THE MECHANISM OF ANTIMALARIAL ACTION OF ELLAGIC ACID ON HAEMOGLOBIN METABOLISM IN *Plasmodium falciparum*

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

~	approximately
%	percent
°C	degree Celsius
=	equal
<	less than
≤	less than or equal to
>	more than
≥	more than or equal to
× *g*	gravitational force
µM	micromolar
µg/mL	microgram per millilitre
µL	microliter
µm	micrometre
e.g.	for example
g	gram
i.e.	that is
kg	kilogram
mg	milligram
mg/mL	milligram per millilitre
mL	millilitre
M	molar
mM	millimolar
mol	mole
mV	millivolt
n	number of subjects
nm	nanometre
nM	nanomolar
pfKelch 13	*Plasmodium falciparum* Kelch 13 gene

pH	potential of hydrogen
pK_a	acid dissociation constant
v/v	volume per volume
w/v	weight by volume
ACTs	artemisinin-based combination therapies
ATP	adenosine triphosphate
AIDS	acquired immunodeficiency syndrome
BMI	body mass index
C	cytostome
CO_2	carbon dioxide
CCM	complete culture medium
CDC	Centers for Disease Control and Prevention
CS	cytostomal number
DFd	degree of freedom denominator
DFn	degree of freedom numerator
dH_2O	distilled water
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DTNB	Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) assay
DV	digestive vacuole
EDTA	ethylenediaminetetraacetic acid
EPM	erythrocyte plasma membrane
ER	endoplasmic reticulum
ET	ellagitannins
E-64	E-64 cysteine protease inhibitor
FAD	the food and drug administration
FP	falcipains
FP-2	falcipains-2
FP-3	falcipains-3
G	Golgi apparatus

G6PD	glucose-6-phosphate dehydrogenase
GMS	Greater Mekong Sub-region
H^+	proton/hydrogen ion
HEPES	hydroxyethyl piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HRPII	histidine-rich protein II
Hz	haemozoin crystals
IC_{50}	half-maximal inhibitory concentration
ICCM	incomplete cell culture medium
INFORMM	institute for research in molecular medicine
JAS	jasplakinolide
K	knobs
LS	large separation
LV	lipid vacuole
MC	Maurer's clefts
Mg^{2+}	magnesium ion
MACS	magnetic-activated cell sorting
MSF	malarial SYBR Green I-based fluorescence
N	nucleus
NaH_2PO_4	phosphate monohydrate
NaCl	sodium chloride
NaOH	sodium hydroxide
NIH	National Institutes of Health
NO	nitric oxide
N_2	nitrogen
O^+	O positive
O_2	oxygen
OsO_4	osmium tetroxide
Pi	inorganic phosphate
PIPs	polyphosphorylated phosphoinositides

PfCRT	*P. falciparum* chloroquine resistance transporter
PPM	parasite plasma membrane
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane
R	ribosomes
RBC	red blood cell
RPMI	Rosewell Park Memorial Institute
RNA	ribonucleic acid
S	segmentation
SD	standard deviation
SNARF	seminaphthorhodafluor
SNP	single nucleic polymorphism
SSC	side scatter
TEM	transmission electron microscope
TF	total fluorescence
TrxR	thioredoxin reductase
USM	Universiti Sains Malaysia
UV	ultraviolet
V-type H^+-ATPase	vacuolar-type proton-pumping ATPase
V-type H^+-pyrophosphate	vacuolar-type proton-pumping pyrophosphatase
WHO	World Health Organization

LIST OF APPENDICES

MEKANISME TINDAKAN ANTIMALARIA ASID ELAGIK KE ATAS METABOLISME HEMOGLOBIN DALAM *Plasmodium falciparum*

ABSTRAK

Malaria yang disebabkan oleh parasit *Plasmodium* telah memberi kesan kepada manusia sejak dahulu lagi, dan ini mengakibatkan kadar morbiditi dan mortaliti yang signifikan. Oleh kerana *P. falciparum* yang rintang terhadap ubat telah tersebar luas, keberkesanan ubat antimalaria piawai telah berkurangan, dan ini memerlukan penyelidikan dalam pembangunan ubat antimalaria baharu dengan sasaran baharu. Asid elagik telah menunjukkan aktiviti antimalaria terhadap *P. falciparum* secara *in vitro* dengan menghalang pembentukan beta-hematin (hemozoin) pada peringkat matang parasit. Kajian ini bertujuan untuk menjelaskan mekanisme tindakan antimalaria asid elagik terhadap pengambilan, pengangkutan dan pencernaan hemoglobin dalam strain *P. falciparum* yang sensitif terhadap klorokuina (3D7) dan yang rintang terhadap klorokuina (W2) secara *in vitro*. Aktiviti antimalaria asid elagik yang merencat 50% populasi parasit (IC_{50}) ditentukan menggunakan ujian malaria SYBR Green I berasaskan fluoresen (MSF) dengan menggunakan artemisinin sebagai ubat piawai. Berdasarkan nilai IC_{50} asid elagik, parasit peringkat trofozoit awal dirawat dengan sebatian ini pada kepekatan yang berbeza selama 8 jam sebelum sapuan darah nipis yang diwarnakan Giemsa disediakan dan diperhatikan dengan mikroskop cahaya. Kepekatan 3.0 dan 3.9 nM dipilih untuk memerhatikan kesan maksimum asid elagik pada parasit 3D7 dan W2 peringkat trofozoit awal menggunakan mikroskop elektron transmisi. Jasplakinolide (7 µM) dan E-64 (µM) digunakan sebagai ubat kawalan.

Keputusan menunjukkan asid elagik menghalang parasit 3D7 dan W2 secara signifikan ($p < 0.001$) dengan nilai IC_{50} masing-masing adalah 1.0 ± 0.7 nM dan 1.3 ± 0.1 nM, berbanding dengan artemisinin ($IC_{50\text{-Parasit 3D7}}$ = 2.2 ± 0.2 nM; $IC_{50\text{-Parasit W2}}$ = 4.3 ± 0.3 nM). Keputusan menunjukkan parasit kelihatan mengecut dalam semua kumpulan parasit 3D7 dan W2 yang dirawat dengan asid elagik. Keputusan menunjukkan pembentukan bahagian sitostoma yang mengandungi hemoglobin 3D7: 3 ± 2 dan W2: 4 ± 2, dan ini menunjukkan terdapat halangan dalam pengambilan hemoglobin dalam parasit 3D7 dan W2 yang dirawat dengan asid elagik. Hanya 3 ± 1 bahagian sitostoma diperhatikan dalam parasit 3D7 yang dirawat dengan jasplakinolida, dan hanya 2 ± 1 bahagian sitostoma diperhatikan dalam parasit W2 yang dirawat dengan sebatian ini. Vesikel pengangkutan yang mengaandungi hemoglobin tidak terlihat dalam parasit 3D7 dan W2 yang dirawat dengan asid elagik dan jasplakinolida. Berbanding dengan kawalan parasit yang tidak dirawat, vakuol pencernaan yang membesar diperhatikan dalam parasit 3D7 dan W2 yang dirawat dengan asid elagik dan E-64. Kesimpulannya, asid elagik mempunyai potensi untuk menjadi ubat antimalaria baharu yang mensasarkan pengambilan, pengangkutan, dan pencernaan hemoglobin dalam *P. falciparum* yang sensitif dan rintang terhadap klorokuina.

