CHANGES IN pH OF DIGESTIVE VACUOLE OF *Plasmodium falciparum* TREATED WITH PIPECOLISPORIN AND ANALOGUE II PEPTIDE

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by

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

Nurul Adila binti Ahmad Fauzi

Date: 26/1/2025

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~	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
µL	microliter
e.g.	for example
g	gram
i.e.	that is
kDa	kilodaltons
kg	kilogram
mg	milligram
mg/mL	milligram per millilitre
mL	millilitre
M	molar
mM	millimolar
mol	mole

n	number of subjects
nm	nanometre
nM	nanomolar
pH	potential of hydrogen
p*K*a	acid dissociation constant
w/v	weight by volume
ACTs	artemisinin-based combination therapies
AIDS	acquired immunodeficiency syndrome
ATP	adenosine triphosphate
BMI	body mass index
CO2	carbon dioxide
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CCM	complete culture medium
DFd	degree of freedom denominator
DFn	degree of freedom numerator
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EPM	erythrocyte plasma membrane
FACS	fluorescence-activated cell sorting
FCS	flow cytometry standard
FITC	fluorescein isothiocyanate
FSC	forward scatter
H^+	proton/hydrogen ion
HIV	human immunodeficiency virus

HEPES	hydroxyethyl piperazineethanesulfonic acid
IC_{50}	half-maximal inhibitory concentration
IL	interleukin
IFN	interferon
ICCM	incomplete cell culture medium
INFORMM	Institute for Research in Molecular Medicine
KCl	potassium chloride
Mg^{2+}	magnesium ion
MACS	magnetic-activated cell sorting
NaCl	sodium chloride
NaOH	sodium hydroxide
NaH_2PO_4	sodium phosphate
NK0	pipecolisporin
NK2	analogue II
PBS	phosphate buffer saline solution
PE	phycoerythrin
PPM	parasite plasma membrane
PV	parasitophorous vacuole
RBC	red blood cell
RNA	ribonucleic acid
Rg/y	fluorescence ratio
RPMI	Rosewell Park Memorial Institute
SD	standard deviation
SSC	side scatter
TNF	tumor necrosis factor

USM	Universiti Sains Malaysia
V-type H+-ATPase	vacuolar-type proton-pumping ATPase
V-type H+-pyrophosphate	vacuolar-type proton-pumping pyrophosphatase
WHO	World Health Organization

PERUBAHAN pada pH VAKUOL PENCERNAAN

***Plasmodium falciparum* yang dirawat dengan**

pipecolisporin dan analog II peptida

ABSTRAK

Malaria adalah penyakit serius dan kadangkala membawa maut yang disebabkan oleh *Plasmodium* spp, kekal sebagai salah satu punca utama kadar morbiditi dan mortaliti global. Kemunculan *Plasmodium falciparum* yang rintang ubat di pelbagai negara telah memerlukan usaha untuk mencari ubat antimalaria baharu dengan sasaran yang berlainan. Kajian mengenai potensi pipecolisporin dan analog II sebagai ejen antimalaria masih terhad. Oleh itu, kajian ini bertujuan untuk mengisi jurang pengetahuan ini dengan menyiasat sifat antimalaria pipecolisporin dan analog II peptida. Potensi antimalaria pipecolisporin dan analog II terhadap *P. falciparum* yang sensitif klorokuina (strain 3D7) telah dinilai menggunakan ujian malaria yang berasaskan pengiraan parasitemia menggunakan smear darah berwarnakan Giemsa. Pipecolisporin dan analog II menunjukkan aktiviti antimalaria yang aktif dengan nilai IC_{50} masing-masing 0.4770 dan 0.1170 µM. Penyiasatan lanjut memfokuskan kepada kesan pipecolisporin dan analog II terhadap pH vakuol pencernaan parasite menggunakan teknik berasaskan sitometri aliran dengan menggunakan fluorescein isothiocyanate-dextran (FITC-dextran) sebagai penunjuk pH. Keputusan menunjukkan tiada peningkatan pH berikutan rawatan pipecolisporin dan analog II peptide. Hal ini menunjukkan bahawa pipecolisporin dan analog II mungkin tiada mekanisma untuk mengubah pH vakuol pencernaan melalui perencatan H^+-ATPase jenis-V yang mengawal pengasidan vakuol. Secara keseluruhannya, kajian ini menunjukkan bukti penting mengenai keupayaan

pipecolisporin dan analog II sebagai calon antimalaria yang diyakini.

