Developing *Plasmodium cynomolgi* culture as a model to investigate antimalarial effects on *Plasmodium vivax*

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Abstract

The ability to culture pathogenic organisms enables researchers to understand the biology of the organism which aids in the development of vaccines and drugs. Hence the establishment of the protocol for the in vitro cultivation of the erythrocytic stages of *Plasmodium falciparum* revolutionized research into this important cause of human malaria. However, it is not possible to cultivate *Plasmodium vivax*, the most widely distributed and difficult to treat malaria parasite, due to its strict preference for reticulocytes. *Plasmodium cynomolgi*, a macaque infecting species phylogenetically close to *P. vivax* has been routinely used in animal models to study the dormant liver-stage forms of *P. vivax*. Cultivation of *P. cynomolgi* erythrocytic stage was reported in the early 1980s, has not been pursued further. In this study, we have revitalized the continuous culture of *P. cynomolgi* and systematically defined the optimal conditions for the tractable in vitro culture of this important *P. vivax* model. Importantly we have discovered that the successful in vitro culture of *P. cynomolgi* is strain dependent, with only one (Berok strain) of the three strains tested, is amenable to long term culture. Morphological and phenotypic characterisation of our *P. cynomolgi* (Berok strain) cultured parasites clearly show similarities with *P. vivax*. In addition to validating the potential of this culture system for high throughput drug screening in erythrocytic stages, a homogenous line was isolated from a single infected cell; paving the way for clean whole genome sequences.

Understanding of hypnozoite biology of *P. vivax* remains elusive due to the lack of sustainable in vitro models for long term cultures of hepatocytes to capture the full liver stage cycle including the transition to blood stage parasites. To define the antimalarial drug effects in the full life cycle of *P. vivax*, we also developed a liver stage model using *P. cynomolgi* infected 3-dimensional (3D) hepatic spheroids which proved to be a predictive radical cure model which corresponds with outcomes using in vivo monkey models. The phosphatidylinositol-4-0H kinase (PI4K) inhibitor- KDU691 was used in this study as the exploratory tool compound to demonstrate the robustness of the 3D hepatic spheroid model in predicting in vivo outcomes. KDU691 was chosen as it was previously proven to be active in the causal prophylaxis model and delayed treatment model carried out in the conventional 2D monolayer based assays but failed in the in vivo radical cure model using rhesus monkeys.
It is hoped that the *in vitro* tools developed using *P. cynomolgi*, will accelerate *P. vivax* vaccine and drug discovery efforts through better understanding of the mechanisms of its pathogenesis.
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This thesis is especially dedicated to my late father, Chua Liang Hock, who moulded me to be the person that I am today. He would have been proud and happy to witness the completion of my doctorate studies. Thank you, Dad, for always encouraging me to be the best version of myself and for always believing in my capabilities through your own unique way.
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<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>ACT</td>
<td>Artemisinin-based combination therapy</td>
</tr>
<tr>
<td>ATQ</td>
<td>Atovaquone</td>
</tr>
<tr>
<td>BPRC</td>
<td>Biomedical Primate Research Centre</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CO2</td>
<td>Carbon dioxide gas</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>DARC</td>
<td>Duffy Antigen Receptor for Chemokines</td>
</tr>
<tr>
<td>DBPs</td>
<td>Duffy-Binding Proteins</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>days post infection</td>
</tr>
<tr>
<td>et al.</td>
<td>et alia (and others)</td>
</tr>
<tr>
<td>ex vivo</td>
<td>Experiment on living tissues outside the organism under artificial condition that mimics natural condition.</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6 phosphate dehydrogenase</td>
</tr>
<tr>
<td>GTS</td>
<td>WHO Global Technical Strategy for Malaria</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Heat Shock Protein 70</td>
</tr>
<tr>
<td>HTS</td>
<td>High-Throughput Screening</td>
</tr>
<tr>
<td>in vitro</td>
<td>Experiment on extracted living tissues outside the living organism.</td>
</tr>
<tr>
<td>in vivo</td>
<td>Biological interactions or experiments that happen within a living organism.</td>
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<tr>
<td>iRBC</td>
<td>Infected Red Blood Cell</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>ITN</td>
<td>Insecticide Treated bed Nets</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>M.</td>
<td>Macaca</td>
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<tr>
<td>Mf</td>
<td>Macaca fascicularis</td>
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<td>mg</td>
<td>Milligram</td>
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<td>min</td>
<td>Minute</td>
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<td>Milliliter</td>
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<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>Mm</td>
<td>Macaca mulatta</td>
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CHAPTER 1

INTRODUCTION
1.1 Malaria

Malaria, a vector borne disease recorded for over 4000 years, still remains among the most devastating infectious diseases of the 21st century. Approximately 3.2 billion people, around half of the world’s population, are risk of contracting malaria. It affects 91 countries globally and accounts for around 445,000 deaths yearly with extensive morbidity mainly in the tropical and sub-tropical countries (Figure 1.1). In 2016, there was around 216 million cases of malaria, an increase of 5 million cases from 2015 [1]. The majority of malaria cases occur in sub-Saharan Africa and Asia. Children and pregnant women are particularly vulnerable to severe and fatal malaria. Certainly, in countries where malaria is endemic, severe malaria is closely linked to the poor and disadvantaged who have limited access to healthcare.

![Figure 1.1 Map of malaria endemic areas (Phillips et al., 2017). *Plasmodium falciparum* is mainly found in the tropical regions as its gametocytes require a temperature of more than 21°C to mate and produce infective sporozoites in the mosquito vector. *Plasmodium vivax* is prevalent globally even in subtropical regions where the parasites are able to develop to infective sporozoites in the mosquito vector at temperatures as low as 16°C.](image)

Due to the high social and economic burden of malaria worldwide, The Roll Back Malaria (RBM) Partnership was launched in 1998 by WHO, United Nations Development Programme (UNDP), United Nations Children’s Fund (UNICEF) and World Bank as a
coordinated global effort to eliminate the disease. The more than 500 partnerships formed between governments, non-governmental organizations, academic institutions, private companies and international organizations worldwide have facilitated malaria-control efforts in both global and national levels. The coordination by the RBM Partnership has prevented duplication and fragmentation of efforts between partners hence ensuring optimal use of resources. The objectives in the recent RBM Strategic Plan 2018-2020 are well aligned with the WHO Global Technical Strategy for Malaria 2016-2030 (GTS) where the goal by 2020 is to reduce global malaria incidence and mortality as compared to 2015 by at least 40%, to eliminate malaria in at least 10 more countries as compared to 2015 and to prevent the re-emergence of malaria in countries declared malaria free in 2015 [2].

US$ 2.7 billion was invested in 2016 in malaria control and elimination globally. Of which the majority of the investment was in sub-Saharan Africa, followed by South-east Asia [1]. The majority of the efforts in controlling malaria are in vector control/bite prevention followed by diagnostic testing and treatment [1]. Malaria transmission is determined by the frequency of contact between infected mosquitoes and humans and vice versa. To reduce or interrupt malaria transmission in endemic areas, appropriate vector control measures such as insecticide treated bed nets (ITN) and indoor residual spraying (IRS) with DDT; and if possible mass drug administration with antimalarials. These combined measures were crucial in reducing malaria cases in parts of Asia, particularly in India and Sri Lanka [3, 4]. Apart from reducing the risk of having a case of malaria developing into a more severe disease, prompt diagnosis and treatment also reduces malaria transmission by eliminating the parasite reservoir in the human host [1].

The malaria parasite was first discovered in 1880 by a French army surgeon, Charles Louis Alphonse Laveran [5]. The disease is caused by the protozoan parasite of the genus *Plasmodium* which is transmitted by the *Anopheles* mosquito vector [6]. Nine *Plasmodium* parasite species are of direct relevance to human health – *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale curtisi, Plasmodium ovale wallikeri, Plasmodium malariae, Plasmodium knowlesi, Plasmodium cynomolgi* [1, 7-10] and more recently, *Plasmodium brasilianum* [11] and *Plasmodium simium* [12]. The simian parasites *P. cynomolgi* and *P. knowlesi* have grown in importance mainly in Malaysia and other parts of southeast Asia due to their zoonotic nature [10, 13] without any evidence of natural direct human to human transmission [8]. Out of the nine species, *P. falciparum* and *P.
*P. falciparum* predominantly found in the African continent and Asia, accounts for the highest mortality while *P. vivax*, with a wider global distribution, causes significant morbidity due to its ability to cause relapsing disease.

The difference in endemicity of *P. falciparum* and *P. vivax* is caused by climate effects (primarily temperature) on the parasite development in the mosquito vector. The global presence of *P. vivax*, in sub-Saharan Africa and Asia, is due to the parasite’s ability to survive lower temperature and higher altitudes in the mosquito vector [14] and the inherent lack of Duffy blood group positive human erythrocytes in the African continent which is required for *P. vivax* infection in erythrocytes [15, 16]. Along with geographical differences, the biology of *P. falciparum* and *P. vivax* has some important differences. As in the less common *P. ovale*, vivax malaria is characterized by a dormant exo-erythrocytic stage – the hypnozoite, which causes relapses sometimes months or years post its primary infection in the human host [17]. This dormant form enables the parasites to survive winter months when *Anopheles* mosquitoes hibernate and are not present for malaria transmission. These relapses serve as a natural reservoir for transmission since it substantially lengthens the period of infection in the host. Another significant difference between *P. falciparum* and *P. vivax* is the latter’s strict preference in invading reticulocytes [18-23] which makes adapting it to *in vitro* culture challenging.

### 1.2 Characteristics of malaria

#### 1.2.1 Life cycle of the malaria parasite

The life cycle of the malaria parasite has a complex life cycle alternating between two hosts - the female *Anopheles* mosquito and its definitive human host. Before laying eggs, the female *Anopheles* mosquitoes require blood meals. During a blood feed, the malaria infected-female *Anopheles* mosquitoes injects *Plasmodium* species sporozoites into the host from its salivary glands. Upon entering the blood stream, the sporozoites traverse through host cells before infecting the hepatocytes in the liver within 30 to 60 minutes post infection. In the human, non-dormant *Plasmodium* species such as *P. falciparum* (Figure 1.2), these sporozoites develop in the infected hepatocytes to form large liver stage known as the liver-schizonts. These further differentiate into merozoites which ruptures to release tens of thousands of merozoites into the blood stream [24].
Figure 1.2 Life cycle of the non-dormant malaria parasite (e.g. *Plasmodium falciparum*).

During a blood meal, the infected *Anopheles* mosquito injects sporozoites into the human host. The sporozoites infect the hepatocytes and develops into liver schizonts which rupture and release merozoites into the blood. The parasites undergo asexual replication in the blood where it progresses from the ring stage to the trophozoite and eventually to schizont which ruptures to release merozoites back into the blood. Some parasites differentiates into the sexual forms of the parasite (gametocytes) which are ingested by the female *Anopheles* mosquito where the gametocytes undergoes fertilization and form infective sporozoites. (adapted from [www.cdc.gov/malaria](http://www.cdc.gov/malaria)).

In relapsing species in humans such as *P. vivax*, *P. ovale* and *P. cynomolgi* - the simian equivalent of *P. vivax*, apart from the liver- schizonts, they develop dormant forms known as hypnozoites which can be reactivated to form liver-schizonts weeks, months or years later to complete its life cycle in the host (Figure 1.3) [17, 25, 26]. Once activated, the hypnozoites mature into liver-schizonts and the infected hepatocytes burst and releases its merosomes into the bloodstream where the released merozoites invade reticulocytes or erythrocytes depending on the species to undergo asexual schizogony.
Figure 1.3 Life cycle of the dormant malaria parasite (e.g. *Plasmodium vivax*). Unlike non-dormant malaria parasite, when the sporozoites infect the liver cell, it can either develop into liver schizonts or dormant hypnozoites which causes relapses after the primary infection (adapted from [www.cdc.gov/malaria](http://www.cdc.gov/malaria)).

Once in the host red cells, the parasites develop within the membrane-bound parasitophorous vacuole (PV) as ring form before it develops into the morphologically distinct and proliferative stages – the trophozoite and schizont. When merozoites in the mature schizonts egress, it results in the destruction of the infected erythrocyte membrane and releases the parasites to invade new host cells. A small part of this asexual erythrocytic population is pre-programmed to undergo gametocytogenesis to produce the sexual forms known as the male and female gametocytes. Once mature, these sexual forms are ingested by the *Anopheles* mosquito during their blood meal where they undergo the sporogonic cycle to form the diploid zygote (ookinete) in the midgut of the mosquito before developing into an oocyst. The motile haploid sporozoites are then released from the oocysts in the midgut of the mosquito and passed into the salivary glands where they can be injected into the next human host to initiate the life cycle again [27].
Plasmodium species are haploid, undergoing mitotic division when they reside in the host. The only diploid stage of its life cycle is the zygote, which is formed when the gametocytes fertilize in the mosquito. Genetic recombination and meiosis occur at the zygote stage in the mosquito. However, meiosis is short lived and occurs only within a few hours of zygote formation followed by mitotic division resulting in the formation of sporozoites (Figure 1.4). Through genetic recombination, various beneficial combination of alleles can spread across the parasite population driven by positive selection. This was first demonstrated by Walliker et al. in rodent malaria where the progeny of genetic crosses between different lines resulted in drug resistance [28]. This similar phenomenon was later described in P. falciparum [29, 30].

![Figure 1.4](image-url) Figure 1.4 Ploidy in the life cycle of Plasmodium species. Malaria parasites are haploid and the only diploid stage in its life cycle is the formation of the zygote, post-gametocyte fertilization in the mosquito host.

1.2.2 Incubation period

Incubation period refers to the time between the bite from an infective Anopheles mosquito and the first signs of clinical symptoms. Generally speaking, the incubation period for human malaria in naïve patients varies between 7 to 30 days. The incubation period for P. vivax infections is around 12-17 days, while in P. falciparum it is slightly shorter at around 9-14 days. However, the incubation period may be extended in patients who have been previously exposed to malaria [31].

Recently, increasing cases of zoonotic malaria due to P. cynomolgi and P. knowlesi have been reported [9, 10, 32]. Humans staying near rainforest areas within the vicinity of
the natural simian hosts and mosquito vectors are at risk of being infected by these simian malarias. It has been reported that *P. cynomolgi* B strain has an incubation period of 10-17 days [33]. While the incubation period of *P. knowlesi* in human is between 9 to 12 days [34].

### 1.2.3 Clinical manifestation of malaria

When a person is first infected with *Plasmodium* species, a sequence of clinical effects may be observed following the bite from an infected mosquito, a pre-patent and incubation period before the patient develops symptoms. Pre-patent period refers to the asymptomatic period which occurs from sporozoite inoculation in the host and the first detection of parasites in the peripheral blood and this usually represents the duration of its exo-erythrocytic stage of the parasite. In general, malaria is a highly preventable and curable disease if diagnosed properly and treated correctly and promptly. Based on the symptoms observed, malaria can be categorized as uncomplicated or severe. Classic clinical symptoms of malaria is closely linked to the erythrocytic cycle of the parasites. It consists of a cold stage which is usually accompanied by shivering, then a hot stage where fever (which is associated the host inflammatory response to the merozoite egress and hemazoin release in the parasites’ erythrocytic cycle) [35] and throbbing headache occurs. This is followed by a sweating stage which is associated with profuse sweating and declining body temperature. Depending on the species of *Plasmodium* infected, the cyclical occurrence of the symptoms may vary from every 2 days for tertian malaria species such as *P. falciparum* and *P. vivax* or every 3 days for quartan malaria species such as *P. malariae* [1].

Severe malaria usually occurs when the primary malaria infection is further complicated by organ failures or changes in the patient’s blood or metabolism. These include symptoms like cerebral malaria, severe anaemia and metabolic acidosis. If left untreated, *P. falciparum* malaria can quickly develop into cerebral malaria due to the ability of *P. falciparum* infected erythrocytes, especially those with mature trophozoites, to adhere to vascular endothelium resulting in the sequestration of infected erythrocytes in the vessels of the brain. If not treated promptly will result in coma and death. Young children of less than 5 years old, pregnant women, the immunocompromised and elderly are at higher risk of developing severe malaria [1]. Patients infected with *P. vivax* and *P. ovale* despite having recovered from the primary malaria infection, they may suffer from relapses from the
primary infection after weeks, months or even years without any symptoms due to the presence of hypnozoites which remains dormant until reactivated to continue the parasites’ life cycle [1]. Relapses can only be prevented by administration of hypnozoitocidal compounds- primaquine and tafenoquine.

1.3 Antimalarial therapy

1.3.1 Background

Antimalarial therapy varies across different malaria-endemic countries, depending on the Plasmodium species, the severity of the disease and the parasite’s sensitivity to first line therapy [36]. Hence, treatment have to be tailored for effective management of the manifestations of malaria.

For the treatment of uncomplicated P. falciparum erythrocytic stage malaria, the first line therapy is usually artemisinin -based combination therapy (ACT), the combination of a fast acting artemisinin derivative with a longer acting antimalarial which has a different mode of action. Artemisinin derivatives include dihydroartemisinin, artesunate and artemether while companion drugs include mefloquine, lumefantrine, amodiaquine, sulfadoxine/ pyrimethamine and piperaquine. Artemisinin was first discovered by Chinese scientists in 1971 and introduced to the world in the 1979 as a potential drug to combat in malaria due to its unique mechanism of action and its ability to kill malaria parasites within minutes [37]. However, due to patients non-compliance to the 7-day artemisinin monotherapy and incomplete parasite clearance due to its short half-life [38], high rate of recrudescence was quickly observed. In the 1990s, artemisinin was no longer used as a monotherapy and ACTs were introduced.

Due to the rapid spread of chloroquine resistant P. falciparum, the first line therapy for uncomplicated P. falciparum malaria (except pregnant women in their first trimester) is the 3-day regimen of ACTs. Commonly used ACTs include artemether with lumefantrine, artesunate with amodiaquine, artesunate with mefloquine, dihydroartemisinin and piperaquine and artesunate with sulfadoxine-pyrimethamine (Figure 1.5). For recurrent P. falciparum infections, the second line treatment which involves the co-administration of tetracycline, or doxycycline or clindamycin together with either artesunate or quinine for 7-days [36]. For special risk groups such as pregnant women in their first trimester and lactating women, they are often treated the 7-day regimen of
quinine with clindamycin. Chloroquine remains as the first line therapy of *P. vivax* erythrocytic stages in areas with no known resistance (e.g. Mexico) [39] while ACTs are used in areas with chloroquine-resistant infections (e.g. Indonesia and Papua New Guinea) [40]. The 8-aminoquinoline - primaquine is often co-administered with chloroquine for the eradication of the dormant forms - the hypnozoites of *P. vivax*.

### 1.3.2 Drugs targeting asexual erythrocytic stages

The last decade has witnessed major advances in antimalarial drug discovery primarily for the erythrocytic stages of the parasites life cycle [41-47]. Due to the almost ubiquitous resistance of *P. falciparum* to chloroquine, ACTs are the preferred mode of treatment for uncomplicated *P. falciparum* erythrocytic infections. The advantages of ACTs are their high efficacy, the reduced risk of resistance developing and their rapid action in clearing the parasites. Chloroquine remains the first line treatment of *P. vivax* and *P. ovale* infections in areas with chloroquine susceptible infections while ACTs are used in chloroquine resistant infections. Drug screening assays for identification of erythrocytic stage active compounds are routinely carried out with *P. falciparum* as it’s the easiest to maintain *in vitro* and amenable for automated liquid handling procedures for high throughput screening. However, erythrocytic stage active compounds in *P. falciparum* sometimes does not translate in efficacy for *P. vivax* [48] hence more physiological relevant erythrocytic stage assay using the sister species - *P. cynomolgi*, may provide a more predictive assessment of clinical outcomes of novel antimalarials treating vivax malaria.

### 1.3.3 Drugs targeting sexual erythrocytic stages

Gametocytes, the sexual stages of the malaria parasite life cycle, are the result of the switch from asexual to sexual replication of the parasite in the host. At the late stages of the intra-erythrocytic life cycle of the parasite, the schizonts release multiple merozoites of which a small ratio (0.2 - 1%) is defined to develop into male and female gametocytes [49, 50]. Since 2007, to complement vector control efforts such as the use of insecticide treated bed nets (ITN) and indoor residual spraying (IRS) with DDT, malaria eradication strategies prioritized antimalarial therapies which combined components capable of blocking transmission of gametocytes from humans to the mosquito vector [51-53].
Figure 1.5 Most commonly used antimalarial drugs used against the three main stages of the life cycle of human malaria (liver stage, blood stage and vector stage) (Delves et al., 2012). The drugs are colored according to their chemical classes and the stars reflect the components of the main ACTs (green: coartem, red: pyramax, orange: eurartesim, blue: artesunate-amodiaquine). Drug discovery strategies in the past century focused on using molecular approaches where target-based screening led to the identification of novel compounds against a validated target. Target-based approaches are dependent on the identification of potential inhibitors against protein “targets” which are vital in the parasites’ survival. Once the “targets” are selected, protein “binders” are synthesized and evaluated in high throughput biochemical screens of chemical or fragment libraries to identify molecules with the appropriate binding affinity.

1.3.4 Drugs targeting exo-erythrocytic stages

While ACTs have been efficient in eliminating non-relapsing, exo-erythrocytic stages of *P. falciparum* parasites, 8-aminoquinoline antimalarials are the only drugs that
prevent relapses in *P. vivax* and *P. ovale* infections. The exo-erythrocytic stages of the relapsing malaria include the liver schizonts and the hypnozoites—a non-dividing yet metabolically active form of the parasite present in *P. vivax* and *P. ovale* in humans as well as in *P. cynomolgi* and *P. fieldi* in non-human primates. Hypnozoites reactivation is responsible for malaria relapse occurring weeks to years following the initial infection [54]. The hypnozoite functions as a natural reservoir of the malaria parasite in the liver, which impedes eradication. For almost a century, primaquine, a rapidly eliminated 8-aminoquinoline, is the only licensed drug known to kill both the liver schizonts and the hypnozoites. Typically, primaquine is administered together with chloroquine for 14 days for chloroquine sensitive *P. vivax* [36].

Unfortunately, primaquine is not a recommended treatment for pregnant woman [55] and patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency [56, 57]. In July 2018, another 8-aminoquinoline drug- tafenoquine was approved by FDA as a single dose radical cure [58]. However, as with primaquine, tafenoquine cannot be used in patients with G6PD deficiency. It took decades for the development of tafenoquine as an alternative radical cure due to the lack of predictive in vitro exo-erythrocytic stages for radical cure. Most of the in vitro models are unable to capture the full cycle of the the exo-erythrocytic stages, including the relapse from the hypnozoites.

