IDENTIFICATION OF ANTIMALARIAL COMPOUNDS AND THEIR MODE OF ACTIONS

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IDENTIFICATION OF ANTIMALARIAL COMPOUNDS AND THEIR MODE OF ACTIONS

by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SCHEMATIC DIAGRAMS

Page

LIST OF FORMULAS

Page

LIST OF SYMBOLS

α	Alpha
Å	Angstrom
β	Beta

LIST OF ABBREVIATIONS

ACT	Artemisinin Combination Therapy
AD	Anno Domini
AGC	PKs A, G and C
ANOVA	Analysis of Variance
ART	Artemisinin
ATO	Atovaquone
ATP	Adenosine triphosphate
BC	Before Christ
BSA	Bovine serum albumin
CaMK	Calmodulin-dependent protein kinase
CBRG	Chemical Biology Research Group
CC	Column chromatography
CCD	Central Composite Design
CK1	Casein kinase 1
CMGC	CDK, MAPK, GSK3, CK2
CPC	Centrifugal Partition Chromatography
CQ	Chloroquine
DBP	Dibutyl phthalates
DHLC	Dihydrolucilactaene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DV	Digestive vacuole
EE	Exoerythrocytic schizogony

eIF2	Eukaryotic initiation factor 2
ePK	Eukaryotes protein kinase
FDA	Food and Drug Administration
FIKK	Phe-Ile-Lys-Lys motif
FSSA	Fakulti Sains dan Sumber Alam
GSK-3	Glycogen Synthase Kinase-3
HPLC	High-performance Liquid Chromatography
HR-ESITOF-MS	High-Resolution electro ionization time-of-flight mass spectrometry
Hs	*Homo sapiens*
iHOPE	in-house-Phenotypic-Evaluation
INFORMM	Institute for Research in Molecular Medicine
IR	Infection rate
LLE	Liquid-liquid extraction
MoA	Mode of action
NMA	Never in mitosis gene
NMR	Nuclear magnetic resonance
OB	Oatmeal broth
OD	Optical density
Pb	*Plasmodium berghei*
PCR	Polymerase chain reaction
PE	Pre-erythrocytic cycle
Pf	*Plasmodium falciparum*
Pf PK	*Plasmodium falciparum* protein kinase

pLDH	*Plasmodium* lactate dehydrogenase
PV	Parasitophorous vacuole
RBC	Red blood cells
RIKEN	Rikagaku Kenkyusho
RSM	Response surface methodology
SAR	Structure-Activity Relationship
SEA	Southeast Asia
SNP	Single nucleotide polymorphism
STE	“Sterile-phenotype” kinases
TKL	Tyrosine kinase-like kinases
TLC	Thin-layer chromatography
UKM	Universiti Kebangsaan Malaysia
UMS	Universiti Malaysia Sabah
UPLC-MS	Ultra-performance Liquid Chromatography-Mass Spectrometry
USM	Universiti Sains Malaysia
WHO	World Health Organization
ZOI	Zone of inhibition

LIST OF APPENDICES

PENGENALPASTIAN SEBATIAN ANTIMALARIA DAN MOD TINDAKANNYA

ABSTRAK

Malaria merupakan salah satu penyakit berjangkit yang paling signifikan di kawasan tropika yang telah menyebabkan hampir setengah juta kematian setiap tahun, terutamanya disebabkan oleh jangkitan *Plasmodium falciparum* (*P. falciparum*). Sehingga kini, *P. falciparum* telah membangunkan rintangan penuh atau separa terhadap ketiga-tiga kelas antimalaria, termasuk artemisinin. Maka, ia boleh menjejaskan keberkesanan Terapi Gabungan Artemisinin (ART) pada masa hadapan. Oleh itu, terdapat keperluan untuk mengenal pasti sebatian antimalaria dengan mod tindakan baharu. Matlamat utama kajian ini adalah untuk mengenal pasti sebatian antimalaria baharu daripada mikroorganisma tanah yang diisolasi daripada Malaysia dan Jepun. Ekstrak mentah strain-strain ini telah menunjukkan aktiviti antimalaria yang poten pada saringan awal terhadap strain *Pf* 3D7 (jenis liar). Ekstrak mentah strain daripada Malaysia juga telah merencatkan aktiviti GSK-3β manusia (*Hs* GSK-3β) (75 % serupa dengan GSK-3 *P. falciparum* (*Pf* GSK-3)). Maka, terdapat potensi untuk mengenal pasti sebatian antimalaria yang merencatkan aktiviti *Pf* GSK-3, iaitu mod tindakan baharu. Selain itu, tiga sebatian tulen baharu yang disimpan di

