Role of *Plasmodium falciparum* genetic backgrounds in tolerance to antimalarial-resistance

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Declaration

I declare that this thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically stated in the text. It does not exceed the prescribed 20,000-word limit for the School of Biological Sciences Degree Committee.

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Abstract

Malaria remains a global public health burden with the highest mortality rate amongst vector-borne diseases, affecting mostly children under five years of age making it the leading cause of child deaths. There has been significant reduction of the malaria burden as a result of global efforts in implementing control interventions to address malaria morbidity and mortality especially since the introduction of artemisinin. However, the burden of malaria remains high in many endemic areas despite the substantial decline in global spatial distribution and burden since 2000, with over 90% of people within sub-Saharan Africa residing in endemic areas. There has been increasing reports of *Plasmodium falciparum* tolerance to several partner drugs currently in use in artemisinin-based combination therapies in certain endemic countries which has been associated with certain genetic backgrounds.

In this study, the impact of resistance-associated mutations on parasite resistance and fitness, and how the genetic background of the parasite affects these phenotypes was investigated. In the first research chapter, the growth phenotypes of a panel of barcoded *P. falciparum* parasites were measured in parallel using barcode sequencing (BarSeq). These barcoded parasites cover different *P. falciparum* strains from different geographic locations, and are grown *in vitro* in competition with each other in the presence of antimalarial compounds. BarSeq was then used to measure the different phenotypes to antimalarials based on the genetic background. Another chapter in the study established the impact of potential antimalarial compounds on genetically modified *P falciparum* parasites harbouring *Pfkelch13* mutations. These experimental compounds were chosen because of their known activity on human *Kelch*-like ECH-associated protein1 (Keap1), and were tested in drug response assays and established to have activity against parasites. Another element in this

chapter established the generation of CRISPR plasmids for editing the *Pfkelch13* gene. These individual donors were deconvoluted from a complex pool of plasmids with a common pDC2 backbone that encoded all 64 possible codons at the critical position 580 of *Pfkelch13* that is the site of the most prevalent artemisinin-resistance variant. The isolation of each possible individual donor was carried out and sub-pools of plasmids were generated. This was done to facilitate future work to examine which amino acid may be more efficient in replacing cysteine at position 580 and the impact of parasite genetic background on the outcome of the allele.

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1.1 Malaria Overview

1.1.1 Epidemiology

Malaria remains a global public health burden with the highest mortality rate amongst vector-borne diseases, affecting mostly children under five years of age making it the leading cause of child deaths, with an estimated 229 million cases and an estimated 400,000 deaths recorded in 2019 alone (World Health Organisation, 2020; Patriani *et al,* 2019). The disease is transmitted by the female *Anopheles* mosquito and caused by several species of *Plasmodium* protozoan parasites in humans including *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium malariae, Plasmodium ovale and Plasmodium knowlesi* (Rocamora and Winzeler, 2020; Su *et al,* 2020). *P. falciparum* remains the most virulent amongst the species of the genus *Plasmodium* accounting for the majority of severe cases and malaria-related deaths in endemic areas (Haldar *et al,* 2018; Kapesa *et al,* 2018).

Transmission was reported to occur in 87 endemic countries in 2019 in which ~94% of the cases are accounted for in the African region and ~3% in South East Asia, with half of the world's population at risk (World Health Organisation, 2020). Areas affected by malaria (Figure 1.1) are mostly low income, tropical and sub-tropical areas, with Africa been the most affected due to a combination of factors including insufficient resources, political and socio-economic instability, which are bottlenecks in implementing efficient malaria control projects (Centres for Disease Control and Prevention, 2020; Fletcher *et al,* 2020).

There has been a significant reduction of malaria burden as a result of global efforts in implementing control intervention to address malaria morbidity and mortality

(Debebe *et al,* 2020). The Global Malaria Eradication Programme, initiated by the World Health Organisation (WHO) in 1955, was a vector control-based campaign using insecticide dichlorodiphenyltrichloroethane (DDT) and chemoprevention with chloroquine. This resulted in elimination of the disease in several parts of Europe, the Americas and Asia, but with no major success in sub Saharan-Africa (World Health Organisation, 2016). Other malaria elimination campaigns have been initiated towards the path to malaria elimination over the past two decades including the Roll Back Malaria initiative, the malaria-focused target of The Millennium Development Goals by the United Nations (UN) and Medicines for Malaria Ventures (MMV) with the aim of dramatically reducing malaria morbidity and mortality (Roll Back Malaria, 2018; Rowe 2017; Wells *et al* 2015). The global increase in intervention has led to a significant decline in malaria mortality by 25% from 2010 to 2016, saving millions of lives (Centre for Disease Control, 2020)

However, the burden of malaria remains high in many endemic areas despite the substantial decline in global spatial distribution and burden since 2000 with over 90% of people within sub-Saharan Africa residing in endemic areas (Weiss *et al,* 2019).

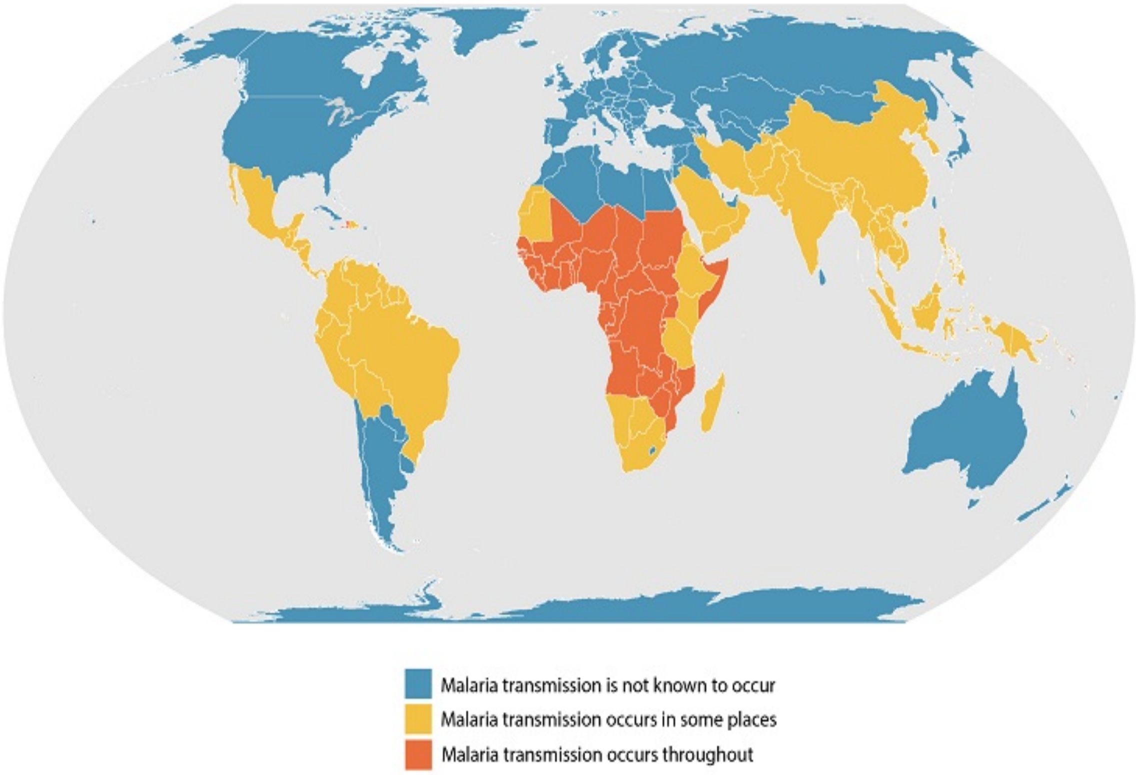


Figure 1.1 Malaria global distribution map showing an approximation of regions of the world with malaria transmission. Figure reproduced from Centres for Disease Control (2020).

1.1.2 Transmission and Vector

Malaria is a vector-borne parasitic disease transmitted by female mosquitoes of several species of the subfamily *Anopheles* during the bloodmeal stage of the reproductive cycle of the mosquito (Liu *et al* 2020; Singh *et al,* 2020). The prevalence of the different *Anopheles* species varies by geographical location with the major vectors being *Anopheles gambiae sensu lato* and *Anopheles funestus.* These are the major species in sub-Saharan Africa where the majority of malaria cases occur with their anthropophilic habits making them a major public health problem (Dahalan *et al,* 2019: Emami *et al,* 2017; Sinka *et al,* 2010).

The difference in behaviour, ecology, physiology and in morphological characters of the mosquito vectors are used for their identification. A combination of these differences is used as criteria to help define species and are the major source of distinction for species identification (Sallum *et al,* 2020). Transmission of parasites to the host by the mosquito occurs during feeding, in which sporozoites are transmitted to the host and sexual forms (gametocytes) are ingested by the mosquito with the blood meal (Yamamoto *et al,* 2016).

1.1.3 *Plasmodium*

Plasmodium spp. which are protozoan parasites belonging to the phylum Apicomplexa are the fundamental agents of malaria with five species of the *Plasmodium* genus causing infection in humans (de Koning-Ward *et al,* 2016). The species that are able to infect humans are *P. vivax, P. malariae, P. knowlesi, P. falciparum* and *P. ovale* which comprises of two genetically variant sympatric species, *Plasmodium ovale curtisi* and *Plasmodium ovale walikeri* (Miller *et al,* 2015). Amongst these species that infect humans, *P. falciparum* has been seen to be the most prevalent and virulent accounting for the majority of severe cases and malaria related mortality (Murphy *et al,* 2020).

1.1.3.1 *Plasmodium ovale*

Plasmodium ovale was first reported as a human-infecting species in 1922 and is geographically distributed across sub-Saharan Africa, the Western Pacific, Timor, and Indonesia with a higher prevalence reported in Nigeria and Papua New Guinea (Kotepui *et al,* 2020). *P. ovale* infections are rarely seen outside of the African region with relatively lower frequency in comparison with other species, thus severity of *P.*

ovale cases are rarely reported (Okafor and Finnigan, 2020). The occurrence of dormant forms of *P. ovale* (hypnozoites) in human infections remains unestablished (Markus, 2015). The two sympatric ovale species, *P. ovale curtisi* and *P. ovale wallikeri* are distinguished by genetic typing but morphologically identical by microscopy (Xia *et al.* 2020).

1.1.3.2 Plasmodium malariae

Plasmodium malariae was first reported as a malaria causative agent in 1880, and occurs in similar geographical locations as *P. ovale* mostly in sub-Saharan Africa, South America, Indonesia, Southeast Asia, and the western Pacific (Collins and Jeffery, 2007). *P. malariae* mono-infections are mostly benign and rarely related to severe cases or mortality, although it mostly occurs as mixed species infections with *P. falciparum* with a higher prevalence of *P. falciparum* than *P. malariae* in these mixed infections. *P. malariae* infections has been associated with long term low-grade chronic infection and nephropathy and anaemia (Yman *et al,* 2019; Lo *et al* 2017).

1.1.3.3 Plasmodium vivax

Plasmodium vivax is the most geographically widespread species and the second primary malaria causative agent in humans that occurs at a higher prevalence in tropical, sub-tropical and temperate zones, mostly in Asia and Latin America although also occurring at significant levels in some parts of Africa (Elgoraish *et al* 2019; Sitali *et al,* 2019). *P. vivax* is not benign despite the high frequency of asymptomatic infections as well as relatively low prevalence estimation and low parasite densities in the peripheral blood (Battle *et al,* 2019; Howes *et al,* 2016). One of the main biological characteristics of *P. vivax* is the formation of undetectable dormant liver stages known as hypnozoites. These forms cause repeated infection relapses that can occur within weeks to several years of primary inoculation thus posing a big challenge for control and elimination of this species (Twohig *et al,* 2019). The low prevalence of *P. vivax* in Sub-Saharan Africa has been linked to the absence of the Duffy antigen receptor, in the majority of that population, which is a crucial red blood cell receptor for the invasion of *P. vivax* into erythrocytes. However, there have been increasing reports of *P. vivax* infections occurring in Duffy-negative individuals and populations indicating the use of alternate receptors for erythrocyte invasion (Popovici *et al,* 2020; Mendes *et al,* 2011). *P. vivax* has been observed to be less virulent in Duffy-negative Africans in comparison to the Africans harbouring the Duffy antigen receptor (Golassa *et al,* 2020).

1.1.3.4 Plasmodium knowlesi

Plasmodium knowlesi malaria is now classified as a zoonotic disease but was initially strictly associated with simian infection of the long-tailed macaque (*Macaca fascicularis*), pig-tailed macaque (*Macaca nemestrina*), and the banded-leaf monkey (*Presbytis melalophos*), which are the parasite's natural reservoir hosts (Amir *et al,* 2018; Singh and Daneshvar, 2013). The first successful experimental infection of 3 human volunteers with *P. knowlesi* malaria by Knowlesi and Das Gupta in 1930, with the first case of a natural infection of *knowlesi* malaria in humans reported in 1965 in an American tourist that travelled to Peninsular Malaysia (Wesolowski *et al,* 2015; Singh and Daneshvar, 2013). Since the landmark report of a large number of *P. knowlesi* cases in Malaysia, in 2004, there were increasing records of the Simian *P. knowlesi* human infections across South East Asia (Zaw and Lin, 2019; Imai *et al,* 2014). The increasing number of *knowlesi* malaria cases are associated with the application of molecular methods for diagnosis, which addressed the challenge of misdiagnosis of *P. knowlesi* as *P. malariae*, as well as deforestation resulting in increased exposure of humans to vector mosquitoes that coexist with the macaque (Jeyaprakasam *et al* 2020; Davidson *et al,* 2019; Imai *et al,* 2014). As a result of the occurrence of the natural hosts in these regions, *P. knowlesi* is suspected to be geographically limited to Southeast Asian countries (Scott, 2020). Similar to *P. vivax, P. knowlesi* also invades the erythrocytes with the use of the Duffy antigen receptors. This invasion pathway is likely a contributory factor for the absence of *P. knowlesi* in the highly Duffy-null African population, in combination with the absence of the reservoir Macaque monkeys in Africa (Muh *et al,* 2018; Onyedibe *et al,* 2016). However, the ability of *P. knowlesi* to exploit alternate mechanisms for erythrocyte invasion is unclear (Antinori *et al,* 2012).

1.1.3.5 Plasmodium falciparum

Plasmodium falciparum is less geographically widespread than *P. vivax* but remains the most virulent amongst the five *plasmodium* species that causes malaria in humans, accounting for the majority of malaria morbidity and mortality (Nureye and Assefa, 2020). The significantly higher incidence of malaria in the WHO African region compared to the other parts of the world is as a result of the predominance of *P. falciparum* in sub-Saharan Africa (Battle *et al,* 2019). Severe complications which can be fatal including coma, transient or permanent neurological effects as a result of cerebral malaria is a major complication of *P. falciparum.* Issues in organs such as lungs, kidneys caused by parasite adherence to tissue cells of different stages is also a major complication of *P. falciparum* (Patel *et al,* 2020; Brazier *et al,* 2017; Rénia *et al,* 2012). The occurrence of mixed infections of multiple distinct strains of *P.*

falciparum species as well as co-infection with other plasmodium species, mostly *P. malariae* and *P. vivax* in hyperendemic regions linked to severity and treatment failure has been reported (Hossain *et al,* 2020; Kotepui *et al,* 2020; Zhu *et al,* 2019).

1.1.4 Life Cycle of *Plasmodium falciparum*

The *P. falciparum* life cycle shown in figure 1.2 involves two hosts, both the intermediate human host and the mosquito vector. The life cycle passes through three major stages (pre-erythrocytic stage, erythrocytic stage and the vector stage), and clinical symptoms are associated with continuous rounds of asexual replication in the blood (Toro-Moreno *et al,* 2020; Venugopal *et al,* 2020). The infection starts with the injection of variable amounts of motile sporozoites from the salivary glands of the mosquito into the bloodstream when an infected female *Anopheles* mosquito is taking up a blood meal from the host, marking the initiation of the pre-erythrocytic stage of the cycle (Vaughan *et al,* 2012).

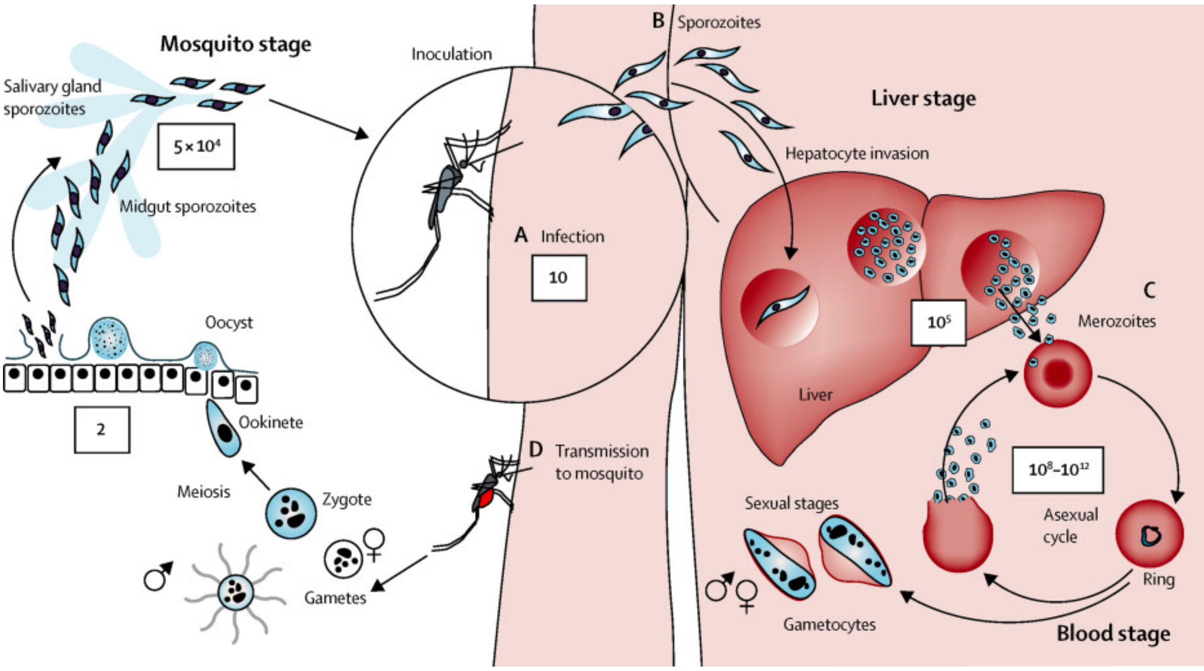


Figure 1.2 Life cycle of *Plasmodium falciparum*. The three main developmental stages of the life cycle: **(A) Pre-erythrocytic stage**: Injection of sporozoites that (**B**) migrate to the liver to generate liver-stage merozoites. **(C) Erythrocytic stage:** Merozoites released into the bloodstream invade RBCs and undergo several rounds of asexual multiplication progressing

through ring, trophozoite and schizont stages. Less than 10% of parasites develop into sexual forms (gametocytes) of the parasite during the erythrocytic stages. (**D**) **Mosquito stage:** Mature gametocytes are taken up by the mosquito, progressing through several developmental stages after fertilisation. Adapted from White *et al* (2014).

1.1.4.1 Pre-Erythrocytic Stage

The sporozoites deposited into the skin by the mosquito migrate to the liver, through the blood circulation, where they invade and develop in the liver cells known as hepatocytes (Osii *et al,* 2020; Putrianti *et al,* 2020). Whilst inside the liver cells, the parasite goes through intra-hepatocytic replication, maturing into hepatic schizonts to produce invasive exoerythrocytic merozoites through a process known as schizogony. (Winer *et al,* 2020; Vaughan and Kappe, 2017; Prudêncio *et al,* 2006).

1.1.4.2 Erythrocytic Stage

During schizogony, merozoites produced after the initial reproductive stage in the liver are released into the bloodstream. These specialised free invasive forms of the parasite of around 1µm in size rapidly invade red blood cells (RBCs) in a multi-process step that involves different phases following initial adhesion to cell membrane through to forming the parasitophorous vacuole membrane (PVM) when the parasite finally enters the host RBC (Blake *et al,* 2020; Paone *et al,* 2020). Interactions between specific parasite ligands and host receptors defines the specific molecular invasion pathway that mediates the invasion of erythrocytes, a key step in parasite growth (Cai *et al,* 2020). The merozoites that successfully invade RBCs develop into ring forms that last for 24 hours before transitioning into trophozoites when RBC contents are taken up, developing a digestive food vacuole visible by microscopy. The trophozoites eventually mature after 12 hours into schizonts after the occurrence of several nuclear divisions. These matured segmented schizonts with multiple nuclei rupture and release between 16 to 32 daughter merozoites which then invade new RBCs marking the beginning of another 48-hour cycle (Burns *et al,* 2019; White *et al,* 2014). During the different stages of development, the *Plasmodium* parasite takes up haemoglobin, as a source of amino acids, which is digested in the digestive food vacuole of the parasite. This leads to the release of iron-containing heme molecules which are toxic to the parasite and are thus detoxified and made inactive by crystallisation into the malaria pigment, hemozoin under the acidic conditions of digestive vacuole (Kapishnikov *et al,* 2019; Pishchany and Skaar, 2012; Dluzewski *et al,* 2008). The host RBC is drastically remodelled as the intracellular parasites grow and replicate resulting in deformation of the cell which is when clinical symptoms are manifested as a result of repeated rupture of RBCs. Diagnosis occur and parasite clearance by antimalarials is targeted at this stage (Amoah *et al,* 2020; Burns *et al,* 2019; Cowman *et al,* 2012). A relatively low proportion (0-20%) of the blood stage parasites differentiate into sexual forms of gametocyte-committed (gc)-ring-stage parasites. These gc-ring-stage parasites go through five morphologically distinct stages (I–V) (Figure 1.3) to become a mature, transmissible stage V gametocyte (Figure 1.3) over a duration of ~8–12 days (Prajapati *et al,* 2020; Venugopal *et al,* 2020). Progression through the developmental stages of gametocytogenesis that leads to the mature infectious sexual form (gametocyte) is driven by a combination of genetic, epigenetic and environmental factors. The presence of *gametocyte development protein 1 (gdv1)* and the *apetela-2 transcription factor (ap2-g)* have been reported to be required in initiating this sexual phase from asexual stages (de Jong *et al,* 2020; Usui *et al,* 2019).

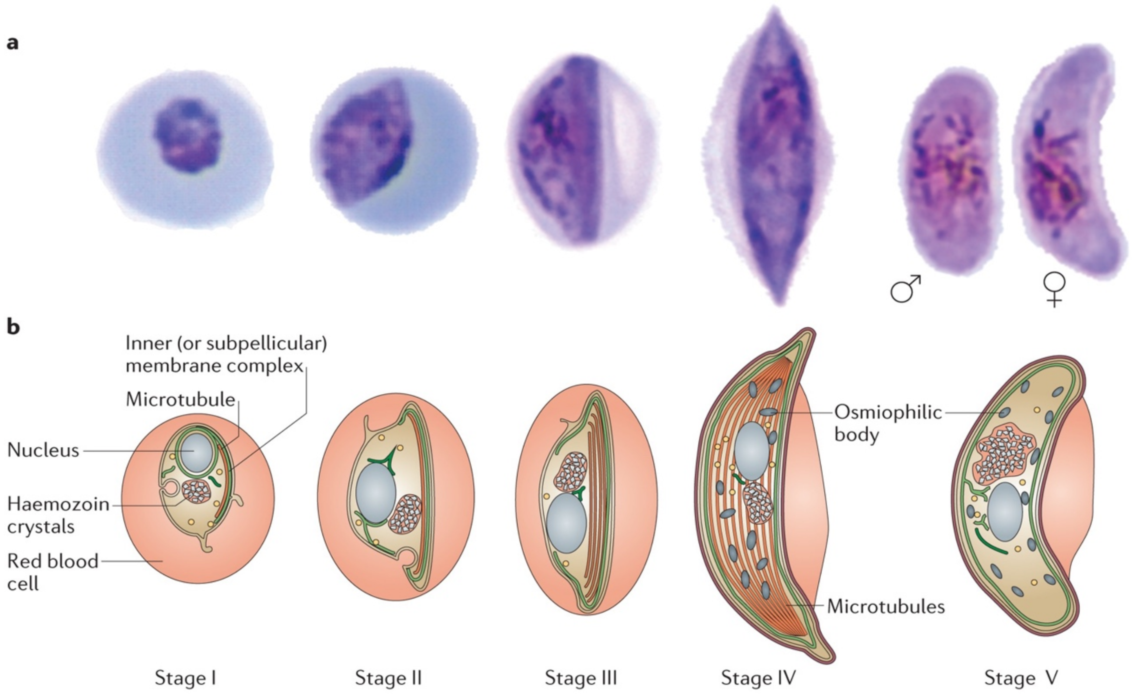


Figure 1.3 Development of mature *Plasmodium falciparum* sexual forms (gametocytes). Gametocytes develop through five distinct morphological stages during the blood stages of the life cycle. Gametocytes differentiate into male and female forms, that are transmissible to mosquitoes at the last stage. Adapted from Josling and Llinás (2015).

1.1.4.3 Mosquito Stage

Sexual differentiation in the blood to form male and female gametocytes during gametocytogenesis is required for transmission of parasites from the human host to the mosquito, and thus is a vital stage in continuity of the transmission cycle. Gametocytes ingested by mosquitoes during a blood meal from the human host mature into gametes, a process that is triggered by environmental factors in the mosquito midgut including exposure to xanthurenic acid, a change in pH, and temperature drop (Josling and Llinás, 2015). Activation and fertilization of male and female gametes occurs forming a zygote that develops into motile ookinete after 16 to 18 hours through meiosis. The mosquito midgut epithelium is invaded by the ookinete that matures into an oocyst on the midgut basal side. This oocyst then produces thousands of sporozoites through asexual replication, which are released and migrate into the salivary gland of the mosquito enabling their transmission to another human host during a blood meal (Volohonsky *et al,* 2020; Ngotho *et al,* 2019; Josling *et al,* 2018).

