DEVELOPING TOOLS TO INVESTIGATE DORMANCY MECHANISMS IN *PLASMODIUM SPP.*

by

ALONA BOTNAR

(Under the Direction of Dennis E. Kyle, Ph.D.)

ABSTRACT

Malaria, caused by protozoa of the genus *Plasmodium*, continues to be a life-threatening disease affecting nearly half the world's population. Our ability to control and eradicate this disease has been hampered largely due to the spread of drug resistance. Further exacerbating this challenge is the parasite's ability to evade drug pressure by entering a developmentally arrested state known as dormancy. *P. falciparum*, the deadliest of human infecting malaria parasites, enters dormancy as a response to artemisinin pressure, whereas *P. vivax*, the most widespread of malaria parasites, has naturally occurring dormant stages in the liver known as hypnozoites. The mechanisms behind both naturally occurring and drug-induced dormancy, and subsequent relapse or recrudescence, remain elusive and we lack the proper tools to study this phenomenon. While dormant cells do maintain low levels of metabolic activity, they do not actively synthesize DNA. Since *Plasmodium* is a purine auxotroph, we developed a novel tool using alkyne modified purines as DNA synthesis markers. We observed that modified adenosine can successfully differentiate *P. vivax* hypnozoites and liver stage schizonts and furthermore that it can capture reactivating hypnozoites. Additionally, modified

adenosine, inosine, and hypoxanthine all incorporated into actively replicating *P. falciparum*, highlighting their utility for cell cycle studies. Alternatively, previous data has shown that exogenous addition of gibberellic acid to dihydroartemisinin-induced dormant *P. falciparum* stimulates early recrudescence. Therefore, we utilized gibberellic acid as a tool to investigate the mechanisms controlling dormancy and recrudescence. We modified gibberellic acid and localized it to the cytoplasm of all asexual blood stages of *P. falciparum*. Furthermore, we conducted pulldown studies and identified 50 proteins of interest involved in transcription, translation, post-translational modification, and protein trafficking. Among the proteins of interest was a putative AP2 transcription factor and several 26S proteasome subunit proteins. These classes of proteins have been associated with artemisinin resistance and could be involved in recrudescence from dihydroartemisinin-induced dormancy. Elucidating the parasite's dormancy mechanisms is crucial towards malaria control and eradication, thus these studies provide the novel techniques necessary for future research of this phenomenon.

INDEX WORDS: *Plasmodium falciparum*, *Plasmodium vivax*, dormancy, hypnozoite, artemisinin, purines, click chemistry, phytohormone, gibberellic acid

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF FIGURES viii

CHAPTER

1 INTRODUCTION 1

Background 1

Discovery & History 2

Plasmodium genome 3

Apicoplast 4

Plasmodium spp. life cycle 4

Symptoms & Diagnosis 8

Global Impact of Malaria 9

Malaria in the US 10

Current Control, Treatments, & Eradication Efforts 10

Antimalarial Drugs & Rise in Resistance 16

Quinolines 17

Antifolates 22

Atovaquone 22

Antibiotics 23

Artemisinins 23

Dormancy....28

Purine Transport & Metabolism....31

Scope of Study....32

2 ALKYNE MODIFIED PURINES FOR ASSESSING ACTIVATION OF *PLASMODIUM VIVAX* HYPNOZOITES AND GROWTH OF PRE-ERYTHROCYTIC AND ERYTHROCYTIC STAGES IN *PLASMODIUM SPP.*....35

Abstract....36

Introduction....37

Materials and Methods....41

Results....48

Discussion....52

3 INVESTIGATING DIHYDROARTEMISININ-INDUCED DORMANCY IN *PLASMODIUM FALCIPARUM* USING A CHEMICAL BIOLOGY APPROACH AND GIBBERELLIC ACID....68

Abstract....69

Introduction....70

Materials and Methods....73

Results....77

Discussion....82

4 Summary....98

REFERENCES....104

LIST OF FIGURES

Page

Figure 2.1: Structures of the alkyne modified purines (EdX)..58

Figure 2.2: Alkyne modified purines incorporate into the replicating asexual blood stage *P. falciparum*..59

Figure 2.3: *P. falciparum* asexual blood stage growth depends on unmodified purines in culture media...60

Figure 2.4: Alkyne modified adenosine (EdA) incorporates in replicating *P. vivax* liver stage parasites, but not in hypnozoites..61