1.4 Antimalarial drug discovery

1.4.1 Identification of novel antimalarial drugs

The emergence of *Plasmodium* species strains with resistance against standard antimalarials [59-61] and the need to find better alternatives to the century old radical cure-primaquine, provide the main driving forces in malaria drug discovery. New antimalarial drugs are often derived from: enhancement of existing drugs, drugs specifically designed against a known target and novel active molecules derived from phenotypic high-throughput screening.

The ideal antimalarial drug should be active against all developmental stages of the parasite which include blood, liver stages and prevents parasite transmission [59]. To ensure patients’ compliance, these compounds should preferably be potent enough to elicit a curative effect in a single dose- also known as single exposure, radical cure and
prophylaxis (SERCaP) treatment [62]. In addition, to ensure accessibility of antimalarials even to patients living in poverty, inexpensive production cost is vital.

However, in the past decade, physiologically relevant phenotypic screens, where often the mechanism of action is unknown, is the main contributor to the discovery of new antimalarials [63-65]. Phenotypic screening or “whole cell-based” approaches in malaria drug discovery involves the evaluation of large compound libraries against the parasite’s phenotype, preferably in physiologically relevant environment where the parasite manifests. Phenotypic high-throughput screening (HTS) for new drug candidates against erythrocytic [43, 63-65] and exo-erythrocytic [42, 66-68] stages of malaria were made possible with the advent of automated liquid handling, high content imaging and analysis, automated assembly of compound libraries and the minute scale which the assays are conducted.

1.4.2 Phenotypic screens for erythrocytic stages

Phenotypic HTS for malaria erythrocytic stage is routinely carried out with P. falciparum as they are easily maintained in vitro. As erythrocytic stages are in suspension, they are well suited for automated liquid handling and other HTS technologies. In 2008, the first large scale phenotypic screen was carried out using asexual P. falciparum erythrocytic stages at a single drug concentration [69]. Only around 650 compounds out of the 1.7 million compounds screened inhibited parasite growth at less than 100 nM. Since then, almost 6 million compounds have been screened where only 0.4% to 1% demonstrated activity against growth of asexual P. falciparum erythrocytic stages [70, 71].

From the results of these screening efforts, a compound library of 400 unique drug entities with antimalarial blood-stage activity was created in 2011 by the Medicines for Malaria Venture (MMV). These compounds were selected from hits generated from the libraries of St. Jude Children’s Research Hospital, GlaxoSmithKline and Novartis [72]. This open access “Malaria Box”, which was made available without cost globally until 2015, enabled biologists who does not have the capacity to resynthesize compounds to participate in the drug discovery process. Developed based on the success of the Malaria Box, the Pathogen Box contained 400 diverse compounds which demonstrated activity against 13 neglected diseases which includes malaria, tuberculosis and cryptosporidiosis. In order to evaluate drug activity against other phenotypes of the parasite, assays were
developed which target alternative phenotypes such as invasion [73], gametogenesis [74] and “delayed death” [75].

Phenotypic screens [76, 77] carried out on the Malaria Box and the Pathogen Box led to the elucidation of several mechanism of actions of compounds [77, 78] which further led to compound optimization for in vivo efficacy. For instance, the 1, 5-naphthyridine MMV024101 was first identified from the Pathogen Box with submicromolar potency against P. falciparum. This paved the way for structure-activity relationship optimization which led to compounds which enhanced in vivo efficacy in the humanized P. falciparum mouse efficacy model. [77].

1.4.3 Phenotypic screens for exo-erythrocytic stages

While initial HTS efforts were mainly targeted on the erythrocytic stages of P. falciparum, recent progress has been in the development of HTS to identify drugs which are active against the exo-erythrocytic stages (also known as liver stages) of the parasite life cycle. Since the establishment of the first exo-erythrocytic stage screen on non-dormant Plasmodium species [42], various groups have developed various exo-erythrocytic assays which utilized sporozoites infection of rodent malaria to either in vivo or in vitro cultured primary hepatocytes [66, 79]. False negatives and false positives may result in HTS exo-erythrocytic stage screens which utilizes rodent parasites as they may not show similar response as the human parasites with the compound of interest. Most HTS exo-erythrocytic stage screens are two dimensional (2D) and utilizes antibody staining of malaria parasites in the infected hepatic cells that requires several liquid-handling wash steps which may sometimes result in the loss of infected hepatic cells. Alternatives to antibody staining of parasites is the use of transgenic parasites [80] which either stably expresses fluorescent reporter protein or luciferase [65, 67] which often lead to a more robust and higher throughput exo-erythrocytic stage screen.

P. vivax and P. ovale are the only human malaria species which form dormant forms known as the hypnozoites which can reactivate randomly weeks, months or years later through mechanisms not currently well understood. In order for malaria eradication, drugs which show activity against these hypnozoites (radical cure) are required.

The typical in vitro radical cure screens utilize standard 2D primary hepatocyte cultures which readily lose their liver specific function and polarity [81]. Hepatic cell lines
are not ideal replacements for primary hepatocytes, since they differ in gene expression, metabolic pathways, and lack some critical cellular functions. Hepatic cell lines occasionally produce contradictory results to what is observed with primary hepatocytes [82], although successful but limited infection of HepG2-A16 and HCO4 cell lines have been reported with *P. vivax* [25, 83-85] and *P. falciparum* [85, 86]. Noteworthy, some drugs such as the 8-aminoquinolines (primaquine and tafenoquine) requires processing by hepatic cytochrome P450 (CYP450) [87] to be active which is often lost in 2D cultures. In the past decade, different strategies have been devised to prolong the viability and functions of primary hepatocytes in culture. Some have already been adapted to malaria research, such as the innovative *in vitro* methods established to maintain the exo-erythrocytic parasite in a monolayer culture using a Matrigel matrix [88] and more recently, micropatterned co-cultures (MPCCs) model [89, 90], and collagen-treated 384-well plates comprising a micro-physiological environment [68]. In addition, *in vivo* human liver-chimeric mice models were also developed [91].

While 2D exo-erythrocytic phenotypic assays are relatively cost effective and amenable to HTS technologies, their cellular responses and phenotypes does not necessary recapitulate the pathology of the disease in the host [92]. Despite the limitations, these 2D exo-erythrocytic phenotypic assays were useful in compounds active against the exo-erythrocytic stages. The first non-8-aminoquinoline compounds, KAI407 and KDU691, were first identified through such 2D assays to be active against the dormant hypnozoites [93] but failed as radical cure in the simian *in vivo* model [94].

Despite animal models being an important aspect in drug discovery, there is a demand for more physiologically predictive models which translate to host biology. Over the past decade, there is an increase in the development of three-dimensional (3D) tissue models [95] which are commonly referred to as spheroid or organoid.

In the presence of microenvironment components such as extracellular matrix (ECM) proteins or partner cells, single cells have the ability to self-aggregate to form spheroids and spheroids from different types of cells have been reported [96, 97]. Physiologically, spheroid models are better than 2D cell culture models in predicting drug efficacy in the human. Various methods have been developed for the formation of spheroids. They include the use of solid scaffold [98-100], bioreactor [101, 102], hanging drop [103, 104], micropatterned plate [105] and microfluidic device [106-108].
1.5 *Plasmodium vivax*

1.5.1 Background

The first report of this human tertian parasite was in 1880 by the French army surgeon, Louis Alphonse Laveran [5] where he unknowingly witnessed the exflagellation of a male gametocyte in the blood of febrile patients in Algeria. Of the nine *Plasmodium* species affecting humans, *P. vivax* is associated with high morbidity while *P. falciparum* is associated with high mortality [1, 109]. While *P. falciparum* is prevalent in sub-Saharan Africa, *P. vivax* is the dominant species in the Americas, Southeast Asia and eastern Mediterranean and in 2016 around 8.5 million new cases of *P. vivax* were reported globally [1]. It was suggested that the activation of the dormant hypnozoites accounts for 90% of relapsing cases of *P. vivax* globally [110].

Unlike *P. falciparum* infections, the level of parasitemia in *P. vivax* infections is typically low partly due to the parasite’s strict preference for reticulocytes [111] which are immature red blood cells formed in the bone marrow after enucleation and released into the circulation (~1-2%). Even though other *Plasmodium* species such as *P. falciparum* preferentially invade reticulocytes, they are still able to invade mature erythrocytes (which has facilitated the establishment of continuous culture in this species). The majority of *P. vivax* patients are asymptomatic with sub-microscopic level of parasitemia [112, 113] which continues to rapidly produce infective gametocytes for transmission [114]. Such sustained transmission, its ability to relapse from dormant liver hypnozoites [54] and its increasing resistance to its first line treatment- chloroquine [61, 115], makes controlling and eliminating *P. vivax* challenging.

1.5.2 Distinct biology of *P. vivax*

Host cells infected by some malaria parasites exhibit various morphological features which are distinct for certain species to facilitate its survival in different environment. In the late stages of *P. falciparum*, they form knob like protrusions on the infected host cell which is involved in cytoadherence [116], while *P. vivax* infected reticulocytes are usually enlarged and exhibit unique Schüffner’s dots associated with caveolae-vesicle complexes which are suggested to be crucial in the parasites nutrient uptake or release of waste metabolite from the parasite infected host cell [117, 118]. Increase in the rigidity of *P. falciparum* infected host cell is associated with splenic
clearance as the parasite matures, while increase in the deformability of \( P. \) \textit{vivax} infected reticulocytes [119] has been associated with the parasite’s ability to avoid splenic clearance and remain in circulation. Gametogenesis between the two species is also markedly different. \( P. \) \textit{falciparum} gametocytes appear after 10 days after first appearance of asexual stages while \( P. \) \textit{vivax} gametocytes are known to appear as early as 3 days post observation of its asexual stages [120]. The gametocytes of \( P. \) \textit{falciparum} are noticeably different from their asexual stages is grouped to five morphologically different developmental stages [121] with the characteristic crescent shaped gametocytes in stage VI [122]. The gametocytes of \( P. \) \textit{vivax} have a distinctly round morphology which fills up the majority of the infected host cell.

The characteristic feature of \( P. \) \textit{vivax} is its ability to relapse from dormant exo-erythrocytic stages known as the hypnozoites [123]. The latter is triggered by unknown signals which activate weeks or months after primary infection to cause malaria relapse. The timing and frequency of these relapse varies on the geographic origin of the strain where strains from temperate regions are associated with longer relapse periods as compared to strains from tropical regions [54, 124].

1.5.3 Rising drug resistance of \( P. \) \textit{vivax}

Resistance is the reduction in effectiveness of an anti-infective agent in curing a disease or condition. Emergence and spread of drug resistance in malaria is a major threat in the control and elimination of malaria. Chloroquine, a member of the 4-aminoquinolines, is widely used for treatment of all human malaria. For the parasite’s survival within the host erythrocytes, the parasite degrades the host haemoglobin to yield essential amino acids necessary for its growth. During this process, the toxic heme produced is converted to non-toxic hemozoin crystals by the parasite. Chloroquine inhibits the parasite’s conversion of heme to hemozoin, hence leading to the accumulation of toxic heme within the parasite.

Currently, it is used as the first line treatment for \( P. \) \textit{vivax} erythrocytic stages in malaria endemic areas which is sensitive to its effect (e.g. India). Chloroquine resistance in \( P. \) \textit{falciparum} was first identified in 1950s [125] in Colombia and the Cambodia-Thailand border and it accelerated globally. The first case of chloroquine resistance in \( P. \) \textit{vivax} was only reported later in 1989 in Papua New Guinea [126] before rapidly escalating throughout Asia and Oceania [40, 61, 127, 128]. While mechanisms of chloroquine resistance remain
elusive in *P. vivax*, it is well studied in *P. falciparum* due to the ability to culture the parasite in the laboratory and the earlier efforts in studying chloroquine resistance in *P. falciparum*. This lead to the identification of mutations in *pfcrt* [129-131] and *pfmdr* [132, 133] responsible for conferring chloroquine resistance in *P. falciparum*. However, mechanisms of chloroquine resistance appear to be distinct between *P. falciparum* and *P. vivax* despite gene synteny between the two species [134].

In order to answer fundamental questions such as mechanisms of drug resistance in *P. vivax*, the development of a robust continuous culture of its erythrocytic stage parasites though vital is fraught with challenges due to the parasite’s strict tropism to reticulocytes. Hence the feasibility of establishing a continuous culture using its sister taxon – *P. cynomolgi* [135, 136], especially from a homogeneous isolate, will greatly impact the understanding of mechanisms of drug resistance in *P. vivax*.

### 1.6 Zoonosis: a rising threat

There are more than 100 *Plasmodium* species, infecting mammals, birds and reptiles. There are 9 known species which infects humans and more than 30 species which have been reported in non-human primates which includes New and Old World monkeys, apes and gibbons. As with human malarias, primate malarias are also transmitted by *Anopheles* mosquitoes. In the past, numerous studies have been conducted to determine if primate malarias were transmissible to man through bites from infected *Anopheles* mosquitoes. They include *P. knowlesi* [13, 137, 138], *P. cynomolgi* [139-141] and *P. inui* [142] from Old World monkeys and *P. brasilianum* [143] and *P. simium* [144] from New World monkeys. This demonstrated with suitable vectors, simian malaria can be naturally transmitted to humans. Diagnostic microscopy was the gold standard in the detection of malaria. Nonetheless, even with trained clinical microscopists, zoonotic malaria is often mistakenly classified as human malaria due to morphological similarities between the species. Using polymerase chain reaction (PCR) based molecular diagnostic tests, zoonotic transmission of *P. knowlesi* to humans was identified in 2004 [145] and is now an important cause of malaria in Malaysia and Southeast Asia [146-151]. With the development of more sensitive and specific detection protocols, other zoonotic malaria has been detected in Asia [10, 32, 152] and also in the Americas [11, 12].
In 1932, Knowles and Das Gupta first identified *P. knowlesi* and its ability to transmit to humans [13]. In their study, they reported its extreme virulence in Indian rhesus macaques (*Macaca mulatta*) while being relatively benign in long tailed and pig tailed macaques—*Macaca fascicularis* and *Macaca nemestrina*. In the three human subjects evaluated, the virulence was varied but all recovered spontaneously without antimalarial therapy. In the same year, Sinton and Mulligan noted its distinct stippling in infected erythrocytes, the 24-hour erythrocytic (quotidian) cycle and the presence of an accessory chromatin dot [153]. With its ability to induce relatively high fevers in humans, “induced malaria” therapy using *P. knowlesi* was explored. In 1935, Van Rooyen and Pile [154] used *P. knowlesi* in the treatment of paresis while Chopra and Das Gupta [155] first demonstrated the use of *P. knowlesi* in the treatment of patients with neurosyphilis in 1936 where they transferred blood directly from an infected *P. knowlesi* monkey to the patients.

The first naturally acquired *P. knowlesi* infection in human was first reported in peninsular Malaysia 1965 by Coatney *et al.* in 1965 [156] and subsequently, Chin *et al.* reported the evidence of transmission of *P. knowlesi* from an infected monkey to man in 1968 [138]. This raised the alarm that infected monkeys could now serve as reservoirs for malaria in humans. The increase in monkey reservoir in malaria parasites thwarts malaria elimination objectives especially when deforestation increases the likelihood of humans contracting zoonotic malaria as they are within the vicinity of natural monkey hosts and mosquito vectors. Naturally occurring zoonotic cases of *P. knowlesi* in humans were initially thought to be rare until a large cohort of human infections were detected by molecular methods in 2004 by Singh *et al.*, *P. knowlesi* is now the main cause of malaria in Malaysia [157].

Further evidence of laboratory-acquired mosquito infections of another simian malaria, *P. cynomolgi* in the 1960s, raised the concerns of zoonosis in public health. [139-141, 158]. However, the first naturally acquired infection of *P. cynomolgi* in humans was only reported in the recent years in peninsular Malaysia [32] and increasing cases were subsequently reported in the region [159]. More recently, Imwong *et al.* demonstrated the prevalence of asymptomatic human infection of *P. knowlesi* co-infected with *P. cynomolgi* in Cambodia [10]. The study also showed genetic similarity between *P. cynomolgi* and *P. vivax* which may account for cross-reactivity in some PCR methods. As with *P. knowlesi*,
*P. cynomolgi* infections in humans may have been occurring for years but might be misdiagnosed by microscopy as *P. vivax* due to their morphological similarities.

### 1.7 Plasmodium cynomolgi

#### 1.7.1 Background

*Plasmodium cynomolgi*, a sister taxon to *P. vivax*, which preferentially infects Old World monkeys (e.g. *Macaca fascicularis* and *Macaca mulatta*) and is one of the six simian malaria known to cause zoonotic infections in humans [32]. It was first identified by Martin Mayer in 1907 in Germany in *Macaca fascicularis* monkeys imported from Java [160]. The following year he described the morphological similarities of the simian parasites with *P. vivax* based on microscopy [161]. As with its human counterpart, the *P. cynomolgi* has a forty-eight hour (tertian) life cycle where Schüffner's stippling, amoeboid cytoplasm and enlarged infected host red blood cells are noted in its late trophozoite stages. Similarly, in the sexual stages, the early gametocytes usually have a large compact nucleus, pronounced Schüffner's stippling, not amoeboid and lack a vacuole. When mature, they resemble the *P. vivax* forms with irregular, diffuse nucleus which the microgametocytes are stained a reddish-purple in contrast to the light blue stain of macrogametocytes (Figure 1.6). There are various strains of *P. cynomolgi* known – *bastianelli* (B) [162, 163], Mulligan (M) [140, 164], Berok [165], Ceylonensis [166], Gombak [167], Cambodian [168], Smithsonian, PT and RO [169, 170] with the possibility of new strains to be isolated from the wild where *Anopheles* mosquitoes are rampant. Even though they were originally categorized distinctly as B and M strains, the laboratory stocks of the B and M strains from the archive sources in the US have recently been reported to be genetically identical [171, 172] probably due to a laboratory mix-up in the early 1960s. It has since been suggested to rename all B and M strains from the US as the B/M strain [171]. The Berok strain was derived from a *Macaca mulatta* monkey infected with parasites which originated from a *Macaca nemestrina* monkey naturally infected with both *P. fieldi* and *P. cynomolgi*. Interesting the *P. cynomolgi* Berok strain appeared to have outgrown *P. fieldi* in the infected macaque [165] despite both species having the same tertian life cycle. In the same study, it was noted that the Berok strain apart from being infectious to Old World monkeys, it’s also known to infect New World monkeys (*Aotus* and *Saimiri*) [165].
Figure 1.6 Representative erythrocytic stages (ring, trophozoite, schizont and gametocyte) of *P. falciparum*, *P. vivax* and *P. cynomolgi*. Erythrocytic stages of *P. cynomolgi* are morphologically similar to *P. vivax*, in contrast to *P. falciparum*.

Apart from the various biologic and morphologic similarities to *P. vivax*, *P. cynomolgi* has been known to be the closest phylogenetically to *P. vivax* (Figure 1.7) [171-173]. The genome size of *P. cynomolgi* B strain is around 26.3Mb spread across 5,722 genes predicted on 14 chromosomes with an estimated 40.5% of GC content. 96% of the genes are orthologous with *P. vivax* [172] which further reinforces *P. cynomolgi* is a good model to study *P. vivax*. In malaria causing parasites, there are two main gene families which is responsible for host cell recognition. The erythrocyte binding-like (*ebl*) genes which encodes for Duffy-binding proteins (DBPs) are responsible for binding to Duffy antigen receptor for chemokines (DARC) on monkey and human erythrocytes. The reticulocyte binding-like (*rbl*) genes which encodes for normocyte-binding proteins (NBPs) in *P. knowlesi* and reticulocyte-binding proteins (RBPs), encoded by the *rbp* gene family in *P. vivax* and *P. cynomolgi* are responsible for the invasion of host erythrocytes. Interestingly, it is hypothesized that *P. vivax* lost its ability to infect Old World monkey erythrocyte as it only has one *dbp* gene while *P. cynomolgi* which infects both humans and Old World monkey erythrocyte has 2 *dbp* genes [172]. Within the species itself, differences were also noted in the *rbp* genes in the different strains of *P. cynomolgi*. For instance, the Berok strain is distinguished by the lack of *rbp2a* gene that’s present in the B and
Cambodian strain, and the presence of *rbp1b* gene which is lacking in B and Cambodian strains and the presence of *rbp2b* gene which is present in all three strains [171, 172].

### 1.7.2 Erythrocytic stages of *Plasmodium* species

The development of a protocol for the routine continuous culture of *P. falciparum* in 1976 [174, 175] liberated malaria researchers from the reliance on *in vivo* observations. This quickly accelerated *P. falciparum* research and led to fundamental and translational advances throughout the parasite’s life cycle. Unlike *P. falciparum* which is responsible for the highest mortality rates globally, the widespread species *P. vivax* causes substantial [7] morbidity hence the urgent need to devise effective measures to control it. However, research on *P. vivax* remains greatly hampered as attempts to maintain these parasites in continuous *in vitro* culture has been hindered, partly due to its strict restriction to invasion of reticulocytes [111, 176, 177]. HTS using *in vitro* cultured *P. falciparum* has enriched the drug discovery pipeline with a plethora of potential novel lead compounds [178]. HTS using *P. vivax* has been challenging due to limitations in availability and diversity in patients’ isolates which renders assay development and standardization impossible. As such, selected compounds have been evaluated on primate models and *ex vivo* drug assays, systems not amenable to HTS. To enable sustainable and consistent study of antimalarial effect in *P. vivax*, having a continuous culture *P. cynomolgi* would be an ideal alternative to screen for active antimalarials against *P. vivax*.

Following the success of the continuous culture of *P. falciparum* in 1976, various groups invested large amounts of effort in the cultivation of the erythrocytic stages in other *Plasmodium* species, notably *P. vivax*, *P. cynomolgi*, *P. fieldi* and other rodent and simian species, especially since this stage is often associated with the clinical symptoms of the disease [179] and an area of interest for vaccine development [180]. In the system developed by Trager *et al.* in 1976 for the continuous culture of *P. falciparum*, the key components for successful adaptation were a well supplemented and buffered growth medium, compatible serum and naïve erythrocytes with regular refreshment of growth medium [174]. The parasites were maintained in petri dishes under these conditions in a candle jar where the gas is around 3% CO₂ and 15-17% O₂. Following the success of cultured *P. falciparum*, few groups were able to successfully adapt other *Plasmodium* species for *in vitro* cultivation [181, 182]. More recently, the zoonotic malaria species *P. knowlesi* was successfully adapted to continuous culture in human erythrocytes [183, 184]
Figure 1.7. Phylogenetic tree of the *Plasmodium* genus (Rutledge *et al*., 2017). Phylogenetic tree of the *Plasmodium* genus depicting the *P. malariae* clade (red) and *P. ovale* clade (blue) alongside the divergence level of the different species. Values at branching points represent bootstrap values.