1.1.5 Pathogenesis

The clinical manifestations of *P. falciparum* have been associated with the asexual erythrocytic stages of the life cycle which can result in either asymptomatic, uncomplicated or severe malaria (Colvin and Joice Cordy, 2020; Zekar and Sharman, 2020). The common unspecific clinical symptoms displayed as a result of host responses include headache, myalgia, dizziness, fever and chills, which may sometimes progress to severe malaria (Su *et al,* 2020). These manifestations are driven by multiple factors including parasite strain and densities in an infected individual, host genetic susceptibility and resistance (immunogenicity) (Loiseau *et al* 2019; Laishram *et al,* 2012; Mendoca *et al* 2012). Development of symptoms in *P. falciparum* infections occur after 3 to 4 cycles when a threshold of 6 to 20-fold exponential increment of parasitaemia per cycle is reached. These symptoms are manifested after an incubation period of 11-15 days*,* which varies in other species (White *et al,* 2017). Periodic fever spikes at 48-hour intervals correspond to schizont bursting at the end of the erythrocytic cycle of *P. falciparum*. Cycle length, which is different in other species, influences timing of these fever spikes that are one of the classical clinical manifestations (Smith *et al,* 2020; Bartoloni *et al,* 2012).

Variation of clinical manifestations have also been observed between children and adults although cerebral involvement, kidney dysfunction, and acidosis has been reported to be independent mortality predictors in both categories (Plewes *et al,* 2018). Severe malarial anaemia, cerebral malaria (CM), and respiratory distress syndrome are the major clinical manifestations or symptoms that have been implicated in severe malaria (Mousa *et al,* 2020). The unique sequestration characteristic of *P. falciparum* infected RBCs to walls of different sized blood vessels is mainly associated with its high virulence compared to the other species (Siciliano and Alano, 2015). The sequestration is a mechanism that protects the parasitised RBCs from filtration by the spleen resulting in host endothelial cell injury and microvascular obstruction thus the development of severe malaria (Lee *et al,* 2019). This leads to severe forms of pathogenesis including cerebral malaria (coma), respiratory distress, severe anaemia, acute renal failure, jaundice, acidosis, hypoxia and hypoglycaemia, which can be fatal. These outcomes are triggered based on the organ involved such as brain, lung, kidney (Ashley *et al,* 2018). It has been reported that aggravated anaemia due to stringent splenic clearance of ring-infected RBCs and uninfected altered RBCs, is associated with reduced risks of severe complications such as cerebral malaria from high parasitaemia. This is as a result of retention of normal and ring-infected RBCs in the spleen, preventing the circulation of a subpopulation of rings to mature and sequester thus leading to reduced parasitaemia (Buffet *et al,* 2011).

Diverse clinical presentations have been observed in *P. falciparum* infections, even with the same individual over time including individuals carrying chronic parasitaemia who are reservoirs for transmission to mosquitoes, due to asymptomatic infection (Gonçalves *et al* 2017). Host genetic factors that have been associated with adaptations in asymptomatic or mild infections, and thus protection against severe malaria, include sickle cell disease (SCD), thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and other red blood cell (RBC) genetic anaemia with Mendelian inheritance as well as blood group O of the ABO type (Cooling, 2015; Mendonça *et al,* 2012). Protection against severe malaria due to a significant reduction in parasite densities is observed in some genotypic variants of these factors. This can result in flawed infected erythrocyte cytoadherence, rosette formation and enhanced infected RBC clearance, leading to decreased parasite invasion ability and growth hinderance (Acquah *et al,* 2020; Archer *et al,* 2018).

1.2 Malaria Control Measures

Due to the significant burden malaria inflicts on global socio-economic and public health, there has been continuous implementation of control measures towards reduction or elimination of morbidity and mortality of the disease over the past decades (Tizifa *et al,* 2018). Prevention of the infection by avoiding bites from mosquitoes carrying the parasites, and prevention of the disease with therapeutic use of antimalarial drugs remains the two major prophylactic strategies for malaria control (Skwarczynski *et al,* 2020). Thus, combination of several measures including the distribution of insecticide-treated bed nets (ITNs), indoor spraying of residual insecticides (IRS) and other vector control strategies; access to early diagnosis such as use of rapid diagnostic tests (RDTs); and more effective antimalarial treatments alongside targeted interventions such as intermittent preventive treatment in pregnancy (IPT) and seasonal malaria chemoprevention (SMC) have been in place by malaria control programmes (Nice *et al,* 2020; Wagman *et al,* 2020; Sy *et al,* 2019; White et al 2011). Development of vaccines for malaria has also increasingly been explored over the past decade (Nureye and Assefa, 2020; Rogerson *et al,* 2020). Current control and elimination campaigns against malaria include the Action and Investment to Defeat Malaria (AIM) by the Roll Back Malaria programme in alliance with the Global Technical Strategy for Malaria 2016 - 2030 (GTS). This Global Malaria Programme of the World Health Organization (WHO) is aimed at reaching reductions of cases of at least 90% as well as elimination in up to endemic 35 countries by 2030 (Nsereko *et al,* 2020; Smith Gueye *et al,* 2016)

1.2.1 Vaccine Development

The enormous complexity of malaria parasite biology and infection cycle, the diversity of the complicated parasite genomes, as well as the ability to evade the host immune response presents a great challenge in the development of an effective vaccine for the highly virulent *falciparum* malaria (Mahmoudi and Keshavarz, 2018). Significant progress has been achieved generally in the efforts made towards the development of effective vaccines with approaches targeted at transmission-blocking, pre-erythrocytic and erythrocytic stages of the parasite life cycle (Molina-Franky *et al,* 2020; Frimpong *et al,* 2018). Vaccines targeting the erythrocytic stage of the parasite aimed at preventing disease development rather than infection have been explored. These vaccines have been developed based on the antigens expressed in the blood stage of infection including Merozoite Surface Proteins (MSP), Apical Membrane Antigen (AMA-1) and glutamate-rich protein (GLURP) (Skwarczynski *et al,* 2020; Pance, 2020). Approaches including synthetic peptides or recombinant proteins that combined sporozoite antigens with blood stages had varied results that were not promising.

The availability of the parasite genome revealed the new prospect of a wide range of malaria antigen-based vaccines (Penny *et al,* 2020, Hill, 2011). One of the first synthetic peptide-based vaccines developed against malaria based on pre-erythrocytic blood-stage fragments involving circumsporozoite protein is SPf66, which demonstrated partial efficacy in humans in South America but not against *P. falciparum* in African trials or in Thailand (Salamanca *et al,* 2019; Schwartz *et al,* 2012). Amongst the current vaccines in development targeting *P. falciparum* malaria, RTS,S/AS01 is the first vaccine that reached completed Phase III clinical trials in 2014. RTS,S/AS01 manifested a protective efficacy of 36% to 50% in young children aged 5 to 17 months and above in late-stage clinical trials. There is evidence of vaccine efficacy against clinical malaria episodes at a 4-dose scheme administered in 1-month intervals from 5 months and a booster dose at 25 months (Hogan *et al,* 2020; Duffy and Gorres, 2020; Mahmoudi *et al,* 2017). RTS,S is the most advanced pre-erythrocytic vaccine targeting *Plasmodium* sporozoites and liver stages aimed at sporozoite clearance from the skin and bloodstream or hepatocyte invasion blockage, as well as stimulating immune response against infected hepatocytes (Duffy *et al,* 2020; Draper *et al,* 2020). RTS,S includes the fusion of a central tandem repeat C-terminal fragment of circumsporozoite (CSP) protein (R) with T-cell epitopes (T) bound to hepatitis B (HBsAg) surface antigen (S) with S protein and adjuvant AS01/ASO2. However, adjuvant AS01 has displayed higher efficacy than adjuvant ASO2 (Sánchez *et al,* 2020; Neafsy *et al,* 2015).

A favourable outcome presenting a better cost-effective conclusion has been suggested by several clinical data that pre-erythrocytic and transmission-blocking vaccines could be incorporated in one construct thus the necessity for continuous investigation of multi-stage approaches (Yusuf *et al,* 2019). However, reduced immunogenicity and efficacy have been reported in some combination multi-stage malaria vaccine trials emphasising the need for further evaluation of alternative schedules or immunization sites for adequate efficacy (Rampling *et al,* 2018).

1.2.2 Vector Control:

Vector control remains a major essential component of malaria control and elimination programmes and has been an effective approach of preventing transmission and thus successfully impacting malaria morbidity and mortality to malaria and other vector-borne diseases (Wilson *et al,* 2020; Lobo *et al,* 2018). Control interventions targeting mosquito vectors mainly include the use of insecticide treated nets (ITNs) that involve the treatment of bed nets with insecticides, and indoor residual spraying of insecticides (IRS) which involves treatment of household walls with chemical insecticides (Choi *et al,* 2019). Other vector control strategies not as widely applied include larval source management (LSM) as well as host-mediated control such as zooprophylaxis in transmission settings involving zoophilic vectors and use of attractive toxic sugar baits that targets a toxin susceptible vector population. Mosquitoes are killed in the process of feeding on toxic sugar meals that they are attracted to, sprayed on plants or used in bait points (Lobo *et al,* 2018; Tusting *et al,* 2013).

Organochlorines, pyrethroids, carbamates, and organophosphates are the four classes of insecticides recommended for IRS by the Word Health Organisation (Yewhalaw *et al,* 2017). The use of dichloro-diphenyl-trichloroethane (DDT) and synthetic pyrethroids which are safe alternatives to DDT, have been the main active insecticides used in IRS and ITN distribution campaigns over the past two decades (N'Guessan *et al,* 2010). However, substitute insecticides with shorter environmental persistence such as carbamates and organophosphates, which are more expensive, are used in settings where widespread vector resistance to DDTs and pyrethroids is observed, thus making IRS approaches more complex and less cost-effective (Kané *et al,* 2020; Wanjala *et al,* 2015; Kleinschmidt *et al,* 2009). The use of carbamates and organophosphates for IRS requires repetitive application due their short residual duration, thus resulting in high cost despite their rapid lethal effects. The use of combined organophosphate pirimiphos-methyl (PM) formulation and other recently developed long-lasting formulations which has demonstrated higher efficacy against pyrethroid resistant vectors and has been a countermeasure to address that limitation (Keita *et al,* 2021; Abong'o *et al,* 2020; Protopopoff *et al,* 2018; Ngufor *et al,* 2017; Hamainza *et al,* 2016).

The use of ITNs which targets the vector population that bites indoors and are susceptible to the insecticide-in-use have been observed to be most effective in protecting against late-night and indoor-biting vectors, demonstrating individual and community wide protection (Levitz *et al,* 2018; Lobo *et al,* 2018). The long-lasting insecticidal nets (LLINs) category of ITNs provides greater prolonged effectiveness due to the embodiment of insecticide into the fibers during manufacturing of the net. These LLINs are thus more effective than bed nets that are repetitively treated with insecticides at six-month intervals (Wangdi *et al,* 2018). Pyrethroid treated LLINs, which have pyrethroid as the main active ingredient, are the current standard across major malaria-endemic regions and are reported to have an estimated life span of 3 years and lasting after up to 20 washes (World Health Organisation, 2020; Fuseini *et al,* 2019). Although the independent implementation of LLINs and IRS has resulted in a dramatic reduction of morbidity and mortality, the combination of these two interventions should be explored further as data from large surveys and trials implementing the combination of the two strategies has suggested higher efficiency in incidence reduction (Guerra *et al,* 2020; Loha *et al,* 2019). However, eliminating malaria transmission in many settings, because of operational limitations, increasing resistance to available insecticides and behavioural evolution of mosquitoes to avoid contact with these interventions, remains a great challenge for these strategies, despite the high efficiency of LLINs and IRS (Killeen *et al,* 2016). Moreover, insecticide resistance has been reported to all four classes of insecticides recommended by the WHO for vector control, with resistance to pyrethroids being the most compromised insecticide in vector resistance (Tangena *et al,* 2020; Fuseini *et al,* 2019; Wanjala *et al,* 2015). One of the major challenges in vector control programmes is the lack of knowledge on geographical distribution and spread of resistance as well as on molecular mechanisms of resistance, which recent developments in sequencing technology is progressively addressing (Clarkson *et al,* 2018). The issues of the negative environmental impact of insecticides has led to the increased development of vector control techniques that are safer and more suitable to the environment such as the use of biorational pesticides. This involves the use of natural or synthetic materials with insecticidal effects that are acquired from animals, plants, bacteria, or minerals on mosquito breeding sites (Ogunah *et al,* 2020).

The mass release of sterilised male mosquitoes is one of the environmentally safe tools that has received great attention. The sterilisation of male mosquitoes known as sterile insect technique (SIT) using radiology or chemicals prevents production of offspring after mating thus leading to a reduction in vector population (Gentile *et al,* 2015). However, limitations reported in this technique include partial sterility during transformation, competition from wild males and insignificant population decrease after successful sterilisation (Khamis *et al,* 2018; Maïga *et al,* 2014; Godfray; 2013). The application of transgenic sterility using genetic manipulation techniques such as CRISPR/Cas9 is another alternate approach. This method aims to introduce genetically edited mosquitoes with a dominant lethal gene that leads to the production of abnormal offspring after mating with wild-type females. Trials of this method have been reported to lead to local population reduction, and is thus a potentially effective control strategy of insecticide resistant vectors (Simoni *et al,* 2020; Yamamoto *et al,* 2019; Kyrou *et al,* 2018; Catteruccia *et al,* 2009). Limitations of these techniques requiring continued investigation include the applicability in larger scale, and technical challenges such as Cas9-based off-target cleavage in CRISPR-based methods (Kandul *et al,* 2019; Hammond *et al,* 2016).

1.2.3 Antimalarials (Drug Discovery and Development)

The use of antimalarial drugs remains a key aspect in the control and elimination of malaria and can be applied for prevention of malaria using different strategies such as chemoprophylaxis, mass drug administration and intermittent preventive therapy (Cui *et al,* 2015). In combination with vector interventions, the extensive distribution of antimalarials has been highly effective in control of the disease and significantly reducing morbidity and mortality rates in highly endemic regions over the past two decades (Lubis *et al,* 2020; Khamis *et al,* 2018). The antimalarial drugs commonly used are categorised into different chemical classes including the antibiotics, antifolate compounds, quinoline-containing compounds, hydroxynaphthoquinone atovaquone and artemisinin and its derivatives. These antimalarials demonstrate differing modes of action and target different malaria life cycle stages (Deshpande and Kuppast, 2016). Antibiotics that have been in use for malaria chemoprophylaxis or in combination with other quinolines include doxycycline and clindamycin (Dahl and Rosenthal, 2008).

1.2.3.1 *Quinolines and Antifolates*

Several natural and synthesized quinoline compounds have been developed over the past years following the isolation of quinine in 1820, which was the first chemically purified compound effective for malaria treatment belonging to the quinoline class of antimalarials that was sourced from the cinchona tree (Tse *et al,* 2019; Golden *et al,* 2015; Raynes, 1999). The antimalarial activity of quinoline compounds has been reported to be exerted by their interference with heme detoxification by preventing the polymerisation of heme to hemozoin (Herraiz *et al,* 2019; Kapishnikov *et al,* 2019; Sullivan *et al,* 2002). Other quinoline-containing compounds have been developed based on the improvement and modification of quinine, comprising a class of heterocycles with varying lengths and nature of their basic amine side chains. These include 4-aminoquinolines such as chloroquine, amodiaquine and piperaquine, 8-aminoquinolines like primaquine, 4-quinoline methanols such as mefloquine, lumefantrine and quinine and hydroxynaphthoquinones such as atovaquone (Baird, 2019; Nqoro *et al,* 2017; Shreekant and Bhimanna, 2016; Bawa *et al,* 2010;).

The development and use of chloroquine in the 1940s, which is a 4-aminoquinoline drug that has been suggested to be trapped in the parasite food vacuole was a major advancement in treatment of malaria. These drugs were widely used for over 4 decades as the gold standard for malaria treatment due to the minimal host toxicity, high clinical efficacy, ease of use as well as cost effectiveness and simplicity of their synthesis (Kapishnikov *et al,* 2019; Cui *et al,* 2015; Bawa *et al,* 2010; Stocks *et al,* 2002). However, the widespread development of parasite resistance to chloroquine and other quinolines in various endemic regions has hindered that success and led to a significant reduction of their efficacy and use in malaria treatment

(Kumar *et al,* 2015; Rajapakse *et al,* 2015). Resistance to quinoline antimalarial drugs has been reported to be as a result of drug efflux out of the food vacuole caused by point mutations mainly on the genes of the membrane transporters *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance-1 (*pfmdr1*). Multiple single point mutations have been observed at codons of the *pfcrt* and *pfmdr* genes. *Pfmdr1* encodes for a P-glycoprotein homolog that is shown to localize to the membrane of the food vacuole, which is the site of action of a number of drugs (Lawrenson *et al,* 2018; Cui *et al,* 2015).

Another vastly used group of antimalarial drugs used in the treatment of malaria is the antifolates which targets enzymes of the folate pathway essential for survival of the parasites thus leading to decreased formation of pyrimidines, purines, and some amino acids. The two major enzymes inhibited by antifolates are dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) (Saifi *et al,* 2013). Antifolate antimalarials targeting these enzymes have extensively been in use over the past decades. These include sulfadoxine, pyrimethamine and proguanil, which are mostly used as a combination of two inhibitors of different targets or in combination with other classes of antimalarials (Posayapisit *et al,* 2020; Yuthavong *et al,* 2014). Development of resistance to antifolates results from point mutations at several codons in the *pfdhfr* and *pfdhps* genes, which have been reported in various endemic regions. These mutations have been implicated in the development of resistance to the most used antifolate combination sulfadoxine-pyrimethamine (SP), a treatment that was effective against chloroquine resistant malaria (Sugaram *et al,* 2020; Haldar *et al,* 2018; Cui *et al,* 2015; Karema *et al,* 2009; Mkulama *et al,* 2008). SP is still widely used in IPT in pregnancy, SMC in children of sub-Saharan Africa, chemoprophylaxis and in combination with other antimalarial classes (Amambua-Ngwa *et al,* 2018).

1.2.3.2 Artemisinin

Artemisinin and its derivatives, which are sesquiterpene lactones, currently remain the frontline treatment of malaria for the past decades due to their highly potent activity against parasites that are resistant to previously developed antimalarial drugs (Abood *et al,* 2017). This class of antimalarial compound is characterised by an unusual endoperoxide bridge that exists in the natural extract, artemisinin, which was isolated from the sweet wormwood (*Artemisia annua*) plant which has been used for fever treatments in China, or the semi-synthesised derivatives (Czechowski *et al,* 2020; Xie *et al,* 2020). Artemisinin was discovered to be highly effective in rapidly reducing parasitaemia. The widespread use of this drug in all regions over the past two decades has led to a significant reduction in malaria mortality (Rathmes *et al,* 2020; Shibeshi *et al,* 2020; Lagarce *et al,* 2016). Although a precise mode of action is still being discussed, the antimalarial activity of artemisinin has been reported to include cleavage of the endoperoxide bond of artemisinin from interaction with heme in the parasite food vacuole, leading to drug activation. This results in the alteration of developing intraerythrocytic parasite proteins and lipid molecules by carbon-centred radicals and reactive oxygen species produced by the activated artemisinin compound (Marapana and Cowman, 2020; Shibeshi *et al,* 2020; Xie *et al,* 2020; Hoppe *et al,* 2004). Other potential modes of action that have been identified in recent studies include interaction of haem and PfATP6 which is a Ca2+ transporter, the unfolded protein response (UPR) pathway in which upregulation was shown to associated with artemisinin and linked to reduced parasite development, as well as the lipid kinase phosphatidylinositol-3-kinase (*Pf*PI3K) (Tse *et al,* 2019; Mbengue *et al,* 2015; Mok *et al,* 2015; Shandilya *et al,* 2013).

Artemisinin derivatives have shown great efficacy as rapidly acting antimalarials and include artemether, artesunate and dihydroartemisinin. Like artemisinin, these derivatives are widely used mostly in combination therapies due to their short half-lives (Rosenthal, 2003). There has been significant reduction in malaria morbidity and mortality, due to the extensive distribution of artemisinin-based combination therapies (ACTs), combined with other control programmes, particularly in Africa (Lubis *et al,* 2020). However, the increasing reports of *P. falciparum* resistance to artemisinin and several partner drugs currently in use in ACTs in several endemic regions poses a global threat. Thus, there is a need for continuous surveillance and a renewed approach to management. As discussed below, various polymorphisms for drug resistance-associated molecular markers in *P. falciparum* genome have been identified. Distinct profiles of these polymorphisms are observed in different endemic regions including molecular markers associated with the resistance to the current frontline antimalarial (ACTs) (Bwire *et al,* 2020; Jiang *et al,* 2020; Yobi *et al,* 2020; Reteng *et al,* 2017;).

1.2.3.3 Antimalarial Resistance

The first resistance to the oldest antimalarial drugs, the quinolines, emerged in South America and Southeast Asia and eventually spread to other highly endemic regions of Africa. Polymorphisms in several transporters including SNPs in *pfmdr1*, *pfcrt*, *pfnhe1* and *pfmrp1* have been associated with decreased sensitivity to quinoline antimalarials which were recommended first line malaria treatment for an extensive period in the past and still are recommended first line treatment in combination with antibiotics for malaria treatment in early pregnancy and some African countries (Tindall *et al,* 2018; Wootton *et al,* 2002). *Pfcrt* was initially identified as a result of mapping a genetic cross between chloroquine resistant and sensitive strains to a 36kb region of chromosome 7 in which point mutations at *pfcrt* were associated with resistant phenotypes (Fiddock *et al,* 2000). One SNP in *pfcrt* in particular, which is highly polymorphic, that remains the main facilitator of chloroquine resistance is K76T (Tola *et al,* 2020). Individual polymorphisms of *pfmdr1* have been reported to result in opposite effects on different drugs. Reduced sensitivity to chloroquine and amodiaquine has been linked with *pfmdr1* N86Y and D1246Y mutations which are common in Africa. These same mutations have been associated with increased sensitivity to lumefantrine, mefloquine, and artemisinin (Cui *et al,* 2015). However, decline in the frequency of chloroquine resistance alleles has been observed after use of chloroquine for *P. falciparum* was discontinued for extended periods in several long-term studies highlighting the effects of fitness costs (Nwakanma *et al,* 2013; Nsanzabana, *et al,* 2010; Mwai *et al,* 2009).

Although point mutations in *pfdhfr* and *dhps* has been strongly associated with hindering SP treatment success, copy number amplifications of *P. falciparum* guanosine triphosphate cyclohydrolase 1 (*pfgch1*), which is an enzyme responsible for coding a vital enzyme in the folate pathway, has also been implicated in antifolate resistance (Sugaram *et al,* 2020). Mutations in *dhfr* that have been commonly implicated in pyrimethamine resistance include S108N, A16V, N51I, C59R and I164L, with I164L reported to result in fast antifolate resistance spread in Africa. Sulfadoxine resistance has been mainly linked to an A437G mutation in *pfdhps* with additional mutations S436A/F/H, A581G, K540E and A613S/T *pfdhps* mutations facilitating elevated resistance (Juma *et al,* 2019). The occurrence of multilocus *Pfdhfr*/*Pfdhps* mutations between N51I, C59R, S108N, I164L / A437G, K540E, S437A A581G, A613S have been widely linked to hindering SP treatment success, with the quintuple *Pfdhfr/Pfdhps* mutant genotype, N51I, C59R, S108N/A437G, K540E, occurring at a higher prevalence (Pacheco *et al* 2020; Quan *et al,* 2020; Okell *et al,* 2017). The increasing occurrence of these haplotypes renders a threat for malaria treatment efficacy in pregnancy and SMC in which SP remains the frontline chemotherapy in Africa, thus emphasising the need for continuous monitoring and evaluation (Turkiewicz *et al,* 2020; Kayode *et al,* 2021).

Increasing prevalence of resistance to artemisinin, which is manifested by delayed parasite clearance, and partner drugs such as piperaquine and mefloquine have been observed mostly in Southeast Asia where it emerged (Pava *et al,* 2020; Imwong *et al,* 2017). Resistance to artemisinin is associated with mutations in the propeller domain of PfKelch13, which was first identified as mediating protection using in vitro evolution (Witmer *et al,* 2020; Ariey *et al,* 2014). *PfKelch13* protein is suggested to have multiple cellular functions involved in intraerythrocytic growth development of asexual parasites, including haemoglobin endocytosis from the host cell, and oxidative stress and unfolded protein responses. *PfKelch13* is suggested to be involved in ubiquitination of specific substrates by functioning as a substrate adaptor for E3 ubiquitin ligases. This leads to proteasomal-mediated degradation when the transfer of ubiquitin from the ubiquitin-conjugating enzyme (E2) to the protein substrate(s) is catalysed by E3 ligase activity (Goel *et al,* 2021; Birnbaum *et al,* 2020; Saddiqui *et al,* 2020; Wu *et al,* 2019). Artemisinin resistant parasites with polymorphisms in *Pfkelch13* have been dominant across the Greater Mekong Subregion over the past decade and only recently emerged in other parts of the world including Guyana, Papua New Guinea and Rwanda where *Pfkelch13* mutations linked to artemisinin resistance were reported (Mathieu *et al,* 2020; Pava *et al,* 2020). A *Pfkelch13* mutation A578S, which was the most common occurring *Pfkelch13* mutation detected in Africa, had previously been reported with no demonstrated effect in artemisinin efficacy thus the continued success of artemisinin malaria treatment in Africa for the past decades. However, the recent detection of R561H *Pfkelch13* mutations in Rwandan isolates demonstrating resistance phenotypes poses a significant threat to the extended success of antimalarial chemotherapy in Africa (Uwimana *et al,* 2020).

Selection pressure for artemisinin resistance is facilitated by reduced effectiveness of partner drugs as well as use of monotherapy thus establishing the cause of resistance between partner drugs and artemisinin may pose a challenge in studying artemisinin failure. Re-emergence of piperaquine and mefloquine resistance in southeast Asia has occurred due to reduced ACT efficacy (van der Pluijm *et al,* 2019; Sá *et al,* 2018). To date there have been over 100 identified *PfKelch13* mutations. Different degrees of delayed parasite clearance in patients treated with ACT have been demonstrated by various *PfKelch13* variants with mutually exclusive SNPs leading to amino acid changes at different codons including F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H and C580Y which are the mutations that have been more frequently reported and linked to artemisinin resistance (Mathieu *et al,* 2020; Witmer *et al,* 2020; Xie *et al,* 2020). The most frequently occurring *Pfkelch13* mutation observed especially in Southeast Asia is the C580Y mutation. In contrast to *Pfkelch13* mutations in Southeast Asia, only parasites with African genetic backgrounds have demonstrated artemisinin resistance linked to mutations in *P. falciparum* adaptor protein complex 2 mu (*Pfap2mu*), and ubiquitin-specific protease 1 *(pfubp1)* encoding the ubiquitin hydrolase (Sharma *et al,* 2020; Henriques *et al,* 2014).

