CHARACTERIZATION AND IN VITRO STUDY OF PROTOPORPHYRIN IX (HAEM) APTAMER IN REVERSING DRUG-RESISTANT MALARIA

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by

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LIST OF SYMBOLS

~	approximately
%	per cent
° C	degree Celsius
=	equal
±	plus, minus
<	less than
≤	less than or equal to
>	more than
≥	more than or equal to
× g	gravitation force
µm	micromolar
µg/mL	microgram per milliliter
µL	microliter
cm	centimetre
dH_20	distilled water
e.g.	for example,
g	gram
i.e.	that is
mg/kg	milligram per kilogram
mg/L	milligram per liter
mg/mL	milligram per milliliter
mL	millilitre
mM	millimolar
nm	nanometer
nM	nanomolar

pH	potential of hydrogen
pM	picomolar

LIST OF ABBREVIATIONS

ACN	Acetonitrile
ACT	Artemisinin-based combination therapy
AP	Atovaquone-proguanil
BD FACS	Becton Dickinson fluorescence-activated cell sorting
CQ	Chloroquine
CQR	Chloroquine resistant
CQS	Chloroquine sensitive
CQ-PG	Chloroquine-proguanil
CQ-SP	Chloroquine-sulfadoxine-pyrimethamine
COL-TEG	Cholesterol triethylene glycol
CI	Combination index
Conc.	Concentrations
CI	Confidence interval
CV	Cyclic voltammetry
DA	Deoxyadenosine
DC	Deoxycytidine
DG	Deoxyguanosine
DT	Deoxythymidine
DV	Digestive vacuole
DMSO	Dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
Em/Exn	Emission/excitation
EtOH	Ethanol
E.U	European Union
$Fe(CN)_6]_3$	Ferricyanide ions

$Fe(CN)_6]_4$	Ferrocyanide ions
FAM	Fluorescein amidites/Carboxyfluorescein
FD	Fluorenes detection
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fa	Fraction affected
Glu	Glutaraldehyde
Hb	Hemoglobin
HPIX	Hemoprotoporphyrin IX
HPLC	High-performance liquid chromatography
HCl	Hydrochloric acid
HCQ	Hydroxychloroquine
HEPES	Hydroxyethyl-1-piperazineethanesulfonic acid
OH	Hydroxyl
iRBC	Infected red blood cells
K_D	Dissociation constant
IC_{50}	Inhibitory concentrations
Idt	Inverted deoxythymidine
Kcal/mol	Kilo calorie per mole
K_{int}	K-internalization
LoD	Limit of detection
LoQ	Limit of quantitation
MFQ	Mefloquine
MMAF	Monomethyl auristatin f
N	Number of times
RSD	Percentage relative standard deviation
PBS	Phosphate buffer saline

PBST	Phosphate buffer saline tween 20
PA	Photodiode array
PFCRT	*Plasmodium falciparum* chloroquine resistance transporter
PLIP	Protein-ligand interaction profiler
QNX	Quinacrine
QN	Quinacrine
RBC	Red blood cells
RF	Retention factor
RT	Retention time
RP-HPLC	Reverse-phase high-performance liquid-chromatography
RMSD	Root mean square deviation
R^2	R-square
S	Slop
SHMCK	Sodium chloride, hepes, magnesium chloride, calcium chloride, and potassium chloride
SWV	Square wave voltammetry
STD	Standard
SD	Standard deviation
SP	Sulphadoxine-pyrimethamine
H_2SO_4	Sulphuric acid
RPMI	The Roswell Park Memorial Institute
3D	Three dimensional
3X	Three times
TEAA	Triethylammonium acetate
UV	Ultraviolet
USA	United States of America
WHO	World Health Organization

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PENCIRIAN DAN KAJIAN IN VITRO APTAMER PROTOPORPHYRIN IX (HAEM) DALAM MEMBALIKKAN KERINTANGAN DRUG MALARIA

ABSTRAK

Cabaran kesihatan global akibat malaria telah diburukkan lagi dengan kewujudan parasit malaria yang rintang terhadap ubat, dimana ia memerlukan pendekatan inovatif bagi meningkatkan keberkesanan rawatan sedia ada. Teknologi aptamer adalah salah satu teknologi yang diharapkan mampu memerangi parasit malaria yang kerintangan ubat kembali menjadi sensitif kembali kepada ubatan khususnya klorokuin (CQ). Kajian awal ke atas aptamer DNA protoporphyrin IX menunjukkan ia mempunyai sifat anti-malaria tetapi tidak mampu menyerap ke dalam sel darah merah yang telah dijangkiti parasit malaria. Susulan daripada itu, kajian ini bertujuan untuk mengkaji potensi aptamer Protoporphyrin IX (hem) yang diubahsuai dengan kolestrol-tri etilena glikol (COL-TEG) bagi menangani masalah yang disebabkan oleh parasit malaria yang rintang ubat. Dalam kajian ini, pelbagai pendekatan telah digunakan termasuk kaedah in-silico untuk meramalkan struktur aptamer dan kajian mengedok molekul bagi menilai interaksi lekatan. Seterusnya, Kromatografi Cecair Berprestasi Tinggi Fasa Berbalik (RP-HPLC) telah digunakan untuk menilai kestabilan aptamer di dalam serum, manakala Titrasi Spektrofotometri Penyerapan UV dan Voltametri Gelombang Segiempat (SWV) memberi keputusan kekhususan (spesifisiti) dan afiniti bagi hem dengan aptamer yang telah diubahsuai. Ujian Internalisasi Sel telah dijalankan dengan menggunakan mikroskop fluoresens dan sitometri aliran bagi menentukan keberkesanan aptamer yang diubahsuai dengan COL-TEG dalam memasuki sel darah merah. Kajian ini juga menilai aktiviti anti-malaria untuk aptamer yang diubahsuai ke atas dua jenis parasit malaria Plasmodium

falciparum iaitu yang sensitif kepada CQ (strain 3D7) dan rintang CQ (strain W2). Analisis pengedokan mendapati bahawa transformasi OKA_26 kepada COL-TEG-OKA_26 tidak mengubah bentuk interaksi dengan menunjukkan nilai tenaga pengikatan dan afiniti yang sama. Sebaliknya, penambahan COL-TEG kepada OKA_24 telah menghasilkan penurunan 23% dalam tenaga afiniti pengikatan. Analisa kestabilan di dalam serum telah menunjukkan kerintangan enzim nuklease bagi kedua-dua aptamer yang diubahsuai dengan COL-TEG iaitu >49% untuk COL-TEG-OKA_26 dan >50% untuk COL TEG-OKA_24 melebihi daripada 24 jam. Lebih penting adalah nilai kekhususan DNA aptamer protoporphyrin-IX untuk hem kekal tidak berubah melalui konjugasi lipid. Pengukuran nilai afiniti SWV menunjukkan bahawa konjugasi kolesterol mengurangkan nilai afiniti COL-TEG-OKA_24. (KD =. 47.13 ± 3.767) dan COL-TEG-OKA_26 (KD = 84.6 ± 8.7 nM) sebanyak tiga kali ganda manakala pengukuran spektrofotometri UV di dalam larutan penimbal, hampir sekali ganda nilai afiniti interaksi hem dapat di lihat bagi kedua-dua aptamer yang telah diubahsuai. Kajian ini juga menunjukkan potensi aktiviti anti-P. falciparum yang kuat dari COL-TEG-OKA_26, dengan nilai IC50 sebanyak 48.5 pM terhadap strain 3D7, sebanyak 300 kaliganda lebih berkesan berbanding COL-TEG OKA_24 dan ubat piawaian CQ (dengan masing-masing menunjukkan IC50s sebanyak 10nM dan 16 nM). Berbanding dengan strain rintang CQ (W2), COL-TEG-OKA_26 adalah empat kali ganda lebih kuat (IC50 sebanyak 100 nM) berbanding COL-TEG-OKA_24 dan CQ (IC50s > 400 nM) menandakan kemampuannya membalikan kerintangan terhadap CQ. Secara umumnya, kombinasi terapi antara aptamer yang diubahsuai dan CQ adalah bersifat antagonistic. Hasilan penemuan kajian ini telah membuktikan bahawa COL TEG-OKA_26 sebagai calon yang berpotensi untuk menangani masalah kerintangan ubat ke atas penyakit malaria dengan menunjukkan kedua-dua sifat

