps with the parasite targets. Since ligand–protein interactions mainly depend on shape, charge and other physical forces, a few drugs originally made for different diseases can also show affinity towards parasite enzymes. This study provides a set of candidates that can be taken forward to biochemical assays and parasite-culture studies to check their actual antimalarial activity.

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INTRODUCTION

Malaria is a vector borne infectious disease caused by protozoan parasites of the genus *Plasmodium* (Alemayehu, 2023). The *Plasmodium falciparum* being responsible for the most severe and fatal forms of the infection in humans among other five species infecting humans (Crutcher and Hoffman, 1996). The transmission occurs via the bite of female Anopheles mosquitoes, introduces sporozoites into humans (Alemayehu, 2023). Sporozoites invade hepatocytes developing into schizonts that release thousands of merozoites into the blood stream (Segireddy *et al.*, 2024). Infected RBCs undergoing asexual replication through ring, trophozoite, and schizont stages(Voß *et al.*, 2023). The destruction leads to clinical symptoms like fever and anemia. Some merozoites differentiate into male and female gametocytes, which are taken up by mosquitoes during blood meals, where sexual reproduction occurs, propagating the cycle (Alemayehu, 2023; Crutcher and Hoffman, 1996; Jeninga *et al.*, 2023) .

Parasite growth and replication depend on a continuous supply of amino acids, largely derived from hemoglobin digestion. The process digest approximately 60-80% of the host hemoglobin. The degradation is occurred in the specialised acidic organelle called digestive vacuole and produce the toxic byproduct heme (Wiser, 2024). In order to survive the parasite, detoxify the heme into hemozoin, the crystallised malaria pigment (Chugh *et al.*, 2013). Disrupting the nutrient acquisition and detoxification of free heme is often fatal to the parasite (Chugh *et al.*, 2013; Coronado *et al.*, 2014).

The aspartic proteases initiate hemoglobin cleavage with plasmepsin I, II and IV being prominent members (Gluzman *et al.*, 1994; Goldberg *et al.*, 1991; Silva *et al.*, 1996). Cysteine proteases include falcipain-2 and falcipain-3 further degrade the hemoglobin fragments (Subramanian *et al.*, 2009). Metalloaminopeptidases such as PfA-M1 and PfA-M17 involved in terminal digestion of oligopeptides to amino acids (Mahanta and Lhouvum, 2024; Mishra *et al.*, 2019). Serine proteases involved in downstream processes and play roles in rupture and invasion (Deu, 2017; Koussis *et al.*, 2009). Threonine proteases essential for parasite survival, their inhibition study proves lethal in different parasite stages (Mahanta and Lhouvum, 2024; Xie *et al.*, 2021).

The essentiality of parasite survival and distinct parasite activity of hemoglobin degrading proteases have long been attractive antimalarial targets (Deu, 2017; Nasamu *et al.*, 2020).

There are several inhibitors are already available to target these proteases displayed a potent activity in vitro and in vivo study (Deu, 2017; Mishra *et al.*, 2019). However, falcipain inhibitors such as vinyl sulfones peptidyl inhibitors have not succeed in clinical trials due to toxicity, lack of sensitivity and poor pharmacokinetics (Shenai *et al.*, 2003). In the case of plasmepsin, the aspartic protease inhibitors analogous to HIV protease inhibitors showed promising efficacy in the preclinical study. They failed in translation study due to redundancy among proteases and compensatory pathways in the parasite (Meyers and Goldberg, 2012). Serine and other proteases are still a preclinical candidate so far, further studies needed to made them a successful target (Deu, 2017; Tehlan *et al.*, 2023).

However, targeting a single protease may trigger compensation by other proteases in the pathway which limits the efficacy of mono-target drugs (Li *et al.*, 2012; Nasamu *et al.*, 2020).

Also, the major concern is off-targeting effects that many inhibitors also inhibit human proteases leads to host toxicity. Oral bioavailability and metabolic stability have been limited; mutation rate and genetic plasticity is high which leads to resistance (Ali *et al.*, 2021). While targeting a protease not all proteases of *P. falciparum* equally druggable. The essentiality and the unique structural features decide whether the protein is valid drug targets or not (Ali *et al.*, 2021; Li *et al.*, 2012). The classic

examples for validated targets are falcipains and certain plasmepsins, while the druggability of other proteases remain uncertain because of lack of essentiality, redundancy and similarity to human homologs (Li *et al.*, 2012; Nasamu *et al.*, 2020).

The limitations in the classical drug discovery against *P. falciparum* proteases have propelled the adaptation of computational strategies including molecular docking to enhance the drug development process (Abdulhameed Almuqdadi *et al.*, 2025; Jimoh *et al.*, 2024). The molecular docking approach predicts the preferred binding orientation between small molecules and target proteins, enable us to identify potential ligand with high binding affinity to the target proteins (Salam *et al.*, 2020). This approach created an impact such as identification and rejection of poorly binding or non-specific molecules to reduce the late-stage failures (Abdulhameed Almuqdadi *et al.*, 2025). Structure based approach differentiate truly essentials from redundant ones by revealing unique and druggable conformation states (Darmin *et al.*, 2024; Ji *et al.*, 2022). The computational prioritisation speeds up the hit identification and minimises the laborious trial and error screens (Abdulhameed Almuqdadi *et al.*, 2025).