Figure 2.5: EdA incorporated into *P. vivax* hypnozoites and liver stage schizonts can be identified and quantified via high content imaging 62

Figure 2.6: Alkyne modified adenosine (EdA) incorporates in replicating *P. berghei* liver stage parasites 64

Figure 2.7: Alkyne modified adenosine (EdA) incorporates in replicating HepG2 mammalian cells 65

Figure 2.8: EdA labels replicating HepG2 mammalian cells and is cytotoxic 66

Figure 2.9: Asexual blood stage parasites recrudescing from DHA-induced dormancy incorporate $^{[3H]}$hypoxanthine and/or $^{[3H]}$adenosine but do not incorporate EdA ...67

Figure 3.1: Structures of GA_3 derivatives..88

Figure 3.2: GAD1566 and GAD1569 contain the same early recrudescence phenotype as GA_3 when supplemented to DHA-induced dormant *P. falciparum* asexual blood stage parasites 89
Figure 3.3: GA_3 derivatives GAD1566 and GAD1569 localize to the cytoplasm in all three asexual blood stages of *P. falciparum* 90
Figure 3.4: GA_3 and GAD1566 interact with the same partners as increasing concentrations of GA_3 compete out the GAD1566 signal 92
Figure 3.5: Structures of biotinylated GA_3 derivatives....93
Figure 3.6: Interacting proteins of GA_3 are involved in cellular processes, regulation of transcription and translation, and cellular protein metabolic processes, and are found in vesicles 94
Figure 3.7: Interacting partners of GA_3 do not present a differential expression profile in K13 mutant parasites versus wildtype parasites but do contain an enriched interactome with clustering for translation & post-translational modifications 96

CHAPTER 1

INTRODUCTION

Background

Malaria is an intracellular, parasitic disease that remains a global health challenge with over 218 million cases and 409,000 deaths reported in 2019. Although there have been great strides in malaria control throughout the years, the data reported in the 2020 World Malaria Report shows that progress is stalling [1]. This disease has plagued mankind for thousands of years, with the malarial fever having been described by human societies as early as the third century B.C. that was attributed to spending time in swamps [2]. Ancient civilizations documented symptoms, such as intermittent fevers, that we now know to be as signs of malaria. The name malaria stems from the Italian name "mal'aria," which means "bad air." This name was used because people associated the intermittent fevers with exposure to vapors from marsh-type areas, such as swamps.

Malaria is caused by *Plasmodium* parasites. Presently, there are over 200 species of *Plasmodium* reported to infect a variety of animals [3]. There are currently six known species of *Plasmodium* that can infect humans which are *P. falciparum, P. vivax, P. ovale, P. malariae, P. knowlesi,* and *P. cynomolgi* [4]. In fact, *P. cynomolgi* was originally thought not to infect humans but recent reports of naturally acquired infections have been reported [5]. About 3.2 billion people are at risk of getting malaria. Of the deaths occurring due to malaria, about 94% of them occur in Africa. *P. falciparum*, which

is the most lethal, is dominant in Africa [1]. Malaria has a disproportionate burden, affecting the poorest countries in the world the most. These countries have the highest risk of malaria and the lowest access to proper healthcare.

Discovery & History

In the nineteenth century, Thomas-Crudeli suggested that malaria was caused by a bacterium and thus named it *Bacillus malariae.* It wasn't until 1880 that Dr. Laveran discovered the malaria parasite, naming it *Oscillaria malariae*. He was working in North Africa on soldiers suffering fevers and found crescent-shaped bodies containing pigmented granules inside their red blood cells (RBCs). He was eventually awarded the 1907 Nobel Prize for determining the role of protozoa in causing disease [6, 7]. In 1897, Ronald Ross showed that mosquitos are responsible for transmitting malaria and was awarded the 1902 Novel Prize for this discovery. In 1885, Camillo Golgi discovered that there were different forms of the disease, where fevers occurred every other day (tertian) or every third day (quartan). He did not, however, realize that more than one species of *Plasmodium* was responsible for human disease. He also found that the fevers overlapped with rupture of erythrocytes and the release of merozoites [6, 7]. In 1890, Giovanni Grassi and Raimondo Filetti differentiated the parasite into two species, naming them *Plasmodium vivax* and *Plasmodium malariae* [6, 7]. Later, William Welch in 1897 named *Plasmodium falciparum* and John Watson in 1922 named *Plasmodium ovale.* To date, *Plasmodium falciparum* is the most lethal parasite infecting humans and is accountable for the bulk of the mortality due to malaria every year. By 1897, the development of *P. falciparum, P. vivax,* and *P. malariae* was described in anopheline