The delayed parasite clearance in artemisinin-treated patients is a measure of loss of drug efficacy. However, this assay is not suitable for in vitro use. Furthermore, the standard 72 h drug assay used for other drugs does not reveal the tolerance phenotype of *pfkelch*-mutant parasites. Due to the short half-life of artemisinin, parasites in vivo need only withstand a relatively short period at high drug concentration. Thus, to more accurately reflect this short exposure artemisinin, the current standard *in vitro* assay is the ring-stage survival assay which measures the percentage of early (0 to 3 h post-invasion) ring-stage parasites that are able to survive a single 6-hour pulse of high concentrations of dihydroartemisinin (DHA). The identification of a number of artemisinin resistance-associated genetic loci and proteins have been facilitated by this approach, in combination with genome-wide association studies of resistant field isolates and gene manipulation experiments to validate variants of interest (Siddiqui *et al,* 2020). These approaches have played key roles in the progress of a detailed understanding of particular treatment failures, mechanisms to manage resistance to these drugs, and the development of more improved and effective antimalarial treatments (Cowell and Winzeler 2019; Blasco *et al,* 2017). Due to the variations of resistance polymorphisms of different *P. falciparum* genetic markers for different antimalarials across different regions (figure 1.4), there is need for continued investigation to determine specificity associated with fitness and genetic backgrounds. This will provide further insight into *P. falciparum* resistance mechanisms (Ross and Fidock, 2019). The genetic diversity of *P. falciparum* has been strongly associated with enabling its adaptation to antimalarials thus the continued success as a parasite (Apinjoph *et al,* 2019).

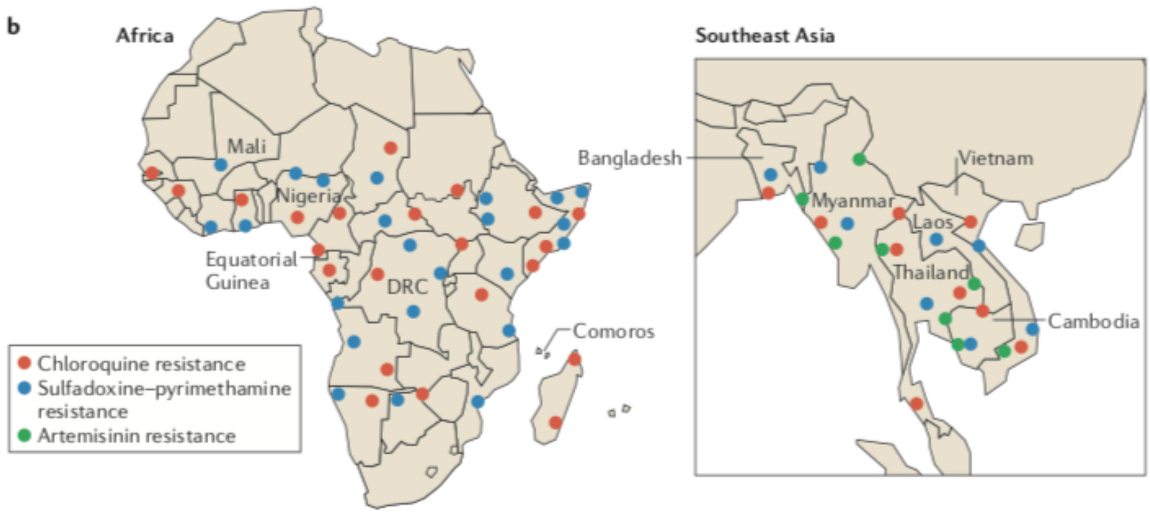


Figure 1.4 Distribution of *Plasmodium falciparum* antimalarial resistance. The distribution of *Plasmodium falciparum* resistance to chloroquine and sulfadoxine-pyrimethamine in Africa and Southeast Asia. (Adapted from Haldar *et al* 2018)

1.3 Genome Editing

Genome editing of malaria parasites has been a new and vital tool for progressive comprehension of genetic mechanisms of *P. falciparum* involved in resistance, as well as the identification of important novel drug targets (Cowell and Winzeler, 2018). In combination with sequencing techniques and the ability to readily culture *P. falciparum,* genome editing is an essential enhancement providing a valuable tool for investigating and validating resistance mutations (Cowell and Winzeler, 2019; Crawford *et al* 2017; Ng *et al* 2016). Various genome engineering methods have been developed for manipulation of the genome of several pathogens which are aimed at making use of DNA repair mechanisms to insert mutations where double-strand breaks (DNA damage) have occurred from exposure to damaging agents (Gopalakrishnan and Kumar, 2013).

Repair of double strand breaks in the majority of eukaryotes can occur by two major pathways as shown in figure 1.5. Homology directed repair (HDR), which is highly accurate and error-free, uses a homologous DNA template from a sister chromatid, a homologous sequence in diploids, a DNA segment like a donor plasmid or an ectopic donor if the double-strand break (DSB) occurs within a repeated sequence, to restore error-free DNA (Lee *et al,* 2014). The alternative DNA repair pathway referred to as non-homologous end joining (NHEJ) is facilitated by rejoining broken ends of the DSB without the use of a DNA template which makes use of DNA ligase IV and Ku70-Ku80 proteins binding to DNA ends, and is prone to error and loss or gain of some nucleotides (Badugu *et al,* 2015; Fleck and Nielsen, 2004). Most of the factors of canonical-NHEJ that are essential for DSB repair in most eukaryotes appear to be absent in the *P. falciparum* genome although there is evidence of DSB repair in *Plasmodium* parasites through an alternative-NHEJ. The prime mechanism for DSB repair in *P. falciparum* is the HDR pathway which has been shown to have its core genes encoded in the *Plasmodium* genome. In addition, point mutations in genes leading to DNA repair functions linked to antimalarial resistance phenotypes have been revealed by whole genome sequencing, suggesting the increasing chances of new polymorphisms emerging (Mathews *et al,* 2018; Gupta *et al,* 2016).

1.3.1 Genome editing techniques

The introduction of single nucleotide polymorphisms into the *Plasmodium* genome has been important for dissecting major roles in essential biological functions and obtaining a detailed understanding of gene functions. In particular, given the prevalence of SNPs in mediating drug resistance, genome editing has been vital in the aims of understanding *P. falciparum* resistance mechanisms. This requires precise *Plasmodium* genome manipulation which various existing techniques have provided significant insights for are still being perfected to fully eliminate these challenges (Straimer *et al,* 2012).

1.3.1.1 Nuclease based genome editing

Nuclease-mediated genome editing has been successfully applied to several organisms based on previous observations of the binding and cleavage properties of the natural type IIS restriction enzyme, *Fok*I (Bibikova *et al,* 2002; Li *et al,* 1992). These approaches utilise site-specific nucleases to trigger DNA repair mechanisms which have been developed for introduction of SNPs in the *P. falciparum* genome by fusing the FokI nuclease with a designable DNA binding protein (Straimer *et al,* 2012).

Prior to CRISPR technologies, zinc-finger nucleases (ZFN) were the most widely used technique to introduce a double strand break in the genome. These breaks would stimulate either the NHEJ pathway (donor free) or HDR pathway in the presence of a donor template to alter target DNA as a result of gene disruption leading to targeted gene replacement or targeted mutagenesis, as shown in Figure 1.5. This genome editing technique makes use of the DNA-binding domain of the zinc finger proteins, which can be engineered for recognition of specific DNA sequences, and the FokI nuclease that induces the targeted double strand break (Straimer *et al,* 2012; Caroll, 2011; Miller *et al,* 2007).

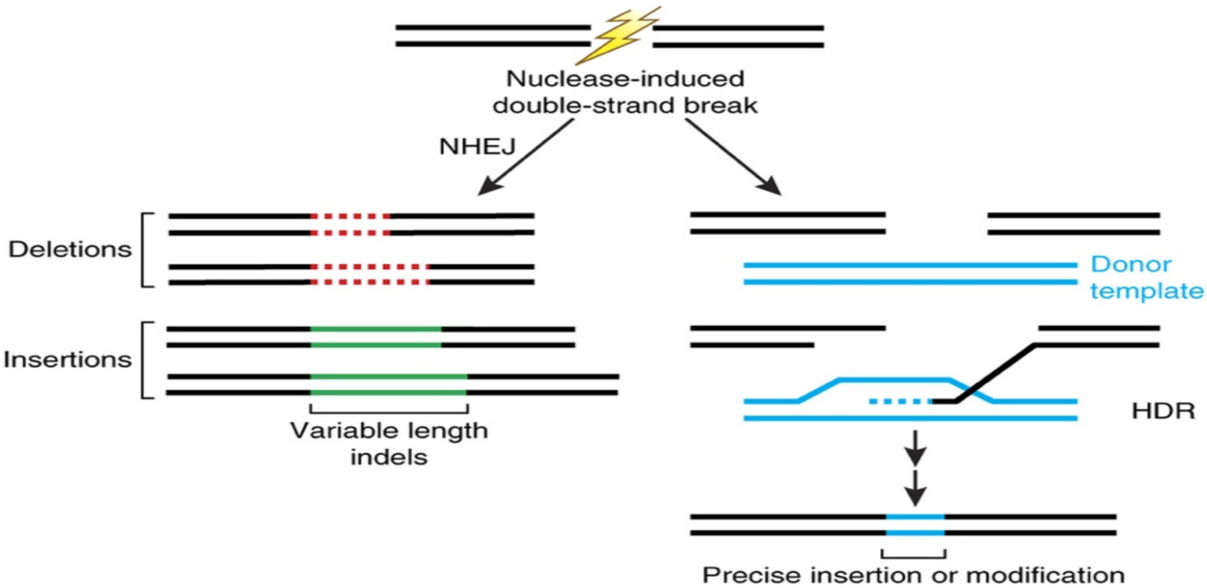


Figure 1.5 DNA repair pathways. Nuclease-induced double-strand breaks (DSBs) are repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). Gene disruption can occur by the introduction of errors during DSB repair to produce variable-length insertion and deletion mutations in the NHEJ pathway. A DNA donor template can be used to introduce point mutations or insertions via the HDR mediated repair pathway (Sander and Joung, 2014)

1.3.1.2 Transcription activator-like effector nuclease-based genome editing

Genome manipulation using transcription activator-like effector nucleases (TALENs) is another nuclease-based genome modification system. TALENs are a less complex system than ZFNs due to the more straightforward array design of the DNA binding element, as well as improved specificity and reduced toxicity. Similar to ZFNs, specific DNA sequences are recognised by TALENS through repeat DNA-binding domains in the presence of two hypervariable amino acid residues required for target site specificity. These residues are engineered to promote site-directed genome editing by generating manipulative DNA-binding proteins which is complicated by the high similarity of TALE recognition sequences. This, combined with the nature of the highly repetitive DNA-binding domain and large size of the nuclease poses a challenge for its use in genome editing of organisms like *P. falciparum* (Gaj *et al,* 2016; Basu *et al,* 2015; Ma and Lui, 2015;).

1.3.1.3 **CRISPR/Cas9 genome editing system**

Genome engineering based on the clustered, regularly interspaced, short palindromic repeat (CRISPR)–CRISPR-associated protein (Cas) (CRISPR-Cas9) system is a powerful tool that has been widely and successfully used in genome manipulation of several organisms accelerating vital gene functional studies (Shinzawa *et al,* 2020; Xu *et al,* 2019). This nuclease-based editing mechanism which has also been successfully applied to *P. falciparum,* is mediated by the Cas9 endonuclease, shown in figure 1.6, that is guided by a single guide RNA (sgRNA) to cause double strand breaks in target sites (Lee *et al,* 2019; Lee and Fidock, 2014). The CRISPR/Cas based technique for *P. falciparum* was first reported by Wagner *et al,* (2014) and Ghorbal *et al* (2014) facilitated by the use of a guide RNA (gRNA), donor template and two-plasmid system for Cas9 delivery.

The CRISPR/Cas9 system evolved as a bacterial immune response against invading viruses and plasmids in which integration of foreign DNA within the CRISPR locus occurs. This foreign DNA is then used by the host to guide the direct sequence-specific degradation of related pathogenic DNA aided by the gRNA-Cas9 protein system. A protospacer-adjacent motif (PAM) (typically -NGG for the *Streptococcus* *pyogenes* Cas9) is also required immediately following the gRNA site in the target DNA for recognition (Gaj *et al,* 2016).

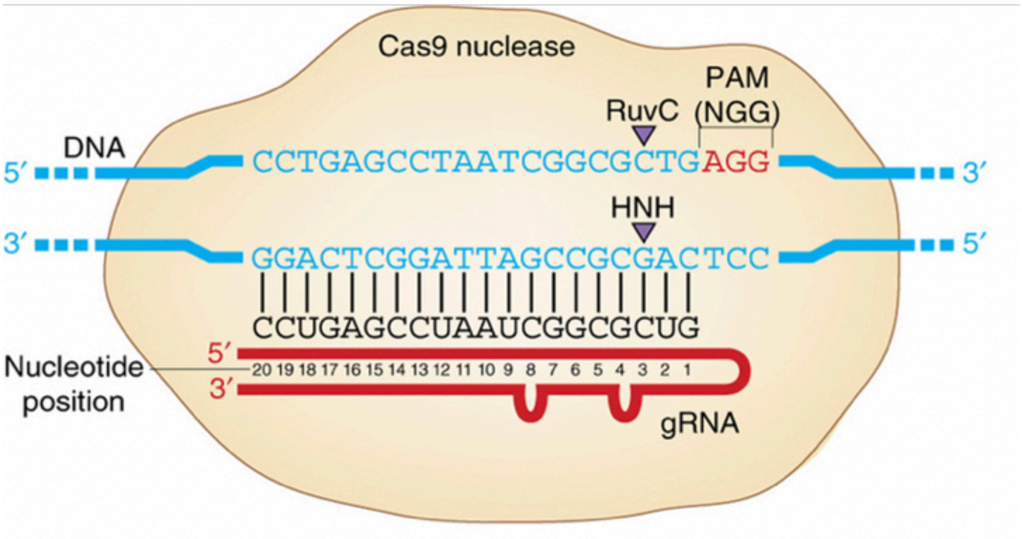


Figure 1.6 Cas9 nuclease. Cas9 nuclease-induced double strand breaks at DNA target sites are directed by complementarity base pairing to the 5′ end of a gRNA. The NGG PAM motif is not included in the guide sequence. (Adapted from Sander and Joung, 2014)

In the recent developments of genome editing in *P. falciparum* with the CRISPR/Cas9 system, the expression of the gRNA and Cas9 is driven by specific promoters, with all components supplied on transfection plasmids. Although modification of the nuclease is not required in the Cas9 system, unlike ZFNs and TALENs, factors to be considered in the experimental design include careful selection and design of the gRNA, which should conform to a 20-nucleotide sequence followed by a (-NGG) protospacer adjacent motif (PAM). Donor design is also crucial in the success of the CRISPR/Cas9 tool for *P. falciparum* editing, which is influenced by a diverse number of factors including length of homology region as well as size and distance of target point mutations to gRNA binding site (Lee *et al,* 2019). In a previous study by Ghorbal *et al* (2014), one of the two plasmids designed harboured the donor sequence and sgRNA cassette with a positive drug-selectable cassette h*dhfr* flanked by homology arms to generate a gene knockout. Another donor for introducing point-mutations was designed for kelch13, with the desired coding mutation as well as a silent mutation to prevent repeated cleavage of the edited locus. All target sites were edited including *Pfkelch13* mutation C580Y with no off-target activity (Ghorbal *et al,* 2014). The application of the CRISPR/Cas9 tool has greatly accelerated insight into studies of various aspects of *P. falciparum* gene investigations including drug resistance mechanisms and epigenetic studies for pathogenesis (Nasamu *et al,* 2021; Xiao *et al,* 2019; Bryant *et al,* 2017).

1.4 Next Generation Sequencing

The prospect of continuous monitoring and surveillance to track developing drug-resistant parasite populations in near real time has significantly enhanced the genomic surveillance and epidemiology of malaria parasites and has become a powerful tool in determining potential drug resistance threats (Kümpornsin *et al,* 2019). The field of genomics has been greatly enhanced by the availability of *de novo* sequencing platforms such as Next Generation Sequencing (NGS) which have been developed for greater genome coverage with higher throughput enabling the identification of crucial polymorphisms involved in *P. falciparum* drug resistance. For genome-scale sequencing, NGS approaches have a significantly lower cost compared to previous sequencing platforms such as Sanger sequencing. This was one of the widely used sequencing methods, that determined each sequence base with the use of deoxynucleotides (ddNTPs) and polymerase, using capillary sequencing since its development in 1970s (Cowell *et al,* 2018; Wang *et al,* 2012; Sanger and Coulson, 1975).

The NGS method developed by Illumina is characterised by the generation of short reads from DNA sequencing and is now the most widely used NGS technology.

Illumina sequencing, which is based on a cyclic reversible termination chemistry (Figure 1.7) is initiated with library preparation that involves the priming of a DNA template by a complementary sequence of the adapter region. Specific adapters are ligated on each DNA molecule and used as substrates cleaved to the double-stranded DNA (dsDNA) region shearing DNA into short length fragments (Goodwin *et al,* 2016). Cluster generation occurs in a glass-surfaced flow cell containing oligonucleotide sequences complementary to library fragment adapters. The single stranded DNA fragments hybridize to these oligonucleotides and go through repeated amplification cycles in a bridge amplification mechanism. This repeated process leads to generation of billions of clusters from clonal amplification of all fragments, with thousands of template copies generated in each cluster. Sequencing takes place by incorporation of fluorescently labelled nucleotides that contain a terminator sequence. These nucleotides that are labelled with a fluorophore specific to each DNA base are detected by light source excitation allowing parallel sequencing of identical fragments. The base calling process upon which alignment of short reads to a reference genome is performed, depends on emission wavelength and signal intensity. Both forward and reverse strands sequences are produced as end product in the Illumina platform (Del Vecchio *et al,* 2017; Slatko *et al,* 2018).

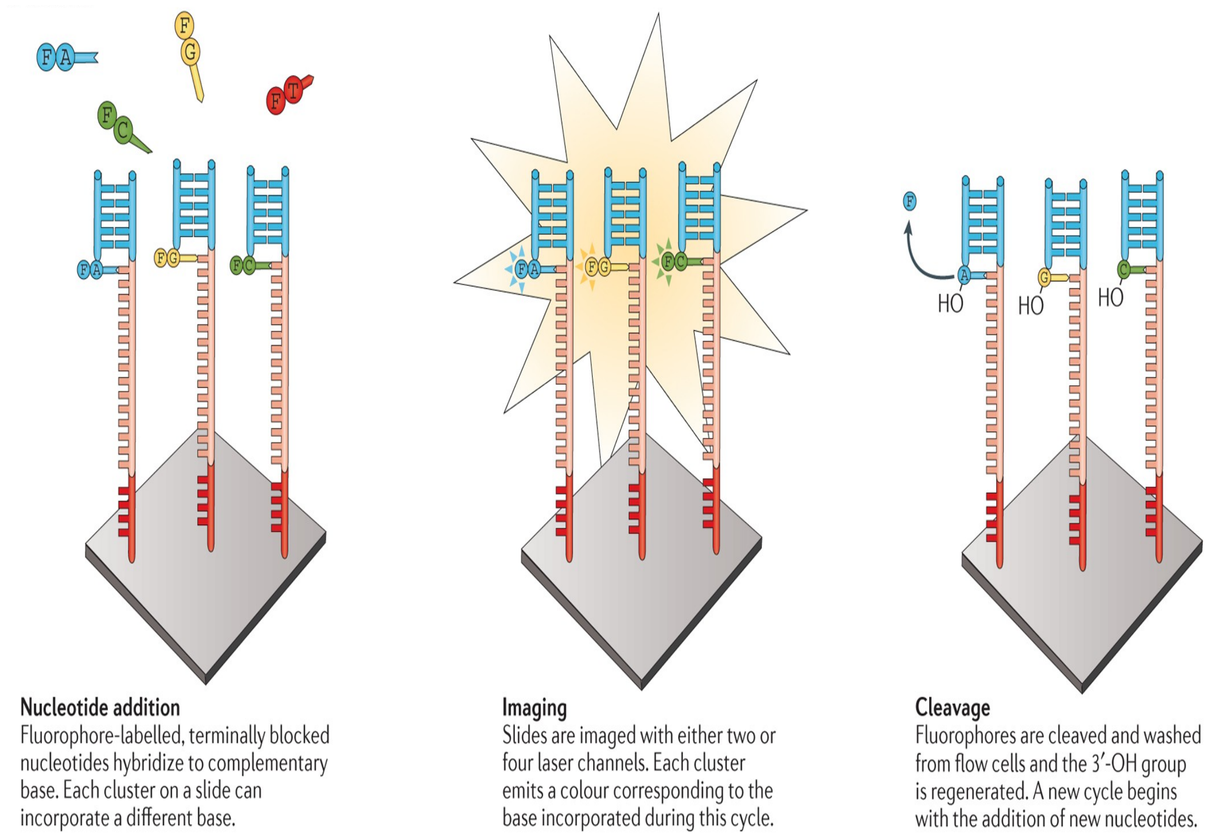


Figure 1.7 The Illumina cyclic reversible termination (CRT) system to determine DNA sequence. Template enrichment is followed by addition of primers, DNA polymerase and modified nucleotides to the flow cell with each nucleotide blocked by a 3′-O-azidomethyl group and labelled with base-specific, cleavable fluorophore (F). Incorporated bases are identified by laser excitation using two or four channels in multiple cycles of nucleotide addition, elongation and cleavage and unincorporated bases are washed away. This repetition of cycles results in determining sequences of the DNA molecules in each cluster. Figure from Goodwin *et al,* 2016.

1.5 Barcode Sequencing (BarSeq)

Barcode Sequencing (BarSeq), which involves the use of genome modified (molecular barcoded) parasites, facilitates high throughput phenotyping of *Plasmodium* parasites by simultaneously assessing multiple uniquely barcoded parasites in a single pool using Illumina NGS. The BarSeq approach has been applied in *P. berghei* to probe gene essentiality and phenotype growth measurements of gene knockouts (KO). Using a reverse genetics approach, pJazz-based *Plasmodium* Genetic Modification (PlasmoGEM) KO vectors were constructed with unique gene-specific 11 base pair molecular barcodes and annealing sites for binding of Illumina indexing primers (Schwach *et al,* 2015; Bushell *et al,* 2017). A high proportion of essential *P. berghei* genes and potential antimalarial drug targets were identified in this study by simultaneously phenotyping 2578 mutants using the BarSeq technique (Bushell *et al,* 2017).

BarSeq and Illumina NGS was used by Carrasquilla *et al*. (2020) as part of a study to accurately quantify the multiplicity of vector uptake in *P. falciparum* transfections in which unique *P. falciparum* transfection vectors were tagged with barcodes derived from the PlasmoGEM resource vectors (Carrasquilla *et al,* 2020). As part of M. Carrasquilla's thesis work, unique barcodes were also inserted into a pseudogene (*Pfrh3*) of *P. falciparum* parasites using CRISPR/Cas9-based genome editing, to allow growth measurements in a similar approach adapted from the previously developed *P. berghei* BarSeq technique (Carrasquilla, 2019). These studies highlight the potential of the combination of genome modification tools and amplicon sequencing by NGS that has significantly enhanced insights in investigations on the *Plasmodium* genome. These continuous advances are providing phenotypic, molecular and genomic information on *P. falciparum* that collectively are highly useful for malaria elimination programs (Nag *et al,* 2017).

1.6 Project Aims

My project is aimed at investigating:

I. How barcode tagging and sequencing approaches could be used to examine how genetic background of parasites affect fitness and resistance.

II. The impact of *Pfkelch13* mutations on parasite resistance and fitness.

III. Potential antimalarial activity of Human keap1 inhibitors on *Plasmodium falciparum*

Chapter 2: Methods

2.1 In vitro cultures of *P falciparum*

2.1.1 Thawing and Freezing of Parasites

Parasite stocks in glycerolyte kept in -80°C were thawed at room temperature and transferred to a 50mL falcon tube. Approximately 100uL of 12% NaCl was slowly added to the thawed parasites dropwise whilst gently swirling the tube to mix. Then 5ml of 1.6% NaCl was slowly added to the parasites whilst swirling and left to sit for 5 minutes before centrifugation at 1500rpm for 3 minutes. The liquid was aspirated and 5ml of 0.9% NaCl, 0.2% Dextrose was then slowly added dropwise to the pellet whilst swirling to mix and centrifuged at 1500rpm for 3 minutes. The pellet was resuspended in 5mL of complete media and supplemented with addition of fresh RBCs.

Parasites stocks were generated by freezing at parasitaemia of ≥ 3% with parasites mostly at ring stage. Parasite cultures were centrifuged at 2000rpm for 5 minutes and the media aspirated. The pellet was resuspended in 1ml of glycerolyte freezing solution (made up of 456 ml Glycerol, 16 g L-Sodium lactate, 300mg KCl, 516.6mg Monobasic sodium phosphate(monohydrate), 1242mg Dibasic sodium phosphate (monohydrate), 544mL MilliQ water, at pH6.8 with Sodium phosphate solution). The glycerolyte was added to the pellet dropwise whilst gently agitating and transferred to a cryovial and stored in -80°C immediately.