pembalikan kerintangan malaria terhadap CQ dan mempunyai kesan antimalaria yang kuat. Walau bagaimanapun, penyelidikan lanjut perlulah dijalankan bagi menilai profil toksisiti dan aktiviti farmakokinetiknya, ke arah pembangunan agen terapeutik malaria yang berpotensi.

CHARACTERIZATION AND IN VITRO STUDY OF PROTOPORPHYRIN IX (HAEM) APTAMER IN REVERSING DRUG-RESISTANT MALARIA

ABSTRACT

The global health challenge of malaria, compounded by drug resistance, necessitates innovative approaches for effective treatment. Aptamer technology is a promising tool towards combatting drug-resistant malaria especially chloroquine (CQ) resistance. Preliminary study on protoporphyrin IX (Haem) DNA-aptamers (OKA_24 and OKA_26) demonstrated an anti-malarial property but lack the ability to internalise into the parasite-infected red blood cell (RBC). This research investigates the potential of cholesterol-tri ethylene glycol (COL-TEG) modified haem aptamers in addressing drug-resistant malaria. The research employs a multifaceted approach, including in silico techniques for predicting aptamer structures and molecular docking studies to assess binding behaviour. Additionally, Reverse-Phase High-Performance Liquid-Chromatography (RP-HPLC) was utilized to evaluate serum stability, while UV-absorption spectral titration and Square Wave Voltammetry (SWV) provided insights into the specificity and affinity of modified aptamers for haem. Cellular internalization assays, conducted using fluorescence-microscopy and flow cytometry, determine the efficiency of COL-TEG-modified aptamers in entering red blood cells. The study also examines the antimalarial activity of modified aptamers against CQ-sensitive (3D7-strain) and CQ-resistant (W2-strain) *Plasmodium falciparum*. Docking analysis reveals that the transformation of OKA_26 to COL-TEG-OKA_26 does not alter binding behaviour, demonstrating similar binding energy and affinity. In contrast, the addition of COL-TEG to OKA_24 results in a 23% decrease in binding affinity energy. Stability assay indicates nuclease resistance for both COL-TEG-modified aptamers,

with >40% for COL-TEG-OKA_26 and >50% for COL-TEG-OKA_24 over 24 hours. Notably, the specificity of Protoporphyrin-IX DNA-aptamers for haem remains unaffected by lipid conjugation. Affinity measurements SWV revealed that cholesterol conjugation decreases the affinity of COL-TEG-OKA_24 (K_D = 47.13 ± 3.767 nM) and COL-TEG-OKA_26 (K_D = 84.6 ± 8.7 nM) by three-fold, yet in a buffer solution by UV spectroscopy, an almost one-fold increase in haem binding affinity is observed for both modified aptamers. The study also underscores the potent anti-*P. falciparum* activity of COL-TEG-OKA_26, with an IC_{50} of 48.5 pM against the 3D7 strain, which is a 300-fold greater efficacy than COL-TEG-OKA_24 and the standard drug CQ (with IC_{50}s of 10 nM and 16 nM, respectively). Meanwhile, on the CQ-resistant W2 strain, COL-TEG-OKA_26 showed four-fold more potency (IC_{50} of 100 nM) than COL-TEG-OKA_24 and CQ (IC_{50}s > 400 nM), signifying its ability to combat resistance. Notably, combination therapy with these modified aptamers and CQ is predominantly antagonistic. These findings show that, COL-TEG-OKA_26 as a promising candidate for addressing drug-resistant malaria, demonstrating both the reversal of CQ resistance and potent antimalarial activity. However, further investigation is warranted to assess its toxicity profile and pharmacokinetics activity, towards its development as a potential anti-malarial therapeutic agent.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is a tropical disease caused by parasitic infection at least from one of the five protozoans of the genus *Plasmodium* (*P*), namely, *P. malariae*, *P. falciparum*, *P. knowlesi*), *P. ovale* and *P. vivax* (Rolling et al., 2023). With humans as a definitive host, the intracellular parasite requires a mosquito as a vector to transmit and complete its complex life cycle (Boudhar et al., 2016a). The World Health Organization (WHO) indicates that malaria claimed the lives of almost 619,000 individuals in the year 2022. The mortality rate was higher (90%) in tropical regions, especially in Africa, while the remaining 10% occurred in Southeast Asia and South America (World Health Organization, 2022). The deployment of malaria prevention and control modalities such as transmission blocking strategy (integrated insecticide management) and antimalarial drug combination therapy have prevented about 6 million malaria-related deaths and 1. 5 billion clinical cases over two decades from 2000 to 2020 (Group, 2019). The evolution of resistance to pharmacological agents used for malaria therapy is growing around endemic areas and thus accounting for a higher global burden of the disease (Blasco et al., 2017, Guggisberg et al., 2018).

Malaria parasites require human blood to thrive and develop normally. At the erythrocytic stage of its life cycle, the parasite, after residing in red blood cells (RBC) cytosol, engulfs haemoglobin into its digestive vacuole (DV), where it undergoes degradation (Bakar et al., 2010). The resultant haem from the later process aggregates and is highly toxic to the parasite. The polymerisation of haem yields hemozoin, which has no toxicity against the malaria parasite (Coronado et al., 2014). Antagonising

hemozoin formation is a mechanism via which some antimalarial agents, like chloroquine (CQ), mediate their therapeutic effect. At the cellular level, CQ is easily transported into the DV, where it is protonated. Protonation of CQ alters its permeability potentials and thus prevents its escape from parasite DV. Double protonated CQ has a high affinity for haem and thus forms a complex with haem, preventing its conversion to hemozoin (Coronado et al., 2014, Chinappi et al., 2010b).

The potency of CQ has declined due to the emergence of resistant strains of malaria parasites worldwide, thereby prostrating the effort toward controlling malaria infection (Tse et al., 2019). In CQ-resistant strains of *P. falciparum*, mutations occur in the *Plasmodium falciparum* CQ resistance transporter (PfCRT) gene. These mutations result in changes to the PfCRT protein's structure and function. Mutated PfCRT is a transporter protein that pumps CQ out of the digestive vacuole and into the parasite's cytoplasm. This efflux of CQ reduces the concentration of the drug within the vacuole, limiting its ability to bind to haem and disrupt hemozoin formation. As a result, the parasite can continue its normal digestion process without accumulating toxic haem, rendering CQ ineffective. The reduced accumulation of CQ within the digestive vacuole due to the efflux mediated by mutated PfCRT results in reduced parasite sensitivity to CQ. This is why CQ, once an effective antimalarial drug, has lost much of its efficacy against CQ-resistant strains of *P. falciparum*. (Henry et al., 2008, Chinappi et al., 2010a) PfCRT-mediated CQ resistance has led to patronage of alternative anti-malarial agents in clinical use.