1.7.3 The *in vitro* culture of *P. cynomolgi*

Of interest to this study was the two reports of continuous *in vitro*-culture of the erythrocytic stages of two strains of *P. cynomolgi* (Berok in one, and Vietnam in the other) in the early 1980s [135, 136] which leveraged on the culture system developed for *P. falciparum*. Apart from the different strains used in their studies, the culture conditions used were also unique.

Nguyen *et al*. [135] was the first to report the successful continuous culture of *P. cynomolgi* in 1981 where the Berok strain was continuously cultured for up to 6 weeks in human serum which supposedly sustained growth better than rhesus serum. Attempts to culture the parasites *in vitro* with human blood proved futile. The cultured parasites remained infective in rhesus monkeys and the reduced amoeboid morphology noted in the trophozoites *in vitro* was reversed *in vivo*. The initial inoculum had to be regularly subcultured with *Macaca mulatta* erythrocytes when grown in a modified candle jar and
also in a continuous flow method. Zhou et al. in 1984 [136] demonstrated the continuous culture of *P. cynomolgi* using another strain isolated from Vietnam where routine subculture with *Macaca mulatta* erythrocytes was required to sustain its growth for more than 140 days. The culture was able to be reinitiated from a frozen stabilate with no apparent difference in amplification. Unlike the Berok strain, the Vietnamese strain retained the amoeboid morphology of the trophozoites *in vitro* and sustained in culture with *Macaca mulatta* serum.

The attainment of continuous culture using different strains of *P. cynomolgi* suggest the success in adaptation for continuous culture of *Plasmodium* species is an eventuality when various strains of the parasite are explored with calculated persistence.

### 1.7.4 Exo-erythrocytic forms of *Plasmodium* species

In 1902, Schaudinn [185] hypothesized that sporozoites infected the erythrocytes directly, a concept that was negated in the avian malaria *Plasmodium relictum* in 1936 [186] and *Plasmodium gallinaceum* in 1938 [187]. It took another 10 years before Shortt and Garnham [188, 189] first described the pre-erythrocytic forms in the liver of monkeys infected with *P. cynomolgi* sporozoites but not erythrocytic stage malaria and was reconfirmed in human subjects infected with *P. vivax* [190, 191] in 1948. Until the late 1940s it was largely assumed that *Plasmodium* infections were strictly erythrocytic. It was not until 1948 where Shortt and Garnham [192] demonstrated experimentally that a pre-erythrocytic or exo-erythrocytic stage existed in *P. cynomolgi* parasites. They were driven by the observations made by James in 1931 [193] where he noted that infections initiated with erythrocytic stage parasites were rapidly associated with clinical symptoms and parasites could be recovered from peripheral blood while infections from sporozoites required an incubation period of several days before any parasites could be detected in the blood. From their investigations, Shortt and Garnham concurred that following sporozoites-induced infection from infected mosquitoes, parasites were not detected in the peripheral blood while for infections induced from infected blood, parasites quickly appeared in the circulation. In addition, infected blood-induced infections could be eradicated with drug treatment while sporozoites-induced infections could not. They carefully catalogued their observations performing daily liver biopsies of the infected monkey and illustrating the progress of the maturation of the liver stage-stage parasite *P.*
cynomolgi in monkeys over a period of 10 days and P. vivax in humans on day 7 post sporozoites induced infections [191].

Following the success of the continuous culture of P. falciparum in 1976, various groups invested large amounts of effort in the cultivation of the erythrocytic stages in other Plasmodium species, notably P. vivax, P. cynomolgi, P. fieldi and other rodent and simian species, especially since this stage is often associated with the clinical symptoms of the disease [179] and an area of interest for vaccine development [180]. In the system developed by Trager et al. in 1976 for the continuous culture of P. falciparum, the key components for successful adaptation were a well supplemented and buffered growth medium, compatible serum and naïve erythrocytes with regular refreshment of growth medium [174]. The parasites were maintained in petri dishes under these conditions in a candle jar where the gas is around 3% CO₂ and 15-17% O₂. Following the success of cultured P. falciparum, few groups were able to successfully adapt other Plasmodium species for in vitro cultivation [181, 182]. More recently, the zoonotic malaria species P. knowlesi was successfully adapted to continuous culture in human erythrocytes [183, 184].

Relapse in malaria is referred to as the re-appearance of erythrocytic parasites in the host from a sporozoite-induced infection which was previously treated with blood schizontocidal therapy [194]. The first recorded history of true relapse in vivax malaria was reported by Thayner in 1897 in which a physician experienced several episodes of chills almost 2 years post initial infection despite not being re-exposed between the two occurrences [195]. In the early 1900s, experimental evidence of relapse arose when Manson [196] and Fearnside [197] independently infected healthy individuals with P. vivax infected mosquitoes which resulted in primary infection which was successfully treated with quinine. Relapse occurred several months post primary infection for both instances.

Relapses can only be prevented through proper diagnosis and treatment with 8-aminoquinoline anti-malarials such as primaquine. Latency period between the primary infection and relapse appear to vary on the epidemiology of the disease. In tropical regions, the latency period is usually shorter (several weeks) as compared to its temperate counterparts (several months) [156]. Apart from the difference geographic zones of P. vivax strains which display different relapse patterns, other speculative factors for hypnozoite reactivation include the recognition of a Anopheles specific protein, host bodily
reaction to a mosquito bite [198], stimulus from a febrile illness [199] and the genetically pre-determined biological clock of hypnozoites [200].

1.7.5 The dormant stages of *P. cynomolgi* and *P. vivax*

As with *P. vivax* in humans, *P. cynomolgi* also cause relapses in its simian hosts [17, 26, 201]. *P. cynomolgi* has been instrumental in the discoveries of the exo-erythocytic stages of the parasite since there was no designated *P. vivax* strain to study the biology of liver hypnozoites and schizonts. The first evidence which demonstrated the utility of *P. cynomolgi* as a relevant model for *P. vivax* research was the study by Shortt *et al.* in 1948 [192] where they identified exo-erythrocytic stages of *P. cynomolgi* parasites as the cause for relapses which is similarly found in *P. vivax*. The model further proved its importance when Eyles *et al.* first demonstrated the zoonotic potential of *P. cynomolgi* B strain in 1960 [139] where the infection in humans retained the tertian periodicity and its distinct Schüffner’s stippling in enlarged host infected erythrocytes. Since then, more cases of zoonotic transmission to humans were reported [140, 141, 158] where the infections were usually low grade. In the early 1980s, Krotoski and colleagues identified that hypnozoites were the cause of the relapses using the B strain [26, 202, 203]. Since then, the *P. cynomolgi* B and M strains have been the most characterized strains as they were widely used in understanding *P. cynomolgi*’s sporogonic cycle, structural studies of the exo-erythocytic stages, and large scale infection studies involving Old World monkeys [156].

1.7.6 *P. cynomolgi* in drug discovery

Animal experimentation is widely used in biomedical research, especially in understanding host-pathogen interactions. Animal studies are usually conducted either using rodent or non-human primate (NHP) models. Rodent models make up the majority of animal studies as they are relatively inexpensive and easier to handle. The usefulness of the data generated from animal experimentation will depend on the clinical and biological relevance of the animal model used. Rodents though useful in the biomedical research, are phylogenetically distinct from human [204, 205] are hence not as useful in predicting behavioral and biological responses in humans. NHPs are phylogenetically and physiologically similar to humans (Figure 1.8) which makes NHP models ideal in studying diseases to gain the relevant insight in humans so as to facilitate drug and vaccine discovery.
Figure 1.8 Primate phylogenetic tree (Rogers and Gibbs, 2014). Phylogenetic tree representing evolutionary relationships among different species of primates. The separation of the human lineage diverged from the non-human primate lineage around 9 to 5 million years ago.

NHP models have been instrumental in malaria research for decades particularly in the identification of hypnozoites in relapsing malaria. These were first identified in NHP models where rhesus macaques were experimentally infected with *P. cynomolgi* [26] and subsequently in chimpanzees infected with *P. vivax*. Apart from understanding the biology of malaria parasites, NHP models are crucial in preclinical development phase of drug discovery where potencies of novel antimalarial drugs are assessed as they provide a more accurate prediction of human efficacy and pharmacokinetics [93, 94, 206-208]. *P. cynomolgi* was vital in the development of primaquine as radical cure [207] and remains important in the search for new radical cure compounds in both experimental *in vivo*
infections in NHP models [209, 210] and \textit{in vitro}-cultured hypnozoite model [88, 211]. Development of \textit{in vitro} primary hepatocyte culture models which sustained the cultivation of both \textit{P. cynomolgi} exo-erythrocytic stages enabled screening of novel anti-hypnozoitocidal drugs [93].

1.8 Aims and objectives of this thesis

In this study, the main objectives were to address the fundamental challenges in \textit{P. vivax} drug discovery- the lack of (i) \textit{in vitro} culture of erythrocytic stages for HTS screening and (ii) predictive radical cure model to evaluate anti-hypnozoitocidal drugs. These were accomplished by using \textit{P. cynomolgi} as the surrogate model to investigate antimalarial effects in all stages (erythrocytic and exo-erythrocytic) of the \textit{P. vivax} parasite in the host.

The first set of results (chapter three) demonstrates the establishment of a robust \textit{in vitro} culture of \textit{P. cynomolgi} erythrocytic stages which retained its infectivity in mosquitoes. This scalable continuous culture was also amenable for HTS drug screening for antimalarials. In addition, to further develop the continuous culture of \textit{P. cynomolgi} as a tool for future techniques such as genotyping and understanding of drug resistance, a homogenous isolate from the continuous culture was isolated.

Chapter four then demonstrates the establishment of a novel \textit{P. cynomolgi} infected 3D spheroid model for \textit{in vitro} radical cure compound profiling. A new method to infect the hepatocytes in suspension prior to 3D spheroid formation to increase infectivity was established, where the infected spheroids were able to recapitulate the entire life cycle of the exo-erythrocytic stages of the parasite which included the transition from liver merosomes to the erythrocytic stages. More importantly, this 3D spheroid model was easily adapted for \textit{P. vivax} infection and its potential use as its \textit{in vitro} radical cure model.
CHAPTER 2

METHODS AND MATERIALS
This section comprises of general cell culture techniques for malaria and experimental details for specific experiments in following chapters of this thesis. The time line (Table 2.1) and experimental details are categorized as:

2.1 Continuous in vitro culture of *Plasmodium cynomolgi* Berok K4 erythrocytic stages

2.2 Establishment of a 3D hepatic spheroid model to study the exo-erythrocytic stages of *Plasmodium cynomolgi*

2.3 Recipes for media and solutions
Table 2.1. Timeline of experiments carried out in this study
CHAPTER 2.1

Continuous *in vitro* culture of *Plasmodium cynomolgi* Berok erythrocytic stages
2.1.1 Ethical committees and animal welfare

Macaca fascicularis (cynomolgous monkeys) were maintained at the Novartis Laboratory Animal Services, New Jersey, U.S.A., (Novartis-LAS) and SingHealth Experimental Medicine Center, Singapore. Both institutions were audited and approved by the Novartis Animal Welfare Compliance. All animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals and the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) Standards. All studies were approved by the Novartis Ethical Review Council and Novartis Institutional Animal Care and Use Committees prior (IACUC) to study initiation. Additionally, work at SingHealth was approved by the SingHealth IACUC. Clinical samples utilized in this study were collected from *P. vivax* infected malaria patients attending the clinics of the Shoklo Malaria Research Unit (SMRU), Mae Sot, Thailand, under the following ethical guidelines in the approved protocol; OXTREC 45-09 (University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, UK) and MUTM 2008-215 from the ethics committee of Faculty of Tropical Medicine, Mahidol University.

2.1.2 *P. cynomolgi* B, M and Berok strains

Three *P. cynomolgi* lines were used in this study, the Berok strain [212], the B strain (*P. cynomolgi bastianelli*) [162], and M strain (*P. cynomolgi mulligan*) [140, 164]. The “B” strain or *P. cynomolgi bastianelli* was isolated by Garnham in 1959 from an infected *M. fascicularis* (previously named *M. irus*) near Kuantan, in the East Coast of Malaysia [162]. The *P. cynomolgi* Berok strain originated from Perak, Malaysia, where it was isolated from an infected *M. nemestrina* monkey [212]. The Berok strain used in this study consisted of a single cryopreserved 1 mL sample passage through *Aotus* monkeys and dated as 1998. The sample was thawed-out as described below and washed prior to a tail-vein administration to a splenectomised *M. fascicularis* monkey. The *P. cynomolgi* “M” strain was first described by Mulligan in 1935 [164] and later shown to be transmitted to man 1961 [140]. The B and M strains were provided by Associate Professor Bruce Russell (University of Otago) and the Berok strain was a kind gift from Professor Dennis E. Kyle (University of Georgia).
2.1.3  *P. cynomolgi* base medium composition for continuous culture

RPMI 1640 (Roswell Park Memorial Institute) supplemented with GlutaMAX (Cat. No. 61870-036, Gibco) containing 30 mM HEPES (Cat. No. H4034, Sigma-Aldrich), 0.2% (w/v) d-glucose (Cat. No. G7021, Sigma-Aldrich) and 200 μM hypoxanthine (Cat. No. 4010BC, Calbiochem). Either 20% (v/v) heat inactivated *M. fascicularis* serum or 20% (v/v) heat inactivated fetal bovine serum (Cat. No. 16000-036, Lot no. 1891471, Gibco) was added prior to use.

2.1.4  Macaque naïve blood and serum extraction and preparation

*M. fascicularis* naïve erythrocytes and serum were obtained from SingHealth Services Pte Ltd. Blood was collected by venous puncture into lithium heparin vacutainers (Becton-Dickinson). The blood collected was adjusted to 50% haematocrit with RPMI 1640 (Roswell Park Memorial Institute) supplemented with GlutaMAX (Cat. No. 61870-036, Gibco) containing 30 mM HEPES (Cat. No. H4034, Sigma-Aldrich) before passing through pre-equilibrated non-woven filters (Antoshin) to deplete leukocytes. The leukocyte-free *M. fascicularis* naïve erythrocytes were pelleted by centrifugation at 1800 rpm (650 rcf) for 5 min (Eppendorf 5804R) at room temperature (RT), washed thrice in RPMI 1640 supplemented with GlutaMax and 30 mM HEPES before it was stored at 4°C in the same medium at 50% haematocrit until use. For collection of *M. fascicularis* serum, the blood was collected by venous puncture into SST serum separation vacutainers (Becton-Dickinson), inverted gently 5 times and allowed to clot in a vertical position. The tubes were then centrifuged at 3000 rpm (1180 rcf) for 10 min at room temperature. The *M. fascicularis* serum supernatant was collected and heat-inactivated for one hour at 56°C and stored at -20°C until use.

2.1.5  Cryopreservation of *P. cynomolgi* Berok K4 and K4-A7 erythrocytic stages

*P. cynomolgi* parasite culture of predominantly ring stages were pelleted by centrifugation at 1800 rpm (650 rcf) for 5 min at room temperature (RT), the supernatant discarded. 0.33 time the volume of the packed red blood cell pellet of Glycerolyte 57
was added drop wise at 30 seconds (s) interval while gently shaking the tube to ensure contents were well mixed. It was then left to incubate for 5 min at RT. 1.33 times the volume of the packed red blood cell pellet of Glycerolyte 57 (Fenwal) was next added drop wise at 15 seconds interval while gently shaking the tube to ensure contents were well mixed. The \textit{P. cynomolgi} parasite-Glycerolyte mixture were then aliquoted to properly labelled sterile cryovials and at kept in the Mr. Frosty™ freezing container overnight at -80°C before the vials were transferred for long term storage in LN tank.

2.1.6 Thawing of frozen stock and routine \textit{in vitro} parasite growth in cynomolgous RBCs with either \textit{M. fascicularis} serum or fetal bovine serum

\textit{P. cynomolgi} Berok K4 or K4-A7 parasite cultures were started from 1 mL stabilate cryopreserved samples. The frozen samples were thawed-out using the sodium chloride method [213]. Briefly, sequentially decreasing concentrations of sodium chloride (NaCl) were added starting with 0.2 mL of 12\% (w/v) NaCl added slowly drop-wise with gentle mixing, incubated at room temperature for 5 min without shaking, and further diluted in 10 mL of 1.6\% (w/v) NaCl added drop-wise with gentle mixing. The samples were pelleted by centrifugation at 1800 rpm (650 rcf) for 5 min (Eppendorf 5804R) at RT, the supernatant discarded and samples were subjected to a final wash in 10 mL \textit{P. cynomolgi} base medium added drop-wise with gentle mixing, pelleted by centrifugation at 1800 rpm (650 rcf) for 5 min at RT (Eppendorf 5804R) and re-suspended in complete medium comprising of \textit{P. cynomolgi} base medium supplemented with either 20\% (v/v) heat inactivated macaque serum or 20\% (v/v) heat inactivated fetal bovine serum (Cat. No. 16000-036, Lot no. 1891471, Gibco).

Naïve macaque blood was added to a final haematocrit of 5\%. The parasites were initially cultured in plates in a modified candle jar (Stemcell technologies 27310) at 37°C comprising of about 3-5\% CO$_2$, and 8-10\% O$_2$ [214, 215]. Cultures were monitored daily by Giemsa-stained thick and thin smears by microscopy and parasitemia adjusted between 1\% and 5\% for routine culture. The medium was changed every other day. To enable larger culture of scale for \textit{P. cynomolgi} Berok K4 and K4-A7 erythrocytic stages, the cultures were adapted to trimix gas (5\% CO$_2$, 5\% O$_2$, 90\% N$_2$). All other conditions were the same as for
the modified candle jar. For cultures in trimix gas, the parasites were cultured and gassed in appropriate non-vented cell culture flasks.

2.1.7 Determination of parasitemia via light microscopy of Giemsa stained slides

The Giemsa stain, consisting of eosin and methylene blue, is used as the gold standard for the diagnosis malaria in blood smears. Parasitemia is the quantification of the percentage of red blood cells which are parasitized and the haematocrit of the culture refers to the amount of red blood cells (usually in v/v percentage) present in the culture. Thick and thin film smears were prepared with 5 µL of packed red blood cells from the culture for Giemsa staining. The thin film smears were fixed with 100% methanol (Cat. No. A412-4, Fisher Scientific) and dried before both the thick and thin film smears were immersed in 10% Giemsa solution (Cat. No. 352603R, VWR) in phosphate-buffered saline (pH 7.2) (PBS) for 30 min prior rinsing. Parasitemia was determined from the thin film under light microscope (NIKON YS100) with 1000X magnification with oil immersion lens. The number of red blood cells per milliliter (mL) was determined using a hemocytometer (inCYTO DHC-N01 Neubauer Improved) to be in the range of \(0.4 \times 10^7\) to \(0.6 \times 10^7\) RBCs/mL. The number of infected red blood cells (iRBC)/µL was calculated using the formula: \((\%\ \text{Parasitemia})/100 \times 5 \times 10^6\) iRBC/µL.

2.1.8 Parasite synchronization by 5% sorbitol

\(P.\ cynomolgi\) parasite cultures at predominantly ring stages were pelleted by centrifugation at 1800 rpm (650 rcf) for 5 min (Eppendorf 5804R) at RT, the supernatant discarded and samples were suspended in 5% sorbitol (Cat. No. S1876, Sigma-Aldrich) to give a final hematocrit of 20%. After incubating for 15 min at 37°C, the parasite culture was pelleted by centrifugation at 1800 rpm (650 rcf) for 5 min at RT (Eppendorf 5804R), the supernatant discarded and samples were washed with at least twice with \(P.\ cynomolgi\) base medium before it was returned to culture in \(P.\ cynomolgi\) complete medium in either (20% (v/v) \(M.\ fascicularis\) serum or 20% (v/v) heat inactivated fetal bovine serum (Cat. No. 16000-036, Lot no. 1891471, Gibco) in trimix gas (5% CO₂, 5% O₂, 90% N₂) at 37°C.
2.1.9 Enrichment of *P. cynomolgi* Berok K4 late stages using MACS LD Columns

The infected RBCs of *in vitro* *P. cynomolgi* Berok K4 were first synchronized with 5% sorbitol (Cat. No. S1876, Sigma-Aldrich) before they were matured to late stages and enriched using MACS LD columns (Miltenyi Biotech Asia Pacific Pte Ltd). The MACS LD column (Cat. No. 130042901, Miltenyi Biotec,) affixed to the MidiMACS Seperator (Cat. No. 130042302, Miltenyi Biotec) was pre-primed with *P. cynomolgi* Berok K4 base medium. The parasite culture was first pelleted by centrifugation at 1800 rpm (650 rcf) for 5 min at RT (Eppendorf 5804R) before the pellet was resuspended to 20% haematocrit with *P. cynomolgi* Berok K4 base medium before it was transferred to the pre-primed MACS LD column. The flow through containing uninfected red blood cells and early stages of the parasite were discarded. The retained hemozoin containing late stages (mid-late trophozoites, schizonts) magnetized to the LD column was eluted and washed three times with RPMI-1640 supplemented with 30 mM HEPES before the concentrated late stages were returned to culture.

2.1.10 Clonal isolation of heterogeneous population of *P. cynomolgi* Berok K4 by dilution method

A mixed stage culture of the heterogeneous *P. cynomolgi* Berok K4 continuous culture was used for the selection of homogeneous parasite populations (clones) by limiting dilution in a 96-well plate. The parasite culture was adjusted to 0.5% parasitemia for column 1 of the plate and was serially diluted 10-fold across the plate to from column 1 to column 11. Column 12 served as a background control of uninfected RBCs. In each well of parasite dilution, it contained 180 μL of *P. cynomolgi* complete medium containing 20% (v/v) of heat inactivated *M. fascicularis* serum at 1.25% haematocrit (Appendix A). Apart from medium refreshment every alternate day, the gas chamber containing the 96-well limiting dilution plate was also gassed with trimix gas every alternate day and 0.1% haematocrit was added to each well every bi-weekly to account for RBC lysis.