perpustakaan kimia RIKEN juga telah dinilai dalam ujian kebolehulangan aktiviti antimalaria untuk memilih sebatian yang paling poten sebelum penentuan mod tindakan. Secara keseluruhannya, 18 sebatian antimalaria telah dikenal pasti/dicadangkan antaranya dibutil phthalates (DBP) (IC_{50} = 14.4 μM), nataxazole (agen antimalaria baharu, IC_{50} = 14.7 μM) dan asid sekalonik F (IC_{50} = 4.1 μM) (daripada strain Malaysia) dan hibarimisin C (agen antimalaria baharu, IC_{50} = ~0.02 μM) (daripada strain Jepun). Kajian *in silico* telah menunjukkan bahawa DBP, nataxazole, dan asid sekalonik F telah membentuk ikatan hidrogen dengan residu asid amino penting pada *Hs* GSK-3β (masing-masing dengan Arg87, Lys85, dan Asp200,) yang juga terdapat pada *Pf* GSK-3 (masing-masing sebagai Arg119, Lys108, dan Asp225). Oleh itu, aktiviti antimalaria sebatian-sebatian ini berkemungkinan hasil daripada perencatan aktiviti *Pf* GSK-3. Sementara itu, dihydrolucilactaene (DHLC, sebatian baharu daripada *Fusarium* sp. 97-94 yang telah didepositkan ke perpustakaan kimia RIKEN) telah menunjukkan aktiviti antimalaria ultrapoten (IC50 = 1.5 nM). Untuk memahami mod tindakan DHLC, profil aktiviti DHLC semasa jangkitan sel darah merah telah ditentukan berdasarkan ujian pendedahan/pembasuhan DHLC pada masa-masa tertentu. DHLC mempunyai profil aktiviti antimalaria yang berbeza (tropozoit > skizon > cincin) dengan klorokuin dan artemisinin, maka cara mod tindakan yang berbeza. DHLC juga melambatkan perkembangan parasit semasa

jangkitan sel darah merah. *P. Falciparum* yang rentang terhadap DHLC telah berjaya dihasilkan melalui pendedahan kepada DHLC secara berterusan. Penjujukan genom keseluruhan menunjukkan gen ditandakan sebagai “gen #6” merupakan satu-satunya gen yang bermutasi dalam genom strain rentang terhadap DHLC. Gen #6 mengenkodkan protein transmembran yang belum diketahui. Maka, protein ini merupakan sasaran baharu untuk pembangunan agen antimalaria. Hubungan struktur-aktiviti menunjukkan bahawa pembukaan cincin tetrahydrofuran dan penyingkiran kumpulan epoksida bertanggungjawab untuk aktiviti antimalaria DHLC. Pengenalpastian DHLC boleh menyediakan agen antimalaria baharu untuk memerangi penyebaran strain rentang antimalaria yang meluas.

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ABSTRACT

Malaria is one of the most significant infectious diseases in the tropics, claiming around half-million lives annually, mainly due to *Plasmodium falciparum* (*P. falciparum*) infections. To date, *P. falciparum* has developed full or partial resistance towards all three classes of antimalarials, including against the artemisinin. Hence, it may jeopardize the efficacy of the current antimalarial treatment regimen, the Artemisinin Combination Therapy (ACT), in the near future. Therefore, there is an utmost need to identify a new antimalarial compound with a novel mode of action (MoA). This study mainly focused on identifying antimalarial compounds from soil microorganisms isolated from Malaysia and Japan. The crude extracts of these strains showed potent antimalarial activity in a preliminary assay against *Pf* 3D7 (wild type). The crude extracts of Malaysian strains also inhibited the activity of human GSK-3β (75 % similar to *P. Falciparum* GSK-3 (*Pf* GSK-3)). Therefore, there is a potential to identify antimalarial activity mediated by *Pf* GSK-3 inhibition, which is as a new antimalarial MoA. In addition, three new pure compounds deposited in RIKEN chemical library were also evaluated in an antimalarial reproducibility test, to select

the most potent compound(s) for MoA determination. Overall, 18 antimalarials were identified/suggested which includes (DBP) (*Pf* 3D7 IC_{50} = 14.4 μM), nataxazole (new antimalarial agent, *Pf* 3D7 IC_{50} = 14.7 μM) and secalonic acid F (*Pf* 3D7 IC_{50} = 4.1 μM) (from Malaysian strain) and koranimine (IC_{50} = ~0.01 μM) (from Japanese strain). Interestingly, *in silico* study indicates that these compounds formed hydrogen bonds with important amino acid residues on *Hs* GSK-3β (Arg87, Lys85, and Asp200, respectively) that are conserved in *Pf* GSK-3 (Arg119, Lys108, and Asp225, respectively). Hence, DBP, nataxazole, and secalonic acid F may exert antimalarial activity by inhibiting *Pf* GSK-3. Meanwhile, dihydrolucilactaene (DHLC, a new compound isolated from *Fusarium* sp. 97-94 that was deposited in RIKEN chemical library) was shown to exert an ultrapotent antimalarial activity (IC_{50} = 1.5 nM). To understand the MoA of DHLC, its erythrocytic stage-specific activity profile was investigated based on a time-specific drug exposure/washout assay against a sorbitol-synchronized *Pf* 3D7 culture. DHLC was indicated to exert a different stage-specific profile (trophozoites > schizont > ring) than chloroquine and artemisinin, hence a different MoA. DHLC was also shown to delay parasite egression during RBC stage infection. A DHLC-resistant strain was successfully generated via continuous drug exposure. Whole-genome sequencing revealed a gene denoted “gene #6” as the only mutated gene in the genome of DHLC-resistant strain. Gene #6 was reported to encode

an unknown conserved *Plasmodium* transmembrane protein. This protein was never reported as the molecular target of any known antimalarials, hence a novel target for antimalarial drug development. Structural-activity relationship revealed that the opening of the tetrahydrofuran ring and the absence of the epoxide group in DHLC were responsible for its antimalarial activity. The identification of DHLC may provide a new drug regimen to combat the widespread of drug-resistant strains.