However, limited studies demonstrated that CQ is still effective in the control of malaria in some regions of the African continent, such as Kenya, Ethiopia, and Malawi (Mekonnen et al., 2014, Kiarie et al., 2015). Therefore, it will not be surprising

if CQ returns to clinical use and the drug market, especially in a drug-combination form, particularly for areas with CQ-sensitive malaria infections.

Various macromolecules have demonstrated the ability to augment the pharmacological effect of CQ on CQ-resistant (CQR) strains without altering its biological efficacy against CQ-sensitive (CQS) strains. Verapamil, a calcium channel blocker used to treat cardiovascular disease, exemplified this. As a chemosensitizer, verapamil increases the parasite DV concentration of CQ via H+- pumping V-type ATPase (Ecker et al., 2012).

Combinatorial therapy, or combination therapy, involves using multiple therapeutic agents to enhance treatment efficacy or target different aspects of a disease. It is widely employed in medicine to tackle complex conditions, prevent the development of resistance to a single agent, and improve treatment outcomes (Kumar et al., 2020). Recent medical research and technology developments have enabled the creation of complexes formed by pharmaceutical drugs and biological molecules such as peptide proteins, antibodies, or oligonucleotides in aptamers (Li and Mahato, 2017). These complexes can take various forms, such as nucleic acid-drug conjugates exemplified by aptamer-drug conjugates or protein-drug conjugates as in anti-body-drug conjugates. Combinatorial therapy involving nucleic acid-drug complexes is particularly promising, and single-stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) molecules with high affinity and specificity toward molecular targets termed as an aptamer (Ecker et al., 2012) is commonly used for this purpose—aptamer in conjugate form with the drug enhanced drug kinetics and dynamics against various disease models. For instance, MMAF (monomethyl auristatin F)-E3 DNA aptamer conjugate possessed more antiproliferative potentials against prostate cancer in mice models (Gray et al., 2018). Conjugated Apt-Lip-DOX significantly reduces

tumour volume with minimal unwanted effects compared to control groups (Dou et al., 2018). In an in vitro study, when used alone, aptamer produced similar or even more potency in antagonising hemozoin formation compared to the effect exerted by CQ (Niles et al., 2009). Therefore, aptamer could be a perfect candidate for combination therapy with drugs when high efficacy and minimal side effects are the therapeutic goal against antibody-drug combinations with high toxicity profiles. Since, aptamer-drug conjugates are a relatively newer class of therapeutics compared to anti-body-drug conjugates, consequently, fewer aptamer-based drugs have progressed through the extensive stages of preclinical and clinical development required for FDA approval compared to the latter. Similarly, aptamers could be designed in variety with others to enhance their pharmacokinetic and pharmacodynamic parameters (Lee et al., 2015a).

1.2 Problem statement.

Although many DNA aptamers with binding affinity to haem molecules have been developed (Okazawa et al., 2000), their potency in antagonising parasite proliferation is affected by their inability to reach the pharmacological site of action. From a previous study, OKA_24 and OKA_26 aptamers preloaded into the RBC showed more promising activity against *P. falciparum* than those just put into growth media. The OKA_24 and OKA_26 DNA aptamer in the growth medium are less permeable to the parasite DV. Therefore, there is a need to make some modifications to enhance the permeation potential of these DNA aptamers, which could be achieved via conjugation with known permeable molecules such as cholesterol, cell-penetrating peptides, liposomes, polymeric nanoparticles and so on. However, cholesterol is commonly used because of its biocompatibility as a naturally occurring lipid in cell

membranes. It is generally well-tolerated by the body, coupled with its low risk of causing adverse reactions or toxicity when used in conjugated form with drugs.

1.3 The rationale of the study

The overwhelming emergence of resistant strains of the malaria parasite to the current antimalaria regimen is increasing and may lead to higher morbidity and mortality. Combination therapy is the mainstay in treating illnesses caused by the malaria parasite. The search for a more potent, cheaper, and safer combined antimalarial regimen is increasing, and the aptamer-drug conjugate is a promising candidate. Research findings indicate that loading the Protoporphyrin IX aptamer into red blood cells (RBCs) resulted in notable inhibition of hemozoin formation, contrasting with its effects when solely included in the growth medium (Niles et al., 2009). At the same time, the protoporphyrin IX aptamer inhibits hemozoin formation. However, its lower permeability to parasite DV is a great setback**.** Therefore, molecular modification of this aptamer through conjugation with membrane-permeabilising agents needs to be investigated to ascertain the potential of its combination with CQ for reversing drug resistance in malaria.

The rationale behind selecting CQ over alternative malaria drugs, such as artemisinin, for inclusion in this study warrants thorough consideration. Despite widespread reports of resistance to chloroquine, it remains a pertinent subject of investigation due to its potential efficacy against specific strains of the malaria parasite. The current study seeks to explore the possibility of synergistic interactions between chloroquine and the protoporphyrin IX aptamer, particularly in the context of reversing CQ resistance. Additionally, the historical significance of CQ in malaria treatment, despite its diminishing effectiveness, underscores its relevance for re-

evaluation and potential repurposing. Practical factors, including CQ's availability, and cost-effectiveness, further justify its selection as the focus of this investigation. Thus, the decision to prioritise chloroquine over other antimalarial agents aligns with the study's objectives and underscores the multifaceted considerations inherent in drug selection for experimental research.

1.4 Hypotheses of the study

H1: A significant difference will exist in silico binding characteristics of cholesterol-conjugated protoporphyrin IX DNA-aptamer and cholesterol-free protoporphyrin IX DNA-aptamer towards haem.

H2: Conjugation of aptamer with cholesterol will significantly increase the cellular internalisation of protoporphyrin IX DNA aptamer.

H2: Conjugation of aptamer with cholesterol will significantly increase binding affinity for haem.

H4: Cholesterol-conjugated protoporphyrin IX DNA aptamer will inhibit parasite growth in vitro.

H5: Combination of cholesterol-conjugated protoporphyrin IX DNA aptamer with CQ will significantly inhibit parasite growth in vitro.

1.5 The objective of the study

1.5.1 The general objective of the study

This study characterizes modified Protoporphyrin IX (Haem) DNA-Aptamers and investigates their in vitro anti-plasmodium effects alone and in combination with CQ toward reversing chloroquine-resistant malaria.