The first FDA approved inhibitor of HIV integrase is emerged from the study that integrates X- ray crystallography, molecular docking, and MD simulations (Gu *et al.*, 2014; Johnson *et al.*, 2012; Summa *et al.*, 2008). Similar approach has been used in recent malaria research to identify the potent inhibitor against threonine protease (ClpQ) (Almuqdadi *et al.*, 2024; Jain *et al.*, 2022; Sharma *et al.*, 2022; Soh *et al.*, 2013).

In this study we have attempted to analyse the binding efficiency of all the proteases involved in hemoglobin degradation pathway. Unfortunately, certain proteins don't have experimentally solved three-dimensional (3D) structure to proceed with. As our entire work is computational based, we need to collect all the primary data from experiments rather than relying on computational models or predictions. Reason, the limited number of templates and quality of template structures makes the homology modelling a failure. Artificial intelligence (AI) based prediction methods failed to predict the quaternary structure, reduce the confidence of using modelled protein in the docking and MD simulation study. Here we have used the high-resolution 3D structure of the proteases to predict the best possible orientation between our compound library. This promising to overcome the historical bottle neck of redundancy, toxicity and other off-target effects seen in earlier antimalarial campaigns.

MATERIALS AND METHODS

Protein structure retrieval and preparation

The three-dimensional (3D) crystal structure of hemoglobin degradation protein (n=12) of *P. falciparum* was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB, https://www.rcsb.org). The selection criteria have prioritised the proteins manually curated by UniProt and structure with high resolution (≤ 3.0 Å) in the case of proteins with multiple available structures. The target proteins included: Plasmepsin II (PDB ID: 5YIC), Plasmepsin III (PDB ID: 3FNS), Plasmepsin IV (PDB ID: 1LS5), Plasmepsin X (PDB ID: 7TBB), Falcipain-2a (2OUL), Falcipain-3 (3BWK), Falcilysin (3S5M), Leucine aminopeptidase (PDB ID: 3KR4), Methionine aminopeptidase (PDB ID: 3S6B), Aspartyl aminopeptidse (PDB ID: 4EME), Serine repeating antigen-5 (PDB ID: 6X42), Subtilisin-like protease 1 (PDB ID: 8POL).

The retrieved proteins were prepared by the protein preparation workflow module in Schrödinger Maestro Suite (Schrödinger, LLC, New York, NY, 2024-1). The preparation step involves removal of co-crystallised water molecules, heteroatoms, and bound ligands.

Missing hydrogen was added to the structure using protonation states at physiological pH (7.4). Bond order was assigned to het groups and missing side

chains were added. To ensure the electrostatic environment around the active sites the protonation states of ionisable residues, particularly histidine, aspartate, glutamate, lysine, and arginine residues, were optimized using the PROPKA algorithm integrated within the protein preparation workflow.

The energy minimisation was done using OPLS3e force field, during the minimisation, hydrogen atoms allowed to move freely while heavy atoms were restrained to their crystallographic positions by following the convergence criterion of 0.30 Å RMSD for heavy atoms.

Active site prediction and receptor grid generation

After preparation the active site of the apo proteins were predicted by the SiteMap module from the Schrödinger Maestro Suite. The SiteMap is a grid-based algorithm to identify potential binding site by calculating energy maps around the protein surface. The algorithm identifies favourable van der Waals and electrostatic interactions that may accommodate small molecules. The predicted sites were ranked based on the druggability scores, basically calculated by Site score and D – Score. The top ranked site was selected for the grid generation.

The receptor grid was generated using the receptor grid generation module in Glide (Schrödinger Suite). The grid box was centred on the predicted active sites; the dimensions were adjusted to accommodate small molecules of various sizes while maintaining computational efficiency. The outer bounding box was set to 20 × 20 × 20 Å where ligand centre of mass could be placed. The inner active site box was set to 10 × 10 × 10 Å where all ligand atoms must be contained. The hydroxyl groups of serine, threonine and tyrosine inside the active sites was allowed to freely rotate during grid generation to ensure the induced-fit binding.

Ligand library preparation

The chemical library of 326 compounds was collected from four different sources to cover the diverse antimalarial chemotypes. The first category, approved antimalarial drugs including quinoline compounds, artemisinin derivatives, and other established antimalarials.

The second group contains, molecules under clinical and preclinical investigation were identified from literatures and therapeutic target database. Third, marine natural products with antiprotozoal and antimalarial activity were collected from the marine pharmacology review series published from the year 1998 to 2022. Fourth, repurposed drug candidates that may show potential antimalarial activity because several targets having homology with approved drug targets.

All the collected ligand structures were prepared using the LigPrep module in Schrödinger Suite. The preparation step included generating ionisation state at pH 7.4 ± 2.0 using Epik, because the small molecule going to interact with a target protein in acidic food vacuole. The enumeration of stereoisomers for compounds with undefined chirality and optimisation of molecular geometries using OPLS3e force field. Further, tautomeric forms were generated and most probable tautomer was selected based on the relative stability calculations.

