Identification and characterization of novel chemical scaffolds to overcome drug resistance in malaria

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**IDENTIFICATION AND CHARACTERIZATION OF NOVEL
CHEMICAL SCAFFOLDS TO OVERCOME DRUG
RESISTANCE IN MALARIA**

TAY WEN JUN DONALD

SCHOOL OF BIOLOGICAL SCIENCES

2020

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RESISTANCE IN MALARIA**

TAY WEN JUN DONALD

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

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Abstract

Till today, malaria remains one of the most prominent infectious diseases in the world. Since the beginning of the decade, the number of malaria cases has drastically decreased, in no small part due to the availability of a plethora of chemotherapeutic strategies. In the last five years however, this progress has seemed to have stalled, with drug resistance, especially to frontline treatments, playing no small part. Without an efficacious vaccine, strong emphasis is placed on discovering and developing new chemotherapeutics with novel mechanisms of action to prevent and control malaria infections.

However, drug development in malaria is a challenging task, faced by the canonical hurdles of drug discovery and further compounded by the lack of understanding of the parasite's complex biology. These issues not only result in prolonged pre-clinical and clinical testing, but also starve the community of starting points for therapeutic design. Therefore, it might take too long before a new antimalarial may reach the hands of clinicians and patients. Hence, we used two distinct drug discovery approaches in an attempt to augment the antimalarial development pipeline. Here, we report two FDA-approved anticancer compounds for repurposing in malaria, as well as a third chemotype, unique to existing antimalarials, both as a promising candidate for drug development and a tool to uncover drug targets. These compounds were identified by screening a collection of anticancer drugs undergoing clinical development as well as an in-house library of compounds previously unintended for biological study. With large number of hits from both screens, we applied a distinct triage workflow to narrow down our candidates from each library. By comparison of pharmacokinetic properties in humans against their efficacy against *Plasmodium falciparum*, this allowed us to shortlist the two candidates which were documented to achieve doses in excess of their minimum inhibitory concentrations for at least 24hrs post administration in a single dose. The extrapolation suggests that these compounds have the potential to be expedited for development into clinical drugs by taking their first step into controlled-human malaria infection studies.

While it may not immediately lead to the next clinical candidate, novel chemical scaffolds may serve as good starting points for anti-drug resistant therapies, as well as to probe the proteome of the parasite for new avenues for therapeutic design. Therefore, our second approach was to biologically characterize the screened hits for suitable candidates displaying as many ideal antimalarial traits possible, and use it as a tool to study the parasite's druggable biology. This led to the identified a chemical scaffold with low nanomolar asexual stage-killing activity and parasite-to-host specificity, herein known as A1. Amongst its ideal antimalarial properties, A1 not only shows to inhibit the growth of not only ring stages of the asexual cycle, but also disrupts gametocyte development. Hypothesizing that its activity against the parasite may disrupt a lesser understood cellular function, we subjected A1 to a target identification screen using Cellular Thermal Shift Assay (CETSA). Interestingly the metalloprotease, Falcilysin (FLN), best described for its critical role in haemoglobin degradation, was identified and subsequently validated as its target in both asexual and sexual developmental stages of the parasite. Our pilot structural activity relation (SAR) studies have also shown that the compound has the potential to be improved into a proper candidate for early clinical testing.

Taken together, we anticipate that the findings of this study have supplemented the antimalarial development community with novel candidates to be seriously considered for therapeutic development, as well as a promising start point for which the next generation of drugs can be designed upon.

Abbreviations

ACT – Artemisinin Combinatorial Therapy

ARMD – Accelerated Resistance to Multidrug

CETSA – Cellular Thermal Shift Assay

CHMI – Controlled Human Malaria Infection

CNV – Copy Number Variant

CYP – Cytochrome P450

DHA – Dihydroartemisinin

DHFR – Dihydrofolate Reductase

DHODH – Dihydroorotate Dehydrogenase

DHPS – Dihydropteroate Synthase

ER – Endoplasmic Reticulum

EXP2 – Exported Protein 2

FACS – Fluorescence Activated Cell Sorting

FLN – Falcilysin

GMS – Greater Mekong Subregion

GTP – Guanosine Triphosphate

HBSS – Hank's Balance Salt Solution

hpi – Hours post-invasion

IP – Intraperitoneal

IPP – Isopentenyl Pyrophosphate

iRBC – Infected Red Blood Cell

IDC – Intraerythrocytic Development Cycle

IP – Immunoprecipitation

ITN – Insecticide-Treated Mosquito Nets

IRS – Indoor Residual Spraying

MAD – Median Absolute Deviation

MDT – Minimal Dose Threshold

MFS – Major Facilitator Superfamily

MIC – Minimum Inhibitory Concentration

MMV – Medicines for Malaria Venture

ORF – Open Reading Frame

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PVM – Parasitophorous Vacuole Membrane

*Pf*ACT – *Plasmodium falciparum* Acetyl-CoA Transporter

*Pf*CARL – *Plasmodium falciparum* Cyclic Amine Resistance Locus

*Pf*PI4K – *Plasmodium falciparum* Phosphatidylinositol 4-Kinase

*Pf*UGT – *Plasmodium falciparum* UDP Galactose Transporter

PK4 – *Plasmodium falciparum* Eukaryotic Translation Initiation Factor 2 α Kinase

PTM – Post-translational Modification

RBC – Red Blood Cell

RDT – Rapid Diagnostic Testing

SAR – Structure Activity Relationship

SERCA - Sarco/Endoplasmic Reticulum Ca^{2+} -ATPase

SERCaP – Single Exposure Radical Cure and Prophylaxis

SNP – Single Nucleotide Polymorphism

SPR – Surface Plasmon Resonance

TRAC – Tracking Resistance to Artemisinin Collaboration

UPR – Unfolded Protein Response

WHO – World Health Organization

Chapter 1: Introduction

1.1 Malaria Epidemiology

Malaria remains one of the world's most prevalent communicable disease, with the World Health Organization (WHO) reporting 219 million cases worldwide which resulted in more than 435 thousand deaths in 2017 alone. These staggering numbers amounts to high economic burden in afflicted countries, resulting in USD\$3.1 billion in control and elimination efforts in a year. While the number of deaths has dropped drastically since the high of 839-thousand in 2000, no significant progress has been made over the course of the past five years. Children under five years of age remain the most afflicted by the disease, accounting for 61% of total deaths [1, 2].

The disease exists predominantly in tropical and sub-tropical regions of the world, where the natural vector thrives on the warmer temperatures, humidity and higher rainfall experienced. This equates to almost half of the world's population being at risk to infection. Africa contributes to the largest proportion of cases (92%), followed by Southeast Asia (5%), and the central and southern Americas (Figure 1). Despite being located at the heart of the Southeast Asian region, Singapore has been declared malaria-free by the WHO since 1982, and cases in recent years have all came from imported cases [3]. However, weakening control programs in countries such as China, Korea, and Thailand has led to the disease making a comeback, placing significant emphasis on control of the disease following eradication [4].

Figure 1. World map of malaria afflicted countries as of 2017. Colours indicate different levels of eradication. Figure obtained from WHO Malaria Report 2018 [1]

1.2 The Malaria Parasite, *Plasmodium*, and its Complex Lifecycle

Malaria is caused by the infection of the protozoan parasite *Plasmodium*, of which five species *falciparum*, *vivax*, *ovale*, *malariae*, and *knowlesi*, are known to infect humans [2]. *P. falciparum* accounts for the highest number of total cases worldwide, while also being the most lethal, responsible for almost all deaths. However, *P. vivax* remains more prominent in South American countries as well as contributing to a substantial share of cases in Southeast Asia [1]. Most recently, the zoonotic species, *P. knowlesi*, was shown to make a significant contribution to clinical cases in Malaysia, with a steep increase in the number of cases between 2015 and 2017 [5].

The parasite lifecycle consists of developmental stages in both a vertebrate host as well as a mosquito vector. (Figure 2) [6, 7]. The female *Anopheles* mosquito first introduces the parasites in the form of a sporozoite into the human host during a blood meal. Through the bite of the mosquito, these sporozoites are introduced into the dermal tissue. Some sporozoites are able to traverse the dermal layers to reach the bloodstream, where they will circulate and eventually amass in hepatocytes of the liver. Upon invading the hepatocytes, the sporozoites undergo multiple rounds of mitosis to form a liver schizont which consists of multiple merozoites. In *P. vivax* and *P. ovale* infections, a proportion

of sporozoites may instead transition to a dormant stage known as hyponozoites (not shown in Fig 2). These cells are capable of “waking up” weeks or months after the initial infection, resulting in relapses of the disease [8]. On the completion of maturation, the merozoites burst out of the hepatocytes and enter the blood circulation, where they invade the host’s red blood cells (RBCs) to begin its intraerythrocytic cycle. Within the RBC, the parasite undergoes a developmental process in which it transitions from the immature ring to the trophozoite stage, followed by a series of mitotic events that lead to it becoming a schizont containing eight to 32 merozoites. Upon rupturing of the RBC, those merozoites are released back into the host circulation in search of the next RBCs to invade. Under certain known conditions, some of the asexual parasites differentiate into the transmissible form, the gametocytes. In *P. falciparum*, these gametocytes sequester and develop mostly in the bone marrow, and only reappear in the peripheral circulation at the end of their maturation [9]. The gametocyte development encompasses a progression from stages I to V and ultimately gives rise to male and female forms. After re-entering the bloodstream, these stage V gametocytes are picked up by the *Anopheles* vector in a subsequent blood meal. The sporogenic development then happens with the mating of the male and female gametes in the mosquito midgut before completing its entire life cycle as the sporozoites take up their position in the salivary glands to be reintroduced into a vertebrate host during the next blood cycle.

Figure 2. The multistage lifecycle of the *Plasmodium* parasite. Figure from Biamonte *et al.* 2013 [6]

1.3 Disease Pathology

The pathology of the disease is attributed to the intraerythrocytic development cycle (IDC), caused by the repetitive sequence of invasion, maturation and subsequently rupturing of the host's RBCs [7]. *P. falciparum*, *P. vivax*, and *P. ovale* have a tertian IDC (48 hr), while *P. malariae* has a quartan IDC (72 hr) and *P. knowlesi*'s a semi-tertian IDC (24 hr) [10]. In *P. falciparum* especially, the duration of the life cycle is reflected in the intervals between symptomatic episodes, although they do not manifest until around one to two weeks, dependent on the establishment of a substantial parasitemia. In the case of other species such as *P. vivax* and *P. ovale*, this incubation period may be even longer due to the formation of hypnozoites prior to liver stage schizogony [11]. Uncomplicated malaria, diagnosed by the early symptoms of the disease, usually consisting of chills, low-grade fever, headaches, nausea and vomiting, and body aches. Severe malaria is almost exclusively caused by *P. falciparum*, largely as a result of the rupturing of RBCs during the IDC as well as the specie's cytoadherence ability, which

allows it to stick to the endothelium of blood vessels in various organs, causing blockages. In severe malaria, the worsening of fever, heavy sweating, and exhaustion are followed by organ failures, and symptoms such as anaemia, hypoglycaemia, seizures or comas may rapidly lead to death [12].

1.4 Measures to Control and Eliminate Malaria

The economic burden that the disease imposes amounted upwards of US\$3 billion in 2017, with similar figures posted in previous years [1]. Disease control and elimination takes up a large proportion of the hefty financial commitment, spanning five arms – surveillance, rapid diagnostic testing (RDTs), vector control, vaccination, and chemotherapy [13].

1.4.1 Surveillance, Diagnosis, and Rapid Diagnostic Tests (RDTs)

Once solely intended for diagnosis of the disease, surveillance has now been integrated as an intervention strategy [14]. Surveillance looks to provide the information of the “who, what, when, where and why” of infection and transmission, subsequently using it to stop and contain the outbreak. At the same time, these records are used to certify and maintain the status of zero-transmission areas [15].

With a significant portion of the world’s population at risk, efficient surveillance can only be achieved by accurate diagnoses. While earlier methods have been able to reliably diagnose symptomatic infections, RDTs aims to do so similarly for asymptomatic patients because of lengthy incubation periods; as well as partially-immune individuals may carry sub-detectable levels of parasites [16, 17]. As these asymptomatic patients may still provide a reservoir for parasite transmission, diagnosing and providing treatment for them is just as important in the move towards malaria elimination [16].

Diagnosis can be accomplished using various methods such as microscopy of thick blood smears, nucleic acid-based assays, and antigenic detection (histidine-rich protein 2 and plasmodium lactate dehydrogenase) [18, 19]. Currently, the gold standard of malaria diagnosis is through examination of blood smears using microscopy. Detection from patient blood smears is able to achieve a detection sensitivity of 50-500 parasites/ μ L of blood (0.001% parasitemia), and at the same time distinguish

between the species of infection [20]. While microscopy is inexpensive and provides the most definitive diagnosis, its effectiveness is highly dependent on the skills and experience of a trained microscopist [21]. Antigen-based diagnoses, known as RDTs, overcome this by being able to detect low levels of parasite antigens such as the *P. falciparum* Histidine-rich Protein II from small amounts of patient blood [22]. Current RDTs are able to achieve up to 100 parasites/ μ L sensitivity, and work has been done to use dried blood spot samples on RDTs [23]. Even though antigen-based diagnoses are less technically demanding, the risk of false negatives are still apparent, as gene deletions and polymorphisms of these antigens in *Plasmodium* hinder the accuracy of antigenic-based tests [22, 24]. There have been applications of nucleic acid-based methods such as polymerase-chain reactions (PCRs) and Quantitative Real Time (qRT)-PCRs for the use of diagnosing infections. These tests specifically detect the 18S ribosomal RNA genes from patient samples, and can be used to distinguish between different *Plasmodium* species due to the interspecies differences in sequence [25]. They are by far the most sensitive, with a detection sensitivity of <5 parasites/ μ L, but are not considered an RDT due to the requirement of high-end equipment not commonly available in the field and time needed for the result to be obtained [26]. With each technique bearing shortcomings, there is a need to develop new methods for to overcome the challenges faced in the clinic, as prompt surveillance and diagnostics remains vital towards the effective deployment of interventive strategies such as vector control, vaccinations, and therapeutics. [27].

1.4.2 Vector Control

Due to the dependence of the *Anopheles* vector for transmission of the parasite, insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) of insecticides play a key role in disease-preventive measures [1]. While these measures target the adult stages of the vector, usage has also been extended towards larval stages by lacing water bodies evenly with thin layers of insecticides, albeit with their health-hazardous consequences [28]. However, monotherapy use of pyrethroids, the most commonly used and approved class of insecticides, has placed a high selection pressure on insecticide resistance in mosquitoes [29]. With resistance now known to be a heritable trait, along

with the increased survivability of mosquitoes, the failure of vector control is becoming a realistic scenario unless more insecticides are approved for use [30, 31].

1.4.3 Vaccination

Malaria vaccine development has come a long way from the first suggestions of using whole irradiated sporozoites, where following a challenge of irradiated *P. berghei* sporozoites, mice were conferred sterile protection to a live sporozoite infection [32]. It was later found that the immunity was conferred by a CD8+ T cell mediated response against the circumsporozoite protein (CSP) [33, 34]. While sterile protection in humans could also be achieved through immunization with radiation-attenuated sporozoites or genetically attenuated sporozoites, its applicability for mass usage is challenged by the need for a high parasite inoculum, sophisticated equipment for cryopreservation and transport, as well as the need for chemoprophylaxis with every dose [35-37].

To date, there are over 20 vaccine programs under clinical development, and while whole organism immunogens form part of this list (genetically-attenuated parasites included), there are also subunit vaccines [38]. The advantages of subunit vaccines are multifold, they; 1) enable good manufacturing practice for consistent quality production; 2) a relatively lower cost than whole pathogen vaccines; 3) can be formulated; 4) are more thermostable and easier to transport, and; 5) can be administered through less invasive routes [39]. The RTS,S/AS01, a subunit vaccine, is closest to obtaining licensing for use, having completed phase III clinical trials. The vaccine consists of a recombinantly-expressed central repeat and thrombospondin domains of the circumsporozoite protein (CSP) fused to the hepatitis B surface antigen (HBsAg) N-terminal region, another unfused HBsAg, and formulated with the AS01 adjuvant [40, 41].

The vaccine is given in a four-dose vaccination regime, where the initial three doses are given at least a month apart, with the final dose given 15-18 months following the third [42]. However, with this strategy, only a modest level of protection was observed amongst the participants of the study over a

duration between 38-48 months, with decreased efficacy over time [42]. RTS,S, being a pre-erythrocytic stage targeting vaccine, requires 100% blockage of hepatocyte invasion in order to be efficacious. Otherwise, the vaccine alone would not be able to prevent the infection even after a single sporozoite manages to escape. Although it is yet to be determined, the protective efficacy may be further compromised by the risk of CSP genetic diversity already observed in the field [43]. On top of this, other socioeconomic factors may play a part in the successful implementation of the vaccine. First, the need for multiple doses for it to attain maximum efficacy makes it difficult for widescale administration, this, even more so critical when the already less ideal protection rate (<75% recommended) decreases even further without the important final dose [44]. Side effects of RTS,S were also observed in the phase III clinical trial, which raises concerns amongst the general public for large scale implementation [42, 45]. Being needed in large numbers in high transmission areas, the cost of implementing it also remains a challenge, with a single dose already costing US\$5, which is multiplied by the requirement of a four-dose regime [46]. RTS,S therefore remains an unfinished article, and the reliance on chemotherapeutics remain apparent.

1.4.4 Antimalarial Chemotherapeutics and its History

Antimalarial drugs have been responsible for curbing rise in the number of cases and deaths, with an arsenal of clinically-approved drugs now available in the market (Table 1). However, the history of the discovery and development process was not without challenges. The first isolated antimalarial, quinine – initially isolated from the bark of the cinchona tree in the year 1820- was used from the 1800s and was the first-choice drug up till the second world war [47]. However, due to the limited availability of the plant, attention shifted towards looking for an easily synthesizable replacement, coming in the form of methylene blue [48]. Yet, despite being tested successful in treating malaria infections in humans, the inability to provide the same potency while also causing toxic effects, such as skin discoloration, halted its potential in replacing quinine [48].

While having a potent antimalarial was important, so too was for it to be readily available. It was then that scientist at Bayer began working on a new synthetic antimalarial [49]. Beginning with two synthetic precursors, a new candidate called resoquin was synthesized, but was soon found to be too toxic for use in humans [50]. It was only through extensive modification of the compound, that toxicity was able to be significantly reduced, resulting in chloroquine, a 4-aminoquinoline [48].

Another compound which was developed from that study was proguanil, an unrelated compound class to chloroquine, later developed into pyrimethamine [51]. Pyrimethamine was highly effective in treating malaria, easy to synthesize and cheap, and was later combined with sulfadoxine as the first combinatorial therapy, being found to have a synergistic effect [51, 52]. Despite concerns of toxicity as a result of usage, sulfadoxine-pyrimethamine, marketed as Fansidar, was soon used as the front-line drug in sub-Saharan Africa, and later, in Southeast Asia [52].

Yet another significant antimalarial discovered to date originated from a natural source [53]. At a time where there was a desperate need for a new and safer antimalarial due to the failure and toxicity risks of sulfadoxine-pyrimethamine, Chinese physicians began reviewing hundreds of old recipes documented to alleviate fever-related illnesses, and eventually identifying an ingredient which recurred within many of them [53]. Isolated from the leaves of *Artemisia annua*, it was the compound *Qinghaosu*, now known as artemisinin, that was found to be the active component [54]. Artemisinin exhibits a rapid parasitic killing rate, while also displaying activity against all asexual and sexual stages of the parasitic life cycle [55, 56]. What stood out for artemisinin was that it possessed potent ring stage activity. This made it a potent weapon for the prevention of severe malaria, where halting parasites from progressing to trophozoite stages prevented sequestration in the blood stream to escape splenic clearance [57, 58].

However, the knock-back of artemisinin was that it is rapidly eliminated through a host of metabolic pathways, giving it a short half-life, which then resulted in recrudescence despite multiple days of treatment [59]. Artemisinin Combinatorial Therapies (ACTs) were proposed, complementing

artemisinin with a partner a drug which has a longer half-life to prevent the recurrence of an infection [60]. Partner drugs currently used include lumefantrine (marketed together as Coartem), mefloquine, amodiaquine, and piperaquine, the last of which is used as the current front-line therapeutic strategy [59, 60]. The potent combination of dihydroartemisinin and piperaquine has been shown to be used as a prophylactic treatment, and is one of the current first line antimalarial in Southeast Asia [61].

1.5 Antimalarials and their Modes of Action

Most of the current antimalarial drugs administered to humans exert their parasite-killing activity on the asexual blood stages of the parasite life cycle, alleviating the symptoms experienced by the patient by reducing the parasite burden (Table 1). Some drugs also act on the gametocyte stages, preventing the transmission of the infection. Apart from the asexual and sexual developmental stages, liver stage schizonts and hypnozoites have also be targeted with chemotherapy to inhibit the advancement into the blood stage infection. The current repertoire of antimalarials are able to achieve developmental inhibition of one to two stages of the lifecycle, depending on their mode of action.

1.5.1 Antifolates

One of the first combinatorial therapies used in malaria treatment, sulfadoxine-pyrimethamine acts synergistically on the folate biosynthesis pathway [62]. Folate is highly required during the replication of genetic material, and the pathway serves as the primary source of synthesis by converting guanosine triphosphate (GTP) to tetrahydrofolate in a cascade of reactions[63]. Enzymes such as the dihydrofolate reductase (DHFR; PlasmoDB geneID: *PF3D7_0417200*) and dihydropteroate synthase (DHPS; PlasmoDB geneID: *PF3D7_0810800*) are part of that pathway, and pyrimethamine and sulfadoxine target the folate synthesis pathway by binding and inhibiting DHFR and DHPS respectively, depriving the parasite of the building blocks required for the synthesis of DNA [64]. Cycloguanil, the active compound of the prodrug proguanil, also targets DHFR in the similar manner [65].

Antimalarial Name (Abbreviation)	Major Clinical Use	Affected Pathway(s) or Mechanism(s)
Antimalarial Class: Endoperoxides		
Artemisinins (ARTs): artesunate (AS), artemether (ATM), dihydroartemisinin (DHA)	First-line treatment as part of ACTs; intravenous artesunate gold standard to treat severe malaria	Pleiotropic; triggers parasite stress response. Alkylates and oxidized heme, multiple proteins, and lipids
Antimalarial Class: 4-Aminoquinolines		
Chloroquine (CQ)	Treatment of non-falciparum malaria	Heme detoxification in digestive vacuole
Amodiaquine (AQ)	Partner drug for ACT (ASAQ)	Heme detoxification in digestive vacuole
Piperaquine (PPQ)	Partner drug for ACT (DHA+PPQ)	Heme detoxification in digestive vacuole
Pyronaridine (PND)	Partner drug for ACT (PA)	Heme detoxification in digestive vacuole
Antimalarial Class: Aryl-Amino Alcohols		
Quinine (QN)	Treatment of <i>P. falciparum</i> uncomplicated malaria in first trimester of pregnancy, or severe malaria	Might include inhibition of hemoglobin import and/or heme detoxification
Lumefantrine (LMF)	Partner drug for ACT (AL)	Might include inhibition of hemoglobin import and/or heme detoxification
Mefloquine (MFQ)	Partner drug for ACT (ASMQ) and prophylaxis (Lariam™ and generic)	Might include inhibition of hemoglobin import and/or heme detoxification
Antimalarial Class: Antifolates		
Pyrimethamine (PYR) + Sulfadoxine (SDX)	Combination (SP) used mostly for intermittent preventive treatment	Folate biosynthesis in parasite cytosol
Proguanil (PG)	see atovaquone-proguanil	Folate biosynthesis in parasite cytosol
Antimalarial Class: Naphthoquinones		
Atovaquone (ATQ)	Used in combination with proguanil (Malarone™ and generic)	Mitochondrial electron transport chain required for pyrimidine biosynthesis
Antimalarial Class: 8-Aminoquinolines		
Primaquine (PQ)	Radical cure and terminal prophylaxis of <i>P. vivax</i> and <i>P. ovale</i> ; gametocytocidal drug for <i>P. falciparum</i>	Unknown
Tafenoquine	Radical cure, terminal prophylaxis, and gametocidal activity for <i>P. vivax</i> and <i>P. falciparum</i>	Unknown

Table 1: List of Current Approved Antimalarials. Table shows the types of usages of these drugs and their mode of action (if understood). Figure from Ross and Fidock, 2019 [66]

1.5.2 4-aminoquinolines

4-Aminoquinolines includes compounds chloroquine, amodiaquine, and piperaquine. Chloroquine is one of the pioneer generation of antimalarials, while amodiaquine and piperaquine are partner drugs in Artemisinin Combinatorial Therapies (ACTs) [59, 60]. Haemoglobin metabolism provides an important source of amino acids for *Plasmodium*, and it occurs in a dedicated compartment in the cell known as the digestive vacuole (DV) [67]. The digestion results in the release of reactive free Fe^{2+} iron compound called hemozoin, which would then undergo oxidation into ferriprotoporphyrin IX, creating oxidative stress [68]. The parasite copes with this by incorporating ferriprotoporphyrin IX into the hemozoin [69]. 4-Aminoquinolines act on the asexual blood stages of the parasites by binding hemozoin, inhibiting its crystallization into an inert crystal called hemozoin, thus preventing detoxification [70]. It has been shown that 4-aminoquinolines, which are weak bases, accumulate in the DV upon being protonated due to the acidity of the compartment [71].