1.5.2 Specific objectives of the study

The specific objectives are as follows:

1. To investigate the binding of lipid-modified aptamer towards haem using a biocomputational approach.
    i. Prediction of aptamer three-dimensional (3D) structures
    ii. Modification of aptamer structure using in silico studies
    iii. Molecular docking of haem with modified aptamer

2. To characterise the produced conjugated aptamers.
    i. To develop and validate an RP-HPLC method for detecting and quantitating the produced conjugate.
    ii. To evaluate the serum stability of the produced conjugate using the validated RP-HPLC method
    iii. To evaluate the characteristic binding behaviour of the conjugate with haem using ultraviolet (UV) spectral titration and an electrochemical-based assay

3. To determine the biological properties of the conjugate
    i. To evaluate the internalisation of the conjugate into red blood cell (RBC)

ii. To evaluate the effect of the conjugate on chloroquine-sensitive (3D7) and chloroquine-resistance (W2), strains of *P. falciparum*.

iii. To determine the effect in combination of the conjugates with CQ on chloroquine-resistance (W2) strains of *P. falciparum.*

1.6 Experimental design

The design of the current study is illustrated in Figure 1.1. The 3D model of protoporphyrin IX DNA-aptamers was predicted and modified before molecular docking with haem using auto-dock vina. The sequence of haem-binding aptamer with instructions for the desired modification was sent to GNETH for subsequent synthesis. The reverse phase HPLC method was developed and validated for detecting and quantifying the cholesterol triethylene glycol- 26-OKA and cholesterol triethylene glycol- 24-OKA modified protoporphyrin IX. Serum stability of the aptamer conjugate was determined by incubating the conjugate in Albumax II spiked medium and subjected to stability analysis by RP-HPLC. A fluorescence detector detected the aptamer-conjugates, RF-20A, connected in series with the HPLC device at 37.5 mins and 40.04 mins retention times. UV absorption spectroscopy was used to determine the kinetics of haem-binding in 500 µl SHMCK buffer pH 5. The shift in peak absorbance wavelength indicating binding was recorded.

To confirm the specificity and the binding affinity of aptamer conjugate for haem, square wave voltammetry electrochemical sensing based on Haem was conducted. Binding was evaluated by immobilising 5 µM of haem onto cysteamine-glutaraldehyde coated gold electrode to construct an electrochemical biosensor. Sensing of varying aptamer conjugate concentrations was carried out. Aptamer cellular

internalisation was assessed by treating 1% haematocrit of RBC in phosphate buffer saline (PBS) with 2 µl of 100 µM fluorescein labelled cholesterol-based haem DNA aptamer and then analysed by acquiring fluorescence image using a 517 nm emission filter of inverted fluorescence microscope or acquiring hundred thousand events via Alexa fluor line on BD FACS Canto II.

The antimalarial activity of the aptamer conjugates was measured using a fluorescence based malarial SYBR Green I assay. In this assay, a mix-culture of *P. falciparum* containing predominantly ring stage of CQ sensitive (3D7 strain) or CQ resistant (W2 strain) was treated with 5% sorbitol to obtain uniformly ring stage parasite. The sorbitol-synchronised parasites were treated with varying aptamer conjugate concentrations for 48 hours. Following 48 hours of aptamer conjugates treatment, the SYBR Green-I working solution in phosphate buffer saline (PBS) was dispensed into the *P. falciparum* suspension. Samples were then subjected to flow cytometry analysis on BD FACS Canto II. Evaluating aptamer conjugate 50% inhibitory concentration (IC_{50}) was determined by acquiring about one hundred thousand events using 488 nm. A similar procedure was repeated with aptamer in combination with CQ to evaluate the effect of combination therapy against *P. falciparum.* Figure 1.1 shows the flowchart of the study.

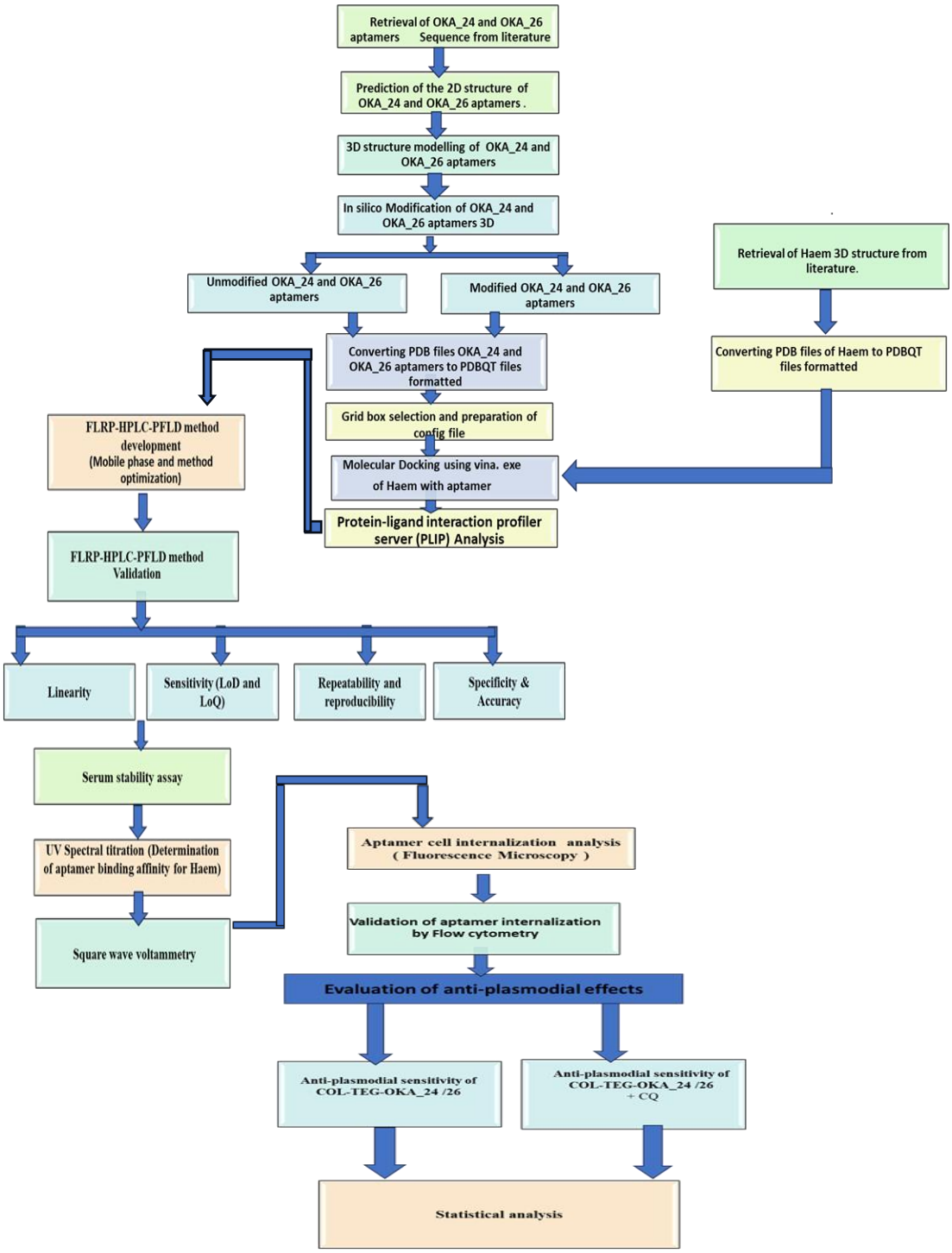

Figure 1.1 Flowchart of the investigations conducted in the current research study.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Malaria

Malaria is a devastating and potentially life-threatening mosquito-borne parasitic infection caused by *Plasmodium*. The disease is transmitted to humans via the bite of the Anopheles mosquito (female) hosting the parasite (World Health Organization, 2022). The parasite multiplies in the liver, from where it proceeds to the systemic circulations and causes various symptoms, manifesting as fever, chills, headache, muscle pains, and emesis. In severe illness, malaria infection can lead to coma, seizures, and eventually death (Blow and Buck, 2022).