Molecular docking studies

Molecular docking calculations was performed systematically using Glide docking program (Schrödinger Suite) in Standard Precision (SP) mode. This protocol provides speed and accuracy for virtual screening of large compound libraries. The screening study was performed with 326 compounds docked against all 12 target proteins. The final docking scores provide the estimate of binding affinity, the more negative value indicating stronger binding affinity. The binding interaction analysis were carried out to identify how well the small molecule interact inside the active site with various bond formation.

RESULTS

We screened 326 compounds against 12 hemoglobin degradation proteases from *P. falciparum* using

molecular docking in Standard Precision mode with Glide software. The docking calculations gave binding scores for all compound-protease combinations. We then sorted and examined the results by grouping proteases according to their functional classes to see which compounds bind best to each target. Table 1 indicates the docking Score of proteases involved in hemoglobin degradation pathway.

Table 1. Docking score of proteases involved in hemoglobin degradation pathway

Class	PDB ID	Protein	Resolution (Å)	Ligand	Docking score (Kcal/mol)
Aspartic protease	5YIC	Plasmepsin II	1.90	Lopinavir	-8.916
				Ritonavir	-8.071
				Lopinavir	-7.957
				Ubiquinone	-7.952
				Psammaplysin	-7.838
	3FNS	Plasmepsin III	2.50	Lapatinib	-8.158
				Pepstatin A	-7.766
				WEHI-842	-7.546
				Amiodarone	-7.546
				Digoxin	-7.511
	1LS5	Plasmepsin IV	2.80	WEHI-842	-11.447
				Saquinavir	-10.839
				WEHI-842	-10.657
				Indinavir sulfate	-10.421
				Saquinavir	-10.198
	7TBB	Plasmepsin X	1.85	Vancomycin	-8.015
				Ritonavir	-7.817
				WEHI-842	-7.751
				WEHI-842	-7.335
				Venetoclax	-7.139
Cysteine protease	2OUL	Falcipain - 2a	2.20	Indinavir sulfate	-7.735
				Mitoxantrone	-6.962
				Flecainide	-6.876
				Fostriecin	-6.815
				Doxorubicin	-6.808
	3BWK	Falcipain - 3	2.42	Pentamidine	-7.779
				Azacytidine	-7.651
				Purfalcamine	-7.420
				Doxorubicin	-7.398
Serine protease	8POL	Subtilisin-like protease 1	3.09	WEHI-842	-8.016
				Ritonavir	-7.817
				WEHI-842	-7.751
				WEHI-842	-7.335
				Venetoclax	-7.139
	6X42	SERA-5	1.20	Dinaciclib	-6.874
				Pepstatin	-6.759
				Vancomycin	-6.625
				Amphotericin	-6.614
				Saquinavir	-6.533
Metallo protease	3S5M	Falcilysin	1.55	Carvedilol	-9.268
				Fostriecin	-9.169
				WEHI-842	-8.871
				Fostriecin	-8.672
				Bestatin	-8.565
	4EME	Aspartyl aminopeptidase	2.60	Aliskiren	-9.919
				Lonafarnib	-9.851
				Ertapenem	-9.588
				DIM-C-PPhOCH3	-9.419
				Mefloquine	-9.399
	3KR4	Leucine aminopeptidase	2.00	Psammaplysin	-9.462

				Amphotericin	-8.639
				Vancomycin	-8.464
				Amphotericin	-8.241
				Mollemycin A	-8.204
	3S6B	Methionine aminopeptidase	1.95	Miglitol	-7.55
				Miglitol	-7.025
				Homogentisic acid	-6.684
				Azacitidine	-6.628
				Azacitidine	-6.512

Aspartic proteases

The aspartic proteases gave very good binding results with many compounds. Plasmepsin IV was the best binding target in our complete screening work. When we docked WEHI-842 with Plasmepsin IV, we got a binding score of -11.447 kcal/mol (Fig. 1). In the 3D structure, WEHI-842 positioned itself deep inside the active site cavity, making multiple contact points with the protease. The 2D interaction diagram showed eight hydrogen bonds with key residues such as GLY36, GLY78, SER79, GLU112, THR217, and SER218 and hydrophobic interactions with surrounding amino acids. The result was the strongest binding we found in the whole study.

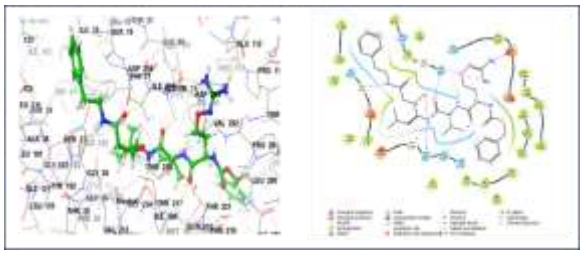

Fig. 1. Molecular interaction between plasmepsin IV and WEHI -842 represented in both 3D and 2D

When we tested Saquinavir with Plasmepsin IV, the binding score exceeded -10.839 kcal/mol (Fig. 2). The 3D structure showed Saquinavir also occupying the active site pocket, but with a slightly different orientation than WEHI-842. The 2D diagram indicated hydrogen bonding with six residues, including ASP34, GLY36, GLY78, SER79, TYR192, and SER218.