mosquitos. It wasn't until 1948 that the parasite was found to develop in livers of rhesus monkeys infected with *P. cynomolgi* [8]. Soon after, the complete life cycles of *P. vivax* and *P. falciparum* were described. Relapsing malaria, or the reappearance of parasetimia following some antimalarial therapies is due to stages called hypnozoites that remain dormant in liver hepatocytes. These occur in *P. vivax, P. ovale,* and *P. cynomolgi.* Hypnozoite research is limited as *in vitro* culture of these *Plasmodium spp.* has proven to be difficult. However, the existence of these stages represent a serious obstacle for malaria control and eradication efforts.

Considering how long this disease has existed, laboratory research with human malaria is relatively recent. It took nearly 100 years to elucidate the *Plasmodium* life cycle. In 1976 methods for the continuous culture of human malaria were published, with the method of Trager and Jensen today being considered as the formative procedure for the development of *in vitro* culture of *Plasmodium falciparum* [9]. With these developments, the malaria scientific community has come a long way in the years following.

***Plasmodium* genome**

In 2002, the *Plasmodium* nuclear genome was sequenced and annotated using the 3D7 strain as the reference [10]. 3D7 was cloned from NF54, an isolate that was obtained from a patient near the Netherlands airport that had never left the country. The genome is 23.33 mega bases with 14 chromosomes and 5720 genes. The genome is unique in that it is adenine and thymine rich (about 80%). *Plasmodium* parasites contain two additional

genomes. The apicoplast organelle contains a 35 kilobase circular genome and the mitochondria contains a 6 kilobase linear genome [11, 12].

Apicoplast

The apicoplast is a relict-like organelle common to most apicomplexan parasites. The complete genome of the organelle was mapped in 1996 and it was found to resemble algal plastid DNA [13]. The circular genome consists of 35kb, which is reduced from 150kb found in an average chloroplast. It is 86% A/T rich and contains 68 genes that encode for less than 50 proteins in the apicoplast. Most apicoplast proteins are nuclear encoded and targeted to the apicoplast and other organelles [13, 14].

The apicoplast is believed to have evolved by two endosymbiotic events. Initially, an ancient cyanobacterium was engulfed by a heterotrophic eukaryote. This was then a new red algae organism which was then engulfed by another eukaryote. Evidence for these events in seen in the apicoplast's four membranes and genome sequence which is similar to that of red algae [15, 16].

The apicoplast is no longer photosynthetic, however, it is essential for survival as disruption leads to parasite death [17-19]. The apicoplast has four known functions: heme biosynthesis, iron-sulfur cluster biosynthesis, fatty acid biosynthesis, and isoprenoid biosynthesis [20, 21].

Plasmodium spp. Life Cycle

The life cycle of *Plasmodium* is comprised of morphological forms that exist in the definitive host (female *Anopheles spp.*) in which the sporogonic cycle occurs, and in

the human intermediate host where asexual reproduction in hepatocytes occurs, followed by erythrocytic schizogony.

Plasmodium in the Mosquito (definitive host)

The *Anopheles* mosquitoes are the definitive host of human-infecting *Plasmodium spp.* and of these about 41 species are capable of transmitting malaria [22]. However, among these, several species have higher efficiency rates in transmitting *Plasmodium.* A typical mosquito life span is 7-14 days. When a female mosquito takes a blood meal on an infected human, she ingests gametocytes, and the parasite's life cycle begins. Male gametocytes (microgametocytes) exflagellate due to environmental cues within the mosquito and fertilize female gametocytes (macrogametocytes), which then form the zygote. Zygotes then develop into mobile and elongated ookinetes at which point they invade the midgut wall of the mosquito and convert to oocysts. As the oocyst matures it releases haploid sporozoites which then migrate to the mosquito's salivary glands. They then wait for the next blood meal at which they are injected through the proboscis of the mosquito into the intermediate host. This entire process in the definitive host takes about 10-18 days [23].