THE MECHANISM OF ANTIMALARIAL ACTION OF ELLAGIC ACID ON HAEMOGLOBIN METABOLISM IN *Plasmodium falciparum*

ABSTRACT

Malaria caused by *Plasmodium* parasites has afflicted humans since ancient times. Due to the prevalence of drug-resistant *P. falciparum*, the effectiveness of the standard antimalarial drugs has decreased, requiring research into the development of new antimalarial drugs with novel targets. Ellagic acid has been shown to have antimalarial activity against *P. falciparum in vitro* by inhibiting the formation of beta-haematin (haemozoin) in the mature stage parasite. The present study aimed to elucidate the mechanism of antimalarial action of ellagic acid on haemoglobin ingestion, transport and digestion in chloroquine-sensitive (3D7) and chloroquine-resistant (W2) strains of *P. falciparum in vitro*. The antimalarial activity of ellagic acid that inhibits 50% of the parasite population (IC_{50}) was determined using a malarial SYBR Green I fluorescence-based (MSF) assay with artemisinin was used as a standard drug. Based on the IC_{50} value of ellagic acid, early trophozoite stage parasites were incubated with the compound at different concentrations for 8 hours before Giemsa-stained thin blood smears were prepared and viewed by light microscopy. The concentrations of 3.0 and 3.9 nM were selected to observe the maximum effect of ellagic acid on early trophozoite stage of 3D7 and W2 parasites, respectively by transmission electron microscopy. Jasplakinolide (7 µM) and E-64 (10µM) were used as control drugs. The results show that ellagic acid significantly inhibited ($p < 0.001$) 3D7 and W2 parasites with IC_{50} values of 1.0 ± 0.7 and 1.3 ± 0.1 nM, respectively as compared with artemisinin ($IC_{50\text{-}3D7\ parasite}$ = 2.2 ± 0.2 nM; $IC_{50\text{-}W2\ parasite}$ = 4.3 ± 0.3

nM). The results show that shrunken parasites were observed in all ellagic acid-treated groups of 3D7 and W2 parasites. The results show the formation of 3D7: 3 ± 2 and W2: 4 ± 2 haemoglobin-containing cytostomal sections, indicating the inhibition of haemoglobin uptake in the ellagic acid-treated 3D7 and W2 parasites. Only 3 ± 1 cytostomal sections were observed in the jasplakinolide-treated 3D7 parasite, 2 ± 1 cytostomal section was observed in the jasplakinolide-treated W2 parasite. The haemoglobin-containing transport vesicle was not observed in the ellagic acid and jasplakinolide-treated 3D7 and W2 parasites. As compared with the control of non-treated parasites, the high contrast and enlarged digestive vacuole was observed in the ellagic acid and E-64-treated 3D7 and W2 parasites. In conclusion, ellagic acid has the potential to be a new antimalarial drug targeting haemoglobin ingestion, transport and digestion of chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum.*

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is an illness caused by *Plasmodium* parasites and afflicts humans since the dawn of time. It causes high morbidity and mortality rates with the World Health Organization (WHO) estimated 219 million infections globally in 2020 from which half a million people were died and many of whom were children under the age of five (WHO, 2021). As a result, many national and international projects aimed at reducing and eliminating malaria have been developed (Garrido-Cardenas *et al*., 2018). *P. falciparum* and *P. vivax*, which account for the majority of malaria cases worldwide, are the most common parasites causing the disease, although there are also *P. ovale*, *P. malariae* and *P. knowlesi* (Rabinovich *et al*., 2017). *P. falciparum*, which causes more than 94% of all cases of malaria, is the most virulent and well-studied species (WHO. 2021).

A female *Anopheles* mosquito transmits the malaria parasites by inoculating sporozoites into the human host (Graumans *et al*., 2020). As the sporozoites enter the liver, they mature to schizonts and release merozoites following the liver cell rupture. The merozoites travel across the bloodstream to invade the erythrocytes where they develop into rings, trophozoites and schizonts before producing more merozoites to invade new erythrocytes (Garrido-Cardenass *et al*., 2019). Some of the merozoites are converted into gametocytes and ingested by the

mosquito when it bites the infected human (Garrido-Cardenas *et al*., 2019). Within the mosquito's midgut, male and female gametocytes fuse to form zygotes and sporozoite-containing oocysts. The sporozoites travel to the mosquito's salivary glands where they are ready to be transmitted into a new human host during the blood meal (Garrido-Cardenas et al., 2019).

The galls of *Quercus infectoria* commonly known as *biji manjakani* in Malaysia are used by the elderly in traditional medicine to treat high fever (Everest and Ozturk, 2005; Jamal *et al.,* 2011), which is one of the clinical symptoms of malaria. Besides the antibacterial (Mustafa *et al.,* 2018), anticandidicidal (Baharuddin *et al.,* 2015), antiviral (Hussein *et al.,* 2000) and antiparasitic properties (Nik Mat Zin *et al.,* 2019), the gall crude extracts have been shown to possess the antimalarial activity *in vitro* (Nik Mat Zin *et al.,* 2020; Nik Mat Zin *et al.,* 2021). The toxicity of the galls has also been reported to ensure they are safe for human consumption (Tayel *et al*., 2018) Nik Mat Zin *et al*., 2020). Major compounds such as tannins, rutin, quercetin, pyrogallol, gallic acid and ellagic acid have been found in the *Q. infectoria* galls (Abdullah *et al.,* 2018; Kheirandish *et al.,* 2016; Tayel *et al.,* 2018). Among these, ellagic acid has been shown to have the antimalarial activity in the nanomolar range and implicated in the haemoglobin metabolism of the malaria parasite (Dell'Agli *et al.,* 2003; Simões-Pires *et al.,* 2009; Sturm *et al*., 2009).