Another pose of WEHI-842 with Plasmepsin IV gave a binding score of -10.657 kcal/mol (Fig. 3), showing that this molecule can bind in multiple orientations to the same protease. Here, the 2D interaction indicated five hydrogen bonds with SER79, TYR77, GLY216, THR217, and SER218 and a pi-cation interaction with the aromatic ring of TYR77.

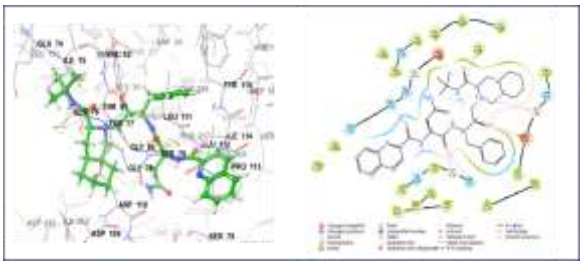

Fig. 2. Molecular interaction between Plasmepsin IV and Saquinavir represented in both 3D and 2D

Plasmepsin II showed binding, but it was weaker than Plasmepsin IV. Lopinavir with Plasmepsin II gave a binding score of -8.916 kcal/mol (Fig. 4). The 3D structure showed lopinavir fitting into the active site with its extended conformation. The 2D interactions showed three hydrogen bonds with GLY218 and SER220 and a pi-pi interaction with TYR79 residues.

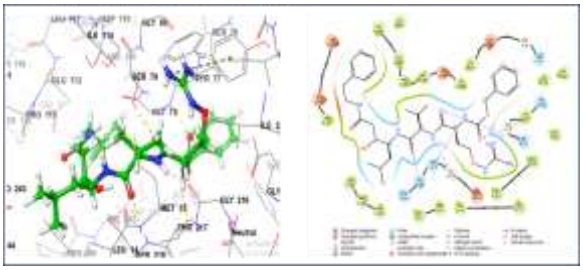

Fig. 3. Molecular interaction between Plasmepsin IV and WEHI-842 (second orientation) represented in both 3D and 2D

Plasmepsin III showed a binding score of -8.158 kcal/mol with Lapatinib (Fig. 5). The structure showed lapatinib contact with the ligand by forming

two hydrogen bonds with GLN191 and ALA280 residues and forming two salt bridges with GLU292.

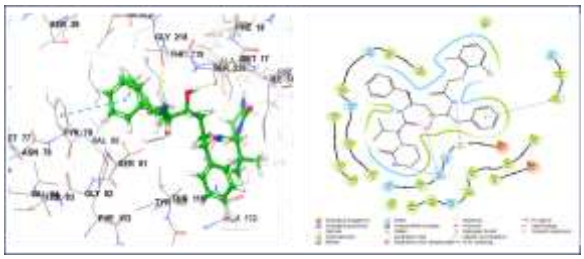

Fig. 4. Molecular interaction between plasmepsin II and lopinavir represented in both 3D and 2D

Plasmepsin X gave a binding score of -7.784 kcal/mol with Mitoxantrone (Fig. 6). The 3D view showed mitoxantrone extending across the active site pocket with its intercalating groups making contact with the protease interior. The 2D diagram showed the target protein making contact with the ligand by forming five hydrogen bonds with residues GLU484, GLU511, GLU512, MET514, and ARG568. Also, they make pi-cation and pi-pi interactions with TYR401 and TYR487, respectively. Further, they form three salt bridges with residues GLU511 and GLU512.

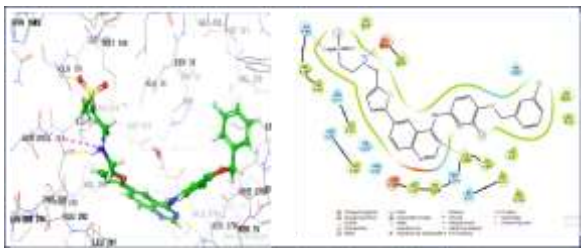

Fig. 5. Molecular interaction between plasmepsin III and lapatinib represented in both 3D and 2D

Cysteine proteases

The cysteine proteases showed weaker binding than aspartic proteases. Falcipain-2a gave the best binding with Indinavir sulfate at -7.735 kcal/mol (Fig. 7). The 3D structure showed Indinavir sulfate in the active site with its inhibitor group positioned near the catalytic site.

The 2D diagram showed hydrogen bonding to the LYS43, ASP137, ASP138, and GLU150 residues; pi-cation interaction with LYS56; and salt bridges with ASP137 amino acids.

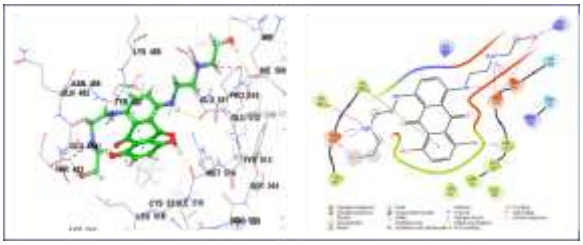

Fig. 6. Molecular interaction between plasmepsin X and mitoxantrone represented in both 3D and 2D

Falcipain-3 showed a binding score of -7.779 kcal/mol with pentamidine (Fig. 8). The 3D structure displayed pentamidine orientation in the binding pocket with its aromatic rings making contacts. The 2D interactions diagram showed two hydrogen bonds with ASP27 and ASP111, four salt bridges with ASP27 and ASP111, a pi-pi interaction with PHE72, and a pi-cation interaction with LYS193. These two falcipain enzymes had different binding patterns with their respective best ligands, indicating they have different active site structures even though both are cysteine proteases.