CHANGES IN pH OF DIGESTIVE VACUOLE OF

***Plasmodium falciparum* TREATED WITH**

PIPECOLISPORIN AND ANALOGUE II PEPTIDES

ABSTRACT

Malaria is a severe and fatal disease caused by *Plasmodium* spp. and remains one of the leading global causes of morbidity and mortality. The emergence of drug-resistant *P. falciparum* in various countries has necessitated an effort to discover new antimalarial drugs targeting different pathways. Research on the potential of pipecolisporin and analogue II as an antimalarial agent has remained limited. Therefore, this study aimed to fill this knowledge gap by investigating the antimalarial activity of pipecolisporin and analogue II peptides. The antimalarial potential of pipecolisporin and analogue II against the chloroquine-sensitive strain (3D7) of *P. falciparum* was assessed based on the calculation of parasitaemia using Giemsa stained-blood smears. The pipecolisporin and analogue II exhibited highly active antimalarial activity with an IC_{50} value of 0.4770 and 0.1170 µM, respectively. A further investigation focused on the effect of pipecolisporin and analogue II towards the pH of the mid trophozoite stage parasite's digestive vacuole, employing a flow cytometry-based technique with fluorescein isothiocyanate-dextran (FITC-dextran) as a pH ratiometric probe. The results revealed no increase in pH following pipecolisporin and analogue II treatment. Suggests that pipecolisporin and analogue II might not have the mechanism to alter the digestive vacuole's pH through the inhibition of V-type H^+-ATPase that regulates the acidification of the vacuole. Overall, this study provides crucial evidence of pipecolisporin and analogue II capability as a promising antimalarial candidate.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is a life-threatening disease spread to humans by infected female *Anopheles* mosquitoes typically found in tropical and subtropical regions of the world. The infection may present with minor symptoms including fever, chills and headache, and severe symptoms including exhaustion, disorientation, convulsions and trouble breathing. *Plasmodium* species caused human malaria are *P. falciparum*, *P. vivax, P. ovale, P. malariae* and *P. knowlesi* (World Health Organization, WHO, 2023). However, most malaria cases worldwide are caused by *P. falciparum*, manifesting acute respiratory distress syndrome, cerebral malaria, severe anaemia and acute renal failure (Zekar & Sharman, 2023).

P. falciparum has a complex life cycle involving two phases: the exoerythrocytic and intraerythrocytic phases. In the exoerythrocytic phase, sporozoites invade the hepatocytes and multiply into schizonts, which then release thousands of merozoites into the bloodstream. The intraerythrocytic phase occurs when merozoites invade the erythrocytes and develop into rings, trophozoites and schizonts (Venugopal et al., 2020). The number of merozoites increases once the erythrocyte rupture, initiating the malaria symptoms. The symptoms vary depending on the malaria parasite's pathogenesis and the host's immune response (Crutcher & Hoffman, 1996)

During the initial immune response, the release of parasite antigens from the rupture of infected erythrocytes stimulates the host's immune system to release cytokines and reactive oxygen species. This immune response leads to the presence of mild symptoms like fever and chills. During the intraerythrocytic phase, the infected erythrocyte ruptures due to the replication of the parasite. This condition, also known as haemolysis, could lead to severe anaemia and haemolytic jaundice due to the destruction of erythrocytes (Chitasombat, 2023). Moreover, the ability of infected erythrocytes to adhereto the endothelium of the blood vessels particularly capillaries and postcapillary venules is one of the key features of *P. falciparum* severity. This condition leads to an obstruction of the microcirculation and tissue anoxia. Additionally, different complications will occur depending on the affected location such as in the brain, causing cerebral malaria, and the kidney, causing acute tubular necrosis and renal failure (Crutcher & Hoffman, 1996).

Malaria causes an enormous number of cases and deaths each year, despite tremendouspreventive efforts. Globally, it reportedly caused 608 000 deaths, with an estimated 249 million cases in 2022 (WHO, 2023). *P. falciparum* is predominantly responsible for severe disease and deaths, especially in African regions where 94% of malaria cases and 95% of deaths came from. In the common highly prevalent regions, drug-resistant parasites are a major challenge in attempts to control and eradicate malaria. Parasite strains associated with *Pf*CRT protein have developed full resistance against the well-established antimalarial drug, chloroquine (Chinappi et al., 2010). The malaria parasite with a mutation of the *Pf*K13 protein has been found to develop partial resistance against the current front-line antimalarial drug, artemisinin (Conrad et al., 2023). The

challenge keeps on growing as there is only one vaccine known as RTS,S/AS01 vaccine, available to prevent malaria. However, this vaccine has limitations as it is only effective for children with an age range of 5 months to 2 years old and ineffective for any individual beyond the age range (Beeson, 2023).

In recent years, there have been a lot of studies regarding the potential of peptide compounds as therapeutic agents in various medical conditions, including diabetes, cancer treatment and infectious diseases. Two well-known peptide drugs that have demonstrated exceptional effectiveness in treating type 2 diabetes and obesity are tripeptide and semaglutide (Bays et al., 2024). Peptide-drug conjugates (PDCs) are a brand-new type of targeted therapy that mixes radioactive or cytotoxic medications with peptides, which highly potential in cancer treatment (Sun et al., 2024). Moreover, some studies have proven certain peptides like PL-18 exhibit strong antimicrobial activity (Rossino et al., 2023). Therefore, due to the increasing number of studies regarding peptides, researchers have started to investigate the antimalarial potential of the compounds.

Pipecolisporin is a cyclic hexapeptide that is obtained from the fungal endophyte, *Nigrospora oryzae*. It has significant therapeutic properties, mostly against parasitic illnesses like malaria and Chagas disease. The key features of pipecolisporin, which contribute to antimalarial activity are the cyclic structure of the peptide. In contrast to linear peptides, pipecolisporin's cyclic structure increases its stability and resistance to enzyme degradation. This structural characteristic is essential for preserving its bioactivity against *P. falciparum.* Additionally, the amino acid composition in pipecolisporin specifically, hydrophobic amino acids like tryptophan and leucine play an

important role in malaria combat by promoting membrane disruption and cell death (Fernández-Pastor et al., 2021). Similarly to pipecolisporin, analogue II is also composed of almost the same structure and components, which could give the same antimalarial potential.