1.5.3 8-aminoquinolines

8-Aminoquinolines are similar in structure to 4-aminoquinolines, where they differ only in the amino group of the 8- position of the quinoline. Antimalarials belonging to this class include primaquine, and Tafenoquine. Apart from treatment, these compounds can also be used for prophylaxis, where primaquine is given with chloroquine to treat liver-stage *P. vivax* and *P. ovale*, and tafenoquine also acts on liver stage *P. vivax* [72]. More importantly, primaquine and tafenoquine are the also the only antimalarials known to kill hypnozoites [73]. Primaquine is also used to prevent transmission by inhibiting Stage V gametocytes [74]. However, their mechanisms of action remain poorly defined.

1.5.4 Aryl-Amino Alcohols

Quinine was the first aryl amino alcohol discovered from a natural source, but served as the basis of which derivatives in the form of mefloquine and lumefantrine was synthesized. The mechanism of action of these drugs remain unclear, with suggestions that aryl amino alcohols may inhibit hemozoin formation [69, 75]. Recently, it has also been shown that mefloquine disrupts protein translation

through binding the 80S ribosomal RNA [76]. However, stage specific assays showed that mefloquine and lumefantrine are actually metabolically active at different stages of the life cycle, suggesting two distinct mode of actions [77].

1.5.5 Naphthoquinones

Atovaquone, a naphthoquinone, is known to target the cytochrome bc1 complex of the mitochondria electron transport chain, the energy hub of the cell. The role of the complex is to facilitate the biosynthesis of pyrimidine for the parasite through the activity of dihydroorotate dehydrogenase, which in turn produces coenzyme-Q that gets re-oxidized by the bc1 complex to maintain the mitochondria membrane potential [78]. Due to the lack of a salvage pathway, cytochrome bc1 is also essential for pyrimidine synthesis [79]. From structural studies, the ionized form of Atovaquone binds the Q_o site, responsible for the oxidase reaction, of the cytochrome bc1 complex, therefore resulting in the inhibition [80]. However, it is now also known that the Q₁ reductase site is also a druggable target, and that development of inhibitors against are in the works [81, 82].

1.5.6 Endoperoxides

Endoperoxide antimalarials used in the field are represented by artemisinin and its derivatives. Artemisinins are now the mainstay in first-line therapeutics, with most combinatorial therapies based on partnering a derivative together with a second antimalarial to take advantage of their rapid killing activity (10, 000 fold reduction of parasites) [83]. The initial step of the mode of action requires an iron (haem)-dependent breakage of the endoperoxide bridge [84]. The active form of the compound then alkylates and covalently-binds multiple targets and causes extensive oxidative damage throughout the cell [84, 85]. In a protein targeted-binding study, it was observed that more than a 120 protein candidates were alkylated by artemisinin [84]. The list consisted of proteins from multiple pathways including haemoglobin catabolism, carboxylic acid metabolism, amino acid metabolism and biosynthesis, ribosome assembly, pH regulation, and protein translation, indicative of a promiscuous parasite-killing activity of the cell. One example, the sarco/endoplasmic reticulum Ca²⁺-ATPase

(SERCA) orthologue in *P. falciparum* (*PfATP-6*, PlasmoDB geneID: *PF3D7_0106300*) was shown to be inhibited by artemisinin in another study [86]. *PfATP-6* regulates the pH in the cells by regulating cytosolic Ca^{2+} levels, and due to the lack of alternate transporters, the inhibition by artemisinin kills the cell [86].

The alkylation and oxidative stress caused by artemisinins has been shown to trigger the unfolded protein response (UPR) in the parasite [87]. The UPR is therefore activated to deal with improperly folded proteins by degradation, where the parasite ubiquitinates these proteins to be dealt with by the 26S proteasome [85]. However, artemisinins are also known to inhibit the proteasome degradation of ubiquitinated proteins, which results in an over-accumulation of unfolded proteins in the cytosol, causing the parasite's death [88]. In addition, it is also seen that the targets are not restricted only to proteins, but nucleic acids as well [89, 90]. Double-stranded DNA breakages were observed in a dose-dependent manner in artemisinin-treated parasites, and irreversible damage leading to cell death was achieved even at low concentrations [90].

1.6 Drug Resistance in Malaria

Despite the number of antimalarials targeting different aspects of the parasite biology, the emergence of drug resistance has been an ever-constant threat (Figure 3) [91]. Earlier treatment regimes consisted of drug monotherapies, and have resulted in a high selection pressure for which resistance was able to develop. At the same time, the use of substandard drugs and poor patient compliance also created the opportunity for recrudescence of infections, and hence resistance [92-95].

Figure 3. A timeline depicting the introduction of antimalarials and the first case of reported resistance. Arrows depict the point in which resistance was first reported, while different colours represent different classes of chemotypes. Figure from Blasco *et al.* 2017. [91]

Pyrimethamine and chloroquine mass administrations were done as part of a global effort for malaria eradication in the 1950s, along with the use of insecticides [96]. However, pyrimethamine and subsequently chloroquine failure, along with the lack of effective insecticide use, quickly became widespread, and plans for eradication had to be shelved [97]. A switch of healthcare policies across

the world to sulfadoxine-pyrimethamine then ensued [97]. To their detriment, sulfadoxine-pyrimethamine resistance arose as early as within the year which it was implemented (Figure 3) [98].

As shown, monotherapy treatment almost guarantees the development of drug-resistant parasites, although chloroquine still remains the treatment policy in certain South American countries such as Guatemala, Haiti, Honduras, and Nicaragua, where it is still effective. [91, 99]. The WHO now recommends a change in therapeutic policy once treatment failure rate rises above 10% [100]. At the same time, the paradigm has shifted towards combinatorial therapy in order to reduce the risk of resistance developing with only the pressure of a single drug [101].

As mentioned earlier, current first-line antimalarials are typically ACTs. In Africa, ACTs such as artemether-lumefantrine, artesunate-amodiaquine, are used, while dihydroartemisinin-piperaquine, artesunate-mefloquine, and artesunate-pyronaridine are also known to be effective [102]. However, resistance to antifolate drugs pyrimethamine and proguanil, as well as sulfadoxine has seen their usage curbed, which is also why the last of the approved ACTs – artesunate-pyrimethamine-sulfadoxine – has ceased to be used in most places [99, 103]. With data as recent as 2018, the efficacy of ACTs has remained well above 90% in Africa since the year 2010 [99]. While there have been individual cases where ACT failure was observed in travelers returning from various parts of Africa, genetic analysis of African patient isolates did not show any indication of the presence of molecular markers of drug resistance in those areas [99, 104-106].

Middle Eastern countries such as Afghanistan, Iran, Pakistan and Yemen, use artesunate-sulfadoxine-pyrimethamine, where treatment failure rates were found to be less than 10% [99]. However, high artesunate-sulfadoxine-pyrimethamine failure rates have been observed in Somalia and Sudan [103]. Artemether-lumefantrine was then implemented as the next first-line treatment in these countries, and its high efficacy pointed to sulfadoxine-pyrimethamine resistance being the cause of the treatment failure, which was later confirmed by the detection its molecular markers of resistance [107, 108].

In contrast, reports of combinatorial therapies failing have been observed in Southeast Asia, predominantly in the Greater Mekong Subregion consisting of Cambodia, Thailand, Vietnam, Myanmar, Laos, and the Yunnan province of China [109]. The region has a reputation for being an epicenter for drug resistance development, where it was also previously one of the origins of chloroquine- and sulfadoxine-pyrimethamine-resistant infections [110, 111]. It was therefore inevitable that these were the countries which first adopted ACTs [110].

Cambodia first introduced artesunate-mefloquine, which subsequently saw a switch to dihydroartemisinin-piperaquine when treatment failure arose [103, 112]. Dihydroartemisinin-piperaquine was phased out of Cambodia entirely by 2014, with piperaquine resistance posing as huge a threat as artemisinin failure [113]. Cambodia then introduced artesunate-pyronaridine and artesunate-amodiaquine, but failure rates have started to increase, last understood to be between 14-23% [103]. Western Cambodia on the other hand, seem to still have artesunate-pyronaridine working above 95%. Artesunate-mefloquine has most recently been reintroduced as the first-line treatment, but with the country experiencing treatment failures to multiple ACTs, a dynamic policy might be needed instead [99].

Thailand similarly began using artesunate-mefloquine, until resistance to artemisinin and later mefloquine meant that even the adoption of a prolonged treatment regime was no longer effective [103, 114]. Dihydroartemisinin-piperaquine was then introduced in 2015, but a failure rate of almost 93% in the north-eastern region of Thailand was observed in 2017, possibly due to imported cases from the Cambodian border [113]. Therefore, the eastern region of Thailand sharing the border with Cambodia currently uses artesunate-pyronaridine [99].

Myanmar first experienced artesunate-mefloquine failure in 2009 at its border with Thailand [112]. Since then, therapeutic failure of artemether-lumefantrine as well as well dihydroartemisinin-piperquine were also detected, predominantly due to artemisinin resistance [115, 116]. However, the

failure rate of these ACTs still remain high, and due to the lack of options, the country still utilizes all three ACTs for malaria treatment while also performing trials on artesunate-pyronaridine [99, 103].

In Vietnam, dihydroartemisinin-piperaquine resistance was observed in 2009 in the district bordering Cambodia, and has later spread to other districts in the southern region since then, attributed to both artemisinin and piperaquine resistance [117]. Vietnam has introduced artesunate-mefloquine, while also monitoring artesunate-pyronaridine and artemether-lumefantrine [99, 110].

Recently, Laos began encountering artemether-lumefantrine failure rates above 10% in towns sharing borders with Thailand and Cambodia [103]. To counter that, dihydroartemisinin-piperaquine was introduced, but to their detriment, the loss of efficacy was observed at the Cambodian border, caused by the spread of artemisinin resistant mutations in parasites in the region [118]. Currently, artesunate-mefloquine and artesunate-pyronaridine are being trialed in the country [103].

However, outside of the Greater Mekong Subregion, ACTs still serve as front-line drugs in Southeast Asian countries such as Bhutan, Nepal, Timor-Leste, Bangladesh and India, where artemether-lumefantrine efficacy is still above 90% in their latest efficacy assessments [99]. Bangladesh also uses artesunate-amodiaquine, artesunate-mefloquine, and dihydroartemisinin-piperquine, and Indonesia adopts dihydroartemisinin-piperaquine as their go-to therapy [103].

1.7 Molecular Markers of Drug Resistance

With the constant emergence of drug resistance, it has become of great importance of understanding the underlying factors responsible for treatment failure. The accumulation of genomic mutations have been the primary effectors behind drug resistance observed across many microorganisms and cancer, and *Plasmodium* is no exception [119-121]. The measured mutation rate from *in vitro* studies estimates an approximate mutation rate of 10^{-9} per base in its 23Mb genome during a single mitotic event, translating to one mutation in every 50 parasites [122]. Statistically, this shows that the rate of which parasites can develop resistance is relatively high. However, not all drug resistance mutations are propagated equally in the sense that they are also limited by environmental factors such as the

diversity of strains present and transmission rates, as well as fitness costs [66]. Currently, gene mutations associated with resistance to drugs such as chloroquine, pyrimethamine, quinine, mefloquine, artemisinin and a few others have been established (Figure 4) [123, 124]. As drug resistance has proven to be inevitable thus far, the identification of genetic molecular markers of resistance has become a vital tool in keeping track of the emergence and spread of resistant infections so as to allow for timely changes in treatment policies [125].

Figure 4. Malaria drugs and their targets. Antimalarials are grouped into various mode of action/classes that act on different aspects of the parasite's biology. Highlighted in red are compounds currently undergoing clinical development. Figure from Wildfire, 2019. [124]

1.7.1 Mechanisms of Drug Resistance

Further leveraging on the information of drug resistance markers, it has become of great interest to understand the underlying molecular mechanisms of resistance in the discovery of future therapeutics [126]. A molecular marker of resistance refers to the genetic mutation for which lead to drug resistant phenotypes to the parasite. These mutations at the genetic level, either single nucleotide polymorphisms (SNPs) or copy number variants (CNVs) result in i) changes in the substrate specificity

of the drug target, ii) destruction or neutralization of the drug, iii) inhibition of cellular uptake of the drug, iv) the increase of the expression of the target, or v) the efflux of the drug away from its intended target or organelle compartment [127, 128]. In some examples discussed later, these molecular markers may be present on the very gene encoding for the protein target itself. However, a target and a molecular marker of resistance be distinguished by the way they interact with the drug. A drug target typically has a direct binding with the drug, resulting in the inhibition of its function and eventually toxicity to the cell. In contrast, the protein product of the gene carrying a molecular marker of resistance often plays a role in negating the binding to the drug in one of the five ways mentioned earlier. Therefore, identification of these molecular markers now also serves as the basis for understanding drug desensitization.

1.7.2 Molecular Markers of Drug Resistance of Current Antimalarials

1.7.2.1 Dihydrofolate Reductase (DHFR)

A well-studied example of resistance caused by the alteration of the drug target is DHFR. Pyrimethamine treatment was introduced following the mass treatment failure of chloroquine as it was able to kill both sensitive and resistance infections [129]. Later deployed together with sulfadoxine, the combination was seen as the next candidate for mass drug administration in the malaria eradication campaign due to the synergistic effect as well as the lesser likelihood of developing therapeutic failure [62]. However, the sulfadoxine-pyrimethamine resistance surfaced within a year of its introduction [130]. Prior to the implementation of the sulfadoxine-pyrimethamine combination, pyrimethamine was used in monotherapy as a therapeutic as well as a casual prophylactic, which already resulted in increasing frequencies of treatment failure due to selection of drug resistant mutants [131-133].

Mutations (such as N51I, C59R+S108M, and I164L) in DHFR resulted structural changes that lower the binding affinity of pyrimethamine to the protein, each to varying degrees [134]. The I164L mutation causes the most drastic in changes to pyrimethamine affinity, and in turn results in the most severe

drug resistance, albeit with a fitness cost which results from altered activity of the enzyme [135]. Despite its fitness cost, this particular SNP was more prevalent in the Southeast Asian region due to the lack of heterogeneity of genotypes in the population and low transmission rates [136]. It was also seen that some mutants such as the C59R+S108M have no distinct differences in enzyme activity to the wild-type enzyme, and hence fitness levels, therefore explaining its higher prominence in high transmission areas such as Africa [137].

1.7.2.2 *P. falciparum* Chloroquine Resistance Transporter (PfCRT)

Chloroquine is one of the oldest antimalarials to be used in treating malaria, dating back to the early 1930s. Together with the use of vector control strategies, the first malaria eradication initiative was started [50]. However, the plan was soon shelved upon the emergence of chloroquine-resistant *P. falciparum* approximately two decades later, with *P. vivax* also following suit soon after [138].

The resistance to chloroquine has been attributed to the mutations in *PfCRT* (PlasmoDB geneID: *PF3D7_0709000*), a transporter localized on the membrane of the parasite's DV [139]. Through sequencing multiple patient samples and *in vitro* drug selected strains, multiple non-synonymous SNPs have been identified in the *pfcr*t loci, although none more critical than the amino acid 76 [140]. K76 is part of the first of ten transmembrane domains of *PfCRT*, the postulated interacting site of chloroquine. The change from the positively charged lysine to the uncharged threonine residue (K76T) allows protonated chloroquine to escape the food vacuole down its concentration gradient, whereas the positively-charged compound would not be able to do so [141]. Two other mutations in *pfcr*t, K76I and K76N, discovered through *in vitro* chloroquine selection also support this model [142]. This justifies earlier findings that the efflux of chloroquine prevented its accumulation in the DV to exert its effect [143].

A total of 32 other SNPs have been identified in patient samples across the world, all of which emerged independently, and it was found that a single *pfcr*t locus can carry anywhere from four up to 10 SNPs [144]. It was only later in Africa that chloroquine-resistance was introduced through an imported

strain from Southeast Asia [145]. However, the common denominator between almost all of the patient samples was the K76T mutation, with the exception of one isolate carrying a rare K76A SNP instead [146]. Therefore, the K76T is currently used as the molecular marker for surveillance of chloroquine resistance in the clinic [93]. The reversion of the K76T to the wild type was observed once chloroquine treatment was withdrawn from Africa, hinting that the mutation confers a substantial loss of fitness [147, 148]. It is still uncertain if there were any environmental factors influencing the accumulation of different *pfcr*t mutations in parasites from different parts of the world. One hypothesis suggests that these multiple SNPs are as a result of a high frequency of mutation events aimed at fine tuning the balance between resistance and fitness cost of the K76T SNP [149].

1.7.2.3 *P. falciparum* Multidrug Resistance Transporter-1 (*PfMDR1*)

It was later discovered that chloroquine sensitivity in *Plasmodium* could be influenced by more than one transporter. When it was shown that Verapamil sensitizes chloroquine-resistant parasites to treatment, parallels were drawn to similar observations in multidrug-resistant cancer cells [150-152]. In cancer, the P-glycoprotein homologue (P-g_h1), an ATP-Binding Cassette (ABC) membrane transporter, was found to be behind this phenomenon [151]. As Verapamil was shown to inhibit P-g_h1 efflux activity, a search for the *p-gh1* homologue in *P. falciparum* was done, leading to the identification of the *pfmdr1* gene (PlasmoDB geneID: PF3D7_0523000) [153, 154]. As its name suggests, *PfMDR1* influences the parasite's susceptibility to multiple antimalarials, namely quinine, mefloquine, piperazine, lumefantrine, amodiaquine, chloroquine as well as artemisinin. It is known to localize to the parasite's DV and functions as a solute carrier [155]. However, different SNPs and/or copy number variants (CNVs) are responsible for conferring resistance or sensitivity to different drugs. [156-159]. A total of five SNPs in *pfmdr1* have been found to alter sensitivity so far, with two found in the N-terminal (N86Y and Y184F) and three in the C-terminal (S1034C, N1042D and D1246Y) end of the protein [160]. Even more interestingly, *PfMDR1* seems to have functional relatedness to *PfCRT* in mediating resistance to chloroquine and many other antimalarials, with a genetic linkage suggested between certain SNPs of both transporters [161].

One of the most prominent examples of the interaction of the two drug resistance genes is the mediation of chloroquine sensitivity. While the K76T mutation in *pfCRT* confers resistance to chloroquine, the presence of the N86Y SNP in *pfmdr1* further desensitizes the parasite to the drug. However, N86Y alone does not confer any resistance at all [162]. Instead, N86Y increases sensitivity to lumefantrine, mefloquine, and artemisinin [160]. In addition, this double mutant combination of SNPs also resulted in the decreased sensitivity to amodiaquine, an ACT partner drug [160].

In the Southeast Asian region, N86Y SNPs were found to be rare, with Y184F instead being highly prevalent [160]. Multiple *pfmdr1* gene copies of up to five were also common in the region. However, even more striking was that the K76T mutation was present in almost all Southeast Asian strains tested in that study [160]. In contrast, African isolates were found to possess a mix of *pfmdr1* N86Y/Y184, N86/Y184F, or N86Y/Y184F haplotypes. The only notable absence was of N86Y/Y184. With regards to *pfmdr1* amplification, it was found to 10 times less common in African isolates, possibly attributed to an associated fitness cost [160]. These serves as precedence for adjustment of chemotherapeutic policies in both areas, in that the partner drug in ACTs were adjusted based on the surveillance of molecular markers in patient samples.

As for SNPs at the C-terminal end, mutations at S1034C, N1042D and D1246Y increases sensitivity to mefloquine, halofantrine and artemisinin but confers resistance to quinine [156, 163]. Another SNP, N86Y, decreases amodiaquine and chloroquine sensitivity but does the opposite for lumefantrine and mefloquine [160]. Evidences then showed that these mutations allowed *PfMDR1* to gain or lose the ability to transport its substrates by altering its substrate specificity [158, 164, 165]. Still, the degree of resistance conferred by these C-terminal SNPs were shown to be comparatively less in contrast to those at the N-terminal ends [160].

1.7.2.4 Kelch Protein (*k13*)

Artemisinins have been used as the main component of first-line combinatorial therapies for more than 10 years. However, resistance to the artemisinin component of the therapy has raised huge

concerns amongst healthcare providers and policy makers. Artemisinin resistance was first reported due to an abnormal rate of recrudescence in *P. falciparum*, the species not known to undergo the latent hypnozoite liver stage [166]. It was subsequently characterized as a delayed clearance of early ring stage parasites, an increase from 48 to 84hrs between sensitive and resistant strains, of parasites despite efficacious doses in the blood [167]. It was attributed to the short half-life (2-6hrs) of artemisinin in the blood, allowing these rings stages to persist during the short exposure [167]. This persistence of ring stages can be shown by a lengthened duration of ring stages, and subsequently an accelerated progression through trophozoite to schizont stages. This phenotype is thought to allow the parasite a higher chance to survive drug exposure [168].

It was originally proposed through genome wide association studies (GWAS) that mutations in the *k13* allele (PlasmoDB geneID: *PF3D7_1343700*) was associated with artemisinin resistance [169] [170]. However, *pfmdr1* mutations alone are also known to confer artemisinin resistance. Still, because it was shown that the knock-in of a *k13* mutation in a sensitive drug can confer resistance, *k13* is still used as an indicative molecular marker for resistance [163]. Originally found in Cambodia, *k13* mutations have now spread across the Greater Mekong Subregion (GMS) [109, 117, 171, 172]. A total of 16 SNPs in the *k13* gene have been identified so far, but only a few, most notably the R539T, I543T and C580Y, have been established to confer resistance to artemisinin [71,72]. Amongst the various resistance-conferring mutants, the C580Y is known to be of the lowest in fitness costs, and is now the most prevalent *k13* SNP across Southeast Asia, replacing other *k13* mutants previously identified in those regions [170].

The true mechanism of how *k13* mediates resistance to artemisinin remains relatively debatable. Perhaps the most widely accepted model is the upregulation of the unfolded protein response (UPR) of the parasite to cope with the protein alkylation and oxidative stress caused by artemisinin [173]. The first evidence pointed out that artemisinin-treated parasites had highly phosphorylated eukaryotic initiation factor-2 α (eIF2 α , PlasmoDB geneID: *PF3D7_1019000*) [174]. eIF2 α activation by

phosphorylation results in the overall suppression of protein translation during drug stresses. It is hypothesized that the UPR of the ER results in the constant phosphorylation of eIF2 α by protein kinase 4 (PK4, PlasmoDB geneID: *PF3D7_0628200*), the *Plasmodium* homologue of the UPR master regulator PERK in mammalian cells. This was further supported by observations of hyper-upregulation of UPR gene in artemisinin-treated *k13* mutant field isolates, which also displayed extended-ring stage phenotype [175].

Recently, it was also shown that *k13* mediates artemisinin resistance through the endocytosis pathway. It was shown that the loss of function of proteins co-localized in the same cellular compartment resulted in conferring artemisinin resistance. These proteins, along with *k13*, were then shown to mediate haemoglobin endocytosis, which was lost when the function of *k13* or the other proteins were ablated. Since degraded haemoglobin products were needed to activate artemisinin, the model for the resistance mechanism was that the lack of haemoglobin resulted in the lack of the active of the drug in the cell [176].

The observation that a vast array of polymorphisms arising in the malaria endemic areas of Southeast Asia has earned the region its reputation as the “hotbed” of malaria drug resistance development. It is hypothesized that different parasite strains harbour varying degrees of an “accelerated resistance to multidrug” (ARMD) phenotype. These traits allow for a higher potential -up to 1000-fold- to accumulate mutations – giving them a better chance of selection under drug pressure [177, 178]. However, this claim has later been rebutted, where it was shown that resistance strains from the region had equal propensity to develop resistance [179, 180]. A more justifiable explanation, as observed in many of the cases discussed, is the allowance of low fitness cost mutations to thrive due to the seasonal, low transmission nature of infections in the region that results in low probability of competing multiple infections [180].

1.7.3 Molecular Markers of Drug Resistance of Antimalarials in Development

1.7.3.1 *P. falciparum* P-type ATPase 4 (*PfATP-4*)

P-type ATPases are transport proteins which translocate cations, heavy metals, and lipids across the plasma membrane to maintain chemical gradients. Structures of this class of transporters have been elucidated and studied extensively, resulting in increased understanding of their function and mechanism of action [181]. Apart from their multi-pass transmembrane domains (six in eukaryotes, seven in prokaryotes), they also possess a cytoplasmic region consisting a nucleotide-binding, phosphorylation, and an actuator domain [182]. These transporters undergo an intermediate phosphorylation state during the active transport of its solute across the membrane in order to prevent the backflow of its solutes following the accumulation of charges [183]. In total, 13 P-type ATPases have been identified in *P. falciparum* based on homology studies [184]. Among them, the *PfATP-4* (PlasmoDB geneID: *PF3D7_1211900*) has been identified as a potential drug target due to the high selectivity against the host cells [185]. *PfATP-4* has been shown to regulate electrochemical homeostasis within the parasite vacuole by serving as a Na⁺ efflux pump [186]. It was shown that the spiroindolone drug cipargamin (a drug under the Medicines for Malaria Venture (MMV) development portfolio, previously known as KAE609 during clinical trials) results in the inhibition of Na⁺ efflux via *PfATP-4* [186].