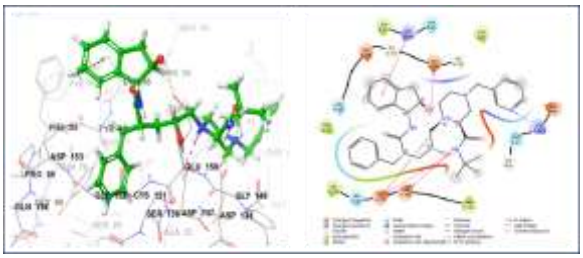

Fig. 7. Molecular interaction between Falcipain-2a and Indinavir sulfate represented in both 3D and 2D

Metal-containing aminopeptidases

The metallo-aminopeptidases showed quite satisfactory binding results. Aspartyl aminopeptidase gave a binding score of -9.462 kcal/mol with Psammaplysin (Fig. 9). The 3D structure showed Psammaplysin deeply positioned in the active site pocket. The 2D diagram showed four hydrogen bonds with ARG391 and ASP519.

Falcilysin showed a binding of -9.268 kcal/mol with Carvedilol (Fig. 10). The 3D view showed that Carvedilol is extended within the active site cavity. The 2D interaction diagram showed three hydrogen bonds with the residues, including HIS133, GLU207,

and ARG1043. Also, two salt bridges were formed between GLU 207 and the ligand.

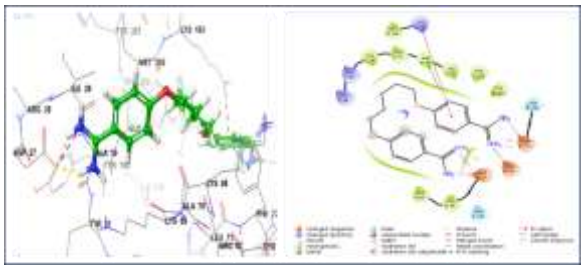

Fig. 8. Molecular interaction between falcipain- 3and pentamidine represented in both 3D and 2D

Falcilysin also showed a binding of -9.169 kcal/mol with Fostriecin (Fig. 11). The 2D interaction showed that residues including HIS133, GLU207, ASN146, and ARG1043 and the surrounded water molecule were in the structure. Also, three salt bridges were formed between MG2000. The structures showed both compounds fitting well in the active site, with fostriecin showing extended conformation.

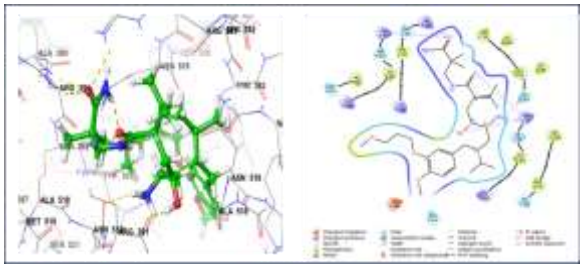

Fig. 9. Molecular interaction between Aspartyl aminoprptidase and Psammaplysin represented in both 3D and 2D

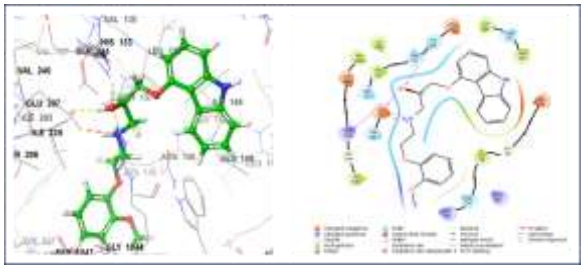

Fig. 10. Molecular interaction between Falcilysin and Carvedilol represented in both 3D and 2D

Serine proteases

The serine proteases showed the weakest binding overall. Subtilisin-like protease 1 gave a binding score of -8.016 kcal/mol with WEHI-842 (Fig. 12). The 3D structure showed WEHI-842 positioned in the active site, with the 2D diagram indicating hydrogen bonding with GLU51, ASP55, GLU187, GLU58, and pi-cation interactions with LYS533 and ARG468 amino acids.

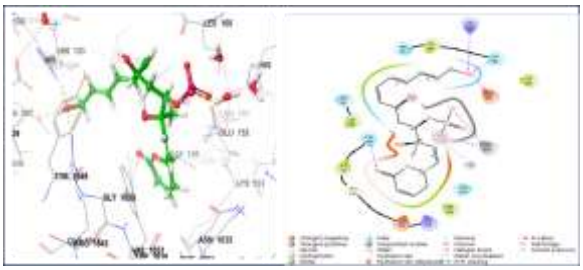

Fig. 11. Molecular interaction between Falcilysin and Fostriecin represented in both 3D and 2D