20 μL aliquots of the plate was first sampled for flow cytometry analysis (FACS) on Day 20 post seeding for determination of total percentage of parasites per well and subsequently repeated every 3 weeks until clones were noted in respective wells. The 20 μL aliquot from each well was harvested into a small curved-bottom tube (Micronic) before 0.5
µL of 1 mg/mL dihydroethidium (Cat. No. D7008, Sigma-Aldrich) and 1 µL of 800 µM of Hoechst 33342 (Cat. No. B2261, Sigma-Aldrich) was added and made up to 100 µL with PBS. The tubes were incubated in the dark at room temperature for 20 min. After incubation, 400 µL of cold PBS was added. 100,000 events were acquired with an Accuri C6 (BD Biosciences, US). The data were analyzed using FlowJo software (Tree Star Inc.).

Giemsa smears were sometimes carried out to determine the total percentage of live parasites in random wells for verification of FACS results. The respective clones (K4-A5, K4-B5, K4-G5, and K4-A7) identified from the positive FACS readout from Day 55 were isolated and amplified in continuous culture before cryopreservation and further studies.

2.1.11 Serum substitution of clone *P. cynomolgi* Berok K4-A7 to complete medium containing 20% (v/v) heat inactivated fetal bovine serum

To minimize the amount of *M. fascicularis* naïve blood needed for the continuous culture of *P. cynomolgi* Berok K4-A7, an asynchronous culture K4-A7 which was amplifying steadily in complete medium containing 20% (v/v) heat-inactivated *M. fascicularis* serum was sub-cultured and maintained in complete medium containing 10% (v/v) heat-inactivated *M. fascicularis* serum and 10% (v/v) heat-inactivated fetal bovine serum (Cat. No. 16000-036, Lot no. 1891471, Gibco). After 2 weeks of steady growth in the complete medium containing the mixed serums, the culture was subsequently maintained in complete medium containing 20% (v/v) heat-inactivated fetal bovine serum (Cat. No. 16000-036, Lot no. 1891471, Gibco).

2.1.12 Micropipette aspiration and RBC sphericity measurement (*collaboration with National University of Singapore (NUS)*)

The micropipette aspiration technique was modified from Hochmuth, 2000 [216]. Briefly, 1 µL of packed red blood cells containing approximately 1% parasitemia and suspended in 1 mL PBS (1% BSA). The samples were mounted onto the Olympus IX71 Inverted Microscope. A borosilicate glass micropipette (~1.5 µm inner diameter) was used to extract the cell membrane under a negative pressure at a pressure drop rate of 0.5 pa/s.
The corresponding cell membrane deformation was monitored using a 100X oil immersion lens. The cell membrane deformation was recorded using the QColor5 High Resolution Color CCD Digital Fire Wire Camera (Olympus) and processed by QCapture Pro 6.0 (Olympus). The cell membrane shear modulus was calculated using the hemispherical cap model [216]. To quantify the binding force between uninfected RBC and a rosetting infected RBC (iRBC), a double pipette aspiration method was used [217]. A rosette was held by a micropipette (diameter = 2.0 ± 0.2 µm). A second micropipette was used to aspirate the uninfected red cell at increased levels of pressure. The force (F) to detach the red cell from the iRBC was calculated as $F = \pi r^2 \times P$; where r is the inner diameter of the second micropipette, and P is the pressure required for cell detachment. The aspiration pressure was measured by a pressure transducer (P61 model, Validyne Engineering) and recorded by USB-COM Data logger (Validyne Engineering). The process was recorded using a Dual CCD Digital Camera DP80 (Olympus®) at one frame/s. Recorded images were analyzed with CellSens Dimension (Olympus®).

2.1.13 Atomic force microscopy (collaboration with NUS)

*P. cynomolgi* Berok K4 continuous culture was harvested at the trophozoite stage and 200 µl of the blood medium mixture was supravitaly stained with 1 µL of DAPI, for 15 min in an incubator and prepared as smears (unfixed and air dried) for atomic force microscopy (AFM). At least 20 iRBCs from each isolate and cell type was scanned using AFM. We were able to conduct serial measurements (with AFM, then and with Giemsa) by using a copper microdisk grid (H7 finder grid, SPI Supplies, PA) attached underneath the glass slide allowing us to locate and image the same cell. These thin smears were first AFM scanned by a Dimension 3100 model with a Nanoscope IIIa controller (Veeco) using tapping mode. The probes used for imaging were 125 µm long by 30 µm wide single-beam shaped cantilevers (Model PPP-NCHR-50, Nanosensors) with tip radius of curvature of 5-7 nm. Images were processed using the Nanoscope 5.30 software (Veeco).

2.1.14 Scanning electron microscopy of infected RBCs
Sorted cells coated on poly-lysine (Sigma) glass coverslips were fixed in 2.5% glutaraldehyde, washed, and treated with 1% osmium tetroxide (Cat. No. 18612, Ted Pella Inc.) before critical point drying (CPD 030, Bal-Tec). Glass coverslips were sputter-coated with gold in a high vacuum (SCD005 sputter coater, Bal-Tec) and imaged with a field emission scanning electron microscope (JSM-6701F, JEOL) at an acceleration voltage of 8 kV[218].

2.1.15 Image stream analysis of rosetting (collaboration with NUS)

We determined the percentage of rosetting of *P. cynomolgi* Berok K4 iRBCs by image stream analysis using a method adapted from Lee *et al*. 2014 [219]. Briefly iRBCs stained with Hoechst and dihydroethidium were suspended in PBS to a haematocrit of 2% and were assayed with the ImageStream 100 (Amnis, Seattle, WA) fitted with a 60X objective. At least 200 untreated parasites for each single-stain condition were used to create a compensation matrix. During screening, 10,000 parasites were acquired and gated using the technique adopted from Malleret *et al*. [220] along with the added selection of cells that were not singlets (one or more cells adhering to an infected cell staining positive for Hoechst). Analysis was performed with the IDEAS software (version 4.0).

2.1.16 Rosetting wet-mount (collaboration with Singapore Immunology Network (SIgN))

As previously described [221], the *P. cynomolgi* Berok K4 culture was sampled at ring, trophozoite and schizonts stages of its life cycle. To determine the number of rosettes and live parasites, the respective sampled culture suspension was stained with Giemsa (final concentration of 5%) for 15 minutes. A minute volume of this suspension was used to make a wet mount with 22 x 32mm (0.17mm thickness) glass cover slip. This wet mount was examined immediately with light microscope under oil immersion magnification. Rosetting rate was evaluated by examining 200 iRBCs.

2.1.17 DNA extraction, PCR amplifications and sequencing
For the comparison of genes rbp1b, rbp 2a and rbp2b via PCR amplifications between heterogeneous P. cynomolgi Berok K4, clonal P. cynomolgi Berok K4-A7 and P. cynomolgi B strain, 200 µL packed cell volume (pcv) of the respective cultures was used for DNA extraction using DNA Blood Mini kit (Qiagen, cat. No. 51104). The respective RBP primers were designed based on the following published sequences - Pcy RBP2a strain B Chromosome 8 GI: 457870682, Pcy RBP2b Berok JQ422039.1, Pcy RBP1b Berok JQ422038.1, Pcy RBP2a B strain GI:389585944, Pcy RBP2a Chromosome 14 strain M GI: LT841392.1 and Pcy RBP2a Chromosome 4 strain M GI: LT841382.1

2.1.18 Preparation of P. cynomolgi Berok K4-A7 for whole genomic sequencing

For extraction of genomic DNA, the P. cynomolgi Berok K4-A7 culture was amplified to 2 billion parasites (i.e. 8 % parasitemia in 100 mL complete medium at 5% haematocrit) before parasite extraction was carried out from the iRBCs via saponin lysis (0.15% saponin and 0.1% bovine serum albumin fraction V in phosphate buffered saline) Briefly, after centrifugation at 1800 rpm (650 rcf) for 5 min at RT (Eppendorf 5804R) the pellet was reconstituted to 10% haematocrit with P. cynomolgi base medium before the required amount of saponin lysis buffer was added on ice. Upon lysis of red blood cells, indicated by a clear red supernatant, the lysed cultures were centrifuged at 1800 rpm (650 rcf) for 5 min at RT (Eppendorf 5804R) and the parasite cell pellets were rinsed twice in chilled PBS. As previously described [222], the DNA was isolated from the parasite cell pellets using a Blood and Cell Culture DNA Mini extraction kit (Cat. No. 13323, Qiagen).

2.1.19 P. cynomolgi Berok K4-A7 whole genome sequencing using Oxford Nanopore

Plasmodium cynomolgi gDNA (2.5 µg) was input to the Oxford Nanopore, SQK-LSK308 (1D2) library preparation workflow according to manufacturer’s instructions. Prepared library of 355.2ng was loaded onto a FLO-MIN107 flowcell on the GridION instrument, running MinKNOW v2.1 with live 1D basecalling via Guppy v1.5.1. The library was sequenced for 48 hours, generating a total of 596,000 reads totalling 2.54GB, with 360,387 reads having Q$\geq$ 7, totalling 1.99GB. FASTQ files containing 596,000 total sequences were used for de novo assembly using Canu (a fork of the Celera Assembler with
specific adaptations for Oxford Nanopore). The assembly consists of 44 contigs totalling 36,839,098bp. To check on the quality of the assembly, the reference genome B strain of the *P. cynomolgi* (RefSeq assembly accession GCF_000321355.1) was used. Mummer4 was used to align the de novo assembly to the reference genome and 90.9% of the reference genome could be mapped to portions of the de novo assembly with an average identity of 88.66%.

2.1.20 PCR Free genomic DNA library for short read sequencing (Illumina)

Genomic DNA (2µg) was sheared using Covaris S2 sonicator in 52.5µl volume using the following parameters: 10% duty cycle, intensity 4 and 200 cycles per burst for 45 seconds to generate DNA fragments that are enriched for 550bp in length. Whole-genome sequencing libraries were prepared using Illumina TruSeq® DNA PCR-Free library prep kit according to manufacturer’s instructions. The libraries were re-suspended in 30 µl of re-suspension buffer. Concentrations of the purified libraries were estimated using the High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA). Subsequently qPCR quantitation was performed using KAPA library quantification kit to ascertain the loading concentration. The resultant libraries were sequenced using Illumina Miseq to generate indexed paired-end reads of 2 × 251bp at a sequencing depth of 60X coverage (3 million reads) per sample.

2.1.21 Compound libraries

The Pathogen Box® (http://www.pathogenbox.org/), modelled after the Malaria Box [72], is an open source library comprising of 400 diverse open source compounds targeting neglected tropical diseases. The Malaria Box was an open source library (until December 2015) which comprised of 400 diverse compounds with antimalarial activity. In addition, the three drug candidates (the phosphatidylinositol-4-OH kinase (PI(4)K) inhibitor KDU691 [66], the imidazolopiperazines GNF179 [42] and the spiroindolone KAF246 [41]) were included as additional controls. The selected 80 compounds in Figure 3.13 were obtained from MMV as a blinded compound screen in *P. cynomolgi* Berok K4 continuous culture to study the effects of serum in drug susceptibility.
2.1.22 Compound plate preparation

Test compounds were prepared by serially diluting a 10 mM compound stock three-fold with DMSO for 10 concentration points in the master compound plate. 100% DMSO was used as the negative control and 10 mM mefloquine was used as the positive control. A thousand-fold dilution (either 100 nL or 50 nL) of compound from master plate were spotted onto a 96-well or 384-well assay plate respectively using either the Mosquito® nanoliter dispenser (Cambridge, UK) or the Echo 550 liquid dispenser (Labcyte, U.S.A). The plates were then sealed with a removable foil seal using PlateLoc Thermal Microplate Sealer (Agilent) until use.

2.1.23 Compound testing with *P. cynomolgi* Berok K4 using SYBR green I proliferation assay (collaboration with NITD)

There are various high-throughput screens based on cultured *P. falciparum* [43, 63-65, 69, 70] or on the hepatic stages of the rodent parasites *P. berghei* or *P. yoelii* [42, 70], described. In this study, we adapted the SYBR green I proliferation assay as described previously [69]. Briefly, 50 µL of the *P. cynomolgi* Berok K4 continuous culture was dispensed manually into the 384-well plate at both final parasitemia of 0.5% (for dose response assay) or 1% (for single point assay) and the haematocrit adjusted to 2.5%. The 384-well assay plates were incubated at 37°C for 72 h in 5% CO₂, 5% O₂ and 90% N₂. After a 72 h incubation, 10 µL of lysis buffer, consisting of 5 mM EDTA, 20 mM Tris-HCl pH 7.5, 1.6% Triton X-100 and 0.16% Saponin were added to each well and the plate was incubated in the dark for 24 h at room temperature. The plate was read for fluorescence using BioTek Synergy™ 4 hybrid microplate reader (Vermont, USA) using a bottom read mode at excitation wavelength of 485 nm and emission wavelength of 528 nm.

2.1.24 Schizont maturation assay with *P. cynomolgi* Berok K4 using flow cytometry analysis

The schizont maturation assay for *P. vivax* was carried-out as previously described [19, 223]; where parasite development was assessed by flow cytometry [220]. 200 µL of
tightly synchronised ring stage culture of *P. cynomolgi* Berok K4 was dispensed manually in the 96-well compound plate at a final parasitemia of 0.5% and haematocrit adjusted to 2%. The assay plates were incubated at 37°C for around 44 h in 5% CO₂, 5% O₂ and 90% N₂ until mid-late schizonts stage (> 5 merozoites) was observed in the drug free control wells via Giemsa staining. Each well was well mixed and 20 µL was harvested into a small curved-bottom tube (Micronic) before 0.5 µL of 1 mg/mL dihydroethidium and 1 µL of 800uM of Hoechst 33342 was added and made up to 100 µL with PBS. The tubes were incubated in the dark at room temperature for 20 min. After incubation, 400 µL of cold PBS was added. 100,000 events were acquired with an Accuri C6 (BD Biosciences, US). The data were analysed using FlowJo software (Tree Star Inc.).

2.1.25 Transmission study from *P. cynomolgi* Berok K4 continuous culture (*collaboration with Biomedical Primate Research Center (BPRC*))

Monkey infections and mosquito feedings were performed as previously described [93].Briefly, cryopreserved in vitro adapted *P. cynomolgi* Berok K4 parasites were thawed as described above and one million parasites were used to infect one *M. mulatta* monkey via intravenous injection, while the monkey was under ketamine sedation. Parasitemia was monitored over time through Giemsa-stained thin smears. Female *Anopheles stephensi* mosquitoes were fed with infected monkey blood using a glass feeder system on days 12 and 13 post infection, after which the monkey was cured from blood stage parasites with chloroquine (3 daily intramuscular injections 7.5 mg/kg). Oocysts were counted in at least 10 mosquitoes at day 7 after the infected blood meal. The remaining mosquitoes were given a second uninfected blood meal to promote sporozoite invasion of the salivary glands.

2.1.26 Sporozoites from *P. cynomolgi* Berok K4 strain (*collaboration with BPRC*)

As previously described [93], sporozoites were isolated from salivary glands of infected *Anopheles stephensi* mosquitoes eighteen days post infected blood meal. Briefly, *Anopheles stephensi* mosquitoes were fed on *P. cynomolgi* Berok K4 infected *M. mulatta* blood. Formation of oocysts was first determined in the infected mosquitoes a week after
the infected blood meal and sporozoites were subsequently isolated on the day of infection of the hepatocytes.

2.1.27 Infectivity of sporozoites derived from *P. cynomolgi* Berok K4 continuous culture in hepatocyte monolayer (*collaboration with BPRC*)

*M. mulatta* hepatocytes from different donors were isolated as previously described [93] and plated onto collagen coated 384-well plates at a density of 28,000 hepatocytes per well in William’s B medium as previously described [93]. Two days later, 20,000 sporozoites were added to each well. Regular medium refreshments were performed, and the infected hepatocytes were fixed in 3.7% paraformaldehyde (PFA) after six days post infection. Parasites were immunostained with anti-*P. cynomolgi* heat shock protein 70 (PcHsp70) antibodies and goat-anti-rabbit Alexa568 red secondary antibodies (Invitrogen A11011), while the nuclei were stained with DAPI before they were visualised using the Operetta high content screening automated microscope. Image analysis was done using the analysis algorithm as designed in previous study [93] with a minor adaptation for the change in fluorescent label of the secondary antibody.

2.1.28 Statistical analysis

The non-parametric data (all data sets failed the D'Agostino & Pearson normality test) presented in Figure 3.8(b, d, f and h) and Figure 3.15(d) was analysed using the Mann-Whitney U-Test with the significance level set at $P < 0.05$. The parametric data presented in Figure 3.15(c and d) were analysed using the Welch’s t-test with the significance level set at $P < 0.05$. The histograms represent means and the error bars the standard deviation (SD). The histograms and lines on box plots and scatter plots represent medians and the error bars the interquartile range (ICQ). All analyses were carried out using GraphPad Prism™ 7 (GraphPad Software Inc, USA).
CHAPTER 2.2

Establishment of a 3D hepatic spheroid model to study the exo-erythrocytic stages of *Plasmodium cynomolgi*
2.2.1 Ethical committees and animal welfare

*Macaca fascicularis* (cynomolgous monkeys) were maintained at the Novartis Laboratory Animal Services, New Jersey, U.S.A., (Novartis-LAS) and SingHealth, Singapore. *M. mulatta* (rhesus monkeys) were maintained at the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand (AFRIMS). All three institutions were audited and approved by the Novartis Animal Welfare Compliance. All animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals and the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) Standards. All studies were approved by the Novartis Ethical Review Council and Novartis Institutional Animal Care and Use Committees (IACUC), protocol Number 100280 Committees (IACUC protocol reference number 100280), prior to study initiation. Additionally, studies at AFRIMS were approved by the AFRIMS’ IACUC (protocol reference number PN13-06) and studies at SingHealth were approved by the SingHealth IACUC (protocol reference number 2015/SHS/1024).

2.2.2 Scanning electron microscopy (SEM) of 3D Cellusponge

Dry sponge discs (approx. 5 mm in diameter and 1 mm in height) were sputter coated with platinum for 60–90 s and the top view of the sponges was captured using a JSM-7400F Field Emission Scanning Electron Microscope at 5 kV.

2.2.3 Confocal microscopy of 3D Cellusponge

The porous structure of hydrated sponges was visualized by confocal microscopy as described previously [224]. Briefly, dry sponges were re-hydrated in PBS overnight at RT. The hydrated sponge was then immersed in 500 µL of propidium iodide (PI) (1 mg/mL in PBS) overnight. The sponge was washed 5 times with PBS to remove excess PI before imaging with a Zeiss 710 confocal microscope.
2.2.4 Young’s modulus measurement of 3D Cellusponge

Young’s modulus (E) was measured by an Instron Model 5848 Bench Top Microtester equipped with an Instron 2530-437 Static Load Cell. Dry sponges were re-hydrated in PBS overnight. The hydrated sponges were compressed to 40% strain at 1 mm/min at RT. Young’s modulus is equal to the slope of the linear region on the stress-strain curve.

2.2.5 Human and simian hepatocytes culture

Cryopreserved human hepatocytes (Lot 358) were purchased from BD Biosciences while simian hepatocytes were either purchased from Invitrogen (CY380 and CY364) or isolated and cryopreserved in-house. Briefly, the cryopreserved hepatocytes were rapidly thawed in 39°C water bath before the contents were transferred to pre-warmed recovery medium (Life technologies, CM7000). Next, they were pelleted and gently resuspended in pre-warmed 36% Percoll before centrifugation. The dead or unhealthy cells in the supernatant was discarded and the hepatocyte pellet was resuspended in plating medium and enumerated. Spheroid cultured hepatocytes were maintained in William’s E media supplemented with 1 mg/mL bovine serum albumin (BSA), 1X insulin-transferrin-selenium, 50 ng/mL linoleic acid, 50 nM dexamethasone and 100 U/mL of penicillin/streptomycin. For infection of human and simian hepatocytes with *P. vivax* and *P. cynomolgi* sporozoites respectively and also for the maintenance of the respective hepatocytes in monolayer, William’s E medium supplemented with 2 mM L-glutamine (Cat. No. 2500-024, Life Technologies), 10% Hyclone FetalClone II Serum (Cat. No. SH30066.03, Hyclone), 5 μg/ml human insulin (Cat. No. I3536, Sigma), 2X penicillin/streptomycin (Cat. No. 15140-122, Life Technologies) and 0.05 μM hydrocortisone (Sigma, H0888) was used.

2.2.6 Urea synthesis
Supernatant was collected from cells cultured in both the cynomolgous hepatocytes cultured in monolayer and in spheroids. Urea synthesis was measured using a Urea Nitrogen kit (Cat. No. 0580, 250, StanBio).

2.2.7 Fluorescein diacetate (FDA) staining

The spheroid cultured human hepatocytes were incubated with 1.5 mg/ml of fluorescein di-acetate (Cat. No. F1303, Thermo Scientific) for 20 minutes before they were washed 5 times with PBS before imaging with confocal microscopy at 60X with an oil lens.

2.2.8 Real-time PCR analysis for hepatocyte specific genes (collaboration with NUS)

RNA was extracted from both monolayer cultured hepatocytes and spheroid-cultured hepatocytes using RNeasy Mini Kit (Cat. No. 4104, Qiagen). Total RNA concentration was quantified by Nanodrop (Thermo Scientific), and 1 μg of RNA was converted to cDNA by high capacity RNA-to-cDNA kit (Cat. No. 4387406, Applied Biosystems). Primers were designed using Primer 3, and real-time PCR was performed by using SYBR Green fast master mix in an ABI 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was plotted as a Log2 transcript over GAPDH.

2.2.9 Sporozoites production and isolation from P. cynomolgi bastianellii (B) strain

P. cynomolgi bastianellii (B strain) sporozoites were obtained by dissection of the salivary glands of infected Anopheles dirus mosquitoes obtained from the insectary in Armed Forces Research Institute of Medical Sciences (AFRIMS, Bangkok, Thailand).

2.2.10 Infection of P. cynomolgi sporozoites with pre-formed 3D simian hepatic spheroids

Simian hepatocytes were seeded at a density of 0.1 million cells per 6 mm 3D Cellusponge in a 48-well plate. After 45 minutes of incubation in a 37°C / 5% CO2 incubator,
280 μL of additional medium was added to each well. Medium was refreshed every alternate day. Four days post seeding of 3D Cellusponge, *P. cynomolgi* sporozoites were added to the spheroid-cultured simian hepatocytes at various MoI (0.7, 2 and 4) in William’s E medium containing 10% serum. After infection in 37 °C / 5% CO₂ for 3 hours, the spheroids were washed 3 times and fresh maintenance medium was added. The infected spheroids were maintained in William’s E without serum with medium refreshment every alternate day before they were fixed with 3.7% PFA at respective end points to detect for liver-stage parasites using immunostaining.