Plasmodium in humans (intermediate host)

Once sporozoites have been injected into the intermediate host (in this case a human), the sporozoites begin to traverse using gliding motility to reach circulation. Once in circulation, the sporozoites arrive at the liver and make their way through liver epithelial and Kupffer cells eventually making their way to, and residing in, hepatocytes.

Plasmodium sporozoites express the circumsporozoite protein (CSP) on their surface, which is essential for the parasite's attachment to the liver cells [24, 25]. It is in the hepatocytes that they undergo multiple rounds of asexual replication. These rounds of asexual replication produce thousands of haploid merozoites per single sporozoite. In *Plasmodium vivax, Plasmodium ovale,* and *Plasmodium cynomolgi,* certain parasites enter a state of dormancy and these parasites do not undergo asexual replication. These are termed hypnozoites and are responsible for relapsing malaria [26]. However, after about 6-10 days, the parasites that did undergo replication and form merozoites are subsequently released from the liver.

The released merozoites then enter the erythrocytic part of the life cycle and begin invading red blood cells (RBCs). Merozoites bind to the surface of an erythrocyte via surface receptor-ligand interactions of the merozoite surface proteins (MSP), apical membrane antigen-1 (AMA-1), the erythrocytic-binding antigen (EBA-175), and PfRh5. The parasite then orients itself to the apical pole and begins to enter the RBC by forming a tight junction and squeezing through, forming a parasitophorous vacuole in the process [27, 28]. Once invaded, the parasite life cycle progresses within the parasitophorous vacuole through the ring stage, followed by the trophozoite stage, and finally the schizont stage. The time for completion of this cycle is species-dependent but is usually about 48 hours. Within these erythrocytic stages, the parasites ingest hemoglobin which is broken down into amino acids and free heme. This molecule is toxic to the parasite, so it is converted into a non-toxic substance termed hemazoin [29].

The haploid erythrocytic stages have a rough similarity to the standard cell cycle format (G_0-G_1-S-G_2-M) seen in other cell types with the merozoite existing in a G_0 state.

Once inside the erythrocyte, the ring stage follows and can be compared to G_1 of the cell cycle in which the preparation for DNA replication and amassing of molecular precursors and RNA levels increase [30]. The breakdown of hemoglobin is aided by proteases in the digestive vacuole and the parasite obtains amino acids in the process. The parasites also ingest the glucose and hypoxanthine needed for glycolysis and the synthesis of purines, respectively [31]. The trophozoite stage represents the S phase during which DNA replication ensues. In this stage, the knob-like KAHRP and *Pf*EMP1 proteins are shuttled to the surface of the red blood cells. These proteins interact with ICAM-1, among many other receptors, to bind to the smooth endothelial cells of the blood vessels in order to sequester the trophozoite and schizont stages. This prevents their removal from circulation via the spleen [32, 33]. In order to disguise the highly immunogenic *Pf*EMP1 protein, the parasite utilizes antigenic variation with about 60 *var* genes encoding *Pf*EMP1 variants [34]. DNA and organelle segregation brings the schizont stage which can segment into as many as 30 daughter merozoites [35]. This stage ends after nuclear segmentation and when the mitochondria have segmented as well. The asymptomatic incubation period of infection in *Plasmodium falciparum* is around 9-14 days. When the mature merozoites rupture from the red blood cell in a synchronized fashion and reinvade new erythrocytes, clinical presentation of symptoms occurs. Of these ruptured merozoites, a small proportion in circulation differentiate into male and female gametocytes which will be taken up by a mosquito upon taking a blood meal, completing the life cycle [33].

Symptoms & Diagnosis

Uncomplicated Malaria

The World Health Organization defines clinical presentation of uncomplicated malaria as consisting of fever, headache, chills, malaise, perspiration, abdominal discomfort, muscle and joint aches, anorexia, vomiting, and is diagnosed with a blood smear or a rapid diagnostic test. Uncomplicated malaria is symptomatic but does not have evidence of vital organ dysfunction [36].

The periodic fever is caused by *Plasmodium* infection and is a direct effect of the asexual stages of the life cycle. When schizonts rupture out of infected erythrocytes, the expulsion of red blood cell debris, hemozoin, and toxins directly into the blood stream causes an inflammatory immunological response that causes fever [37]. All human infecting *Plasmodium spp.* can cause uncomplicated malaria. However, the incubation period varies. The length of the asexual cycle duration correlates to the length of time between periodic fevers. A fever of 48 hours is consistent with infections of *Plasmodium falciparum, Plasmodium vivax, Plasmodium cynomolgi,* and *Plasmodium ovale.* Fevers of 24-hour periodicity are associated with *Plasmodium knowlesi* and fever of 72-hour periodicity are associated with *Plasmodium malarie* [38, 39].