Huge volumes of haemoglobin from the erythrocyte cytosol are internalised as the parasite develops from the immature ring to the mature trophozoite and eventually to the replicating schizont (Elliott *et al*., 2008; Tougan *et al*., 2020). The ingested haemoglobin is eventually digested in the digestive vacuole compartment

(Abu Bakar *et al.*, 2010; Nasamu *et al*., 2020). The poisonous haem moiety produced by haemoglobin digestion is detoxified by polymerisation and sequestration as haemozoin, an inert crystalline deposit (De Villiers and Egan., 2021). The haemoglobin metabolism of the parasite is therefore important for its growth and survival. In addition to this, the vital pathways and metabolic adaptations of the parasite's haemoglobin metabolism provide the potential targets for ellagic acid (Simões-Pires *et al*., 2009; Soh *et al*., 2009)

1.2 Rationale of the study

Phytochemical studies have found that phenolic compounds like ellagic acid are abundantly quantified in the *Q. infectoria* galls (Amira Raudhah *et al*., 2018; Baharuddin *et al*., 2015). Ellagic acid has been shown to have the antimalarial activity against *P. falciparum in vitro* (Soh *et al*., 2009) by inhibiting the formation of beta-haematin (haemozoin) in the mature stage parasite (Simões-Pires *et al*., 2009; Soh *et al*., 2009). Up till now, no comprehensive studies have demonstrated the effect of this compound on the haemoglobin metabolism of both chloroquine-sensitive and chloroquine-resistant strains of the malaria parasite and established a downstream target. The haemoglobin metabolism of which the parasite ingests a huge amount of haemoglobin, transfers to and digests in the digestive vacuole has always been the focus of the antimalarial drug development (Wendt *et al*., 2016; Dalal and Klemba, 2015; Nagaraj and Padmanaban, 2017; Tang *et al*., 2018). Ellagic acid might inhibit one or more of these metabolic processes since this compound has been associated with the inhibition of haem polymerisation (Dell'Agli *et al*., 2003). Thus, the ultrastructural study of the effect of ellagic acid on these pathways by using

transmission electron microscopy will give a better understanding of the role of this compound on the haemoglobin ingestion, transport and digestion.

1.3 Objectives of the study

1.3.1 General objective

The study aimed to elucidate the mechanism of antimalarial action of ellagic acid on the haemoglobin metabolism of chloroquine-sensitive (3D7) and chloroquine-resistant (W2) strains of *P. falciparum in vitro*.

1.3.2 Specific objectives

1) To determine the antimalarial activity of ellagic acid against the parasites by malaria SYBR Green I- based assay.
2) To investigate the effect of ellagic acid on haemoglobin ingestion from the erythrocyte cytosol via the formation of cytostomal section within the parasite's cytostol.
3) To ascertain the effect of ellagic acid on haemoglobin transport to the digestive vacuole of the parasites via the formation of haemoglobin transport vesicles.
4) To determine the effect of ellagic acid on haemoglobin digestion of the parasites via the enlargement and contrast of the digestive vacuole.

1.4 Hypothesis of the study

1) Ellagic acid has a promising antimalarial activity against the parasites.
2) Ellagic acid inhibits haemoglobin ingestion from the erythrocyte cytosol by the parasites.
3) Ellagic acid inhibits haemoglobin transport to the digestive vacuole of the parasites.
4) Ellagic acid inhibits haemoglobin degradation in the digestive vacuole of the parasites.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria

Malaria is a parasitic disease that spreads through the bites of infected female *Anopheles* mosquitoes. The disease affects more than 200 million people and kills over 400 000 people worldwide each year, particularly children in sub-Saharan Africa (National Institutes of Health (NIH), 2022). Despite public health efforts such as the use of insecticide-treated bed nets and antimalarial drugs to reduce the burden of malaria (Centers for Disease Control and Prevention (CDC), 2022), 241 million malaria cases were reported in 2020 as compared with 227 million cases in 2019 (WHO, 2022). The number of malaria deaths raised to 627 000 in 2020, which is an increase of 69 000 deaths over the previous year of 2019. As malaria poses a significant economic and health impact, the discovery of new antimalarial drugs for the treatment and prevention of the disease is urgently needed (Gaudinski *et al.*, 2021).

2.2 Causes of malaria

Malaria is the result of infection by unicellular parasites belonging to the *Plasmodium* genus. More than 200 distinct *Plasmodium* species have been identified, each targeting specific hosts (Sato, 2021). *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* are among the *Plasmodium* species commonly found to infect humans (Rabinovich *et al*., 2017). The initial four species are malaria parasites

specifically affecting humans, whereas *P. knowlesi* is typically prevalent in macaque monkeys, leading to zoonotic malaria across Southeast Asian countries (Anstey and Greg, 2019). Among the five human malaria species, *P. falciparum* stands out as the most lethal parasite, accounting for the majority of malaria cases and fatalities globally (WHO, 2022).

2.3 *Plasmodium falciparum*

Studies into many aspects of the biology of human malaria parasites have been facilitated by the establishment of an *in vitro* culture of asexual intraerythrocytic stages of *P. falciparum* (Trager and Jensen, 1976). Several *P. falciparum* strains and clones have been cultivated from varying geographical regions, allowing researchers to determine differences in parasite phenotypes as diverse as the invasion of host erythrocytes (Gaur *et al.,* 2003) to the development of drug resistance (Fidock *et al.,* 2000). Table 2.1 lists the widely studied *P. falciparum* strains and clones that have been used for more than two decades. Many of these parasite lines originate from Asia and the Americas, although the majority of malaria cases and deaths are reported in Africa (van Schalkwyk *et al.,* 2013). Drug resistance, particularly to chloroquine, is one of the challenges to controlling *P. falciparum*. Chloroquine is thought to act by accumulating in the digestive vacuole of the parasite and inhibiting the mechanism of haem detoxification, leading to parasite death. It was reported that chloroquine-resistant parasites expelled chloroquine from erythrocytes more rapidly than chloroquine-sensitive parasites. Many studies demonstrated that a *P. falciparum* chloroquine (Egan *et al.,* 2002).