However, *PfATP-4* was also identified as the molecular marker of resistance of cipargamin [185]. Through exposure of the multidrug-resistant *P. falciparum* Dd2 cell line to prolonged sublethal doses of cipargamin, non-synonymous SNPs (D1247Y and the I398F + P990R double mutation) and CNVs of *PfATP-4* were observed from whole genome sequencing of the resultant mutant parasites [185]. Interestingly, *PfATP-4* mutants were also identified to confer cross-resistance to other classes of antimalarials, namely aminopyrazoles, dihydroisoquinolones as well as 28 other compounds in the malaria box, an open-source library of 400 chemically-diverse antimalarial compounds [187]. This characterizes *PfATP-4* as a multidrug-resistance marker [188, 189]. However, the mechanism by which the mutant *PfATP-4* confers resistance to cipargamin and all other *PfATP-4* inhibitors is still being

highly debated. A hypothesis drawn from a cell-free assay suggests that these compounds may exhibit an inhibitory effect on PfATP-4 through direct binding [190]. This binding model is also supported from *in silico* docking studies of the clinical trial dihydroisoquinolone compound, (+)-SJ7733 to the transporter [189].

1.7.3.2 *P. falciparum* Acetyl CoA Transporter (PfACT)

Despite not yet being introduced into the market, *in vitro* evolutionary approaches have been deployed to anticipate potential drug resistance when new drugs are tested in the clinic [178]. Most recently two new transporters, previously not known to be related to treatment failure, were identified as possible molecular markers of drug resistance; the PfACT (PlasmoDB geneID: PF3D7_1315400) and PfUGT (PlasmoDB geneID: PF3D7_1113300) [191]. Both transporters were identified through a spontaneous resistant mutant selection of GNF179, the more potent analogue of the clinical trial phase IIB drug KAF156. Another multidrug resistance marker, PfCARL (PlasmoDB geneID: PF3D7_0321900; an integral transmembrane ER protein) previously identified in another similar study with KAF156, was also identified from the selection [191, 192]. From sequencing the genome of each limiting dilution clone, a total of 12 different nonsynonymous SNPs was identified across those transporters, eight of which came in the *pfact* alone (Figure 5A). However, when the experiment was conducted in an *in vivo* model using *P. berghei*, SNPs were identified only in *pfact* locus of the resulting parasite clones, adding onto its significance in the clinical context (Figure 5A, B).

The acetyl-CoA transporter belongs to the Major Facilitator Superfamily (MFS) of transporters. These transporters exhibit an MFS core fold, typically consisting of two multi-pass transmembrane domains (N and C) in an almost symmetrical manner about the transport axis, while some members carry additional cytoplasmic domains [193]. They facilitate the transport of a range of ubiquitous solutes without the use of a cotransporter [193]. The human homolog AT-1 is shown to transport the highly-charged acetyl-CoA from the cytoplasm into the Endoplasmic Reticulum (ER) lumen to serve as the substrate for N-acetylation of the peptides to transit into the Golgi [194]. N-acetylation, a post-

translational modification (PTM) identified in the ER, are known to play an important role in protein folding, stability, and subsequently into trafficking [195-197]. Mutations of AT-1 are related linked to spastic paraplegia, a disease encompassing a subset of neurological disorders [198]. However, apart from evidence that other MFS transporters have shown to confer drug resistance in *Candida* and *Aspergillus spp*, there has not been any evidence to show how the acetyl-CoA transporter in particular is involved such a role [199, 200]. To the best of our knowledge, the molecular mechanism of resistance to imidazolopiperazines by *PfACT* remains unknown. However, as discussed with previous examples of drug resistance-conferring transporters, a change in efflux of either the drug or a secondary substrate may be the most probably explanation. In the published study, particular attention was drawn towards the D165N mutation, in which the mutant cell line exhibited a 224-fold increase in IC₅₀ compared to the parent cell line. This amino acid resides in a highly conserved motif of a cytoplasmic gating loop (encircled in Figure 5B, C) [191]. It was therefore hypothesized that these mutations affect the transport regulatory function of *PfACT*, similar to what was described earlier in *PfCRT* and *PfMDR1*.

A

B

C

Figure 5. Molecular markers of resistance against a new class of antimalarials: the imidazolopiperazines. **A)** Graphical represent of non-synonymous SNPs identified via forward genetic screen using the imidazolopiperazine compound, GNF179. **B)** Ribbon cartoon structural model of *Pf*ACT and *Pb*ACT. Amino acid mutations identified from spontaneous resistant mutants are highlighted in atomic spheres. The putative cytoplasmic gating hinge region is circled in blue. **C)** Diagram representation of conservation of the amino acids 160-170 of the putative cytoplasmic gating loop. Figures from Lim *et al.* 2016 [191].

1.8 Overcoming Drug Resistance with the next Antimalarial Candidate

Despite the arsenal of antimalarials available, the bane in the goal towards malaria eradication is the constant emergence of drug resistance [1]. With reported incidences of decreased patient response to ACTs, the risk of losing our front-line treatment against the disease is looming large. Despite reports of failure to the artemisinin component of ACTs, they remain a vital part of malaria chemotherapeutics due to their rapid-killing activity, especially during the ring stage of the IDC [201]. Hence, the search for new antimalarials with novel mechanisms of action and/or partner drugs to artemisinin grows ever more urgent [202].

Ideally, a new candidate drug would 1) prevent transmission by preventing the development of gametocytes or killing them altogether; 2) block sporozoite development within the mosquito; 3) eliminate dormant liver stage (hypnozoites of *P. vivax* and *P. ovale*) infections; 4) provide a single dose radical cure for all parasite stages; 5) have good pharmacokinetic and pharmacodynamics properties; 6) cure asymptomatic infections; 7) be suitable for prophylactic use; 8) be suitable for safe use across a wide range of patient medical profiles and; 9) be inexpensive to produce, amongst others. All these, while being able to kill drug-resistant parasites effectively. [203-205]. Although the current available antimalarials are able to achieve one or more of these on their own, a treatment strategy which can perform most, if not all, of the above would greatly enhance the chances towards the elimination of the disease and decrease the likelihood of treatment failure due to resistance.

1.9 Accelerating Drug Discovery through Repurposing

The preclinical identification and development of a novel chemotype involves a laborious, costly, and time-consuming process which could amount to years before delivering a candidate ready for human use. This excludes the already high attrition rate of drugs making it past the clinical testing [206]. In dealing with malaria however, time is especially a scarce commodity when it comes to curbing the spread of drug resistance infections [202]. However, exploring approved drugs for other diseases with the aim of utilizing them for malaria speeds up this process greatly, a process termed “drug repurposing” [207].

The advantages of repurposing allow for 1) increased success rates of passing safety requirements having been approved for human administration; 2) shortened development process as drugs have typically reached formulation development and optimization and; 3) lower risk of failure required having been through both preclinical and clinical developments, depending on the stage of advancement of the drug (Figure 6) [208, 209]. Adding onto these, repurposing also adds to the process of expanding the arsenal of antimalarials to deal with resistance. Repurposing of antimalarials for other implications has already seen its fair share of successes, with chloroquine and pyrimethamine also being used to treat amoebic liver abscess and toxoplasmic encephalitis respectively [210, 211]. As for malaria, Doxycycline, a broad-spectrum antibiotic, was approved for prophylaxis against malaria, where it is known to be active against the liver parasites while it is also slow-acting against the asexual blood stage [212]. Another antibiotic, Clindamycin, has also shown to be a slow-acting compound against malaria. Clindamycin was also tested in combination with quinine to treat drug-resistant infections, and has also shown to be efficacious against Toxoplasmosis [213, 214].

Figure 6. Comparison of A) a typical drug discovery process versus B) drug repurposing. The shortening of the duration between both processes is highlighted by the lack of need for discovery and screening and lead optimization prior to IND submission, as well as the need for Phase I clinical trials for safety validation in humans. Figure from Farha and Brown 2019 [209].

1.10 Discovery and Development of Novel Antimalarial Chemotypes

Cellular, chemical, *in silico*, and target-based approaches have been taken to unearth the next antimalarial candidates [202, 215]. *In silico* screenings provide cost-effective ways of large-scale testing, although it only serves as a decent springboard onto *in vitro* validation [216, 217]. Target-based approaches are typically limited by the poor understanding of the parasite's unique biology, while cellular phenotypic assays are the most direct form of screening assays with the ability to be applied in a mid- to high-throughput fashion [218-221]. A simple and classical approach would be to determine parasite growth inhibition, where quantification is based on a cell counting assay through

a reporter dye/protein [220, 221]. Libraries consisting of a variety of novel chemical scaffolds typically have the biggest potential for identifying compounds which fit the profile of an ideal antimalarial, with examples of breakthroughs derived from such sources [185, 222, 223].

As overcoming drug resistance is of great importance, chemical substructure analyses serves as an important step in sifting out compounds which might potentially encounter cross-resistance [224]. Once shortlisted, candidate hits are evaluated through multiple phenotypic assays for desired properties to be prioritized and brought forward for more complex characterization assays. Lead optimization by Structure Activity Relationship (SAR) studies are then applied to further optimize the lead candidate to attain more favourable pharmacologic and efficacious properties (Figure 7) [202]. Of note, research into discovery and development of novel antimalarials has identified a number of potent candidates which have advanced deep into clinical testing [6]. However, the developmental pipeline of antimalarials faces not only the canonical challenges of drug discovery, but is also further compounded by unique challenges posed by the disease. Of these unique challenges, drug resistance tops the list of concerns, although this is also commonly seen in antibiotic development [225]. This is reflected by the lack of novel antimalarial classes introduced in the last decade, with the most recent antimalarial being Tafenoquine, which is actually an improvement of the 8-aminoquinoline Primaquine [226]. Furthermore, the lack of follow-up healthcare in countries endemic to the disease sets even more stringent requirements of up-and-coming antimalarials. These include the need to be orally bioavailable, tolerable, require few doses for effective therapy, and cheap to produce [227]. Still, the lessons from these failures have served to shape the guidelines for which drug design for malaria are based on [228].

Figure 7. A typical antimalarial drug discovery and development process. Beginning with high throughput screens, large compound sets or naturally derived samples are tested in vitro or in vivo in mouse models for activity against the parasite. Depending on whether a known target is available, cell-free assays can then be used to complement the screens. Subsequently, *in vitro* *P. falciparum* assays such as drug resistance profiling, screening for drug-resistant mutants, stage-specific activity, and other compound characterization studies are done. Following which, selected compounds are then put through in vivo assays that include the *P. berghei* four-day test for suppression, followed by basic toxicology, pharmacokinetics/pharmacodynamics, and even detailed toxicology studies.

Medicinal chemistry and lead optimization then follow to address the shortfalls and enhance the properties of lead compounds. All these before taking a lead candidate into preclinical and clinical development. Figure from Fidock *et al.*, 2004 [229].

Apart from the antimalarials and ACT combinations approved for use, the malaria drug development pipeline has amassed a number of promising candidates over the years [230]. The MMV creates partnerships with big pharmaceuticals and academic institutes alike with the network of expertise and resources to accelerate product development [231]. This has resulted in a portfolio of candidates which meet the profiles of the next-generation antimalarials (Figure 8) [231].

Figure 8. The MMV antimalarial pipeline. Drug are classified under three broad categories based on the stage of their development: translational, product development, and access. Each box represents a therapeutic candidate, underscored by their discovering institute. Figure from Hooft van Huijsduijnen *et al.* 2019 [231].

This translational framework anchored by the MMV has garnered the success of producing some of the most clinically advance drugs which were discovered from the vast collection of novel chemical

compounds in the laboratories. High throughput systems have allowed for rapid screening and identification of novel chemotypes with antimalarial activity. Compounds such as the ACT-451840, identified via a collaboration between Actelion Pharmaceuticals and the Swiss Tropical and Public Health Institute in a chemical compound library screen, have successfully reached and completed phase I and is moving into human efficacy studies [232-235]. Another screen conducted by the University of Dundee on their own library led to the discovery of M5717, then known as DDD107498 [223]. Using the quinoline-4-carboxamide compound to select for resistant parasites *in vitro*, it was found that it inhibited the elongation factor 2 [223]. Preclinical studies then used M5717 in a combination with pyronaridine, to prevent recrudescence *in vivo* [236]. Ultra-high throughput screens developed by the then Novartis' Genome Research Foundation screened their collection of millions of compounds against the asexual stage parasites [237]. That served as the base for the discovery of KAE609 and KAF156, now known as cipargamin and ganaplacide respectively [185, 231, 238]. As described earlier, generation of resistant mutants against these compounds identified novel molecular markers of resistance [185, 191]. Both cipargamin and ganaplacide have cleared phases I and IIa as monotherapies, while also shown to be efficacious against *P. vivax*. [185, 239-241].

Some of these compounds came from a more targeted approach. Imatinib, a known tyrosine kinase inhibitor was identified from a functional screen against the erythrocyte tyrosine kinase inhibitor [242]. It was shown to inhibit parasite egress, and because it is known to be safe for children and cancer patients with little side effects, was considered to be a prime antimalarial candidate [243, 244]. Sevuparin, a heparin analogue with low anticoagulant activity which has shown to inhibit parasite cytoadhesion and rosetting, has reported good phase I data [245, 246]. Despite showing lower than expected efficacy in tandem with atovaquone-proguanil, it was later used in a clinical trial for severe malaria, with results still being evaluated [247]. More prominently, a multi-institutional collaboration to develop a synthetic endoperoxide which rivals the activity of artemisinins led to the discovery of the trioxolane, OZ277 [248]. Being a synthetic compound, it is able to simplify and drive down the production cost of artemisinin and their derivatives, yet also made with significantly improved

pharmacokinetic properties [248]. The compound was later then taken forward later to produce a new clinical candidate with longer plasma half-life and higher exposure in hopes of being part of a single-dose cure, leading to the development of OZ439, now known as artefenomel [249]. Currently, artefenomel is trialed in a combination with ferroquine, having passed phase I and IIa studies [250]. With concerns over its lack of activity against *k13*-mutant parasites, a new analogue, the OZ609 was developed [251]. Now called MMV052, it is undergoing clinical safety testing [231].

1.11 Identifying and Characterization of Novel Mechanisms of Action

In comparison to other approaches in drug candidate discovery, target-based identification methods in malaria is currently limited due to the lack of identified druggable targets in *Plasmodium* [202]. In fact, following the successful completion of sequencing the *Plasmodium* genome, almost half of the genes remain annotated as hypothetical due to the lack of knowledge or homology to known proteins [252]. This indicates that much is still left to be understood about the biology of these parasites, while also suggesting there may be a large untapped pool of potential targets for antimalarial development [252, 253]. Characterizing proteins as drug targets can either provide information on the optimization of the chemical scaffold, or provide the platform for the discovery of other chemical entities [254, 255]. Discovering novel antimalarial chemical scaffolds also serves a secondary purpose of identifying new antiplasmodial targets [205]. This can be achieved by coupling drug development with the current advancements in molecular biology techniques [256]. One such example is the recently established Cellular Thermal Shift Assay (CETSA). One of the common ways a drug exerts its pharmacodynamic effect is through binding directly to one or multiple protein targets. This binding typically occurs at a functional site, thus enhancing or inhibiting the protein's function and subsequently leads to the physiological perturbations in the cell. However, as drug-target association is difficult to observe *in situ*, it is difficult to identify binding targets, all the more so amongst eukaryotic proteomes made up of thousands of proteins [257]. Through the use of CETSA, this issue may be overcome. Based on thermal stability of proteins during heating, CETSA is modelled upon the increase of protein melting temperatures upon binding to its ligand, characterizing the engagement of the drug to its target

(Figure 9) [258]. Coupled with mass spectrometry, the method allows for a non-biased monitoring of drug-protein binding against the entire proteome to be done in a single reaction [259]. Recently, the CETSA method has been developed to be applied in *Plasmodium*, critically where the protein melting temperature profile of the entire parasite proteome was assessed to determine optimal thermal conditions for use to study thermal stabilisation of a wide range of proteins [260].

Figure 9. Graphical abstract of the thermal shift basis of CETSA. The “melt curve” of a protein (blue solid line) is demonstrated as its relationship between percent folded protein (or thermal stability) and temperature, where its T_m (blue dotted line) is indicated as the temperature in which 50% of the proteins are denatured. The assay is based on the principal that a protein would be more thermostable upon ligand binding, in this case, a chemical compound. This can be observed with an increase in the T_m of the protein when bound to it ligand, and an overall increase in proportion of denatured protein in response to heat (pink dotted line). Figure obtained from Friman 2020 [261]. In our review of molecular markers of drug resistance, we have discussed the identification and characterization of the

*Pf*ATP-4 as the target of cipargamin, a clinical drug undergoing development by the MMV. Below, we review two more examples of antimalarials under the MMV portfolio where despite their advances down the drug development pipeline, the understanding of their targets was not neglected, and as a process, aided in their development as potent antimalarials.

1.11.1 MMV048

The 2-aminopyridine, MMV048, was identified from a high-throughput screen on asexual stage parasites *in vitro*. It was shown to be a potent asexual stage antimalarial, with a dose of only 1mg/kg needed to control a rodent model infection. MMV048 was initially found to be the most active in the schizont stages, but later further characterized to be active against all other stages of the life cycle except hypnozoites. More importantly, MMV048 has good pharmacokinetic properties, making it an ideal candidate for single exposure radical cure and prophylaxis (SERCaP) [262].

The target of MMV048 identified via protein affinity pull-down using the compound, in which a single protein, the *P. falciparum* Phosphatidylinositol 4-Kinase (*Pf*PI4K, PlasmoDB geneID: *PF3D7_0509800*). While the binding affinity of the compound to *Pf*PI4K were not in agreement with the *in vitro* efficacy, there was a correlation between *in vitro* efficacy and the efficacy of the MMV048 analogues. Together with the cell-free functional inhibition of *Pf*PI4K activity by MMV048, this confirms the protein as the cellular target [262]. While MMV048 has already progressed into clinical trials, the identification of the *Pf*PI4K has led to efforts to optimize the next generation of inhibitors to achieve better potency across the targeted stages of the parasite's life cycle as well as improved pharmacokinetic properties [263].

1.11.2 DSM265

DSM265 was discovered through a target structure-based screen [264]. The target in mention is the parasite's Dihydroorotate Dehydrogenase (DHODH, PlasmoDB geneID: *PF3D7_0603300*), which is part of the pyrimidine biosynthesis pathway that is an essential source for nucleoside synthesis [78]. Being structurally different from the human DHODH, targeting the parasite's enzyme makes for drug design

with good selectivity [265]. Earlier attempts to identify chemical scaffolds had already yielded two other different compound classes which were established to bind sub-optimally to DHODH. These compounds already exhibited potent enzyme inhibition activity, but did not have achieve good enough *in vivo* efficacy for clinical trials.

Using the X-ray structure of DHODH to identify the binding sites of previous inhibitors, a structural-guided approach was conducted to design a compound with optimized binding affinity that also possesses good drug-like physiochemical properties [264]. It was through this approach that the Trizolopyrimidine structure of DSM265 was designed and synthesized. Subsequent studies showed that DSM265 possessed potent antimalarial activity correlative to it binding affinity not only in the parasite IDC, but also in liver stage schizonts [266]. At the same time, a long half-life made it a candidate suitable for consideration not only for treatment, but also prophylaxis. [266]

Aims of this Study

The benefits of identifying novel chemotypes for drug development spans two arms: the discovery and development of the next antimalarial candidates; as well as uncovering new druggable pathway or target for which therapeutic strategies can be developed upon. Using libraries with diverse chemical scaffolds, we aim identify novel chemotypes which display as many desirable traits as described with the vision to bring forth a candidate to be considered for advancement into clinical testing.

In order to have the highest chance of success in finding a clinically-ready candidate for field testing, we reach into the Selleck Chemicals Anticancer Library to identify a compound with activity against the symptomatic asexual stage malaria. With an expected number of hits to assess, we will perform literature reviews of clinical data to triage and prioritize these compounds to eventually obtain a select few for proposal into human infection trials.

We also aim to uncover new chemical scaffolds with antimalarial properties from screening the biologically-untested Nanyang Chemical Compound Library. Using guidelines as described earlier, we would characterize our hit(s) with efficacy, toxicity, and phenotypic assays and identify the candidates displaying the most ideal properties. We would then leverage on one of them as a tool to shed light on new druggable target(s) and/or mechanisms of action against the *Plasmodium* parasite, and functionally characterizing them to the best of our abilities.

Chapter 2: Materials and Methods

2.1 *P. falciparum* *in vitro* continuous culture and synchronization

P.falciparum 3D7, Dd2, K1, T994, W2, and W2mef cell lines were obtained from Malaria Research and Reference Reagent Resource Center (MR4), BEI Resources. Artemisinin-resistant and -sensitive parasite field isolates from Ratanakiri, Cambodia were obtained by request from the Tracking Resistance to Artemisinin Collaboration (TRAC). *In vitro* parasite culture was performed as described by Maier and Rug 2013 [267]. Briefly, cultures were maintained with RPMI medium supplemented with Albumax II; 81g/L RPMI-1640 (Gibco, Life Technologies), 2.5g/L Albumax II (Gibco, Life Technologies), 2.3g/L Sodium Bicarbonate (Sigma-Aldrich), 50mg/L Hypoxanthine (Sigma-Aldrich), 10mg/L Gentamicin Sulphate (Gibco, Life Technologies), in 2-3% haematocrit human RBCs collected via patient donation at National University Hospital, Singapore. Cultures were maintained with a special mixed gas of 3% O₂, 5% CO₂, balanced with N₂ (SOXAL, Air Liquide, Singapore). For cultures grown in plate format, plates were kept in a sealed bag filled with mixed gas. Parasitemia and parasite stages were checked by microscopy of blood smears fixed in Methanol (Merck), stained in Giemsa (Sigma-Aldrich) in 1x Tris-Base, Acetic Acid, EDTA (TAE) buffer (analytical grade, Fischer US)

Synchronization of ring stage parasites (0-20 hpi) was done by incubating packed RBC culture with 5% (w/v) D-Sorbitol (Sigma-Aldrich) at 37°C for 20 min. Cultures were washed with incomplete RPMI (iRPMI), 900 xg, 3 min, low brake to remove sorbitol and cell debris. Late stage parasites (30-48 hpi) were isolated using a 68% Percoll (MP Biomedicals) gradient prepared with 1x Phosphate Buffer Saline (PBS) and iRPMI. Packed parasite cultures were diluted with iRPMI 1:5, then laid carefully atop of the 68% Percoll gradient in a 15mL Falcon tube. Tubes were spun at 900 xg for 20 min, no brake. RBCs collected at the iRPMI-Percoll interface were carefully removed and washed in iRPMI twice.

2.2 Chemical Compound Library Screen

2.2.1 Chemical Compound Libraries

The Selleck Chemicals Anticancer Library was purchased from the Genome Institute of Singapore (GIS). 0.1µL of 10mM library compounds in DMSO were “pre-printed” onto flat-bottom 96-well plates via echo-pulsing. Plates were stored in -80°C and pre-warmed at room temperature before use. The Nanyang Compound library was obtained courtesy of Prof Tan Choon Hong’s lab, School of Physical and Mathematical Sciences, NTU. It consisted of a total of (at that point) seven 96-well plates of compounds pre-dissolved in DMSO at a concentration of 10mM. Compound plates were stored at -20°C until use.

2.2.2 Mid-throughput Screening

Compounds were screened for antiparasitic activity by growth rate over a duration of 60hrs (slightly over one IDC). ~1% parasitemia of 0-3hpi ring stage culture in 2% haematocrit were treated with 10µM of library compound (0.1% final DMSO concentration), and seeded into 96well flat bottom plates. Controls of 0.1% DMSO- and 10µM chloroquine-treated cultures, and 2% haematocrit uninfected RBCs were also done. Culture volume per well was 100µL. Plates were sealed in air-tight bags with gas mixture and incubated at 37°C. After 60hr of treatment (to allow ring stages to develop a more defined morphology), taken for Fluorescent-activated Cell Sorting (FACS) read out.

Assay read out was done by staining with Hoechst 33342 (Excitation 350nm, Emission 461nm; Molecular Probes, Life Technologies), a cell permeable DNA staining dye. Staining solution was prepared by diluting DMSO stock of Hoechst 33342 1:1000 in 1x PBS (16.2µM). Plates were spun at 2,200rpm for 5min, brake 1 to pellet cells. Culture supernatant was removed, and 100µL of staining solution was added to each well and incubated at 37°C in the dark for 20min. 200µL of cold 1x PBS was then added to the wells to dilute the dye and stop the staining. Cells were read via a High Throughput Sampler (HTS)-coupled FACS machine (BD Biosciences) in the UV laser excitation channel (Excitation 355nm).