2.1.2 Parasite Culture Maintenance

Different *P. falciparum* parasite strains of various genetic background (Table 2.1) were maintained *in vitro* in routine culture adapted from Trager and Jensen (1976) for the experiments performed in this study. Parasites were routinely cultured with complete growth medium at a final haematocrit of 3% in O+ red blood cells (RBCs) obtained from healthy donors with informed consent from the National Health Services Blood and Transfusion service (NHSBT). Human RBCs were used in accordance with approval from the NHS Cambridgeshire Research Ethics Committee and the Wellcome Sanger Institute Human Materials and Data Management Committee. Parasites were kept in incubators at 37^{o}C and routinely supplied with a gas mixture with concentrations of 1% oxygen, 3% carbon dioxide and 96% nitrogen. The parasitaemia of all parasites in routine culture was kept at under 5% to keep parasites fit and growing. Parasitaemia of cultures was measured by making smears from 2uL of culture on glass microscope slides which were fixed with 100% methanol and then stained with filtered 10% Giemsa solution for 10 minutes. The dried smear was observed by light microscopy at 100X magnification and parasitaemia was obtained in percentage by counting the number of parasitized RBCs (iRBC) against the total RBCs in 10 fields.

Table 2.1 *Plasmodium falciparum* strains used for experiments performed in the project. The origin and year that the different strains were isolated and adapted into culture (Lee and Fidock 2016; Walliker *et al,* 1987; Green *et al,* 1985; BEI Resources, 2020; Heinberg *et al,* 2013).

Strain	Origin	Year Isolated
3D7	West Africa, Isolated in Netherlands (NF54 clone)	1970s
Dd2	Laos, Southeast Asia (W2 clone)	1982
FCR3	Gambia, West Africa	1976
Tanzania (200708)	Tanzania, Africa	2006
V1/S	Vietnam, Southeast Asia	1980
PH0212-c (CAM)	Pursat, Western Cambodia	2010

2.1.3 Red Blood Cells and Culture Medium

Complete growth medium used in the routine culture of parasites was prepared using Roswell Park Memorial Institute (RMPI)-1640 as supplement. RPMI-1640 containing Albumax was made to completion by supplementing 0.5M HEPES, 1X Glutamax and 25ug/mL gentamicin. Alternative supplements were made in the media preparation for drug assays and selecting transfections where the relevant drug was added to the growth medium.

Red blood cells of blood group O and rhesus positive (O+) were washed twice with growth medium by centrifugation at 3000rpm for 5 mins during each wash to pellet RBCs. RBCs were then re-suspended in an equal volume with growth medium at 50% haematocrit supplemented with 10% (v/v) citrate-phosphate-dextrose plus adenine (CPDA) to maintain fresh RBCs kept at 4^{o}C. RBC stocks for culture maintenance were replaced with a new batch of O+ blood on a weekly basis with every batch undergoing the same routine wash steps previously mentioned.

2.1.4 *Plasmodium* Stage Synchronisation

Loss of synchrony in *P. falciparum* erythrocytic stages occurs after a few life cycles *in vitro*, leading to asynchronous growth with the different asexual stages present. The elimination of distinct parasite stages by various parasite synchronisation methods are described to address these challenges and allow synchronisation of parasites (Ranford-Catwright *et al,* 2010).

Synchronisation aimed at the isolation of mature segmenting schizonts using a Percoll gradient was performed to enrich late trophozoites or schizonts (Ressurreição *et al.* 2020). A 63% (v/v) Percoll solution was made from neat Percoll in filter-sterilised 10X PBS and serum-free RPMI-1640 and pre-warmed. Parasite pellet from 3% haematocrit culture was collected by centrifugation at 900g for 5 minutes and resuspended in complete media to haematocrit of 30-40% and maximum volume of 5mL. The resuspended pellet was then gently layered onto 10mL of the Percoll cushion in a 15mL falcon tube avoiding mixture of the two phases. The Percoll gradient was then centrifuged at 1300g for 11 minutes with zero brake to obtain efficient gradient and separation of the stages. The thin brown band between the Percoll and media, containing the late stages, was carefully collected. The collected late stages were washed and resuspended in pre-warmed complete media and returned to culture. Cultures were assessed microscopically over the next few hours, and reapplied to a fresh Percoll cushion once substantial invasion of RBCs was seen. After centrifugation, the late stages and media were discarded, and the pellet consisting of uninfected RBCs and early rings was collected and sorbitol treated to further tighten synchronisation.

Synchronisation by enrichment of ring stages was done using the sorbitol lysis method originally described by Lambros and Vanderberg (1979) in which culture was treated with filter-sterilised 5% (w/v) sorbitol in water, killing the mature forms of the asexual stages. The infected RBC pellet was obtained by centrifugation at 2000rpm for 5 minutes in Falcon tubes. Parasite pellets were then resuspended in 10 volumes of pre-warmed 5% sorbitol and incubated for 10 minutes at 37^{o}C. Sorbitol was removed by decantation upon centrifugation at 1500g for 3 minutes followed by 2 wash steps with pre-warmed complete media centrifuged at 1500rpm for 3 minutes. The washed pellet was resuspended in pre-warmed complete media and adjusted to the appropriate haematocrit by addition of fresh RBCs to make up for RBCs ruptured with matured parasites.

2.2 Making a Pool of Barcoded Parasites

Six *P. falciparum* lines (Table 3.1), previously barcoded at the *Pfrh3* locus by Manuela Carrasquilla in her thesis work (Carrasquilla, 2019) and described in section 3.3.1, were maintained in culture and mixed to generate a pool with equal representations for barcode sequencing. Three independent mixes (pools) were made to conduct experiments in triplicate. The parasitaemia of each line was measured by flow cytometry using the CytoFLEX Flow Cytometer (Beckman Coulter). Staining of cells was done with both SYBR green and mitotracker deep red (ThermoFisher) so that live parasites can be distinguished from dead parasites for accurate counting. The haematocrit of each culture was measured using the cell counter (Cellometer auto 1000; Nexcelom Bioscience). To ensure that equivalent proportions for the different lines were added, the number of parasites per mL of each culture was calculated by multiplying the parasitaemia obtained on the flow cytometer by the number of cells per mL obtained with the cell counter. Equal absolute number of parasites for each strain was mixed and the parasitaemia of the pool was adjusted to 1% parasitaemia

Synchronisation of these pools were performed and continuous culture in the absence and presence of drugs at different concentrations, listed in Table 2.2, was carried out. Drug pressure was applied when parasitaemia was at least 1% over a duration of time. Parasitaemia was monitored and cells were harvested at intervals.

Table 2.2 List of compounds used for Barcode sequencing phenotypic assays and the range of concentrations. The pools of the different strains were grown in different concentrations of each these drugs in the range listed, in triplicates.

Drug	Concentration range (nM)
Chloroquine	20-500
Piperaquine	10-500
Mefloquine	10-500

2.3 Parasite Phenotypic Assays

2.3.1 Antimalarial Drug Sensitivity Assay

To assess parasite growth inhibition in the presence of drugs, standard malaria drug sensitivity assays were performed on parasites using the malaria SYBR Green I-based fluorescence (MSF) assay developed by Smilkstein *et al* (2004). Different concentrations of the relevant drugs (Table 2.3) were prepared in a two-fold serial dilution or 3-fold serial dilution in a 96 well tissue culture plate format accommodating 10 dilution points of each drug as shown in figure 2.1. Parasites in routine culture were prepared by diluting with uninfected RBCs to a haematocrit of 2% and parasitaemia of 1% and assessed in duplicates for each dilution point. A column of no drug and uninfected RBCs of 2% haematocrit were included in the plate as control wells which were used for the calculation of parasite growth inhibition as well as growth medium only wells as control for background fluorescence. 50uL of each of the drug concentration dilutions done with growth medium was added to each well except the uninfected RBCs wells and the no drug wells. 50uL of prepared parasite culture was added to all the wells except the media only wells containing 100uL of media. The assay plates containing the drug suspension and parasites with a total volume of 100uL in each well were then put into routine culture gas and incubation conditions previously described for 72 hours.

Table 2.3 List of compounds used for 72-hour drug assays to obtain inhibitory concentrations and the range of concentrations used in the serial dilution for drug plate set up. (A) Drug concentration used for Barcode Sequencing experiments (B) Compounds and concentration used in testing *P. falciparum* parasites with Keap1-Kelch inhibitors

A

Drug	Concentration range (nM)
Chloroquine	0-5000
Piperaquine	0-3000
Mefloquine	0-600
Dihydroartemisinin	0-100

B

Drug	Concentration range (µM)
KI696	0-250
RA839	0-500
ML334	0-500

2.3.2 SYBR Green Assay

The drug assay plates were removed from routine culture conditions after 72 hours. A 2X SYBR lysis buffer with SYBR green stain was prepared for the assessment of parasite growth. The lysis buffer containing 10mM Tris pH 7.5, 3mM EDTA, 0.1% saponin solution and 2% Triton X-100 solution was used to make a 1:5,000 dilution of the 10,000X stock SYBR Green to 2X. 100uL of this solution was added to the 96 well drug-parasite plate, and then incubated for 30 minutes at 37^{o}C in the absence of light. Fluorescence from stained DNA in parasitised RBCs correlating to growth was measured on the FLUOstar Omega microplate reader (BMG Labtech) using a 485-nm excitation filter and a 535-nm emission filter. Parasite growth and inhibitory concentration (IC50) values were determined by results derived from the analysis of drug assays reads as well as statistical tests done on GraphPad Prism.

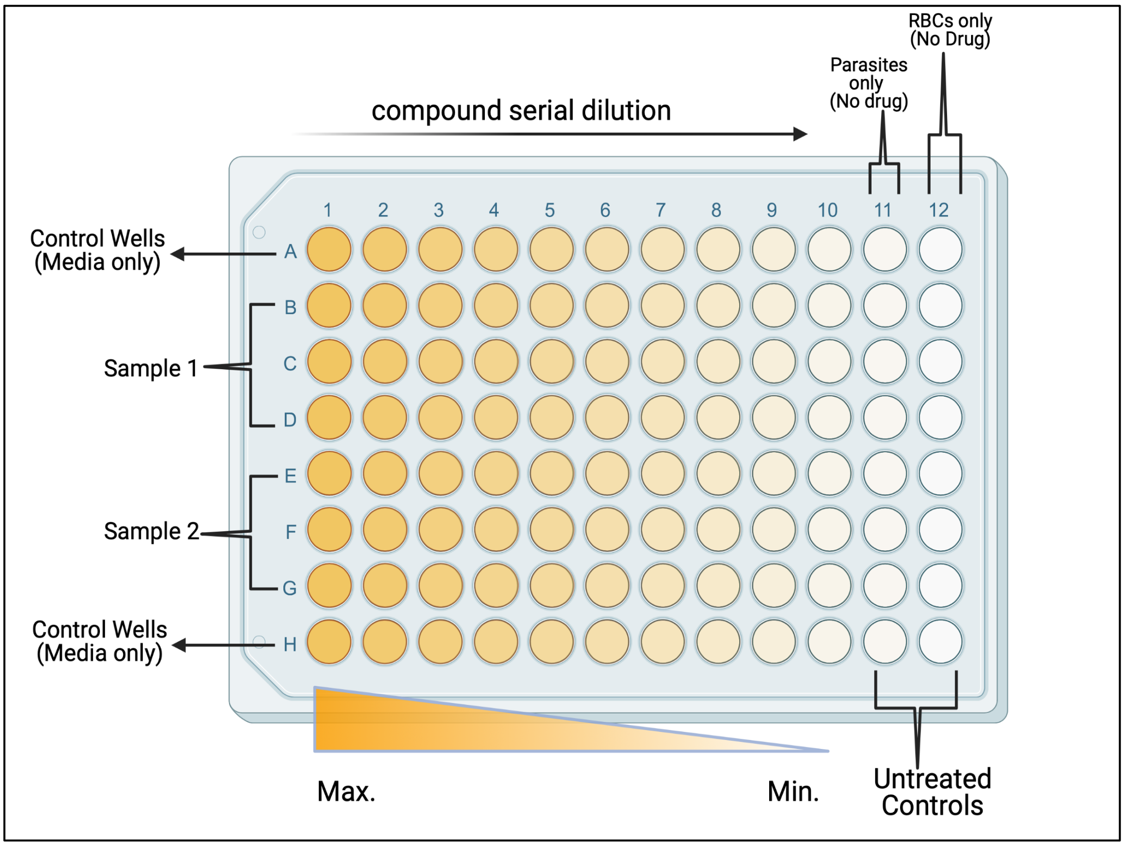


Figure 2.1 Plate layout up for drug sensitivity assays. Sample are loaded in triplicate. Maximum concentration of the selected drug is loaded on column 1 and a 2-fold serial dilution per column is done up to column 10. Column 11 loaded with untreated parasites and column 12 loaded with RBCs only are the negative controls used for the analysis of the dose response curve.

2. 4 Transfection of *P. falciparum* by Electroporation of Infected Red Blood Cells

Standard episomal transfections were carried out for CRISPR/Cas9 genetic editing of parasites listed in table 2.1 using methods adapted from (Fidock and Wellems, 1997). Parasite cultures at >5% parasitaemia were harvested and pelleted by centrifugation of 1500pm for 3 minutes to discard growth medium. <150uL pellet was resuspended in a volume of cytomix that was equal to volume of harvested culture. The suspension was centrifuged and the supernatant discarded. The parasite pellet was resuspended in cytomix with 50ug of pooled plasmid DNA, described in 2.5.3, 2.5.4 and table 4.1, in a total volume of <450uL. The suspension was loaded into

0.2cm Biorad electroporation cuvettes and cells were electroporated at 0.31 kilovolts (Kv), 950 microfarad (μF) and maximum capacitance using the BioRad Gene Pulser Xcell electroporator and immediately resuspended in complete growth media followed by a 1-hour incubation to allow cells to recover. The suspension was then washed and resuspended in complete media with fresh RBCs added and put in routine culture conditions previously described. Transfected cells were continuously cultured in complete media with WR99210 drug at 5nM for Dd2 and 2.5nM for FCR3 and Tanzanian strains, for selection at day 1 of post-transfection. Transfected parasites were continuously cultured in the presence of the WR99210 until recovery of parasites occurred and routinely monitored by microscopy at least twice a week.

2.5 Molecular Assays

2.5.1 Genomic DNA Extraction from Parasite Cultures

Parasites cultures were harvested for extraction of genomic DNA (gDNA) using 5 to 10mL of culture volumes, at 3 to 5% parasitaemia and 3% haematocrit. Parasite cultures were centrifuged and the pellet was lysed by resuspension in 0.15% saponin in PBS and incubated for 5 minutes at room temperature. Washing of the lysed cells was done twice with 10mL of sterile PBS and gDNA extraction was carried out directly from the pellets using Qiagen's DNeasy Blood and Tissue Kit. The pellet was first resuspended in 200μL PBS and incubated at 56^{o}C for 10 minutes following addition of 20μL of proteinase K and 200μL of lysis buffer AL. The sample was applied to the column, which was washed twice with 500μL buffers AW1 and AW2 respectively provided in the kit and eluted in 50μL of Elution Buffer. The gDNA was stored in -20˚C for subsequent library preparation for Barcode Sequencing

2.5.2 Barcode Sequencing

To amplify barcodes for library generation for Illumina sequencing (MiSeq), a three-step PCR was performed on gDNA isolated from the cultures using CloneAmp 2X Mix (CloneAmp™ HiFi PCR Premix, 639298), nuclease-free water and relevant primers at 10µM, as described below. The PCR was done in a total reaction volume of 25µL for 30 cycles of 98°C for10 s, 55°C for 15 s, 72°C for 45 s, finishing at 4°C. A water-only control was included in all PCR runs to check for potential contamination of reagents. An aliquot of all PCR products was separated on a 2% agarose gel to confirm amplification of the barcode amplicon, and the remainder purified using AMPure SPRI paramagnetic bead-based chemistry (Beckman Coulter). An initial PCR was done using primers p191/p194 for amplification of outside the homology arms of pfrh3 to ensure amplification of only integrated barcodes. A second PCR was performed on the purified products using adapter primers p1356/p1357 (150bp) for BarSeq at 30 cycles of 98°C for10 s, 55°C for 15 s, 72°C for 45 s, finishing at 4°C in 25uL reaction volume. A third PCR was performed to introduce Illumina indexes using forward primers i501 or i502 (Paired-end 1.0 from Illumina, p2085/p2086) and Illumina index primers (1-96, p1359-p1454) listed in chapter 3. The reaction was performed for 10 cycles of 98 °C/10 s, 55 °C/15 s, 72°C/15 s, finishing at 25°C in a 10uL reaction volume. These products from this PCR were quantified using the PicoGreen dsDNA quantitation fluorescence assay kit by Invitrogen in a 96 well plate format. Fluorescence was measured using the FLUOstar Omega microplate reader and diluted to 4nM for Illumina sequencing.

2.5.3 Deconvolution of Plasmid Pool for Plasmid Selection

Plasmids used in this project were selected from a complex pool of pDC2-CAM-Cas9-U6-hDHFR plasmids containing a codon-optimised Cas9 under the control of the *P. falciparum* calmodulin promoter and a single guide RNA (gRNA), expressed from the U6 promoter, designed to target *Pfkelch13* at position 580 as shown in figure (2.3.1). The plasmids were generated by a former PhD student, Manuela Carrasquilla (PhD, 2019) as a complex pool with up to 64 different Pfkelch13 donors, with each plasmid differing by a single codon at position C580 of the locus. As part of my project, I intended to generate defined sub-pools of these plasmids, and thus needed to isolate individual plasmids. Deconvolution of this complex pool of plasmids with a common pDC2 backbone encoded with 64 possible codons, was carried out in order to isolate each possible individual donor with the aim of constructing sub-pools of donors with only viable amino acids that has been previously seen to be more efficient in replacing cysteine at position 580. The bacterial glycerol stock of the complex plasmid pool was grown overnight and then several dilutions were plated to isolate single colonies. Approximately 96 single colonies were picked and cultured overnight in 37^{o}C in 96 deep well plate in NZY + Ampicillin at 240rpm illustrated in figure 2.5 Minipreps of these individual colonies were carried out using the standard protocol as per the QIAprep 96 Plus Miniprep Kit. Sanger sequencing with primer p282 was performed on the isolated plasmid DNA.

2.5.4 DNA Isolation of Plasmid with single codons

Chromatograms from the Sanger sequencing results was used to identify plasmids with single codons at position 580 encoding for the amino acids of interest described in chapter 4. Plasmids harbouring these codons were further expanded into bigger bacterial cultures in 200mL of Luria-Bertani (LB) broth + Ampicillin (100µg/ml) and cultured overnight. Midipreps were performed using the manufacturer's protocol (NucleoBond Xtra Midi/Maxi) kit by MACHEREY-NAGEL. The final plasmid pellet was eluted in sterile cytomix and quantified using the nanodrop and then stored in -20˚C for use in transfections of parasite cultures.

2.6 Data Analysis

The data generated from the standard drug assays was analysed on GraphPad Prism and curves were fit using the non-linear regression model (variable slope).

For barcode sequencing of parasite lines, the barcodes, day, assay condition and the barcode read counts were included in the sequencing output file. The barcode proportion was used to measure the abundance of each line in a pool, and the change in barcode proportion over time used to measure the growth rate of each parasite over time, either in the presence or absence of drug. The BarSeq data presented in section 3.3.4 was analysed on GraphPad Prism to plot the relative growth curves of the parasites and analysis of variance (ANOVA) with Tukey test was performed to compare the mean difference of the change in barcode proportion over time across the different parasites.

Chapter 3: Assessing *Plasmodium falciparum* fitness and antimalarial resistance using Barcode sequencing

3.1 Summary

The aim of this chapter is to assess how barcoded parasite lines could be used to measure the fitness and antimalarial resistance of *P. falciparum*. To investigate how the genetic background of parasites affect these phenotypes, I used barcoded *P. falciparum* strains from different geographic locations, described below, in pooled growth competition assays. Parasite growth in the presence and absence of drugs was measured over time and each barcode proportion in the pools were measured. These parameters were measured at different time points using Barcode sequencing (BarSeq).

3.2 Background

3.2.1 *Plasmodium falciparum* genetic background, fitness and resistance

Increasing artemisinin resistance remains a major challenge in malaria treatment but despite this being a widely studied area there is not yet an established understanding of the mechanism. However, point mutations in *Pfkelch13* are associated with resistance, with one of the major *PfKelch13* mutations, C580Y, linked with decreased haemoglobin uptake by the endocytic machinery (Birnbaum *et al,* 2020; Sharma *et al,* 2020). Mutations in *Pfcrt,* the determinant of chloroquine resistance, are now also associated with resistance to other antimalarials currently in use and in development such as piperaquine (Martin, 2020). The association of parasite fitness, that is the parasite's ability to survive and replicate to the next generation, with drug resistance has been reported in various studies. The presence of acquired mutations leading to decreased susceptibility of *P. falciparum* to antimalarials has been observed to negatively affect parasite fitness in the absence of drug pressure (Froberg *et al,* 2013).

The fitness cost of *P. falciparum* incurred by resistance-associated mutations has been observed with polymorphisms of different genes including *pfmdr1, pfcrt and* other genes, in various endemic locations. This has led to a drastic decrease in prevalence of resistant parasites over time in the case of chloroquine where the drug had been withdrawn from malaria treatment for a long period (Hayward *et al,* 2005; Nursing *et al,* 2009; Fohl and Roos, 2005; Kublin *et al,* 2003). However, observations of enhanced fitness in the presence of acquired drug resistance associated mutations as a result of strong selective pressure have been reported in other studies (Gabryszewski *et al,* 2016). The co-existence and interaction of these multiple genes in epistasis can confer varying phenotypic outcomes. This can also be distinctly impacted by parasite genetic backgrounds that can demonstrate varying tolerance to resistance-associated polymorphisms. Thus, understanding the aspects of parasite genetic backgrounds and co-segregating compensatory mutations is vital. This can have varying impacts on the observed fitness costs of artemisinin resistant mutations which is important in understanding mechanisms of resistance to artemisinin and partner drugs in ACTs (Tirrell *et al,* 2019; Amato *et al,* 2018).

3.2.2 Measuring *Plasmodium falciparum* fitness

Various approaches that are based on parallel comparison of different strains are applied in assays to evaluate resistance and fitness of *P. falciparum*. The quantification of different protein markers, metabolic products or DNA that are overall indicators of parasite biomass are mostly relied on in current in vitro phenotypic assays. This is aided with the use of intercalating fluorescence dyes or radioactive markers, microscopic assessment of parasite development and detection of parasite specific proteins (Molnar *et al,* 2020). In most studies a maximum of two lines are mixed in head-to-head competition assays in multiple experiments performed in parallel with two competing parasite lines compared in each experiment.

Several studies have assessed parasite growth and resistance by fluorescence microscopy and quantitative analysis using genetically edited *P. falciparum* lines labelled with fluorescent and bioluminescent proteins such as Green Fluorescent Protein (GFP) and mCherry (Marin-Mogollon *et al,* 2019; Mbengue *et al,* 2015; Istvan *et al,* 2019). A study by Gabryszewski *et al* (2016) utilised wild type and transgenic Dd2 and Cam734 lines edited with *pfcrt*-specific zinc-finger nuclease in co-culture assays with a GFP-tagged wild type competitor line. This study revealed the impact of *pfcrt* mutations on parasite physiology and haemoglobin catabolism, and the capacity of compensatory mutations in neutralizing parasite fitness costs and associated drug resistance impact (Gabryszewski *et al,* 2016).

Recent studies have made use of growth competition assays with sequencing to assess parasite fitness and resistance *in vitro*. In a study by Straimer *et al* (2017) assessing the impact of *Pfkelch13* mutations on ozonide susceptibility and parasite fitness, pyrosequencing was used to determine growth rates of *Pfkelch13* mutant lines i*n vitro*. The fitness costs conferred by the resistance-associated polymorphisms in genetically edited resistant mutants was validated in this study (Straimer *et al,* 2017). A similar approach was performed in another study by Nair *et al,* (2018) investigating fitness outcomes in *Pfkelch13* mutations, that utilised genome editing strains in competition assays and deep sequencing. This study validated the higher fitness cost of C580Y mutation compared to the R561H *Pfkelch13* allele (Nair *et al,* 2018). However, one limitation of these co-culture methods is that only two lines, the test line and control competitor, can be examined at once.

Drug sensitivity assays remain a crucial aspect in surveillance and monitoring of drug efficacy and resistance, and for the identification of important resistance-associated molecular markers (Dhingra *et al,* 2019). These antimalarial drug susceptibility techniques are mainly based on development and maturation of parasites in the presence of a titration of drug concentrations for which several methods have been developed and measured *in vitro* or *ex vivo* (Maji, 2018). An essential basis for drug discovery by mimicking the *in vivo* situation is facilitated by application of *in vitro* drug sensitivity assays of parasites that allows the effective penetration of compounds inside cellular membranes of parasites to a measurable level (Sinsha *et al,* 2017). Assays that have been developed to specifically target the ring stage of the parasites include the ring-stage survival assay ($RSA^{0\text{–}3\,h}$) that measures parasite survival rates after a short-term exposure to artemisinin. This method, which aims to simulate the short half-life of artemisinin in vivo, has been vital in determining delayed parasite clearance linked with *Pfkelch13* and the current goal standard for identifying artemisinin resistance (Niaré, *et al,* 2018; Witkowski *et al* 2013). For the majority of antimalarials, however, standard 48-hour or 72-hour drug sensitivity assays are widely used to monitor overall drug susceptibility phenotype of *P. falciparum* parasites for current antimalarial drugs (Chaorattanakawee *et al,* 2015). Understanding drug resistance dynamics and evolution patterns in a competitive environment that is crucial for drug discovery and surveillance, is determined by measuring parasite fitness and drug resistance experiments in which *in vitro* assays are mostly performed (Rosenthal *et al,* 2013).

In this chapter, I assessed a method to capture both the fitness and drug sensitivity profiles in genetically edited strains of different genetic backgrounds. These lines had been CRISPR-edited with unique molecular barcodes, allowing their co-culture and quantification using barcode sequencing (BarSeq) by next generation sequencing.

3.3 Methods and Results

3.3.1 Making a Pool of Barcoded Parasites

Six *P. falciparum* lines with unique barcodes were previously generated by Manuela Carrasquilla (PhD thesis, 2019), as described in section 2.2. The thawed lines were maintained in culture until growing well and then mixed to generate a pool with equal representations for barcode sequencing. The lines were each edited with a unique barcode inserted at the *Pfrh3* locus, which is a non-essential gene that does not have any impact on parasite growth (Duraisingh *et al,* 2002). Three independent mixes (pools) were made to perform experiments using the barcoded *P. falciparum* strains (Table 3.1) of different genetic backgrounds with varying drug resistance profiles. The parasitaemia of each line was measured as described in section 2.2. The individual barcoded lines were synchronised as described in 2.1.4 and equal proportions of each were added in each pool following the descriptions in section 2.2. The parasitaemia of each pool was adjusted to 1% parasitaemia and synchronisation of the pools done in 50mL culture flasks. The co-cultures of the independent pools were maintained in the absence and presence of chloroquine, mefloquine and piperaquine at different concentrations as described below and samples were harvested on different days over an 18-day period. Drug pressure was applied when parasitaemia was at least 1% and maintained for the duration of the experiment.

