REPURPOSING NATURAL PRODUCTS AGAINST *PLASMODIUM KNOWLESI* LACTATE DEHYDROGENASE VIA *IN SILICO* APPROACH FOR ANTIMALARIAL DRUG DEVELOPMENT

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ABSTRACT: Malaria cases have increased globally, which is due to the emergence of zoonotic malaria parasites that infect human, along with the existence of artemisinin-resistant parasites. Hence, there is an urgent need to find new therapeutics to counter these issues. As a vital enzyme in the glycolytic pathway that serves as the parasite's primary energy source during its intraerythrocytic stage in human, lactate dehydrogenase from *Plasmodium knowlesi* (*Pk*-LDH) can be a potential drug target. This project aims to screen for existing natural products that have progressed to preclinical or advanced drug development phases against *Pk*-LDH via ligand-based virtual screening (LBVS) and to evaluate the potentials of these bioactives as repurposed drugs by binding energy estimation through structure-based virtual screening (SBVS). The LBVS method, which was conducted *via* LiSiCA and ChemMine, are based on shape-based molecular similarity calculations to screen for analogues of the query molecules, which are lactate and pyruvate. Subsequently, PyRx simulation software were utilised for docking studies with the aid of PyMOL and PLIP software. This study discovered that Compound 7, α-hydroxyisovaleric acid, and Compound 2, α-ketoisovalerate are structurally similar to compounds that directly involved in the metabolic pathways of *P. knowlesi*, lactate and pyruvate, with a similarity score of 0.75. Both compounds also formed strong interactions with *Pk*-LDH, resulting in strong binding affinities of -4.6 kcal mol^{-1} and -4.3 kcal mol^{-1}, respectively. These findings open possibilities for using natural products in drug repurposing as anti-malarial agents.

KEY WORDS: *Malaria, Plasmodium knowlesi, Lactate dehydrogenase, Glycolysis, and Drug repurposing*

1. INTRODUCTION

Malaria, a persistent global health challenge, continues to exact a significant toll, particularly in regions such as Asia and Africa. The disease is predominantly transmitted through the bites of *Plasmodium*-infected *Anopheles* mosquitoes [1]. The emergence of drug-resistant parasites, notably in Southeast Asia, has magnified the complexities of malaria treatment, which subsequently jeopardising the efficacy of widely used antimalarial

drugs. This includes Artemisinin (ART) and its derivatives, which is one of the most effective antimalarial treatments [2]. Hence, addressing the resistance issue is of paramount importance, compelling the exploration of novel antimalarials.

In the pursuit of innovative solutions, repurposing natural products stands out as a promising strategy. This approach involves identifying FDA-approved drugs or natural compounds and assessing their potential as antimalarial agents. Natural products, characterised by their diverse chemical structures, have garnered attention for their capacity to modulate biological functions across therapeutic sectors [3].

This study is driven by the urgent necessity to combat malaria by targeting *Plasmodium knowlesi* lactate dehydrogenase (*Pk*-LDH) by using repurposed natural products through *in silico* approaches. *Pk*-LDH, a crucial enzyme in the parasite's glycolytic pathway, plays a vital role in energy generation, facilitating *Plasmodium* replication within the host's blood cells [4]. Hence, targeting *Pk*-LDH presents a promising strategy to disrupt the parasite's metabolic processes and hinder its proliferation.

To identify potential *Pk*-LDH inhibitors, this research employs virtual screening strategies, by integrating ligand-based and structure-based approaches. These methods enable screening of databases for compounds that are similar to known ligands, followed by docking studies to evaluate binding energies of the compounds interacting with *Pk*-LDH, thereby assessing their potential as antimalarials. Notably, two specific natural products were explored in this study: α-hydroxyisovaleric acid (Compound 7) and α-ketoisovalerate (Compound 2). The first compound, α-hydroxyisovaleric acid is a derivative of valine, featuring a substitution of the amino group with a hydroxyl group and commonly used as a biomarker [5]. The second compound, α-ketoisovalerate serves as both a human and *Saccharomyces cerevisiae* metabolite, playing significant roles in various biological contexts, while also serving as an adjuvant and biomarker [6].