1.2 Problem statement

Malaria remains a major global cause of disease and mortality. *P. falciparum* has become resistant to antimalarial medications such as chloroquine (Chinappi et al., 2010). The demand for continuously looking for novel and promising antimalarial drugs has been highlighted by recent reports of parasites becoming resistant to artemisinin and the limited effectiveness of a vaccine that is currently on the market (Conrad et al., 2023; Beeson, 2023).

Peptide compounds have become an exciting focus in modern medicine due to their unique ability to interact with specific biological targets, mimicking natural processes in the body. These small chains of amino acids play diverse roles, such as hormones, growth factors and neurotransmitters, making them valuable in treating a wide range of conditions. The specificity, potency and relative safety have led to significant advancements in therapeutic applications, especially in areas like cancer, metabolic disorders and infectious diseases like malaria (Rossino et al., 2023). However, research on the antimalarial potential of pipecolisporin remains limited to date while the antimalarial potential for analogue II remains somewhat unexplored in terms of malarial research.

The acidic environment of the digestive vacuole, where haemoglobin breakdown and subsequent haemoglobin detoxification take place, is crucial to the parasite's survival and growth within the host's erythrocyte (Edgar et al., 2022). In this mechanism, ion pumps and proteases work together to maintain the low pH of the digestive vacuole. Therefore, the purpose of this study was to investigate the potential and effect of pipecoliporin and analogue II on the pH levels in the digestive vacuole of *P. falciparum.*

1.3 Rationale of the study

In modern medicine, peptide-based drugs have been widely used to address various health challenges. Despite their potential, the current understanding of pipecolisporin and analogue II peptides' antimalarial capabilities are noticeably lacking. Although these peptides contain a wealth of bioactive substances, such as amino acid sequences, it is yet unknown how exactly they work against *P. falciparum*. It has not been fully studied how these peptides affect the digesting vacuole, a vital organelle in *Plasmodium* species and a known target of several antimalarial medications. By assessing pipecolisporin and analogue II peptides' potential as antimalarial options, our study filled up these knowledge gaps. This study aims to provide light on their mode of action and examine their potential as innovative strategies for treating drug-resistant malaria by examining their impact on the pH of the gastric vacuole.

1.4 Objectives of the study

1.4.1 General objective

To determine the antimalarial activity of pipecolisporin and analogue II against *P. falciparum.*

1.4.2 Specific objectives

i. To determine the concentration of pipecolisporin and analogue II that demonstrate a 50% inhibition of *P. falciparum* growth (IC_{50}).

ii. To measure the effect of pipecolisporin and analogue II on the pH level of the digestive vacuole of *P. falciparum.*

CHAPTER 2

LITERATURE REVIEW

2.0 Overview of malaria

Malaria is a life-threatening infectious disease caused by *Plasmodium* species. It has been a significant worldwide health issue throughout human history and a major source of illness and mortality in many tropical and subtropical nations. It is the most prevalent disease in Africa and several Asian countries and transferred from endemic regions into the developed world.

2.1 Prevalence of malaria

According to the World Malaria Report 2023, in 2022, there were approximately 249 million cases in 85 malaria-endemic countries, significantly increasing as compared to 247 million cases in 2021 (WHO, 2023). The main contributor country for the increase in cases was Pakistan with 2.1 million cases. Between 2000 and 2019, malaria cases decreased from 81 to 57 per 1 000 at-risk individuals. Incidence rates did not change over the last three years, except for a 3% increase in 2020. The incidence of malaria cases in 2022 was 58 per 1 000 at-risk individuals. The WHO African Region had the highest cases (94%) globally.

Globally, the mortality of malaria decreased steadily from 864 000 in 2000 to 586 000 in 2015 and 576 000 in 2019. In 2020, an estimated 631 000 people died from

malaria, a 10% increase from 2019. In 2022, the estimated number of deaths dropped to 608 000 (WHO, 2023). In the WHO African Region, estimated deaths declined to 580 000 in 2022 from 604 000 in 2020. Pregnant women and children under five are particularly at risk. The WHO African Region has a high incidence of malaria cases and deaths due to several factors such as humidity, socioeconomic instability and mosquito vectors.

2.3 Aetiological agents and vectors of malaria

The aetiological agents of malaria are *Plasmodium* species. About 156 species of *Plasmodium* have been identified to be able to infect different kinds of vertebrates (Centers for Disease Control and Prevention, CDC, 2024). Only five species of *Plasmodium* naturally infect humans and cause malaria in vast regions of the world: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Sato, 2021). Among these five, *P. falciparum* is the most pathogenic species and predominantly causes severe and fatal malaria (Zekar & Sharman, 2023). In the last half-century, *P. falciparum* has become resistant to all antimalarial medications and recent years have seen a danger to all significant progress in the control of malaria due to resistance to artemisinin derivatives and the failure of artemisinin-based combination therapies (ACTs) (Thu et al., 2017).