Cultures maintained in the absence and presence of drugs were adjusted to a 1% parasitaemia after collection of 10mL that was then lysed for DNA isolation and library preparation. Parasitaemia was monitored and cells were harvested at intervals on different days. Genomic DNA was extracted from harvested parasites as described in section 2.5.1.

3.3.2 Generating libraries for Illumina Sequencing

To amplify barcodes for library generation for Illumina sequencing (MiSeq), a nested PCR reaction (Figure 3.1) was performed on gDNA isolated from the harvested cells using reagents and cycling conditions described in section 2.5.2. A water-only control was included in all PCR runs to check for potential contamination of reagents. An aliquot of all PCR products was separated on a 2% agarose gel to confirm amplification of the barcode amplicon, and the remainder purified using AMPure SPRI paramagnetic bead-based chemistry (Beckman Coulter). An initial PCR was performed using primers p191/p194, producing a 3kb product (figure 3.2 A) that extends outside the homology arms of the original *Pfrh3* editing donor, to ensure amplification of only integrated barcodes and not episomal donors. A second PCR described in section 2.5.2 was performed on the purified 3kb products to generate a short 150bp amplicon, using the primers p1356/p1357 (figure 3.2 B). Illumina indexes were introduced in a third PCR using forward primers i501 or i502 (p2085/p2086) and Illumina index primers (1-96, p1359-p1454) with conditions previously described in section 2.5.2. The libraries generated from this PCR were purified and diluted to 4nM for Illumina sequencing. BarSeq samples were multiplexed in one MiSeq run.

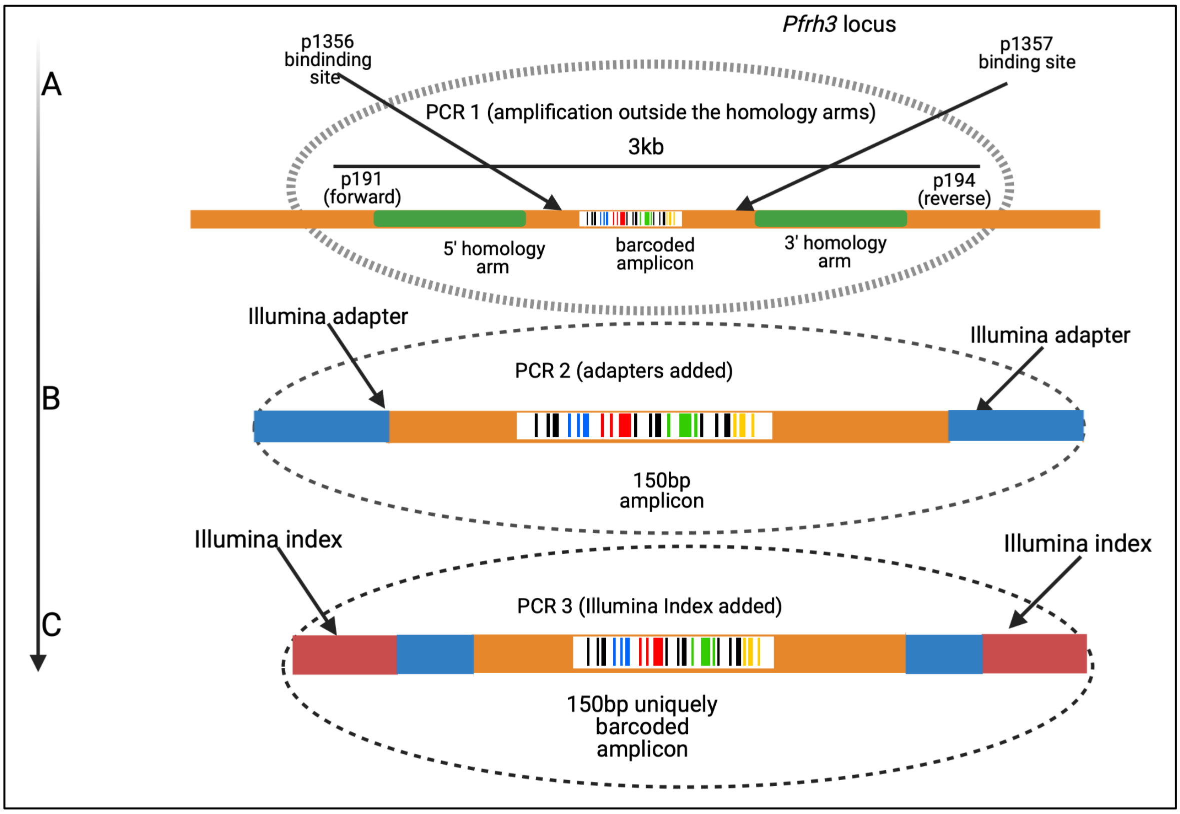


Figure 3.1 Library generation workflow for Barseq A) The 3kb product from the first PCR using primers 191/194 that extends outside the homology region to avoid episomal amplification. **B)** A short 150bp amplicon is produced from the second PCR using primers p1356/p1357 that flank the barcode. **C**) Illumina adapters added in the third PCR using i501 or i502 (p2085/p2086) and Illumina index primers (1-96, p1359-p1454).

Table 3.1 *P. falciparum* strains used in BarSeq assay. Each clone is barcoded with a unique sequence (barcode ID) from chloroquine-sensitive and multidrug-resistant strains of different backgrounds (Pinheiro *et al,* 2018).

Strain	Clone	Barcode Sequence	Resistance Profile
3D7	A	TAGCAGAAGTT	Sensitive
3D7	B	TAATAGGGAGC	Sensitive
3D7	C	GTCTCCGTATT	Sensitive
V1/S	A	TCTCTGAATCA	Chloroquine, pyrimethamine
V1/S	B	GACATGGGATT	Chloroquine, pyrimethamine
PH0212-c(CAM)	A	GTGAGAGTATC	Artemisinin, pyrimethamine, chloroquine

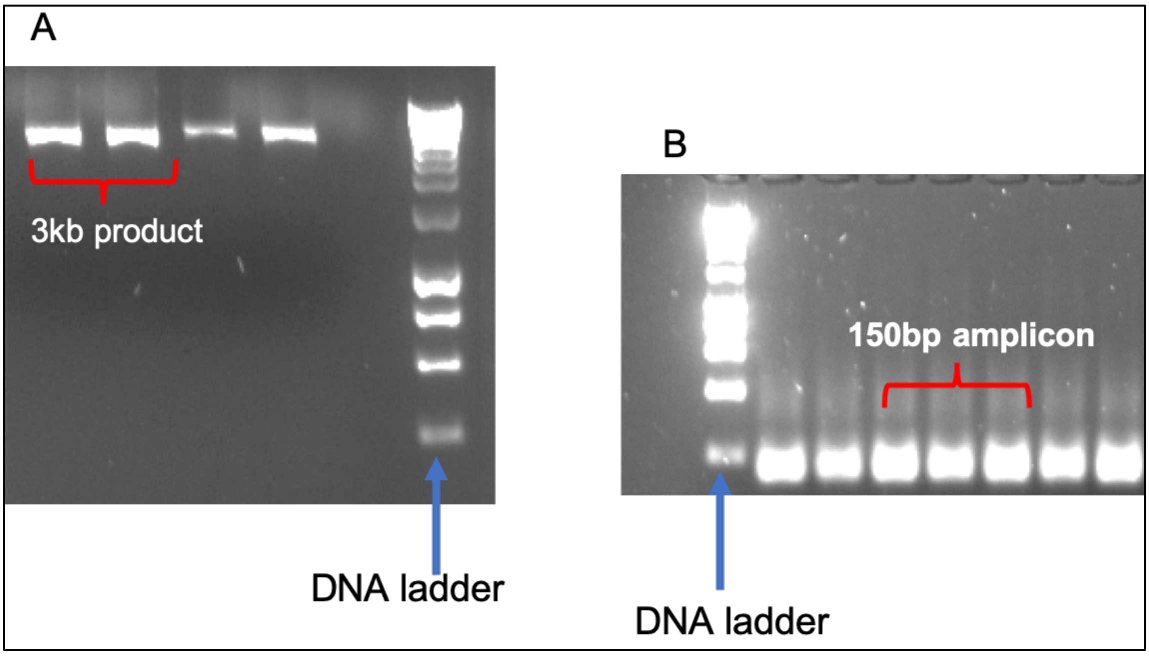


Figure 3.2 PCR products from the nested PCR in Illumina library generation. A) The 3kb product from the first PCR using primers 191/194. B) The 150bp amplicon produced from the second and third PCR described above. 1kb DNA hyperLadder (Bioline) was loaded alongside products.

3.3.3 Drug sensitivity assays to determine drug concentrations for BarSeq

One of each strain (3D7, V1/S and CAM), were used to perform standard 72-hour drug sensitivity assays to determine IC_{50} values of the different drugs listed in Table 2.2. The drug sensitivity assays were performed in biological replicates following methods described in section 2.3.1 and 2.3.2 to determine the concentration range of the drugs used in *in vitro* assays for the BarSeq assays. The standard inhibitory curves are shown below in figure 3.3. As expected, there was a >10-fold shift in IC_{50} for chloroquine between the sensitive 3D7 strain and the resistant V/1S and CAM strains. In contrast, there were smaller differences in IC_{50} fold-change across strains for mefloquine and piperaquine.

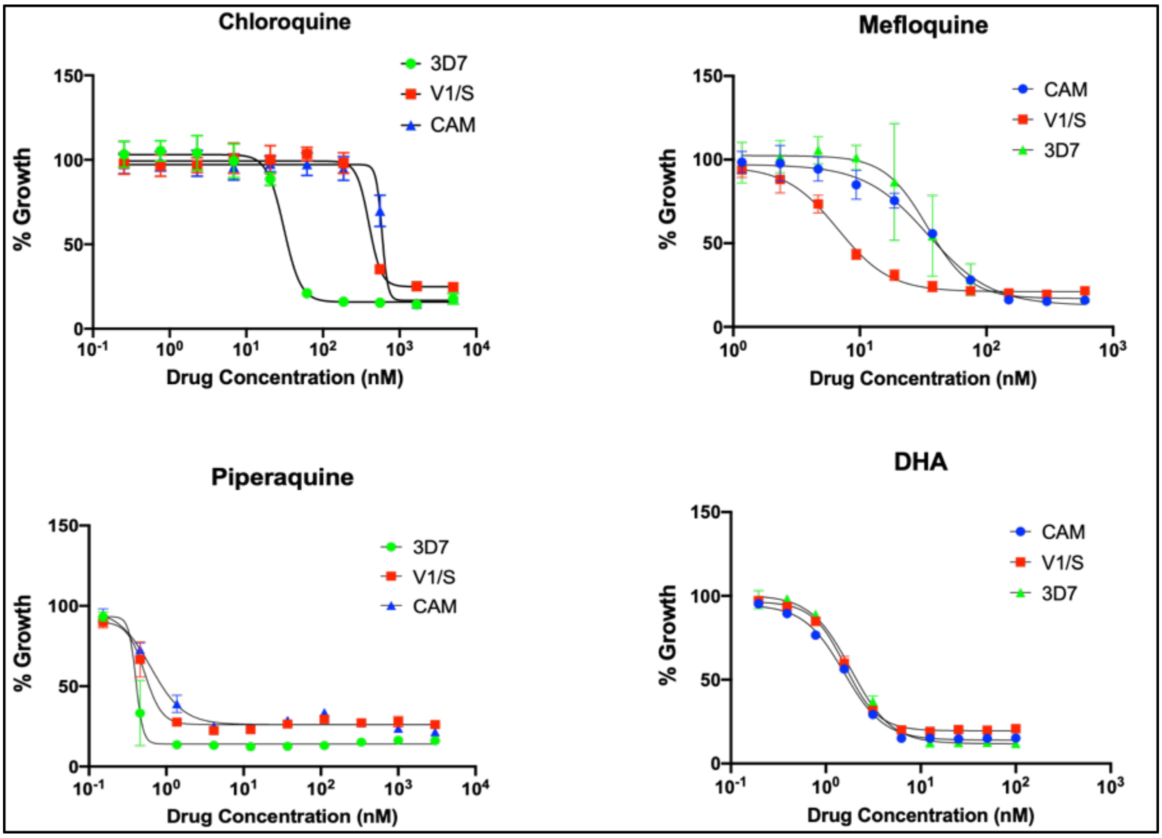


Figure 3.3 Standard dose response curve for chloroquine, mefloquine, piperaquine and DHA. Dose response curve for strains used in competition assays. IC50 curves obtained from assays performed in triplicate. The IC_{50} values are shown in table 3.2 below

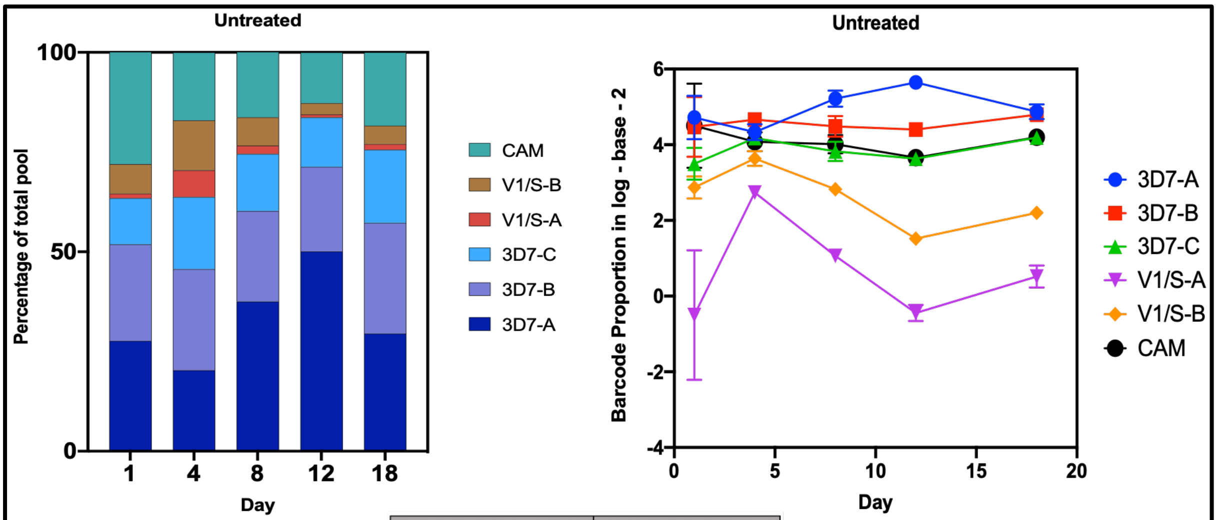
Table 3.2 The IC_{50} values obtained for chloroquine, mefloquine, piperaquine, DHA. Standard 72-hour drug assays were performed and the mean IC_{50} values and standard deviation from three biological replicates of each drug.

Drug	3D7 IC50 (nM)	V1/S IC50 (nM)	CAM IC50 (nM)
Chloroquine	23.2 ± 6.8	332.4 ± 57.2	576.4 ± 64.3
Mefloquine	32.7 ± 4.8	7.2 ± 3.8	29.2 ± 5.2
Piperaquine	0.4 ± 0.03	0.9 ± 0.7	2.7 ± 3.3
DHA	1.9 ± 0.2	1.6 ± 0.6	2.1 ± 1.7

3.3.4 Drug resistance phenotypes determined by BarSeq

To determine if the BarSeq co-culture method could detect differences in fitness and drug resistance phenotypes across strains *in vitro*, competition assays were carried out in triplicates as described in section 3.3.1.

In the absence of drugs, in normal culture conditions, the 3D7 and CAM strains moderately outcompeted the V1/S strains in an 18-day co-culture, although both V1/S lines were present at a lower initial proportion in the pool (figure 3.4). The growth rates of the two independently barcoded V1/S clones (V1/S-A and V1/S-B) was similar across the 18 days co-culture. Similar growth rates were also observed across the independent internal replicates of 3D7 clones (3D7A-C). One-way analysis of variance (ANOVA) with Tukey test was performed using the barcode proportion change over time to determine the mean difference between strains. There was a significant difference between V1/S and the other strains. No significant difference was observed between 3D7 and CAM when the barcode proportion change was compared across all three strains over time.



Comparison	Adjusted P-value
3D7 vs. V1/S-A	<0.0001
3D7 vs. V1/S-B	<0.0001
3D7 vs. CAM	ns
V1/S-A vs. CAM	<0.0001
V1/S-B vs. CAM	0.0414

Figure 3.4 Growth rates of all strains over 18 days in absence of drug. Competition assay of three strains with two internal replicates for V1/S and three internal replicates of 3D7 each uniquely barcoded. The percentage of each barcode in the total pool at different days shown in the left bar graph and log barcode proportion over time shown in the right plot. The statistical difference of barcode proportion changes over time across the different strains using one-way analysis of variance (ANOVA) with Tukey test is shown in the bottom panel.

To examine how the presence of different drug selections impacted growth, the pool was exposed to different concentrations of chloroquine, mefloquine and piperaquine. Drug resistance phenotypes were determined in triplicate cultures using BarSeq as described above. The relative growth of each barcoded line in the presence of different concentrations of chloroquine is shown in figure 3.5. The growth curves are shown as the log proportion of each barcode over time. The range of chloroquine concentrations was selected to start at the equivalent of approximately 1X the IC_{50} of 3D7 (20nM) up to the approximate 1X IC_{50} of the CAM strain (500nM). The differences in drug IC_{50} correlated with the phenotypes observed by BarSeq showing a significant difference in the means of barcode proportion change between the sensitive and chloroquine resistant strains, p-values obtained by performing ANOVA with Tukey to measure differences across strains is shown in table 3.3. There was no significant difference between the internal 3D7 clones at exposure to all different concentrations and between the internal V1/S lines across the concentration range of 1X IC_{50} to 10X. A significant difference was observed between the 3D7 and both V1/S and CAM strains at the lowest concentration of chloroquine of 1X IC_{50}. Between the two resistant strains, V1/S demonstrated intermediate tolerance with increasing chloroquine doses compared to the CAM strain that showed a higher tolerance even at 5X and >10X IC_{50}. The 3D7 strain showed relative sensitivity to chloroquine at the lowest concentration of 1X IC_{50}, as expected. At higher doses of chloroquine of up to >10X IC_{50} of 3D7, the CAM strain maintains a strong relative growth, consistent with the high IC_{50} for this strain demonstrated in the standard drug assays (Table 3.2).

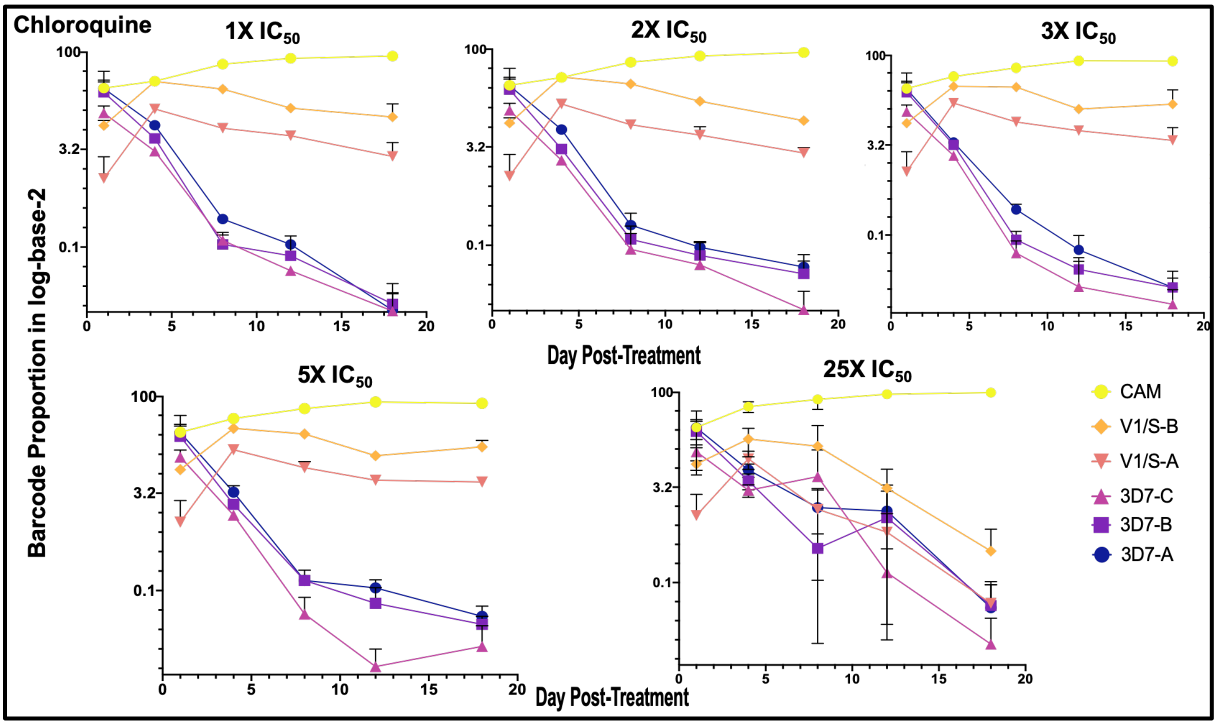


Figure 3.5 BarSeq of barcoded clones of *P. falciparum* strains with increasing concentrations of chloroquine. Competition assays of barcode pools in the presence and absence of chloroquine at different concentrations. The barcode proportion in the total count at every time point is represented on the y-axis

Table 3.3 Mean comparison of barcode proportion change across strains exposed to different chloroquine concentrations. The statistical difference between the change in proportion relative to day 1 of the different strains in presence and absence of chloroquine. ANOVA with Tukey test showed significant difference between chloroquine sensitive and resistant strains in the presence of CQ. Non-significant (ns) difference between groups is $p>0.05$. There was no significant difference between CAM and V1/S

Mean Comparison	Chloroquine concentration (Adjusted P-value)					
	Untreated	20nM	50nM	60nM	100nM	500nM
3D7-V1/S-A	ns	0.0005	0.0004	0.0002	0.0002	ns
3D7-V1/S-B	ns	0.0025	0.0019	0.0036	0.0010	0.0414
3D7-CAM	ns	0.0021	0.0018	0.0013	0.0009	ns

Based on the IC_{50} value obtained for mefloquine in the standard drug assays, the BarSeq assays for mefloquine were set up with concentrations ranging from a sublethal concentration for 3D7 to >10xIC50. The relative growth curves from the proportions of each barcode are shown in figure 3.6. Contrary to results obtained for chloroquine, V1/S showed a sensitive phenotype even at low concentrations of mefloquine and was outcompeted by the other strains. The 3D7 and CAM strains showed higher tolerance to mefloquine than V1/S at sub-lethal doses which correlates with the differences in drug IC_{50} values for mefloquine. A relatively unchanging barcode proportion over time was observed across the different strains at >10X $1C_{50}$, and the assay was stopped at day 8 due to the cultures not growing. This reflects the amplification of DNA present from dead parasites and does not represent genuine growth change. The statistical summary of ANOVA with Tukey test for mean comparison across groups is shown in table 3.4.

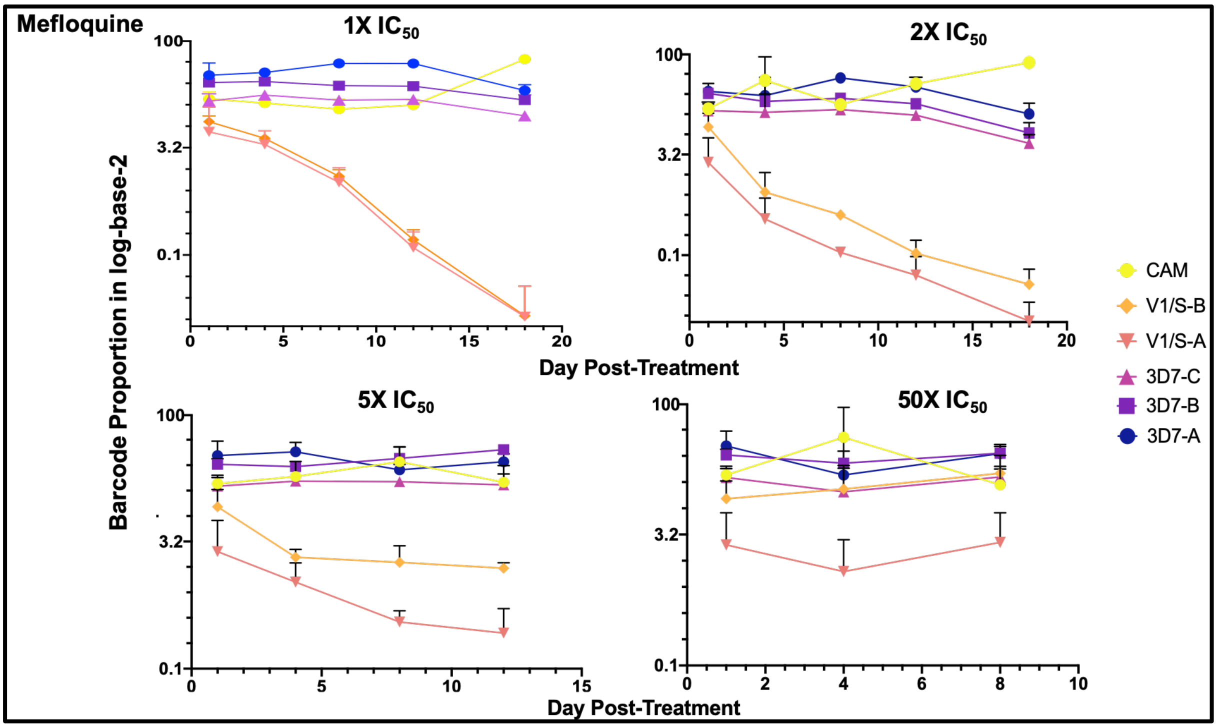


Figure 3.6 BarSeq of barcoded clones of *P. falciparum* strains with increasing mefloquine concentrations. Competition assays of barcode pools without drug treatment and with mefloquine treatment at different concentrations. The log2 of each barcode proportion in the total count across different time points is represented. Note that at 500nM, all strains are killed.