Table 2.1: List of *P. falciparum* strains and clones that are widely used for *in vitro* studies

Strain/Clone	Origin	Region	Year Reported
Chloroquine-sensitive			
NF-54	Netherlands	Europe	1981
3D7 (Cloned from NF-54)	Netherlands	Europe	1987
D10 (Cloned from FC27)	Papua New Guinea	Oceania	1983
HB3 (cloned from Honduras I/CDC)	Honduras	Central America	1984
D6 (cloned from Sierra Leone I/CDC)	Sierra Leone	Africa	1988
T9-96 (cloned from T9)	Thailand	Asia	1981
GB4 (cloned from Ghana III/CDC)	Ghana	Africa	2003
ITG2F6 (cloned from Ituxi 084)	Brazil	South America	1979
Chloroquine-resistant			
K1	Thailand	Asia	1981
FCR3	The Gambia	Africa	1981
W2 (cloned from Indo III/CDC)	Indochina	Asia	1988

Table 2.1: Continued

W2mef (derived from W2)	Indochina	Asia	1988
Dd2 (cloned from W2mef)	Indochina	Asia	1988
7G8 (cloned from IMTM22)	Brazil	South America	1984
V1/S (cloned from V1)	Vietnam	Asia	1990
Malayan Camp	Malaya	Asia	1965

resistance transporter (PfCRT) was involved in this efflux (Sanchez *et al.,* 2003; Howard *et al.,* 2002). Mutations of PfCRT have been described in all chloroquine-resistant *P. falciparum* isolates. The genetic profile of chloroquine resistance in the malaria parasites showed a particular mutation in PfCRT (K76T) that was associated with chloroquine resistance in genetically modified *P. falciparum* strains and field isolates (Fidock *et al.,* 2000; Howard *et al.,* 2002). Due to the ability of *P. falciparum* to develop resistance to chloroquine and other antimalarial drugs, a search for a new compound such as ellagic acid against chloroquine-sensitive (3D7) and chloroquine-resistant (W2) strains of *P. falciparum* was initiated in the present study.

2.4 Life cycle of *P. falciparum*

The life cycle of *P. falciparum* is similar among *Plasmodium* species that infect humans. It involves the asexual cycle within a human and the sexual cycle within a female *Anopheles* mosquito (Votýpka *et al.,* 2016).

2.4.1 Sexual cycle within a mosquito

The sexual cycle begins when a mosquito ingests the blood containing gametocytes from an infected human (Figure 2.1A). In the mosquito midgut, microgametocyte (male) and macrogametocyte (female) develop into eight microgametes and one macrogamete, respectively (Bennick *et al.,* 2016). The microgamete fertilises the macrogamete to form a zygote a few minutes after entering the midgut (Venugopal *et al.,* 2020). The zygote undergoes meiosis and differentiation into a motile ookinete. The ookinete penetrates the midgut wall and forms an oocyst

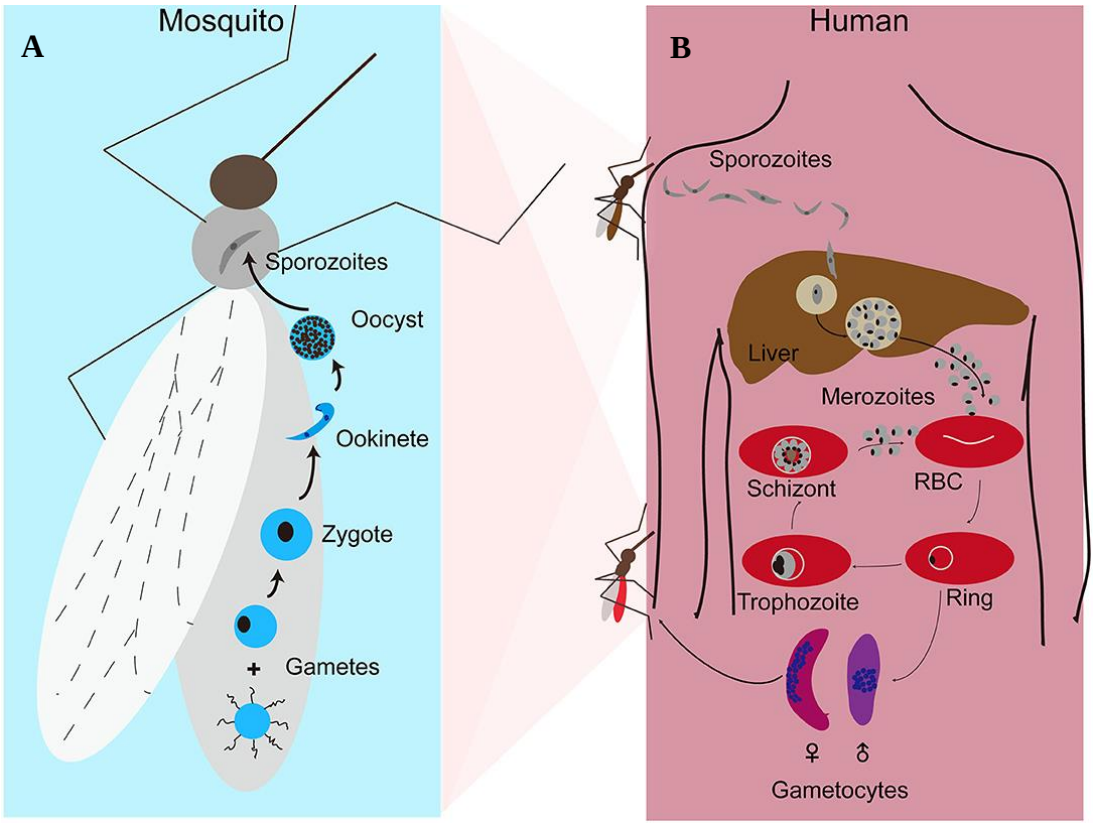


Figure 1.1: Life cycle of *P. falciparum* within a mosquito vector and a human host

(A) In a mosquito, microgametocyte and macrogametocyte form eight microgametes and one macrogamete, respectively. They fuse to form a zygote in the midgut and become an ookinete. The ookinete forms an oocyst where thousands of sporozoites are produced. The sporozoites travel to the salivary gland until being transferred to a human during a blood meal. (B) In a human, the injected sporozoites migrate to the liver and invade hepatocytes to form schizonts. Merozoites released from the schizonts travel through the bloodstream and invade erythrocytes. The parasite develops from the ring to the trophozoite and finally to the schizont. The schizont ruptures and releases merozoites that invade new erythrocytes (Su *et al*., 2020).

on the outer side (Siciliano *et al.,* 2020). In the oocyst, numerous sporozoites are produced by several rounds of mitosis (Araki *et al.,* 2020). When the oocyst ruptures, the sporozoites are released into the haemolymph and migrate to the salivary gland where the parasites acquire the ability to infect human cells (Smith and Jacobs-Lorena, 2010).