Given the escalating malaria cases, which signifies the threats of drug-resistant *Plasmodium* strains, the urgency to explore alternative antimalarials has never been more critical [7]. Artemisinin, known as the most potent antimalarial drug, has served as the primary treatment for *P. falciparum*, with Artemisinin-based Combination Therapies (ACTs) developed and recommended as the first-line treatments in endemic areas. Nevertheless, the emergence of drug resistance, particularly along the Thai-Cambodian border, has raised global concerns due to the lack of equally effective alternatives [8]. The conventional drug discovery process is intricate, time-consuming, and costly [9]. However, the application of *in silico* techniques offers a rapid and cost-effective alternative, particularly in repurposing existing natural products for antimalarial properties.

By focusing on repurposing natural products against *Pk*-LDH, this study aligns with Sustainable Development Goal (SDG) 3, which aims to ensure good health and well-being of the nations. Identifying potential inhibitors against *Plasmodium* holds promises for advancing malaria elimination efforts *via* virtual screening approaches.

2. MATERIALS AND METHODS

2.1. Ligand-Based Screening

Ligand-Based Virtual Screening (LBVS) was performed to identify repurposable natural products that resemble the query molecules; pyruvate and lactate, which are associated with *Pk*-LDH enzyme in the glycolytic pathway. LiSiCA and ChemMine programmes were utilised to analyse multiple databases, including PubChem and other

databases, to identify potential natural product-derived compounds for further screening. Both LiSiCA and ChemMine use the Tanimoto technique as a scoring function to measure the structural similarity between compounds. A higher Tanimoto coefficient indicates a greater degree of similarity between the compounds.

Initially, OpenBabel, a chemical toolbox, was employed to convert the SDF file from the databases to the MOL2 format. The screening process starts with the initial screening of unknown compounds in LiSiCA, where compounds with Tanimoto scores of 0.4 or above were identified. To further enhance the similarity scores, the MCS Tanimoto function provided in ChemMine was utilised. The results were narrowed down to natural product-derived compounds that have reached the pre-clinical or advanced drug development phases. In this analysis, chemical databases such as PubChem, CheMBL, Drugbank, and Clinicaltrials were used to identify these compounds. Finally, natural products with high similarity scores, determined by setting a cutoff of 0.75 using the MCS Tanimoto score, were selected as ligand candidates for further evaluation.

2.2. Structure-Based Screening

Structure-Based Virtual Screening (SBVS) was employed to identify potential ligands from LBVS that are capable of binding to *Pk*-LDH proteins through molecular docking analysis *via* PyRx software, with subsequent analysis of their interactions using PyMOL and PLIP programmes. The 3D structure model of the *Pk*-LDH protein, obtained from a prior work [10], was used for this screening.

Compound candidates from LVBS that exhibit significant similarities to one of the query molecules with a high MCS Tanimoto score derived from ChemMine, were chosen for further assessment in the structure-based screening. Initially, the protein structure of *Pk*-LDH underwent modifications to ensure proper preparation before the docking process in PyRx. This preparation encompassed necessary adjustments such as the removal of water molecules from the cavity, charge stabilization, completion of absent residues, and construction of side chains according to defined parameters [11].

For the preparation of the ligand candidates, conversion from SDF to PDBQT format was carried out using OpenBabel, an integrated tool within PyRx. Subsequent steps involved energy minimisation of the ligands, facilitated by PyRx functionalities. The compounds were then categorised as protein or ligand, and the grid box was set to encompass all protein residues.

In the docking process facilitated by PyRx, flexibility was allowed for the compound candidates, while the protein structure remained rigid. Docking results were visualised using the software, displaying poses along with their respective binding affinities and additional parameters such as RMSD. The most favorable poses with the lowest binding energies were selected among the nine poses generated for further analysis. These chosen compounds, along with *Pk*-LDH protein, were subjected to detailed binding affinity assessments using PyMOL and PLIP software to explore atomic binding interactions within the ligand-enzyme complexes. Furthermore, for additional validation, ADME analyses were conducted to evaluate the drug-likeness of the selected ligand candidates.