Female *Anopheles* mosquitoes are the main vectors for malaria. Around 400 known species of *Anopheles* mosquitoes, and about 70 of them may be human malaria vectors. According to Akeju et al. (2022), in Africa, *An. funestus* and *An. gambiae* are the main vectors of malaria parasites. The transmission of malaria is also linked to environmental factors such as warm temperatures, high humidity, regular rainfall, soil

quality and proximity to water bodies (Castro, 2017).

2.4 Life cycle of the malaria parasite

The life cycle of the malaria parasite involves two hosts: female *Anopheles* mosquitoes and humans in which multiple distinct stages exist (Figure 2.1).

2.4.1 Sexual cycle of the malaria parasite

The malaria parasite is transmitted through a female *Anopheles* mosquito, which ingests gametocytes during blood feeding on an infected human. According to Nureye & Assefa (2020), after the ingestion, male and female gametocytes undergo gametogenesis. Zygotes are produced when female macrogametes are fertilised by flagellated male microgametes produced by the exflagellation process. The zygotes mature into ookinetes, which subsequently mature into oocysts, where numerous sporozoites are formed by the nucleus division. As soon as the sporozoites are completely developed, the oocyst ruptures, releasing them into the mosquito haemocoel. The sporozoites complete the sexual (sporogony) life cycle by migrating to the mosquito salivary glands. The life cycle of the parasite is sustained when the sporozoites from the salivary glands are inoculated to a new human host.

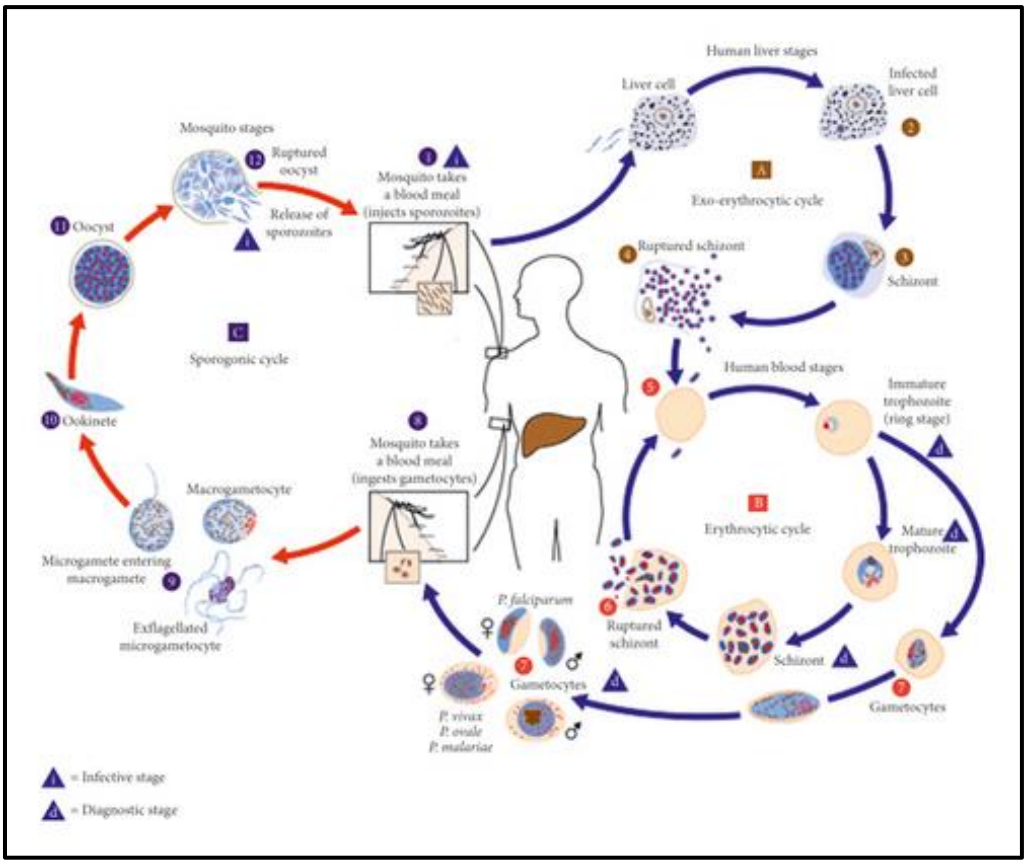

Figure 2.1: Life cycle of the malaria parasite

(A) The asexual life cycle of the malaria parasite begins when a female *Anopheles* mosquito transmits sporozoites into the human host. The sporozoites migrate to the liver, invade hepatocytes and then produce merozoites. (B) The merozoites infect erythrocytes and progress into different stages (ring, trophozoite and schizont stages). Some merozoites develop into gametocytes, which are taken up by a mosquito to initiate the sexual life cycle. (C) The sexual life cycle begins when male and female gametes fuse to form a zygote. The zygote develops into a motile ookinete and migrates to the epithelial layer of the mosquito's midgut wall to transform into an oocyst. Sporozoites are released once the oocyst ruptures and invade the mosquito's salivary gland. Adapted from Nureye and Assefa (2020).