Severe Malaria

Plasmodium falciparum can progress into severe malaria if left untreated. Severe malaria is indicated in patients that experience organ failure and is often accompanied by a high mortality rate approaching 100% when left untreated [36]. Severe malaria can occur for non-immune individuals, people who didn’t receive or delayed treatment, or

those who received ineffective antimalarial drugs. Severe malaria is life-threatening and requires hospitalization. Clinical signs of severe malaria include coma, prostration, convulsions, respiratory distress, significant bleeding, shock, and pulmonary edema. Clinical signs of severe malaria include severe anemia, hypoglycemia, jaundice, renal impairment, hemoglobinuria, metabolic acidosis, and hyperparasitemia. Cerebral malaria caused by *Plasmodium falciparum* accounts for a majority of severe malaria cases. It accounts for about 10% of hospitalizations and 80% of malaria deaths. Cerebral malaria is caused by infected erythrocytes that have adhered to the cerebral microvasculature which causes a blockage, leading to a shortage of oxygen and nutrients to the brain. The risk of death due to severe malaria intensifies with the presentation of multiple complications [36].

Global Impact of Malaria

Malaria affects about 3.2 billion people, most of which are present in the poorest countries in the world. While malaria control has come a long way, malaria still accounts for about half a million deaths per year [1]. A large percentage of these deaths occur in Africa, but malaria is also endemic in southeast Asia, the South Pacific, parts of South America, and sub-continental India. Of the species known to infect humans, *Plasmodium falciparum* causes the majority of cases and deaths in Africa. Furthermore, a large percentage of these deaths in Africa are in children under the age of five [1, 40].

Although *Plasmodium falciparum* is the most lethal and is concentrated in Africa, *Plasmodium vivax* is more widely distributed across the globe. About 80 million cases of

P. vivax occur each year and more than 50% of malaria cases (excluding Africa) are caused by *P. vivax* [41].

Over the years there has been a discrepancy in disease burden reports that may stem from several reasons. These include, but are not limited to, the fact that not all infections result in progression to disease, many people elect to self-medicate and do not seek care and thus their cases are not reported, and inaccurate diagnoses might be used to report disease rates. Furthermore, while we have seen a decline in mortalities due to malaria over the past few decades, we are now seeing a plateau [1].

Malaria in the US

Malaria used to be endemic in the United States, specifically in the southeast. The Centers for Disease Control (CDC) was originally established as the Malaria Control in War Areas (MCWA) to prevent malaria on military bases and prevent its spread to the general public [42]. By 1949, about 5 million houses had been sprayed with DDT, and by 1951 the CDC began withdrawing from active participation and shifted its interest to surveillance. By 1951, malaria was considered eradicated from the United States. Today, the majority of cases diagnosed in the US are acquired in endemic areas. However, the mosquito responsible for transmitting this disease remains prevalent today and thus, a risk of reintroduction is always present.

Current Control, Treatments, & Eradication Efforts

After the discovery of effective antimalarials in the 1950s, malaria eradication seemed possible. In 1955, the World Health Organization established the Global Malaria

Eradication Program. This program failed to interrupt transmission completely in many countries and eventually this, among other factors, led to its demise. By 1969, the World Health Organization admitted the campaign's failure and abandoned it in favor of control programs.

Mortality and morbidity due to malaria began to increase again in the 1980s due to the increase in parasite and vector resistance to antimalarial drugs and insecticides and the weakening of malaria control programs. This led to the Global Strategy for Malaria Control program in 1992 [43].