2.4.2 Asexual cycle within a human

The asexual cycle begins when motile sporozoites are injected into the skin dermis when an infected mosquito feeds on human blood (Figure 2.1B) (Amino *et al.,* 2006). The sporozoites migrate to the liver and invade hepatocytes where they multiply by a process known as schizogony (Prudêncio *et al.,* 2006). A multinucleated exoerythrocytic schizont of *P. falciparum* comprising 8-24 merozoites develops for two to several days (Gerald *et al.,* 2011). The merozoites released from the hepatocytes travel through the bloodstream and quickly invade erythrocytes. The parasite undergoes the asexual development from the ring stage to the trophozoite stage and finally to a multinucleated intraerythrocytic schizont. The *P. falciparum* schizont ruptures, releasing merozoites that invade new erythrocytes and repeat the cycle every 48 hours. It is during the intraerythrocytic stages that most of the symptoms of malaria are occurs (Shahpudin *et al.,* 2020). The parasite adopts several important metabolic pathways and adaptations during the intraerythrocytic stages that provide potential drug targets and strategies to prevent the disease (Ginsburg, 2016). Many studies have mainly focused on haemoglobin metabolism of the parasite (Klonis *et al.,* 2011; Abu *et al.,* 2010; Francis *et al.,* 1997).

2.5 Haemoglobin metabolism in *P. falciparum*

The malaria parasite needs approximately 80% of the haemoglobin to develop within the host erythrocyte (Lee *et al.,* 2018). As a soluble protein of the erythrocyte, haemoglobin serves as a rich source of nutrients for parasite metabolism (Counihan, Modak and de Koning-Ward, 2021). There is a specific organelle within the parasite known as the digestive vacuole where degradation of most haemoglobin takes place (Abu Bakar *et al.,* 2010). The process starts with the ingestion of haemoglobin followed by the transport of haemoglobin-containing vesicles to the digestive vacuole before massive haemoglobin digestion occurs (Wunderlich, Rohrbach and Dalton, 2012).

2.5.1 Haemoglobin ingestion

Although the precise mechanism of haemoglobin ingestion is still a matter of debate (Elliot *et al.,* 2008; Lazarus *et al.,* 2008), the cytostome-dependent endocytosis is thought to be the key mechanism for the uptake of host cell cytoplasm in the early intraerythrocytic stage of the malaria parasite (the ring stage) (Abu Bakar *et al.,* 2010; Beck and Ho, 2021) (Figure 2.2). In mature parasites (the trophozoite and schizont stages), it is likewise the main pathway. The cytostome is morphologically identified as a double-membrane invagination formed by the parasitophorous vacuolar membrane (PVM) and the parasite plasma membrane (PPM). This distinctive structure is characterized by the presence of electron-dense material at the neck of the cytostome, as observed through serial thin-section electron microscopy (Figure 2.3, black arrow) (Milani, Schneider and Taraschi, 2015).

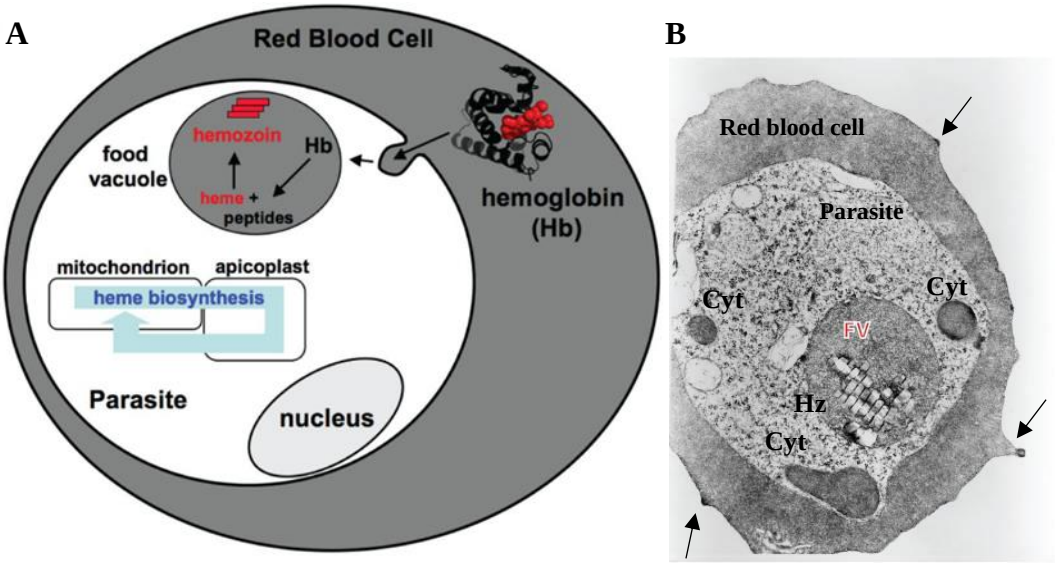


Figure 2.2: General mechanism of haemoglobin ingestion, transport and digestion in *P. falciparum*

(A) Haemoglobin enters the parasite via a cytostome. Haemoglobin-containing vesicles pinch off from the cytostome and then are transported to the digestive vacuole. In the digestive vacuole, haemoglobin is digested into haem and globin. The toxic haem is metabolised into the malarial pigment called haemozoin. (B) In transmission electron micrograph, the erythrocyte membrane has small distinct knobs (arrows) that are characteristic of the infection of *P. falciparum*. The parasite has a distinct digestive vacuole (FV) with haemozoin pigment granules (Hz). Cytostomes (Cyt) with the similar contrast to the erythrocyte cytoplasm are present (Bonilla *et al.*, 2007; Francis *et al.*, 1997).