CHAPTER 1

INTRODUCTION

1.1 Malaria: a brief background

Malaria is a prominent mosquito-borne (*Anopheles* sp.) disease in the tropics (Lee et al., 2020; Li et al., 2020). In 2020, around 241 million malaria cases were reported in 85 malaria-endemic countries, with 627,000 deaths, a slight increase from the previous year (World Health Organization, 2021). Most deaths were recorded in Africa (96 %), especially children under five years old and pregnant women, contributed by their more compromised immunological and hormonal responses than the general adult population (Chua et al., 2021; World Health Organization, 2021).

In humans, malaria is caused by 9 *Plasmodium* parasites species belonging to apicomplexan protozoa, that require two hosts to survive, mosquito for transmission and sexual stage (in the midgut), and vertebrate (divided into liver and blood asexual stage). *Plasmodium falciparum* (*P. falciparum*) is the most prevalent and virulent species (Ansari et al., 2016) since complications of severe malaria such as cerebral malaria, anemia, acute renal failure, pulmonary edema, and bleeding are almost entirely caused by this species. These symptoms may rapidly lead to coma and death within hours or days after manifestation (World Health Organization, 2000).

Over the past decade, *P. falciparum* continues to be the most prevalent and virulent case of malaria, followed by *P. vivax* (Amambua-Ngwa et al., 2019; World Health Organization, 2021). Meanwhile, mild or uncomplicated yet persistent malaria cases are caused by *P. malariae* (Bartoloni & Zammarchi, 2012). *P. ovale curtisi* and *P. ovale wallikeri* (closely related but distinct malaria parasite species) received the least attention as it is considered uncommon and only causes mild infection. Although *P. ovale* infection can be easily treated with chloroquine, it can evolve into a more severe or fatal strain (Mueller et al., 2007; Lau et al., 2013). In recent years, *P. knowlesi* (a zoonotic *Plasmodium*) has been the prominent cause of malaria in Southeast Asia (SEA), especially in the east of Malaysia (Zaw & Lin, 2019; Cooper et al., 2020). Moreover, possible infection from the other zoonotic *Plasmodium* species (*P. inui*, *P. cynomolgi* and *P. coatneyi*) has been reported in Malaysia (Yap et al., 2021).

1.2 Problem statement

1.2.1 Drug resistant development

SEA continues to be the hotspot for the emergence of strains resistant against frontline antimalarial starting with CQ (1957) and sulphadoxine-pyrimethamine resistance strains (in the 1960s) (Trape, 2001; Roper et al., 2004; Singh & Sharma, 2016; Bushman et al., 2018). The resistance emergence is mainly driven by poor quality antimalarials widely used in SEA (Dondorp et al., 2004; Nayyar et al., 2012).

More than 40 % of antimalarials sold in SEA are either did not contain any active ingredients or contained <10 % of the active compounds (Dondorp et al., 2004). The subtherapeutic dosage increases the risk of resistance emergence (prolonged drug exposure), which eventually favors the selection/spreading of the resistance strains (Newton et al., 2006).

As a consequence, the ART-resistant strains have emerged in SEA since 2009 and recently in Africa (2019) (may also be due high use of counterfeit drugs) (Nayyar et al., 2012; Boddey, 2017; Tahghighi et al., 2020; Balikagala et al., 2021). Its emergence in Africa will likely cause a malaria outbreak if proactive actions to curb the spreading are not taken since ART is the final effective frontline antimalarial drug (Ouji et al., 2018; World Health Organization, 2021).

1.2.2 Low antimalarial drug diversity

Although natural and synthetic antimalarials were introduced in the late 1800 and 1940s, only a small number of compounds reached the clinical stage (Grimberg & Mehlotra, 2011). Moreover, these compounds belong to only three broad classes; aryl amino alcohol compounds, antifolate compounds (Luzzatto, 2010), and ART compounds (White, 2008; Patrick, 2020). Since *P. falciparum* has developed resistance phenotypes against all classes of antimalarials, new drug classes that exert

a different mode of action (MoA) than the existing drug regimen need to be explored for effective treatment (Flannery et al., 2013; Burrows et al., 2017).

1.2.3 Antimalarials with unknown MoA

Determining the exact target of a drug is valuable knowledge as it can prevent late-stage failures that increase the chances of drug approval. In general, information on the actual target leads to better dosing, monitoring potential side effects of a drug, and stratifying better clinical trials on suitable patients. Hence, many promising drugs failed at an early stage trial due to cytotoxic activity, considering a wide array of cell signaling processes, enzymes, and isoforms functioning in the human system, (Medina-Franco et al., 2013; Peters, 2013).

Similarly, most frontline antimalarials were developed without knowing their target since antimalarial therapeutic potency was primarily identified via cell-based assay (Fidock et al., 2004; Schlitzer & Ortmann, 2010). As a result, it is difficult to establish hit optimization due to pharmacokinetics or toxicological issues (Hallyburton et al., 2017). For example, primaquine is the only clinically approved antimalarial that can eliminate hypnozoite to date. However, since primaquine's target is unknown, further studies have been limited only to related analogs such as tafenoquine (Vale et al., 2009). Therefore, a drug with a known MoA is preferable for the next generation of antimalarials.

1.3 Strategies to solve malaria-associated problems

1.3.1 Drug discovery from nature

Natural products are genetically encoded in the genome of their producer, resulting from the evolution via natural selection. The products are usually beneficial to the producer as they are produced to engage with their respective biological targets. Hence, natural products are naturally equipped with chemical features required for biological activity, such as higher counts of chiral centers, sp^3-hybridized carbon and oxygen content, low nitrogen cod aromatic rings, and larger macrocyclic aliphatic rings. These features create complex 3-D structures and are stereochemically superior to synthetic compounds to effectively interact with biological components (Rodrigues et al., 2016).