Parasitemia obtained from test and control wells were used for the calculation of % growth inhibition of each well. % growth inhibition was calculated as $100\% \times \left(1 - \frac{\text{Screen } P\% - \text{CQ } P\%}{\text{DMSO } P\% - \text{CQ } P\%}\right)$, where P% is parasitemia. Average % growth inhibition between replicates were calculated and plotted in Microsoft Excel. Hits displaying $\geq 95\%$ growth inhibition were considered as hits

2.3 *P. falciparum* Dose Response Assay

2-fold serial dilutions of compounds were done in 50 μ L starting compound treatment concentration in the 96well flat bottom plate in replicates. *P. falciparum* 0-3hpi ring stage parasite cultures were adjusted to 1% parasitemia in 4% haematocrit, then 50 μ L of culture stocks were added to the treatment wells. Controls of 0.1% DMSO- and 10 μ M chloroquine-treated cultures, and 2% haematocrit uninfected RBCs were also done. Cells were incubated for 60hr before staining with Hoechst 33342 for FACS reading as per described in compound library screen. Parasitemia read outs between bioreplicates were averaged and plotted on the IC Estimator (ICE) 1.2 online software for calculation of Inhibitory Concentration 50 and 99 (IC₅₀ and IC₉₉) values (<http://www.antimalarial-icestimator.net/MethodIntro.htm>).

2.4 *In vitro* Toxicity Assay

2.4.1 *In vitro* Culture of Mammalian Cell Lines

A549 (human lung carcinoma), HEK 293 (human embryonic kidney), HepG2 (human liver cancer), and AGS (human stomach gastric adenocarcinoma) cell lines were obtained from ATCC. Cultures were grown in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS; Gibco), and 10,000U Penicillin-Streptomycin in 5% CO₂ at 37°C. Culture media was changed every 2-3 days. Confluency was monitored under microscopy at 40X magnification. At around 70-90% confluency, cells were passaged by resuspending in culture media following trypsin-EDTA (Gibco) treatment, and then diluted to be seeded at a lower density.

2.4.2 Determining Cell Viability of Mammalian Cells in Dose-Dependent Toxicity Assay

Mammalian cell cultures were trypsinized between 80-90% confluency and washed in culture medium. Cells were diluted in culture medium and counted on a haemocytometer, then further diluted in culture media to obtain 10,000 cells/mL. 1,000 cells were seeded per well and left at 37°C overnight to settle and adhere.

Treatment plate containing 2-fold serial dilutions of compounds dissolved in DMEM culture media were prepared. DMSO, artemisinin, and staurosporine (Sigma-Aldrich) – a protein kinase inhibitor which induces apoptosis in mammalian cells – served as controls. Before treatment, culture media in all plates were aspirated, and then replaced with media from the treatment plate, and incubated at 37°C for 72hr.

Following the 72hr treatment, cell viability was assessed by cellular redox potential. 20µL of CellTiter 96 Aqueous One Solution (Promega) was added to each well. Plates were incubated at 37°C for 1-2hrs in the dark. Absorbance readings were taken at 490nm over intervals of the incubation period.

2.5 Artemisinin Resistance 6hr Ring Stage Survival Assay

Ring stage survival assay for investigation of artemisinin resistance was done as per previously described but modified for a high throughput format [167]. Dd2 carrying the k13^{I543T} mutation (obtained as a gift from Xiong A. of Singapore-MIT Alliance Research Institute), and its parent culture were synchronized for 0-3hpi ring stage parasites by sorbitol as mentioned earlier. Synchronized cultures were adjusted to 1% parasitemia, 2% haematocrit. Cultures were treated with either 1µM of A1 or dihydroartemisinin (DHA; Sigma-Aldrich), and seeded in 100µL volumes in triplicates. Five 2-fold serial dilutions of each treatment were made. Treatments were incubated for 6hr, then washed with cRPMI twice to remove the drug. Fresh cRPMI was added to each well, and incubated for 66hr.

Following the 66hr incubation, the cells were washed twice with iRPMI. Staining was done using 0.4x SYBR Green and 1.8 μ M MitoTracker Deep Red FM (Thermo Fischer Scientific) in 1x HBSS supplemented with 2% FBS. Cells were stained at 37°C for 30min in the dark. Cells were washed twice with 1x PBS, and resuspended with 1x PBS before reading by HTS-coupled FACS. % Survival was calculated and plotted in a %survival vs dose curve on Microsoft Excel.

2.7 *In vitro* Generation of Spontaneous Resistance

Prior to drug selection, IC₅₀ and IC₉₉ of test compounds A1 and Atovaquone (Sigma-Aldrich) were re-determined in 1% parasitemia for *P. falciparum* Dd2 and W2 cell lines. Inhibitory concentration values were determine as mentioned above.

In order to obtain a 10⁹ number of parasites, 1mL of packed infected Dd2 or W2 cultures was adjusted to 1% parasitemia. Drug treatment was done in doses of 10x IC₅₀, 2x IC₉₉ or 10x IC₉₉ as performed in previously published experiments [178, 191]. Drug wash-offs were done by centrifuging cultures followed by two more washes with iRPMI. Cultures were maintained by changing media, with or without drug every other day, and parasites are monitored for by microscopy of Giemsa-stained blood smears during every passage. Cultures are split 1:2 once every two weeks to maintain freshness of blood.

2.8 Stage Specific Activity Assay

3D7 0-3hr ring stage parasite cultures were adjusted to 1% parasitemia in 2% haematocrit. Individual cultures were either treated with compound 10 μ M A1, DMSO or artemisinin, at 0, 12, 24, or 36hr of the IDC. Smears of each well were made at every 12hr intervals post treatment (Figure 7). Giemsa-stained blood smears were visualization under a light microscope for morphological changes

2.9 Late Stage (Stage III) Gametocyte Development Inhibition Assay

2.9.1 *P. falciparum* Gametocyte Induction and Purification

Gametocyte induction was done as per described with minor adjustments using 3D7 parasites [268]. Briefly, a culture of 3% parasitemia at schizont stage in 3% haematocrit was given fresh cRPMI. On Day 1, when parasitemia would reach 9-12%, culture media was changed with 30mL fresh cRPMI and 20mL spent media. On Day 2, culture was split 1:3, and 1mL packed volume of RBCs were added to obtain 3% haematocrit. 40mL of fresh cRPMI with 10mL of spent media was added. On Day 3, when parasites would have been gametocyte-committed, media would be replaced entirely with 10.01mg/mL N-acetyl-D-glucosamine (Sigma-Aldrich) containing media to eliminate remaining asexual stage parasites. NAG media was replaced once every two days. Gametocytes were purified of cell debris by purification on 68% Percoll and washed thoroughly as described previously.

2.9.2 Gametocyte Development Inhibition Assay

Late gametocyte inhibition assay was done with minor adjustments described previously [60]. Gametocytes purified at Day 9 of the gametocyte induction were counted for gametocytemia from a Giemsa smear. Gametocytemia was adjust to 2%, in 2% haematocrit using cRPMI and fresh uninfected RBCs, and seeded 100 μ L per well in a 96-well plate. Cultures were treated with either 0.1% DMSO, 500 or 5000nM lumefantrine (Sigma-Aldrich), or 1000, 5000, or 10000nM A1. Treatment media was changed once every two days, and on Day 15, cultures were washed twice with cRPMI and Giemsa smears were made. Gametocytemia for each treatment was determined by counting 2000 cells per treatment.

2.10 Cellular Thermal Shift Assay (CETSA)

Protocol by Dziekan J. *et al.* 2020, manuscript accepted. Same procedures were used in Ring and Trophozoite Stage lysate CETSA and In-Cell Gametocyte CETSA unless otherwise stated.

2.10.1 2x Lysis Buffer

CETSA Lysis buffer contains 100mM HEPES (Goldbio), 10mM β -glycerophosphate (Merck), 0.2mM activated Na_3VO_4 (Merck), and 20mM of MgCl_2 (Merck), dissolved in water. Na_3VO_4 was activated by boiling 200mM stock solution for 10-15min until it turns colorless and cooled to room temperature. Prior to resuspending the lysates, 1x solution is made by mixing 1:1 with water and adding 1:1000 EDTA-free protease inhibitor cocktail (Naclai-Tesque). 1M Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sinopharm Chemical Reagent Co, Ltd) was added into lysis buffer for trophozoite lysis buffer.

2.10.2 Parasite Sample Preparation

2.10.2.1 Parasite Lysates

Synchronous parasites of either 10-15hpi or 26-32hpi (prepared by Dziekan J.) were pelleted from culture and washed once with 1x PBS. Packed RBC volume was estimated, and 9 volumes of fresh ice-cold 0.1% Saponin (Sigma-Aldrich) was added to the RBC volume. Lysis was incubated on ice for 5min with intermittent vigorous shaking every minute. Parasite lysates were pelleted at 2,500 xg for 5min to remove supernatant. Pellets were washed at least two times or more with ice-cold 1x PBS until supernatant is not red.

2.10.2.2 Live Stage III Gametocytes

NF54 iGP2 high gametocyte-producing cell line was obtained as a gift from Till Voss, Swiss Tropical and Public Health Institute/University of Basel, Switzerland. Asexual stage parasites were grown in cRPMI supplemented with 2.5 μ M D-glucosamine (Sigma-Aldrich) at 2-2.5% haematocrit and synchronized to a 6hr window. To induce parasites for sexual stage commitment, ring stage parasites were grown in media without D-glucosamine for 1 cycle, following which D-glucosamine is then replaced and media

was supplemented with N-acetyl D-glucosamine. At Day 8 post induction, Stage III gametocytes were enriched for by Percoll and washed thoroughly to remove cellular debris. Gametocytemia and cell counts were done to determine number of cells.

2.10.3 Cell Lysis by Freeze-Thaw and Mechanical Sheering

This procedure was not done yet for in-cell CETSA. Cells were freeze-thawed for three cycles in liquid N₂ and room temperature water. Mechanical sheering was then done by passing cells through a 26g needle followed by a 31g needle 10-20 times each while on ice. Cell suspension was centrifuged at 20,000xg for 20min at 4°C. Supernatant was collected. 1:1 volume of 2x Lysis buffer was added to the remaining pellet and resuspended, then subjected to the repeat of the freeze-thaw and mechanical sheering procedure. Cell suspension was centrifuged at 20,000xg for 20min at 4°C, and supernatants from both lyses were pooled together. Protein concentrations of soluble lysates were quantified with the BCA Protein Quantification kit (ThermoScientific) as per manufacturer's instructions

2.10.4 Drug Treatment and Melt Curve

2.10.4.1 Lysates

Eight 4-fold serial dilutions of 10mM A1 in DMSO were done. A non-treated (DMSO only) well was also prepared. Parasite lysates were diluted to a 2.5mg/mL protein concentration and brought to room temperature. Starting with the undiluted 10mM compound up to the DMSO, 3uL was carefully transferred into each well of a PCR plate. 300μL of the prepared lysate stock was then added to each compound-containing well. Treatment was incubated for 3min at room temperature. During incubation, treatments were split into three equal (two for trophozoite CETSA) 100μL wells on different plates. Each plate was then sealed and subjected to 3min heat challenge at 37, 51, or 57 °C (57°C not done in trophozoite CETSA), followed by 3min cooling at 4°C. Heat treated samples were then centrifuged at 20,000xg for 20min at 4°C to pellet denatured proteins. Equal volumes of supernatant were collected while being careful not to disturb denatured protein pellet. Samples were frozen at -80°C until processing.

2.10.4.2 Intact Cell

Eight 4-fold serial dilutions of 10mM A1 in DMSO were done. A non-treated (DMSO only) well was also prepared. Parasite cells were diluted to 10^7 cells/mL in cRPMI. Starting with the undiluted 10mM compound up to the DMSO, 3 μ L was carefully transferred into each well of a PCR plate. 300 μ L of the prepared cell stock was then added to each compound-containing well, then put into a bag and gassed. Treatment was incubated for 1hr at 37°C. After treatment, three equal 100 μ L wells for each treatment were made in different plates. Each plate was then sealed and subjected to 3min heat challenge at 37, 51, or 57°C, followed by 3min cooling at 4°C.

Thermal challenged cells were freeze-thawed for three cycles in liquid N₂ and room temperature water. Mechanical sheering was then done by passing cells through a 26g needle followed by a 31g needle 10-20 times each while on ice. Cell suspension was centrifuged at 20,000xg for 20min at 4°C. Equal volumes of supernatant were collected while being careful not to disturb denatured protein pellet. Samples were frozen at -80°C until processing.

2.10.5 Haemoglobin Depletion

Done only for in-cell Gametocyte CETSA. Protein amounts were quantified by BCA assay as per previous. 150 μ g of protein from each sample was transferred into a new tube, and added with 15 μ L of HemoglobindTM resin suspension (Biotech Support Group). Top up volume with 20mM K₃PO₄ (pH6.5) until 2x original volume is reached. Tubes were vortexed to mix resin, then put on constant rotation for 15min at 4°C.

Tubes were centrifuge at 8,000xg for 1min at 4°C. Protein-resin mix was resuspended and applied onto a 96well 0.22 μ m filter plate with an attached collection plate below. Plate was centrifuged at 800xg for 3min at 4°C. Protein concentrations of each sample were measured again using a Reducing Agent Compatible BCA Assay Kit (ThermoScientific) as per manufacturer's protocol.

2.10.6 Protein Reduction, Alkylation, and Digestion

30µg (15µg for Gametocyte CETSA, remaining volumes adjusted accordingly) of protein from each sample were added with 1M TCEP, 1% RapiGest (Waters), and 1M TEAB (Merck) to a final concentration of 100mM TEAB, 20mM TCEP, and 0.05% RapiGest. Reactions were incubated for 20min at 55°C. Chloroacetamide (CAA, Merck) was added to a final concentration of 55mM and vortexed, followed by incubating in the dark for 30min at room temperature. 100mM TEAB was added to reduce RapiGest to 0.025%. 3µL of 2.33AU/µL LysC (Fujifilm Wako Chemicals) solution was added the samples, and incubated for 3hrs at 37°C. 3µL of 0.5µg/µL Trypsin (Mass Spec Grade, Promega) was added to each sample and incubated for 18hrs at 37°C.

One-tenth reaction volume of 10% Trifluoro Acetic Acid (TFA, Merck) was added to each sample and vortexed to mix, then incubated for 45min at 37°C. Samples were then centrifuged at 20,000xg for 15min at room temperature. 80% of supernatant was recovered and dried on a centrifugal vacuum evaporator. Dried peptide samples were resuspended in 200µL of 200mM TEAB pH8.5 and dried again. Dried peptides were then resuspended in 24µL 200mM TEAB pH8.5.

2.10.7 Quality Check of Sample Processing

2.10.7.1 Stage Tip Preparation

Desalting Stage Tips were prepared by stacking a 10µm filter paper (Mobitec) followed by HLB 30µm powder (Oasis) in a 200µL sterile pipette tip. Column was then activated by pushing through 100µL of 100% Acetonitrile (Merck) by air.

2.10.7.2 Preparation of Minicurves

0.5µL of each sample of a curve was combined and added into 95µL ammonium formate (Merck). Stage Tip was first washed with 100µL 10mM ammonium formate, then minicurve was pushed through by air. Stage Tip was then washed again with 100µL 10mM ammonium formate. Peptides were then eluted by pushing through 100µL 10mM ammonium formate in 50% acetonitrile (Merck). 20µL of 0.1% formic acid, 60% acetonitrile was added to the eluted peptides. Peptides were dried in a

centrifugal vacuum evaporator at 60°C, then resolubilized in 10µL 0.5% acetic acid, 0.06% TFA, 1% acetonitrile.

2.10.7.3 Digestion Quality Check by MS

An equivalent of 1µg was injected into the LC-MS/MS system and analysed. Raw data file of each minicurve was processed with Proteome Discoverer software to ensure a maximum of <10% missed cleavages was seen in the minicurve.

2.10.8 TMT Labelling

10µL of each sample of the curves (~10µg peptides) were added with 10µL TMT10plex reagent (Thermo Fisher Scientific) of different isobaric mass tags. Labelling reaction was incubated overnight in the dark at room temperature.

2.10.9 Quality Check of TMT Labelling

2.10.9.1 Stage Tip and Preparation of Minicurve

Done as per described for digestion quality check.

2.10.9.2 TMT Labelling Efficiency Quality Check by MS

On Proteome Discoverer, the search for labelled and unlabelled peptide masses were conducted. For good labelling efficiency, 90% labelling should be achieved.

2.10.10 Sample Desalting and Fractionation

TMT labelling reaction was quenched by adding 50µL of 1M Tris (pH7.4). Quenched reactions of each curve were then combined with 490µL of 0.5% acetic acid, then 10µL of TFA was added to each pool. An HLB column (Oasis) for each curve was equilibrated with 1mL 100% acetonitrile, then washed once with 1mL 0.5% acetic acid. Pooled curve samples were then pushed through by air, followed by one wash with 1mL 0.5% acetic acid. Samples were then eluted with 1mL of 0.5% acetic acid, 50% acetonitrile. Samples were then dried in a centrifugal vacuum evaporator at 60°C, then resuspended in 1mL of 5% acetonitrile, 5% ammonia. Samples were centrifuged at 12,000xg for 5min at room

temperature to pellet solid debris (if any). Sample was fractionated by high pH reverse phase fractionation protocol on a HPLC. Fractionation by HPLC done by Wirjanata G. for gametocyte in-cell CETSA, Go K. D. for ring lysate CETSA.

2.10.11 Sample Concatenation and Mass Spectrometry

Sample is injected into the ÄKTAmicro system and separated on a high-pH reversed-phase Zorbax 300 Extend C-18 column. Detailed parameters for peptide fractionation and gradient elution are described in Table 1. Fraction collection on a 96-well plate begins after the initial 5 min of the gradient elution, starting with well A1, and lasts for 94 min to collect all fractions. Only fractions from wells B3–H12 are used for subsequent processing. Collected fractions for each curve were pooled into 20 separate tubes based on the pooling scheme and dried in a centrifugal vacuum evaporator at 60°C. Each dried fraction was then washed with 100µL of 0.1% formic acid, 60% acetonitrile twice by drying. Wirjanata G. helped with the pooling for gametocyte in-cell CETSA, Go K. D. helped with the pooling for ring lysate CETSA.

Dried fractions were then resolubilized in 10µL of 0.5% acetic acid, 0.06% TFA, 1% acetonitrile. Fractions were plated onto a 96well autosampler plate, injecting 2µL of peptide from each fraction into the LC-MS/MS. Each sample is loaded onto the trap column at a flow rate of 25 µL/min for 5 min and eluted over the analytical EASY-Spray LC column (50 °C) for 70 min with a flow rate of 0.3 µL/min. The instrument's nanospray-ionization (NSI) settings are as follows: spray voltage 2.1 kV, capillary temperature of 300 °C, and S-lens radiofrequency level of 60.0. MS/MS scan parameters are as such:

Parameter	Value
General	
Run time	0–70 min
Polarity	Positive
Default charge	2
Full MS	
Resolution	70,000
AGC (automatic gain control) target	3.0×10^6
Maximum ion time (IT)	100 ms
Scan range	350–1,550 m/z
dd-MS²	
Resolution	35,000
AGC target	1.0×10^5
Maximum ion time	80 ms
Loop count	12
TopN	12
Isolation window	1.6 m/z
Fixed first mass	110.0 m/z
Normalized collision energy/stepped normalized collision energy	34
dd settings	
Minimum AGC target	8.0×10^3
Charge exclusion	Unassigned, 1, 8, >8
Peptide match	Preferred
Exclude isotopes	On
Dynamic exclusion	45 s

2.10.12 Data Analysis

Raw data files from all 20 fractions of a curve were merged into a single list on Proteome Discoverer for protein quantification. Treatment concentrations corresponding to each isobaric mass of the TMT labels were indicated. Finally, protein quantification data was then processed in the RStudio-mineCETSA package for normalization, curve-fitting, and graph plotting. Analysis done by Dziekan J., Wirjanata G, and Go. K. D.

2.11 Cloning of *Pf* Zinc Finger (CCCH-type) Protein Tagging Construct

2.11.1 PCR

Polymerase Chain Reaction (PCR) was done using the KOD-Plus-Neo (Toyobo) reagents. PCR Reactions were done in 50µL reactions; 5µL 10x PCR Buffer for KOD-Plus-Neo Buffer (1x), 5µL 2mM each dNTPs

(0.2mM each), 3μL 25mM MgSO₄ (1.5mM), 0.15μL each 100μM Forward Primer (0.3μM), 1μL 50ng/μL genomic DNA (gDNA) or complementary DNA (cDNA) library (50ng), 1μL 1U/μL KOD-Plus-Neo Polymerase (1U/50μL), made up with water. The typical thermal cycling conditions for PCRs were 94°C for 2min; 25 cycles of 98°C for 10sec, X°C for 20sec, 68°C for 1min; 68°C for 4min, where X is the lower melting temperature (T_m) of the two primers. Primers used for the amplification of the *Pf* Zinc Finger (CCCH-type) Protein recombination sequence are F – TGACACTATAGAATACTCGCGGCCGC TGCATCTACCTTCATCAGATGCATCAAC and R – CACCAGCAGCAGCACCTCTAGCACGCGT TTCTTTGGTTTCCCATTTCCAAACCTTTTG. Primers used to validate the 5' integration site are F – GATTTTCGTACCTGGGGAGGGTAATAAAG and R – CTTGAGCCTGGCGAACAGTTC. Primers used to validate the 3' integration site are F – GTACGTTTGAAATTGGGTATATTGGGGAC and R – CCATGATTACGCCAAGCTATTTAGGTG. PCR products were run on a 1% agarose gel (1st BASE-Asia) with ethidium bromide for visualization under ultraviolet (UV) light. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) as per manufacturer's recommended protocol

2.11.2 Plasmid and Construct Cloning

The pSLI (selction-linked integration) plasmid was a kind gift from Prof Tobias Spielmann of Bernhard Nocht Institute for Tropical Medicine, Germany. 4μg of the plasmid was digested with 1.5μL each of NotI and AvrII (Fermantes) at 37°C for 8hrs. PCR product of the *Pf* Zinc Finger (CCCH-type) Protein recombination sequence was ligated by incubating the fragment with the digested plasmid in a 3:1 molarity ratio together with the NEBuilder HIFI Master Mix (NEB) for 15min at 50°C. Half of each ligation reaction was transformed into XL-10 Gold Ultracompetent cells (Stratagene, Agilent). Cells were plated on 100μg/mL Ampicillin (Sigma-Aldrich) LB agar plates (10g/L Bacto Tryptone, 5g/L Bacto Yeast Extract, 10g/L NaCl, 15g/L Agar; BD Difco, Merck). Plates were incubated at 37°C overnight. Bacteria colonies were picked and grown in 100μg/mL Ampicillin LB media at 37°C overnight. Plasmids were extracted using Favorprep Plasmid Extraction Mini Kit (Favorgen Biotech Corp) as per manufacturer's protocol. 3μL of the eluted products were restriction digested using NotI and AvrII at

37°C for 1hr, and then ran on a 1% agarose gel for confirmation of the positive ligation. Sequencing of positive clones was done by 1st BASE DNA sequencing services.

2.12 Parasite Transfection and Selection

Late stage parasites (42-48hr IDC) are put to shake in 37°C for 5 hr to avoid multiple reinvasion of RBCs. 200µL of packed infected RBCs of above 5% parasitemia (counted by blood smear) were mixed with 50µg of pSLI *Pf* Zinc Finger HA Tagging plasmid resuspended in 50µL 1:10 TE-EF and 250µL 2x Transfection Cytomix. Transfection mix were put into a 0.2cm cuvette (BioRad) and electroporated at 310V, 950F, $\infty\Omega$. Electroporated cells were transferred into pre-warmed flasks containing cRPMI and freshly drawn uninfected RBCs. Cuvettes were washed with media to obtain maximum number of cells. Drug selection media containing 2.5nM WR99210 (Jacobus Pharmaceutical) was changed once daily for the first five days, and subsequently once every other day until parasites were observed. Subsequently, the WR99210 selected parasites are then grown in the presence of 125µg/mL G418 (Gold Biotech) until parasites were observed.

2.13 Validation of Transfection Integration

2.13.1 Western Blot Validation of pSLI tagged *Pf* Zinc Finger (CCCH-type) Protein

Mixed stage parasite cultures were centrifuged at 900 xg. Saponin lysis of the culture was done as described earlier. The resulting parasite pellet was resuspended in 4 volumes of 1x PBS, then added with 6x SDS Loading Buffer with 5% β -mercaptoethanol (Sigma-Aldrich) and boiled for 5min at 95°C. Sample was run on a 12% SDS-PAGE gel at 120V and subsequently transferred onto a PVDF membrane (Biorad) at 100V for 60min with an ice pack.

Western Blotting was done first by blocking the membrane with Odyssey Blocking Buffer (PBS; Licor) for 40min at room temperature. The membrane was then incubated with 1:2000 Rat anti-HA and 1:3000 Rabbit anti-Glyceraldehyde-3-Phosphate Dehydrogenase primary antibodies overnight at 4°C. Membrane was then washed three times with 1x PBS with 0.1% Tween20 for 10min each. Secondary antibodies of 1:15000 Goat anti-Rat 800 and Goat anti-Rabbit 680 (Li-cor) were then incubated with

the membrane for 1hr at room temperature, followed by another three washes. Membrane was then scanned on the Li-cor Odyssey CLx.