2.2.11 Infection of *P. cynomolgi* sporozoites with suspension simian hepatocytes prior to spheroid formation

Simian hepatocytes and *P. cynomolgi* sporozoites (MoI: 2) were added to low attachment plate and co-incubated for 3 hours with shaking at 100 rpm in a 37 °C / 5% CO₂ incubator with built-in shaker to facilitate sporozoites infection of the hepatocytes. The infected hepatocytes in suspension were then collected, washed extensively with PBS to remove the excess un-invaded *P. cynomolgi* sporozoites and seeded at a density of 0.1 million cells per 6 mm 3D Cellusponge and maintained as described above.

2.2.12 Dissociation and plating of *P. cynomolgi* infected spheroids (*collaboration with NUS*)

Uninfected and infected spheroids were harvested from the 3D Cellusponge, dissociated enzymatically using TrypLe™ (Cat. No. 12604013, Thermo Scientific) and plated on collagen coated 96-well plates overnight in 37 °C / 5% CO₂ incubator. Samples were then fixed with 3.7% PFA before they were immunostained and imaged using confocal microscopy.

2.2.13 Drug treatment of *P. cynomolgi* and *P. vivax* exo-erythrocytic stages in spheroid-cultured hepatocytes
At selected time points post infection with *P. cynomolgi* and *P. vivax* sporozoites, the infected spheroid-cultured hepatocytes were incubated with medium containing 0.1% DMSO, 500 nM primaquine biphosphate (Cat. No. 156507, Sigma-Aldrich), 200 nM atovaquone (Cat. No. 1044651, Sigma-Aldrich) and 2 μM of Novartis compound (KDU691) respectively.

2.2.14 Complete maturation of *P. cynomolgi* liver-stages and blood stage transition in spheroid-cultured hepatocytes

Naïve *M. fascicularis* red blood cells were added to *P. cynomolgi* infected spheroid-cultured hepatocytes at 0.1% hematocrit on day 7, 10 and 14-post infection. Every day for five days, fresh red blood cells were added and samples of the red blood cells from the previous day’s culture were transferred to a separate well and cultured for another 48 hours before they were harvested. Thin blood smears were prepared, stained with 10% Giemsa for 30 minutes and examined for asexual stage parasites.

2.2.15 *P. vivax* sporozoites production and isolation

For *P. vivax* mosquito infections from infected patients, isolates of *P. vivax* were collected from symptomatic patients with their informed consent at the Shoklo Malaria Research Unit (SMRU) in Tak province, Mae Sod Thailand. Briefly, *Anopheles dirus* and *Anopheles cracens* mosquitoes were fed on freshly collected blood samples from a *P. vivax* infected patient. Formation of oocysts was first determined in the respective infected mosquitoes and *P. vivax* sporozoites were isolated on the day of infection of the hepatocytes.

2.2.16 Infection with *P. vivax* sporozoites of suspension human hepatocytes prior to spheroid formation

Human hepatocytes and *P. vivax* sporozoites (Mol: 2) were added to a low attachment plate and co-incubated for three hours with shaking at 100 rpm in a 37°C/ 5% CO₂ incubator with built-in shaker to facilitate sporozoites infection of the hepatocytes. The
infected hepatocytes in suspension were then collected, washed extensively with PBS to remove excess un-invaded *P. vivax* sporozoites and seeded at a density of 0.1 million cells per 6 mm 3D Cellusponge and maintained as described above.

2.2.17 Immunostaining of *P. cynomolgi* and *P. vivax* infected and uninfected spheroid-cultured hepatocytes

Sporozoites infected and uninfected spheroids were fixed with 3.7% PFA for 15 minutes at 37°C followed by washing with PBS and blocking with 2% BSA/0.2% Triton-X 100 for 2 hours. After which the spheroids were incubated overnight at 4°C with either mouse anti-human CD-81 Clone JS-81 (Cat. No. 555675 BD Pharmingen), rabbit anti-MRP2 (Cat. No. M8316, Sigma-Aldrich), mouse anti-CD147 (Cat. No. ABCA2022492, Serotec), rabbit anti-PcHsp70, mouse anti-PcUIS4 or mouse anti-PvUIS4. Secondary antibodies used were goat anti-rabbit 488 and goat anti-mouse 555 (1:1000) respectively (Invitrogen). Nuclei stain was performed using mounting medium containing DAPI stain (Vecta Shield). All samples were blinded before it was sent for imaging and analysis. Images were captured using Olympus Fluoview FV1000 or Zeiss LSM 710 with a 60X water lens. Images were analysed using IMARIS (Bitplane scientific solutions, United Kingdom) and images assembled using Adobe illustrator CS2.

2.2.18 Image analysis of *P. cynomolgi* and *P. vivax* infected spheroid-cultured hepatocytes (collaboration with NUS)

The images of *P. cynomolgi* and *P. vivax* infection in 3D spheroid-cultured hepatocytes were pre-processed using the Fiji (Fiji ImageJ2) processing plugins to demarcate the spheroid boundaries (either 3D transformation of DAPI channel or creation of a pseudo-channel using summation of the DAPI and the GFP channels). The Imaris images analysis software (Bitplane), was used to determine the (1) GFP intensity summation within spheroid and the (2) DAPI intensity summation within spheroid. The Cell Module of Imaris was used, wherein the spheroids were treated as cells and the nuclei were treated as vesicles. The results were filtered to exclude spheroids that are too small or composed of cells without nuclei.
2.2.19 Statistical analysis

All data were tested for normality using the Shapiro-Wilk normality test. The comparison of urea production between 3D Cellusponge and collagen monolayer and the comparison of the different *P. cynomolgi* treatment models were performed using two-tailed $t$-test. Comparison on the rate of *P. cynomolgi* infections for two distinct methods of infection and the rate of *P. vivax* infections were carried out using two-tailed $t$-test.
CHAPTER 2.3

Recipes for media and solutions
2.3.1 Continuous in vitro culture of Plasmodium cynomolgi Berok K4 erythrocytic stages

**P. cynomolgi base medium**

RPMI 1640 (Roswell Park Memorial Institute) supplemented with GlutaMAX (Cat. No. 61870-036, Gibco) containing 30 mM HEPES (Cat. No. H4034, Sigma-Aldrich), 0.2% (w/v) d-glucose (Cat. No. G7021, Sigma-Aldrich) and 200 μM hypoxanthine (Cat. No. 4010BC, Calbiochem). Base medium was filter-sterilized over 0.22 μm filter and stored in 4°C and used within 1 month.

**P. cynomolgi complete medium**

To prepare complete medium, either 20% (v/v) heat inactivated *M. fascicularis* serum or 20% (v/v) heat inactivated fetal bovine serum (Cat. No. 16000-036, Lot no. 1891471, Gibco) was added directly to sterile *P. cynomolgi* base medium. Complete medium was stored 4°C and used within 2 weeks.

**M. fascicularis naïve blood washing medium**

30 mM HEPES (Cat. No. H4034, Sigma-Aldrich) was added to RPMI 1640 (Roswell Park Memorial Institute) supplemented with GlutaMAX (Cat. No. 61870-036, Gibco). Wash medium was filter-sterilized over 0.22 μm filter and stored 4°C and used within 1 month.

**10% Giemsa working solution**

Add 9 volumes of phosphate buffer (pH 7.2) to 1 volume of Giemsa (Cat. No. 352603R, VWR) solution. It is always prepared fresh before use.
5% (w/v) sorbitol

5g of sorbitol (Cat. No. S1876, Sigma-Aldrich) was dissolved in 100 mL of distilled water and sterilized by filtration through a 0.22 µm filter. Store in 4°C until use.

12% (w/v) NaCl and 1.6% (w/v) NaCl

To prepare 12% (w/v) NaCl and 1.6% (w/v) NaCl solution, 12g and 1.6g of NaCl (Cat. No. S9888, Sigma-Aldrich) was dissolved in 100 mL of distilled water respectively and sterilized by filtration through a 0.22 µm filter. Store in 4°C until use.

2.3.2 3D hepatic spheroids to study *P. cynomolgi* exo-erythrocytic stages

Medium for sporozoites-hepatocytes infection and for the maintenance of monolayer cultured hepatocytes

William’s E (Cat. No. 12551-032, Life Technologies) medium supplemented with 2 mM L-glutamine (Cat. No. 2500-024, Life Technologies), 10% Hyclone FetalClone II Serum (Cat. No. SH30066.03, Hyclone), 5 µg/ml human insulin (Cat. No. I3536, Sigma-Aldrich), 2X Penicillin/Streptomycin (Cat. No. 15140-122, Life Technologies) and 0.05 µM hydrocortisone (Cat. No. H0888, Sigma-Aldrich) was prepared and sterilized by filtration through a 0.22 µm filter. The medium was stored in 4°C and used within 1 month.

Maintenance medium for spheroid cultured hepatocytes

William’s E medium (Cat. No. 12551-032, Life Technologies) supplemented with 1 mg/mL BSA (Cat. No. A2153, Sigma-Aldrich), 1X Insulin Transferrin and Selenium (Cat. No. 51300044, Life Technologies), 50 ng/mL linoleic acid (Cat. No. L1012, Life Technologies), 50 nM dexamethasone (Cat. No. D4901, Sigma-Aldrich) and 100 U/mL of Penicillin
/Streptomycin (Cat. No. 15140-122, Life Technologies) was prepared and sterilized by filtration through a 0.22 μm filter. The medium was stored in 4°C and used within 1 month.

**Plating medium for monolayer cultured hepatocytes**

William’s E medium without serum and phenol red (Cat. No. A1217601, Life Technologies) was supplemented with thawing/ plating supplement pack (Cat. No. CM3000, Life Technologies,) and sterilized by filtration through a 0.22 μm filter. The medium was stored in 4°C and used within 1 month.

**36% (v/v) Percoll**

To prepare 100ml of 36% (v/v) Percoll, 36 mL of Percoll (Cat. No. 170891-01, GE Healthcare,) and 4 mL of 10X PBS (Cat. No. 70011-044, Life Technologies) was added to 60 mL of William’s E medium (Cat. No. 12551-032, Life Technologies) before sterilization through a 0.22 μm filter. The solution was stored in 4°C and used within 1 month.

**Sporozoites dissection medium**

Schneider insect medium (Cat. No. S9895, Sigma-Aldrich) supplemented with 4X Penicillin/ Streptomycin (Cat. No. 15140-12200, Life Technologies) and 0.5 μg/ mL amphotericin B (Cat. No. A2942, Sigma-Aldrich) before sterilization through a 0.22 μm filter. The medium was prepared on the day of usage and used within the day.

**3.7% (v/v) paraformaldehyde**

To prepare 50 mL of 3.7% (v/v) paraformaldehyde, 11.6 mL of 16% paraformaldehyde (Cat. No. 15710, Electron Microscopy Sciences) was added to 38.4 mL of 1X PBS
CHAPTER 3 - RESULTS

Development of the continuous in vitro culture of P. cynomolgi Berok K4 erythrocytic stages
3.1 Propagation of *P. cynomolgi* strains

Three *P. cynomolgi* lines were available to us: Berok strain [212] cryopreserved in 1998 from *Aotus trivirgatus*, B strain (*P. cynomolgi bastianelli*) [162], and M (*P. cynomolgi mulligan*) strain [140]. Separate *Macaca fascicularis* monkeys were successfully infected with one or other of these lines and blood samples were collected to initiate cultures. We conducted preliminary experiments using the Berok strain, to test various culture conditions and materials in short-term cultures initiated from cryopreserved stocks prepared from infected monkey blood (Figure 3.1). This allowed us to define an optimal working protocol that was then tested on Berok strain parasites from different animals, and on B and M strain parasites freshly collected from infected macaques. The robust continuous culture of *P. cynomolgi* Berok K4 parasites were subsequently used for further work involving molecular, rheological, morphological, drug screening and transmission studies.
Figure 3.1. Schematic diagram of the successful adaptation of *P. cynomolgi* Berok from *in vivo* to *in vitro* culture.
3.2 Robust multiplication of the continuous culture of *P. cynomolgi* Berok K4 strain

Blood was collected when the parasites were predominantly mature (late trophozoites and schizonts) and depleted of leukocytes before enrichment of the mature parasites on a Percoll gradient. *In vitro* cultures were initiated at parasitemia > 1% (50,000 parasites/µL) and monitored daily by microscopic examination of Giemsa-stained smears. In repeated independent experiments the parasitemia of the B and M strain cultures declined to become undetectable within a few days (Figure 3.2a). The parasitemia of the Berok strain parasites obtained from one of the monkeys (K4), but not the others, increased such that regular dilution of this culture became necessary within two days post-seeding in contrast from the *P. cynomolgi bastianelli* strain (Figure 3.2b). For these Berok K4 parasites, ring stages were regularly observed as were a few gametocytes from day 6 post thaw.
Figure 3.2. *In vitro* culture of *P. cynomolgi* (Berok K4 strain, *bastianelli* (B) and Mulligan (M) strain) erythrocytic stages initiated from infected monkeys. (a) Pilot *ex vivo* culture of *P. cynomolgi* Berok, B and M strain. (b) Multiplication of the *P. cynomolgi* Berok K4 strain necessitated regular dilution of this strain which was in contrast to the *P. cynomolgi* B strain.
3.3 Biological and morphological characteristics of *in vitro* cultured Berok K4 line

A culture was initiated, using sorbitol synchronised and MACS columns-concentrated mature blood stage parasites, and then sampled at two hourly intervals for 48 h (Figure 3.3). The morphology of the parasites was indistinguishable from that observed or previously described from infected animals. Eight to sixteen merozoites were observed in fully mature schizonts around 46-48 h. Gametocytes were observed *in vitro*, albeit in low numbers, which probably accounts for the low level of transmission of the parasite from continuous culture to mosquitoes. It was not clear at this point whether these laboratory- and culture-adapted parasites had thereby lost their infectiousness to their insect hosts, as had been previously observed for some culture-adapted parasites, such as the *P. knowlesi* H strain that lost the capacity to produce gametocytes [225]
Figure 3.3. Tertian cycle of *the P. cynomolgi* Berok K4 continuous culture. Mature schizonts of culture adapted *P. cynomolgi* Berok K4 were enriched and allowed to re-invade fresh red blood cells that were then monitored every two hours to document the complete asexual erythrocytic cycle *in vitro.*
3.4 Culture characteristics of the heterogeneous *P. cynomolgi* Berok K4 line

The initial Berok K4 line culture (multiplication rate ranging from 2 to 4-fold over more than 5 cycles) was used to constitute a working cryopreserved stock. Successful propagation of the line was maintained for up to 180 days in cultures initiated with thawed parasites. Culture conditions were further refined, with robust growth best observed under reduced oxygen environment (5% CO$_2$, 5% O$_2$ and 90% N$_2$) to reach parasitemia beyond 10% (i.e. 500,000 parasites/µL) after sub-culturing (Figure 3.4a). Growth rates were unaffected when different culture vessels were used (24-well plate, 6-well plate, T25 or T75 flasks). The *P. cynomolgi* Berok K4 parasites retained their distinct morphological features throughout the 30 days of *in vitro* culture. The robustness of the *P. cynomolgi in vitro*-grown Berok K4 stabilates was independently validated by successful *in vitro* cultivation in six additional laboratories (the National University of Singapore, the University of Otago in New Zealand, the University of Malaya in Malaysia, the Shoklo Malaria Research Unit in Thailand, the Université Pierre et Marie Curie in France and the Novartis Institute of Infectious Diseases in United States of America).

The parasitemia of the Berok strain parasites obtained from only one of the monkeys (K4), but not the others, increased at such a high rate that such that regular dilution of this culture became necessary within two days post-seeding (Figure 3.4a). In contrast from the *P. cynomolgi* B and M strains never showed substantial increases in parasitemia. For these Berok K4 parasites, ring stages were regularly observed as were a few gametocytes from day 6 post thaw. Like the laboratory adapted strains of *P. falciparum*, the *P. cynomolgi* Berok K4 stain retains its morphological characteristics through the duration of continuous culture *in vitro* (Figure 3.4b).

One crucial aspect to ensure the successful cultivation of *P. cynomolgi* Berok K4 *in vitro* is not to include any antibiotics in its culture medium. Unlike *P. falciparum* continuous culture where antibiotics are commonly added to prevent contamination, the addition of any antibiotics (e.g. gentamicin and penicillin-streptomycin) even at low concentrations caused the *P. cynomolgi* Berok K4 continuous culture to ultimately die off. In contrast, the culture which was grown in the absence of antibiotics grew robustly with regular sub-culturing required (Figure 3.4c). This phenomenon was consistently observed when antibiotics were added in the culture medium.
Figure 3.4. Robust continuous culture of *P. cynomolgi* Berok K4 (a) High amplification rate of the parasites over 30 days in continuous culture (b) Similar morphology of the ring, trophozoite and schizonts stages of the *in vitro* culture of *P. cynomolgi* Berok K4 strain over 30 days in continuous culture (c) Effect of antibiotics on the continuous culture of *P. cynomolgi* Berok K4 (red arrows signify days when cultures were sub-cultured)
3.5 Selection of homogeneous parasite populations (clones) from heterogeneous population of \textit{P. cynomolgi} Berok K4 continuous culture

3.5.1 Isolation of \textit{P. cynomolgi} Berok K4 clones

With the establishment of Berok K4 strain continuous culture, our next objective was to isolate single parasite clones from the heterogeneous parent culture by limiting dilution method. Obtaining single parasite clone facilitates the conduct of various techniques in malaria research such as genotyping [226], drug resistance testing [222], genetic manipulation [227]. Limiting dilution is a standard method for the \textit{in vitro} cloning of \textit{P. falciparum}, particularly after genetic manipulation [228]. The method entails the dilution of the heterogeneous parasite population with uninfected erythrocytes in 96-well plates at less than one infected erythrocyte per well. Conventionally, positive wells used to be determined after a few weeks of culture by microscopic determination of Giemsa smears. However, various methods have since facilitated this laborious effort through the detection of parasite DNA [220, 229], enzymatic activity [228] or secreted proteins [230].

In this study, we carried out limiting dilution and isolated 5 positive clones (K4-A5, K4-B5, K4-G5, K4-A7 and K4-F10) from the parental Berok K4 strain on Day 55 post clonal dilution through FACS detection (Appendix B). The remaining wells in the clonal plate was monitored for another 3 weeks to prevent the possibility of discarding slow growing clones before the clonal plate was discarded. All of the isolated clones were amplified \textit{in vitro} and cryopreserved except for clone K4-F10. As anticipated, the respective clones demonstrated different rates of amplification in the well (Figure 3.5a and 3.5b) where clones K4-A5, K4-B5, K4-G5 and K4-A7 were detected via FACS on day 45 while clone K4-F10 was only detected in Day 55 of culture. With the clonal dilution method, clones derived from columns 6 to 11 (i.e. K4-A7 and K4-F10) in the clonal plate should technically arise from a single parasite in the well. Even though both clones were isolated on Day 55, only K4-A7 continued to show robust growth when isolated from the clonal plate while the growth of K4-F10 stagnated with no amplification observed via Giemsa smears.

Whole genomic sequencing using Oxford Nanopore and Illumina sequencing was next carried out for the homogeneous isolate of K4-A7.
Figure 3.5. Clonal isolation of homogenous populations of *P. cynomolgi* Berok K4 via dilution method. (a) Different growth rates of positive clones (b) Cumulative amplification of the respective clones.
3.5.2 Morphology and serum adaptation of *P. cynomolgi* K4-A7 clone

Morphologically, *P. cynomolgi* Berok K4-A7 parasites grown in complete medium containing 20% *M. fascicularis* serum in continuous culture retains characteristic features (Figure 3.6) similar to its original heterogeneous population from *P. cynomolgi* Berok K4. The trophozoites (T1 to T8) from the K4-A7 clone has the distinct amoeboid cytoplasm which occupied half of the RBC. Like the original heterogeneous population, Schüffner’s stippling was also observed in both trophozoites (T1 to T8) and schizonts (S1 to S8) where eight to sixteen merozoites were commonly observed in the latter. Like the heterogeneous population from *P. cynomolgi* Berok K4, enlargement of the infected host cells were observed in the later stages of the parasites.

To limit the use of naïve monkey blood in the continuous culture of the *P. cynomolgi* Berok K4-A7 clone, it was adapted to grow in complete medium containing 20% fetal bovine serum (FBS). The morphology of the Berok K4-A7 clone grown in complete medium containing 20% FBS shared similar distinct features (Figure 3.7) to its counterpart grown in 20% *M. fascicularis* serum. Interestingly, enlargement of the infected host cells was not noted in the later stages of the FBS adapted culture.
Figure 3.6. Representative images of asexual erythrocytic stages of *P. cynomolgi* Berok K4-A7 clone grown in complete medium containing 20% *M. fascicularis* serum
Figure 3.7. Representative images of asexual erythrocytic stages of *P. cynomolgi* Berok K4-A7 clone grown in complete medium containing 20% fetal bovine serum
3.6 Phenotypic and genotypic characterization of heterogenous \textit{P. cynomolgi} Berok K4 continuous culture

3.6.1 Nanostructure and rheology of \textit{P. cynomolgi} Berok K4-infected RBCs (collaboration with NUS and SIgN)

\textit{P. cynomolgi} Berok K4 dramatically alters the nanostructural and rheological properties of the iRBC in a manner similar to that observed for \textit{P. vivax}. Caveolae (~90 nm-diameter openings) appear on the surface of the infected RBCs. While these caveolae, that are generally associated with vesicle complexes, have the same dimensions as those noted in \textit{P. vivax}, they are present in significantly lesser densities (Figure 3.8a-d), our data on caveolae agree with previous in-depth analyses conducted on \textit{P. cynomolgi} Berok [231]. The \textit{P. cynomolgi} Berok K4-infected RBC increases in size with maturation (rings ~5 µm and schizonts ~8 µm) with rosettes forming approximately 20 h post-invasion (Figure 3.8e and 3.8f), a timeline similar to that previously noted for \textit{P. vivax} [221]. It is interesting that rosettes had not been observed in earlier studies on the B and M strains [232]. The \textit{P. cynomolgi} Berok K4 rosettes formed highly stable adhesive bonds requiring around 400 pN (piconewton) to affect a separation (Figure 3.8g and 3.8h), an adhesive force similar to that recorded for \textit{P. vivax} [233].