Plant natural products are a rich source of prolific antimalarial compounds with unique structures. The first antimalarial was quinine, isolated from cinchona bark (*Cinchona cordifolia*) in 1820 and fully synthesized in 1854. Quinine remained in the frontline regimen until replaced by CQ in the 1940s. A few decades later, ART was identified from sweet wormwood (*Artemisia annua*) as another plant-based antimalarial. ART bears a sesquiterpene lactone with an endoperoxide group, a unique natural product structure that is responsible for ART antimalarial activity (Dagen, 2020). Although numerous experimental antimalarials were continuously reported

from plants such as 34,5-tri-*O*-caffeoylquinic acid (Nugraha et al., 2022) (*Pluchea indica*) and 8ß,13ß-kaur-15-en-17-al (*Podocarpus polystachyus*) (Amir Rawa et al., 2022), in recent years, no plant-based antimalarials even reached preclinical tests (Saha et al., 2022).

One of the significant problems with plant natural products is the low production of active compounds (including artemisinin) since external factors such as sampling location and environmental factors may influence the recovery rate of the natural products. Although synthesis of the active compound could solve the problem, unfortunately, not every compound can be produced via total synthesis (Katiyar et al., 2012). To overcome this problem, larger quantities of samples are needed to acquire enough pure compounds, which will increase the production cost (Habibi et al., 2019). Often, this problem cause plant-based antimalarials to be economically impractical for development and commercialization. It will also provide a market for counterfeit drugs, especially in less-developed regions (Karunamoorthi, 2014).

Since the discovery of microorganisms as a prolific source of active compounds in the 1920s, microorganism-derived compounds have been used extensively in biopharmaceutical sectors, scientific research, food industry, and agriculture (Sanchez-Garcia et al., 2016; Pham et al., 2019). The ability to culture them under controlled parameters or to be engineered to activate silent genes in the

laboratory gives microorganisms an edge over plants for the mass production of new active compounds (Song et al., 2015; Pérez-Moreno et al., 2016; Matsumura et al., 2018; Wright, 2019). Therefore, microbial natural products are an excellent source to search for new antimalarials.

1.3.2 Targeting host enzymes

Antimalarials targeting host enzymes are an attractive therapeutic strategy to prevent resistant development. It is because, unlike viruses, parasites cannot mutate enzymes encoded in the host genome since no genome integration occurs between *Plasmodium* and humans during their life cycle (Fidock et al., 2004; Kesely et al., 2016). For instance, Band 3 (AE1, SLC4A1, anion transporter) and ankyrin are predominant erythrocyte membranes. It maintains red blood cell (RBC) integrity via connecting the cell membrane to its cytoskeleton (Anong et al., 2009; van den Akker et al., 2010).

Tyrosine phosphorylation of band 3 causes detachment of ankyrin, destabilizing the RBC membrane and leading to hemolysis (Ferru et al., 2011; Pantaleo et al., 2011). Interestingly, *P. falciparum* infection stimulates a significant increase in band 3 tyrosine phosphorylation despite the absence of tyrosine kinase in *P. falciparum* kinome (Pantaleo et al., 2010). It indicates that *P. falciparum* utilizes host

tyrosine kinase (activating) to facilitate its egression from ring to trophozoites and schizont. The weakened cell membrane finally ruptured towards the end, releasing merozoites and infecting new RBC (Kesely et al., 2016).

Through drug repurposing (a process of finding new medical uses for existing drugs/compounds), imatinib (Food and Drug Administration (FDA)-approved tyrosine kinase inhibitor to treat cancer) was identified as a potent antimalarial that reached phase II in a clinical trial. Imatinib inhibits erythrocyte tyrosine kinase activity that causes parasite entrapment and termination (Talevi & Bellera, 2020; Chien et al., 2021). Hence, targeting host enzymes paired with drug repurposing is a powerful approach moving forward in antimalarial development. It offers a lower failure rate as the repurposed drug has already been tested for human safety (Pushpakom et al., 2019).

1.3.3 Targeting essential cell components for *Plasmodium* survival

A recent PiggyBac mutagenesis analysis uncovers numerous cell components that can be targeted to develop antimalarial agents against *P. falciparum.* The AT-richness of the *P. falciparum* genome was exploited to generate over 35,000 *P. falciparum* mutants. These mutagenesis lead to the identification of essential genes (non-mutable genes). Eventually, almost 3,500 essential genes were identified for *P.*

falciparum in vitro asexual blood-stage growth (Zhang et al., 2018). Hence, numerous unexplored malarial parasite cell components can be targeted for drug development.

1.4 The rationale of the study

In this study, antimalarial compounds from microbial sources were explored, and the MoA of the most potent compound(s) was determined as an effort to identify new malaria treatment that may curb the spreading of drug-resistant *P, falciparum*. It may prevent major malaria breakout and enrich our knowledge of the parasite's biology (Weiwer et al., 2010; World Health Organization, 2021).