2.13.2 IFA Validation of pSLI tagged *Pf* Zinc Finger (CCCH-type) Protein

Thin blood smears of the HA-tagged *Pf* Zinc Finger (CCCH-type) Protein culture at approximately 12, 30, and 44hpi were fixed in methanol for 3min. After drying, the slides were blocked with 1% Bovine Serum Albumin (BSA; Sigma-Aldrich) for 1hr at room temperature. Blocking solution was poured away and primary antibody solution containing 1:200 Rat anti-HA and 1:400 Rabbit anti-EXP2 antibody in 1% BSA was applied onto the slides. Primary antibody incubation was done for 1hr at room temperature. Slides were then washed with 1x PBS with 0.1% Tween 20 for three times for 3min each. Secondary antibody solution containing 1:500 Goat anti-Rat 594, 1:500 Goat anti-Rabbit 488 antibody, and 16.2μM Hoechst 33342 in 1% BSA was applied onto the slides. Secondary antibody incubation was done for 1hr at room temperature. Slides were then washed with 1x PBS with 0.1% Tween 20 for three times for 3min each, then imaged by fluorescent microscopy.

2.14 Domain Prediction of *Pf* Zinc Finger (CCCH-type) Protein

Amino acid sequence of *Pf*3D7_1315400 was retrieved from PlasmoDB (<https://plasmodb.org/plasmo/>). Amino acid sequence was then put into the sequence bar of Interpro protein classifications predictor (<https://www.ebi.ac.uk/interpro/>).

2.15 Western Blot Validation of CETSA Results

2.15.1 CETSA Assay

Parasite lysates and CETSA procedures were done as per previous with the screen. However, thermal challenge was done using temperatures 51, 52, 54.1, 59.6, 61.8, 63.6, 65.8, and 71.8°C with 10μM A1 or DMSO.

2.15.2 Western Blot Quantification

Band intensities for the HA-tagged protein and the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase were measured on the Image Studio software (Li-cor). *Pf* Zinc Finger (CCCH-type)

Protein relative band intensity was obtained by normalizing against the internal Glyceraldehyde-3-Phosphate Dehydrogenase band intensity.

2.16 Falcilysin (FLN) Proteolytic Cleavage Assay

Assay was done as described in publication [269]. Recombinant FLN and the Dabcyl-HKRHSFRMRG-Edans fluorescent-quencher peptides were a gift from Zbynek Bozdech of School of Biological Sciences, NTU. Briefly, recombinant FLN at a final concentration of 0.3 µg/ml in assay buffer (50 mM Bis-tris, pH 7.2) was prepared. A final concentration of 5µM Dabcyl-HKRHSFRMRG-EDANS was mixed with either 10µM A1, 1mM ZB1, 1mM Cyclohexamide, 0.1% DMSO, 0.1% Methanol, or water, and was added to each well of a black flat-bottom polystyrene 96-well plate (Greiner). Just prior to the read out, recombinant FLN in assay buffer was added. V₀ was determined by monitoring fluorescence (λ_{ex} = 340 nm, λ_{em} = 490) over 7min on a Tecan Infinite M200 Pro plate reader. Assays were done with two technical replicates each. Data of three biological replicates were averaged and plot on Microsoft excel to compare the slope of the fluorescence signal.

2.17 In vivo Studies of A1 in *balb/c* Mic Model

2.17.1 Rodent *P. berghei* Infection

P. berghei ANKA parasite line expressing Green Fluorescence Protein (GFP) throughout its life cycle was donated by Prof Christiane Ruedl, School of Biological Sciences, NTU. 4-week-old male, *balb/c* mice (In Vivos) were infected with 100µL thawed frozen vial of *P. berghei* intraperitoneally (IP). Parasitemia was monitored through obtaining blood samples via tail snips. 1.5µL of the tail snip would be added into a plate with 180µL 2mg/mL Heparin Sulphate (Sigma-Aldrich) solution were and read via FACS to determine parasitemia.

2.17.2 Formulation of A1

Compound A1 was dissolved up to 37.5mg/mL in DMSO, and serial diluted with DMSO for lower concentrations. DMSO stock solutions were then dissolved in sterile 1x PBS-diluted TWEEN-80 (1:8:1).

2.17.3 *In vivo* Efficacy Study of A1

Once parasitemia reached 3% or above, mice would be treated with either 5, 10, 15, 20 or 30 mg/kg of A1, 30mg/kg of artemisinin, or vehicle (n=5) daily for four days. Parasitemia was monitored daily by FACS, and mice were terminated by euthanasia when parasitemia reaches above 10%.

2.17.4 *In vivo* Toxicity Determination of A1

Uninfected animals were given dose of either 40, 50, 60 or 70mg/kg A1, once daily for four days and monitored for signs of poor drug tolerability and or toxicity over the first 15min of treatment and once daily for three days after final treatment.

2.17.5 *In vivo* Pharmacokinetics of A1

2.17.5.1 Dose Administration and Sample Collection

4-week old mice were given 30mg/kg doses of A1 in formulation and were sacrificed (by Pentobarbital overdose) at time-points of 0 min, 15 min, 30 min, 1hr, 2hr, 4hr, 8hr, 16hr and 24hr post administration for blood collection via cardiac puncture. Each timepoint consists of an n=5 sample size. Collection of blood will be done with heparinized needles. Blood samples will be centrifuged at 1000 g for 10 min at room temperature within 1 h of collection to obtain plasma. Plasma samples will be stored at -80°C until quantification analysis. Blood from undosed mice will also be collected.

2.17.5.2 Sample Preparation

Sample preparation was performed by Pang Y. Y. Protein precipitation was done using methanol at the ratio of 1 part sample to 3 parts methanol. Internal standard solution was prepared at 200 ng/mL in methanol. 150 µL of internal standard solution was added to 50 µL of sample and vortexed. Samples were centrifuged at 17,000 rpm for 10 min. Supernatants were carefully transferred out into LCMS glass vials and analysed on the same day via LC-MS/MS.

2.17.5.3 LC-MS/MS Analysis

LC-MS/MS was done by Pang Y. Y. Analysis of plasma samples will be carried out via LC-MS/MS. Briefly, calibration standards and QC samples will be generated via compound and internal standard spiked plasma samples. Calibration curves will be generated by plotting response against concentration. Collected mouse plasma samples will be run through the same sample treatment and LC-MS/MS analysis as calibration standards and QC samples. Concentration of compound in collected mouse plasma will be determined from the calibration curve.

2.17.5.4 Data Plotting and Calculation of Pharmacokinetics Parameters

All data acquisition and analysis were obtained and processed using MassHunter Workstation Software. Concentration of compounds were plotted against time to generate the PK concentration-time curve. PK parameters calculated by PKSolver (2010): AUC₀₋₂₄, AUC_{0-∞}, C_{max}, T_{max}, elimination t_{1/2}, CL, V_d, MRT.

Chapter 3: Results

3.1 Identification of Novel Growth Inhibitory Compounds via Mid-throughput Screening

With the spread of resistance rendering current antimalarials ineffective, the healthcare sector cries out for novel therapeutics. The efforts in this study spans two arms, 1) identifying clinically-established drugs to repurpose them for antimalarial use; and 2) discovering new biologically active scaffolds with a novel antimalarial activity. We aim to achieve these by screening two distinct types of chemical compound libraries. The first objective explores a subset of the Selleck Chemicals Anticancer Library for clinically tested active compounds of cancer therapeutics. The library consists of 414 active ingredients which currently span from preclinical up till Clinical Trial Stages I to IV, presenting a huge opportunity to study clinically-ready compounds for fast-tracking the drug development process. The second approach taps on the biologically-untested potential of the Nanyang Chemical Compound Library, a collection of 700 compounds collated by Professor Tan Choon Hong of the School of Physical and Mathematical Science in NTU.

Using an established FACS based readout of parasitemia, we screened each compound in a single-shot dose of 10 μ M in the drug sensitive *P. falciparum* strain 3D7 over one IDC [270]. The resulting parasitemia after drug exposure was used to calculate growth inhibition. Applying a 95% growth inhibition cut-off, we have identified 131 and 38 hits from the Selleck Chemicals Anticancer Library (Figure 10A) and Nanyang Chemical Compound Library (Figure 10B) respectively.

Figure 10. Performance of mid-throughput compound library screens. Screen data of each individual compound is represented as a single dot on a % growth inhibition plot, where growth inhibition is

expressed as $100\% \times (1 - \frac{Screen\ P\% - CQ\ P\%}{DMSO\ P\% - CQ\ P\%})$. Orange line corresponds to the cut of value deemed as complete growth inhibition, set at 95%.

3.2 Validation and Prioritization of Screening Hits

3.2.1 Evaluation and Prioritization of Selleck Chemicals Anticancer Hits

The primary purpose for screening the Selleck Chemicals Anticancer library was to rapidly identify and propose clinically tested drugs for repositioning for malaria. In order to triage the 131 hits for the next step of evaluation, we reviewed the compounds on their 1) current clinical trial phase; 2) mode of administration; 3) maximum tolerable dose (human); 4) clearance rate; 5) half-life; 6) target/mode of action. We structured a hierarchy in order to rank the compounds more favourably for those which were the most clinically advanced, followed by the easiest to administer for better patient compliance, then having the highest tolerability in patients, and possessing the best pharmacokinetic properties in humans. We also conducted literature searches to eliminate compounds which have already been tested on *Plasmodium*. This enabled us to rank and shortlist 50 of the 131 compounds to be brought forward for *in vitro* efficacy study (Table 2).

Pharmacokinetic Data of Top 50 SelleckChem Anticancer Compound Library Hits					
Compound	MOA	Trial Phase	Max tolerated	Clearance	Half Life(h)
1	Oral	IV	2mg daily	3.21 L/hr	127
2	Oral	IV	400mg	N.D.	13
3	Oral	IV	400mg	N.D.	10
4	Oral	IV	600mg/kg/day	N.D.	2
5	Oral	III	1600mg	0.85L hr/kg	1200
6	Oral	III	1.3mg/m ²	102-112L/hr	193
7	Oral	III	40mg/day	N.D.	168
8	Oral	III	300mg	N.D.	135
9	Oral	III	2.97 mg/m ²	N.D.	96
10	Oral	III	80mg/d	14.2 (±0.3) L/hr	48
11	Oral	III	60mg	N.D.	45
12	Oral	III	900mg/day	N.D.	31
13	Oral	III	200mg/day	63.1L/hr	29
14	Oral	III	25mg/day	4.2–7.1 L/hr	28
15	Oral	III	1800mg	N.D.	24
16	Oral	III	250mg/twice daily	100 L/hr	22
17	Oral	III	12 mg/m ² /day	N.D.	20
18	Oral	III	400mg	84000L/hr	15
19	Oral	III	90mg	N.D.	12
20	Oral	III	600mg/dose	37.8 L/hr/m ²	5

21	Oral	II	40mg/day	N.D.	264
22	Oral	II	0.5mg	6.3±2.3 L/hr	216
23	Oral	II	600mg/day	0.56 ± 0.17 L/hr	192
24	Oral	II	60mg	N.D.	80
25	Oral	II	680mg	24.2 L/hr	67
26	Oral	II	37.5 mg/day	19.9L/hr/m ₂	60
27	Oral	II	24 mg/m ² twice weekly	18.9 L/hr	54
28	Oral	II	160mg/m ²	N.D.	38
29	Oral	II	600mg	40 to 60 L/kg•hr.	28
30	Oral	II	200mg/day	N.D.	16
31	Oral	II	9mg/m ²	N.D.	16
32	Oral	II	400mg/day	1.511 ± 0.863L/hr/kg	15
33	Oral	II	200mg/twice daily	N.D.	15
34	Oral	II	12mg	N.D.	12
35	Oral	II	200 mg/m ²	N.D.	11
36	Oral	II	100mg/day	0.62 L/hr/m ₂	10
37	Oral	II	100mg/m ²	4.8 to 7.8 L/hr	8
38	Oral	II	5 mg/m ² twice daily	N.D.	4
39	Oral	II	1000mg/m ² /day	N.D.	2
40	Oral	II	N.D.	N.D.	N.D.
41	Oral	II	N.D.	N.D.	N.D.
42	Oral	II	450 mg/day	N.D.	N.D.
43	Oral	II	N.D.	N.D.	N.D.
44	IV(Subcutaneous)	II	10 mg/m ²	74 L/hr	33
45	IV injection	II	N.D.	N.D.	216
46	IV injection	II	N.D.	N.D.	216
47	IV	II	17-18 mg/m ²	10.5 L/hr/m ²	4
48	I.P Injection, potential oral	II	450 mg/m ²	12.76 to 17.28 L/hr/m ²	3
49	I.P Injection	II	1000mg	3.3L/hr	20
50	I.P Injection	II	50 mg/m ² /day	N.D.	13

Table 2. Pharmacokinetic properties of the top 50 ranked validated hits. Blacked-out boxes indicate no parameters obtained for compound.

3.2.2 *In vitro* efficacy of Selleck Chemicals Anticancer Library Leads

We used a dose response assay to serve as a validation for our screen, as well as to obtain one of the critical hit prioritization parameters: efficacy. Beginning at the highest dose of 10μM, the same dose used in the screen, up to 11 two-fold serial dilutions of the compounds were tested. Compounds were deemed as false-positives should they not exhibit 100% growth inhibition at the highest dose. We identified 2 false positives from our shortlisted 50. Of the other 48 hits, the IC₅₀s of five compounds

could not be accurately determined due to poor sigmoidal curve R^2 values, while 20 of our 50 hits had IC_{50} values lower than $1\mu M$ (Table 3, Figure 11). Based on the typical $1\mu M$ cut-off for antimalarial screens, we will move forward with the next analysis with those 20 compounds.

<i>In vitro</i> IC_{50} of Top 50 Hits			
Compound Number	IC_{50} (nM)	Compound Number	IC_{50} (nM)
19	4.6	29	1244.6
11	14.1	48	1312.2
40	87.1	21	1349.0
36	197.9	47	1381.8
33	227.0	39	1397.1
8	228.1	49	1477.6
26	256.3	32	1995.5
3	262.9	37	2245.7
30	331.4	35	2308.6
38	358.6	1	2600.1
44	487.5	9	2641.3
46	666.7	2	2700.0
10	760.5	7	2899.5
4	765.2	34	2990.2
6	776.4	31	3115.7
45	852.6	14	3243.0
22	879.9	23	3511.8
43	886.6	42	4011.5
15	957.3	13	-
24	972.2	25	-
28	1029.0	16	-
12	1047.7	17	-
27	1056.6	18	-
20	1135.1	50	-
5	1221.6	41	-

Table 3. *In vitro* efficacy comparison of 50 validated anticancer compound hits. Compounds with IC_{50} s which cannot be determined due to the low R^2 curve or incomplete inhibition at the highest dose are indicated by “-”. Highlighted in red are compounds which were deemed false positives due to incomplete inhibition at $10\mu M$.

Figure 11. Dose response curves of anticancer compound hits. Each line represents the dose-dependent inhibition of each compound. Curves were plotted using normalized data points by applying the $100\% \times \left(1 - \frac{\text{Screen } P\% - \text{CQ } P\%}{\text{DMSO } P\% - \text{CQ } P\%}\right)$ formula.

3.2.3 Assessing Suitability of Selleck Chemicals Anticancer Library Leads for Clinical Trials

With the IC_{50} efficacy values of the compounds, we extrapolated the minimum inhibitory dose (MIC) required to be achieved in humans. This is done by converting the nanomolar IC_{99} values of the compounds into ng/mL for comparison against human pharmacokinetic data. From the comparison, two compounds, compounds 40 and 26 (IC_{99} of 105.62 and 255.56 ng/mL respectively) were able to achieve blood concentration above the MIC over a 24hr period, deeming them suitable candidates for consideration for human testing (Figure 12).

Compound 40 IC₉₉: 181.78nM or 105.62ng/mL

Compound 26 IC₉₉: 487.08nM, or 255.56ng/mL

Figure 12. Comparison of extrapolated minimal inhibitory doses of anticancer compounds 40 and 26 against blood plasma levels achieved in humans over 24hr period. A) Compound 40, with an *in vitro* IC₉₉ of 181.78nM, is able to achieve drug concentrations higher than the extrapolated minimal inhibitory dose for *P. falciparum* over 24hr post administration with a single dose of 300 (solid black line), 400 (dotted black line) or 500mg (solid grey line) of drug. Lowest limit of quantification is indicated as a dotted grey line. Figure adapted from Pardanani *et al.* 2015 [271] **B)** Compound 26, with an *in vitro* IC₉₉ of 487.08nM, is able to achieve also able to achieve blood concentrations higher than its extrapolated minimal inhibitory dose over 24hr post administration. In that study, Compound 26 was administered with a co-drug (light grey line) or alone (darker grey line), and plasma concentrations were not significantly different. Figure adapted from Storniolo *et al.* 2008 [272] Data obtained from phase II clinical trials in human patients. Red lines indicate the extrapolated IC₉₉ dose of the compound, indicated below each graph.

3.2.4 *In vitro* Efficacy and Toxicology of Nanyang Chemical Compound Library Hits

Since no biological data was available on the Nanyang Chemical Compound Library, we proceeded directly to validate and determine the IC₅₀ values for the 38 hits from the Nanyang Chemical Compound Library. Similar to what was conducted for the anticancer compounds, we used the highest concentration of 10μM to identify false positives. Only 6 compounds exhibited dose dependent growth inhibition in 3D7, giving 32 false positives (Figure 13A, false positives not shown).

Subsequently, four human cell lines, namely A549 (human lung carcinoma), HEK 293 (human embryonic kidney), HepG2 (human liver cancer), and AGS (human stomach gastric adenocarcinoma) were exposed to each of the six hits over 72 hours. Only compounds A1, C1, and C4 did not display toxicity to any of the cell lines up to 10μM, whilst A2, A3, and A4, were all found to be toxic to all cell lines (Figure 13B). Staurosporine, an apoptotic inducer, was used as a positive control in all experiments. Taken together with their *in vitro* efficacy, compound A1 has the strongest selectivity

(>100 times) for *P. falciparum*-infected to mammalian cells, and looks a promising candidate for further characterization and development.

Figure 13. Comparison of efficacy and cytotoxicity of Nanyang Chemical Compound Library validated hits. A) Comparison of IC_{50} values of the six true positive hits determined from dose response curves. IC_{50} values are indicated (in μM) above each compound. A1 is the only compound amongst the six hits with an IC_{50} in the nanomolar range. **B) Toxicity of hits towards various human cell lines.** Table displays the highest dose of which 100% viability of cells were observed following 72hr exposure to the compounds. Doses shown are in micromolar (μM). Only compounds A1, C1, and C4 showed no toxicity even at 10 μM . The apoptotic inducer, Staurosporine, was used as a control, and only showed toxicity at all concentrations.

3.3 Characterization of A Novel Antimalarial Chemical Scaffold: A1

3.3.1 Assessment for Cross-Resistance to Existing Antimalarials

In order to validate that A1 does not face cross-resistance with any existing antimalarials, we tested its efficacy in established drug-resistant cell lines. In some resistant phenotypes, such as chloroquine, resistance can be defined by a 10-fold or higher shift in the IC_{50} of the compound in a resistant cell line [273]. Dose response assays were conducted against a panel of drug-resistant parasite lines which consisted of *Dd2*, *K1*, *T994*, *W2mef*, as well as field isolates from The Tracking Resistance to Artemisinin Collaboration (TRAC). Compound A1 was found to be equally potent, if not better, against all cell lines tested (Figure 14A)

Artemisinin resistance in comparison, cannot be characterized by a mere shift in IC_{50} following continuous exposure to treatment over one cycle. Artemisinin is typically characterized by the delayed ring stage clearance phenotype. In order to properly investigate artemisinin-type cross resistance, the 0-3hr ring stage survival assay was adopted for compound A1 [167]. *Dd2*^{*Kelch13/543T*} mutant parasite survival was observed even in high doses of DHA-treatment. However, compound A1 exhibited dose-dependent killing following a 6hr treatment (Figure 14C). In contrast the artemisinin-sensitive *Dd2* background strain showed a dose-dependent killing in relation to both treatments (Figure 14B). Taken together, compound A1 makes for a suitable antiparasitic strategy against a host of drug-resistant phenotypes.

A

B

C

Figure 14. Cross-resistance studies against drug-resistant strains. A) IC_{50} comparison between 3D7 and panel of drug resistant clones and clinical isolates. Orange line indicates the *in vitro* IC_{50} of A1. **B)** RSA of A1 (blue) vs dihydroartemisinin (red) in parental *Dd2* or **C)** the *kelch13* I543T artemisinin resistant cell line. Both compounds were able to completely inhibit *Dd2* growth after 6hr exposure, but only A1 was able to do so in the resistant strain.

3.3.2 Generation of Spontaneous Resistant Mutants Against A1

While it is essential to test for cross resistance, it is also necessary to predict any potential underlying genetic factors which may result in increased drug tolerance. This would also serve to predict and anticipate any potential outbreaks of resistance in the field, and hence provide the information for effective deployment of a drug [178]. At the same time, it can be used as a tool to investigate the nature of drug resistance.

In order to determine if there may be a risk of developing resistance to A1, we attempted to generate *in vitro* spontaneous resistant mutants using *P. falciparum* cell lines with a higher propensity for developing resistance [177]. The *W2* and *Dd2* cell lines were both seeded at 10^9 cells/flask, and subsequently exposed to either high doses of the drug for up to two cycles or near-lethal

concentrations for prolonged periods. In all cases, we applied the same treatment regime with Atovaquone, an antimalarial control due to its high propensity for selecting resistant mutants [274]. At the end of each treatment period, we were able to obtain Atovaquone resistant mutants but not that of A1 (Table 4). Only from a single condition, the 2x IC₉₉ treatment for 24hr, did we initially see parasites before the end of the two-month duration (Table 4, highlighted yellow). However, upon repeat of the same treatment, we never obtained parasites again. This bodes well for A1 from a drug development aspect in that resistant mutants are difficult to generate, even when using such a large parasite inoculum.

Strain	Treatment Dose	Treatment	Atovaquone Mutants (Control) Produced?	Status
Dd2	2x IC ₉₉	2 days wash-off	Yes	Terminated after 2 months without parasites
	10x IC ₅₀	Constant pressure	Yes	Terminated after 2 months without parasites
W2	2x IC ₉₉	2 cycles wash-off	Yes	Terminated after 2 months without parasites
	10x IC ₅₀	Constant pressure	Yes	Terminated after 2 months without parasites
	2x IC ₉₉	1 cycle wash-off	Yes	Terminated after 2 months without parasites
	2x IC ₉₉	1 day wash-off	Yes	Terminated after second selection
	1x IC99	Constant pressure	Yes	Terminated after 2 months without parasites

Table 4. List of conditions used for selecting spontaneous resistant mutants against A1 *in vitro*.

Highlighted in yellow is the condition of which spontaneous mutants were obtained once.

3.3.3 Stage Specific Activity in the Asexual Lifecycle

Since our earlier efforts have not yielded any resistance mechanisms against A1, we turned our attention to characterizing its antimalarial properties to shed more light of its function. It is understood that the expression of the parasite genome occurs in a “just-in-time” manner over the *Plasmodium* IDC, and that the biology of the parasite differs greatly across the different stages of the development [275]. We treated highly-synchronous parasites at 12hr intervals of the IDC to determine the window of which A1’s antiparasitic activity is observed, potentially narrowing down the number of possible targets or mechanisms of action. From the parasite stage-specific assay, compound A1 was seen to be active during a large proportion of the *P. falciparum* asexual life cycle. Differential morphologies were observed 12 hr after treatment with 10 μ M of A1 at 0, 12, 24, and 36hr of the IDC. Ring stage-treated parasites (0 and 12hr) showed a pyknotic structure after 12 hr of treatment (Figure 15, 2nd and 3rd row), whilst trophozoite stage parasites (24hr) displayed a condensed nucleus morphology after the same duration (Figure 15, 4th row). In all of these cases, the parasites did not continue to develop normally even at the end (48hr time point) of the experiment (Figure 15, 5th column). However, treatment starting at the 36hr of the IDC did not seem to affect parasite growth, where reinvasion of RBCs was observed 12 hr after (Figure 15, 5th row).

Figure 15. Stage specific activity of compound A1 over the 48hr *P.falciparum* IDC. Panels in the top row depict the morphology of the parasite over 12hr intervals of the life cycle. Subsequent rows are smears taken from cultures every 12hr post-treatment starting at different intervals of the life cycle.

3.3.4 Stage Specific Activity in the Gametocyte Development

While the asexual stages are entirely responsible for the causing the disease symptoms, they are not the form which are taken up and subsequently transmitted by the *Anopheles* vector [7]. Instead, some of the intraerythrocytic asexual parasites undergo differentiation into gametocytes (Figure 2). Gametocytes would undergo five distinguishable stages of development, the last of which are able to continue their development in the mosquito midgut into sporogonic forms ready to infect the next human host [276]. It is therefore critical to also inhibit gametocyte development in order to curb the transmission of the disease between hosts, which also stops the spread of drug resistance.