2.4.2 Asexual cycle of the malaria parasite

The asexual life cycle begins when sporozoites are inoculated into a new human host during blood feeding of an infected mosquito. The circulatory system carries the sporozoites to the liver within 60 minutes of the inoculation. The sporozoites evolve into schizonts over 7-12 days, producing up to 30 000 merozoites that are released from the rupture of the hepatocytes. However, in some cases of *P. vivax* and *P. ovale*, the sporozoites develop into hypnozoites, which can cause relapses in infected individuals and remain dormant in the liver for months or years.

The merozoites released from the hepatocytes invade erythrocytes. Depending on the malaria parasite species, they develop within the erythrocytes from the early ring stage to the late trophozoite stage and then, following mitotic divisions, to the late schizont stage, comprising 6-32 merozoites. The merozoites released from the ruptured erythrocytes invade new erythrocytes to continue their life cycle. In this intraerythrocytic cycle, cyclic malaria fevers occur before or during haemolysis. In cases of tertian malaria, it happens every 48 hours, while quartan malaria happens every 72 hours (Figure 2.2). Some merozoites undergo this recurring cycle by maturing into gametocytes, which are single-nucleated male and female sexual forms, waiting to be ingested by a mosquito during blood feeding (Nureye & Assefa, 2020).

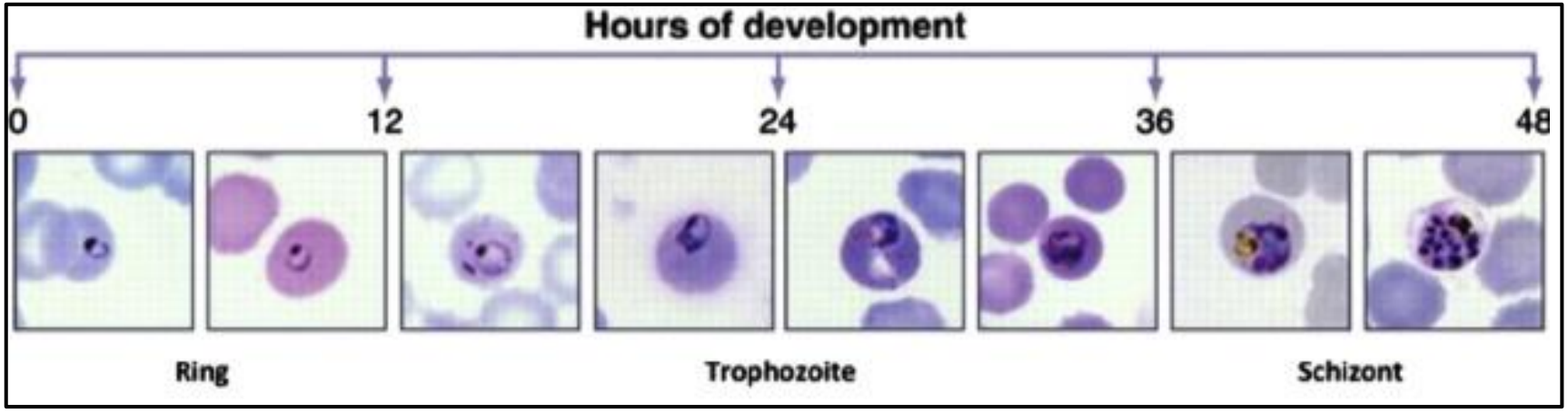

Figure 2.2: Asexual developmental stages of *P. falciparum* throughout the 48-hour intraerythrocytic life cycle

The parasite's life cycle involves multiple stages in the host erythrocyte from an immature tiny ring form to mature trophozoites and schizonts that include merozoites. Modified from Cheng and Yansouni (2013).

2.5 Clinical manifestation of malaria

The majority of infected patients often have a silent initial rupture and invasion, but as the asexual cycle repeats itself for the following 24-48 hours, parasitaemia and the immune response escalate. Interleukin 10 (IL-10) and interferon-gamma (IFN-gamma) are two inflammatory markers in the cascade that are typically linked to the manifestation of malaria symptoms, along with an increase in TNF alpha (TNF-α). There is a wide range in the connection between higher parasitaemia and a more severe clinical presentation. According to Zekar & Sharman (2023), the clinical manifestation of malaria can be divided into two categories: uncomplicated and severe. Uncomplicated malaria usually presents with non-specific symptoms such as fever, chills, myalgia, headache and anorexia. Patients could also present with jaundice and gastrointestinal and respiratory symptoms. Severe malaria, which is often associated with *P. falciparum*, includes several severe signs like respiratory distress, impaired consciousness, severe anaemia and acute kidney injury.

2.6 The digestive vacuole of the malaria parasite

During parasite growth and replication, large volumes of the erythrocyte cytosol are taken up by the parasite, which then breaks down haemoglobin in a specialised acidic organelle known as the digestive vacuole. Rudzinska and Trager discovered the structure and role of the digestive vacuole in 1957 (Figure 2.3). The digestive vacuole contains hydrolytic enzymes of several different molecular types that break down haemoglobin. Aspartic proteases, known as plasmepsins, and cysteine proteases, known as falcipains, have been identified in biochemical research as important mediators of this

degradative process. The ideal pH range for these proteases is between 4.0-5.5 comparable to the pH range of 3.7-6.5 found in the digestive vacuole (Moura et al., 2009; Muchtar et al., 2022).