Table 3.4 Mean comparison of barcode proportion change between strains exposed to different mefloquine doses. The statistical difference between change in barcode proportion relative to day 1 across strains measured by one-way analysis of variance with Tukey test. Non-significant (ns) difference between groups is p>0.05

Mean Comparison	Mefloquine concentration (Adjusted P-value)			
	10nM	20nM	50nM	500nM
3D7-V1/S-A	ns	ns	ns	ns
3D7-V1/S-B	ns	0.0418	ns	ns
3D7-CAM	ns	ns	ns	ns
V1/S-A-CAM	ns	0.0178	ns	ns
V1/S-B-CAM	ns	0.0057	ns	ns

The BarSeq approach was performed to determine phenotypes of the different strains in the absence and presence of piperaquine concentrations ranging from 10nM to 500nM which is approximately 25X IC_{50} to 1250X IC_{50} of 3D7, respectively, over a 13-day period shown in figure 3.7. This concentration range was based on previous IC_{50} data derived in the lab with a piperaquine stock that was subsequently discovered to be significantly less potent. Consistent with the results shown in figure 3.4, there was a significant difference between V1/S and 3D7 in the competition assays in the absence of the drug with V1/S outcompeted by 3D7 and CAM strains. In the presence of the lowest piperaquine concentration tested (25X IC_{50}), the change in proportion over time observed does not correlate with differences in drug IC_{50} across the strains in the standard 72-hour assay. The barcode proportion for the CAM strain, which had the highest IC_{50} for piperaquine, showed a greater drop by day 13 indicating greater sensitivity of CAM at this concentration and higher doses. Similar barcode proportion to the total input over time was observed across the different strains from 75X IC_{50} and dead parasites were confirmed by microscopy. This suggests these assays are not meaningful and represent the presence of DNA from dead parasites. As noted above, the initial concentration range was selected based on historical lab data for piperaquine IC_{50}, however the stock used for these BarSeq assays was subsequently discovered to be considerably more potent, reflected in the sub-nanomolar IC_{50} values shown in Table 3.2. Thus, all the concentrations tested were already several fold above the IC_{50} of the strains in the pool.

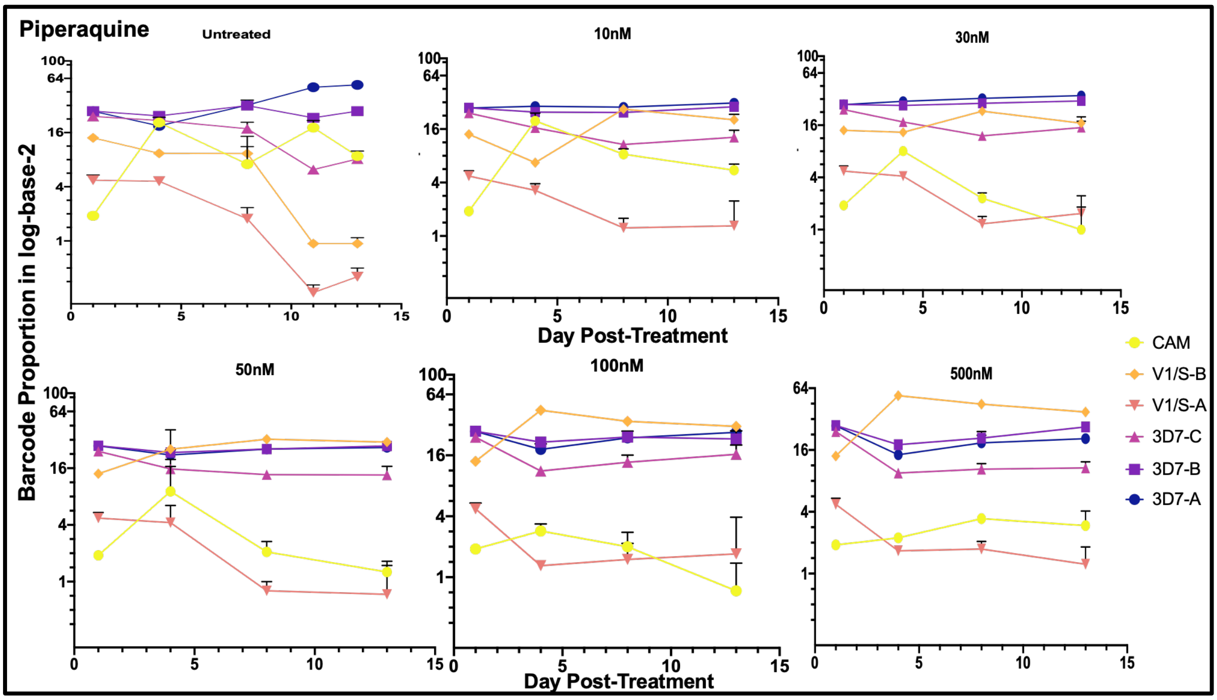


Figure 3.7 BarSeq of barcoded clones of *P. falciparum* strains with increasing piperaquine concentration. Competition assays of barcode pools without drug treatment and with piperaquine at different concentrations with each barcode proportion in the total count at every time point represented.

Table 3.5 Mean comparison of barcode proportions across strains in the different assays. The statistical difference between the means of the different groups and clones in presence and absence of CQ, measured by one-way analysis of variance with Tukey test. There was statistical difference in mean comparison across groups with a p-value of <0.0001 of the ANOVA summary. Non-significant (ns) difference between groups is p>0.05. Note that at 500nM, all strains are dead

Mean Comparison	Piperaquine BarSeq (Adjusted P-value)					
	Untreated	10nM	30nM	50nM	100nM	500nM
3D7-V1/S-A	ns	ns	ns	ns	ns	ns
3D7-V1/S-B	ns	ns	ns	ns	ns	0.0110
3D7-CAM	ns	ns	ns	ns	ns	ns
V1/S-A-CAM	0.0045	0.0108	ns	ns	ns	0.0224
V1/S-B-CAM	0.0050	ns	ns	ns	ns	ns

3.4 Discussion

Studies involving *in vitro* selection of drug resistant *P. falciparum* remains a crucial technique for identification of the mechanisms and genetic markers of antimalarial drug resistance and parasite fitness (Mwai *et al,* 2012). Informative analyses of genetic markers of drug resistance and fitness cost associated with resistance have been facilitated by the combination of *in vitro* assays, gene editing and genome sequencing (Ng and Fidock, 2019, Tirell *et al,* 2019; Hott *et al,* 2015).
The results obtained from the experiments in this chapter demonstrate the ability of assessing the resistance and fitness profiles of multiple *P. falciparum* strains using the BarSeq approach. The V1/S strain demonstrated a fitness disadvantage relative to the CAM strain, which in turn appears less fit than 3D7, which has been cultured in the lab for decades. It is evident from the results that there is a fitness advantage in some strains over others in the absence of drug pressure *in vitro*. Clearer differences in overall fitness may be revealed by extending the assay period. This BarSeq approach allows studying the fitness profile of multiple strains at the same time at a large-scale contrary to previous methods that independently measure fitness in head to head assays mostly with two strains co-cultured at a time, limiting scalability.

The expected phenotypes for the strains that have been well profiled with regards to chloroquine sensitivity was observed, as were the drug sensitivity profiles of these strains for mefloquine and piperaquine that are partner drugs in the ACTs. An increase in copy number of the *Pfmdr1*, resulting in an amplification of cellular transcripts had been suggested to directly modulate mefloquine resistance (Mwai *et al,* 2012; Llinàs *et al,* 2006). This highlights the importance of the sensitivity of V1/S to low concentration of mefloquine that is observed in the results. V1/S has *Pfdhfr* and *Pfcrt* mutations causing resistance to pyrimethamine and chloroquine, respectively.

However, the absence of *Pfmdr1* copy number amplification that affect sensitivity to several drugs including mefloquine is indicated by the phenotype observed in the results (Yongkiettrakul *et al,* 2020; Ding *et al,* 2012, Fidock *et al,* 2000). In a study by Chugh *et al* (2015) identifying cross-resistance signals from antimalarial compounds using multidrug-resistant strains, the *Pfmdr1* copy number for V1/S was 1 (Chugh *et al,* 2015). However, *Pfmdr1* copy number is unlikely to be the sole reason for differences in sensitivity, as 3D7 was less sensitive than V1/S despite also having a single copy of *Pfmdr1*. The intermediate phenotype of the artemisinin resistant CAM strain to mefloquine and piperaquine obtained by the Barseq approach highlights the importance of its ability to assess multiple strains of different resistance profiles. The results obtained shows sensitivity of CAM to mefloquine by BarSeq demonstrating efficacy of mefloquine which is one of the main partner drugs in ACTs to counteract the challenge of multi drug resistance in Southeast Asia where the CAM strain originated (Hamilton *et al,* 2019; Ménard and Fidock 2019). However, there is a lack of correlation between the standard drug assay and BarSeq approach in the drug profiles across the strains for piperaquine, likely due to the high concentrations of piperaquine used relative to the IC_{50} values of the strains. This illustrates that the BarSeq approach may not give meaningful data at concentrations appreciably above the IC_{50}, with counts coming from dead parasites. Alternate approaches would be to use a version of the *in vitro* piperaquine survival assay (PSA) in the BarSeq approach for piperaquine sensitivity, as PSA has been reported to detect piperaquine resistance and treatment failure more reliably than classic dose–response assays (Duru *et al,* 2015).

In subsequent work, I aim to assess the phenotype of sensitivity to artemisinin which remains the frontline antimalarial. This will be done by performing DHA drug response by BarSeq with RSA (0-3h) in which parasites are left to grow into the subsequent cycle before microscopy assessment after tightly synchronised early rings (0–3h) are exposed to a ‘pulse’ of DHA and washed (Witkowski *et al,* 2013). I also aim to perform this approach with an additional selection of strains of other backgrounds to have a greater diversity representation of strains.

For longer term growth experiments or maintenance of drug-screening pools, rapid out-competition of slow growing mutants by strains that grow faster has been pointed out by Bushell *et al,* (2017) in a *P. berghei* gene function study using BarSeq (Bushell *et al,* 2017). This point will be considered in future work by ensuring that the different strains to be mixed in a pool for competition assays will have relatively comparable growth rates.

Chapter 4: Investigating the role of *Plasmodium falciparum* genetic background in tolerance to artemisinin-resistance *Pfkelch13* mutations and assessing human kelch inhibitors for antimalarial activity on *Pfkelch13*

4.1 Summary

The first aim of this chapter is to test the effect of different genetic backgrounds on the acquisition of artemisinin resistance. The hypothesis is that strains with existing artemisinin resistance will have a higher likelihood of acquiring a resistance allele of *Pfkelch13* compared with sensitive parasites, due to previous adaptations. This question will be tested by triggering a double-strand break at the key C580 codon using Cas9, and providing multiple possible template for repair. To create the mix of donor templates, I aimed to generate new pools of *Pfkelch13* donor plasmids for CRISPR editing of the C580 codon. From a comprehensive pool of all 64 possible codons, I aimed to isolate plasmids of donors that encode for only viable alleles incorporated in 580 of *Pfkelch13* and exclusion of plasmids with "non-compatible" donors in the pool based on previous transfection data. Using sub-pools of only donors that are compatible with viability, this should lead to increased transfection efficiency at a larger scale thus allowing the investigation of tolerance of resistance-associated mutations in different genetic backgrounds.

The second aim of this chapter was to determine the potential antimalarial activity of inhibitors of human kelch-like ECH-associated protein 1 (KEAP1) on *P. falciparum* parasites including whether there was differential activity on parasites harbouring *Pfkelch13* mutations.

4.2 Background

4.2.1 Parasite fitness and drug resistance:

The reduction in malaria morbidity and mortality has been achieved by use of ACTs as frontline treatment which consist of artemisinin and partner drugs. This progress in malaria control is however threatened by the increasing prevalence of *P. falciparum* parasites with decreased sensitivity to artemisinin that has emerged at various locations in South East Asia over the last decade, and recent emergence in Africa (Siddiqui *et al,* 2020). The efficacy of previous frontline antimalarials such as chloroquine has historically been hindered by emergence of drug resistance with a similar pattern of resistance emergence and spread in different endemic regions (Uwiman *et al,* 2020). The occurrence of point mutations in the Kelch propeller domain of *Pfkelch13*, has been associated with artemisinin resistance. However, there is no definitively established mechanism of artemisinin resistance, which has been linked to an activated unfolded protein response, altered DNA replication, increased levels of phosphatidylinositol 3-phosphate, reduced protein translation and increased cellular stress (Birnbaum *et al,* 2020). Assessing the prevalence of molecular markers associated with therapeutic efficacy of artemisinin and its partner drugs for continuous systematic, conscientious monitoring of anti-malarial drug resistance is necessary to ensure the continuous use of efficacious ACTs (Raman *et al,* 2019).

Several *Pfkelch13* mutations have been identified in various malaria endemic regions with only a few validated to incur artemisinin resistance from *in vitro* experiments. The C580Y allele has become the most prevalent allele, replacing other circulating mutations of *Pfkelch13* in multiple Southeast Asian regions, and has also been detected in Africa and South America. The mutation has been validated both *in vitro* and *in vivo* as having a role in artemisinin resistance (Stokes *et al,* 2021; Miotto *et al,* 2020). Mutations in other genes, which have been suggested to either contribute to a multigenic basis of resistance or fitness, or serve as genetic markers of founder populations, have also been associated with artemisinin resistance in *Pfkelch13* mutant parasites. This includes mutations in ferredoxin *(fd),* multidrug resistance protein 2 *(mdr2)* and *plasmepsin 2-3* that is associated with piperaquine resistance in dihydroartemisinin-piperaquine treatment failure (Apinjoh *et al,* 2019; van der Pluijm *et al,* 2019).

Plasmodium kelch13 mutations have been observed at a low frequency in regions including Africa and South America but have been occurring at an increasing prevalence in Southeast Asia over the past decade (Cerqueira *et al,* 2017). Several non-synonymous mutations with multiple independent origins have been associated with artemisinin resistance since its emergence in early 2000s. The C580Y allele is one of the four Asian mutations (C580Y, R539T, I543T, and Y493H) validated *in vitro* and *in vivo* that are rarely reported in other endemic regions. The C580Y mutation has become the most implicated allele that has occurred and spread in higher frequency in Asia (Anderson *et al,* 2016; Mernard *et al,* 2016). A multidrug-resistant lineage harbouring the *Pfkelch13* C580Y has been reported to spread and nearly reached fixation in Southeast Asian regions such as Cambodia, East Thailand and Vietnam, indicating the rapid invasion of the C580Y allele of the population (Coppée *et al,* 2019; Ariey *et al,* 2013). The C580Y SNP in the *Pfkelch13* gene has been shown to play a dominant role in artemisinin resistance in multiple regions with independent origins of the mutation reported (Miotto *et al,* 2020; Zaw *et al,* 2020). The enhanced fitness of *Pfkelch13* C580Y compared to other *Pfkelch13* mutations is suggested to be the main factor of its dominance instead of the level of resistance conferred. This is suggested by *in vitro* studies in isogenic backgrounds in which parasites harbouring *Pfkelch13*

R539T or I543T mutations that conferred the highest levels of artemisinin resistance were outcompeted by C580Y mutated parasites (Gnädig *et al,* 2020; Straimer *et al,* 2015).

Understanding the aspects of parasite genetic background and co-segregating compensatory mutations with varying impacts on observed fitness costs of artemisinin resistant parasites is essential in understanding mechanisms of resistance in ACTs (Tirrel *et al,* 2019; Amato *et al,* 2020). Factors driving the increased substitution rate of tyrosine with cysteine at position 580 in the *Pfkelch13* gene resulting in the C580Y allele, are yet to be well established. However, the success of C580Y and impact on parasite fitness may be explained by epistatic interactions with other variants and genetic background that has been suggested to play an important role in frequencies of some *P. falciparum* SNPs (Nair *et al,* 2018; Straimer *et al,* 2017).

4.2.2 Kelch-like ECH-associated protein 1 (KEAP1)

Kelch-like ECH-associated protein 1 (KEAP1) is a highly conserved dimeric protein, consisting of 624 amino acids, that is rich in cysteine and shares 92% sequence homology among the mammalian species (Naidu *et al,* 2020). The human KEAP1 is a component of the Cullin 3 (CUL3)-based E3 ubiquitin ligase complex that is involved in the activation of nuclear factor erythroid 2-related factor 2 (NRF2) protein (Taguchi and Yamamoto, 2017). NRF2 and KEAP1 are the key signalling proteins in the KEAP1-NRF2 pathway that regulates cytoprotective responses resulting from oxidative stress (Kansanen *et al,* 2013). The KEAP1-NRF2 protein-protein interaction is involved in several diseases of oxidative stress and inflammation including lung, liver, kidney, gastrointestinal (GI) tract and cardiovascular system diseases, neurological conditions, metabolic, inflammatory and autoimmune disorders. Expression of critical enzymes involved in the synthesis and use of redox buffer reduced glutathione, and many ancillary proteins within the redoxin family, are regulated by NRF2 thus controlling production of reactive oxygen species (Baird and Yamamoto, 2020; Cuadrado *et al,* 2019). However, enhanced survival and proliferation of cancer cells has also been reported to occur due to elevated NRF2 activity.

Due to the major involvement of the KEAP1-NRF2 signalling pathway in these diseases, this system has become a potential therapeutic target. In addition to the development of modulators of NRF2 for this purpose, compounds that target KEAP1 as a main regulator of NRF2 degradation have also been developed (Cuadrado *et al,* 2019; Qin *et al,* 2019; Deshmukh *et al,* 2016). Some of these molecules developed to target KEAP1 have been reported to have very high affinity for, and are inhibitors of, the C-terminal Kelch/β-propeller domain of KEAP1. This KEAP1 Kelch/β-propeller domain through which KEAP1 binds to NRF2 is one of the major functional domains of KEAP1 (Lu *et al,* 2019; Robledinos-Antón *et al,* 2019). There is significant similarity in the structures (Figure 4.1) of the KEAP1 Kelch/β-propeller domain and kelch13 of *P. falciparum* that is associated with resistance to artemisinin derivatives. The potential activity of reported KEAP1 inhibiting compounds against *P. falciparum* was assessed in this chapter.

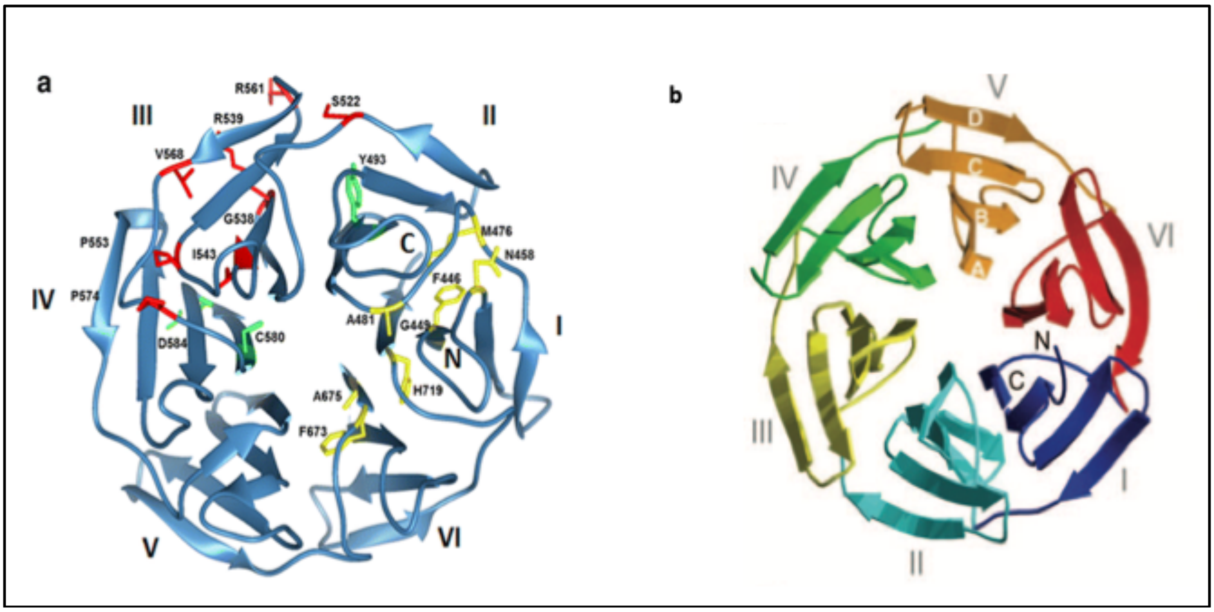


Figure 4.1 Schematic representation of KEAP1 kelch and *Pfkelch13* domains consisting of 6 blades (I – VI). a) The propeller domain of *Pfkelch13* and artemisinin resistance mutations in the propeller domain. Residues in clusters I and II are coloured red and yellow respectively; green coloured residues are not in any cluster. **b)** the kelch domain of KEAP 1; the four β-strands labelled A-D on each blade and the N and C termini located in blade I. Figures from Singh *et al* (2016) and Li *et al* (2004)

4.2.3 CRISPR/Cas9 plasmids design

As part of Manuela Carrasquilla's PhD project (2019), CRISPR/Ca9 plasmids harbouring different donor templates were designed to perform saturation mutagenesis at position 580 of *Pfkelch13* in parasites of different strains. The donor sequences of the plasmids were designed to also include synonymous 'shield' mutations at the cleavage site close to the PAM. This protects the modified sequence to prevent further cleavage by the Cas9 nuclease with a gRNA designed and cloned to target *Pfkelch13*. These plasmids contained a common pDC2 backbone with a codon-optimised Cas9 driven by a calmodulin promoter from *P. falciparum*, a hDHFR selectable marker, a U6 cassette for the expression of a single gRNA and a K13 donor sequence for homologous recombination (pDC2-coCas9-U6-K13-C580NNN-hDHFR). This template was synthesised to contain a homology sequence with the potential to encode any codon at position 580 to generate all possible mutant

Pfkelch13 lines, in effect mimicking spontaneous mutations due to random codon repair at this position. This was facilitated by synthesis of a gBlock template with NNN specified at position 580. All possible 64 donors were represented within the pool of plasmids where NNN represents any possible codon to replace 580. This complex mixture of plasmids representing all possible amino acids was assessed using amplicon sequencing to determine the proportions of each of the 64 codons in the pool.

Manuela Carrasquilla used this complex pool of plasmids with mutations at position 580 of *Pfkelch13* to transfect parasites of different strains. Transfections initially measured by PCR and Sanger sequencing, were assessed by Next Generation Sequencing to determine the proportion of each amino acid for each transfection. Tyrosine (Tyr) was only obtained in a Cambodian isolate (CAM) of the KEL1 lineage described by Amato *et al,* 2018, and not in any of the other genetic backgrounds. Multiple codons including the reference allele, cysteine (Cys), were successfully incorporated at position 580 in the other genetic backgrounds using this approach (Figure 4.2) performed by Manuela Carrasquilla. However, the vast majority of transfections were unsuccessful, likely due to the presence of non-viable codons in the mix. I intended to deconvolute this complex pool of plasmids to isolate individual donors to be used in generating new sub-pools of *Pfkelch13* C580 plasmids.

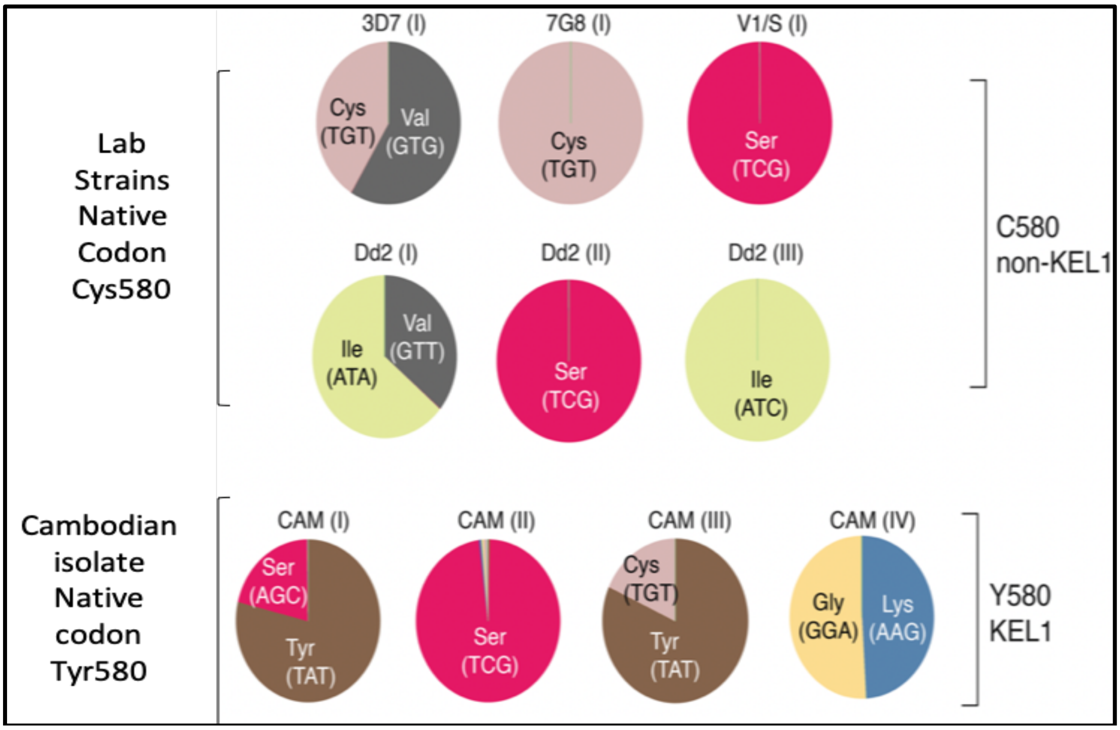


Figure 4.2 Codon substitutions at position 580 from transfections with the complex pool of plasmids. The proportion of each codon observed in independent transfections across the different *P. falciparum* lab strains (3D7, 7G8, V1/S, and Dd2) harbouring cysteine (C580) and CAM strain harbouring tyrosine (Y580) as the original haplotype at position 580. Each pie chart represents the outcome from a single transfection, and shows the proportion of each residue observed in the bulk culture. Thus 3D7(i) indicates that this transfection of 3D7 returned predominantly valine and to a lesser extent cysteine. Tyrosine substitution and phenylalanine (not shown in the picture) were only observed in the CAM strain that is artemisinin resistant. Figure adapted from Manuela Carrasquilla (2019).