In order to investigate if A1 possesses any gametocidal activity, we treated stage III-V gametocytes with A1 and compared it to a known gametocyte active drug, lumefantrine. The assay showed that following four days of treatment with 5 μ M of A1 or lumefantrine, no gametocytes were seen to develop into the elongated form characteristic of the stage IV and V morphology. (Figure 16A). In fact, A1 treated cells, most gametocytes either showed lysis of the membrane or a condensed, rounded morphology (Figure 16B).

A

B

Figure 16. A1 inhibits late gametocyte development. A) Distribution of live and dead parasites upon treatment of either A1 or lumefantrine. Orange and blue represent dead and live parasites respectively, based on morphological-determined counts from giemsa smears. **B)** Morphology of A1-treated stage III gametocytes. Compared to the elongated oval shape of mature stage IV and V gametocytes (1st row), treated parasites appear either condensed (2nd row) or lysed (3rd row).

3.4 Target Identification of A1

3.4.1 Identification of Asexual Blood Stage Target via Cellular Thermal Shift Assay (CETSA)

Innovations and advances in molecular biology has led to ground breaking techniques which have allowed us to better understand the nature of the multistage life cycle of the parasite. One such example, the *in vitro* selection of resistant parasites, has been coupled with whole genome sequencing to allow the identification of SNPs and CNVs which give rise to the decreased drug susceptibility [178]. This method could also be applied to discover drug targets should there be a target-resistance mechanism relationship [277]. While we have shown that our earlier efforts have failed to yield any such information, we switched our attention towards to the non-evolutionary-based, protein engagement identification, CETSA, in search of A1's target (Table 4).

We incubated 30hr IDC parasite lysates of the drug-sensitive 3D7 cell line with A1 in series of four-fold serial dilutions as well as an untreated control, and subsequently subjected the samples to thermal challenges of 37 or 51°C. Using the 37°C melt curve of each protein as the baseline, a protein would be deemed to be thermal stabilized by A1 should there be a dose-dependent increase of at least 0.3-fold peptide abundance in the corresponding 51°C melt curve. From our results, five proteins were observed to exhibit thermal stabilization with increasing doses of A1. Amongst these hits, four belong to ribosomal RNA proteins and the last of which is the putative *P. falciparum* Zinc Finger (CCCH-type) protein (PlasmoDB geneID: PF3D7_1315400) (Figure 17A). Being potentially interesting from a functional perspective, we did a protein domain prediction using the PF3D7_1315400 amino acid sequence on various online databases. As expected, the results revealed a Zinc Finger (C-x8-C-x5-C-x3-H) domain (Figure 17B, red arrow). Zinc finger domains of this ion coordinating fold have been characterized to be RNA-binding, functioning as gene expression regulators at the mRNA level [278, 279]. In addition to the zinc finger domain, a WD40-repeat domain was also identified in the C-terminal of the protein (Figure 17B, blue arrow). WD40 domains have been shown to be made up of four to 16 multimeric beta sheets, which then fold together into a ring-like secondary structure reminiscent of propeller blades [280]. These domains serve as the scaffold for the formation of protein

complexes involved in a variety of cellular pathways ranging from gene regulation to signal transduction [281].

Figure 17. Identification of the putative *Pf* Zinc Finger (CCCH type) Protein as the intracellular asexual stage target of A1 via CETSA. **A)** Protein thermal stabilisation curve of the *Pf* Zinc Finger (CCCH-type) protein at 51°C . Data points depict the fold-change in protein abundance in the gradient of drug concentrations relative to the DMSO control. **B)** Protein domain search of the amino acid sequence confirms a Zinc Finger (C-x8-C-x5-C-x3-H) domain (red arrow) as well as a series of WD40 protein scaffolding domain repeats (blue arrow).

3.4.2 Target Validation of the *Pf* Zinc Finger (CCCH-type) Protein

We next sought to validate the result of the *Pf* Zinc Finger (CCCH-type) Protein thermal stabilization through a targeted approach. In order to do so, we repeated the CETSA experiment by quantifying the *Pf* Zinc Finger (CCCH-type) Protein abundance following heat challenge with lysates from an endogenously-tagged protein cell line.

Up till a while ago, gene editing in *Plasmodium* has proven to be a challenging task until CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) was established [282]. The method relies on the single-strand RNA-guided *Streptococcus pyogenes* Cas9 enzyme to the desired locus to create

double-stranded breakages in protospacer adjacent motifs (PAM, made up of an NGG sequence), which are then subsequently repaired via homologous recombination [283, 284]. However, the nature of the AT-rich genome of the parasite means that PAM sites are of poor abundance, especially in the untranscribed regions up- and downstream of open reading frames (ORFs) [285]. This makes gene tagging via insertion of tag sequences in terminal ends challenging. We therefore overcome this using a selection-linked integration system to insert a triple hemagglutinin (HA) tag at the 3' end of the protein. The method involves a two-step selection process, the first of which uses WR99210 for selection of parasites carrying the episomal editing plasmid which contains the human dihydrofolate reductase cassette. Subsequently, a second drug selection uses the antibiotic G-418 to select for parasites harbouring the correct integration of the plasmid. This selection is dependent on the neomycin-resistance gene being co-expressed only under the endogenous promoter of the target protein (Figure 18A) [286].

We cloned a 400bp homologous gene fragment of the *Pf* Zinc Finger (CCCH-type) Protein DNA sequence into this plasmid to allow the single-crossover at the 3' end of the ORF. Following successive drug selections, the parasites obtained were validated for the correct integration of the plasmid. By performing PCR using primers flanking the 5' and 3' recombination junctions of the integration sites, it was observed that there were only bands present in the transgenic cell line, but not in the untransfected parental 3D7 (Figure 18B). We then collected parasite lysates of these transgenic cell lines for detection of the tagged protein on a western blot. We observed a HA-tagged protein of approximately 51kDa in size, corresponding to the predicted molecular weight of the the *Pf* Zinc Finger (CCCH-type) Protein (Figure 18C). We then performed immunofluorescence assays (IFAs) of the transgenic cell line at 12, 30, and 44hr timepoints of the IDC to establish the localization of the protein at various stages. The IFA showed that the protein was present in all stages of the life cycle, present in a punctate pattern. (Figure 18D, 3rd column). Despite the evidence showing that C-x8-C-x5-C-x3-H type Zinc Finger proteins being associated with RNA regulation, our IFAs showed that the protein did not co-localized with the nucleus (Figure 18D, 5th column) [279]. At the same time, the protein did not

seem to be exported outside of the parasitophorous vacuole membrane (PVM), as none of the signal was seen beyond the exported protein 2 (EXP2) signal (Figure 18D, 6th column). This shows that the protein is localized within the internal parasite cytoplasm. Taken together, these give an indication of a successful knock-in of the triple-HA tag at the 3' end of the *Pf Zinc Finger (CCCH-type) protein* locus.

Figure 18. Validation of the endogenously-tagged *Pf* Zinc Finger (CCCH-type) Protein cell line. A)

Schematic of the selection-linked integration plasmid construct. Figure obtained from Birnbaum *et al.*

2017 [286]. **B)** PCR validation of the integrated cell line. **C)** Western Blot detection of the HA-tagged

protein in cell lysates. Green band represents the approximately 51kDa HA-tagged *Pf* Zinc Finger (CCCH-type) Protein (indicated by white arrow), while the red band was the internal parasite loading control glyceraldehyde-3-phosphate dehydrogenase (PlasmoDB geneID: *PF3D7_1462800*) of 37kDA.

D) IFA of the tagged *Pf* Zinc Finger (CCCH-type) protein shows the presence of the protein across all stages of the IDC. Row 1: Ring; Row 2: Trophozoite, Row 3: Schizont. *Pf* Zinc Finger (CCCH-type) protein does not colocalize with the nucleus (5th column), but does not seem to be exported beyond the PVM (6th column).

After successfully obtaining an endogenously-tagged cell line, we proceeded to use it to validate the observation of the CETSA screen by replicating the experiment with the transgenic parasite lysates. Instead of requiring the need for a mass spectrometry, we attempted to validate this stabilisation via Western Blot by measuring relative band intensity against a loading control. Indeed, we observed an

increase in stabilisation of the HA-tagged protein in the presence of A1 with increasing temperature, giving confidence to the mass spectrometry screen results (Figure 19).

B **C**

Temperature	Relative Band Intensity	
	Treated	Untreated
51	876	-
52	1100	576
54.1	966	647
59.6	737	591
61.8	704	400
63.6	267	240
65.8	89.1	56.4
71.8	15.8	16.9

(P < 0.05)

Figure 19. A1 binds to *Pf* Zinc Finger (CCCH-type) protein. A) Western Blot images of treated *Pf* Zinc Finger (CCCH-type) Protein HA-tagged cell line lysates either treated with A1 (top) or DMSO (bottom) after gradient heat challenge. *Pf* Zinc Finger (CCCH-type) Protein in green, *Pf* Aldolase in red. **B)** Graphical representation of relative band intensity of *Pf* Zinc Finger Protein, normalized against *Pf* Aldolase. **C)** Table comparing relative band intensity between A1 and DMSO treated samples.

3.4.3 Identification of Ring Stage Target via CETSA

The parasite prioritizes different cellular processes at each different phases of the asexual cycle, characterized by evidence of the differential gene expression that happens across the life cycle [275, 287]. Therefore, despite obtaining a putative target from lysates of 30 hr post-invasion (hpi) trophozoites, we wanted to investigate the possibility that A1 may act on a different target in ring stage. From the time at which the initial trophozoite lysate screen was performed, the workflow of the CETSA experiment was further improved, where sample preparation procedures such as checks for haemoglobin content was added. It was also later established that the *Plasmodium* proteome consists of a subset of proteins which possess T_m s higher than 51°C, which might have led to the loss of coverage of more thermal-stable proteins during the single temperature screen (data from Dziekan, J. and Go, K. D., unpublished). Therefore, in order to accommodate a wider range of the proteome in the analysis, an additional melt curve of 57°C was added into the protocol. Incorporating both the aforementioned advances in the method development, we conducted another CETSA screen using lysates prepared from ring stage parasites, the stage of which A1 was shown to have the most extensive effect.

The current screen was performed on parasite lysates generated from 10-16hr post-invasion parasites, with both 51 and 57°C melting curves in addition to 37°C. We applied a three median absolute deviation (3 MAD) cut-off to narrow down the list of hits to proteins with the highest significant stabilization. Similar to the earlier screen, we observed multiple hits belonging to the ribosomal protein complex (Figure 19A). From this experiment however, we did not observe the *Pf* Zinc Finger (CCCH type) Protein in our hits, even when applying a 2 MAD cut-off (Supp Figure 1). Instead, we obtained 3 other non-ribosomal protein subunit hits: the putative Dipthine Synthase (PlasmoDB geneID: *PF3D7_1009000*), Falcilysin (FLN, PlasmoDB geneID: *PF3D7_1360800*), and a conserved *Plasmodium* protein (PlasmoDB geneID: *PF3D7_1022100*) (Figure 20B). The most significant hit was the proteolytic enzyme FLN, where its thermal stabilisation curve at 57°C gave the highest R^2 value amongst the non-ribosomal protein hits (Figure 20B). At this point however, with the molecular basis

of A1's mode of action being completely unknown, it is difficult to speculate on which is/are the true targets of the compound.

Figure 20. Identification of the ring stage target of A1 via CETSA. A) Whole ring stage proteome analysis of 0-100 μ M A1 ITDR at 51 (red circles) and 57°C (blue triangles). X-axis is a scale of protein

stabilisation measured in terms of R^2 value, while y-axis plots ΔAUC values which were obtained after normalization against nondenaturing (37°C) conditions. Significant hits are labelled and named with PlasmoDB geneID. Hits obtained are of three median absolute deviation (MAD) of ΔAUC with a $R^2 = 0.8$ threshold, indicated by the vertical dotted line. **B)** Peptide abundance curves of non-ribosomal protein stabilized hits identified in A). 37, 51, and 57°C curves plotted in black, blue, and red respectively. Data points depict the fold-change in protein abundance across the gradient of drug concentrations relative to the DMSO control.

3.4.4 Identification of Gametocyte Stage Target via CETSA

Other than being derived from the ring stages, gametocytes undergo a different biological development as compared to the rest of the asexual lifecycle, where it is fully committed to the chance of being taken up by the mosquito during feeding rather than replicating [287]. However, when we tested for the extensiveness of A1's activity on the *Plasmodium* life cycle, we also observed that A1 was able to efficiently inhibit gametocyte development (Figure 16). We therefore wanted to investigate if A1 either acts on a shared biological target between asexual and sexual development, or if it is pharmacodynamically different in two distinct maturation phases.

Following further improvements of the CETSA assay, the in-cell thermal challenge was also augmented into the optimized protocol. This required an additional step of haemoglobin depletion to the sample processing due to the high abundance of haemoglobin present in the intact cell mass, which would mask peptide signals of less abundant parasite proteins. Performing CETSA on intact live cells adds additional dimensions to the assay, taking into consideration factors such as drug accumulation, metabolism, and the presence of protein complexes as well as potential competitive binders, all of which would not be obtained from parasite lysates.

For this, we performed the thermal challenges on 8-10day post commitment gametocytes, similar to those used in the stage specificity tests. The results showed highly significant stabilisation (3 MAD) of a single protein, the proteolytic enzyme Falcilysin (FLN, *PF3D7_1360800*) (Figure 21). FLN has been

prominently characterized to be part of the haemoglobin digestion pathway in asexual parasites, while there has also been suggestions of an alternate role in processing proteins for apicoplast-targeting [288]. The evidences from these earlier studies, along with identifying FLN from CETSA performed in both asexual and sexual parasites, gives us greater confidence that A1 may exert it's pharmacodynamic effect in both stages through Falcilysin, possibly in two different pathways.

Figure 21. In-cell CETSA of A1 in gametocytes identified a single thermal-stabilized hit, Falcilysin. A)

Gametocyte stage proteome analysis of 0-100 μ M A1 in-cell ITDR at 51 (red circles) and 57°C (blue triangles). X-axis is a scale of protein stabilisation measured in terms of R^2 value, while y-axis plots Δ AUC values which were obtained after normalization against nondenaturing (37°C) conditions. Significant hits are labelled and named with PlasmoDB geneID. Hits obtained are of 3 MAD of Δ AUC with a $R^2 = 0.8$ threshold,. **B)** Peptide abundance curves of Falcilysin and HSP90, the protei stabilized hits identified at 3 and 2 MAD respectively. 37, 51, and 57°C curves plotted in blue, red and green respectively. Data points depict the fold-change in protein abundance in the gradient of drug concentrations relative to the DMSO control.

3.4.5 Validation of FLN inhibition as the mode of action of A1

Since FLN was present in both the CETSA screens in asexual and sexual stages, we sought to functionally validate this observation. Previously, an assay to characterize and understand the selectivity of FLN was developed, leading to the identification of the specific peptide substrates and hence the role of the protease [289]. We therefore used recombinant FLN in a cell-free proteolytic cleavage assay using fluorescent label-quencher peptides to validate the activity of A1 over its function. An inhibition of the proteolytic activity would see a slower increment of the fluorescent signal over time. Indeed, when the assay was performed with 10 μ M of A1, peptide cleavage activity was severely reduced, comparable to that of the ZB1 control (Figure 22A, ZB1 data unpublished). In order to show that this was not a non-specific inhibition, we performed a dose response enzymatic inhibition assay

of A1. Indeed, we observed a dose-dependent inhibition of FLN activity in the assay, despite the inhibition of the protease still happening below the parasitic *in vitro* efficacious levels (Figure 22B). We also do not obtain an IC_{50} that corresponds to the *in vitro* efficacy assay. Taken together this shows us that A1 acts on FLN by inhibiting its proteolytic function, but hints that there may be other secondary factors influencing the interaction between the compound and its target.

Figure 22. A1 inhibits the proteolytic activity of FLN. A) Fluorescence emission of cleaved peptide substrates were measured at 490nm over 7min. FLN activity was abolished with 10μM A1 (green), similar to that of the positive control 1mM ZB1 (dark blue). No inhibition of proteolytic activity was observed in cyclohexamine (CHM, red), similar to the solvent controls (DMSO, purple; methanol, light blue; water, orange). B) Dose response curve of A1 in the FLN inhibition assay. Enzymatic assay was performed using two-fold serial dilutions of A1. The curve does show a dose dependent relationship, although even at the lowest concentration, inhibition of the enzyme activity was still observed.

3.5 Lead Candidate Optimization of A1

3.5.1 *In vivo* efficacy and toxicity of A1

One key area of lead optimization in the improvement of the efficacy of the candidate compound. Structural Activity Relation (SAR) studies are usually driven by a target-based approach once the mode of action of the candidate is identified and well characterized. Another aspect of lead optimization is to improve the pharmacokinetic properties of the candidate in order to lower the required efficacious dosage to avoid toxicity. While we know that A1 has a low IC_{50} from a starting point, its chemical structure is very hydrophobic in nature. It has a predicted partition coefficient (xlogP) of 9.008, which

demonstrates its high hydrophobicity (ideal $xlogP \leq 5$). This might translate to poor bioavailability due to a high tendency of hydrophobic compounds to be distributed into tissues, and hence result in higher than expected therapeutic doses. Since we have yet to reach a good understanding of the pharmacodynamics of A1, the lead optimization approach will be guided by the aim of improving the pharmacokinetic properties of A1, at the same time indirectly improving its efficacy.

In order to assess the efficacy of A1 in an *in vivo* setting, we performed a Peter's 4-day Test in the rodent *P. berghei* infection model. The test is conducted over a five-day period, with a once-daily dosing regime for four days. The first dose is administered intraperitoneally at day 0 when parasitemia is 3% or above. Using a transgenic GFP reporter *P. berghei* strain, parasitemia in each mouse was measured by FACS daily before administering a dose of either 5, 10, 20, or 30mg/kg A1, vehicle, or artemisinin. The results showed that a daily dose of 5mg/kg could not reduce parasitemia, where growth rate was observed to be similar to that of the vehicle treated control (Figure 23, dark and light blue lines). 10mg/kg treatments appeared to control parasitemia for two days (Figure 23, red line), without significantly reducing it, but parasite growth resumed on day 2 despite administering the third dose. A1 was initially able to control and reduce parasitemia at 20mg/kg dose, but the increase in parasitemia resumed on day 4. However, with a 30mg/kg dose, no detectable parasitemia was seen in all mice by day four, and continued to remain so on day 5 (Figure 23, yellow line, day 5 data not shown). Interestingly, recrudescence was observed in each mouse, varying between days 6 to 8 (Data not shown).

A generic toxicity test in the *Balb/c* model was conducted to assess the suitability of administering a higher dose of compound A1. At 60mg/kg, some mice showed mild signs of toxicity, but were able to complete the 4-day treatment course. However, at 70mg/kg, all mice were dead following the first dosage of the treatment (Data not shown). Taken together with the efficacy study, A1 only has a maximum of two-fold selectivity *in vivo*.

Figure 23. *In vivo* efficacy and pharmacokinetic study of compound A1. Parasitemia levels across a 4-day, once daily, dose regime of compound A1 in *P. berghei*-infected Balb/c mice. Doses of 5, 10, and 20mg/kg were not able to reduce parasitemia completely, while 30mg/kg was able to reduce parasitemia to at least below the level of detection over the treatment period.

3.5.2 *In vivo* Pharmacokinetic Profile of A1

We hypothesize that the lack of bioavailable A1 may result in incomplete clearance of the parasite load, resulting in the recrudescence in the 30mg/kg group. A short *in vivo* half-life ($t_{1/2}$) may also lead to an insufficient exposure duration of the compound, leading to survival of the parasites. We therefore sought to establish the pharmacokinetic profile of A1 *in vivo* as a means to investigate the cause of the poor efficacy. In doing so, we would use the data as a means to direct SAR studies later on.

To understand if poor bioavailability was the cause of incomplete treatment of the infection, we conducted the pharmacokinetic study using a 30mg/kg single-dose IP administration and collected blood samples from mice at 0, 0.25, 0.5, 1, 2, 4, 8, 16, and 24hr post administration and quantified plasma concentrations over time (Figure 24A, B). Using the concentration-time curve generated, we were able to calculate the Area Under Curve₀₋₂₄ (AUC₀₋₂₄), Area Under Curve_{0-∞} (AUC_{0-∞}), maximum achieved concentration (C_{max}), time to reach maximum concentration (T_{max}), half-life ($t_{1/2}$), clearance of drug observed (CL/F_{obs}), volume of distribution of drug observed (V_z/F_{obs}), and the mean residence time of drug observed (MRT 0-inf_{ob}) (Figure 24C). The C_{max} was approximately 750ng/L, which translates to approximately 1.4nM, 100-fold lower than the *in vitro* IC₅₀. T_{max} was observed at 15min, and therefore a Tlag of 0min. From the concentration-time curve, it is observed that A1 was cleared rapidly from the blood, with a calculated $t_{1/2}$ of only 6.11hr. However, this is not the sole responsible factor which may explain the poor A1 *in vivo* efficacy. The results also show that a 30mg/kg dose only resulted in a 1.4nM maximum plasma concentration. The high V_z/F_{obs} , which meant that there is a high distribution of A1 to body tissues, which was also a consequence of a low plasma concentration. Taken together, these results show that from the pharmacokinetic perspective, A1 has much to be desired.

A

B

C

Parameter	Value	Unit
Lambda_z	0.113400298	1/h
t1/2	6.112392916	h
Tmax	0.25	h
Cmax	749.2405297	ng/L
Tlag	0	h
Clast_obs/Cmax	0.01407786	
AUC 0-t	1545.065575	ng/L*h
AUC 0-inf_obs	1638.078586	ng/L*h
AUC 0-t/0-inf_obs	0.943218224	
AUMC 0-inf_obs	10053.04703	ng/L*h^2
MRT 0-inf_obs	6.137096908	h
Vz/F_obs	161.4999248	L/kg
Cl/F_obs	18.31413966	L/h/kg

Figure 24. A1 has sub-optimal *in vivo* pharmacokinetic properties. Blood plasma concentrations were plotted against time to generate **A)** concentration-time curve and **B)** log-liner concentration-time curve. **C)** Pharmacokinetic parameters calculated from concentration-time curves using PKsolver software.

3.5.3 SAR Optimization of A1

3.5.3.1 Testing Commercially Available Analogues

At this moment, we have yet to fully elucidate the exact nature of A1's biological activity such as the interacting site with its target, which makes it difficult to pinpoint the active chemical groups of the compound to direct SAR studies. A quick way to rapidly identify modifiable chemical groups on the compound is to obtain structurally similar chemical analogues. Following a search of the Sigma-Aldrich catalogue for compounds with a similar scaffold core, we acquired nine analogues and followed them up with *in vitro* efficacy testing. Unfortunately, none of those compounds exhibited IC₅₀s lower than 10µM against 3D7 (data not shown). While the central scaffolds were similar, these compounds differed extensively in many areas, making it difficult to identify groups which are likely to be suitable for modifications.

3.5.3.2 Synthesizing Chemical Analogues by Modification of Individual Chemical Moieties

From the efficacy and pharmacokinetics data, it can be hypothesized that poor pharmacokinetic properties of A1 may result in the incomplete clearance of the infection. At first glance, the structure of A1 is highly hydrophobic, which would result in poor solubility. In the bloodstream, solubility affects the amount of bioavailable drugs, where hydrophobic compounds may accumulate in fat and other tissues [290]. We therefore looked to direct our medicinal chemistry approaches to improve this physiochemical property of A1 while also indirectly improving its efficacy.

For the medicinal chemistry approach, modifications to the structure to improve solubility were done by either adding or substituting with polar chemical groups, reducing the number of hydrophobic groups, or generating truncated forms of the compound. Modifications were made to either one of the five chemical moieties of the scaffold at a time (Figure 25A). Additionally, in an attempt to reduce the overall hydrophobic bulk, some of these modifications involved removing the moiety altogether. A molecular weight limit of 600 Da was set during the planning of analogue structures.

Due to the limitations of acquiring certain chemical precursors for synthesis, we were only able to synthesize 26 analogues either with substitutions in either moiety 1 or 3 (series 1 & 3 respectively), or truncated analogues of A1 (series T, with the removal of moiety 4 & 5, Figure 25A). In order to triage these compounds in a high-throughput manner, we screened them using *in vitro* efficacy assays. We found that the three compounds in series 3 showed at least two-fold decrease in IC_{50} , while compounds in the other series achieving IC_{50} s no lower than 548.11nM (Figure 25B). Between these three compounds, two of them possess a substitutions at either the 1' and 4' carbon of a benzene ring, while the third and most potent has a substitution for a carbonyl group.

Subsequently, we tested the series 3 compounds for toxicity on HEK and HepG2 cells and did not observe any toxicity even at the highest test concentration of 10 μ M (data not shown). Taken together, this indicates that these compounds are an improvement of the parent A1 compound in terms of

hydrophobicity, efficacy, and at the same time provided an indication of a malleable chemical moiety of the chemical scaffold.