In 2007, the Bill and Melinda Gates Foundation classified priority aims to work towards in eradication of malaria. This renewed vigor towards Malaria eradication inspired the global malaria research community. The malaria research efforts have been transformed with the revolutionized desire to fund projects in transmission blocking, chemoprotection, and new drug development. As a result, there have been considerable collaborative efforts to speed up the malaria drug discovery pipeline and thus, many new promising classes of drugs have been discovered. In order to make the eradication of malaria possible, several goals need to be met. These include: a. new drugs need to be constantly developed in order to combat resistance; b. funding must be maintained in countries endemic to malaria; c. insecticides, larvicides, and insecticide treated bed nets must be used broadly, and d. drugs for chemoprotection be administered to vulnerable populations, although this last one depends on funding [44]. There are additional goals that are desirable to be met such as developing a drug that can provide a single-dose radical cure. This would reduce the concern of patient compliance. Furthermore, many current drugs are unsafe for pregnant women, unsafe for patients with a glucose-6-

phosphatase dehydrogenase (G6PD) deficiency, or are failing due to resistance. Making new chemopreventative drugs that would combat these issues is necessary.

Prevention/Prophylaxis

Prophylactic drugs that target liver stage parasites can be taken in order to prevent malaria. These only need to be taken while in malaria endemic regions. These are better than suppressive prophylactic drugs that kill blood stages, as those require treatment for weeks after leaving an endemic area to clear any possible emerging parasites in circulation. Non-immune people traveling to malaria endemic regions are recommended to take several prophylactic drugs such as: atovaquone/proguanil, chloroquine, doxycycline, mefloquine, primaquine, and/or tafenoquine [45]. However, some of these drugs (atovaquone/proguanil, doxycycline, primaquine, and tafenoquine) are not suggested for pregnant women. Furthermore, chloroquine and mefloquine have widespread resistance. Doxycycline, mefloquine, primaquine, and tafenoquine are also known to have serious side effects. Primaquine and tafenoquine have shown to be life-threatening in causing hemolysis in people with a G6PD deficiency [46, 47]. Mefloquine has shown to cause severe psychiatric events [48]. These issues highlight the need for novel drug development.

Treatments

Treatment of malaria depends on factors such as disease severity, the species of malaria parasite that caused infection, and the part of the world in which infection was

acquired. Determining the probability of the organism's resistance to certain antimalarial drugs is vital. Age, weight, and pregnancy status should also be factored in.

The current gold standard treatment for uncomplicated malaria recommended by the World Health Organization as front-line therapy is an artemisinin combination therapy (ACT). The current ACTs recommended by the World Health Organization are: artesunate and mefloquine, artemether and lumefantrine, artesunate and sulfadoxine/pyrimethamine (SP), artesunate and amodiqauine, and dihydroartemisinin and piperaquine [36]. All of these ACTs are given daily over a three-day period, with some being given twice a day. If women are pregnant and in their first trimester, they are treated for seven days with quinine and clindamycin. Women in their second and third trimesters are given the standard ACT treatments. Unfortunately, ACTs are beginning to fail clinically and new partner drugs or longer courses of treatment are now being evaluated. For *P. vivax, P. ovale,* and *P. cynomolgi* to prevent relapse of infection from hypnozoites, an additional recommendation of a 14-day course of primaquine or tafenoquine is recommended. However, this is only recommended for G6PD normal individuals.

For second-line therapies, an alternative ACT artesunate and tetracycline/ doxycycline/clindamycin or quinine and artesunate and tetracycline/doxycycline/ clindamycin can be used. These combinations should be given for seven days.

Severe malaria is treated with either intravenous or intramuscular artesunate for at least 24 hours and until the patient can take oral medication. Once they can take oral medication, they complete a three-day course of an ACT with an added dose of primaquine in areas of low transmission. Adults, pregnant women, and children all

receive this course of treatment. Supportive care is required, and treatment is often dependent on the complications that present themselves in severe malaria [36].

Vaccines

There are several challenges to the development of a successful malaria vaccine that is capable of providing immunity to patients. These challenges are mainly due to the highly-adaptive evasive responses the parasite employs in order to hide itself from the host immune system. An issue with malaria is that unlike other infectious diseases that have successful vaccines, people who get malaria never acquire complete immunity from the disease, but they can acquire some protective immunity. People who have been infected with malaria do have a lessened degree of severity for ensuing infections, which provides partial immunity but is not completely protective. Another issue is that there are many transitions in morphology and stage specific antigens throughout the parasite’s life cycle. This makes the development of a single vaccine extremely difficult. In order to create a perfect malaria vaccine, key goals would need to be met. These are: to generate a robust immune response to multiple stages of the parasite’s life cycle, overcome antigenic variation (*Pf*EMP1, *var* genes), and induce both cell-mediated and humoral immune responses in the host [49]. Currently, there are three approaches to vaccine development that focus on different stages of the *Plasmodium* lifecycle. These include transmission-blocking vaccines, pre-erythrocytic vaccines, and blood stage vaccines [50, 51].