4.3 Methods and Results

Multiple techniques, illustrated in figure 4.3, were performed to generate new sub-pools of plasmids with only compatible donors of “viable”, previously observed alleles from the complex pool for enhanced transfection efficiency of parasites of different genetic backgrounds This was aimed at facilitating assessment of tolerance and impact on fitness of *Pfkelch13* mutations in these strains.

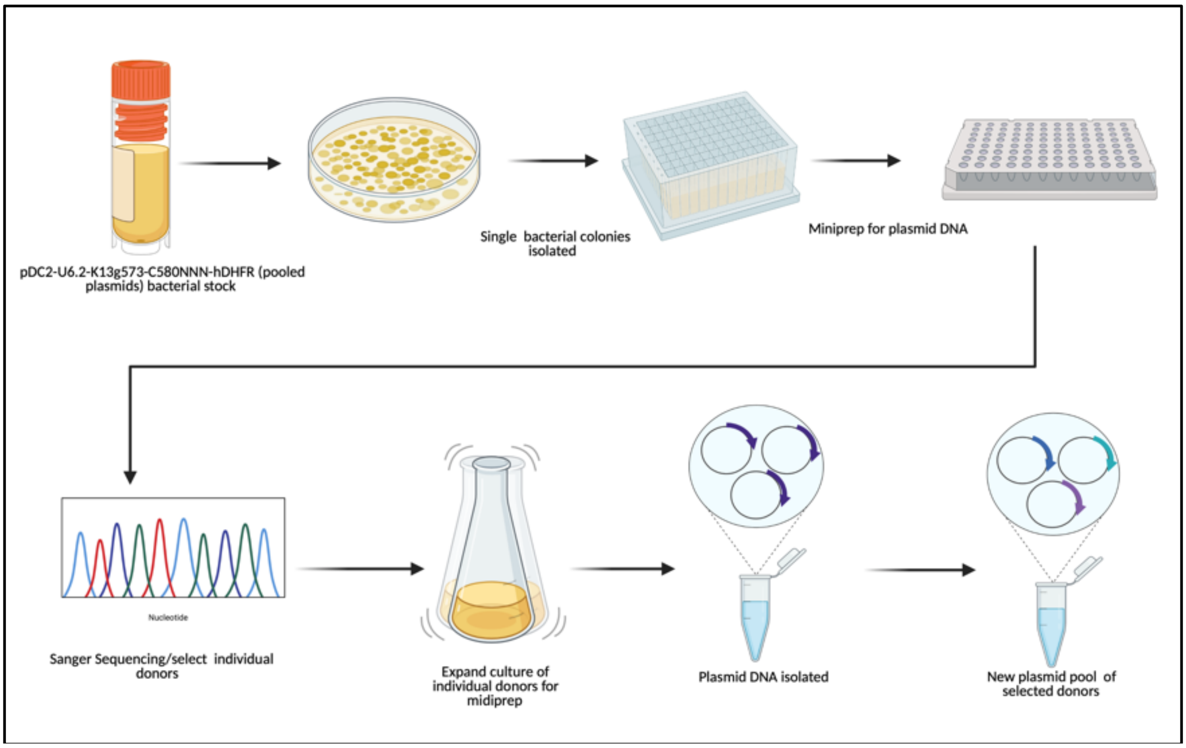


Figure 4.3 Schematic representation of the generation of new plasmid sub-pools. The workflow of deconvoluting the complex pool of plasmids from bacterial stock to isolate and select individual donors is shown

4.3.1 Deconvolution of a complex pool of plasmids

To deconvolute the complex pool of plasmids, plasmid DNA was isolated from 116 single individual bacterial colonies from the bacterial glycerol stock of the complex pool using the methods described in section 2.5.3. In order to identify individual plasmids with amino acids encoding for the donors of interest (Table 4.1), Sanger sequencing was performed on isolated plasmid DNA and the reads were aligned to K13-C580NNN donor sequences and analysed using SeqMan Pro software on DNASTAR Navigator. The chromatograms of the region around 580 of *Pfkelch13* were used to determine the codon replacing 580 in each isolated plasmid (examples shown in Figure 4.4). Plasmids with any of the target codons with no other mutation in the sequences in the *Pfkelch13* region were selected for use in generating the new pool.

Besides the donors with the codons of interest, several other codons encoding for various amino acids were obtained (Figure 4.5), including multiple codons encoding for the same amino acid. The occurrence of multiple codons from the deconvolution of the pooled plasmids supports the efficiency of design of the previously mentioned gBlock for introduction of spontaneous mutations.

The isolated plasmids with the donor of interest were inoculated in larger volumes and Midipreps performed for plasmid DNA isolation, described in section 2.5.4, with final elution in sterile cytomix ready for transfection.

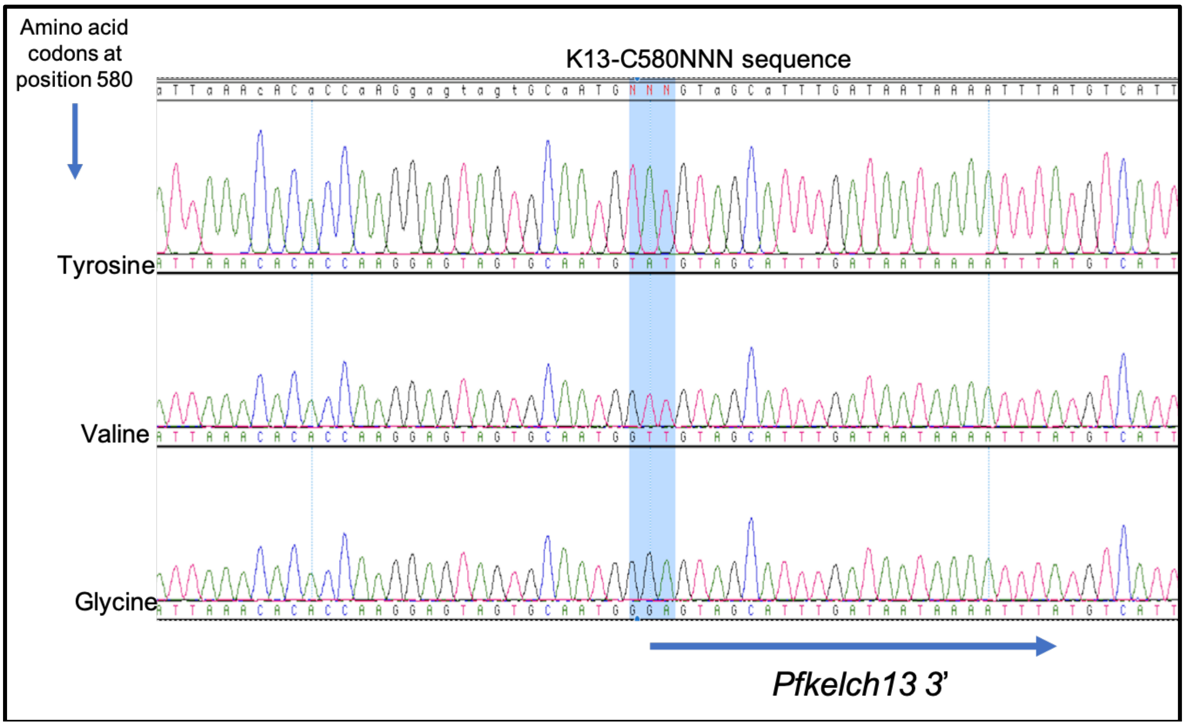


Figure 4.4 Chromatogram from Sanger sequencing of isolated plasmids. Donor sequence, with position 580 highlighted in blue, encoding for some of the donors of interest. The sequences were aligned with the K13-580NNN donor sequence (above).

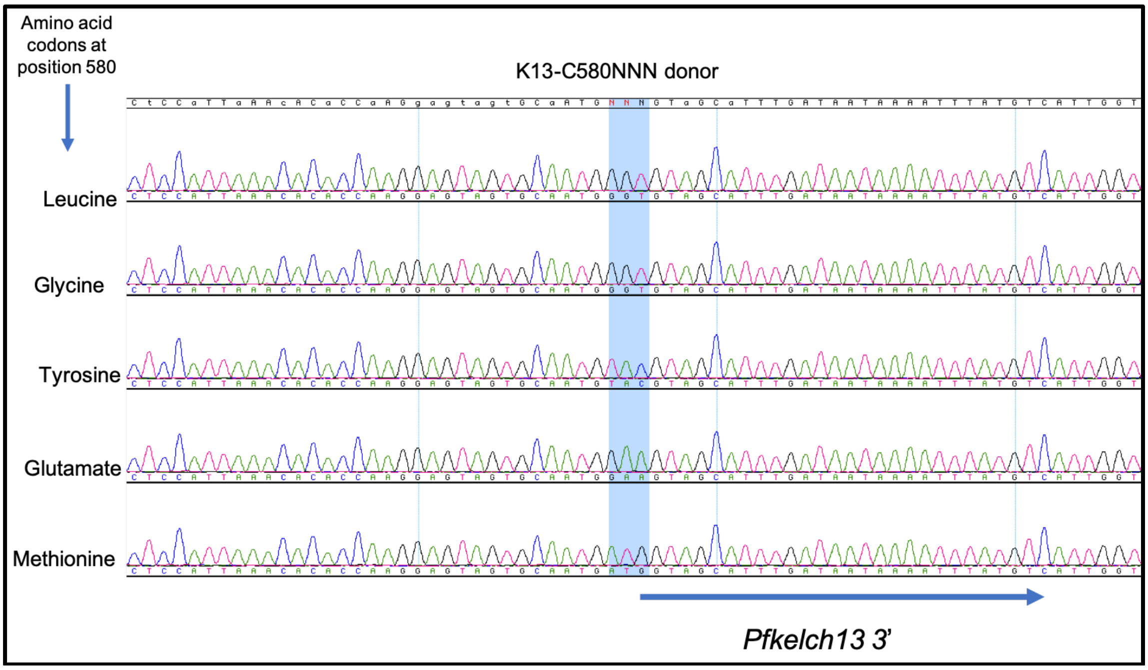


Figure 4.5 Chromatogram from Sanger sequencing of individual plasmids. Donor sequence at position 580 encoding for some of the donors of interest and other codons not included in the sub-pool of plasmids.

4.3.2 Identification of individual donors to generate a plasmid sub-pool

Donor plasmids with multiple codons encoding for various amino acids were isolated upon deconvolution of the complex plasmid pool. The results obtained for frequencies of the different amino acids from the deconvoluted complex pool indicates the representation of different codons in the original donor pool. This supports the findings by Manuela Carasquilla in which most codons were found to be represented fairly equally by amplicon sequencing of the original donor pool. Only a subset of codons encoding for leucine was found at a lower abundance (Manuela Carrasquilla, PhD 2019). In addition to the target donors of interest, a number of other donors with diverse codons (Table 4.1) at position 580 were also isolated, with some occurring at relatively high frequency. Donors encoded for by a multiple different codons and donors encoded for by a single codon were all represented in the total number of individual plasmids isolated. Altogether, 51 codons out of the possible 64 were identified. The individual codons encoding for the amino acid serine (C580S allele) and glycine (C580G), which were two of the target donors for the sub-pool, occurred at the highest frequency, consistent with their being encoded by six and four codons respectively and their relative abundance in the original pool.

Table 4.1 Amino acid codons obtained from isolated individual donors. The different amino acids and their codons from each of the individual donors after isolation from the mixed pool. Targeted amino acids that represent known viable codons used to make a new plasmid sub-pool are highlighted in bold

Amino Acid	Codon
Glutamate (Glu)	GAG, GAA
Arginine (Arg)	AGA, CGT, CGA, CGG, AGG
Termination (Ter)	TAA, TGA, TAG
Proline (Pro)	CCA, CCC
Threonine (Thr)	ACA, ACT, ACG
Leucine (Leu)	TTG, CTA
Histidine (His)	CAC, CAT
Asparagine (Asp)	AAT, AAC
Phenylalanine (Phe)	TTC, TTT
Aspartate (Asp)	GAC, GAT
Glutamine (Gln)	CAA
Isoleucine (Ile)	ATC
Tryptophan (Trp)	TGG
Alanine (Ala)	GCA, GCC
Valine (Val)	GTG, GTT, GTA, GTC
Methionine (Met)	ATG
Cysteine (Cys)	TGT, AGT
Lysine (Lys)	CGA, AAA, AAG
Glycine (Gly)	GGG, GGT, GGA, GGC
Serine (Ser)	TCA, TCG, TCC, AGT
Tyrosine (Tyr)	TAT, TAC

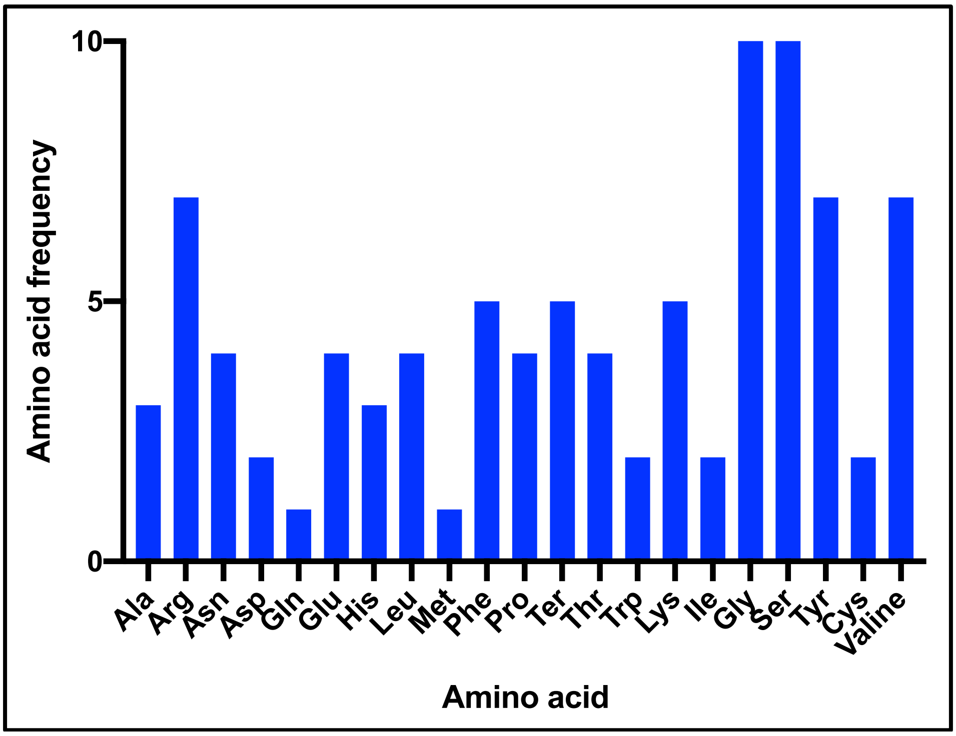


Figure 4.6 Frequency of the different amino acids in the isolated plasmids. The occurrence of the different amino acids from isolated donors with codons for glycine and serine occurring the highest frequency. Termination/stop codons were also isolated.

4.3.3 Transfection of *P. falciparum* parasites with plasmid sub-pool

Plasmid DNA was quantified using the NanoDrop ND-1000 UV-Vis Spectrophotometer (ThermoFisher Scientific) and a new pool of the plasmid consisting of donors encoding the known viable codons (cysteine, lysine, glycine, valine, isoleucine, phenylalanine, tyrosine and serine) was prepared with each plasmid at equal stoichiometric amounts, with a final concentration of 50µg/mL in the final mixture. *P. falciparum* parasites have been shown to have the ability of efficiently taking up multiple plasmids in co-transfections (Carrasquilla *et al,* 2019; Ghorbal *et al,* 2014).

In addition to testing Southeast Asian backgrounds, I planned to examine the impact of *Pfkelch13* mutations in African parasite backgrounds (see Table 2.1 in methods). Transfections were performed with the Southeast Asian lab line, Dd2, African strains FCR3 (from The Gambia) and a recent isolate from Tanzania (Tanzania-200708). These parasites were transfected using the plasmid sub-pool of multiple *Pfkelch13* 580 donors generated. The transfections were performed in two duplicates of two independent batches. To assess transfection efficiency, these same strains were transfected in parallel with a control plasmid pDC2-ef1a-Vps4wt-mRFP/hDHFR, expressing the mRFP fluorescent reporter. Transfections of parasites were performed using the method described in section 2.4. Transfected parasites were maintained in culture with WR99210 to select for plasmid-containing parasites. WR99210 is a compound that blocks production of tetrahydrofolate in the folate pathway by acting against parasite bifunctional dihydrofolate reductase thymidylate synthase enzyme (DHFR-TS) but not human DHFR. WR99210 is widely used for selection of transfectants due to elevated IC_{50} in *P. falciparum* parasites transformed with expressing episomal expression of the hDHFR coding sequence. This has made hDHFR a powerful selectable resistance marker for transfection of *P. falciparum* parasites (Remcho, *et al,* 2020; Fidock and Wellems, 1997).

Culture of the transfected lines with the plasmid sub-pool were maintained as described in section 2.14 up to day 32 and day 12 post-transfection for the two independent transfections done with the C580 plasmid sub-pool, respectively. However due to the closures of the laboratories as result of the Covid-19 outbreak these transfections were discontinued. Dd2 parasites transfected with the control plasmid pDC2-ef1a-Vps4wt-mRFP/hDHFR were however recovered at day 16 post-transfection when parasites became visible by microscopy.

There was no recovery of parasites in the transfected field strains FCR3 and Tanz 200708 in both transfections done with the control plasmid and the plasmid pool of K13 mixed donors, although this was challenged by the disruption of the experiments from the closure of laboratories. However, while Dd2 returned parasites on day 16 with the control plasmid (pDC2-ef1a-Vps4wt-mRFP/hDHFR, an episomal plasmid expressing an mRFP-tagged protein), the other two lines transfected with control plasmid in parallel with Dd2 did not return parasites by day 57 post-transfection suggesting that they are more challenging to transfect.

4.3.4 Assessing antimalarial activity of Kelch-like ECH-associated protein 1 (KEAP1) inhibitors

Due to time constraints upon reopening of the laboratory, this part of the project was refocused to explore whether small molecules would be able to differentially target wild type and mutant *Pfkelch13*. Although no compounds targeting *Pfkelch13* have yet been described, human Kelch-like proteins have been the subject of drug development efforts as described in the Introduction. In order to assess if compounds known to inhibit the Kelch-like ECH-associated protein 1 (KEAP1) have potential antimalarial activity on *P. falciparum* parasites, standard 72-hour drug sensitivity assays were performed.

4.3.4.1 Drug Sensitivity Assays using Keap1 inhibitors

Standard 72-hour antimalarial drug sensitivity assays were performed using compounds that are known to inhibit the kelch domain of Keap1 following methods described in section 2.3.1 and 2.3.2. This was performed to investigate the potency of these compounds against *P. falciparum* parasites. The IC_{50} value, which is the concentration of the compound required to inhibit 50% of parasite growth to determine response to a drug, was measured for compounds KI696, RA839 and ML334 as described below. The assay was performed to determine IC_{50} values against laboratory adapted *P. falciparum* strains 3D7 and Dd2 wild type (WT) and Dd2 *Pfkelch13* edited mutants harbouring R539T and C580Y mutations. This rapid and cost-effective method has been applied over the past years, to determine potency of drugs by measuring growth inhibition in vitro using several DNA intercalating dyes like SYBR Green 1. SYBR Green dye binds directly to double stranded DNA allowing the measurement of DNA amount as a result of the detection of fluorescence signals from the SYBR Green intercalation with parasite DNA. Parasite growth which is inhibited by therapeutic concentrations of antimalarial drugs is measured by this fluorescence intensity (Dery *et al,* 2015; Karl *et al,* 2009).

The first KEAP1 kelch inhibiting molecule tested was KI696 that has been shown to demonstrate tight and selective binding to the kelch domain of KEAP1 by Davies *et al,* (2016) using fragment-based drug discovery. This molecule exhibited high potency and activation of the in human cell-based assays NRF2 pathway in vivo (Davies *et al,* 2016). The second compound RA839, with the chemical name (3S)-1-[4-[(2,3,5,6-tetramethylphenyl) sulfonylamino]-1-naphthyl] pyrrolidine-3-carboxylic acid, was reported to demonstrate non-covalent binding characteristics to the kelch domain of KEAP1 by Winkel *et al* (2015). RA839 was observed to be a selective inhibitor of the Keap1/Nrf2 interaction with a high affinity between the naphthalene ring system of RA839 bound to the central solvent channel of the kelch domain. Other KEAP1 kelch binding sites of RA839 formed by residues including Tyr334, Phe577, and Tyr572 at the hydrophobic and aromatic pocket and were also observed (Winkel *et al,* 2015).

The third KEAP1 inhibiting compound tested against *P. falciparum* was ML334 that is also referred to as LH601A with the chemical name (1*S*,2*R*)-2-[[(1*S*)-1-[(1,3-Dihydro-1,3-dioxo-2*H*-isoindol-2-yl) methyl]-3,4-dihydro-2(1*H*)-isoquinolinyl] carbonyl] cyclohexanecarboxylic acid. The ML334 molecule, a stereoisomer (SRS)-5, was reported to demonstrate an inhibiting characteristic of Keap1-Nrf2 interaction by Hu *et al* (2014) dissociating NRF2 from KEAP1. The presence of the free carboxylic group of ML334 was observed to result in high affinity of ML334 isomer to the Nrf2 peptide binding site kelch domain of KEAP1 (Hu *et al,* 2013). The chemical structures of KI696, RA839 and ML334 are shown in Figure 4.7.

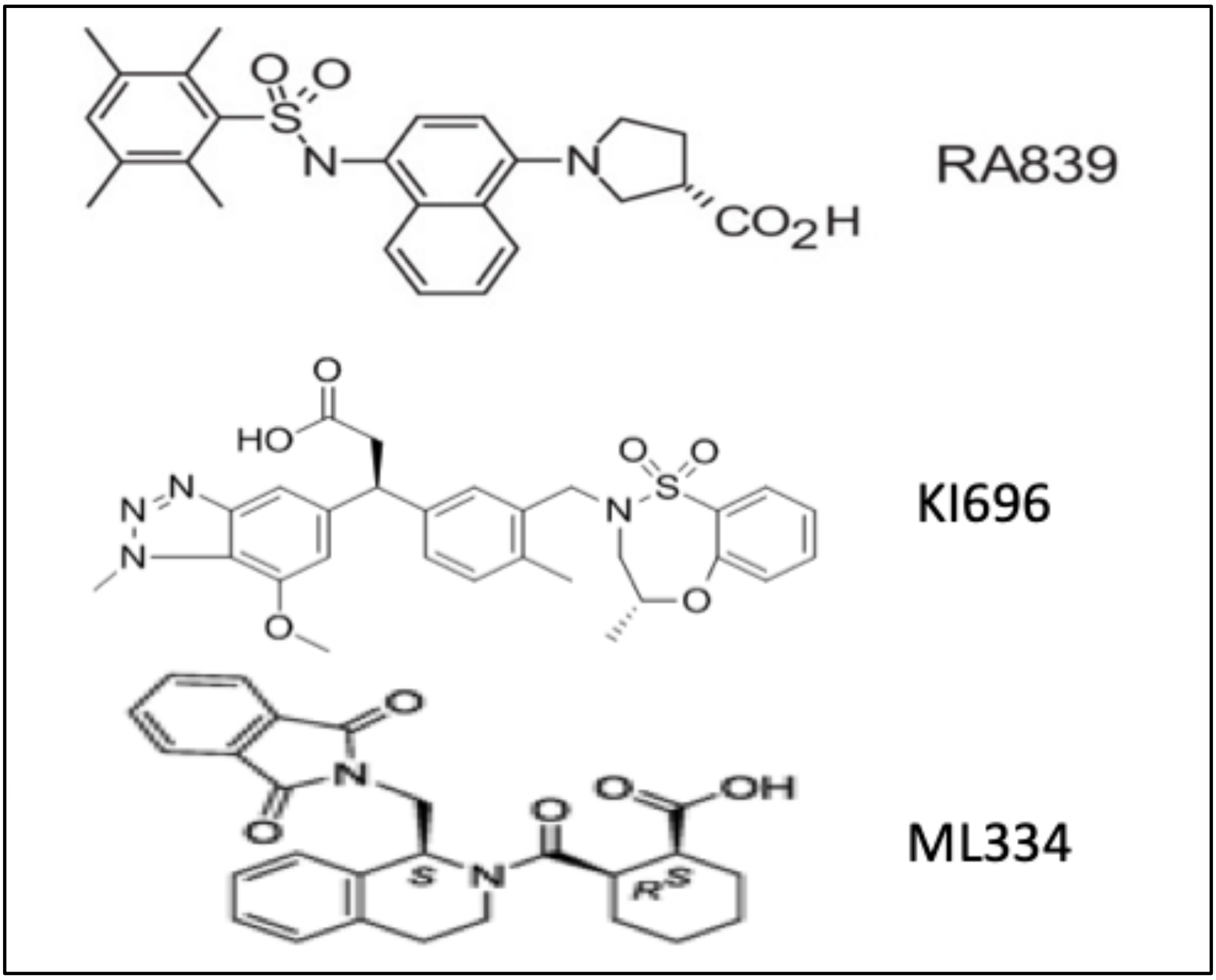


Figure 4.7 Chemical structure of KEAP1 kelch-inhibiting compounds. The structural difference of RA839, KI696 and ML334 (LH601A). (Davies *et al,* 2016; Winkel *et al,* 2015; Hu *et al,* 2104)

4.3.4.2 *Plasmodium falciparum* sensitivity to KEAP1 Kelch inhibitors

The drug-response curves for KI696, RA839 and ML334 were experimentally obtained in triplicates. Analysis was done on GraphPad Prism software and IC_{50}

values were established with a non-linear regression analysis generating the drug dose response curve. Activity against parasite growth in *P. falciparum* strains was demonstrated by all three compounds that were tested. Of the three compounds, ML334 was the most potent, with an IC_{50} of 1.7µM against 3D7, although the multi-drug resistant Dd2 strain had a higher IC_{50} of 5.2µM (Table 4.2 and Figure 4.10). In contrast, the other two compounds KI696 and RA839 showed only a slight shift with the IC_{50} for 3D7 marginally higher than Dd2 (Table 4.2, and Figures 4.8 and 4.9).