B

Series	Compound	IC ₅₀	Std Dev
1	1	10000>x>5000	-
1	2	4686.67	2181.38
1	3	906.50	201.13
1	4	1113.33	270.37
1	5	10000>x>5000	-
1	6	828.52	87.68
1	7	10000>x>5000	-
1	8	2799.50	427.28
1	9	3725.76	1291.74
1	10	10000>x>5000	-
3	1	77.50	7.06
3	2	103.04	41.73
3	3	104.86	27.75
T	1	548.11	20.85
T	2	2390.24	521.12
T	3	5854.29	7.67

Figure 25. SAR studies of A1 identified 3 analogues which exhibited 2-fold or better IC₅₀. **A)** Schematic of the A1 structure, with each sphere numbered one to five representing a chemical moiety of the compound. **B)** Synthesized analogues and their *in vitro* IC₅₀s. First column indicates the compound moiety which is modified, second column is the analogue series number. Last two columns indicate *in vitro* IC₅₀ (nM) and standard deviations. Data was generated based on three biological replicates.

Chapter 4: Discussion

4.1 Identification of Novel Growth Inhibitory Compounds via Mid-throughput Screening

The average hit rate of a high throughput library screen lies within the range of 0.27-0.98%. The Selleck Chemicals Anticancer Library yielded 131 hits out of 414 compounds, while the Nanyang Chemical Compound Library gave an initial 38 out of 672, resulting in hit rates of 31.6 and 5.6% respectively. Typically, the growth inhibitory cut-off for library screens are set at 80%, but because we had a high number of hits, we used an 95% threshold instead to decrease the chances of encountering a larger proportion of false positives [224]. Even after subsequent validation, the Selleck Chemicals Anticancer Library had a hit rate of 31.1% (2 false positives) and the Nanyang Chemical Compound Library 0.89% (32 false positives). This stark contrast in hit rate is possibly down to the nature of the sources of both libraries. While the anticancer library consists of biologically active and optimized compounds, the Nanyang Chemical Compound Library is made up of scaffolds only intended for catalysing chemical synthesis or were the end-product of those reactions. However, this made the latter unique in the sense that they have a high potential to provide novel mechanisms of action, a valuable trait when discovering new drugs against the rapidly evolving drug-resistant *Plasmodium* parasites.

We also encountered a highly unusual false positive hit rate in the Nanyang Chemical Compound library screen. This high error rate could simply be a result of experimental error. The anticancer library was prepared with compounds pre-printed on assay plates and in triplicates, significantly reducing the error rate of the screen. However, the Nanyang Chemical Compound library compounds were provided in individual stock form. In order to accelerate the screening process, we attempted to dovetail the hit screening and dose response assays together by performing the screen over a single repeat, following which, gather the initial hits for dose response as a form of validation. It is probably through this, and also the handling of multiple compounds at one go, where the error rate was escalated.

The high hit rate of the anticancer library also suggests that there may be many shared biological pathways which are common between *Plasmodium* and cancer cells. One such trait is a rapid replication rate, a common hallmark of cancer cells [291]. In the asexual IDC, rapid replication and growth is exuberated the most in the trophozoite stages, where multiple rounds of mitosis occurs over a short period to when generating its progeny [292]. It will therefore not be surprising should the cell cycle regulating protein-targeting drugs of this library be effective against *Plasmodium*, especially in these fast-growing phases.

4.2 Validation and Prioritization of Screening Hits

4.2.1 Evaluation and Prioritization of Selleck Chemicals Anticancer Hits

A classical drug discovery approach usually takes more than a decade to produce a lead compound ready for clinical testing. Even so, the risks of failure during clinical trials remain high, ending the process with huge amounts of resources spent and time wasted. The repurposing approach allows for a fast trajectory of new compounds into preclinical and clinical trials, the latter especially so if the drug is already FDA-approved [207]. Despite shortening the process of drug discovery, overcoming drug resistance is a critical consideration in malaria therapeutics. With poor patient compliance being one of the major contributors to the selection of drug resistance, it would be ideal if a drug can be readily taken without much supervision of a clinician [94]. Drug discovery stories have also created lessons in drug resistance, the most famous being artemisinin, where its short half-life resulted in rapid recrudescence of infections, giving rise to the emergence of resistant phenotypes [92]. In order to leverage on a quick drug discovery approach to deliver an ideal antimalarial candidate, we picked the Selleck Chemicals Anticancer Library, consisting of clinically-studied and well-documented compounds and drugs for screening in a *P. falciparum* *in vitro* growth assay.

As mentioned, drug screens typically yield a hit rate of <1%. However, our screen garnered 131 initial hits, and with the large number to evaluate and limited automation capabilities and resources, there was a need to scrutinize these hits with existing information available from existing data. We therefore

organized our selection criteria to a) take advantage of bypassing preclinical testing by using well-tested and FDA-approved drugs with the least risk of toxicity; b) selecting drugs which would run into the least issues with patient compliance. This led to the formation of our list of triaging factors in the order of 1) clinical trial phase, 2) mode of administration, 3) maximum tolerable dose, 4) clearance rate, and 5) half-life. Despite not being able to obtain the full range of information for every compound, we were still able to establish the selection of our top 50 candidates for further evaluation. This was in part due to the number of repeated compounds in different formulations in the library, of which the compound with better properties were picked (data for other compounds not shown).

4.2.2 *In vitro* Efficacy of Selleck Chemicals Anticancer Library Leads

After narrowing down the list to 50, the next step was to assess their efficacy in *Plasmodium*. We observed two false positives from this assay where incomplete inhibition of parasite growth was observed at 10 μ M. At the same time, we also could not accurately determine the IC₅₀s of five other compounds due to the insufficient data points at the end of the higher end of the concentration curve, resulting in poor R² curves. Since these were compounds which would give high IC₅₀ values, we excluded them from any further analysis.

4.2.3 Assessing Suitability of Selleck Chemicals Anticancer Library Leads for Clinical Trials

Under the recommended guidelines, a <95% cure of the blood stage infection in every patient is required to be achieved by the active compound, but at the same time required to prevent recrudescence due to insufficient dosing [293]. We therefore first used the IC₉₉ values of the *in vitro* efficacy assay as well as the available pharmacokinetic data of these compounds to determine the suitability of conducting clinical testing in humans. We identified two compounds, 26 and 40, which have extrapolated IC₉₉ (in ng/L) values lower than the lowest concentration achieved in humans over a 24hr post-administration study to ensure that the MIC is achieved. Although the current available literature on these compounds only display data no longer than a 24hr period, we can take confidence in that these compounds can achieve plasma concentrations higher than the MIC for that duration.

This is already better in comparison to quinine and artemisinin, in which the latter is known to have a half-life no longer than 6hrs [59, 294]. Therefore, these compounds are in a good position for plans to conduct controlled human malaria infection (CHMI) studies with.

4.2.4 *In vitro* Efficacy and Toxicology of Nanyang Chemical Compound Library Hits

We initially obtained 38 hits from the screening of the Nanyang Chemical Compound Library, while only ending up with six following the subsequent validation. This large error rate was not observed in the Selleck Chemicals Anticancer Library screen (2 false positives out of 50 validated compounds). Barring the low-likelihood of a change in culture conditions, this discrepancy could be down to the methodology of the conduct of the screen. The Nanyang Chemical Compound Library came in ready-dissolved stock volumes of 20-30 μ L, requiring individual transfer of each compound into the test cultures. In contrast, the Selleck Chemicals Anticancer Library was obtained from the Genome Institute of Singapore, which provided the compounds “pre-printed” on 96-well plates from automated echopulsing of small volumes of DMSO-resuspended compounds, removing the potential of pipetting error occurring while handling a large number of compounds at a go.

One of the first and most direct parameter to triage hits from chemical compound screens is the comparison of efficacy and toxicology [224]. These parameters would then be used to obtain a selective index, expressed as a fold-difference between efficacy and toxicity. From our six hits, five compounds exhibited low micromolar IC₅₀s, and the sixth 170nM. Three candidates showed similar toxicity levels as their antiparasitic efficacy, while the remaining three had no toxicity to all four of the mammalian cell lines used in the study. Therefore, by comparing the selectivity of the three compounds, A1 becomes the top candidate with a selectivity of over 50 folds, as compared to C1 and C4 which average approximately four-fold each. It has been suggested that compounds to be taken forward into the drug discovery pipeline are required to have IC₅₀ <1 μ M and <10-fold IC₅₀/toxicity selectivity [295]. In this regard, A1 is the only candidate which meets both the criteria, and is by definition, a potent antimalarial scaffold.

4.3 A1 – A Novel Antimalarial Scaffold that Targets FLN

4.3.1 Assessment for Cross-Resistance to Existing Antimalarials

One of our key aims of conducting our compound screening was to identify a novel chemical scaffold for development into a candidate for antimalarial therapy. The importance of discovering antimalarials which overcome most, if not all, drug resistance phenotypes present in the field could not be further emphasized. Therefore, testing novel chemical compounds against established drug resistance strains and clinical isolates have become a fundamental part of lead evaluation in all antimalarial drug discovery programs [296].

Our panel encompassed *Dd2*, *K1*, *T994*, *W2mef*, as well as field isolates from TRAC, lab-adapted patient samples gathered from the GMS [297]. None of the strains showed decreased sensitivity to A1, indicating that the compound would be suitable for treating infections not susceptible to chloroquine, cycloguanil, pyrimethamine, mefloquine, sulfadoxine, and lumefantrine [178, 273, 298]. We also used the RSA to test for cross-resistance against the artemisinin, where A1 was able to fully inhibit the growth of the *kelch13*^{L543T} *Dd2* strain over a 6hr ring stage exposure.

However, this list is still not exhaustive of all drug resistant phenotypes encountered in the field. For example, the atovaquone resistance is characterized by the *cytochrome bc1*^{Y268S} mutation [299]. Atovaquone is part of the first-line antimalarial Malarone, where it is formulated together with proguanil. This combination is used to treat uncomplicated malaria in children and adults while also being deployed as a prophylactic in frequent travellers to afflicted African regions [300]. Resistance to Atovaquone has been well documented in the clinic [301]. Despite this resistant phenotype not being present in our current panel, it can be argued that resistance is not conferred by the same pathway. This is seen where our selection for Atovaquone mutants *in vitro* yielded very high success rate, while the same could not be achieved with A1 when done in parallel. Therefore, with our current data, we can conclude that A1 does not show cross-resistance to Atovaquone.

4.3.2 Generation of Spontaneous Resistant Mutants Against A1

While we have yet to observe cross-resistance in A1, it does not mean that the compound is immune to the susceptibility of drug resistance. Time and time again, the *Plasmodium* parasites have shown their ability to develop resistant phenotypes when faced with the challenge of various chemotherapeutics [91]. Therefore, it would be ideal if the probability of the selection of resistant parasite be as low as possible, if not at all. One such strategy to anticipate the potential risk of developing resistance mechanisms from novel chemotypes is the use of *In vitro* selection assays [178]. This selection method also serves to improve the understanding of the parasite biology and druggability by identifying molecular markers of resistance through whole genome sequencing of the resultant resistance strains [302].

To evaluate the possibility of selecting for unique resistance phenotypes with A1, we adopted the aforementioned method to predict the possibility of such an outcome. Seeing that our initial efforts did not obtain any resistant mutants, we also included discontinuous drug exposure regimes to account for the likelihood that the resistance could not develop under permanent exposure [303]. However, none of our selection efforts yielded any surviving parasites. One theory that could explain the difficulty of obtaining resistant mutants might be because more than a single point mutation is required to increase the chances of developing a resistant phenotype to A1. In other words, a pre-existing genotype background might be required in order for the selection of a second SNP, where only then would the parasite be able to achieve resistance. Most examples of drug resistant phenotypes require only a single SNP in order to achieve decreased drug sensitivity. [273]. However, an exception has been seen in the selection of artemisinin-resistant mutants in the clinic. There, genome-wide association studies associated mutations in underlying genetic markers such as *ferredoxin* to the increased likelihood of *kelch13* mutations accumulating in Southeast Asian strains [304]. It might be possible that such underlying genetic predisposition is absent in the two strains which were used in our study. The fact that the rate of occurrence of an SNP is 10^{-9} at any particular

point in the genome, makes chances of the occurrence of two SNPs highly unlikely within the parasite inoculum [305].

Another goal for the use of the *in vitro* resistance selection method is to determine the resistance selection frequency [274]. This approach uses multiple increments of inoculum (i.e 10^5 up to 10^9 parasites) treated with the same drug dose to monitor for the lowest inoculum for which surviving parasites can be obtained. This parameter gives an indication of the propensity of resistance to happen, and is interpreted as a high risk should the drug enter the clinic. For example, the minimum inoculum for Atovaquone resistance selection is 10^5 in *Dd2*, while a more recent clinical trial drug, the GNF179, seems to be at most 10^9 if not lower [191, 274]. In comparison, we have yet to be able to obtain surviving parasites at an inoculum of 10^9 regardless of the selection doses and exposure durations, deeming A1 to an ideal antimalarial candidate with a low risk of selecting for resistance.

4.3.3 Stage Specific Activity in the Asexual Lifecycle

While our efforts have not been able to identify a genetic marker of resistance of A1, we looked towards trying to understand the pharmacodynamics of A1. In order to narrow down the vast number of possibilities, we wanted to identify the developmental stages of the life cycle which A1 perturbs. We exposed the parasite to A1 at every 12hr interval of the asexual lifecycle to observe for morphological changes over time. It was observed that A1 exhibited non-viable morphologies when treated at 0, 12, and 24hr of the IDC, while it continued to develop normally when treated from 36hr, despite compound concentrations being at $10\mu\text{M}$. This shows that A1's asexual blood stage activity occurs during at least the first 24 hr of the IDC.

From the perspective of an antimalarial trait, the ring and early trophozoite stage activity that A1 exhibits ideal, especially in treating *P. falciparum* infections. *P. falciparum*, unlike other human infecting species, are able to cause cytoadherence of the infected RBC to the endothelial layer of blood capillaries and veins in various tissues, and the ability to do so is the underlying cause of the symptoms of severe malaria [306]. The parasite tries to achieve sequestration in the later stages of the IDC

(trophozoites onwards) in order to avoid being cleared by the spleen due to its less-deformable state [307]. In order to achieve sequestration, the parasite has to export a host of proteins, such as the variant antigen *Pf* Erythrocyte Membrane Protein 1 (*PfEMP1*), to the RBC membrane surface to interact with the endothelial receptors [308]. Other variant surface antigens that *P. falciparum* displays on the host cell surface also include the Repeated Interspersed Families of Polypeptides (RIFINs) and Subtelomeric Variant Open Reading Frame (STEVARs) multigene families [309, 310]. These types of variant surface antigens are understood to cause rosetting, a form of sequestration in which the parasite binds to and surrounds itself with multiple uninfected RBCs [311, 312]. These rosettes are capable of blocking microvasculature, which also lead to the development of severe malaria [311]. This process of remodelling the host membrane surface with the parasite's own proteins peaks at the onset of trophozoite development, and having a ring and early trophozoite stage-killing drug would help the prevention of complications of the disease [313].

4.3.4 Stage Specific Activity in the Gametocyte Development

Gametocyte inhibitory drugs are few and far between, with Primaquine being the only approved antimalarial capable of effectively blocking transmission [314]. With Primaquine administration deemed unsuitable to G6PD-deficient patients because of the haemolytic activity caused by its metabolites, there is a strong need to add on to the arsenal of gametocyte-killing drugs [315]. We therefore tested A1 for gametocyte inhibitory activity. After treatment of Stage III to V gametes with A1, we did not observe any gametocytes with viable morphologies. However, the gametocyte stage efficacy of A1 is less than ideal compared to that of the asexual stages, with live parasites observed when an exposure of 1 μ M was used (approximately 10-fold difference in IC₅₀). Still, since the data was generated off a microscopic count, there may be unaccounted biases which may lead to an inaccurate count, and a more automated, quantitative count is required to accurately quantify the gametocidal efficacy. For example, if we compare the IC₅₀ of lumefantrine in our assay to that obtained from a high throughput imaging-based readout, there is a discrepancy of 100-fold [316]. Therefore, the IC₅₀ estimation from this experiment should be taken with a pinch of salt, while a more robust

quantification assay should be used to determine the true value. With our current results however, A1 does exhibit the exemplary traits of an antimalarial in terms of its ability to inhibit transmission and prevent the onset of severe malaria.

4.4 Target Identification of A1

4.4.1 Identification of Asexual Blood Stage Target via CETSA

Drug discovery constantly experiences a high attrition rate of lead candidates [206]. One of the causes behind this failure is attributed to the lack of understanding of the pharmacodynamics of the compound [317]. In many cases, the advancement of antimalarial development based on efficacy derived from phenotypic assays has resulted in the neglect of understanding their mode of action [229]. This might also indirectly result in unexpected treatment failures should drugs be deployed with insufficient understanding of their modes of action or resistance mechanisms [318].

We capitalized on the recent establishment of CETSA for use in drug-target engagement screening in *P. falciparum*. The workflow was adapted using 30hpi trophozoite lysates and the heat treatment temperature of 51°C used in the protocol was based on the proteome melt curve profile. Peptide abundance between 37 and 51°C was compared and a 0.3-fold increase in peptide abundance was used as a cut-off to determine thermal stabilized hits. This hit selection criteria led to the identification of *Pf* Zinc Finger (CCCH-type) Protein and four other ribosomal subunit proteins. Since the 80S ribosomal translation is an essential function throughout the entire life cycle, while A1's activity spans only the rings and early trophozoite stage, we deprioritized the ribosomal hits from our study and chose to focus on the *Pf* Zinc Finger (CCCH-type) Protein hit [319].

A concern over the suitability of this protocol was the use 30hpi lysates to screen for A1's potential binding target. As it was described earlier, the transcriptional profile of the parasite differs greatly over the course of the asexual life cycle, and further evidence showed that regulation of mRNA was done in a stage-specific manner. This time-dependent regulation is an observation that has been unprecedented in other organisms [275, 320]. This phenomenon has been shown to be reflected in

the regulation of protein levels as well, where proteins also showed a single abundance peak across the IDC [321]. Therefore, there is a possibility that the pharmacodynamic target of A1 may not be present in the 30hpi lysate, and a secondary validation of our hit(s) is required.

4.4.2 Validation of the CETSA Target *Pf* Zinc Finger (CCCH-type) Protein

After evaluating the CETSA screen, we obtained the *Pf* Zinc Finger (CCCH-type) Protein as the primary hit. The approach to perform this validation was to replicate the thermal stabilization assay of the *Pf* Zinc Finger (CCCH-type) protein for quantification on a Western Blot, and in order to do so, we created an endogenously-tagged cell line using a co-expression selection system. This enabled the protein to be expressed by its endogenous promoter and retain normal physiological levels throughout the IDC. IFA results then showed that the *Pf* Zinc Finger (CCCH-type) Protein was detected in all stages of the life cycle, indicating that the *Pf* Zinc Finger (CCCH-type) Protein may be the binding target in ring stage parasites. We were then also able to repeat the thermal stabilization using 30hpi lysates of the transgenic parasites, giving better confidence to the results.

However, there is an outstanding issue as to whether the *Pf* Zinc Finger (CCCH-type) Protein truly serves as the target of A1 remains to be answered. Multiple target engagement hits have demonstrated minimal dose threshold (MDT, the lowest dose of the compound required for high-confidence protein stabilization) values similar to *in vitro* IC₅₀s of the drug [260, 322]. However, the MDT of *Pf* Zinc Finger (CCCH-type) Protein was 2.5μM, which is in disagreement with A1's efficacy of 170nM, more than 10-folds in excess. Secondly, how *Pf* Zinc Finger (CCCH-type) Protein serves as a drug target is not understood. Evidences from random mutagenic gene knock-out studies using the *piggybac* transposon system have shown that the *Pf* Zinc Finger (CCCH-type) Protein is non-essential for asexual development (data obtained from PlasmoDB) [323]. Therefore, our current inference that this is the drug target of A1 still requires the support of functional validation experiments.

From our stage-specific assays, we observe that A1 is active from the beginning of the asexual life cycle up till at least 24 hpi. However, the assay which identified the *Pf* Zinc Finger (CCCH-type) Protein

was the one performed using lysates of 30hpi trophozoites, where the activity of A1 could be waning. Also, should A1 really bind to the *Pf* Zinc Finger (CCCH-type) Protein, the binding could be inconsequential. Therefore, there is a possibility that the protein was identified as an artefact, rather than an actual binding target.

Another explanation to this hypothesis could be that the *Pf* Zinc Finger (CCCH-type) Protein may be a sticky protein. Sticky proteins are proteins with significant proportion of hydrophobic residues and have the potential to form aggregates with other proteins when unfolded or degraded, and therefore the levels of the protein observed were influenced by its clumping with other proteins [324]. The familiarity and improvements made to the CETSA protocol over the sample processing (such as maximizing the conduct of cell lysis steps over ice, or avoiding prolonged handling of sample tubes by hand, etc.) may also decrease the chances for protein degradation/unfolding to occur when conducting the ring stage CETSA screen later. As for the gametocyte stage, the experiment having being done in live cells would have the *Pf* Zinc Finger (CCCH-type) Protein exist in its native environment, and hence less prone to stability issues during handling [325]. There is also the possibility that the *Pf* Zinc Finger (CCCH-type) Protein is too low in abundance in gametocytes, shown in transcriptomics and proteomic data, that they would not be detectable during the mass spectrometry [326, 327].

4.4.3 Identification of Ring Stage Target via CETSA

Despite the in-cell protocol already being established at the point of this experiment, the method was not yet optimized for ring stage parasites. This limitation was compounded two-fold by the following reasons. First, the enrichment of sufficient ring stage parasites (up to 80-95%) remains challenging for the demands of CETSA. While methods are available for selectively-lysing uninfected RBCs, it would be laborious and too resource-consuming to obtain sufficient material even for mass spectrometry analysis [328]. Secondly, even if the ring stage enrichment could be achieved, the haemoglobin content in a ring stage-infected RBC would still pose a hindrance to the sample analysis due to its abundance [329]. This is still the case despite having a haemoglobin depletion method, as the resins used for haemoglobin depletion also non-specifically binds other proteins. Using high volumes of the resin will run the risk of losing too much *Plasmodium* proteins for subsequent detection on the mass spectrometer (data from Go, K. D., unpublished). Therefore, current CETSA experiments on ring stages can only be done using protein lysates.

In order to conduct a thorough, non-biased target-engagement screen using a representative parasite proteome for a compound with unknown mode of action, the improved protocol incorporated an additional 57°C melt curve on top of the two temperatures used in the earlier screen. We then performed the CETSA on A1 using 12hpi ring lysates to also eliminate potential false-positive proteins not present during the efficacious stages of A1. During this screen, we observed four and 16 proteins which are significantly thermal-stabilized by A1 from the 51 and 57°C. When comparing the hits to the earlier assay done with trophozoite lysates, it was most noticeable that the *Pf* Zinc Finger (CCCH-type) Protein was not amongst the hits. This draws speculation that the earlier result may either be of a false-positive or of low confidence.

Among the non-ribosomal hits, we obtained three proteins with thermal stabilization in high confidence, the highest of which was the metalloprotease FLN. The melting temperature of FLN is higher than 51°C, and hence explains why it did not appear as one of the hits in the trophozoite CETSA

(data by Dziekan J., data unpublished). FLN, an essential asexual stage protein, was first discovered to play a role in breaking down the by-products of haemoglobin degradation [288]. Based on this function, it casts some doubt over the reliability of FLN being one of the hits in early ring stages. This is because the early ring stages, where A1 is seen to have an activity on, are typically not associated with haemoglobin metabolism. Instead, it is in the late rings to trophozoite stages that haemoglobin digestion begins to peak, and a distinct haemazoin can be detected in many forms [330, 331]. However, evidences of haemoglobin digestion in early ring stages have been well documented, and this is supported by the theory that the artemisinin activation in early rings is attributed to the presence of haem [332, 333]. Therefore, it is justifiable that FLN is present throughout the ring stages, and that it may be possible that A1 acts on the essential haemoglobin metabolic pathway through FLN.

The second of these hits is the putative *Pf* Dipthine Synthase. Dipthine synthases are involved in the formation of dipthamide, a post-translational modification unique to the translational elongation factor 2 for proper function [334]. The protein in particular, functions as a methyltransferase in the second step of the modification, the lack of which results in attenuation of protein translation. This could mean that A1 may work by attenuating protein translation. The presence of ribosomal subunit proteins amongst the hits may also support the notion that A1 affects protein translation. Similar to the results of the trophozoite CETSA, we observed multiple ribosomal subunit proteins showing thermal stabilization with high confidence, this time with a total of 16 different proteins spanning the 40 and 60S subunits. They consist of two and 15 proteins in the 51°C and 57°C melt curves respectively. There was a total of 14 from the 60S and two from the 40S ribosomal subunit protein. One of these subunit proteins, the 60S ribosomal protein L36 (PlasmoDB geneID: *PF3D7_1109900*) was observed not only between both melt curves, but also in the trophozoite lysate CETSA (Supp Figure 1). At this moment, the evidence gathered seemed to indicated that A1 engages many proteins of the 60S ribosomal subunits (14 of 44 known) [335]. This would suggest that A1 may exert its mode of action by disrupting protein translation via the 80S ribosome. Studies have suggested the 80S ribosome of *Plasmodium* as a druggable target, with structural evidence of the ribosome binding with mefloquine

[76]. In that study, it was shown that the binding site of mefloquine was in the GTPase-associated center of the 60S subunit, interacting with the L13 protein which was also identified as a hit in our screen. However, two other proteins of the subunit were also shown to be involved in this interaction, and were not identified from our screen. At the same time, the structure of A1 differs greatly to that of mefloquine, which makes it highly unlikely that A1 engages the ribosome in the same manner. Despite the *Pf* Dipthine Synthase also playing a role in protein translation, further evidence from functional experiments is required to support the claim that A1 disrupts this pathway. Despite the evidences presented that support each of the hits being a druggable target, there ultimately is still a need to further deconvolute the mechanism of action of A1.