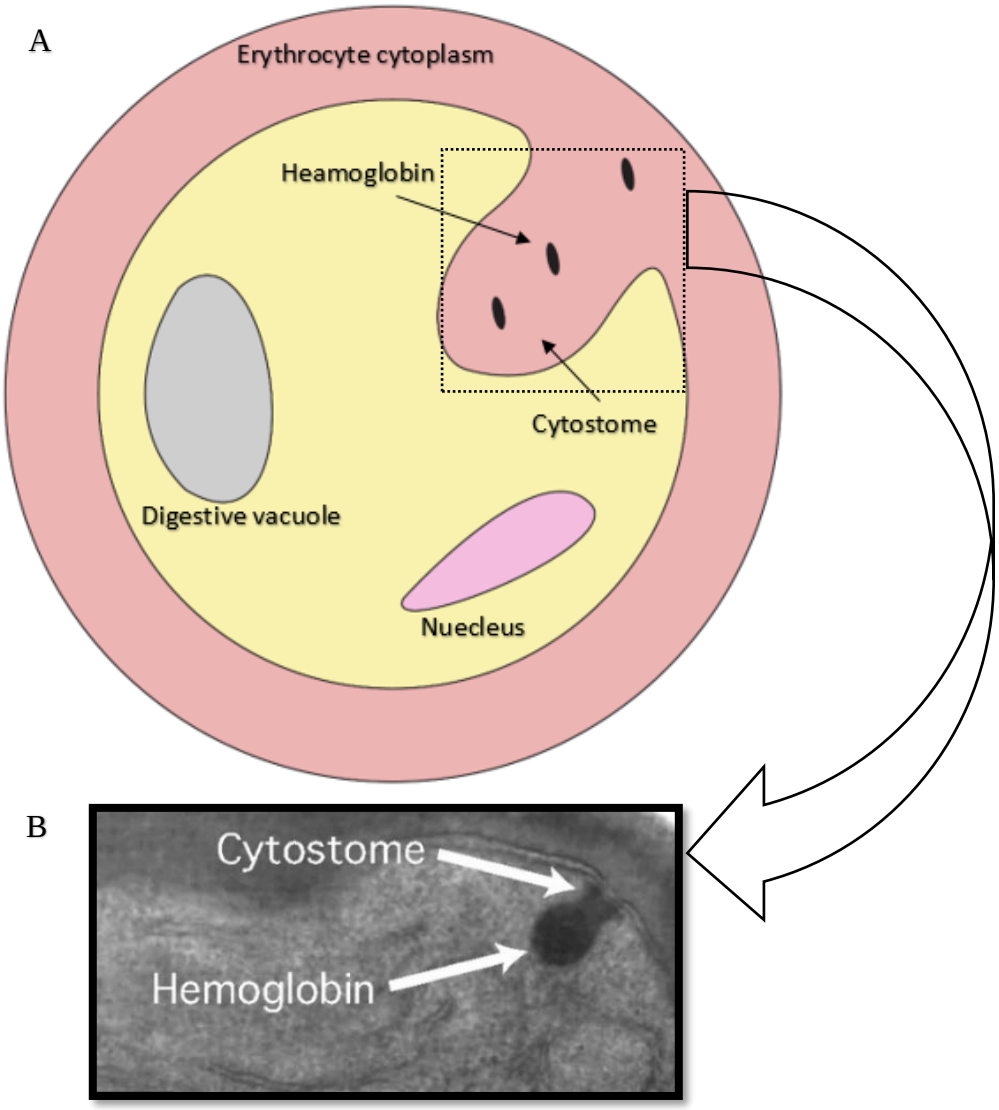


Figure 2.3: Mechanism of haemoglobin ingestion in *P. falciparum*

(A) The host cell cytoplasm containing haemoglobin is ingested by the parasite by cytostome. (B) The cytostome is evident in transmission electron micrograph as an electron-dense ring that surround the neck of the endocytic structure called the cytostomal invagination of haemoglobin (Elliott *et al.*, 2008).

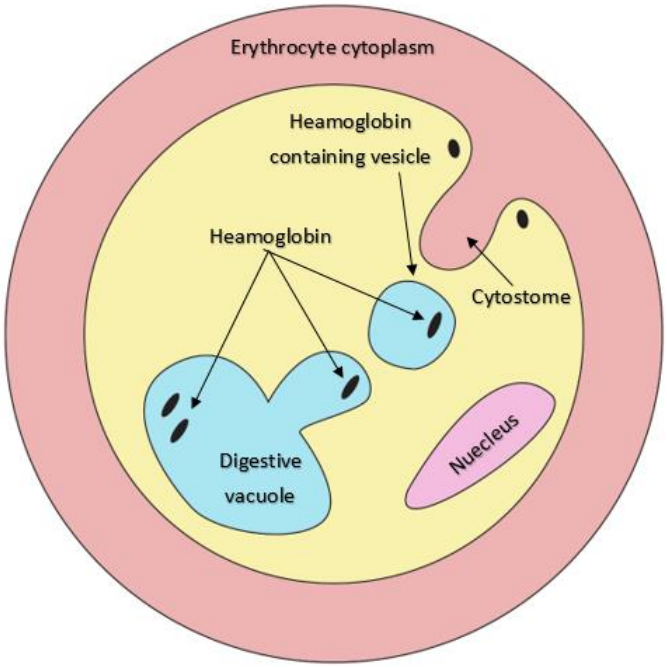
Polyphosphorylated phosphoinositides (PIPs) of parasite origin are thought to be involved in the endocytosis from the host cell (Vaid, 2010; Tawk, 2010).

2.5.2 Haemoglobin transport

The budding process of cytostomes results in the creation of vesicles enveloped by two membranes: the outer membrane originates from the parasite plasma membrane (PPM), while the inner membrane is derived from the parasitophorous vacuolar membrane (PVM) (Figure 2.4) (Adisa *et al.*, 2003). The transport of haemoglobin-containing vesicles to the digestive vacuole has been reported to involve the mechanism of actin and myosin interaction (Xie, Ralph and Tilley, 2020). Two distinct actin isoforms expressed by *Plasmodium* species differ significantly from each other and from conventional actins. The parasite's life cycle is characterised by the expression of actin-I. Actin-II is exclusively present in *Plasmodium* species and only expressed during the gametocyte, gamete and sporozoite phases, which are involved in transmission via the mosquito. This motility system is thought to be heavily dependent on actin-I. Actin-I is also considered to have a role in vesicle trafficking and endocytosis, ring stage morphogenesis and the spatial organisation of genes in the nucleus in the intraerythrocytic stages of *P. falciparum*. The inheritance of intracellular organelles including the apicoplast, mitochondria and secretory vesicles during intracellular parasite replication has recently been demonstrated to be significantly influenced by actin dynamics (Hliscs *et al.*, 2014).