3. RESULTS

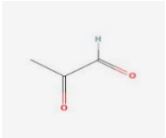
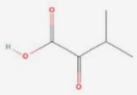
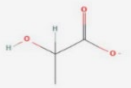
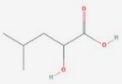
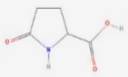
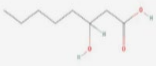
3.1. Ligand-based Virtual Screening

In the Ligand-Based Virtual Screening (LBVS), the performance of both ChemMine and LiSiCA was compared for similarity screening, aiming to evaluate their effectiveness. Initially, LiSiCA software was utilised to rank compounds based on their 2D structural resemblances to pyruvate, oxamate, and lactate. LiSiCA was chosen as the primary screening tool due to its capability to screen diverse compound databases from various sources, a feature lacking in ChemMine. Among various databases that have been screened, only databases namely PubChem, NP ATLAS, and AnalytiCon Discovery possess compounds with a similarity score of 0.4 or above with at least one of the three reference molecules. Notably, ChemMine displayed a more promising similarity score compared to LiSiCA, attributed to its employment of the Maximum Common Substructure (MCS) Tanimoto coefficient for assessing structural similarity. In this screening, 24 unknown compounds that have MCS Tanimoto scores of 0.4 and above with at least one of the three reference molecules have been screened using LiSiCA.

The subsequent phase of screening targeted the identification of natural products with potential as anti-malarial agents. Compounds sourced from natural origins and those in advanced drug development stages were referenced from chemical databases such as PubChem, CheMBL, Drugbank, and Clinicaltrials. In this phase, compounds with lower structural similarity scores were eliminated, setting a cutoff value of 0.75 to identify natural products potentially repurposable as antimalarials. While previous research recommended a cutoff of 0.85 [12], the initial scores obtained did not consistently meet this threshold. Lowering the cutoff to 0.75 enabled the inclusion of compounds exhibiting reasonable similarity, enhancing the chances of identifying potential candidates for repurposing. This adjustment balanced capturing significant similarity while expanding the pool of potential candidates. These natural products with high similarity scores, presented in the Table 1, are indicative of ligands that may possess similar biological activity to the reference molecules.

Subsequently, three ligand candidates meeting the predefined criteria were identified after the rigorous screening process. Ligand candidates with PubChem CIDs 49 and 880 exhibited notable resemblances to pyruvate, while ligand with PubChem CID 99823 displayed high similarity to lactate. These compounds bear substantial structural likeness to pyruvate and lactate, essential metabolites involved as the substrate and product, respectively, in the LDH enzyme's activity in *P. knowlesi*. Targeting these metabolites or interfering with *Pk*-LDH enzymatic activity presents a potential avenue to disrupt the parasite's energy metabolism and inhibit its growth.

Table 1: The analogues of the query molecules (Pyruvate and Lactate)

Compound	Ligand Structure	PubChem CID	Drug Development Phase	Natural Source	MCS Tanimoto Score
			Pyruvate		
3		880	Preclinical	*Arabidopsis thaliana, Sesamum indicum*	0.833
2		49	Preclinical	*Aloe africana, Euglena gracilis*	0.75
1		1662	Clinical but unknown phase	*Lemna perpusilla,Lotus creticus*	0.417
			Lactate		
7		99823	Preclinical	*Vitis vinifera, Saccharomyces cerevisiae*	0.75
8		92779	Preclinical	*Annona muricata, S. cerevisiae*	0.667
5		743	Preclinical	*Escherichia coli* (strain K12, MG1655)	0.5
6		499	Preclinical	*Stellaria palustris, Synechocystis*	0.5
4		26613	Investigative	*Streptomyces, Fragaria*	0.417

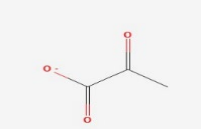
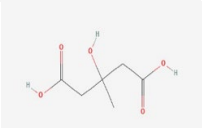
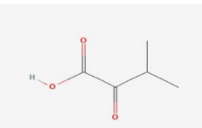
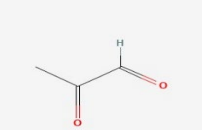
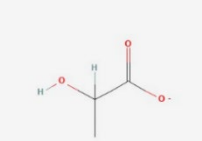
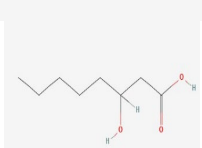
3.2. Structure-Based Virtual Screening

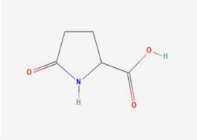
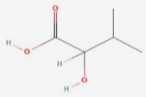
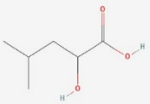
To further assess the potential of these compounds, Structure-Based Virtual Screening (SBVS) methods were subsequently employed for in-depth analysis and exploration of their interactions with *Pk*-LDH enzymes.