4.4.4 Identification of Gametocyte Stage Target via CETSA

The in-cell CETSA is currently the most sophisticated form of the thermal challenge assay, allowing for *in situ* cellular processes to elucidate results that reflect the pharmacodynamics of the drug. We therefore conducted last of the CETSA screen in gametocytes using the in-cell method. From the hits observed, ribosomal proteins were most noticeably absent. At the same time, the *Pf* Zinc Finger (CCCH-type) Protein, *Pf* Dipthine Synthase, as well as the hypothetical protein PF3D7_1022100 were all absent from both 2- and 3MAD plots. Amongst the proteins, the Heat Shock Protein 90 (Hsp90, PlasmoDB geneID: PF3D7_0708400) was shown to pass the 2MAD cut-off. More interestingly though, FLN was observed to be thermal stabilized with high confidence, passing the 3MAD cut-off.

We have earlier discussed that haemoglobin metabolism occurs over a large part of the asexual IDC, and that A1 could possibly act on the rings and trophozoite stages through this biological pathway. This would not be likely in the case of the transmission-focused gametocytes, especially in the late stages of developments where haemoglobin digestion does not occur [336]. However, according to other studies, there is more to FLN than simply haemoglobin catabolism. Firstly, using recombinant FLN, the protein was observed to have different substrate preferences depending on the pH of its environment [289]. The pH for most of the internal cellular compartment is 7.2, while the food vacuole

differs greatly at 5.2 [288]. These hinted that FLN may be localized in parts of the parasite other than the DV. Indeed, it was observed that FLN was seen to be associated with vesicular structures in asexual parasites, which were later found to be apicoplasts [289, 337]. Because FLN is phylogenetically similar to other M16 metalloprotease proteins which function to cleave organelle-bound transit peptides, it was then hypothesized and subsequently shown that FLN does the same in apicoplasts [337]. The function of FLN and other M16 metalloproteases are vital to the parasite as it prevents uncleaved transit proteins from non-specifically inserting into and disrupting the organelle membrane, resulting in the loss of overall function [338]. The apicoplast is known to encode 30 of the 545 proteins found in the organelle [339, 340]. This means that in order to carry out its essential role for parasite development, the import of its remaining proteome from the cytosol is critical. With the need to cleave off the transit peptide of these imported proteins is needed prior achieving their function, disrupting the apicoplast protein-processing function of FLN will achieve parasite killing.

The previous discussions have only served to explain how disrupting FLN function in the apicoplast may be a mode of action of A1 in the asexual IDC. However, as FLN was also a hit identified in the gametocyte in-cell CETSA screen, there may be a possibility that a single target disruption of both developmental stages may hold. Indeed, the apicoplast is known to be vital in the development of gametocytes. Parasites depleted of apicoplasts developed abnormal morphologies, which in turn, also affected the oocyst count [341]. However, the abnormal morphologies of apicoplast-depleted gametocytes differed from what we observed from A1 treatment of stage III gametes. Although apicoplasts are shown to have functional relevance in gametocytes, there is still some doubt as to whether there is a possibility that the disruption of the organelle function through FLN will also lead to the same defects observed in the apicoplast-depleted cells.

4.4.5 Validation of FLN Inhibition as the Mode of Action of A1

The observation that FLN was identified as a target in rings as well as gametocytes warranted validation of the enzyme as the target of A1. We therefore pursued the validation of FLN as the biological target

of A1 in a functional assay. Indeed, when we adopted the fluorescent-quencher system to determine FLN activity, the recombinant protein's activity was almost abolished at doses similar to the MDT in the in-cell CETSA, while also observing a dose-dependent inhibition of the enzyme. However, the cell-free enzymatic assay's and *in vitro* efficacy assay's IC₅₀s are far apart, although such disagreements are not unheard of. We hypothesize that there could be a need for an accumulation of a high concentration of the compound in the vacuolar compartments where the protein resides may be required in order to exert its antimalarial effect. While this has not been proven to be the case of apicoplast-targeting drugs, it is known that Chloroquine accumulation in the digestive vacuole is needed for the exertion of its antimalarial effect [342]. Still, this result is promising with regards to the understanding of A1's mode of action, and further cellular phenotypic assays are required to confirm this finding.

Still, the *Pf* Zinc Finger (CCCH-type) Protein stabilization experiment and the proposal of the ribosomal translational inhibition model makes us ponder over these as the possible mechanisms of action other than FLN inhibition. However, our experimental data gives evidences apart from this functional validation to argue that FLN is more likely to be the target of A1. More often than not, drug molecules and their targets follow the lock-and-key concept of a single target specificity [343]. The observation that FLN appears as a hit in both asexual and sexual stage CETSA screens gives us higher confidence that it is the engagement target of A1, especially so when CETSA was done in-cell for gametocytes. The stage-specificity assays done are in agreement, A1 activity is observed in the rings and gametocytes, while we are less confident about activity in mid trophozoites. However, we are not ruling out that A1 inhibition of FLN does not occur in 30hpi trophozoites, simply because the CETSA done at that point would not have given FLN as a hit.

Current knowledge shows that FLN is known to have more than one biological function in the parasite, with evidence showing that it plays a role in apicoplast protein processing on top of the previously more established involvement in the haemoglobin digestion pathway [289]. It was shown that at

different pHs, FLN has different substrate specificities which is shown by the higher enzymatic activity of the preferred substrate over the other. Our functional validation assay was performed in a pH7.2 buffer with the preferred substrate at the neutral pH. At the same time, when FLN was not incubated with inhibitors, enzymatic activity observed in our assay seem to be comparable to that of the published data. It therefore leans towards the hypothesis that A1's activity on FLN disrupts its role in apicoplasts over that of the DV.

4.5 Lead Candidate Optimization of A1

4.5.1 *In vivo* Efficacy and Toxicity of A1

We attempted to determine the efficacy of A1 *in vivo* using a rodent infection model. It is difficult at this point to determine an efficacious dose as recrudescence was observed even at the highest administered dosing regime. However, this sub-lethal dosing may also suggest that the selection of A1-resistant mutants may be happening. It is seen that from day 0 to day 2, parasitemia decreased in the mice with each subsequent treatment. However, despite administering a third dose on day 2, the parasitemia instead increased on day 3 when supposedly the lower parasite burden should have been reduced by the same dose. The resulting parasites of the 20mg/kg may serve as a platform to identify a potential molecular marker of resistance which our *in vitro* approaches have failed to yield so far.

Following a quick assessment of the structure of A1, it was apparent that the hydrophobic chemical structure might lead to issues with bioavailability, which may in turn translate to a lower than expected *in vivo* compared to *in vitro* efficacy levels. Indeed, a dose as high as 30mg/kg was required to effectively reduce the parasite burden below FACS detection limits. Based on our calculations, a dose of 10mg/kg will give a plasma concentration of about 260nM, higher than the estimated IC₅₀ of A1. A dose of 20mg/kg would therefore already be in excess of the required MIC. However, even a four-day once daily dosage of 30mg/kg could not fully get rid of the infection. A 30mg/kg dose would equate to approximately 4.5-fold higher plasma concentrations than the *in vitro* IC₅₀, which is theoretically sufficient to kill all the parasites in a single dose, let alone four. This suggests that there may be some

agreement with the initial hypothesis on the lack bioavailability. Hence, we needed a detailed *in vivo* pharmacokinetic analysis of A1 to ascertain this claim.

4.5.2 *In vivo* Pharmacokinetic Profile of A1

We conducted an *in vivo* pharmacokinetic analysis of A1 to investigate its behaviour in the blood stream, and at the same time attempt to find an explanation to the poor *in vivo* efficacy. $C_{\max}$ was reached within 15min post-IP administration, with a T_{lag} of 0min. The time needed for $C_{\max}$ to be achieved reflects the nature of intraperitoneal administration, where the compound gets absorbed directly into the venular network in the peritoneal spaces, and drains into the liver by the hepatic portal vein before reaching other organs [344]. It is therefore likely that A1 may be subjected to the first-pass metabolism (also known as first-pass effect) of the liver [345]. Liver first-pass metabolism is known to severely reduce bioavailability due to degradation, especially to drugs, by the enzymes in the liver such as the cytochrome P450 (CYP) family [346]. While CYP enzymes play different roles in homeostatic pathways in many other tissues of the body, the ones in the liver are responsible for approximately 75% of the enzymatic activity in the liver [346]. These enzymes may either have specific or multiple substrates, collectively delivering a wide range of metabolic reactions. Despite the structural studies done to improve the understanding on the CYP enzyme functions, it is still difficult to predict whether compounds are subjected to CYP degradation, and it cannot be ruled out whether A1 is similarly affected.

Despite the poor bioavailability of A1, a dose 4.5-fold above the estimated IC_{50} is still able to severely reduce parasites. Another possible explanation of how an equivalent of 1.4nM blood plasma concentration would be able to kill parasites is tissue distribution. Due to the highly hydrophobic nature of A1, it is anticipated that a large portion compound would accumulate in tissues. It has been established that RBCs also serve as such reservoirs for hydrophobic drugs, although to a much lesser extent compared to those such as adipose cells [347]. Our pharmacokinetics studies only monitored plasma concentrations, where removal of the RBC fraction was part of the sample processing step.

Therefore, should A1 have accumulated in the RBCs in levels sufficient to kill that parasites, our pharmacokinetics data may still be in agreement with the efficacy results if whole blood concentrations were measured instead.

4.5.3 SAR Optimization of A1

Our lead optimization efforts through SAR studies in A1 have delivered 3 analogues with improved IC_{50} s and hydrophobicity profiles. From our efficacy and toxicity tests, it is shown that all analogues have improved selectivity over A1. Since these assays were done *in vitro*, the results were independent of pharmacokinetic factors, and hence it can be claimed that these compounds have better efficacy over the parent compound. However, lead optimization efforts should not stop here. While the predicted $xlogP$ values of each of these analogues fall below that of A1 (ranges from 6 to 8), they are not yet ideal. While we have determined that it is highly unlikely to modify moiety 1, there are other chemical groups of the scaffold yet to be explored. The truncation of moieties 4 and 5 all seemed to have diminished the parasite-killing efficacy to varying degrees. This suggests that there is a likelihood that these structures are required for the target engagement as well. Overall, the data serves as critical information to future SAR strategies, while the compounds may later be used as tools for target validation studies.

Chapter 5: Future Directions

5.1 Identification of Anticancer Lead Compounds for Repurposing in Malaria

From comparing the data we have generated and subsequent literature reviews, we have identified two lead candidates from the Selleck Chemicals Anticancer Library for repurposing in malaria treatment. The suitability on whether they can be tested on humans was based on the extrapolated MIC being achieved with a single dose of the drug formulation. However, even for the purpose of repurposing, the *in vitro* efficacy data on its own is insufficient to cause serious consideration of taking these compounds further. Further confidence in these compounds can be instilled by bringing them through a similar assessment and characterization route which A1 has undergone, with the added benefit that human testing is more within reach should the compounds fulfil the criteria of a next-generation antimalarial drug.

5.1.1 Assessment for the Risk of Resistance

Similarly done as with A1, these compounds should be tested against our panel of drug resistant strains. In this regard, it would also give more confidence if cross-resistance is not seen in clinical isolates obtained from the same geographical region. While the TRAC initiative provides patient isolates from the Southeast Asian region, other consortiums have also done likewise in Africa [348]. Being one of the countries most in need of novel antimalarials to overcome the drug resistance countries, having our lead candidates tested against representative isolates from different pandemic states would relieve the worry of treatment failure. At the same time, we can complement this by attempting to select for drug resistance in the ARMD cell lines. Resistance markers identified (if any) can then be used as a surveillance marker before deployment for treatment.

5.1.2 Identifying the Molecular Target of Compounds 26 and 40

We have shown through A1 that target identification can be achieved through CETSA. We therefore have the means to do likewise for these compounds. Getting an understanding of their target may

also allow for the creation of in-roads into target-based rationale design of new inhibitors, or develop a novel phenotypic screen for which compound libraries can be tested for activity against these targets.

5.1.3 Human Trials

Having passed multiple rounds of safety testing and regulatory approval, clinical testing of these anticancer drugs is now within reach. In order to conduct these trials, we would have to obtain these drugs in GMP-produced formulations from the existing license holders to be able to safely conduct these tests. With the drugs, we will then be able to contact the agencies which carry out controlled human malaria infection studies to have a preliminary assessment of the efficacy in humans, as well as to establish a suitable dosing regime for each drug [349].

One common side effect observed in patients

5.1.4 Identification of Partner Drugs for Combinatorial Therapy

With the resistance encountered by the use of monotherapy, WHO now highly recommends the use of combinatorial therapy for treatment of all malaria cases [1]. Therefore, the likelihood of either candidates being used individually is very low. With a huge repertoire of antimalarials available in the market, there are many options for which we can propose for the use in combination with. One method to determine the most suitable partner drug simply by looking for synergistic effects. This can be done *in vitro* by testing co-treated samples in a dose-dependent manner with different antimalarial drugs, without the knowledge of how the compounds may work together mechanistically (Figure 25) [350]. As synergistic effects would also aid in reducing the risk of resistance, identifying drug combinations that complement each other would favour one drug over another.

Figure 25. A model isobologram showing possible dose-dependent relationship during co-treatment.

By testing dose-dependent co-treatments by changing concentrations of either drug at a time, the plots of the IC₅₀s would give the shape of a **1**) additive, **2**) synergistic, or **3**) antagonistic effect. Figure from Bell, A. 2005 [350].

5.2 Biological Characterization of A1

5.2.1 Assessment for Cross-Resistance to Existing Antimalarials

Despite already testing negative for cross-resistance against a large panel of resistant strains, we should remain vigilant with regards to screening for the possibility of a loss of therapeutic efficacy in other resistant phenotypes. For example, the *cytB*^{Y268S} atovaquone resistance-conferring mutant is one which remains elusive in our efforts. The addition of the TM90C2B strain carrying this SNP would therefore aid in testing for atovaquone cross-resistance [299]. In addition, as proposed for the anticancer compounds, including additional clinical isolates from the African region would provide a more current assessment A1's suitability for the clinic.

5.2.2 Selection of A1 Drug Resistant Mutants with Different Approaches

So far, our attempts of generating resistant mutants have not yielded any surviving parasites, which indicates that there is a low risk of selecting for mutants should A1 be eventually given as an antimalarial. However, this is based off the ideal situation that the A1 treatment regime successfully kills 100% infections. In the body, drug levels decrease over time, where it eventually reaches sub-therapeutic doses. This is when the selection of resistant mutants is at its prime. Therefore, we can replicate this *in vitro* by applying drug pressure at IC₅₀ doses or lower instead of IC₉₉ to replicate this condition. *In vivo*, we may already have observed this scenario unfolding (Figure 20). We can investigate if that is true by subjecting the 20mg/kg-treated parasites with gradual increment of doses until the parasites are no longer responsive to A1, and subsequently sequence the genome of those parasites to identify for molecular markers.

5.3 Investigating A1's Mode of Action

5.3.1 Functional validation of the *Pf* Zinc Finger (CCCH-type) Protein as the Asexual Stage Target

Currently, we only have preliminary evidence showing that A1 does indeed engage and thermal-stabilizes the *Pf* Zinc Finger (CCCH-type) Protein. However, this engagement is observed only in the trophozoite stage lysate CETSA, and not in rings and gametocytes where A1 shows its most promising antimalarial activity. Therefore, a functional validation would aid in decipher the truth in this conflict.

5.3.1.1 RNA Co-Immunoprecipitation (Co-IP) of *Pf* Zinc Finger (CCCH-type) Protein Substrates

The first part of the approach uses the established HA-tagged cell line we have generated for the initial western blot validation. By using anti-HA antibodies on beads, we can IP the protein from cell lysates. Since evidence has shown that zinc finger proteins with the C-x8-C-x5-C-x3-H coordinating domains are RNA binding, we can then subsequently purify the RNA bound to the precipitated proteins for RNA sequencing. By comparing the sequencing data against a negative condition (i.e non-tagged parental 3D7), we can then identify the *Pf* Zinc Finger (CCCH-type) Protein's substrates based on enrichment.

5.3.1.2 Drug Response Transcriptomic Profiling

One of the tools which has enabled us to uncover gene targets for therapeutic development is through transcription-level profiling. Under drug pressure, it has been shown that parasites would attempt to maintain the homeostatic balance in the cell, and gene regulation serves as the basis of these responses [175]. This has been used to understand parasite stress responses, treatment responses, as well as resistance mechanism responses [351]. The molecular approach compares the gene transcription changes in parasites subjected to drug treatment vs untreated through techniques such as RNA sequencing or microarrays. In doing so with A1, we can identify the stress response the treated parasites exhibit during exposure to the compound. The data would serve to complement the RNA-IP experiment in that we may observe the altered expression of *Pf* Zinc Finger (CCCH-type) Protein's target genes. Alternatively, should there be a lack of cross-talk between these two experiments, the

transcriptomic profiling would still serve to uncover the true cellular responses which the parasite undergoes, leading to a better understanding of A1's mode of action.

5.3.2 Study of FLN as the Effector of A1's Mode of Action

5.3.2.1 Validation of the Binding to FLN

So far, the evidences gathered suggests that A1 disrupts the parasite development through FLN's function. This was shown by the inhibition of the peptide proteolytic activity using recombinant FLN protein. However, this engagement has to be further validated. It may be possible to achieve this first with the use of surface plasmon resonance (SPR). The assay would require the immobilization of the recombinant protein on the sensor surface. Subsequently, a solution of the drug will be run over the surface, and refractive index changes is measured to obtain real-time binding kinetics and affinity. The target engagement can be further validated by performing SPR to compare the binding kinetics of A1 and its more potent analogues, where a correlation of K_m vs IC_{50} would give further confidence that the FLN binding results in better efficacy. Another technique to validate the binding is to perform co-crystallization of A1 with FLN. Like in SPR, doing so with our analogues would increase the confidence of the target engagement data. Structural data of the binding site of A1 would also then serve to provide information to conduct a target-based SAR strategy for better analogues.

5.3.2.2 Elucidating the Downstream Effects of FLN Binding by A1

As described, FLN plays two biologically different roles in the parasite. In the asexual stage, FLN is present in the DV as a catabolic enzyme of short peptides generated from haemoglobin digestion [24]. At the same time, evidence has been shown that FLN has a second specificity for positively charged peptides, characteristic of transit peptides that targets proteins to the apicoplast [289]. There, the cleavage of the transit peptide is required to generate the mature protein for its function in the apicoplast [337]. At the same time, the apicoplast is important for gametocytogenesis to occur [341]. However, we have earlier concluded that it is more likely that the apicoplast hypothesis hold true. In order to investigate if FLN-targeting compromises apicoplast function, a complementation assay using

Isopentenyl Pyrophosphate (IPP) can be done. It has been shown that parasites lacking the entire apicoplast can be rescued simply by supplementing growth media with IPP [341]. Therefore, if A1 treatment can be reversed through IPP supplementation, it can be said that the compound's function targets the apicoplast FLN.

5.4 Improving A1 as a Clinical Candidate

5.4.1 SAR Strategies

With poor pharmacokinetics accounting for the lack of *in vivo* efficacy, the overall plan to improve the physiochemical properties of A1 remains as the basis of the SAR studies. A target-based approach would be the most ideal, but that would be subjected to the advances of structural studies with FLN. After testing a series of analogues, we have only been able to identify one moiety which further modifications can be based on. While this serves as a stepping stone towards creating a more drug-like molecule, there is more potential in the structure yet to be tapped on. The straightforward move would be to attempt modifications in A1 at sites other than moieties 1 and 3. Any successful modifications can also then be combined with moiety 3 modifications to test if they can be additive.

5.4.2 *In vivo* pharmacokinetics

Achieving a measly $C_{\max}$ of 750ng/L in plasma concentration to control a 3% parasitemia infection may be a little farfetched. Therefore, there is a need to identify the underlying cause behind this observation. Due to the hydrophobic nature of the compound structure, we hypothesized that the compound may be highly distributed to the tissues. One of these compartments is potentially the RBCs. Therefore, a quantification of A1 in lysed RBC supernatant would help to investigate that.

The second hypothesis behind such low levels of compound may be the first-pass metabolism present in the liver. The liver microsomes are the major source of CYP enzymes in the organ. Purified liver microsomes of mice and humans are available to be used in cell-free assays. This can be used to test for CYP metabolism, and any evidence of it would suggest the need for structural modifications to block degradation by these enzymes to improve bioavailability.

Chapter 6: Summary and Conclusion

The stagnation of the number of cases and deaths reported over the last five years is an indication that no significant process towards the goal has been made. Drug resistance has been reported to play a central role behind the slowing down of eradication efforts, with treatment prompting for newer control strategies. Having a multifaceted approach to malaria control is vital in the efforts towards curbing the spread of the disease, but approaches other than chemotherapeutics either do not directly reduce the number of cases or have started to have waning effects. Despite having a war chest of antimalarials are available for deployment, resistance even towards combinatorial therapies have been reported. Therefore, the need to discover novel antimalarials remains the way forward, even if it may take years to achieve the next deployable drug.

In our efforts to contribute to the drug discovery pipeline, we screened two libraries with different agendas. With an aim of speeding through the drug development process, the first library we screened, the Selleck Chemicals Anticancer Library, contains hundreds of compounds already in advanced clinical stages for cancer, needing little development before repurposing. From there, we identified two compounds which have been shown to be able to achieve human plasma concentrations higher than extrapolated *in vitro* efficacy indexes. We therefore propose these lead candidates for preliminary clinical testing in human controlled malaria infection studies before full-scale clinical studies.

Following the recommendations which outline the ideal traits of an antimalarial candidate, we set out to identify a novel chemical scaffold which is deemed suitable for development into a new drug. The second library we screened was the Nanyang Chemical Compound Library, consisting of a diverse set of chemical scaffolds previously not involved in biological research. From there, we identified a lead compound, therein known as A1, to have low nanomolar efficacy and good selectivity *in vitro*. It was important that A1 did not exhibit cross-resistance to existing antimalarials and did not have the propensity to develop resistance following drug exposure. These made for an ideal chemical scaffold

of a drug candidate, with a low risk of aggravating the current drug resistance problem. Still, further evaluation of the likelihood of encountering drug resistance is needed in order to fully assess the risk of therapeutic failure should A1 go develop into a clinical drug.

A1 also exhibited other desirable traits of an antimalarial. It was shown to kill asexual parasites at ring stages, which would help in preventing parasite sequestration and the subsequent complications that follows in *P. falciparum* infections. A1 also inhibited gametocyte development from stage III onwards, which will contribute to stopping the transmission of the disease. Therefore, it checks off the criteria of having a potent activity against multiple stages of the parasite life cycle.

In order to understand the mode of action of A1, we used CETSA to identify the putative target(s) of A1, and validated them using various methods. With the development of the CETSA protocol over time, we managed to identify and validate, to certain degrees, the *Pf* Zinc Finger (CCCH-type) Protein and FLN as the engagement targets of A1. FLN was identified as a hit with high confidence in CETSA done in both ring and gametocyte stage samples, and along with the functional validation assay showing that it inhibits FLN proteolytic activity, makes it the primary target to investigate. Based on the essentiality of the FLN's function in the apicoplast, we hypothesize that A1 disrupts parasite development through inhibiting the protein's proteolytic activity. This makes for a tantalizing prospect in which A1 acts on a highly important pathway of the parasite biology. Further studies planned on investigating its interaction with the protein would not only serve to improve our understanding of a druggable pathway in the parasite, but also lead to structural improvements of the compound.

Finally, we have also initiated a medicinal chemistry approach in parallel to improve the pharmacokinetic properties as well as the efficacy of A1. The *in vivo* efficacy of A1 was poorer than expected, where a higher than expected dose was required to control the infection. This might have been explained by the poor bioavailability of the compound, observed during a subsequent pharmacokinetics assay. We attributed these observations to the highly hydrophobic nature of A1's chemical structure, and developed the initial SAR strategy to improve the compound in this aspect.

We obtained three candidates in which we have shown to have better *in vitro* efficacy, showing the potential of the chemical scaffold to be optimized further to become a serious candidate for clinical testing.

Supplementary Figures

Figure S1. Protein abundance plots of all thermal stabilized proteins (≥ 0.3 fold increase) identified from the trophozoite lysate CETSA screen. Apart from the validated *Pf* Zinc Finger (CCCH-type) Protein, four other ribosomal proteins belonging to the 80S ribosome were also found.

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