1.4.1 Soil actinomycetes, an inexhaustible source for bioactive compounds

As of 2020, around 75,000 bioactive compounds originated from natural resources were identified, by which microorganisms (actinobacteria/actinomycetes, fungi, and eubacteria) are the major contributor with 34,000 (46 %) of bioactive compounds, followed by plants (higher and lower plants) (31,000), marine invertebrates (10,000) and terrestrial animals (1,000). Soil actinomycetes are known as an inexhaustible source of bioactive compounds. Out of ~2000 genera, only *Streptomyces* is well studied (easy to be cultured in the laboratory) for drug discovery as 60 % of bioactive compounds from actinobacteria were identified from this genus

alone. Hence, more bioactive compounds are to be discovered from the underexplored genus or the “rare actinomycetes” (Ding et al., 2019).

Soil microorganisms, significantly contribute to natural drugs due to their high therapeutic index and significant pharmacological activities (Adegboye & Babalola, 2013). In addition, compounds from soil microorganisms are an excellent natural product source of antimalarial agents due to; 1) their sizeable structural diversity, and 2) they are still not widely explored for the discovery of antimalarial drugs (Pérez-Moreno et al., 2016). The type of forests was suggested to determine soil actinomycetes distribution and the variety of secondary metabolites produced since their growth are influenced by organic materials mainly from plants (Hackl et al., 2005; Ghorbani-Nasrabadi et al., 2013; Tripathi et al., 2016; Law et al., 2019; Mahmud et al., 2022).

Tropical forests are regarded as the best sampling location for drug discovery, hence known as the “world’s largest pharmacy” (Guariguata et al., 1998; Temu, 2015). As part of Borneo Island, Sabah is one of the twelve mega biodiversity hotspots globally. More than ten forest types can be found in Sabah, which covers approximately 60 % of its landmass (Kulip et al., 2010; Payne, 2011; Yen et al., 2021). In general, 15,000 plant species were recorded in Borneo, of which 34 % are endemic species (WWF, 2022). Hence, the existence of diverse species of flora and fauna in

Sabah, Malaysia, may lead to the isolation of novel strains and bioactive compounds resulting from soil actinomycetes-plant interaction.

In a previous study, more than 1000 culturable actinomycetes strains were isolated from different forest types of Sabah. Some of these strains showed potent activities against human glycogen synthase kinase-3 (*Hs* GSK-3β) and malaria parasite, *P. falciparum* 3D7 strain (*Pf* 3D7, wild type (WT)) (Mahmud et al., 2022). The most potent strains were selected to identify active compounds in this study. To further diversify the tested scaffolds, microbial strains isolated from Japanese soils and synthesized compounds deposited at the Chemical Biology Research Group (CBRG) at Rikagaku Kenkyusho (RIKEN) microbial and chemical libraries were evaluated for their antimalarial activities. In addition, a series of new lucilactaene derivatives, a biosynthesis product of *Fusarium* sp. 97-94, was also tested for their activity against *Pf* 3D7. Lucilactaene is an interesting antimalarial drug lead that was previously reported with potent antimalarial activity (Kato et al., 2020).

1.4.2 *Hs* GSK-3β inhibitor may lead to identifying new antimalarial compounds, a target-based approach.

Previously, a yeast-based assay system utilizing a mutant yeast strain expressing *Hs* GSK-3β was applied to screen for *Hs* GSK-3β inhibitors from soil microorganisms and plant extracts of Sabah (Cheenpracha et al., 2009; Ho et al., 2009).

It was shown that most of the active extracts also exert potent antimalarial activity with no cytotoxic effect against Chang's liver cells. Notably, these samples also were indicated to inhibit host GSK-3 activity of *P. berghei* infected mice in the liver (Dahari et al., 2016). Hence, dual inhibition of host and parasite GSK-3 is an exciting approach in malaria treatment.

Based on these premises, the potential of using mutant yeast expressing *Hs* GSK-3β as a preliminary assay to narrow down the sample for the antimalarial activity was further evaluated. The crude extracts that showed potent inhibitory activity against *Hs* GSK-3β and *Pf* 3D7 were subsequently fractionated to identify the active compounds.

1.4.3 Spontaneous drug resistance development, an effective strategy to identify the antimalarial molecular target

The development of drug resistance ability in *Plasmodium* has been described as unique compared with other infectious diseases. They can induce exact resistance in the cellular target of the antimalarial drugs instead of random mutations (Goldberg et al., 2012). Although the precise mechanisms are still not clearly defined, resistance in *Plasmodium* has a genetic basis (Sibley, 2015). Hence, the molecular target of antimalarials can be identified via generating drug-resistance parasites in the laboratory, then comparing the genomes of the resistant clones to the WT (Meister et al., 2011). This method has led to the identification of numerous novel targets,

including *Pf* ATP4 and *Pf* CARL (Crowther et al., 2011). In this study, the spontaneous mutation approach was applied via continuous drug exposure; a) to identify the MoA/molecular target of compounds identified/deposited in RIKEN and b) to confirm if active compounds identified from the target-based approach can be confirmed via the generation of mutant *Pf* GSK-3.

1.5 Research objectives

1.5.1 General objective

The study aimed to the assess antimalarial potential of soil microorganisms and to explore new chemical scaffolds, possibly with a different mode of action than the current antimalarial frontline.