Through this analysis, compounds shortlisted from the earlier LBVS underwent docking simulations to assess their binding affinity. These compounds were docked specifically to the active site of the *Pk*-LDH protein. Through this process, compounds exhibiting the most negative binding energy indicating stronger binding affinity were listed as top candidates, which is a practice corroborated previously [13] [14].

To ascertain compounds with acceptable binding affinity *via* the docking technique, a comparative analysis was conducted between the docking scores of the selected compounds and the binding affinity values of their closest reference molecules. This comparative approach aimed to prioritise compounds demonstrating similar or superior binding affinity to the reference molecules, suggesting their potential as regulators of *Pk*-LDH enzyme. The PyRx docking approach highlighted pyruvate with the highest docking score of -4.1 kcal mol^{-1} and lactate with a score of -3.8 kcal mol^{-1}. Based on these scores, eight ligands exhibiting acceptable binding affinities were identified. These ligands, detailed in Table 2, showcased binding affinities ranging from -5.5 to -3.6 kcal mol^{-1}, indicating their potentials to interact effectively with the *Pk*-LDH protein and potentially evoke similar biological responses as the query molecules.

Table 2: The binding affinities of selected compounds and the query molecules

Compound	Structure	PubChem CID	Binding Affinity [kcal/mol]
Pyruvate		107735	-4.1
1		1662	-5.5
2		49	-4.3
3		880	-3.6
Lactate		91435	-3.8
4		26613	-4.8
5		743	-4.8

6		499	-4.7
7		99823	-4.6
8		92779	-4

4. DISCUSSION

Through both LBVS and SBVS analyses, Compound 2 (CID 49, α-ketoisovalerate) and Compound 7 (CID 99823, α-hydroxyisovaleric acid) emerged as the top candidates following a thorough analysis encompassing both screening approaches. The combination of screening strategies yields a comprehensive insight into the candidates' structural resemblance to known active molecules and their favorable interactions with the target protein. The integration of data acquired from both screening approaches enhances the overall reliability of the findings. As tabulated in Table 3 and Table 4, both compounds 2 and 7 exhibit noteworthy similarity scores with their respective reference molecules, which are pyruvate and lactate, while also demonstrated favorable binding affinities of -4.3 kcal mol^{-1} and -4.6 kcal mol^{-1}, respectively. These compounds have been identified as potential inhibitors that specifically target the *Pk*-LDH enzyme.

Table 3: Comparison of Compound 2 binding affinities with its respective reference compound (Pyruvate)

Compound	**PubChem CID**	**Binding Affinity [kcal/mol]**
Pyruvate	107735	-4.1
Compound 2	49	-4.3

Table 4: Comparison of Compound 7 binding affinities with its respective reference compound (Lactate)

Compound	**PubChem CID**	**Binding Affinity [kcal/mol]**
Lactate	91435	-3.8
Compound 7	99823	-4.6

Subsequently, docked complexes interaction analysis was performed, which centers on the identification of precise amino acid residues involved in the interactions between the selected compounds and *Pk*-LDH, to comprehend the pivotal binding sites and molecular interactions essential for inhibiting the enzyme's activity. Diverse interaction types, including hydrophobic contacts, hydrogen bonds, π-stacking, salt bridges, amide stacking, and cation-π interactions, collectively contribute to the stability and binding affinity of the ligand-protein complex. Notably, highly efficient ligands often exhibit a higher prevalence

of hydrophobic interactions, while fragment inhibitors commonly used in drug development demonstrate increased occurrences of hydrogen bonds [15, 16].

The first phase of the analysis involved visualising the lactate-*Pk*-LDH and Compound 7-*Pk*-LDH complexes. A comparative assessment between these complexes, depicted in Fig. 1, highlights that Compound 7-*Pk*-LDH complex did not involve salt bridges, which are not considered crucial for overall binding stability [17]. Despite forming fewer interactions, Compound 7, structurally akin to lactate, displayed a higher binding affinity (-4.6 kcal mol^{-1}) to *Pk*-LDH, compared to *Pk*-LDH-lactate interactions (-3.8 kcal mol^{-1}). Compound 7 interacted notably with ALA-80A and PHE-82A *via* hydrophobic interactions, underscoring the significance of these residues in the binding process.