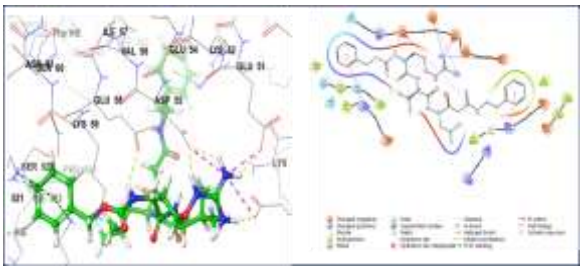

Fig. 12. Molecular interaction between Subtilisin-like protease 1 and WEHI-842 represented in both 3D and 2D

SERA-5 showed the weakest binding in our complete study, giving a score of -6.874 kcal/mol with Dinaciclib (Fig. 13). The 3D structure showed dinaciclib positioned in the binding pocket with limited contact points observed in the 2D interaction diagram, including hydrogen bonds with ASN73, LEU585, and TYR798. Salt bridges were observed between the ligand and ASP795 and LYS800 residues.

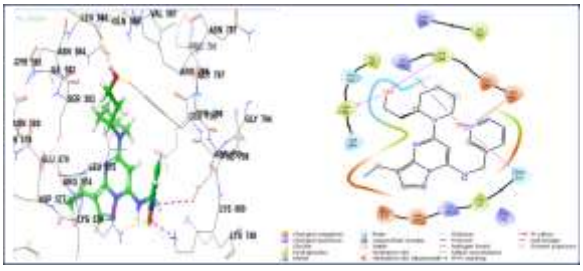

Fig.13 Molecular interaction between SERA – 5 and Dinaciclib represented in both 3D and 2D

DISCUSSION

This study aimed to compare binding of a diverse library of 326 compounds against twelve experimentally solved proteases from the *Plasmodium falciparum* hemoglobin-degradation pathway using structure-based docking (Glide-SP) and modern preparation tools (LigPrep, OPLS3e force field). Malaria caused by *P. falciparum* remains a major health problem and the parasite depends heavily on hemoglobin digestion in the acidic digestive vacuole for amino acids and survival (Gluzman *et al.*, 1994). Proteases that carry out this sequential cleavage; plasmepsins (aspartic), falcipains (cysteine), aminopeptidases (metallo) and serine proteases are therefore logical drug targets because blocking them impairs nutrient supply, causes buildup of toxic heme, and can kill the parasite (Deu, 2017; Qidwai, 2015). Our computational pipeline (high-resolution PDB structures → protein preparation with protonation/PROPKA and OPLS3e minimisation → SiteMap active-site detection → Glide/SP docking) is well aligned with current best practice in virtual screening and gives a rapid, reproducible way to prioritise ligand–target pairs for follow up (Halgren *et al.*, 2004; Roos *et al.*, 2019).

Target-specific inhibitors reduce the chance of wasting resources on compounds that bind poorly or non-specifically. In the haemoglobin-degradation cascade, individual proteases occupy distinct structural niches and act at ordered steps hence a compound that binds tightly to an essential, non-redundant protease is more likely to show parasite killing (Deu, 2017; Gluzman *et al.*, 1994). Computational approaches such as the one used here are important for three main reasons. First, they allow screening of many ligands rapidly and cheaply and point out likely high-affinity poses that deserve experimental validation (Halgren *et al.*, 2004).

Second, structure-based analysis can reveal subtle differences between parasite enzymes and human homologs (pockets, flap dynamics, conserved motifs), helping to select compounds with better selectivity (Deu, 2017; McGillewie and Soliman, 2016). Third, computation documents hypotheses that experimental work can test docking score and interaction maps guide mutagenesis, biochemical assays, and focused medicinal chemistry. These advantages are not merely theoretical: many drug discovery efforts that progressed to clinical candidates used an integrated structural and computational workflow (Halgren *et al.*, 2004; Roos *et al.*, 2019).

Drug repurposing uses approved or late-stage compounds for new indications. It is attractive because safety and many ADME parameters are already known, lowering both cost and time to the clinic (Ashburn and Thor, 2004; Pushpakom *et al.*, 2019). Historical successes (e.g., sildenafil, thalidomide derivatives) show the value of paying attention to unexpected bioactivities and side effects (Ashburn and Thor, 2004; Pinzi *et al.*, 2024). In malaria specifically, several non-antimalarial drugs have shown antiplasmodial activity in vitro or in vivo and some clinical signals exist: HIV protease-inhibitor regimens (lopinavir/ritonavir) were associated with reduced malaria recurrence in a randomized trial in children (Achan *et al.*, 2012), and many HIV-PIs show *in vitro* activity against *P. falciparum* (Porter *et al.*, 2012). These observations support the logic of screening approved drug libraries against parasite targets a route we have followed here. Still, success is mixed: repurposing can fail if pharmacokinetics, toxicity or mechanistic mismatch make the exposure in the parasite-relevant compartment inadequate (Pushpakom *et al.*, 2019).

Our results show a clear pattern: aspartic proteases (plasmepsins) gave the strongest docking scores overall, with plasmepsin IV showing the top hits (WEHI-842: −11.447 kcal·mol^{-1}; Saquinavir: −10.839 kcal·mol^{-1}). Cysteine proteases (falcipains) and metallo-aminopeptidases gave intermediate results, and serine proteases produced the weakest scores in our screen. Below we discuss each major protein class, their role, and how the docked drugs could be relevant.