1.5.2 Specific objectives

i) To evaluate the antimalarial activity of crude extracts previously identified as *Hs* GSK-3β inhibitors via *in vivo* yeast-based assay from Malaysian soil microorganisms.

ii) To identify antimalarial compounds produced by Malaysian and Japanese soil microorganisms.

iii) To determine the antimalarial MoA of the most potent antimalarial compounds in this study.

iv) To predict the importance of the antimalarial molecular target of the most potent antimalarial compound in this study via stage-specific activity evaluation.

v) To establish the structure-activity relationship of the most potent antimalarials identified in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Brief historical data on malaria

Malaria is an ancient infectious disease that remains endemic in the tropics until today. The term “malaria” was derived from medieval Italian words “mal (bad)” and “aria (air)” due to the connection of this disease with the swamp area in Italy. However, the origin of this disease can be traced back to Egyptian papyrus (3,500 BC), The Chinese Canon of Medicine, *Nei Ching* (3,000 BC), ancient Indian sculptures (1,000 BC), and Cuneiform tablet in Mesopotamia (500 BC), all describing fever associated with rigors and enlarged spleen, certainly attributed to malaria (Bruce-Chwatt, 1988). Meanwhile, malaria spread into Europe, Scandinavia, and Britain during the Neolithic Period (~4,000 BC) via the Nile Valley (Carter & Mendis, 2002; Suh et al., 2004; White, et al., 2014; Piperaki & Daikos, 2016; Dagen, 2020).

A more detailed description of malaria was recorded by Celsus in 1 AD (a Roman writer), who described three types of malaria fever in *De Medicina* as quartan, tertian, and lethal semitertian fevers. These malarial fevers are now known to be inflicted by *P. malariae*, *P. vivax,* and *P. falciparum*, respectively (Cox, 2002). However, the *Plasmodium* parasites were not identified until almost 2,000 years later. Giovanni Lancisi was one of the first to suggest the relation between swamp areas and

mosquitoes with malaria in 1716 (Italy) as the draining of the swamp area reduced malaria cases and suggested that mosquitoes spread the disease. Lancisi also performed a postmortem study on the body of a periodic fever victim and identified a dark-colored pigmentation in the tissue (Klaassen et al., 2011). In 1847 Rudolph Virchow (Germany) confirmed that the colored pigmentation observed by Lancisi in 1716 came from the blood (Brabin, 2021).

Almost 30 years later, the causative candidates of malaria were finally proposed. First, in 1878, Corrado Tommasi-Crudell and Theodor Klebs proposed a bacterium (named *Bacillus malariae (B. malariae*)) isolated from Pontine Marshes, a former marshland in Italy, as responsible for malaria. This assumption was based on symptoms such as fever and spleen enlargement observed in *B. malariae*-injected rabbits (Haas, 1999). At about the same time, Charles Laveran, a French military doctor, performed a postmortem of patients who succumbed to malaria in Algeria. At first, Laveran observed pigmentation in organs and tissues as previously reported by Lancisi but failed to determine the cause. He then examined wet blood film (without staining) of leukocytes and erythrocytes and observed pigmented cells. Upon further inspection, he also discovered spherical and crescent-shaped (now known as the *Plasmodium* gametocytes) bodies containing pigment in those cells (Tan & Ahana, 2009; Dagen, 2020).

In 1880, Laveran observed pigmented spherical bodies with flagella-like structures in the blood of malaria patients that were motile and capable of infecting new RBC. He also found that the crescents can be removed when treated with quinine (the first antimalarial compound purified from the bark *Cinchona cordifolia* in 1820). Based on his study, two monumental findings were obtained: 1) Laveran reported parasite (initially named *Oscillaria malariae* (*O. malariae*)) as the causative agent of malaria, a remarkable finding on its own as it was the first protozoan parasite ever identified in humans, and 2) he proposed possible egression of the parasite in blood and its pathological implication. First, a clear spot was observed in RBC upon infection. Then, the spot grows and acquires the pigment until the erythrocyte ruptures, which causes fever in malaria patients (Cook, 2007; Dagen, 2020).

Laveran's finding was met with many oppositions, including from distinguished scientists such as Louis Pasteur and Robert Koch, because the "parasite" observed by Laveran was possibly the breakdown product of RBC. Hence, the idea of *B. malariae* as a malarial causative remained accepted until 1884. However, in the same year, Pasteur was finally convinced and supported Laveran's proposal once he directly observed the motile and flagellated spheres under the microscope (Brey, 1999). In 1884, Ettore Marchiafava and Angelo Celli identified a distinct structure to the one observed by Laveran, characterized as an active "amoeboid ring" (now known as the

ring stage). They thought it was an entirely different microorganism and was named *Plasmodium*, which eventually overrides *O. malariae* (Garnham, 1988; Dagen, 2020).

In 1885, Camillo Golgi identified two distinct species of parasite protozoa; *P. malariae* (responsible for quartan fever) and *P. vivax* (that cause tertian fever) (Tognotti, 2007). In 1890, Marchiafava and Golgi made another breakthrough discovery as they identified *P. falciparum,* which also causes tertian fever but is more fatal than *P. vivax*. Hence, *P. falciparum* is accounted as the cause of malignant tertian fever, while *P. vivax* causes benign tertian fever (Capanna, 2006). In 1894, Laveran (now associated with Pasteur Institute) stated that he had failed to find malaria parasites in the water, air, or even soil. Hence, he believed that *Plasmodium* might exist in another host outside humans. Laveran suggested the mosquito as another *Plasmodium* host, based on Patrick Manson's finding in 1877 that the mosquito spread filarial worms, acting as its vector (Dagen, 2020).