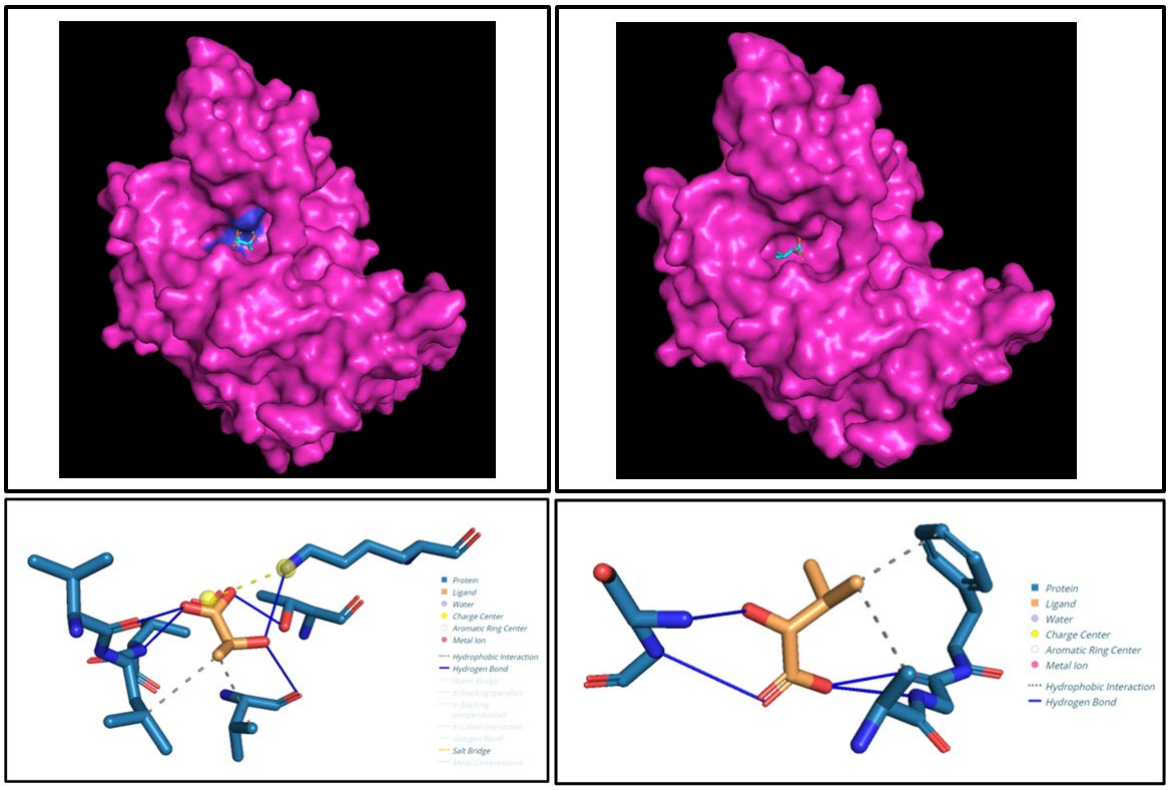


Fig 1. Visualisation of lactate-*Pk*-LDH (left) and Compound 7-*Pk*-LDH (right) complexes using PyMOL sofware. The specific interactions are also shown for both complexes.

Subsequently, visualisation of pyruvate-*Pk*-LDH and Compound 2-*Pk*-LDH complexes were employed. As illustrated in Fig. 2, Compound 2, resembling pyruvate structurally, formed a greater number of interactions compared to pyruvate. These included five hydrophobic interactions and one salt bridge. These findings corroborate the higher binding affinity exhibited by Compound 2 (-4.3 kcal mol^{-1}) in contrast to pyruvate (-4.1 kcal mol^{-1}), indicating a more robust binding with *Pk*-LDH. Further evaluation of hydrogen bonds unveiled the interaction of both Compound 2 and pyruvate with the specific amino acid residue HIS-182A, suggesting a common binding site within the *Pk*-LDH enzyme. The shared interaction with HIS-182A in both Compound 2 and pyruvate points toward a conserved binding region for these ligands within the *Pk*-LDH enzyme.