The digestive vacuole must maintain its acidic pH level to promote effective haemoglobin breakdown and detoxification. Additionally, proton pumps, the vacuolar H^+-ATPase (V-type H^+-ATPase), and pyrophosphatase (V-type H^+-pyrophosphatase) strictly regulate the acidity of the digestive vacuole (Kapishnikov et al., 2017). The acidity of the digestive vacuole is critical for the occurrence of several biological activities. It has been claimed that even small changes in the acidic environment may interfere with haemoglobin breakdown and haem detoxification, resulting in the inhibition of parasite growth and proliferation (Mohd Zamri et al., 2017).

According to Schrezenmeier & Dörner (2020), a common antimalarial drug, hydroxychloroquine, was demonstrated to accumulate in the digestive vacuole and prevent the breakdown of cargo originating from the endocytosis pathways. To carry out this process, the pH of the digestive vacuole increases, which stops the enzymes from functioning. The digestive vacuole is a desirable target for antimalarial drugs due to its characteristics and vital function. One important strategy in the creation of antimalarial drugs is the disturbance of pH control within the digestive vacuole.

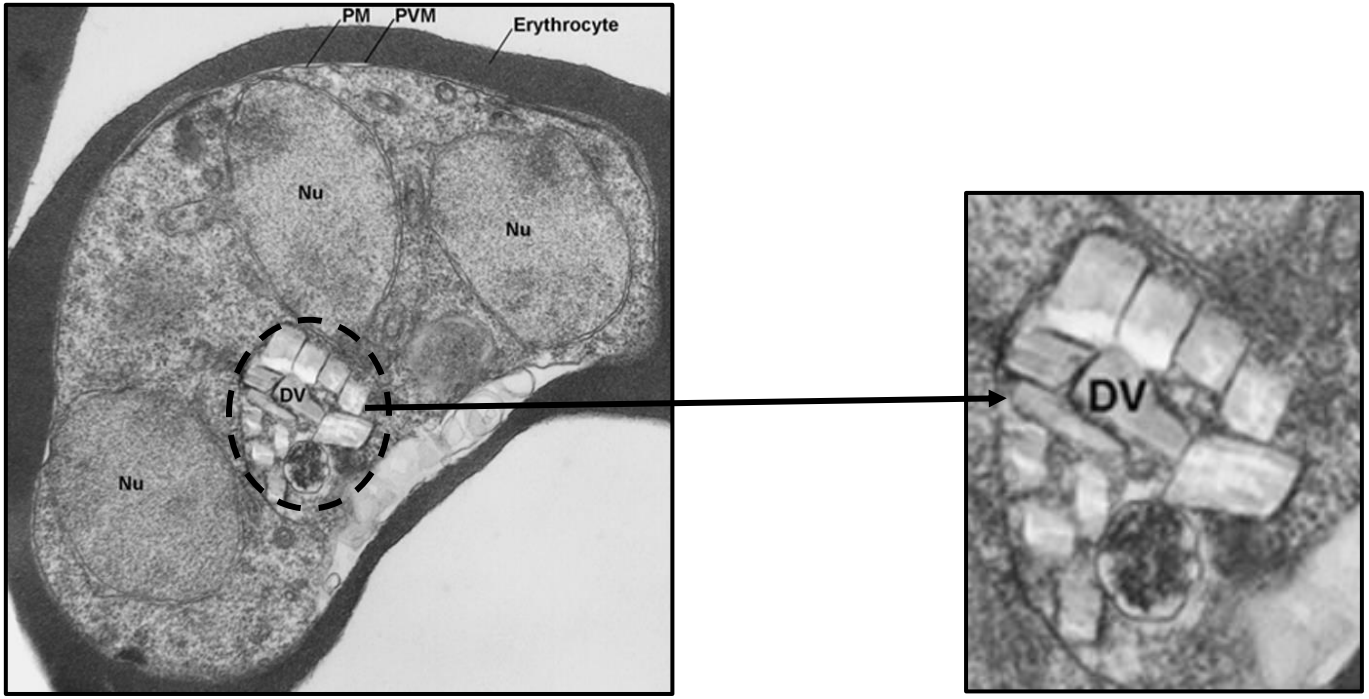

Figure 2.3: Transmission electron micrographs of the digestive vacuole of *P. falciparum*

The Zeiss EM10 transmission electron microscope was used to capture images of *P. falciparum* in its early schizont stage. Haemozoin crystals (Hz) were prevalent in the parasite's digestive vacuole. Nu stands for nucleus; DV for digestive vacuole; PM for parasite plasma membrane; and PVM for parasitophorous vacuole membrane. Adapted from Wunderlich et al., 2012.

2.7 Antimalarial drugs and vaccines

The antimalarial drugs and vaccines are significant initiatives in the global strategy to combat malaria. According to Cui & Su (2009) and Kakolwa et al. (2018), fast-acting medications, known as artemisinin-based combination treatments (ACTs) decreased the global death rate of malaria. However, the therapeutic efficacy of artemisinin and its derivatives dropped dramatically, which has been linked to Kelch13 (K13) mutations and a high rate of parasite recurrence caused by four other genes (i.e. arps10, fd, mdr2 and crt) (Birnbaum et al., 2020; Muller et al., 2019; Nima et al., 2022). Furthermore, the effectiveness of ACTs is in jeopardy due to resistance to partner drugs, including piperaquine and lumefantrine (Hamilton et al., 2019; Okell et al., 2018).