Transmission-blocking vaccines are focused on preventing the formation of viable sporozoites. This vaccine strategy doesn’t prevent humans from getting malaria, but it

does prevent the infected individual from transmitting malaria to others, and thus provides protection to the community. The pre-erythrocytic vaccine strategy is focused on targeting the liver stages in order to prevent malaria in the human host. This would prevent the parasites from proliferating and entering circulation. Blood stage vaccines are focused on intervening in the malaria cycle by blocking merozoites from invading red blood cells. Several blood stage erythrocyte surface antigens are being evaluated as potential vaccine candidates. In 2015, the pre-erythrocytic *Plasmodium falciparum* vaccine candidate's RTS,S phase 3 clinical trial results were reviewed and in 2019 this vaccine was introduced into national pilot programs, in spite of generating only about 30% protection [52].

Vector control

Vector control is used to reduce transmission from mosquito to human and vice versa. Historically, this method has been the most effective weapon against malaria. Countries that have succeeded in malaria elimination achieved it largely due to the use of larvicides and pesticides such as DDT (dichlorodiphenyltrichloroethane). However, eliminating water sources where female mosquitoes lay eggs also proved to be important [53]. The widespread use of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) is currently the primary method of vector control. Using these interventions in combination represents nearly 60% of global funding toward malaria control. Aggressive vector control makes a considerable impact in endemic areas [54].

Larvicides and pesticides, although successful in the fight against malaria, are potentially toxic. In the 2001 Stockholm Convention, DDT was classified as a persistent

organic pollutant and thus, use was discontinued. However, in 2006 the World Health Organization continued to recommend DDT use for IRS in regions where the mosquitos remained DDT-susceptible.

ITNs are used not only to create a physical barrier between nighttime feeding mosquitos and sleeping humans, but to also kill these mosquitos. Pyerthroids are used on ITNs due to their low toxicity to humans and long-lasting residual activity. ITNs are two times more effective in malaria prevention compared to untreated nets. Due to the fact that net washing and lack of retreatment of ITNs reduces their efficacy, long-lasting insecticidal nets (LLINs) have generally replaced them. LLINs have insecticide resin bound to the fibers or incorporated into the fibers directly. These last much longer and can tolerate repeated washing [55, 56].

Indoor residual spraying is the application of long-lasting insecticide residue to the interior of domestic structures. When a female mosquito is looking for a blood meal, it often resides on the walls and ceilings of homes while in her search. These surfaces are covered with residual insecticide and thus, the mosquito absorbs it and is killed. This reduces the transmission of malaria. However, this method of control is dependent on the entire community to receive residual spraying, otherwise there is minimal protection obtained from an individual home [57].

Antimalarial Drugs & Rise in Resistance

Malaria is a curable disease if it is promptly diagnosed and treated correctly. Antimalarial drugs are vital in global malaria prevention. However, most of the drugs currently available have been in use for decades and are becoming severely limited by the

emergence and spread of drug resistance. Therefore, many endemic countries are facing a situation in which there are no affordable and effective antimalarial drugs. Although there is a tremendous global effort towards malaria drug discovery, the progress is not keeping pace with the emergence of drug resistance and issues such as distribution, accessibility, cost, and correct usage have only intensified the situation. The constant emergence of drug resistance has made it imperative to understand the mechanisms of resistance. In order to do so, it's essential to review the history of antimalarial drugs and to identify current knowledge of the mechanisms of drug activity.

Quinolines

Quinolines are aromatic nitrogen compounds that are characterized by a central solid-ring structure. Essentially, they are a benzene fused to a pyridine at two adjacent carbon atoms. Quinoline molecules can have various functional groups that add to their solubility and specificity of drug activity. The main quinolines used as antimalarial drugs can be grouped based on their chemical attributes. Type-1 quinolines are weak bases, deprotonated, and hydrophilic at pH 7. Type-2 quinolines are weaker bases and lipid soluble at neutral pH [58].