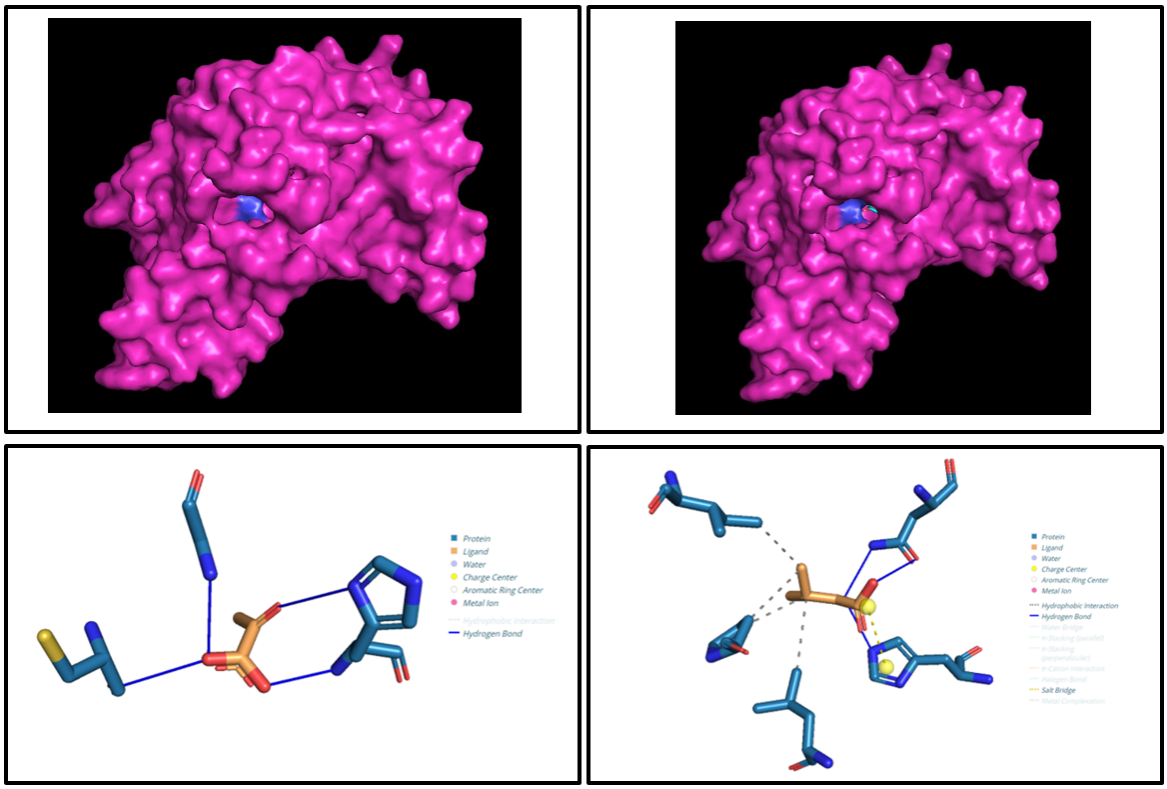


Fig 2. Visualisation of pyruvate-*Pk*-LDH (left) and Compound 2-*Pk*-LDH (right) complexes using PyMOL software. The specific interactions are also shown for both complexes.

5. CONCLUSION

The implementation of drug repurposing approaches presents promising avenues in identifying potential treatments for artemisinin-resistant malaria parasites. This approach not only expedites research endeavors but also significantly reduces the associated costs in the drug design pipeline. Through employing virtual screening and molecular docking techniques, this study has pinpointed α-hydroxyisovaleric acid (Compound 7) and α-ketoisovalerate (Compound 2) as potential inhibitors of lactate dehydrogenase from *P. knowlesi* (*Pk*-LDH). Compound 7 demonstrated stronger binding affinity despite fewer interactions, while Compound 2 displayed enhanced binding affinity over pyruvate due to specific interactions with interacting residues in *Pk*-LDH. These findings highlight the potential of repurposing Compound 7 and Compound 2 as alternative treatment options for artemisinin-resistant malaria. In the future, further research avenues should encompass rigorous validation, structural optimisation, potential combination therapies, mechanistic elucidation, and pathways for effective clinical translation, aiming to pave the way for the development of viable treatments against artemisinin-resistant malaria.

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