The complexity of the life cycle of the malaria parasite and the lack of understanding of how the host immune system responds make it challenging to develop the malaria vaccine. It took more than 30 years for RTS,S/AS01, the only candidate suggested by the WHO, to receive regulatory approval and begin pilot implementation studies. Approximately 30% less hospitalised cases of severe malaria were reported in Ghana, Malawi and Kenya when the vaccine was introduced through routine vaccination programmes in large WHO-coordinated pilot deployments (Hamilton et al., 2023). This vaccine, however, does have significant drawbacks, despite its promising nature. The effectiveness of the vaccine is temporary and weak. For children between the ages of 5 and 17 months, the effectiveness was 55% throughout the first 12 months following the three doses of the main immunisation. It is advised to have a booster dosage after 18 months following the completion of the original vaccination due to the rapid waning of protective immunity. Over four years in the Phase 3 trial, the RTS,S vaccine

only provided 36% vaccination efficacy against symptomatic malaria and 29% efficacy against severe malaria when a booster dose was administered. Due to the limited efficacy, this vaccine is only recommended for children at age 5 or 6 months for the first dose and at 2 years old for booster administration (Beeson et al., 2022). Given the escalating issue of drug resistance and the limitations of the present vaccine, it is apparent that alternative treatments such as the hunt for novel antimalarial drugs must be explored.

2.8 Therapeutic peptide compounds

The study of therapeutic peptides began with basic research on human hormones such as insulin, oxytocin, vasopressin and gonadotropin-releasing hormone (GnRH), and their distinct physiological actions in the human body. These peptides are a unique type of pharmaceutical agent made up of a sequence of well-ordered amino acids, typically having molecular weights of 500-5 000 Da. The early part of the 20th century witnessed the discovery of several bioactive peptides, including insulin and adrenocorticotrophic hormone, which were initially researched and isolated in nature. The discovery and development of insulin, a 51-amino acid peptide, has been regarded as one of the most significant scientific triumphs in medication research. It was first isolated by Frederick Banting in 1921, then further refined by Frederick and Charles Best, and was available to diabetic patients a year later (Wang et al., 2022).

Antimicrobial peptides (AMPs), referred to as host defence peptides, are short, mostly positively charged peptides that are present in a broad range of species, including humans and microbes. The majority of AMPs can directly destroy microbial infections, whereas some work indirectly by altering the host's defence mechanisms. The

effort to introduce AMPs into clinical practice is moving more quickly against a rapidly rising resistance to traditional antibiotics worldwide (Mahlapuu et al., 2016). One of the key components of antimicrobial peptides is amino acid composition. The specific arrangement and kinds of amino acids in a peptide are critical to its antimicrobial properties. Positively charged amino acids such as lysine (Lys) and arginine (Arg), increase the peptide's capacity to interact with negatively charged bacterial membranes, allowing for membrane breakdown (Torres et al., 2018). Several AMPs derived from natural sources showed promising antimalarial activity such as cecropins, which are produced by insects. An-cecB peptide, derived from *Anopheles* mosquito showed significant antimalarial activity against *P. falciparum* strain with an IC_{50} value of 12.4 µM. However, due to its cytotoxicity to mammalian cells, the therapeutic application is limited (Lou et al., 2024).

2.8.1 Pipecolisporin

Pipecolisporin is a novel cyclic hexapeptide derived from the endophytic fungus, *Nigrospora oryzae*. This compound was found during research aimed at identifying new antiparasitic drugs from microbial sources, particularly those that may efficiently target *P. falciparum*. Reversed-phase high-performance liquid chromatography (HPLC) is one of several chromatographic procedures used to extract the chemical as a white amorphous solid. Nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS) are used to determine the structure of pipecolisporin, confirming its chemical formula, $C_{37}H_{53}N_7O_6$. The specific structure of the amino acids in pipecolisporin is Ile-βAla-Trp-Pip-Leu-Pro (Figure 2.4), which is essential for both its general antimalarial activity and its interaction with

biological membranes (Fernández-Pastor et al., 2021; Kurniaty et al., 2023). According to Fernández-Pastor et al. (2021), with an IC_{50} value of 3.21 μM, pipecolisporin has notable activity against *P. falciparum,* suggesting that it may be advantageous for the treatment of malaria.