Malaria is a significant public health concern in many parts of the world. There were around 249 million cases of malaria worldwide in 2022, with nearly 619,000 malaria-related deaths. (Organization, 2022). Most of the malaria cases and deaths occur in the region of Africa, where the disease is a significant cause of morbidity and mortality in younger children below the age of five, immunocompromised patients and pregnant women (Greenwood et al., 2022).

According to the World Malaria Report 2021, the African region plays a significant role, accounting for 94.7 per cent of the global malaria caseload and 95.8 per cent of global malaria-related mortalities. The global aggregate of malaria cases increased by 0.8 per cent between 2020 and 2021, with a 6.5 per cent increase between 2019 and 2021. The disruptive ramifications of the COVID-19 pandemic during the years 2020 and 2021 resulted in an approximately 11.2 per cent increase in malaria-associated fatalities and a concurrent 5.6 per cent increase in malaria occurrences at a global level, with most of these occurrences manifesting within the African Region.

Similarly, according to the 2022 report, there were an expected 5.4 million cases of malaria in Southeast Asia, with approximately 9,000 malaria-related deaths. This represents 2% of the global malaria burden; many malaria cases in Southeast Asia occur in India, which accounts for 79% of the total. Other countries with high malaria burdens in the region include Indonesia (15.6%), Myanmar (1.6%), Thailand (1.4%), and Vietnam (1.1%) (Word Health Organization, 2022).

2.1.1 Current Malaria Status in Malaysia

Malaysia achieved a significant milestone in public health as it successfully reported zero cases of human malaria for a continuous period of five years. This noteworthy accomplishment reflects the effectiveness of the country's comprehensive malaria control and elimination programs. The sustained absence of human malaria cases signifies a triumph in the efforts to combat the disease, showcasing the success of various interventions and strategies implemented by health authorities (Alemayehu, 2022).

During this period, Malaysia have focused on a multifaceted approach, including robust vector control measures and prompt diagnosis and treatment of malaria cases (Organization, 2021, Organization, 2023). Surveillance systems were likely strengthened to detect and respond to any potential resurgence swiftly. The collaborative efforts of healthcare professionals, researchers, communities, and governmental and non-governmental organizations likely played a crucial role in achieving and maintaining this malaria-free status (Organization, 2023).

This accomplishment not only highlights Malaysia's commitment to public health but also demonstrates the feasibility of malaria elimination with sustained efforts and a well-coordinated approach. It provides a foundation for continued

vigilance, as maintaining a malaria-free status requires ongoing surveillance, research, and a resilient healthcare infrastructure to prevent the re-emergence of the disease. The Countries with indigenous cases in 2000 and their status by 2022 are illustrated in the world malaria map, as shown in Figure 2.1 below.

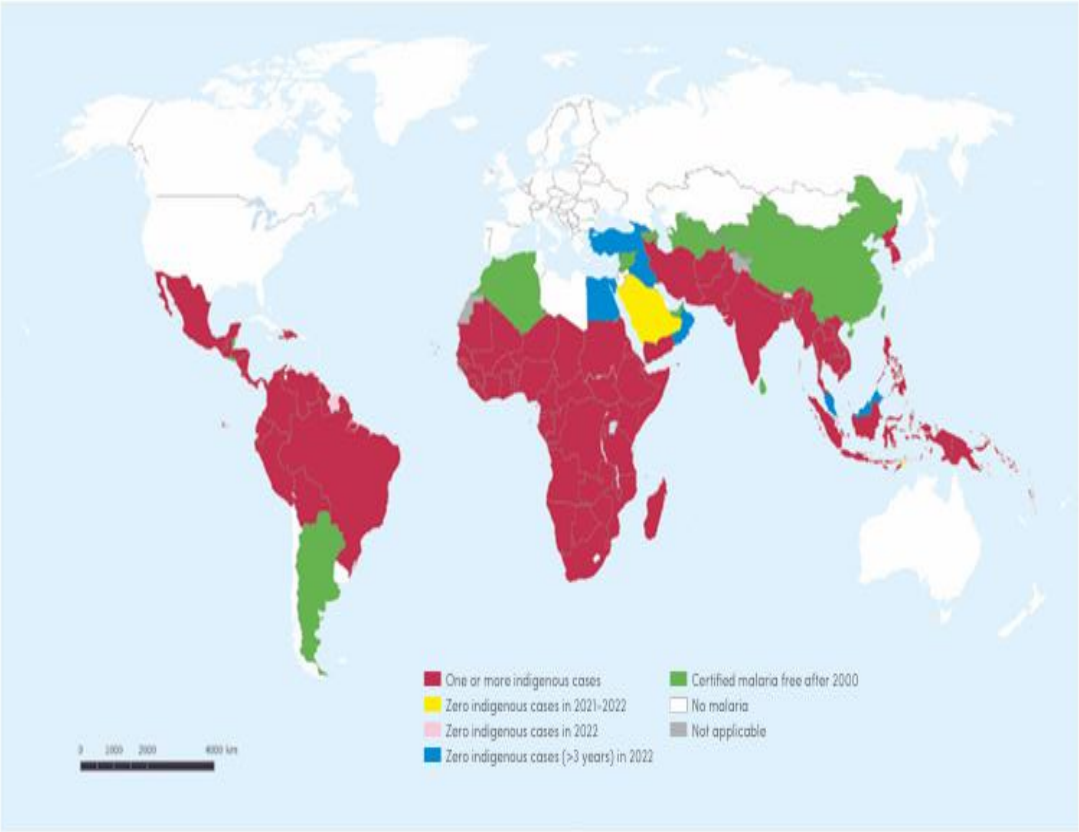

Figure 2.1 Countries with indigenous cases in 2000 and their status by 2022 adopted from Malaria - world map.

The *Plasmodium genus* from Malaria encompasses several species, with *Plasmodium falciparum (P. falciparum*), *Plasmodium vivax*, *Plasmodium malaria*, *Plasmodium ovale*, and *Plasmodium knowlesi* being the primary species responsible for human infections (Xu et al., 2020). Each species exhibits distinct characteristics, including differences in clinical manifestations, distribution, and response to treatment (Yu et al., 2020). Compared to other *Plasmodium* species, *P. falciparum* generally causes more severe disease. While other species can also lead to complications, *P.*

falciparum remains the primary focus due to its prevalent distribution and drug resistance impact (Wahlgren et al., 2017).

2.2 *Plasmodium falciparum*

The *P. falciparum* is renowned for its virulence, often causing severe and life-threatening malaria. It invades many erythrocytes and reproduces rapidly, yielding high parasite densities in the bloodstream. This characteristic is linked to the severity of symptoms and complications that may arise, including cerebral malaria, severe anaemia, and organ dysfunction (Wahlgren et al., 2017). Regarding global distribution, P. falciparum is particularly prevalent in sub-Saharan Africa, accounting for most malaria cases and deaths (Kojomx,,,,,,, and Singh, 2020). Its global distribution largely depends on factors such as female anophe les' mosquito and vulnerable host (susceptible humans), leading to its dominancy in each region.

Diagnosing *P. falciparum* infection presents unique challenges due to its ability to sequester in deep tissues, evading detection by routine blood tests. This can lead to missed or delayed diagnoses, complicating timely treatment (Gendrot et al., 2019). One of the most pressing challenges posed by *P. falciparum* is its propensity to develop resistance to antimalarial drugs. Resistance has emerged to various medications over time, including CQ and Sulfadoxine-pyrimethamine, necessitating the adoption of artemisinin-based combination therapies (ACTs) as the frontline treatment (Rout and Mahapatra, 2019, Wicht et al., 2020).