Since *P. falciparum* has no known intermediate filaments and microtubules do not develop until the schizont stage, which is well after the majority

A



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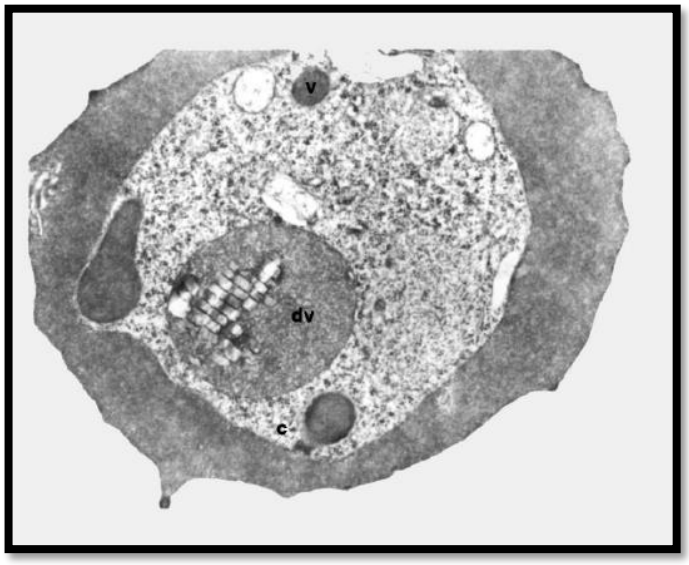

Figure 2.4: Mechanism of haemoglobin transport in *P. falciparum*

(A) Haemoglobin-containing vesicle pinches off from a cytostome and are delivered to the digestive vacuole. (B) TEM image of haemoglobin transport. c, cytostome; v, vesicle; dv, digestive vacuole (Francis *et al*., 1997).

of haemoglobin internalisation and destruction has taken place. *P. falciparum* actin (Pfactin) assembles as short, flaky filaments *in vitro*, despite the fact that the dynamics of actin in the intraerythrocytic stage parasites are poorly understood. Actin may have a function in the endocytic trafficking of haemoglobin in *P. falciparum* according to a recent study. (Lazarus *et al.,* 2008). Motility of *P. berghei* ookinetes inhibited by the F-actin-stabilising drug, jasplakinolide (JAS), which also prevents the invasion of *P. falciparum* merozoites. JAS significantly lowers the critical actin subunit concentration required to promote filament stabilisation (Pospich *et al.,* 2017).

2.5.3 Haemoglobin digestion

The fusion of endocytic vesicles with the membrane of the digestive vacuole releases the cargo containing haemoglobin therein (Figure 2.5). The parasite digests about 80% of the haemoglobin (Lee *et al.,* 2018), but only 16% of the amino acids derived from the digestion are used for protein synthesis (Krugliak, 2002). Given that haemoglobin contains a less small amount of methionine, cysteine, glutamine and glutamate, and lacks isoleucine, the malaria parasite degrades haemoglobin for purposes other than just nutrition (Naughton *et al*., 2010). A mathematical model suggests that this degree of permeabilization would normally cause premature RBC lysis driven by osmotic water influx. It was proposed that excessive haemoglobin processing by the parasite lowers the concentration of haemoglobin in the RBC cytoplasm and thus reduces colloid-osmotic pressure and water influx (Matz, 2022). It is believed that the main purpose of haemoglobin degradation is to regulate osmotic pressure in order to prevent the parasite from early lysis (Esposito *et al.,* 2008).

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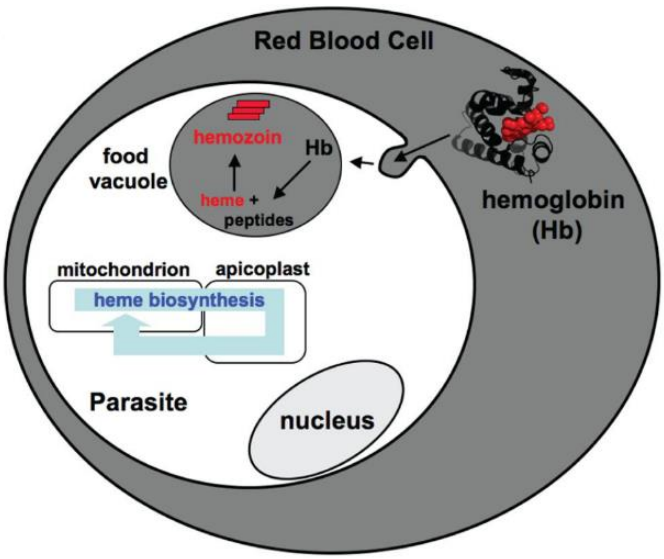

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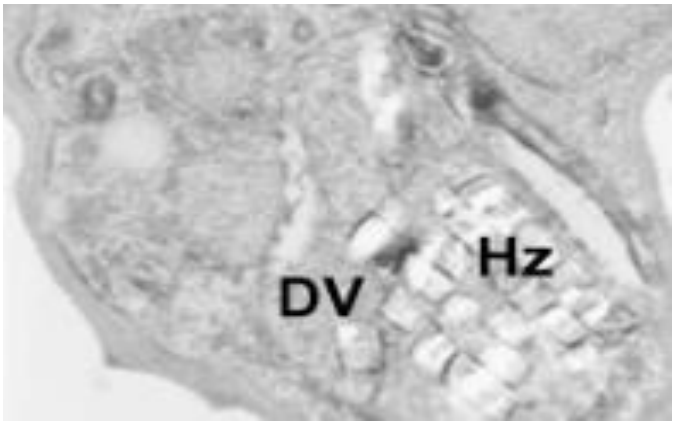

Figure 2.5: Mechanism of haemoglobin digestion in *P. falciparum*

(A) Inside the digestive vacuole, haemoglobin is metabolised into the haemozoin. (B) Haemozoin crystals (Hz) are evident inside the digestive vacuole (DV) (Bonilla *et al.*, 2007).

2.5.3(a) Enzymatic mechanism of haemoglobin digestion

Aspartic (plasmepsin I and II) and cysteine (falcipain) proteases have been implicated in the haemoglobin degradation pathway of *P. falciparum* (Kumar *et al.,* 2018). Plasmepsin I makes the initial cleavage between the α33Phe and 34Leu residues of haemoglobin, which plays a conformational role of this molecule before proceeding with the subsequent cleavages elsewhere (Coronado *et al*., 2014). This exposes other sites for later cleavage by aspartic and cysteine proteases. In the ring stage of the malaria parasite, exclusive expression of plasmepsin I occurs, and this expression persists into the subsequent stages (Nasamu *et al.,* 2020). Conversely, plasmepsin II is predominantly expressed during the trophozoite stage, characterized by heightened metabolic activity. A study done by Francis *et al.* (1994) showed that the inhibition of plasmepsin I activity by the plasmepsin inhibitor (SC-50083) led to parasite death. This suggests that while the quantity of plasmepsin II is elevated in the trophozoite stage compared to the ring stage, it is insufficient to compensate for the absence of plasmepsin I during the trophozoite stage. Cysteine proteases, falcipains continue the degradation process to degrade the denatured haemoglobin to small peptides (Eggleson *et al.,* 1999). Research demonstrated that the use of cysteine protease inhibitors, including leupeptin, chymostatin, and E-64, resulted in the buildup of undigested globin within the digestive vacuole (Rosenthal, 1995; Semenov *et al.,* 1998). Each of these proteases function most effectively within the pH range of 4.5-5.5, aligning with the acidity of the digestive vacuole (Hayward, Saliba and Kirk, 2006).