Findings made circa 1900 have an unprecedented impact on malaria study during the postmodern era that further enriched scientific understanding of *Plasmodium* infection treatment, vector control, and biology (Cox, 2010). In 1934, IG Farbenindustrie developed a series of antimalarial based on the structure of quinine, including CQ (4-aminoquinolines), a frontline antimalarial drug in the 1950s before the emergence of the CQ-resistance strain. Meanwhile, the life cycle of the

Plasmodium parasite was finally understood in the early 1980s (Krotoski et al., 1982; Josling & Llinás, 2015), which led to a new drug regimen by pairing drugs targeting different *Plasmodium* life stages and MoA (Nosten & White, 2007).

2.2 *Plasmodium* and its life cycle

In general, *Plasmodium* parasites require two hosts to complete their life cycle. The first host is *Anopheles* sp. mosquito, vital for *Plasmodium* sexual diploid cycle (in the midgut) and acts as their transmission vector (haploid sporozoites in salivary glands) to infect their second host (vertebrates such as mammals, reptiles, or birds), for the haploid asexual cycle (Henriquez, 2020). Almost 40 *Anopheles* sp. mosquitoes are known to transmit *Plasmodium* parasites to humans (Nicoletti, 2020). *Anopheles gambiae* was identified as the most efficient vector responsible for spreading *P. falciparum* (Koella et al., 1998).

In humans, *Plasmodium* asexual life cycle is divided into exoerythrocytic (in the liver) (Vaughan & Kappe, 2017) and intraerythrocytic (in the RBC) (Wallqvist et al., 2016) stages. Saliva injected by an infected mosquito will transfer sporozoites into the bloodstream and enter liver cells. The entry of sporozoites into the hepatocytes is highly precise due to the circumsporozoite protein that bears a ligand that only binds specifically to the liver basolateral domain (hepatocyte cell membrane). The entry of sporozoites will initiate the asexual reproduction, known as the pre-erythrocytic cycle

(PE) or primary exoerythrocytic schizogony (EE) stage. Sporozoites then metamorphose into trophozoites and transform into schizont and merozoites through schizogony (Ménard et al., 2008; Duffy et al., 2012; Texeira et al., 2021).

The erythrocytic cycle starts once merozoites leave the liver cell to penetrate RBC. During the invasion, the merozoites initiate invagination of the RBC membrane to form a membrane sack known as parasitophorous vacuole (PV) (Koch & Baum, 2016). Although the function of PV is not fully understood, it is known to act as an intraerythrocytic shelter necessary for nutrient acquisition (Bullen et al., 2012), facilitate the export of virulence factors (Pellé et al., 2015) and subcompartmentalization (Adisa et al., 2003; Matz et al., 2020).

Within this capsule, the merozoites transform into trophozoites (early trophozoites or ring-stage and late trophozoites). Next, the late trophozoites developed into blood-stage schizont. They were released from ruptured RBC together with metabolic waste and residual bodies to stimulate the release of macrophages and cytokines responsible for synchronous fever, a common malaria pathology. After an indeterminate number of asexual cycles, some of the merozoites will enter erythrocytes and become macro- and microgamonts to be ingested by the mosquito for sexual reproduction, and the cycle is repeated (Henriquez, 2020) (Figure 2.1).

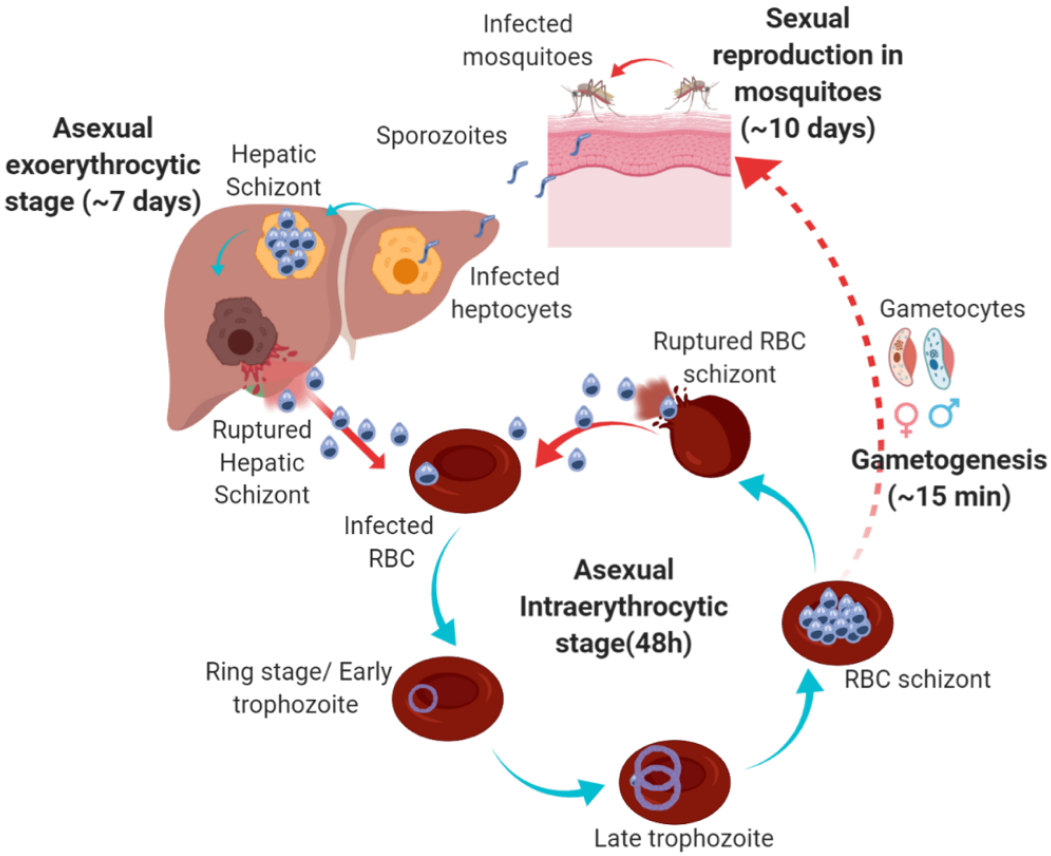

Figure 2.1 The life cycle of *P. falciparum* (Matthews et al., 2018). Created with BioRender.com