The distinct characteristics of *P. falciparum*, including its rapid replication and virulence, pose challenges for malaria control and elimination efforts. Its ability to cause severe disease emphasises the urgency of effective treatment and preventing transmission through vector control measures.

2.3 Life cycle of *Plasmodium falciparum*

The intricate life cycle of *P. falciparum*, the most virulent malaria-causing parasite, encompasses both mosquito and human hosts. Beginning with the mosquito's blood meal from an infected human, the cycle involves sexual reproduction in the mosquito's midgut, forming sporozoites. These sporozoites mature and migrate to the mosquito's salivary glands, subsequently transmitted to a definitive human host through mosquito bites during another blood meal. Within humans, sporozoites invade liver cells, leading to asexual replication and the release of merozoites into the systemic circulation, causing fever and other manifestations of malaria infections. The parasite then differentiates into male and female gametocytes, ingested by mosquitoes during bites, initiating the sexual phase. In mosquitoes, gametocytes transform into gametes, fertilising and forming ookinetes, leading to oocyst development, meiosis, and the release of sporozoites into the mosquito's salivary glands. This intricate cycle Figure 2.2 exploits distinct host environments, perpetuating transmission and posing challenges for control efforts (Wahlgren et al., 2017).

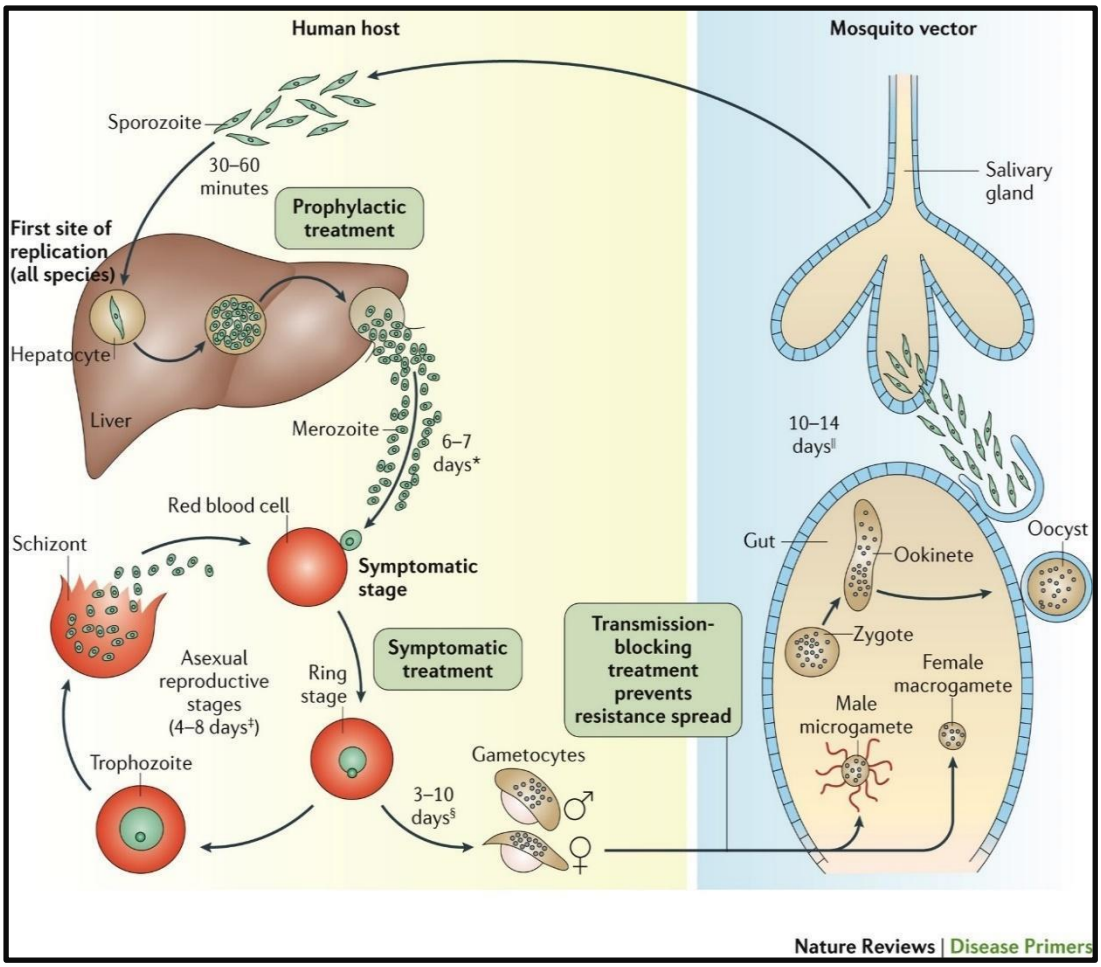

Figure 2.2 Complex life cycle of P. falciparum adopted from (Phillips et al., 2017)

2.4 Malaria-Resistant Parasites

Malaria-resistant parasites exhibit distinctive characteristics that enable their survival in the face of antimalarial interventions (Menard and Dondorp, 2017). Firstly, these parasites display a significant degree of genetic diversity, facilitating rapid adaptation to selective pressures imposed by antimalarial drugs. Specific genetic mutations within key proteins targeted by these drugs confer resistance (Stofberg et al., 2021). Secondly, altered metabolic pathways are a common feature, allowing resistant parasites to modify their energy metabolism and nutrient acquisition strategies, enhancing their survival under drug pressure. This adaptation helps them bypass or minimize the impact of antimalarial drugs (Capela et al., 2019). Lastly,

enhanced drug efflux mechanisms are employed, where resistant parasites possess the ability to pump drugs out of their cellular compartments, thereby reducing the effective drug concentration (Garrido-Cardenas et al., 2019). This efflux process is facilitated by the presence of efflux pump proteins, contributing to the overall resilience of these malaria-resistant parasites.

2.4.1 Mechanism of Malaria-Resistant

Malaria-resistant parasites exhibit a diverse array of mechanisms aimed at ensuring their survival in the face of antimalarial interventions. One key aspect is genetic diversity, exemplified by the presence of multiple alleles in the PfCRT gene (Dhingra et al., 2019). This genetic variability contributes significantly to the development of chloroquine resistance, particularly in malaria-endemic regions where the selective pressure is high. Additionally, altered metabolic pathways play a crucial role in the resilience of parasites against specific antimalarial drugs. For instance, parasites resistant to atovaquone, a drug targeting the mitochondrial electron transport chain, undergo modifications in their respiratory pathways to circumvent the drug's impact (Chen et al., 2021, Burnell, 2020), allowing them to maintain viability and resist treatment.

Another noteworthy resistance mechanism involves enhanced drug efflux, where resistant parasites possess mechanisms, such as overexpressing P-glycoprotein encoded by the *Plasmodium falciparum* multidrug resistance 1 (pfmdr1) gene. This overexpression results in the pumping out of antimalarial drugs from the parasites' cellular compartments, reducing the effective drug concentration and conferring resistance (Muhammad et al., 2023). These multifaceted mechanisms underscore the

adaptability of malaria-resistant parasites, emphasizing the challenges faced in developing effective antimalarial strategies.