Plasmepsins start the haemoglobin cleavage cascade and also have other roles (e.g., protein processing for export), so they are attractive targets (Gluzman *et al.*, 1994; Qidwai, 2015). Our strongest single docking result was WEHI-842 with Plasmepsin IV (−11.447). Important caveat: WEHI-842 is a well characterised peptidomimetic inhibitor developed against plasmepsin V (PMV) it mimics the PEXEL motif and blocks protein export; crystal structures show tight binding to PMV and very low IC_{50} (Hodder *et al.*, 2015). That literature makes clear WEHI-842's designed specificity for PMV, and pocket conservation or similarity may explain why WEHI-842 also docks well to Plm IV in silico. Plm IV binding could reflect real cross-reactivity (pocket similarity) or be an artefact of docking to an apo structure; experimental follow up (biochemical inhibition of Plm IV, cell assays) is necessary to confirm. Structural and dynamics work on Plm V shows that inhibitor binding strongly stabilises the flap and reduces flexibility a mechanism likely to be shared across plasmepsins and relevant to inhibition (Hodder *et al.*, 2015; McGillewie and Soliman, 2016). Saquinavir and other HIV-protease inhibitors also scored well against Plm IV in our work; this is consistent with several studies showing HIV PIs can inhibit plasmepsins and sometimes reduce malaria burden in vivo or in clinical settings (Achan *et al.*, 2012; Porter *et al.*, 2012). Mechanistically, HIV PIs were developed to target the viral aspartyl protease; plasmepsins are aspartyl proteases too, this shared catalytic chemistry explains the cross-reactivity and repurposing potential.

Falcipains are the major cysteine proteases that further digest hemoglobin peptides produced by plasmepsins (Deu, 2017). Our top docking hits here were HIV-PI related (Indinavir sulfate for Falcipain-2a: −7.735) and pentamidine for Falcipain-3 (−7.779). Pentamidine and related diamidines have known antiprotozoal and antimalarial activity (Stead *et al.*, 2001). For pentamidine, literature suggests accumulation in infected erythrocytes and interaction with heme/hemozoin pathways rather than a single protease mechanism; nevertheless, docking to falcipains may indicate an additional interaction worth testing in biochemical assays (Stead *et al.*, 2001). Cysteine proteases have been difficult to progress clinically because covalent inhibitors or peptidyl vinyl sulfones suffered toxicity or poor pharmacokinetics (Deu, 2017; Mishra *et al.*, 2019). Our docking scores here are modest compared with the best plasmepsin hits, suggesting less immediate priority unless medicinal chemistry can raise affinity and selectivity.

Aminopeptidases perform the terminal trimming to free amino acids. They are metal-dependent and have different active-site geometry from aspartic/cysteine proteases, often making them druggable with chelating scaffolds (Deu, 2017), Our docking gave respectable scores for several metallo targets (for example, Aspartyl aminopeptidase with Aliskiren −9.919; Falcilysin with Carvedilol −9.268). Aliskiren is a renin inhibitor (peptide-mimetic) its high docking score could reflect its peptidic, polar nature that matches large peptide-binding pockets. However, being active against human renin means off-target toxicity is a big concern; any repurposing hypothesis should be followed by selectivity assays versus human homologs (Pushpakom *et al.*, 2019).

These scored weakest overall in our screen (e.g., SERA-5 with Dinaciclib −6.874). SERA proteins and subtilisin-like proteases have roles late in the lifecycle (egress/invasion) and are under active preclinical study (Deu, 2017). Low docking affinity suggests either the screened compounds are not well matched, or that these pockets require different chemotypes. Below we summarise the principal drug classes that showed up in our top hits and how plausible repurposing is, citing available evidence.

WEHI-842 (peptidomimetic PMV inhibitor)

Class: peptidomimetic aspartyl protease inhibitor designed for Plasmepsin V. Evidence: crystallography and cellular inhibition show WEHI-842 potently blocks PMV and parasite protein export (Hodder *et al.*, 2015). Our docking shows strong binding to Plm IV as well; this suggests possible cross-plasmepsin

activity, but literature points to WEHI-842 being optimised for PMV cleavage motif recognition — experimental assays should confirm activity on Plm IV before strong claims (Hodder *et al.*, 2015; McGillewie and Soliman, 2016).

HIV protease inhibitors (Saquinavir, Lopinavir, Ritonavir, Indinavir)

Class: viral aspartyl protease inhibitors (peptidomimetics). Mechanism in HIV: inhibit HIV-1 protease, preventing gag/pol maturation. Relevance to malaria: many HIV PIs show *in vitro* antiplasmodial effects and some clinical studies (children on LPV/r) showed reduced malaria recurrence mechanisms proposed include direct parasite killing and pharmacokinetic interactions raising antimalarial partner drug exposure (Achan *et al.*, 2012; Porter *et al.*, 2012). Our docking scoring of HIV-PIs to plasmepsins (strong to moderate) supports a possible molecular basis for these observations. But clinical translation is complicated by safety, dosing, and drug–drug interactions (Achan *et al.*, 2012).