As rosetting was only observed in the Berok K4 strain and not in the B or M strain of \textit{P. cynomolgi}, we further evaluated the rate of rosetting across the different erythrocytic stages of Berok K4 (Figure 3.9a). Rosette was not found in the ring stages, which is a similar observation from \textit{P. falciparum} and \textit{P. vivax} [221]. Rosetting was noted at the early trophozoite stage, and the rosetting rate increased with the maturation of the parasites until it reached plateau at the late trophozoite stage. Interestingly, as the mature schizonts are ready for rupture and release (segmenter), the rosetting rate decreased, where most of the segmenters (Figure 3.9b) noted were non-rosetting.
Figure 3.8. Phenotypic and rheological characterization of the heterogeneous Berok K4 strain from *in vitro* culture. (a) *P. cynomolgi* Berok K4 infected RBCs exhibit caveolae structures that are similar to those in *P. vivax* infected RBCs (Scanning Electron
Microscopy, scale bars represent 1 µm and 100 nm for area shown at higher magnification in white box). (b) An atomic force microscope scan of trophozoite infected blood cells, revealed caveolae occurred at lower frequency when compared to *P. vivax*. (c,d) The median (+/- IQR) dimensions of these caveolae were similar. (e, f) Amnis flow imaging clearly shows that the mature erythrocytic stages *P. cynomolgi* Berok K4; readily formed rosettes with uninfected red blood cells, which are also a key feature *P. vivax*. (g, h) A dual micropipette aspiration method was used to demonstrate the rheological stability of the *P. cynomolgi* Berok K4 rosettes. As observed in *P. vivax*, *P. cynomolgi* rosettes are tightly attached and the cells require around 400 pN (piconewton) to disrupt the adhesion. The non-parametric data in (b, d, f, h) was analysed using Mann-Whitney U-Test with the significance level set at p < 0.05.
Figure 3.9. Rate of rosetting across different asexual erythrocytic stages of Berok K4 strain
(a) Development of rosetting along the maturation of *P. cynomolgi* Berok K4 erythrocytic
maturation. (b) a mature segmenter of *P. cynomolgi* Berok K4
3.6.2 Molecular characterization of in vitro cultured *P. cynomolgi* Berok K4 strain and clonal strain *P. cynomolgi* Berok K4-A7

Genotyping of the reticulocyte binding protein (*rbp*) genes from the parasite lines used confirmed that parasites in the heterogeneous population of *P. cynomolgi* Berok K4 and the clonal strain *P. cynomolgi* Berok K4-A7 were indeed derived from the original Berok stock (Figure 3.10). Copy number variations had been observed for *rbp1b* and *rbp2a* with the genetically related Berok and Gombak strains, and in accordance to previous studies [171, 172], *rbp1b* was found in the Berok strain only, *rbp2a* in the M and B strains only, while *rbp2b* was found in all 3 strains.
Figure 3.10. Genotyping of the reticulocyte binding protein (rbp) genes of the *P. cynomolgi* Berok K4, the clonal strain A7 and B strain.
3.6.3 Whole genomic sequencing of *P. cynomolgi* Berok K4-A7 clone (*collaboration with SIgN*)

In order to enable future technologies or genomic studies to be carried out on *P. cynomolgi* Berok K4-A7, whole genomic sequencing was carried out using Oxford Nanopore and Illumina. This combination was used so that the long sequence reads derived from Oxford Nanopore can be complemented with the short sequence reads from Illumina. Sequence analysis from both methods requires a considerable amount of time and effort. However, preliminary results from Oxford Nanopore sequencing using Sanger/ Illumina 1.9 encoding showed a 36% GC content of the genome with lengths of read ranging from 5 to 144, 963bp (). This GC content is in agreement with previous study where the GC content in *P. cynomolgi* is around 40.5% [172]. Currently (as of date of submission of thesis), the complete analysis of the sequencing data generated from both Oxford Nanopore and Illumina is still underway.
3.7 *Plasmodium cynomolgi* Berok K4 *in vitro* culture as a platform for drug susceptibility testing

3.7.1 Drug susceptibility testing in *P. cynomolgi* Berok K4 asexual erythrocytic stages using a high-throughput screening assay (collaboration with NITD)

We opted to validate the SYBR green I proliferation assay [69, 234], used routinely for anti-*P. falciparum* drug screening, for use with the *P. cynomolgi* Berok K4 line to establish dose response and single point screens based on 96 and 384 well-plate assay formats (Figure 3.11a-c). Using the BioTek Synergy™ 4 hybrid microplate reader, the range of the 72 h fluorescent read-out in the SYBR green I proliferation assay showed a linear correlation with parasitaemia in the range of 0.3-2%.

A SYBR green I dose response assay was performed in a 384-well plate format using a set of reference compounds (Figure 3.11a) and selected compounds (Figure 3.11b) from the Malaria Box. Culture medium was supplemented with 20% macaque serum for the Berok K4 line, whereas 0.5% Albumax was used for *P. falciparum*. The reference compounds included the licensed antimalarials chloroquine, lumefantrine, pyrimethamine, artemisinin, atovaquone, and artesunate, as well as three drug candidates: the phosphatidylinositol-4-OH kinase (PI(4)K) inhibitor KDU691 [66], the imidazolopiperazines GNF179 [42] and the spiroindolone KAF246 [41] (GNF179 and KAF246 are analogues to KAF156 and KAE609 currently in phase 2b clinical trials [235]). The IC₅₀ values measured for both species were broadly concordant, though *P. cynomolgi* proved more sensitive to artesunate, artemisinin and atovaquone (Figure 3.11a). The assay was also conducted for 38 in-house synthesized compounds from the Malaria Box [72] (Figure 3.11b) and identified some that had differential inhibitory activity against the two parasites species. For example, MMV000563, MMV007839 and MMV008294 were highly active against *P. falciparum* but not *P. cynomolgi*.

In order to evaluate whether the type of culture supplement (serum or Albumax) influences inhibitory activity, the Pathogen Box® chemical library (Medicines for Malaria Venture, Switzerland) was screened in a 384-well format as a single point assay at 10 µM (Figure 3.11c) using *P. cynomolgi* (20% serum) or *P. falciparum* (20 % human serum or 0.5% Albumax). The Pathogen Box® comprises 400 compounds, of which 125 are antimalarial tool-compounds, while the rest include 26 reference compounds active against
tuberculosis (n= 116), kinetoplastids (n= 70), helminths (n= 32), cryptosporidiosis (n= 11), toxoplasmosis (n= 15) and dengue (n= 5) (http://www.pathogenbox.org/). Assay data for *P. cynomolgi* in 20% macaque serum (Column A) proved to be highly comparable to that obtained for *P. falciparum* in 20% human serum (Column B), but both assays differed significantly from the *P. falciparum* assay performed in 0.5% Albumax (Column C). This observation was not surprising given the high protein content of 20% serum-supplemented media with the likely consequent effect on protein binding. As above, the rate of inhibition of nine anti-malarial compounds (MMV676380, MMV023388, MMV026550, MMV007625, MMV023949, MMV007638, MMV676442, MMV006833, MMV020289) were significantly different for both parasite species in the presence of 20% serum (Figure 3.11 c). Overall, the SYBR green I proliferation assay demonstrated robust reproducibility, at single point and for dose response, for both *Plasmodium* species in serum-supplemented cultures.
A: *P. cynomolgi* in 20% Mf serum
B: *P. falciparum* in 0.5% albumax

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**IC₅₀** (µM) in 20% Mf serum

**IC₅₀** (µM) in 0.5% albumax

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**Pathogen Box**

**Anti-malarial compounds**

- Atovaquone
- Artemisinin
- Pyrimethamine
- KAF246
- Lumefantrine
- KDU691
- KAF179
- Mefloquine
- Chloroquine
- Proguanil
- Artesunate
- Artemisinin

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**A: *P. cynomolgi* in 20% Mf serum**
**B: *P. falciparum* in 0.5% albumax**

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**Pf/ Pc (in 20% serum) > 4 fold**

**Pf/ Pc (in 0.5% albumax) > 4 fold**
**Figure 3.11.** Drug susceptibility testing using *P. cynomolgi* Berok K4 *in vitro* culture. (a) Correlation of *P. cynomolgi* Berok K4 and *P. falciparum* IC₅₀ values of common antimalarial reference compounds in a SYBR green I proliferation assay. The potency of the compounds was comparable between the two species except for artemisinin, atovaquone and artesunate which were more potent in *P. cynomolgi* as compared to *P. falciparum*. A X=Y line indicates equal inhibition towards compound. (b) Heat map showing IC₅₀ (µM) of representative set of compounds from the Malaria Box. Majority of the compounds showed activity against both *P. falciparum* and *P. cynomolgi* except for 6 compounds - MMV000563, MMV006250, MMV007839, MMV008127, MMV008270 and MMV008294 which displayed an IC₅₀ >10 µM for *P. cynomolgi*, and <5 µM for *P. falciparum*. (c) Heat map showing percentage of inhibition of compounds from the Pathogen Box® in *P. cynomolgi* Berok K4 and *P. falciparum* in different serums and concentrations. Nine compounds (MMV676380, MMV023388, MMV026550, MMV007625, MMV023949, MMV007638, MMV676442, MMV006833, MMV020289) from the anti-malarial subset of the Pathogen Box® showed more than 4-fold difference in inhibition between *P. cynomolgi* Berok K4 and *P. falciparum* in their equivalent serums.
3.7.2 Evaluating the effect of serum on *P. cynomolgi* Berok K4 based high-throughput screening assay for asexual erythrocytic stages

From the request from Medicines for Malaria Venture, we next evaluated the effect of serum used in drug screening for malaria asexual erythrocytic stages. Using the established *P. cynomolgi* Berok K4 line-based high-throughput screening assay for asexual erythrocytic stages, we compared the serum conditions (0.5% albumax) routinely used in the *P. falciparum* SYBR green I proliferation assay [69, 234] with the more physiologically relevant serum condition (20% *M. fascicularis* serum) for 80 blinded compounds. As expected, the *P. cynomolgi* Berok K4 high-throughput screening assay was reliable in predicting the IC\(_{50}\) values of the positive and negative controls of compounds which were included in the screen. For instance, the screen showed inactivity for negative controls such as primaquine, riboflavin and deoxyDHA (IC\(_{50}\) > 10 \(\mu\)M) while showing high potency in positive controls such as artemether, artemisinin and atovaquone (in single-digit nM range). 10% of the compounds demonstrated 5-fold difference in potency between the 2 serum conditions (highlighted as red dots in Figure 3.12). The compounds which were more potent in 20% serum were hydroxychloroquine, sulfamethoxazole, UCT944 and DB-75 while compounds which were less potent in 20% serum were DSM265, KAF-156, 21A092 and cladosporin. The compounds which showed decreased potency in 20% serum is of particular importance as they tallied with *P. vivax* clinical outcomes. For instance, the dihydroororate dehydrogenase inhibitor (DSM265), a phase II clinical candidate which blocks the pyrimidine biosynthesis of the parasite, demonstrated 10-fold less potency in 20% serum condition (~60 nM) compared with 0.5% albumax (~6 nM). This result corresponded with clinical outcome as it was more active on *P. falciparum* field isolates than *P. vivax* field isolates [44, 236]. While its improved sister compound (DSM421) demonstrated equal potency in both serum conditions (~60 nM) in the *P. cynomolgi* Berok K4 line-based high-throughput screening assay which also tallied with the clinical outcomes in both *P. falciparum* and *P. vivax* field isolates [236]. Such results further validated the robustness and potential of the use of *P. cynomolgi* Berok K4 line-based high-throughput screening assay for predicting *P. vivax* clinical outcomes of potential drugs.
Figure 3.12. Correlation of IC50 values of 80 selected MMV compounds in *P. cynomolgi* Berok K4 *in vitro* culture via SyBr green I proliferation assay in different serum conditions (20% *M. fasicularis* serum versus 0.5% albumax). Red dots signify compounds which showed at least 5-fold difference in the IC50 values between the two serum conditions.
3.7.3 Comparative schizont maturation assays using *P. cynomolgi* Berok K4 and *P. vivax*

*In vitro* cultured *P. cynomolgi* is ultimately to be used as a surrogate for *P. vivax* in drug sensitivity assays. Therefore, we carried out the standard schizont maturation assay routinely used for the drug susceptibility determination of *P. vivax* with tightly synchronized Berok K4 ring stage cultures as well as several clinical *P. vivax* isolates. The Berok K4 parasites were seeded on plates containing serially diluted chloroquine, and parasite growth was assessed about 44 hours later by flow cytometry [220] (Figure 3.13a). In parallel, the SYBR Green I proliferation assay was carried out for the same drug using Berok K4. Each dot represents individual *P. vivax* clinical isolate and replicates for Berok K4 line respectively. The IC$_{50}$ values for chloroquine were similar for Berok K4 in the schizont maturation assay (Figure 3.13b) and the SYBR Green I proliferation assay (Figure 3.13c), and equally comparable to the IC$_{50}$ values (~50 nM) obtained from the *P. vivax* clinical isolates (Figure 3.14d).
Figure 3.13. Correlation of chloroquine IC$_{50}$ values of *P. cynomolgi* Berok K4 continuous culture with *P. vivax* clinical isolates. (a) Flow cytometry dot plots (Hoechst/Ethidium) of chloroquine treated *P. cynomolgi* Berok K4 continuous culture gated for schizonts population in replicates. (b) IC$_{50}$ determination of chloroquine in *P. cynomolgi* Berok K4 continuous culture using the schizont maturation assay. (c) IC$_{50}$ determination of chloroquine in *P. cynomolgi* Berok K4 continuous culture using the SYBR green I proliferation assay. (d) IC$_{50}$ of chloroquine in *P. cynomolgi* Berok K4 continuous culture and *P. vivax* clinical isolates using the schizont maturation assay. The median (+/- IQR) values for the IC$_{50}$ of *P. vivax* clinical isolates and of *P. cynomolgi* Berok K4 continuous culture were similar.
3.8 Transmission study of *P. cynomolgi* Berok K4 continuous culture (collaboration with BPRC)

*In vitro* cultured Berok K4 parasites were used successfully to infect a naive intact rhesus (*M. mulatta*) monkey, where asexual and sexual stages were observed 6 days post-infection (dpi) and thereafter. *Anopheles stephensi* mosquitoes fed at 11 and 12 dpi (0.85% and 1.4% parasitaemia respectively) showed oocysts a week later (feed 11 dpi: in 40% of mosquitoes, average of 3.5 oocysts/mosquito; feed 12 dpi: in 90% of mosquitoes, average of 37.6 oocysts/mosquito). Eighteen days after feeding, lots of 100,000 sporozoites isolated from salivary glands of infected mosquitoes (feed 12 dpi: ca. 31,000 sporozoites per mosquito) were successfully used to infect two naive *M. mulatta* monkeys and the course of infection was monitored by microscopy. Both monkeys became patent 11 dpi (Figure 3.14a), and were later administered a 5-days chloroquine treatment to eliminate blood stage parasites in order to monitor for relapses as previously described [94]. Monkey 1 relapsed 31 dpi and 52 dpi, after which radical cure was administered (chloroquine and primaquine), while the second monkey remained negative for the whole duration of follow-up (102 dpi). The time to patency was similar to that noted for *P. cynomolgi* M strain infections (using the same sporozoites inoculum and infection route), as was that for the first relapse (around day 27.5 ± 3.25 dpi in eight monkeys infected by the M strain [94]). Usually, monkeys infected with *P. cynomolgi* M strain relapse at least once, regardless of inoculation dose, which contrasts with the relapse episodes noted in only one of two rhesus monkeys inoculated with the *P. cynomolgi* Berok K4 sporozoites.

Sporozoites were also used to infect *in vitro*-cultured *M. mulatta* primary hepatocytes as previously described [211], and six days later schizonts and uninucleate parasites (possibly hypnozoites) were detectable (Figure 3.14b). The average infectivity of the sporozoites derived from *P. cynomolgi* Berok K4 was lower, around 16 pre-erythrocytic (PE) forms per 10,000 inoculated sporozoites, than those derived from the *P. cynomolgi* M strain, around 23 PE forms per 10,000 inoculated sporozoites (Figure 3.14c). Enumeration of the two hepatic forms suggested that *P. cynomolgi* Berok K4 generated half the number of hypnozoites (around 30%) compared to the *P. cynomolgi* M strain (around 60%) (Figure 3.14d). Further studies are required to ascertain whether the failure of one of the monkeys infected with the Berok K4 line to relapse reflects with the lower numbers of uninucleate forms observed *in vitro*. 
Rhesus hepatocytes and strains

Hypnozoites (% of total PE forms)

Donor 1 Donor 2 Donor 3

p < 0.05

P. cynomolgi PE forms per well

Rhesus hepatocytes and strains

50 µm

50 µm

(b)
Figure 3.14. Transmission study from *P. cynomolgi* Berok K4 continuous culture. (a) *In vivo* blood stage parasitemia in 2 rhesus monkeys infected with 100,000 *P. cynomolgi* Berok K4 sporozoites (because of the use of a log scale for parasitemia, negative smears are shown as 0.0001% parasitemia). Both monkeys became blood stage patent on day 11 post-infection. Arrows indicate drug treatment (black arrows: 5-day chloroquine (CQ) treatment, red arrow: 7-day primaquine (PQ) treatment). Monkey 1 was bled for stocks on day 15 and relapsed (measured as thin smear positivity) on days 31 and 52 post-infection, after which it was treated with CQ and PQ. Monkey 2 was bled on day 19 post-infection and did not relapse during the follow-up period of 102 days post-infection, after which it was treated with PQ. (b) *In vitro* infection of primary rhesus hepatocytes with *P. cynomolgi* Berok K4 sporozoites produced both hypnozoites (left panel) and developing liver stage schizonts (right panel). Cultures were fixed at day 6 post-infection and stained with anti-PcHsp70 and a secondary antibody labelled with Alexa 568 fluorescent dye. Nuclei were stained with DAPI. An average of 16 PE forms per 10,000 inoculated *P. cynomolgi* Berok K4 sporozoites were observed. (c) The total pre-erythrocytic forms of *in vitro* infection rate of various primary rhesus hepatocytes with *P. cynomolgi* M strain sporozoites and *P. cynomolgi* Berok K4 strain sporozoites. (d) The percentage of hypnozoites observed *in vitro* using primary rhesus hepatocytes from different donors infected with *P. cynomolgi* M strain or *P. cynomolgi* Berok K4 strain sporozoites.
CHAPTER 4 - RESULTS

Establishment of a 3D hepatic spheroid model to study the exo-erythrocytic stages of *Plasmodium cynomolgi*
4.1 3D Cellusponge characterization

Once the sporozoites derived from the continuous culture of the *P. cynomolgi* Berok K4 proved to be infectious, we next looked into the feasibility of developing a predictive radical cure model for drug screening of exo-erythrocytic stages of *P. cynomolgi*. The disc-shaped 3D Cellusponge (Invitrocue) [100] (Figure 4.1a) was chosen as previous studies have shown its robustness in the formation of spheroids derived from rodent hepatocytes and human hepatocytes which the latter spheroids were subsequently infected with hepatitis C virus (HCV) [98]. Fabricated from inert hydroxypropyl cellulose, 3D Cellusponge exhibited an interconnected porous structure in the dry state as observed under SEM (Figure 4.1b). This porous structure was maintained after the scaffold was rehydrated as shown by bright-field microscopy (Figure 4.1c). To further verify the macroporosity in its hydrated state, the sponge was stained with propidium iodide (PI), which was absorbed unspecifically by the scaffold. Confocal image clearly showed that the hydrated PI-stained sponge contained pores from 50 to 200 µm in diameter (Figure 4.1c and 4.1d). Taken together, the results confirmed the macroporous structure of the sponge in both dry and hydrated states. Young’s modulus of the hydrated sponge was determined to be 25.4 ± 2.1 kPa (n = 2).