In addition to these mechanisms, resistance in malaria parasites extends to alterations in drug targets and activation processes. Target modification is observed in resistance to artemisinin, a key component of artemisinin-based combination therapies (ACTs). Mutations in the Kelch13 gene impact the parasite's response to artemisinin, contributing to reduced drug efficacy (Erhunse and Sahal, 2021). Furthermore, drug activation alterations are evident in cases such as proguanil resistance, linked to mutations in the *Plasmodium falciparum* dihydrofolate reductase (pfdhfr) gene, affecting the activation of the drug (Daskum et al., 2021). The complexity and diversity of these resistance mechanisms emphasize the ongoing challenge in developing strategies that effectively combat malaria.

2.4.2 Recent Developments

Genomic surveillance plays a pivotal role in monitoring the spread of antimalarial resistance, with initiatives like the MalariaGEN consortium utilizing advanced genetic tracking methods. By identifying novel genetic markers associated with resistance, these efforts inform malaria control strategies and contribute to the ongoing battle against drug resistance (Mayor et al., 2022). Another avenue of progress involves the exploration of novel antimalarial compounds, such as KAF156 (Guerra and Winzeler, 2022). This promising compound has demonstrated efficacy against artemisinin-resistant parasites, offering hope for future malaria treatment options.

In the realm of treatment strategies, combination therapies have proven effective in mitigating resistance risks. Dihydroartemisinin-piperaquine (DP), an artemisinin-based combination therapy (ACT), combines the rapid action of

artemisinin with a longer-acting partner drug. This approach enhances treatment efficacy and helps reduce the likelihood of resistance development (Schäfer et al., 2024). Additionally, vaccination strategies contribute to the arsenal against malaria. Despite partial efficacy, the RTS,S/AS01 malaria vaccine represents a significant advancement in malaria prevention, complementing other control measures(Taddese, 2023).

The integration of artificial intelligence (AI) and big data analytics stands out as a contemporary approach. Machine learning models analyze extensive datasets to predict emerging drug resistance patterns. This proactive insight allows for the adjustment of treatment strategies, fostering a more adaptive and effective response to the evolving landscape of malaria resistance (Pasrija et al., 2022). Lastly, community engagement and education initiatives play a vital role in malaria-endemic regions. Community-based programs emphasize the importance of completing antimalarial treatment courses, contributing to reduced resistance risk through informed and empowered communities (Awasthi et al., 2024).

2.5 Haem in malaria parasite biology

Haem plays a central and complex role in the biology of the malaria parasite (*Plasmodium* species). This role is particularly pronounced during the intraerythrocytic stage of the parasite's life cycle when it resides within red blood cells (RBCs). As the parasite invades RBCs (Volz et al., 2016), it embarks on a crucial process of haemoglobin. Haemoglobin, the predominant protein within RBCs, is a rich source of nutrients for the parasite. During digestion, haem, globin peptides, and amino acids are released (Xie et al., 2016). Although vital as a nutrient, this haem poses a significant challenge due to its inherent toxicity to the parasite. Haem toxicity arises

from its potential to generate harmful reactive oxygen species (ROS) and disrupt various essential cellular processes. To circumvent this threat, the malaria parasite has evolved an ingenious mechanism for haem detoxification. This process occurs within specialised organelles known as food vacuoles or digestive vacuoles. Within these vacuoles, haem is converted into an inert crystalline structure called hemozoin or malaria pigment. Hemozoin formation involves the polymerisation and sequestration of haem molecules into crystalline structures, effectively neutralising the toxic properties of haem (Pena and Pamplona, 2022, Niles et al., 2009).

2.5.1 Haem biosynthesis

Haem biosynthesis is a complex and highly regulated metabolic pathway responsible for producing haem, a crucial molecule in numerous biological processes (Ajioka et al., 2006). This pathway occurs primarily in the liver and bone marrow and comprises several distinct enzymatic steps. It begins with the synthesis of delta-aminolevulinic acid (ALA) from glycine and succinyl-CoA, followed by a series of reactions that lead to the formation of protoporphyrin IX (Ajioka et al., 2006). The final step involves the insertion of an iron atom into protoporphyrin IX, resulting in the formation of haem. The pathway's regulation is crucial to maintain a balance of haem and prevent the buildup of toxic intermediates. Feedback inhibition, negative feedback by haem, iron availability, and gene expression regulation are fundamental mechanisms controlling haem biosynthesis (Layer et al., 2010). Disruptions in haem biosynthesis can lead to porphyria, a group of rare genetic disorders characterised by the accumulation of haem precursors (Phillips, 2019). These conditions manifest with symptoms such as skin sensitivity to light and neurological problems. Understanding haem biosynthesis is essential for managing porphyria and developing therapies for related disorders like iron metabolism disorders and anaemia. Furthermore, haem

biosynthesis has broader implications for studying cellular metabolism and the roles of haem-containing proteins in various vital biological processes (Erwin and Balwani, 2021). Figure 2.3 shows the haem biosynthesis pathway.

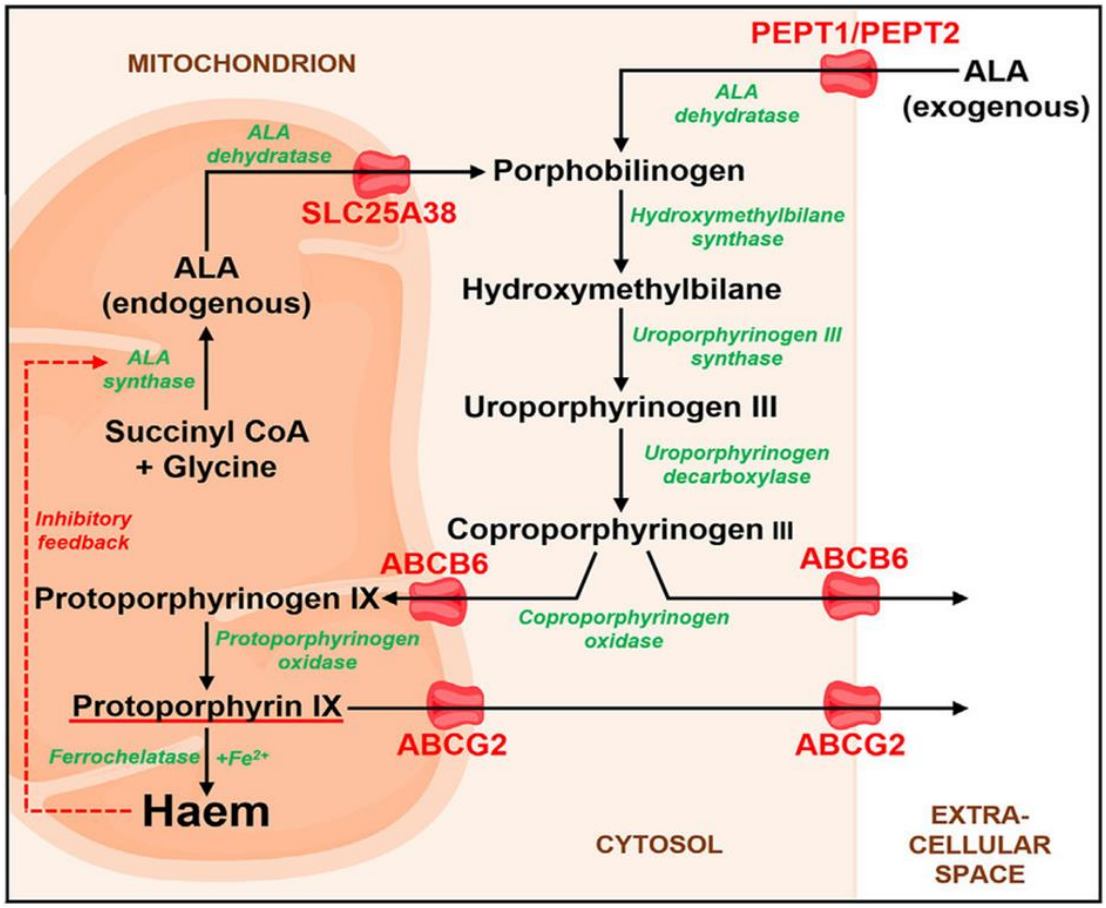

Figure 2.3 Haem biosynthesis adopted from (Labib et al., 2021)