Pentamidine and other diamidines

Class: diamidines (antiprotozoal). Known activity: pentamidine concentrates in infected erythrocytes and inhibits hemozoin formation and shows activity against *P. falciparum* (Stead *et al.*, 2001). Our docking to falcipain-3 suggests another possible interaction, though the dominant mode for pentamidine is likely heme binding/food-vacuole accumulation.

Mitoxantrone, Doxorubicin, Fostriecin, Carvedilol, etc. many of these are large, often polycyclic or highly polar drugs developed for other indications (anticancer, cardiovascular). Docking scores (e.g., Mitoxantrone to Plm X −7.784; Carvedilol to Falcilysin −9.268) indicate these molecules can fit protease pockets in silico, but repurposing into antimalarials is not straightforward: pharmacokinetics, toxicity, and target exposure in infected erythrocytes are real hurdles. For instance, carvedilol is a beta blocker with anti-parasite activity only suggested in other protozoa (Rivero *et al.*, 2021), not robustly in malaria. Thus, these in silico hits are hypothesis generators, not immediate repurposing candidates.

New compounds / marine natural products (Psammaplysin, Mollemycin A)

Natural products often show strong docking because they occupy large pockets and form several interactions. Psammaplysin docked well to Aspartyl aminopeptidase and Leucine aminopeptidase (scores ~ −9.4 to −9.46). Natural scaffolds are good starting points for lead generation, but require rigorous follow up for potency, selectivity, and ADME.

It is important to be clear about limits. Docking (even with careful protein preparation, PROPKA protonation, OPLS3e minimisation and SiteMap site selection) predicts plausible poses and gives a relative score, but it cannot alone prove binding or activity. False positives occur when: (a) docking overestimates enthalpic complementarity but neglects entropy, water networks, and induced fit beyond side-chain rotations; (b) compound cannot reach the target in the parasite cell because of permeability, metabolism, or subcellular localisation; (c) parasite has redundant enzymes or compensatory pathways that bypass the blocked step (Deu, 2017). Therefore, top docking hits must be tested biochemically (enzyme inhibition assays), in parasite culture (growth inhibition and stage specificity), and checked for selectivity against human homologs to rule out host toxicity.

At molecular and atomic level, binding is governed by physics: complementary shapes, electrostatics, hydrogen bonding, hydrophobic packing and van der Waals forces decide whether a ligand will stick to a protein pocket at the timescale of interest (Friesner *et al.*, 2004). Molecules in the cell move by diffusion and sample many potential partners; they will preferentially dwell in sites where the free energy of binding is favourable. This basic physical picture explains why a drug developed for one target can bind another with similar pocket geometry or chemistry that is off-target binding. If the off-target is abundant,

highly expressed, or more accessible (for example located in a compartment the ligand reaches easier than the intended target), then that off-target binding may dominate the compound's cellular activity or its side effects (Pushpakom *et al.*, 2019). This is also why some off-target interactions become repurposing successes: a known safe drug may "fit" another disease-relevant protein well enough to provide benefit (Ashburn and Thor, 2004; Pinzi *et al.*, 2024). Conversely, unwanted off-targets explain many adverse effects and the high attrition of new drug candidates hence the emphasis on both structural selectivity and expression/localisation data in modern drug discovery (Deu, 2017; Pushpakom *et al.*, 2019).

Overall, our computational screening has produced useful hypotheses: some approved drugs and known bioactive molecules fit the hemoglobin-degrading protease pockets well in silico, and that supports the idea of targeted repositioning for malaria. But docking is a first step.

Because binding is ultimately ruled by physical chemistry (shape, charge, hydrogen bonding, hydrophobic contacts and dynamics), and because cellular context (expression, compartment, abundance) strongly modulates which interactions matter in real life, experimental validation is essential. This balanced workflow use computation to narrow space, then test in biochemistry and cell biology remains the most pragmatic route to repurposing or new antimalarial leads (Friesner *et al.*, 2004; Pushpakom *et al.*, 2019; Hodder *et al.*, 2015).

CONCLUSION

In this study, we screened a large set of approved drugs and known bioactive compounds against the key proteases of the *Plasmodium falciparum* hemoglobin-degradation pathway using standard docking methods, and the results showed that several molecules, especially WEHI-842 and some HIV-protease inhibitors, can strongly fit into plasmepsin pockets, while a few compounds also showed reasonable binding to falcipains and aminopeptidases. These findings suggest that computational screening can guide antimalarial work by quickly identifying promising candidates and supporting drug-repurposing ideas for molecules that already have known safety profiles. Some compounds in our list also appear as completely new entries for malaria research and may be useful for future testing. At the molecular level, all ligand–protein interactions follow simple physical principles like shape complementarity and non-covalent forces, which also explains why drugs sometimes bind to more than one protein and show unexpected effects. Overall, this work provides first-level evidence that some of these compounds can be explored further through biochemical assays and parasite culture studies to confirm their real antimalarial potential.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Rashtriya Uchchatar Shiksha Abhiyan (RUSA), Government of India, for financial support to Dr. E. Balamurugan under the Rashtriya Uchchatar Shiksha Abhiyan (RUSA) Scheme [File Ref. No. 306]. Mr. V. Sethupathi, serving as a project fellow, carried out this work. The Schrodinger software used in this study was licensed by the Department of Biochemistry and Biotechnology, Annamalai University.

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