2.3 Druggable stage of *Plasmodium* life cycle

Based on the *Plasmodium* life cycle, different life stages can be targeted to develop antimalarial drugs, such as targeting asexual and sexual stages (Wadi et al., 2019), hypnozoites (dormant schizont stage)/liver stage (Derbyshire et al., 2011), and transmission-blocking (Birkholtz et al., 2022). However, most of the antimalarial act during *Plasmodium's* RBC life cycle since the screening requires the least labor and specialized material. Besides, an extensive compound library can be tested simultaneously. Hence, the RBC stage will continue to be the main target in drug development. (Gamo et al., 2010; Meister et al., 2011; Baragaña et al., 2015; Vos et

al., 2015; Swann et al., 2016). Antimalarial agents known to inhibit *Plasmodium* at the RBC stage are CQ, amodiaquine, quinine, sulfadoxine-pyrimethamine, ART and its derivatives, and lumefantrine (Zarchin et al., 1986; Loria et al., 1999; Joof et al., 2021; Famin & Ginsburg, 2002; Krishna et al., 2004).

CQ and ART are the most effective antimalarial compounds identified so far. Both compounds exert a unique MoA and can target a broad range of essential biochemical functions in *Plasmodium*. CQ is a weak diprotic base found in three forms in cells (un-protonated, mono-protonated, and di-protonated). The un-protonated form of CQ is a membrane-permeable form that can diffuse into the infected erythrocyte and digestive vacuole (DV) (lysosomal isolated acidic compartment formed by *P. falciparum* in erythrocytes) (Slater et al., 1991; Ehlgen et al., 2012).

In RBC, *Plasmodium* continues to multiply by ingesting the host cell cytosol into peptides and heme. Heme is a toxic material to *Plasmodium* and must be converted into an inert and harmless crystalline polymer hemozoin (malaria pigmentation) and deposited in the DV (Slater et al., 1991; Ehlgen et al., 2012). Once inside the DV un-protonated CQ become protonated (not permeable) and trapped in the DV (Homewood et al., 1972; Yayon et al., 1984). Protonated CQ binds with haematin (oxidized heme), preventing its further conversion into hemozoin crystal which eventually damages the *Plasmodium* membrane (Sugioka et al., 1987; Bray et al., 1998; Pagola et al., 2000).

Meanwhile, ART is a highly effective antimalarial agent as it affects *Plasmodium* at all stages of growth and acts on multiple targets (Ismail et al., 2016). In the infected RBC, ART become activated from the bond cleavage of its endoperoxide bridge by Fe^{2+}-heme (originated from hemoglobin degradation by *Plasmodium*). In this form, ART is lethal to the parasite due to biomolecules alkylation (heme, protein, and lipids) that cause oxidative stress and cellular damage (Tilley et al., 2016). It now becomes clear that the activity of ART is directly proportional to the amount of Fe^{2+}-heme. It makes ART unique compared with most antimalarial as it also can kill *Plasmodium* at the early ring stage (hemoglobin degradation begins with several hours of RBC invasion) (Portugaliza et al., 2020).

ART antimalarial activity is at its peak during trophozoites as hemoglobin catabolism at the highest rate (Klonis et al., 2013; Xie et al., 2016). It can also reduce 10,000-fold *Plasmodium* every 48-72 hours, providing rapid clearance but having a short half-life (thus requiring the second drug for complete *Plasmodium* elimination). Moreover, ART has a consistent antimalarial activity that is reproducible, wide therapeutic index, and low toxic effect on human cells (Dondorp et al., 2009; Maude et al., 2010; White et al., 2014).

2.4 Malaria: an ongoing battle

Despite numerous efforts to eradicate the *Plasmodium* parasite, malaria remains one of the most widely spread and fatal diseases today. More than 200 million new clinical cases with half a million death have been reported annually over the past two decades (Cibulskis et al., 2016; World Health Organization, 2021). Overall, malaria cases were reported to be increasing in 2020 (World Health Organization, 2021). Modeling analysis suggested that the increase was contributed by COVID-19. The pandemic indirectly disrupts malaria treatment and prevention worldwide (Weiss et al., 2021). In addition, a more worrying development was reported in certain regions, such as Laos, by which malaria cases have been steadily increasing since 2012, possibly linked to the emergence of drug resistance malaria (Imwong et al., 2015).

Moreover, only three broad classes of chemicals were identified as the antimalarial agent in clinical use. They are arylamino alcohol compounds, antifolate compounds, and ART compounds. The MoA exerted by these drugs can be divided into three mechanisms which are tissue schizonticides (primaquine), blood schizonticides (CQ, ART, quinine), and sporonticides (proguanil, pyrimethamine) (Cui et al., 2015).