2.5.2 Haem as a signalling molecule

Haem is a versatile signalling molecule with significant implications for cellular processes and health. Recent research has highlighted haem's involvement in various signalling pathways, particularly in cellular response to oxidative stress, inflammation, and cellular homeostasis (Mense and Zhang, 2006, Shimizu et al., 2019). Here, we discuss the multifaceted role of haem as a signalling molecule.

i. **Redox Signalling:** Haem's iron atom can undergo reversible redox reactions between its ferrous (Fe2+) and ferric (Fe3+) states. This redox

activity allows haem to serve as a sensor of oxidative stress within cells. When exposed to oxidative stress, the haem can release its iron atom, producing reactive oxygen species (ROS), which act as signalling molecules (Carter et al., 2017). These ROS can modulate various signalling pathways, affecting cell survival, proliferation, and gene expression (Paes et al., 2011).

ii. **Haem as a Gasotransmitter:** Haem has been recognised as a Gasotransmitter, like nitric oxide (NO) and carbon monoxide (CO). When haem is degraded by the enzyme haem oxygenase (HO), it produces carbon monoxide, biliverdin, and free iron. Carbon monoxide plays a signalling role in various physiological processes. It can activate the soluble guanylate cyclase (sGC) enzyme, leading to the production of cyclic guanosine monophosphate (cGMP) and downstream signalling, including vasodilation and anti-inflammatory responses (Mustafa et al., 2009, Huang et al., 2021).

iii. **Regulation of Gene Expression:** Haem can directly modulate gene expression by binding to specific transcription factors known as haem-regulated inhibitors (HRI) and haem-regulated eIF2α kinase (HRIK) (Abdel-Nour et al., 2019). These factors regulate genes responsible for haem biosynthesis, iron homeostasis, and antioxidant responses. Haem can also bind to nuclear receptors, such as the nuclear factor erythroid 2-related factor 2 (Nrf2), which plays a critical role in cellular defence against oxidative stress (Mense and Zhang, 2006, Abdel-Nour et al., 2019).

iv. **Inflammatory Signalling:** Inflammation triggers a release of haem from haemoproteins, such as haemoglobin and myoglobin, due to tissue damage or haemolysis. Extracellular haem can activate Toll-like receptors (TLRs) and the NLRP3 inflammasome, producing pro-inflammatory cytokines and exacerbating inflammatory responses. This pro-inflammatory effect of haem has implications for various diseases, including sepsis and atherosclerosis (Lin et al., 2012).

v. **Role in Immune Responses:** Haem has been found to modulate immune responses by influencing immune cell function and cytokine production. For example, haem can enhance the production of pro-inflammatory cytokines and affect the polarisation of macrophages, leading to either pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes, depending on the context (Colin et al., 2014).

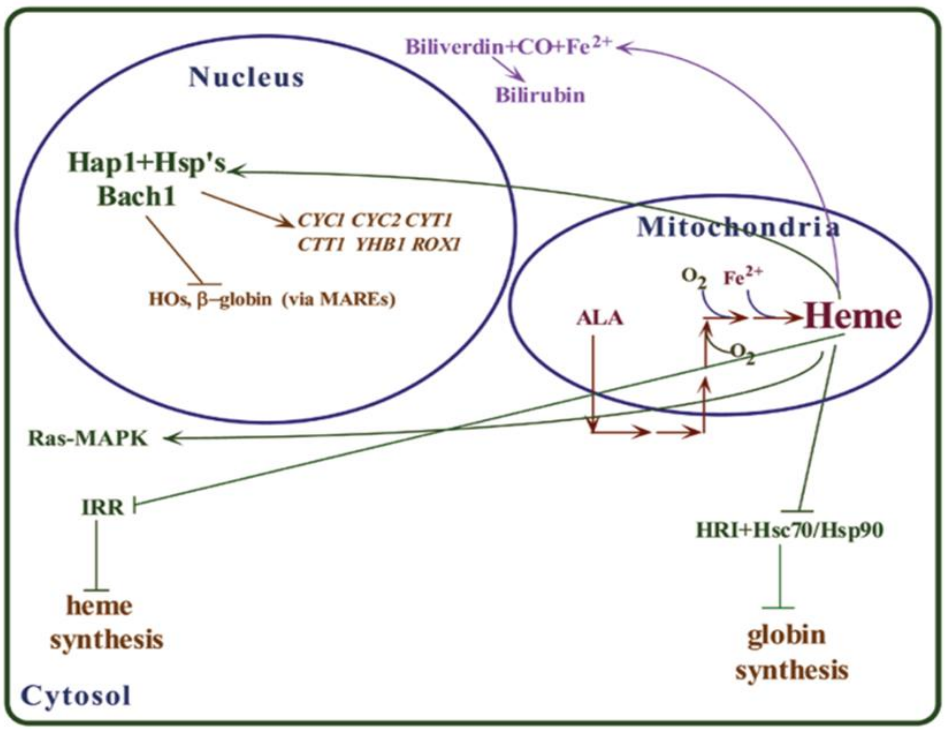

Figure 2.4 Haem regulation of cellular and molecular processes adopted from (Mense and Zhang, 2006)

2.6 Malaria control challenges

The target goal of malaria control involves reducing the disease's impact to a point where it's no longer a public health threat. On the other hand, malaria elimination aims to completely stop local mosquito-driven malaria transmission in a specific area (Organization, 2020). The WHO's Global Technical Strategy (GTS) 2016-2030, starting in 2016, aims to decrease malaria cases and malaria-related deaths by at least 40% by the year 2020, and then 75% - 90% by 2030 (World Health Organization, 2020, Patouillard et al., 2017).

While progress has been made in reducing malaria cases, challenges persist due to complex transmission dynamics influenced by factors such as the environment, stability of governance, healthcare infrastructure, and economic development (World Health Organization, 2020). Current malaria control strategies focus on eradicating mosquito breeding sites, effective management of mosquito vectors, swift diagnosis, and proper treatment. This multi-pronged approach is further strengthened by community education and engagement, as well as research into novel antimalarial drugs and the development of vaccines. (World Health Organization, 2019, Gujjari et al., 2022). Despite concerted efforts to control, diagnose, and prevent the disease, significant challenges persist, hindering progress toward its eradication (World Health Organization, 2022). This discourse elucidates the multifaceted difficulties encountered in malaria control, diagnostics, and prevention, underscoring the intricate nature of combating this pervasive global health concern.

2.6.1 Drug resistance

A significant drawback to effective malaria control is the emergence and spread of multidrug-resistant P. falciparum (Jagannathan and Kakuru, 2022).