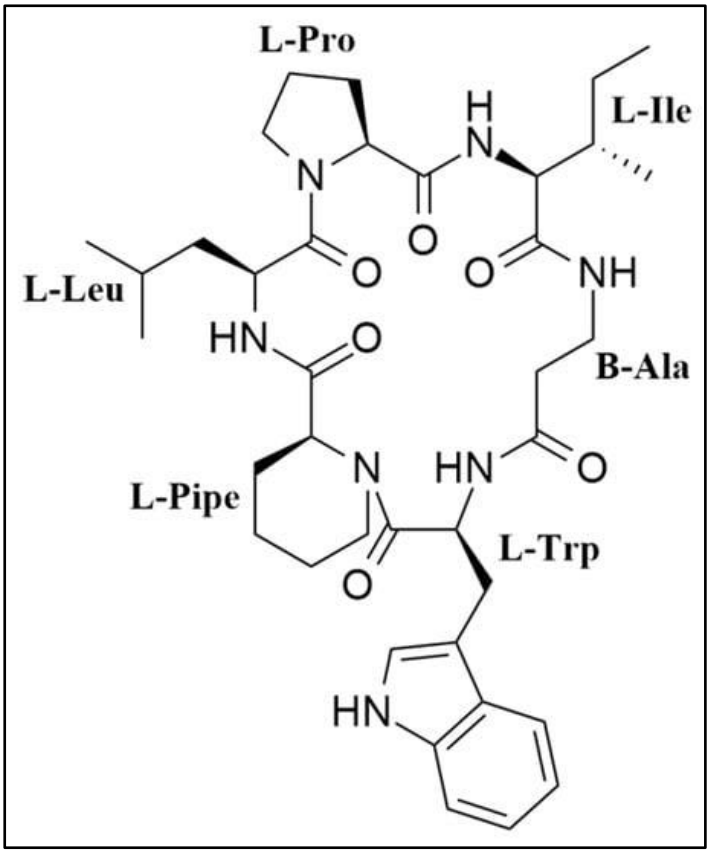

Figure 2.4: Molecular structure of pipecolisporin

The molecular formula of pipecolisporin is $C_{37}H_{53}N_7O_6$ with an amino acid sequence of Ile-βAla-Trp-Pip-Leu-Pro.

2.8.2 Analogue II

Analogue II is a novel peptide compound that is still being reviewed as a new candidate for a new antimalarial agent. Analogue II is synthesised based on pipecolisporin with a protein sequence of Ile-βAla-Trp-Pip-Lys-Pro. As the amino acid sequence has a similarity with the pipecolisporin peptide, the antimalarial activity of this analogue II is worth to be studied.

2.9 FITC-dextran

Maintaining an optimal acidic pH is crucial for the normal activity of various enzymes, hence certain antimalarial drugs have targeted it. Innovative techniques have been used to examine the significance of intracellular pH in biological processes, including dextran conjugates. The are numerous variations of pH-sensitive probes conjugated to dextran on the market including, FITC, Oregan Green, and LysoSensor.

Previous researchers have used dextran-labelled fluorescein isothiocyanate (FITC) probes for quantitative pH assessment within parasites (Bakar, 2015; Ibrahim & Abu Bakar, 2019; Muchtar et al., 2022). Fluorescein isothiocyanate (FITC)–dextrans is available in a range of molecular weights, from 3-2,000 kDa (Hoffmann et al., 2011). The pH of the particular cellular compartment containing FITC-dextran can be estimated by dividing the fluorescence intensities at these two emission wavelengths. Quantitative analysis with fluorescent probes that only have one emission feature is challenging because a variety of factors will interfere with accurate analysis. Ratiometric fluorescent probes, on the other hand, are induced by analytes and will significantly increase the

sensitivity and selectivity of detection (He et al., 2024; Yue et al., 2024). Furthermore, because ratiometric fluorescence self-calibrates against background signal interference, it increases the precision and dependability of sensing detection (Liu et al., 2022).

This probe can be used to measure the pH of the digestive vacuole during the trophozoite stage of the malaria parasites. The trophozoite stage, which has a unique endocytic structure known as cytostomes, is the most active stage during the uptake of host cytoplasm. Following the consumption of FITC-dextran and haemoglobin, vesicles formed from the cytostome and moved to and fused with the acidic digestive vacuole (Bakar et al., 2010). Following that, the use of flow cytometry with FITC-dextran provides an efficient method for measuring pH within the digestive vacuole. Flow cytometry is a suitable technology for precise pH measurements since it can swiftly process and evaluate a large population of mature stage parasites. As a result, the present work used a flow cytometry-based approach with FITC-dextran to evaluate the pH of the malaria parasite's digestive vacuole treated with pipecolisporin and analogue II peptide compounds.

CHAPTER 3

MATERIALS AND METHODS

3.1 General reagents and equipment

All chemicals, reagents, equipment and software used in this study are listed in Table 3.1, 3.2 and 3.3 respectively.

Table 3.1: List of chemicals and reagents

Chemical and reagent	Manufacturer
Adenosine 5'-triphosphate (ATP)	Vivantis. Malaysia
Albumax II	Gibco Laboratories, USA
Artemisinin	Sigma-Aldrich, USA
Carbonyl cyanide m-chlorophenyl hydrazone	Sigma-Aldrich, USA
Dimethyl sulfoxide (DMSO)	Vivantis, Malaysia
D-glucose	Sigma-Aldrich, USA
D-sorbitol	Sigma-Aldrich, USA
Fluorescein isothiocyanate-dextran (FITC-dextran)	Thermo Fischer Scientific, USA
Gentamicin	Duopharma, Singapore
Giemsa stain solution	Sigma-Aldrich, USA

4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES)	Sigma-Aldrich, USA
Hypoxanthine	Sigma-Aldrich, USA
Phosphate buffer saline (PBS) solution (1x)	Sigma-Aldrich, USA
Roswell Park Memorial Institute (RPMI) 1640 Medium	Gibco Laboratories, USA
Sapogenin	Sigma-Aldrich, USA
Sodium dihydrogen phosphate monohydrate (NaH_2PO_4)	Sigma-Aldrich, USA