Spheroid formation in the 3D Cellusponge platform has been established for rodent and human hepatocytes but not for simian (*M. fascicularis*) hepatocytes [98, 100]. Herein, the feasibility of spheroid formation using simian hepatocytes was evaluated. Both uninfected simian hepatocytes (Figure 4.2a) and sporozoites pre-infected simian hepatocytes (Figure 4.2b) were evaluated for their ability to form spheroids in the 3D Cellusponge. Next, we evaluated adherent simian hepatocyte viability in standard monolayer culture seeded on collagen-coated plates [237] and subsequently on spheroids formed in the 3D Cellusponge platform. The ability of the spheroids formed from pre-infected simian hepatocytes (Figure 4.2b) was further investigated for its ability to complete the parasites life cycle into the erythrocytic stages from the exo-erythrocytic stages (Figure 4c) and the potential use as a platform for liver stage drug screening (Figure 4.2d).
Figure 4.1. Characterization of 3D Cellusponge. (a) Photograph of 3D Cellusponge. (b) SEM image of a dry Cellusponge. (c) Bright-field image of a hydrated Cellusponge in PBS. (d) Confocal image of PI-stained Cellusponge at the same field of view as (c).
Figure 4.2. Schematic diagram showing the work flow of *P. cynomolgi* sporozoite infection. (a) Spheroids formed using uninfected hepatocytes (b) Spheroids formed using sporozoites pre-infected hepatocytes and subsequent studies for (c) Reinfection in blood erythrocytes and (d) Models for drug evaluation against liver stages.
4.2 Maintenance of hepatocyte synthetic function in spheroid-cultured simian hepatocytes

In both systems of using uninfected simian hepatocytes (Figure 4.2a) and sporozoites pre-infected simian hepatocytes (Figure 4.2b), compact spheroids of simian hepatocytes started forming from day four post seeding in the 3D Cellusponge platform. Conversely, hepatocyte detachment was noted from day four in the monolayer cultures (Figure 4.3a). After a week in culture, the hepatocytes in the monolayer culture dedifferentiated to a fibroblast like phenotype, while the spheroid-cultured hepatocytes formed in the 3D Cellusponge platform maintained their structural integrity for up to 60 days (Figure 4.3a). Viability of the spheroids was assessed using calcein-AM/PI staining (calcein acetoxyethyl ester- propidium iodide) (Figure 4.3b). We then evaluated the urea production on adherent simian hepatocytes in monolayer culture as compared to spheroid-cultured hepatocytes (Figure 4.3c). Urea production is used as a marker to determine hepatocyte functionality [238]. Hepatocytes maintained in the monolayer culture had a rapid loss of urea function while the spheroid-cultured hepatocytes demonstrated elevated urea synthesis for up more than 2 weeks in culture (Figure 4.3c).
**Figure 4.3.** Cell morphology and hepatocyte specific functions in spheroid-cultured simian hepatocytes. (a) Bright field images of cell morphology of simian hepatocytes in spheroid culture in 3D Cellusponge and monolayer cultures on days 1, 4, 14 and 60 (20X). (b) Live/dead staining of hepatocytes using calcein-AM/PI in spheroids cultured in 3D Cellusponge on day 5 and day 30 respectively (c) Hepatocyte specific function of urea production in monolayer culture and spheroids on 3D Cellusponge for days 2, 4, 6, 8, 10, 13 and 22 post cells seeding. *p <0.05 for two-tailed t-test against collagen monolayer samples. Data expressed as mean ± SEM.
4.3 Characterization of spheroid-cultured human primary hepatocytes (collaboration with NUS)

Unlike the simian hepatocytes spheroid system, which is novel, spheroid-cultured human primary hepatocytes have already been extensively studied and shown to generate stable and functional hepatocytes to sustain an *in vitro* viable HCV infection [98]. We next evaluated the functionality of the spheroid-cultured human hepatocytes as they are the target platform for studying relapses of *P. vivax* infections. To establish the expression and localization of Plasmodium entry marker (CD81) and apical and basolateral markers (MRP2 and CD147), immunostaining was carried out after 5 days of culture. The spheroid-cultured human hepatocytes were positive for CD81, MRP2 and CD147. The CD81 and CD147 were localized at the basolateral domain and MRP2 was localized at apical domain (Figure 4.4a and b). The localization of the markers was similar to that observed in the *in vivo* liver. We next evaluated the functionality of the spheroid-cultured human hepatocytes by incubating the spheroids in the presence of fluorescein diacetate (FDA) to determine the presence functional esterases. The fluorescein produced from FDA by esterases accumulated between the hepatocytes on day 5 of culture appeared as punctuated spots which demonstrated the presence of functional bile canaliculi and functional hepatocytes (Figure 4.4c). The basal transcript and metabolism of the human hepatocytes cultured in the 3D Cellusponge and collagen monolayer were evaluated next. The human hepatocytes cultured as spheroids in the 3D Cellusponge demonstrated similar mRNA levels of CYP1A2 and CYP 3A4 compared to collagen monolayer (Figure 4.4d).
Figure 4.4. Cell morphology and hepatocyte specific functions in spheroid-cultured human hepatocytes (a) Spheroid-cultured human hepatocytes stained for both MRP2 and CD147, which are apical and basolateral markers of polarity. Scale bar at 20 μm (b) The spheroid cultured hepatocytes were also stained for CD-81, a basolateral marker and also a plasmodium entry marker. Scale bar at 20 μm. (c) The spheroids stained with fluorescein di-acetate (FDA) demonstrating punctuated distribution of fluorescein between the hepatocytes, thus proving the presence of functional bile canaliculi and apical polarity. Scale bar at 20 μm. (d) The human hepatocytes cultured in spheroid cultures demonstrated similar transcript levels of CYP1A2 and CYP 3A4 in comparison to monolayer cultures. This demonstrated the presence of functional metabolic activity necessary for drug metabolism and activity.
4.4 *P. cynomolgi* infection of spheroid-cultured simian hepatocytes

The susceptibility of pre-formed (four days old) spheroid-cultured simian hepatocytes in the 3D Cellusponge to *P. cynomolgi* sporozoites was examined. To determine the most suitable sporozoites load for optimal infection in spheroid-cultured hepatocytes, three different MoIs (multiplicity of infection) of 0.7 (as per monolayer culture), 2 and 4 were assessed on spheroids (Figure 4.5). In this study, MOI refers to the ratio of sporozoites to primary hepatocytes infected. Immunostaining using antibody specific for *P. cynomolgi* HSP70 (anti-PcHSP70) were carried out to determine the level of infection by *P. cynomolgi* sporozoites. A MoI of 2 was selected for infection as it gave the most efficient rate of infection as it gave comparable level of infection as with a MoI of 4. As sporozoites are extracted from the salivary glands of infected mosquitoes, they are usually accompanied by microbes and debris from the mosquitoes. Using half the number of sporozoites load reduced the risk of contamination associated with large sporozoites inoculum. The analysis of exo-erythrocytic stages in the 3D spheroids proved challenging due to the distribution of the schizonts and hypnozoites along all 3 axis of the spheroid and macroporous scaffold. Coupled with the lack of z-resolution in current confocal microscopy techniques, it was difficult to quantify the infection rates in the spheroids formed in the scaffold. In this study, to evaluate the infectivity of the infected 3D spheroids, the relative fluorescence units (RFU) of the anti-PcHSP70 antibody on the exo-erythrocytic forms were determined for respective treatments. Next, the system was validated for potential drug screening purposes. Four days post-infection (dpi), the infected spheroids were treated with 0.1% (v/v) DMSO (negative control), 200 nM atovaquone and 500 nM of primaquine [211]. Atovaquone is known to inhibit liver schizonts but not hypnozoites while primaquine is a potent inhibitor of both liver schizonts and hypnozoites [211]. The inhibitory concentrations employed were pre-determined using the standard six days liver-stage assay on monolayer culture as previously described [88, 211].

To rule out non-specific binding of anti-PcHsp70 antibody and the presence of remnant sporozoites attached on the surface of hepatocytes in 3D culture, immunostaining of both treated and untreated samples was carried out as alternative antibodies against *P. cynomolgi* exo-erythrocytic forms were limited (Figure 4.6a). Relative fluorescence units (RFU) of anti-PcHSP70 antibody were quantified for the respective treatments. A strong signal was found in infected samples treated with 0.1% DMSO control (RFU of 0.09) while
infected samples that received treatment with atovaquone and primaquine resulted in a reduced (RFU of 0.018) or near the limit of detection signal (RFU of 0.003) respectively (Figure 4.6b). The results were reproducible in every subsequent experiment (n > 8) and in agreement with the report on *P. vivax* causal prophylactic experiments on human liver-chimeric mice [91].
**Figure 4.5.** Determination of multiplicity of infections (MoI) for optimal *P. cynomolgi* infection in spheroid cultured simian hepatocytes. Four days old spheroid cultured simian hepatocytes were infected with different MoIs and fixed on day 6 post-infection. Parasites were visualized with the confocal microscope (Olympus FV-1000 at 60X magnification with oil lens) using the *P. cynomolgi* specific antibodies PcHsp70. DNA was labelled using DAPI. Scale bar at 20 μm.
**Figure 4.6.** *P. cynomolgi* liver-stage parasites in pre-formed, infected spheroid-cultured simian hepatocytes. Parasites were visualized by confocal microscopy (60X) using the *P. cynomolgi* specific antibodies PcHsp70. DNA was labelled with DAPI. Scale bar at 20 μm. (a) *P. cynomolgi* infection in pre-formed spheroids in 3D Cellusponge 6 days post-infection. Four days old pre-formed spheroids were infected with *P. cynomolgi* sporozoites (MoI: 2), exposed to 0.1% DMSO control, 200 nM atovaquone or 500 nM primaquine from d4 to d6 post-infection. (b) Rate of *P. cynomolgi* infections in pre-formed, infected spheroids. Fluorescence intensity was quantified using Imaris and normalized to DAPI fluorescence intensity *p < 0.05 for two-tailed *t*-test against 0.1% DMSO samples. Data expressed as mean ± SEM.
4.5 *P. cynomolgi* infection in simian hepatocytes maintained in suspension increases yield

To improve the rate and consistency of infection while decreasing the overall duration of the experiment, we set out to infect the primary simian hepatocytes in suspension prior to seeding in the 3D Cellusponge. The rationale for infecting the simian hepatocytes in suspension originated from previous studies demonstrating increased rate of hepatitis B virus (HBV) infection [239] and also enhanced rate of transduction in suspension primary hepatocytes [240, 241]. Previous attempts at seeding pre-infected hepatocytes onto collagen-coated 96-well plates failed to yield confluent cultures. Furthermore, only few scattered infected cells were detected via an immunofluorescence assay (IFA) at six days post-infection suggesting that infection in suspension compromised the integrity of surface proteins required for cell adhesion and attachment in monolayer. In contrast, the seeding of pre-infected hepatocytes onto the 3D Cellusponge proved highly permissive and reproducible (Figure 4.7a and 4.7b). The infected spheroids were treated with 0.1% DMSO, 200 nM atovaquone and 500 nM of primaquine at four days post-infection and fixed at ten days post-infection. Relative to 0.1% DMSO controls (RFU of 0.3), the atovaquone treated samples (RFU of 0.2) showed a reduction in the parasitic load in the spheroids while infections in the primaquine treated samples were mostly eradicated (RFU of 0.04) based on anti-PcHsp70 immunostaining (Figure 4.7b). Next, the different rate of infectivity between the two different methods of infection was assessed. *P. cynomolgi* infection was around 4-fold higher when the simian hepatocytes were infected in suspension before seeding and spheroid formation as compared to infection in pre-formed spheroids for 0.1% DMSO control (Figure 4.7b).

To demonstrate the presence of viable parasites in the infected hepatocytes within the spheroids, infected and uninfected spheroids were dissociated and plated on collagen-coated 96-well plates. The samples were then immunostained with anti-PcHsp70 and antibodies specific to *P. cynomolgi* Upregulated in Infective Sporozoites 4 protein (anti-PcUIS4) and visualized for *P. cynomolgi* liver stages (Figure 4.8a). Apart from colocalizing within the infected cell the HSP expressing *P. cynomolgi* parasites, the anti-PcUIS4 antibody could clearly demarcate the parasitophorous vacuole membrane (PVM) circumference of both the liver schizonts and hypnozoites which were represented in the top panel of Figure 4.8a. A distinct and well-defined PVM was only seen in the
hypnozoites. PVM prominence is a distinct feature of *P. vivax*, the human equivalent of *P. cynomolgi*; which shares a similar liver-stage life cycle. Accordingly, the PVM is usually observed in the initial stages of infection [91]. The dimensions noted for both liver stages concurred with those observed by other groups [91, 211]. This clearly demonstrated that not only did the infection of suspension hepatocytes increased the infectivity of the hepatocytes, the parasites were viable and able to develop within the infected cell.

### 4.6 3D Cellusponge captures *P. cynomolgi* pre-erythrocytic merozoites reinvasion *in vitro*

Once established that the *P. cynomolgi* sporozoites infect the spheroid-cultured hepatocytes in the 3D Cellusponge, the next aim was to determine whether maturing liver schizonts and hypnozoites could develop pre-erythrocytic merozoites and proceed to invade simian erythrocytes. Simian erythrocytes were used based on previous work, which determined that in contrast to *P. vivax*, *P. cynomolgi* preferentially infect simian erythrocytes followed by simian reticulocytes and to a lesser extent human reticulocytes but not human erythrocytes [242]. Successful infections of the spheroids were first confirmed by immunostaining with anti-PcHsp70 antibody (data not shown). At seven, ten and 14 days post-infected spheroids were overlaid with simian erythrocytes. The overlaid blood was sampled and evaluated via Giemsa staining for viable parasites after 48 hours of co-incubation with the spheroids. Infected RBCs were recovered from the 48 hours co-incubated blood overlays harvested on days nine and 16 (Figure 4.8b). Ring and trophozoite stage parasites were detected in the infected erythrocytes from samples on days nine and 16 but not 12. These blood stage parasites detected might result from the burst of pre-erythrocytic merozoites from the schizonts on day nine and from the reactivated hypnozoite in day 16 as it has been previously been reported that schizonts mature and burst within day 11 post-infection [211] Overall these results indicated that the liver-stage parasites were viable, able to develop into multiple pre-erythrocytic merozoites and complete the liver-stage life cycle by invading the erythrocytes.
Figure 4.7. *P. cynomolgi* liver-stage parasites in suspension-infected spheroid-cultured simian hepatocytes. Parasites were visualized by confocal microscopy (60X) using the *P. cynomolgi* specific antibodies PcHsp70. DNA was labelled with DAPI. Scale bar at 20 µm.

(a) *P. cynomolgi* liver-stage parasites in suspension-infected spheroids in 3D Cellusponge 10 days post-infection. The infected spheroids were exposed to DMSO control, 200 nM atovaquone or 500 nM primaquine from day 4 to day 10 post-infection. (b) Comparison of the rate of *P. cynomolgi* infections in the two distinct methods of infection for spheroid cultured simian hepatocytes. Fluorescence intensity was quantified using Imaris and normalized to DAPI fluorescence intensity. *p < 0.05, **p < 0.001, ***p < 0.0001 for two-tailed t-test between samples infected by the different methods undergoing the same drug treatment. Data expressed as mean ± SEM.
Figure 4.8. Progression of exo-erythrocytic forms in the suspension-infected spheroid-cultured simian hepatocytes. (a) *P. cynomolgi* liver-stage parasites cultured one day in monolayer culture, after dissociation of infected spheroids from 3D Cellusponge 10 days post-infection (MoI: 2). Scale bar at 20 µm. (b) Complete liver-stage cycle of *P. cynomolgi* in *vitro*. Ring stage and trophozoite stage of *P. cynomolgi* in erythrocytes from blood overlay initiated on day seven and day 14 post infected spheroid-cultured hepatocytes on 3D Cellusponge.
4.7 Malaria-infected spheroids in 3D Cellusponge is a predictive platform for anti-relapse drug evaluation for exo-erythrocytic stages

The spheroid-cultured hepatocyte model allowed us to widen the capabilities for long-term culture of infected simian hepatocytes from the established monolayer culture for simian liver-stage compound-screening assay [88, 93, 211]. We next questioned whether the model could be used to study invasion of RBCs from maturing liver schizonts and relapse from hypnozoites (radical cure) \textit{in vitro}. The same conditions (seeding of pre-infected hepatocytes) and controls (atovaquone, primaquine and DMSO) were used. In addition, the phosphatidylinositol-4-OH kinase (PI(4)K) inhibitor-KDU691 (2 \( \mu \text{M} \)) [66] was used as an exploratory tool compound to evaluate the potential of the spheroid infection model for radical cure determination. KDU691 was chosen because it was previously proven to be active in the 2D collagen coated plates but failed in the \textit{in vivo} radical cure model using rhesus monkeys [93, 94]. A high inhibitory concentration of KDU691 (2 \( \mu \text{M} \)) was chosen to demonstrate its maximum inhibitory effect in the system while being non-cytotoxic to the simian hepatocytes. Three different experimental models were set up (Figure 4.9a) using the same batch of simian hepatocytes and \textit{P. cynomolgi} sporozoites: (\textit{i}) a causal prophylaxis model whereby treatment was initiated at the time of seeding of the infected hepatocytes and terminated on day five, (\textit{ii}) a delayed-treatment model whereby the drugs were added on day four and refreshed every second day (days six and eight) followed by termination on day ten and (\textit{iii}) a radical cure model based on the delayed-treatment model except that drugs were removed by three consecutive washing on day 16 and the experiment was terminated five days later (day 21).

The spheroid causal prophylaxis model (Figure 4.9b) mirror in-house and published literature on the standard six days monolayer culture based assay [66, 93]. In this model, atovaquone had poor inhibitory activity, while primaquine and KDU691 eradicated the parasites compared to the untreated DMSO negative control in the delayed-treatment model (Figure 4.9c), atovaquone once again displayed poor inhibition while primaquine eradicated the parasite. Noteworthy and in contrast to the causal prophylactic model, there was an increase in parasites in the KDU691 treated samples. In the radical cure model (Figure 4.9d), some parasites reappeared in the primaquine treated samples. The extent of parasites observed in the KDU691 treated samples was similar to the staining profile of the DMSO negative control. Consistent with the \textit{in vivo} data, these results confirmed that
KDU691 did not inhibit hypnozoites [94]. Once KDU691 was removed on day 16, hypnozoites could mature into liver-stage schizonts and merozoites as indicated by the observed by immunofluorescence with the anti-PcHsp70 antibody.
Figure 4.9. Models for studying *P. cynomolgi* liver-stages *in vitro*. Parasites were visualized with the confocal microscope (60X) using the *P. cynomolgi* specific antibody PcHsp70 (b, c, d). DNA was labelled with DAPI. Scale bar at 20 μm. (a) Summary of models for studying *P. cynomolgi* liver-stages *in vitro*. (b) *P. cynomolgi* infected spheroids in 3D Cellusponge used in a causal prophylaxis model. Suspension infected simian hepatocyte (MoI: 2) were exposed to DMSO control, 200 nM atovaquone (ATQ), 500 nM primaquine (PQ) or 2 μM of KDU691 from the day of infection to day 5 post-infection. (c) *P. cynomolgi* infected spheroids in 3D Cellusponge used in a delayed treatment model. Infected spheroid-cultured simian hepatocytes (MoI: 2) were exposed to DMSO control, 200 nM atovaquone, 500 nM primaquine or 2 μM of KDU691 from the day 4 to day 10 post-infection. (d) *P. cynomolgi* infected spheroids in 3D Cellusponge used in a potential radical cure model. Infected spheroid-cultured simian hepatocytes (MoI: 2) were exposed...
to DMSO control, 200 nM atovaquone, 500 nM primaquine or 2 μM of KDU691 from the day four to 16 post-infection and fixed on day 21 post-infection. Fluorescence intensity of *P. cynomolgi* infection in the three experimental models under various drug treatments was quantified using Imaris and normalized to DAPI fluorescence intensity *p < 0.05, **p < 0.001 for two-tailed *t*-test of different drug treatments against 0.1% DMSO samples within the respective treatment models. Data expressed as mean ± SEM.
4.8 P. vivax infection validates the liver-stage model using spheroid-cultured hepatocytes

To demonstrate the relevance of the P. cynomolgi spheroid-cultured simian hepatocyte model in drug screening for human P. vivax exo-erythrocytic stages, the model was repeated using human primary hepatocytes and P. vivax sporozoites. To secure fresh supply of P. vivax sporozoites, the experiment was carried out on-site at the SMRU, Thailand. The infection of the monolayer culture was carried out 24 hours post-seeding as previously described [93, 211]. The same human primary hepatocytes used in the monolayer system were infected in suspension prior to seeding in the 3D Cellusponge at a Mol of 2. Like in the P. cynomolgi 3D spheroid model, the P. vivax infected spheroids were treated with 0.1% DMSO, 200 nM atovaquone and 500 nM of primaquine at four days post-infection and fixed at ten days post-infection. Immunostaining using antibody specific for P. vivax UIS4 (anti-PvUIS4) were carried out to determine the level of infection by P. vivax sporozoites (Figure 4.10a). Like in the P. cynomolgi 3D model, the level of P. vivax infection was highest in the 0.1% DMSO samples followed by the atovaquone and primaquine treated samples (Figure 4.10b). Overall, these results verified that the P. cynomolgi spheroid-cultured simian hepatocyte model is reproducible using the human P. vivax spheroid-cultured human hepatocyte model.
Figure 4.10. Spheroid-cultured human hepatocytes used for *P. vivax* infection (a) Parasites were visualized with the confocal microscope (60X) using anti-PvUIS4 antibody specific for *P. vivax* and DAPI for DNA staining. Scale bar at 30 µm (A to D) and 100 µm (E to H). Infected human hepatocytes in spheroids (B, C, D) and in monolayer culture (F, G, H) were exposed to 0.1% DMSO control, 200 nM atovaquone or 500 nM primaquine from day 5 to day 8 post-infection. (b) Fluorescence intensity of *P. vivax* infection in spheroid-cultured human hepatocytes under various drug treatments was quantified using Imaris and normalized to DAPI fluorescence intensity, *p* <0.05 for two-tailed *t*-test against 0.1% DMSO samples. Data expressed as mean ± SEM.
Chapter 5

Discussion and Conclusion
5.1 Overview

In 1912, the first protocol for *in vitro* cultivation of the erythrocytic stages of malaria parasite was made for *P. falciparum*, *P. malariae* and *P. vivax*, though growth was restricted to a few cycles [243]. Sustained multiplication over extended periods (months), i.e. continuous cultures, proved elusive and was only achieved in 1976 for *P. falciparum* [174, 244] after persistent efforts which spanned across three decades. Within a few years, the continuous cultivation of four macaque parasites, *P. knowlesi*, *P. cynomolgi*, *P. inui* and *P. fragile* were reported [245]. More recently the continuous culture, and humanization of *P. knowlesi* was subsequently exploited to carry out genetic and biological studies [183, 246].

In the case of *P. falciparum* and *P. knowlesi*, the establishment of tractable long term *in vitro* culture has led to significant gains in our understanding of these important pathogens. In stark contrast, the continued failure to develop a continuous culture system for *P. vivax* has greatly hampered efforts to develop drugs and vaccines capable of eliminating relapsing malaria. The success of 2 other groups in the 1980s to culture *P. cynomolgi*, encouraged us to reexamine and exploit this neglected approach to understanding its important sister species *P. vivax*.

This study was set out to achieve two main objectives:

I. Re-establish the continuous culture of *P. cynomolgi* erythrocytic stages to facilitate its use as the physiologically relevant model to understand its human counterpart, *P. vivax*.

II. Develop an *in vitro* model using *P. cynomolgi* exo-erythrocytic stages for better evaluation of hypnozoitocidal compounds.

5.2 Continuous culture of *Plasmodium cynomolgi* Berok K4 erythrocytic stages

The original *P. cynomolgi* Berok strain was originally extracted from a mixed *P. cynomolgi*-*P. fieldi* infection in a wild *M. nemestrina* isolated in peninsular Malaysia during the early 1960s, and subsequently maintained by blood and/or sporozoite inoculation mainly in *M. mulatta* and later also in *Aotus* monkeys [165]. Clonal diversity is well known to exist even in single isolate of a parasite species [247-249]. It is unknown whether the
Berok line derived from monkey K4 that thrived in this study was selected from a genetically heterogeneous *P. cynomolgi* population, or as a phenotypic variant from an otherwise clonal population. Deciphering the mechanism for this preferential growth could provide fundamental insights on the cultivation of blood stage malaria parasites. It is possible that other *P. cynomolgi* lines, or from other malaria species, would also be amenable to continuous blood stage cultivation, provided that the initial failures are met with a patient and systematic study of various culture conditions. The heterogeneous Berok K4 and homogenous Berok K4-A7 lines from *P. cynomolgi*, a species proven as an excellent surrogate for *P. vivax* research will be disseminated globally so as to increase the scope of investigations using this physiologically relevant surrogate to propel *P. vivax* blood stages research.

Since the first reports of *in vitro* culture of *P. cynomolgi* by Nguyen *et al.* and Zhou *et al.* in the 1980s, the potential of the system was neither repeated nor further exploited probably due to the almost universal focus on the newly developed *P. falciparum* culture method, developed in 1976 [174]. In this study, through meticulous testing of various culture conditions we were able to re-establish and improve the continuous culture of *P. cynomolgi*, we were also able to utilise its robust *in vitro* growth for a range of genotypic and phenotypic studies.