2.5.3(b) Maintenance of acidic pH of the digestive vacuole

The responsibility for maintaining an acidic digestive vacuole is attributed to the vacuolar-type proton-pumping ATPase (V-type H^+-ATPase) (Saliba *et al.*, 2003). This proton pump has 14 subunits that are divided into two domains. The V_0 domain is responsible for proton translocation, while V_1 domain is responsible for ATP hydrolysis (Parra, Chan and Chen, 2014). In *P. falciparum*, the V-type H^+-ATPase has been localised to the PPM, digestive vacuole and small clear vesicle compartments (Hayashi *et al.*, 2000). Apart from acidifying the digestive vacuole, this proton pump also regulates the parasite cytosolic pH for enzyme function and pH gradient generation across the PPM and internal organelle membranes (Solebo *et al.*, 2021). The pH gradient across the PPM is critical for the transport of nutrients and protons and removal of waste products. The V-type H^+-ATPase is also implicated in generating an inside-negative membrane potential (~ -95 mV) that is used by the parasite to regulate the concentration of ions (e.g. potassium ions) in the cytosol and the uptake of nutrients (e.g. phosphate and choline) (Lehane *et al.*, 2004).

2.5.4 Haem and haematin detoxification

Upon digestion of each haemoglobin tetramer by the malaria parasite, four molecules of haem (ferroprotoporphyrin IX) are released into the digestive vacuole (Fong and Wright, 2013). Under the acidic condition of the digestive vacuole, haem is immediately oxidised to haematin (ferriprotoporphyrin IX) (de Villiers and Egan, 2021). Haematin is a toxic waste product of haemoglobin digestion that the parasite needs to destroy. Due to the lack of haem oxygenase in *Plasmodium* spp. (Dalapati

and Moore, 2021), the parasite converts the haematin monomer into the inert biocrystal malarial pigment known as haemozoin (Coronado *et al*., 2014). The crystallisation of haematin is a spontaneous process (Stiebler *et al.,* 2010), although studies have shown that this process might be facilitated by lipid catalysts (Stiebler *et al.,* 2014).

Quinoline antimalarial drugs such as chloroquine are thought to exhibit their activity by interacting with haem and haematin (Herraiz *et al.,* 2019). Another drug class, endoperoxides such as artemisinin are thought to exert their activity upon reaction with haem (Zhu and Zhou, 2022) or ferrous iron (O'Neill, Barton and Ward, 2010). However, since the 1960s, resistance to chloroquine and other quinoline-based drugs has made malaria treatment more problematic (Schlagenhauf *et al.,* 2019). The pharmacokinetic limitations and emerging resistance to artemisinin have also threatened the efforts to control malaria (Talisuna *et al.,* 2012). Therefore, the discovery of novel antimalarial drugs especially from medicinal plants that can interfere with the haemoglobin metabolism and its metabolic pathways is likely to provide successful therapeutics against the disease (Renslo, 2013).

2.6 Past and present antimalarial drugs

The past decades have witnessed remarkable efforts in the antimalarial drug discovery, which has made possible in part by the vision of organisations such as the Wellcome Trust and the Bill and Melinda Gates Foundation that has led to the emergence of pharmaceutical industries (Renslo, 2013).

2.6.1 Quinoline-containing antimalarial drugs

Primaquine (Figure 2.6A) is an 8-aminoquinoline, the only potent gametocytocide against *P. falciparum* malaria (Graves, Gelband and Garner, 2015) and the only drug effective to prevent relapse against *P. vivax* and *P. ovale* infections (Ashley, Recht and White, 2014). However, the use of primaquine has been reported to cause haemolytic toxicity in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Chu *et al.,* 2018). Fourteen deaths were recorded in six decades from the use of this drug among 200 million people worldwide (Ashley, Recht and White, 2014).

Quinine (Figure 2.6B) is an alkaloid that was first isolated from cinchona bark in 1820 (Achan *et al.,* 2011). Despite growing drug resistance in the malaria parasite and limited access to newer antimalarial drugs in low-income countries, quinine has been used for more than four centuries as a first-line treatment for severe malaria (Achan *et al.,* 2011). Since quinine has a limited therapeutic index, precautions have to be taken as any major changes in the drug distribution may increase the likelihood of adverse effects such as the formation of hyperinsulinaemic hypoglycaemia (Adehin *et al.,* 2019).

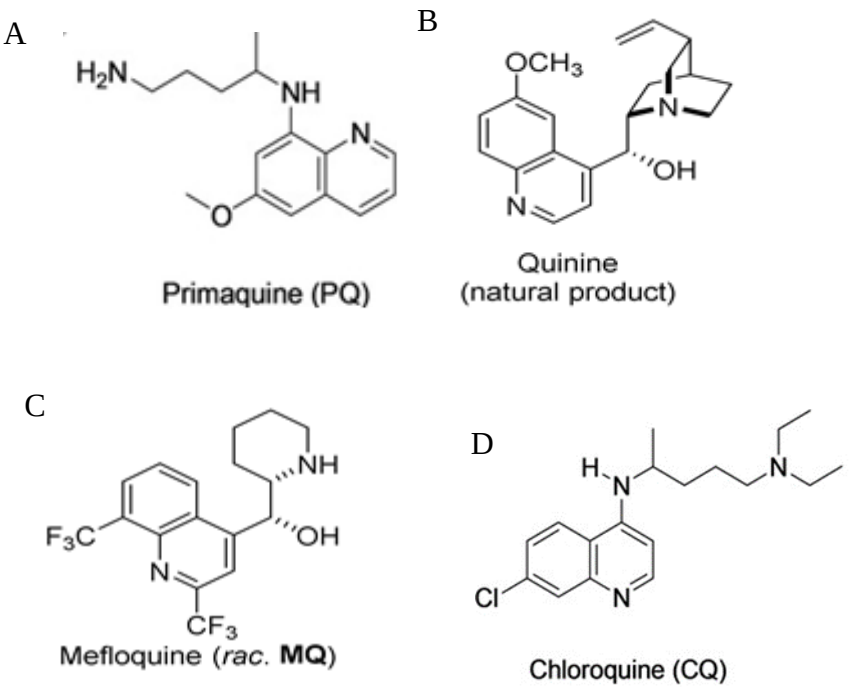

Figure 2.6: The molecular structure of quinoline-containing antimalarial drugs

(A) primaquine, (B) Quinine, (C) mefloquine and (D) chloroquine are one of the quinoline drugs that have been used for decades to treat malaria (Kucharski, Jaszczak and Boratyński, 2022).