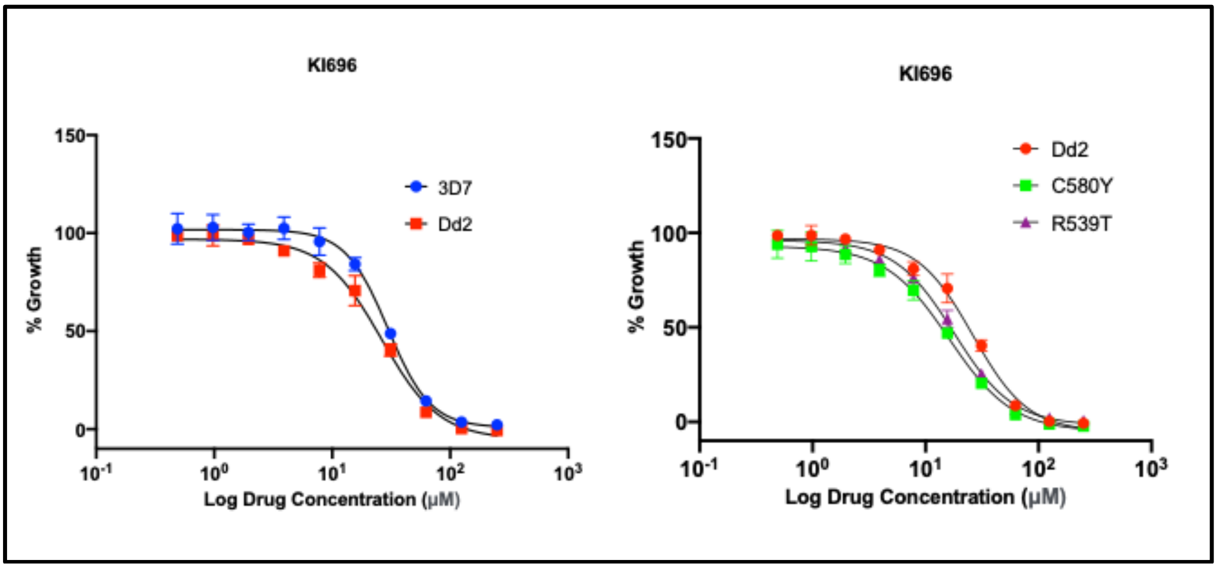


Figure 4.8 KI696 standard dose response curve. Dose response curves for the wild types (3D7 and Dd2) and Dd2 *Pfkelch13* mutants. A representative assay (technical triplicates) is shown. Similar IC_{50} curves were observed in the two biological replicates.

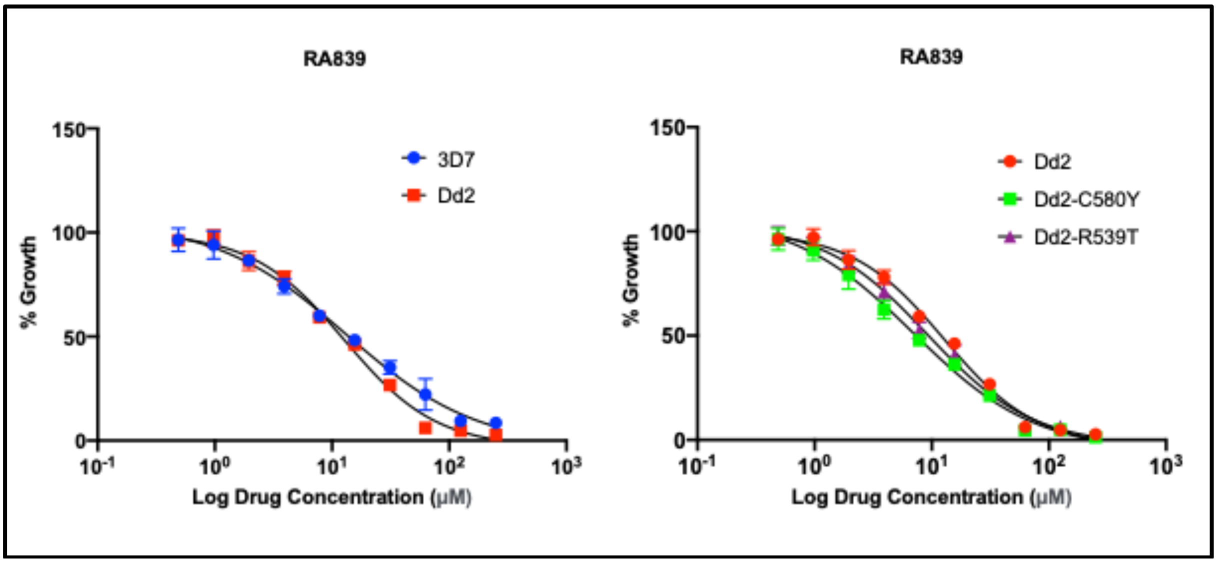


Figure 4.9 RA839 standard dose response curve. Dose response curves for the wild types (3D7 and Dd2) and Dd2 *Pfkelch13* mutants. A representative assay (technical triplicates) is shown. Similar IC_{50} curves were observed in the two biological replicates.

Table 4.2 The IC_{50} values obtained for compounds KI696, RA839 and ML334. Standard 72-hour drug assays were performed and the IC_{50} values obtained from biological duplicates of wild type strains and Dd2 mutant lines harbouring *Pfkelch13* mutations.

Compound	3D7 IC_{50} (μM)	Dd2 IC_{50} (μM)	Dd2-C580Y IC_{50} (μM)	Dd2-R539T IC_{50} (μM)
KI696	30.3	23.2	18.2	19.6
RA839	27.6	18.3	17.4	13.2
ML334	7	14	20.1	20.2

Interestingly, the Dd2 *Pfkelch13* mutant parasites, Dd2-C580Y and Dd2-R539T, showed increased sensitivity to KI696 and RA839, with the IC_{50} values lower than for the wild type Dd2. In contrast, the results obtained with the ML334 compound (Figure 4.10) demonstrated higher sensitivity by the wild type parasite than the Dd2 *Pfkelch13* mutant lines. Overall, however the highest fold shift of IC_{50} values in the Dd2 mutants compared to wild type Dd2 was < 2-fold for all the three compounds. The lowest IC_{50} value (7µM) across all three compounds tested was obtained with ML334 for 3D7 indicating some aspect of the Dd2 genetic background, such as amplification of *Pfmdr1* or the chloroquine-resistance mutations in *Pfcrt,* may affect susceptibility to the ML334 compound.

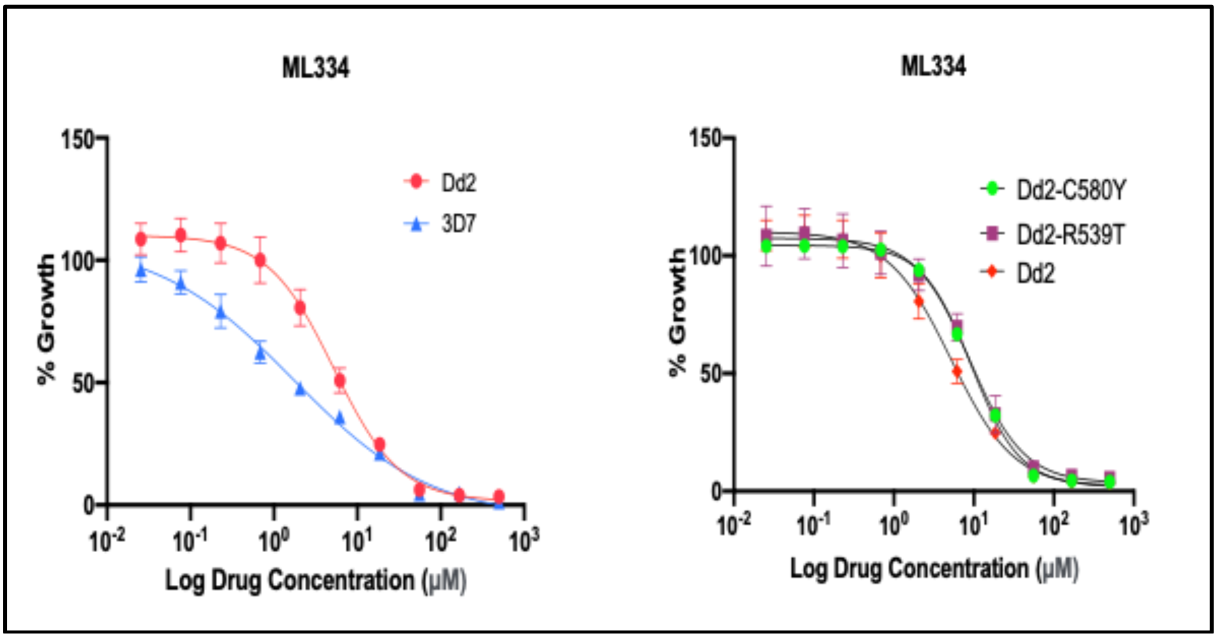


Figure 4.10 ML334 standard dose response curve. Dose response curves for the wild types (3D7 and Dd2) and Dd2 *Pfkelch13* mutants in assays performed in technical triplicates to obtain IC_{50} values

4. 4 Discussion

Resistance to antimalarial drugs that have emerged in *P. falciparum* parasites for different classes of antimalarials have been shown to occur upon exposure to strong selection with antimalarial treatments. Based on these previous trends, there is the expectation of the absence of resistance mechanisms for antimalarial candidates of classes different to the current ones (Ravenhall *et al,* 2019; Kidgell *et al,* 2006). Thus, the demonstrated sensitivity of the Dd2 mutant parasites Dd2-R539T and Dd2-C580Y to the kelch inhibiting compounds supports the importance of studying these compounds as drug candidates. This is highlighted by the activity against parasites growth in both wild type and mutant strains, demonstrated by all three KEAP1 kelch inhibiting compounds as observed in the results.

However, the drug resistance profiles that have been developed for the different antimalarials over the past years as well as geographical origin vary across different *P. falciparum* strains (Zhao *et al,* 2019; Anthony *et al,* 2016; Llinás *et al,* 2006). This can explain the varying results observed between 3D7 and Dd2 across the different compounds although both strains demonstrated moderate sensitivity to the compounds. These two strains have been shown to have varying drug resistance profiles. 3D7 is generally a sensitive strain but is however resistant to some antifolates (sulfadoxine), whereas Dd2 is a multi-drug resistant strain with *pfmdr1* copy number amplification and *pfcrt* point mutations (Corey *et al,* 2016).

This study describes the identification of human KEAP1 kelch inhibitors as having antimalarial activity, albeit with limited potency. However, the importance of these compounds to be studied as potential antimalarial drug candidates is based not on their overall potency but whether they could show a proof-of-principle differential activity against mutant *Pfkelch13*. The high level of structural similarity between

KEAP1 kelch and *Pfkelch13* highlights the importance of validating the activity of these compounds against *P. falciparum* parasites (Coppée *et al,* 2019; Li *et al,* 2004). C580Y and R539T are one of the major *Pfkelch13* mutations that have been implicated in artemisinin resistance (Chenet *et al,* 2017). Therefore, their sensitive phenotypes in the presence of two of the compounds tested indicates the potential to develop kelch inhibitors against resistant strains that may antagonise artemisinin resistance.

The occurrence of pre-existing resistance mechanisms and of rapid occurrence of resistance acquisition to new antimalarial compounds was shown in a previous cross-resistance study by Corey *et al,* (2016). These findings and existing antimalarial resistance patterns pose a big threat to both existing drugs and new drug candidates (Molnar *et al,* 2020; Corey *et al,* 2016). Screening of current antimalarial agents and compounds in development with parasites harbouring resistance-associated mutations facilitated by gene editing have provided important insights in drug resistance mechanisms and drug development (Ng and Fidock, 2019). Furthermore, there is a need for continued progress in identification of potential candidates for use in combination therapies due to the rapid resistance development pattern in malaria monotherapies. This has highlighted the importance of combination therapies in malaria chemotherapeutics which is the current recommendation for treatment, particularly ACTs (Gorka *et al,* 2013). Therefore, further work is needed in validating the potential of kelch inhibiting compounds as potential antimalarial candidates. The next steps towards this validation would be to perform experiments to assess if these potential kelch-interacting compounds are directly acting on the parasites through *Pfkelch13* or alternative modes of mechanism. This could be done using *Pfkelch13* knock down parasites (Birnbaum *et al,* 2020) that should sensitise the parasite to kelch13 inhibition, or more direct biochemical assays to look at binding such as surface plasmon resonance. Techniques for assessing drug combination efficacies such as isobologram analyses, a widely used classic method for determining drug interactions (Huang *et al* 2019), could be performed to assess the interaction of these compounds and current frontline antimalarials such as artemisinin. Application of this method will help establish whether KEAP1 kelch inhibiting compounds tested in these experiments will exhibit antagonistic or synergistic drug efficacy properties when in combination with artemisinin. This will determine the potential suitability of *Pfkelch13* inhibitors as potential antimalarial candidates.

Although the general experiments for genome editing of the *P. falciparum* parasites were not completed due to Covid-related interruptions, parasites were recovered in transfections carried out on the Dd2 laboratory strain using the control plasmid by 16 days. The main limitation in the genome editing of field isolates is the length of time it takes for parasites to be recovered (Caro *et al,* 2012). This combined with the closure of the laboratories from the nationwide lockdown did not allow the continuity of the transfections. However, further work will be done to continue efficient transfection of different *P. falciparum* strains with the generated plasmid sub-pools of *Pfkelch13* 580 donors. This will facilitate the investigations on determining the factors driving the dominance of certain *Pfkelch13* mutations such as C580Y in certain backgrounds. The assessment of whether tolerance of the different mutations is driven by genetic backgrounds or co-existence of different mutations is needed. This assessment will be aided by successful transfection of field strains and genomic analysis. In the next steps, these experiments to provide important insight in understanding resistance patterns as well as enhancing transfection efficiency will be continued.

Chapter 5: Conclusion and future work

Advances in understanding population dynamics, drug resistance, gene function and molecular evolution of malaria parasites has been greatly enhanced by population and genetic and genomics studies (Su *et al,* 2019). Identification of drug development and diagnostics targets is strengthened by the availability of the *P. falciparum* genome sequence that underpins our understanding of the complex biology of *Plasmodium* parasites (Govindarajalu, *et al* 2019). *In vitro* resistance can be generated for most *de novo* candidates of antimalarials that have been placed in the drug development pipeline suggesting the continuous restoration of the antimalarial pipeline with novel targets (Medicines for Malaria Venture, 2020; Antonova-Koch *et al,* 2018; Flannery *et al,* 2013).

Rapid emergence of resistance to antimalarials, including previous and current drugs, remains a major problem in malaria chemotherapeutics. There are increasing reports of resistance to the frontline antimalarial artemisinin and its derivative, and partner drugs in ACTs, mostly in Southeast Asia and recently in other regions including Africa (Uwimana *et al,* 2020; Rosenthal, 2018). Mutations in *Pfkelch13* have been implicated in artemisinin resistance with some polymorphisms such as the C580Y allele dominant over other associated mutations in Southeast Asia. This dominance has been linked to enhanced fitness conferred by resistance associated mutations. Inherent fitness differences across different strains has also been observed in strains of different origin *in vitro* (Goel *et al,* 2021; Ménard and Fidock, 2019). Gene editing tools such as CRISPR/Cas9 and modern sequencing methods such as NGS have immensely accelerated advances made in unravelling these essential aspects of the complex biology of *P. falciparum* and identification of markers associated with resistance, drug targets and development. Developments in the CRISPR/Cas9 system allows the advanced genetic manipulation of *P. falciparum* using multiple plasmids in a transfection (Carrasquilla *et al,* 2020). I deconvoluted a complex pool of CRISPR/Cas9 plasmids to isolate individual viable donors of *Pfkelch13* mutations at position 580 and made up a new sub-pool. This plasmid sub-pool was generated with the aim of enhancing transfection efficiency and was used to transfect strains of different genetic backgrounds in order to address the question of how genetic background impacts parasite fitness with different *Pfkelch13* alleles. This would build on previous work from the lab that showed that different *Pfkelch13* alleles were returned by different genetic backgrounds when CRISPR-edited parasites were offered a range of potential donors. A major limitation of this work was the discontinuation of the experiments from the closure of the laboratories. However, this can be done in the next steps with a broader selection of parasites of varying origin, as these viable plasmids are readily available. I tested two lines of African origin that did not return transfected parasites with the control plasmid, in contrast to the well-established Dd2 line indicating some parasites are more easily transfectable than others. Thus, additional work will also require identification of strains that are more readily transfected and cultured.

In this study I was able to determine the resistance phenotypes and fitness of different *P. falciparum* strains of different genetic background in the absence and presence of drugs using barcode sequencing revealing the fitness disadvantage of resistant strains in the absence of drugs. Although the specific drug resistance phenotypes of these lines were already known by conventional drug assays, this work was to demonstrate proof-of-principle that this barcode sequencing methodology could be applied to mixed parasite pools to accurately reveal differences in drug susceptibility. In future work, this can be applied in larger scale settings to allow comparison of lines from different epidemiological regions, to understand resistance associated mechanism in genetic background and fitness in resistant parasites by using the BarSeq approach to tag parasites of the same genetic background with different mutations in genes involved in these mechanisms. Significance of the barcode readout for the growth assays was analysed using a one-way ANOVA test combined with a Tukey post-hoc test to identify differences between lines. However, analysis was performed on the change in proportion rather than the absolute proportion that is plotted in figures 3.5-3.7 because the starting proportion of the individual lines was not exactly equal. In some cases, apparently large differences in the behaviour of the lines was not significant (e.g. 10nM mefloquine, Fig. 3.6); this likely results from variability in particular from the V1/S lines, which were at low abundance in the pool by the time the assay was initiated due to losses during synchronisation. Thus, relatively small fluctuations in absolute proportion of these lines yielded large apparent changes in proportion, contributing to variability. Future assays would aim to initiate the experiment with more equal representation of each line, and would avoid synchronisation post mixing. An application of this BarSeq approach can be used to test the effects of different alleles of a particular gene in parasites of different genetic background. This could be an essential approach in assessing the major alleles of *Pfkelch13* in different genetic backgrounds particularly C580Y, R539T, Y493H and I543T alleles that have been genetically confirmed to confer high level of resistance *in vitro* in Southeast Asia where artemisinin resistance and chloroquine emerged. Understanding the basis of the dominance of the C580Y major resistance allele will provide insight into whether this form of the protein might be a viable drug development target. But more importantly, understanding the spread of C580Y would help dissect the contribution of both resistance and fitness of different Pfkelch13 alleles, and help address the challenges of preventing spread or emergence of artemisinin resistant parasites on different genetic backgrounds and to other regions where artemisinin resistance is not prevalent. Determining the effect of these mutations in parasites from other regions where artemisinin resistance linked with these major *Pfkelch13* alleles has not been established can be achieved with this BarSeq approach. Additionally, the barcoding approach can be utilised to tag progeny from genetic crosses, in order to allow mixing and competition among the progeny to understand the effect of different loci in specific traits in the progeny, as well as the parents.

Resistance studies included in the early stages of the development process of *de novo* antimalarial candidates are important in establishing molecular mechanisms involved in increased parasite tolerance to drugs and progressive targeted drug discovery efforts (Schlott *et al,* 2019). One area of interest is the identification of antimalarials that antagonise resistance by displaying greater potency against specific resistance alleles, a processed termed collateral sensitivity. As examples, compounds that are more potent against resistant versions of dihydroorotate dehydrogenase and chloroquine-resistant *pfcrt* have been identified (Ross *et al,* 2018; Lukens *et al,* 2014). One potentially important discovery would be compounds that are more effective against mutant *Pfkelch13*, in order to protect artemisinin combinations. I therefore tested human KEAP1 kelch inhibiting compounds for antimalarial activity using wild type and mutant lines and established the efficacy of these compounds on mutant lines harbouring artemisinin resistance-associated mutations. Two of the KEAP1 kelch inhibiting compounds I tested were observed to demonstrate moderate potency against artemisinin resistant parasites suggesting that further target-based chemistry programmes might yield more specific compounds with higher potency against parasites resistant to current antimalarials. This highlights the importance of developing antimalarial candidates of different classes to the current ones. As these assays were done in duplicate, I aim to expand this work to perform additional biological replicates of these assays to measure the statistical significance of these results.

Continuous surveillance of drug resistance markers and mechanisms is required to tackle the potential spread of artemisinin to other endemic regions such as Africa where reports of artemisinin resistance lines are now emerging. Moreover, genomic studies looking at resistance markers, as well as parasites of the different endemic regions in joint studies making use of gene editing and sequencing as highlighted in this project should be applied more. This will greatly accelerate progress in malaria control and treatment efforts due the great potential insights that can be achieved with the approaches applied in this project.

6. Appendix

List of primers and sequences

Primer	Sequence
p191	GGGTAAACTAGAATATGCTATACCGG
p194	ACCATCACGGGATAAAGTAACTGG
p282	AACATATGTTAAATATTTATTTCTC
p1356	TCGGCATTCCTGCTGAACCGCTCTTCCGATCTGTAATTCGTGCGCGTCAG
p1357	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTTCAATTTCGATGGGTAC
p1358	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCT
p1359	CAAGCAGAAGACGGCATACGAGATTGCTAATCACTGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1360	CAAGCAGAAGACGGCATACGAGATTAGGGGGATTCGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1361	CAAGCAGAAGACGGCATACGAGATAGTTTCCCAGGGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1362	CAAGCAGAAGACGGCATACGAGATCCTGGGAGGTAGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1363	CAAGCAGAAGACGGCATACGAGATATACCACAAATGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1364	CAAGCAGAAGACGGCATACGAGATGATCTCTCGGGGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1365	CAAGCAGAAGACGGCATACGAGATACCCTATACTCGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1366	CAAGCAGAAGACGGCATACGAGATCTCAATTAAGAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1367	CAAGCAGAAGACGGCATACGAGATCGACAGAACGTGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1368	CAAGCAGAAGACGGCATACGAGATTCGCCATTATGGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1369	CAAGCAGAAGACGGCATACGAGATATGTTCCGGCCGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1370	CAAGCAGAAGACGGCATACGAGATTCTTGAAGTGAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1371	CAAGCAGAAGACGGCATACGAGATGAAGGCCAGCTGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1372	CAAGCAGAAGACGGCATACGAGATCCAATGTGCAGGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1373	CAAGCAGAAGACGGCATACGAGATATCGAAGGACCGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1374	CAAGCAGAAGACGGCATACGAGATTCGGGTGCGAAGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1375	CAAGCAGAAGACGGCATACGAGATGTAATTTACGGGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1376	CAAGCAGAAGACGGCATACGAGATATATCGACTACGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1377	CAAGCAGAAGACGGCATACGAGATTGATTCTTACAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T

p1378	CAAGCAGAAGACGGCATACGAGATACGGCGGGCCTGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1379	CAAGCAGAAGACGGCATACGAGATCTTGCGTGGAGGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1380	CAAGCAGAAGACGGCATACGAGATTAATCAAAGACGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1381	CAAGCAGAAGACGGCATACGAGATGGCGGGCTCTAGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1382	CAAGCAGAAGACGGCATACGAGATCCTCCATTTCTGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
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p1384	CAAGCAGAAGACGGCATACGAGATTATTCGTCAACGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1385	CAAGCAGAAGACGGCATACGAGATGCGCTGATGCAGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1386	CAAGCAGAAGACGGCATACGAGATCTCATATGGCTGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
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p1388	CAAGCAGAAGACGGCATACGAGATGGTTTTATACCGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1389	CAAGCAGAAGACGGCATACGAGATGCATGACTTTAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1390	CAAGCAGAAGACGGCATACGAGATTTCTGAGTTCTGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
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p1392	CAAGCAGAAGACGGCATACGAGATTCTCTTAAGCCGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
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p1397	CAAGCAGAAGACGGCATACGAGATGGAGTTCAACAGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1398	CAAGCAGAAGACGGCATACGAGATACTGCGTATATGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1399	CAAGCAGAAGACGGCATACGAGATTACGTCGTGCGGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
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p1401	CAAGCAGAAGACGGCATACGAGATATACTTGTTAAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1402	CAAGCAGAAGACGGCATACGAGATTACCCAGGAGTGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T

p1403	CAAGCAGAAGACGGCATACGAGATGGGGTTTTCTGGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1404	CAAGCAGAAGACGGCATACGAGATACTGCTCGTGCGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1405	CAAGCAGAAGACGGCATACGAGATGAAGCATAATAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1406	CAAGCAGAAGACGGCATACGAGATATACAGTCGCTGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1407	CAAGCAGAAGACGGCATACGAGATCTGTCGCAAGGGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1408	CAAGCAGAAGACGGCATACGAGATTGCAATCTAACGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1409	CAAGCAGAAGACGGCATACGAGATGATGGTATTTAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
p1410	CAAGCAGAAGACGGCATACGAGATCTAAAAAATATGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
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p1416	CAAGCAGAAGACGGCATACGAGATTAACGCGTGGCGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
p1417	CAAGCAGAAGACGGCATACGAGATCCGTATCTATAGAGATCGGTCTCGGCATTC CTGCTGAACCGCTCTTCCGATC*T
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p1420	CAAGCAGAAGACGGCATACGAGATAAGCCGAGATCGAGATCGGTCTCGGCATT CCTGCTGAACCGCTCTTCCGATC*T
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p1428	CAAGCAGAAGACGGCATACGAGATTTGTATCATTTGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
p1429	CAAGCAGAAGACGGCATACGAGATAAATAATGGGGGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T
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p1454	CAAGCAGAAGACGGCATACGAGATCTGAACCTGACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T

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