For the growth medium used in our study, apart from combining the supplements used by both Nguyen *et al.* and Zhou *et al.*, we also replaced L-glutamine with GlutaMAX due to its enhanced stability [250] (Table 5.1). One novel and critical finding, is that antibiotics are not added to the growth medium (which is a standard practice in *P. falciparum* continuous culture). We repeatedly observed that antibiotics have a negative effect on the growth of *P. cynomolgi* Berok K4 *in vitro* (Figure 3.4c) which ultimately eradicates the culture.

Unlike the study by Nguyen *et al.*, we were unsuccessful in growing the parasites in human serum (despite ensuring agglutination was not observed with the combination of different batches of human serum used with *M. fascicularis* erythrocytes). Our initial success with continuous culture of Berok K4 was possible using suitable donors of *M. fascicularis* serum and erythrocytes despite being non-autologous. However, it is important to note only serum collected from certain *M. fascicularis* donors were able to sustain parasite amplification while serum from other donors gave lower rates of amplification.
Despite the fact that serum collected from all donors were processed and stored in a standard manner. Such phenomenon was also noted for the naïve *M. fascicularis* erythrocytes used to sustain the cultures. To reduce batch-to-batch variation of serum and also minimize the reliance on macaque samples, the *M. fascicularis* serum was successfully substituted with fetal bovine serum for the continuous culture of the homogeneous Berok K4-A7 strain. However, it was still necessary to retain the use of suitable *M. fascicularis* erythrocytes for continuous culture.

Even with growth conditions optimized for *P. cynomolgi* Berok K4 continuous culture, robust growth appears to be limited to certain *P. cynomolgi* strains. Not only did M or B strains failed to grow *in vitro* in our hands, but it appears that there’s a sub-population (K4) from the heterogeneous Berok strain which was more adaptable for *in vitro* cultivation.

<table>
<thead>
<tr>
<th>In this study</th>
<th>Nguyen et. al. (1981)</th>
<th>Zhou et. al. (1984)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. cynomolgi strain</strong></td>
<td>Berok K4</td>
<td>Berok</td>
</tr>
<tr>
<td></td>
<td>Berok K4-A7</td>
<td></td>
</tr>
<tr>
<td><strong>Days of in vitro culture</strong></td>
<td>&gt; 180</td>
<td>42</td>
</tr>
<tr>
<td><strong>Growth medium</strong></td>
<td>RPMI1640 with Glutamax, 30 mM HEPES, 200 M hypoxanthine, 0.2% (w/v) glucose</td>
<td>RPMI1640 with 30mM HEPES</td>
</tr>
<tr>
<td><strong>Gas Phase</strong></td>
<td>Trimix (5%CO₂, 5%O₂, 90%N₂)</td>
<td>Modified “candle jar”/Continuous flow method (7%CO₂, 5%O₂, 88%N₂)</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td>Heat-inactivated 20% <em>Macaca fascicularis</em></td>
<td>10% human O+ serum</td>
</tr>
<tr>
<td></td>
<td>20% fetal bovine serum</td>
<td></td>
</tr>
<tr>
<td><strong>Hematocrit/ Erythrocytes used</strong></td>
<td>5% <em>Macaca fascicularis</em></td>
<td>8% <em>Macaca mulatta</em></td>
</tr>
<tr>
<td><strong>Trophozoite morphology</strong></td>
<td>Amoeboid</td>
<td>Less amoeboid</td>
</tr>
<tr>
<td><strong>Amplification fold change</strong></td>
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<td>2 to 3</td>
</tr>
<tr>
<td><strong>Culture restart from frozen stabilate</strong></td>
<td>Yes</td>
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</tr>
<tr>
<td><strong>Amplification fold change of thawed stabilate</strong></td>
<td>3 to 8</td>
<td>n.a.</td>
</tr>
<tr>
<td><strong>Reproducibility</strong></td>
<td>Yes (across 6 labs)</td>
<td>No</td>
</tr>
</tbody>
</table>

**Table 5.1.** Summary of culture conditions used for *in vitro* cultivation of *P. cynomolgi*
Routine cultivation of the continuous culture of *P. cynomolgi* Berok K4 can be easily initiated from cryopreserved stocks using conditions and methodology that were optimized in this study to enable robust growth and large-scale production of the parasite when necessary. The parasites of the continuous culture of *P. cynomolgi* Berok K4 are indistinguishable from *in vivo* parasites, and they retain their infectivity to monkeys and to mosquitoes to generate infective sporozoites. The cultured *P. cynomolgi* Berok K4 share several of the morphological, phenotypic and rheological characteristics of *P. vivax*-infected erythrocytes. The trophozoites and schizonts of Berok K4 formed rosettes with uninfected erythrocytes, which were as tightly bound to each other as those of *P. vivax*.

The homogeneous isolate (cloned from a single infected cell) *P. cynomolgi* Berok K4-A7, was also amenable to robust *in vitro* cultivation (even in fetal bovine serum substituted complete medium), where majority of the distinct morphological characteristics seen in parental Berok K4 were retained. The isolation of a homogeneous isolate of *P. cynomolgi* greatly facilitates techniques such as target deconvolution, deep sequencing and genetic manipulation, all which may help in elucidating important questions relevant to vivax malaria. Of these questions, one of the most important is to identify the molecular basis for chloroquine resistance in *P. vivax* [40, 251]. Despite the fact chloroquine remains a first line drug against vivax malaria in many countries (i.e. India), its mechanism of action and resistance in *P. vivax* is poorly studied and controversial [134, 252, 253]. Once we have the whole genome of the homogeneous isolate K4-A7 elucidated, we hope that our continuing efforts to induce chloroquine resistance will select for a resistant mutant daughter clone which can be then re-sequenced in an effort to identify molecular polymorphisms associated with this important resistance phenotype.

The last decade has witnessed some successes in the search for new antimalarial compounds, with 17 new drug candidates developed since 2010 [59, 178]. One of the key contributions to these discoveries was the development of a standard *in vitro* *P. falciparum* asexual blood stage SYBR green I proliferation assay adapted to automated screening technologies [234]. Such strategies are not possible for *P. vivax* due to the inability to grow the parasite *in vitro*, and high throughput screening of compounds *in vivo* in the surrogate macaque model would require an excessively large number of *P. cynomolgi*-infected macaques. We validated the potential of the *P. cynomolgi* Berok K4 continuous culture for high throughput screens to possibly identify lead compounds active against *P. vivax*. It was
interesting to note that some of the compounds from the Malaria Box and the Pathogen Box® used for the validation of the assay were inhibitory to one but not the other of the parasite species used for the validation (P. cynomolgi and P. falciparum). Hence, it is likely that drug susceptibility data from in vitro-based P. cynomolgi screens may provide a reliable predictor of activity against P. vivax. This is particularly important for high throughput search of compounds active against the P. vivax liver stages, which currently relies on P. berghei and P. yoelii, two parasites of rodents [234] which does not form relapsing hypnozoites. This led to the development of in vitro-based P. cynomolgi and P. vivax liver stage assays [68, 88, 89, 211] for profiling of schizontocidal drugs. One of the major logistical challenges in high-throughput screening of the P. cynomolgi liver stages is the availability of infective sporozoites which can only be obtained from mosquitoes fed on blood collected from P. cynomolgi-infected macaques. The fact that Berok K4 line parasites maintained in vitro for extensive duration retain infectiousness to mosquitoes and produce gametocytes while in culture promises its eventual use of such in vitro cultures for mosquito infections, hence reducing the use of primates while possibly increasing sporozoite production. We are currently optimizing mosquito-feeding protocol with the emphasis to promote gametocytogenesis, a process known to be highly sensitive to culture conditions, which were only defined for P. falciparum over many years [254].

It is important to highlight that the P. cynomolgi model provides the malaria research community the possibility to understand fundamental biological, immunological and pathological responses as well as therapeutic approaches derived from in vitro observations and correlate with in vivo experimentations in the natural host. The ability to refine or terminate a candidate compound or vaccine formulation through such in vitro-in vivo interpretations, significantly enhances the value of this pre-clinical model.

It is hoped that with the availability of easily maintained in vitro erythrocytic P. cynomolgi Berok K4 and K4-A7 parasites which can now be exploited to conduct critical fundamental and translational research to develop drugs and vaccines against P. vivax, a widespread species whose control will impact the eradication of malaria.

5.3 3D hepatic spheroid model for radical cure prediction

The study of P. vivax and search for anti-relapse drugs is often hampered by the
lack of physiological relevant \textit{in vitro} models that are able to capture the entire liver-stage life cycle of the parasite including relapses caused by the hypnozoites. In this study, we explore the potentials of the 3D hepatic spheroid model that is amenable to research on the biology of the liver stage and for anti-relapse drug screening assay for \textit{P. vivax}.

The development and use of the 3D hepatic spheroid model has accelerated in recent years, driven primarily by toxicology and metabolism studies [255-257]. Recently, spheroids have been used in for modeling infectious diseases [98]. Here, we first adapted the 3D Cellusponge system originally developed with human, rat and mouse hepatocytes to simian un-infected/infected hepatocytes. In this system, 3D hepatic spheroid model proved to be a physiologically relevant \textit{in vitro} model to study relapses arising from the hypnozoites. The seeding of pre-infected hepatocytes proved to be more efficient, homogenous and enabled the complete liver-stage cycle of the parasite with RBC invasion by nascent merosomes. More importantly, this platform proved to work equally well with \textit{P. vivax} infection in human hepatocytes.

Robust predictable \textit{in vitro} models for radical cure remain a challenge. A six-day monolayer culture system using the collagen-coated plates has been used extensively for IC$_{50}$ determinations of potential anti-liver stage malaria parasites [45, 47, 66, 93]. To date, liver-stage drug screens have failed to determine whether drugs inhibit the onset of infection (causal prophylactic) or kill the dormant hypnozoites (radical cure). The standard \textit{in vitro}-2D based \textit{P. cynomolgi} liver-stage assay [93, 211] can identify liver-stage active compounds but not discriminate causal prophylactic drugs from radical cure active compounds. This is because treatment is administered within the first three hours of infection and not when the infection has developed. The \textit{in vivo} \textit{P. cynomolgi} model in combination with other surrogate liver-stage systems using the rodent malaria parasites \textit{P. berghei} and \textit{P. yoelii} have successfully been used to screen for antimalarial active compounds [42, 67, 258]. These systems have allowed for the identification of several compounds active on the phosphatidylinositol 4-kinase (PI(4)K). The PI(4)K inhibitors KAI407, LMV599 and KDU691 showed promising antimalarial activity in the \textit{P. cynomolgi} and \textit{P. yoelii} \textit{in vitro} liver-schizonts systems and in the \textit{P. berghei in vivo} models [66, 93, 259]. In the \textit{in vivo} macaque model, KDU691 and LMV599 were found to be potent causal prophylactic compounds when co-administered at the time of infection of the macaques, but failed to prevent relapse in monkeys that were first infected with \textit{P.
cynomolgi sporozoites and subsequently administered the compounds [94]. In accordance, using the 3D hepatic spheroid model described here, KDU691 proved to be an efficacious compound in the causal prophylactic model but not in the radical cure model. In the latter model, KDU691 appeared to have lost its cidal activity against the non-dividing hypnozoites.

The liver-stage platform presented here is amenable for use in both P. cynomolgi and P. vivax. This will facilitate the study of the respective parasites’ liver-stage cycle and selection of efficacious hypnozoitocidal compounds. Not only can this system be used as the penultimate screen for potential radical cure compounds prior to P. cynomolgi sporozoites infection in rhesus monkeys, it could also greatly reduce the use of primates for compound evaluation and the accompanying cost.

Much is known today on the biology of the malaria parasites; nevertheless, a profound comprehension of the non-dividing phase of the hepatic stage, the hypnozoites, remains elusive. The existence of the pre-erythrocytic liver-stage that is associated with relapse of P. cynomolgi and P. vivax was first proposed in 1948 [188] and its presence shown in humans and non-human primates in the early 1980s [26, 202, 203]. It has taken another three decades to witness other breakthroughs on the biology of the malaria liver-stage, with a few notable exceptions such as the infection of cultured hepatocytes [83, 215, 260, 261]. A major limitation attributed to the absence of working models has in part been overcome by the recent Matrigel monolayer culture [88], the micropatterned co-cultures (MPCCs) model [89, 262], human liver-chimeric mice [91] and a high throughput plate culture system [68]. Of these, only the micropatterned co-cultures model and the high throughput plate culture systems have been shown to be capable to sustain the invasion into red blood cells [68, 89, 262]. One advantage of using P. cynomolgi as a surrogate model for P. vivax is its ability to invade equally well normocytes and reticulocytes [242]; hence facilitating the merosomes invasion into red blood cells. Discrimination between schizonts and schizonts originating from hypnozoites could be achieved by treating with KDU691 during the intermediate phase (days four to ten).

While the 3D hepatic spheroid model in this study proved to be a suitable model to distinguish liver-stage schizontocidal versus hypnozoitocidal compounds and offers an interesting system to study the liver-stage biology of the malaria parasites, it is not without limitations. The major limitation of the 3D hepatic spheroid model in its present form is
quantification of the parasitic load. While analysis of the 3D spheroids by high content imaging is possible, it is time-consuming and data intensive. Alternative detection methods by RNA-FISH (fluorescent in situ hybridization) or a biochemical assay could be adapted for detection. The construction of a transgenic fluorescent reporter *P. cynomolgi* strain [80] could be used as an alternative system for parasite quantification. Here, to evaluate the level of *P. cynomolgi* and *P. vivax* infection in the cultured spheroids, we quantified the relative fluorescent units (RFU) of antibodies specific to either *P. cynomolgi* HSP70 (anti-PcHsp70) or *P. vivax* UIS4 (anti-PvUIS4). To further verify the strong fluorescent signal emitted from immunostaining with anti-PcHsp70 was born out of viable hepatic parasites in the spheroids and not debris or artefact, the infected spheroids were dissociated and plated on 2D collagen coated plates for imaging. DAPI staining in combination with immunostaining using anti-PcHsp70 and anti-PcUIS4 clearly demarcated hypnozoites and liver schizonts confirming that the signal emitted was primarily from viable parasites. The study was further limited in the optimization process for RBC invasion from merosomes due to the restricted number of attempts at this experiment which reduced the opportunities for trouble-shooting, including media optimization and sampling. Detection of infected RBCs would be an ideal alternative assay readout for anti-relapsing drugs.

The establishment of a 3D hepatic spheroid model provided long-term hepatic functions and enhanced permissiveness and development of both *P. cynomolgi* and *P. vivax* infections. Consequently, the model was also able to capture the full liver stage cycle starting with *P. cynomolgi* sporozoites invasion in the spheroid-cultured hepatocytes and the release of the hepatic merozoites resulting in the invasion of simian erythrocytes in vitro. It is hoped that this 3D hepatic spheroid model, can be used to evaluate the liver-stage biology of the parasite and provide a method for discriminating between causal prophylactic and cidal antimalarials activity in vitro in both *P. cynomolgi* and *P. vivax* infections.

### 5.4 Overall conclusions and future directions

*P. vivax* research has been fraught by the lack of predictive in vitro culture models to study the parasite’s life cycle of throughout its erythrocytic and liver stages in the human host. In this study, we chose to work with *P. cynomolgi* since it shares various phenotypic
and genetic similarities to *P. vivax*. Furthermore, in preclinical studies of drug candidates, the *in vivo* *P. cynomolgi* macaque model has been used as the gold standard for the prediction of the pharmacokinetics and the drug’s efficacy in humans. This thesis further demonstrates the utility of *P. cynomolgi* as a model to study *P. vivax*; by our *in vitro* recreation of its full life cycle using the optimized *in vitro* culture of its erythrocytic stages, and development of a predictive radical cure liver stage model for hypnozoitocidal compounds.

It is hoped that with the continuous culture of *P. cynomolgi* Berok K4 and K4-A7 erythrocytic stages, we will be able exploit our *in vitro* system to minimize the reliance on macaques. Certainly, one of our goals was to replace the source of infected blood from the macaques and generate sporozoites using *in vitro* erythrocytic stages. However, to ensure success, additional studies would be needed to determine the rate of infectivity and also the percentage of hypnozoites formed by both the heterogeneous K4 strain and the homogeneous K4-A7 strains. To evaluate the rate of infectivity, we need to infect more macaques with both strains to evaluate patency and relapse patterns so as to expand on our initial work (Figure 3.14a). Our preliminary transmission study (Figure 3.14d) suggested the Berok K4 strain produced a lower percentage of hypnozoites compared to the M strain, such evaluation would have to be repeated with the K4-A7 strain and perhaps the other likely clonal strains (K4–A5, K4-B5 and K4-G5) which were obtained to identify a strain which produces a higher rate of hypnozoites.

One of the most interesting outcomes from this study, is a single cell derived clone of *P. cynomolgi* (K4-A7) which can be readily cultured and manipulated. With the whole genome sequence of K4-A7, it is hoped that it can potentially serve as the genetic backbone for future work in target deconvolution and genetic manipulation in addressing drug resistance, particularly in defining the cause of chloroquine resistance in *P. vivax*. This is especially important since mechanisms of chloroquine resistance appear to be distinct between *P. falciparum* and *P. vivax* despite gene synteny between the two species [134].

While the radical cure liver stage model using *P. cynomolgi* infected 3D hepatic spheroids might allow for the study of the liver stage cycle of the parasite and selection of hypnozoitocidal compounds *in vitro* before preclinical testing in macaque models, some limitations exist, particularly for higher throughput screens. To accurately evaluate the potency of the liver stage active compounds, it is crucial to develop a quantifiable endpoint
measurement of the parasites within the infected 3D hepatic spheroids. Although identification of the liver stages of the parasites in the infected 3D hepatic spheroids by high content imaging is possible, they are very slow and require large amount of computer memory. Alternative detection methods by fluorescently tagged parasites, RNA-FISH (fluorescent in situ hybridization) or a biochemical assay could be adapted for a more quantifiable detection.

Apart from direct quantification of liver stage parasites in the infected 3D hepatic spheroids, another method of determining the potency of the hypnozoitocidal compounds is the measurement of the parasite’s transition from the liver stage life cycle into the erythrocytic life cycle through the release of merosomes from the liver stages for invasion into naïve host erythrocytes. Though such phenomenon was observed in vitro in this study, various conditions can potentially be further optimised to increase the rate of erythrocyte invasion which can subsequently be quantitated with FACS.

The successful establishment of *P. cynomolgi* in vitro models for both the erythrocytic and exo-erythrocytic stages not only enables the malaria community to better understand the effects of antimalarials against both the blood and liver stages of *P. vivax*, it can also be used as the model to study other areas of interest in *P. vivax* such the elucidation of mechanism of drug resistance, the generation of recombinant *P. cynomolgi* parasites to facilitate high throughput drug screening and the mechanism of parasite invasion (summarized in Figure 5.1).
Figure 5.1 *P. cynomolgi* continuous culture as a surrogate model to investigate antimalarial effects on both the blood and liver stages of *P. vivax*. 
REFERENCES


APPENDICES
Appendix A. Plate layout for the isolation of homogenous population of *P. cynomolgi* Berok K4

<table>
<thead>
<tr>
<th>Serial dilution</th>
<th>Neat (0.5% P)</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
<th>10⁻⁷</th>
<th>10⁻⁸</th>
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<th>10⁻¹⁰</th>
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</thead>
<tbody>
<tr>
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<td>625</td>
<td>62.5</td>
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<td>Transfer over (µL)</td>
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### Summary of FACS readouts (normalized to maximum count of uninfected control wells)

#### 1. FACS readout on 5 June 2017

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#### 2. FACS readout on 30 June 2017

#### 3. FACS readout 6 July 2017

#### 4. FACS readout 21 July 2017

#### 5. FACS readout 31 July 2017

### Appendix B. FACS readout of clonal dilution plate over time

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**Appendix C. Table of contribution for collaborative efforts**

<table>
<thead>
<tr>
<th>Figure 3.8</th>
<th>Phenotypic and rheological characterization of the heterogeneous Berok K4 strain from <em>in vitro</em> culture</th>
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<tbody>
<tr>
<td>Contributors</td>
<td>A.C.Y.C generated the <em>P. cynomolgi</em> Berok K4 sample and was involved in all the experiments listed, inclusive of data analysis. Collaborators in NUS conducted the experiments involving specialised equipments (e.g. SEM, AFM, AMNIS).</td>
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<table>
<thead>
<tr>
<th>Figure 3.9</th>
<th>Rate of rosetting across different asexual erythrocytic stages of Berok K4 strain</th>
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<tr>
<td>Contributors</td>
<td>A.C.Y.C generated the <em>P. cynomolgi</em> Berok K4 sample and was involved for the wet mount rosetting experiment, inclusive of data analysis together with collaborator from SIgN.</td>
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<tr>
<th>Section 3.6.3</th>
<th>Preliminary results of Oxford Nanopore sequencing of <em>P. cynomolgi</em> Berok K4-A7 isolate</th>
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<td>Contributors</td>
<td>A.C.Y.C generated the <em>P. cynomolgi</em> Berok K4-A7 DNA used in the whole genome sequencing using both Oxford Nanopore and Illumina sequencing. Collaborators in SIgN (bioinformaticians) were responsible for assembling and annotating the genome.</td>
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<tr>
<th>Figure 3.11</th>
<th>Drug susceptibility testing using <em>P. cynomolgi</em> Berok K4 <em>in vitro</em> culture</th>
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<tr>
<td>Contributors</td>
<td>A.C.Y.C generated the <em>P. cynomolgi</em> Berok K4 sample used for its drug susceptibility testing and the subsequent data analysis on both <em>P. falciparum</em> and <em>P. cynomolgi</em> results. Collaborator in NITD was responsible for <em>P. falciparum</em> drug susceptibility testing.</td>
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<table>
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<th>Figure 3.14</th>
<th>Transmission study from <em>P. cynomolgi</em> Berok K4 continuous culture</th>
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<td>Contributors</td>
<td>A.C.Y.C generated the <em>P. cynomolgi</em> Berok K4 sample used for the experiment, shared protocols and taught the collaborators in BPRC the method for successful continuous culture and analyzed all data generated from the transmission study. Collaborators in BPRC carried out the transmission study where the yield and infectivity of the sporozoites generated from the continuous culture were evaluated.</td>
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<th>Figure 4.4</th>
<th>Cell morphology and hepatocyte specific functions in spheroid-cultured human hepatocytes</th>
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<td>Contributors</td>
<td>A.C.Y.C generated the spheroid-cultured human hepatocytes used in the experiment, carried out the respective experiments and analysed the data together with the collaborator in NUS.</td>
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