Quinine (QN)

This antimalarial is a natural product found in the native South American bark of the Cinchona tree. In 1820, QN was isolated by Joseph Pelletier and Jean Biename Caventou [59]. QN is used today for uncomplicated malaria but is not recommended for prophylaxis or routine treatment due to toxicity and cinchonism (a syndrome associated

with dizziness, impaired hearing, nausea, vertigo) [36]. QN is normally combined with an antibiotic and is useful for first-line treatment in areas with multidrug-resistant malaria. Furthermore, QN can be used to treat severe malaria because it can be administered intravenously and is highly effective. It is primarily active against mature trophozoites and does not prevent sequestration or further development of circulating rings. It's also not active against pre-erythrocytic stages of *Plasmodium falciparum.* It kills sexual stages of *Plasmodium vivax, Plasmodium malariae,* and *Plasmodium ovale*, but not mature gametocytes of *Plasmodium falciparum.* The exact drug mechanism of QN action has yet to be elucidated, but it is believed to interfere with heme detoxification or nucleic acid synthesis in the parasite [60, 61]. Unlike many other drugs, quinine resistance has been relatively slow, but reports of resistance began to emerge from Brazil in the early 1900s [62]. Quinine resistance is associated with polymorphisms in several transporters such as SNPs in the *pfmdr1*, *pfcrt,* and *pfmrp1* genes [63, 64].

Chloroquine (CQ)

In 1934, German research produced CQ as an alternative antimalarial drug to quinine and in 1946 US/British scientists recognized it as an effective and safe antimalarial [65]. CQ is easy to use and costs less than 10 U.S. cents per treatment, enabling poorer regions to treat more cases of malaria. It has become the most widely used antimalarial drug in the world and has utility for both treatment and prophylaxis. Although historically CQ has been the first-line drug of choice for treatment, widespread resistance to the drug made it rather ineffective [66]. CQ is a weak base that accumulates in the parasite's acidic digestive vacuole. The mechanism of drug action is quite

complex, but essentially CQ becomes deprotonated once it enters the acidic digestive vacuole and thus becomes trapped inside. It then acts to directly inhibit the polymerization of toxic heme molecules into hemozoin. The heme accumulates to levels that then kill the parasite [67]. CQ is not active against pre-erythrocytic stages and has low activity against young gametocytes [36]. Although CQ has become ineffective due to the rise of resistance, it is still effective against *Plasmodium falciparum* in the Caribbean, and Central America [36]. CQ remains the first line of treatment for *Plasmodium vivax* across the globe. CQ is well-tolerated against *Plasmodium vivax* and its long half-life provides protection from early relapses. However, *Plasmodium vivax* resistance to CQ is growing in many endemic areas [68]. Chloroquine resistance is linked to multiple point mutations in the *pfcrt1* gene, with K76T playing a central role in resistance [69].

Mefloquine (MQ) and Primaquine (PQ)

Initiated in the 1960s, the US Army Antimalarial Drug Development Program began to screen over 200,000 compounds for antimalarial activity. This effort took over 40 years and MQ was one of two licensed drugs that was developed as a result of this large effort [70]. It was first synthesized in 1969 for chemoprophylaxis in the military as it is effective against CQ or multidrug-resistant strains of *Plasmodium falciparum.* In 1977, it was introduced for general use. MQ is recommended for prophylaxis when traveling in CQ resistant malarious areas. However, there are areas in the world where MQ is also ineffective due to resistance [70]. MQ has a long half-life, allowing it to be used in combination therapies. A disadvantage of this drug is that it is known to cause neuropsychiatric episodes [48].

Primaquine (PQ) was part of a search for antimalarial drugs that preceded the discovery of CQ [71]. It was first used to prevent relapsing *Plasmodium vivax* in US soldiers returning from war. PQ is effective against all stages of parasites, except for sporozoites, making it an attractive drug. Until recently, it was the only FDA licensed drug that kills dormant *P. vivax* hypnozoites. It is used to provide radical cure, as it eliminates relapse, in combination with a blood schizonticide for the erythrocytic parasites [36, 72]. However, PQ is contraindicated in people that have a G6PD deficiency because of the risk of hemolytic anemia [36]. This drug cannot be used widely as an antimalarial drug as it displays toxicity. The exact mechanisms of action for MQ and PQ are unknown but it is hypothesized that these drugs inhibit membrane trafficking events involved in uptake of metabolites in RBCs, or that they are involved in hemoglobin degradation similar to CQ [73]. A recent theory for PQ mechanism involves a two-step biochemical relay which involves the reduction of the drug by cytochrome P450 NADPH:oxidoreductase and generation of hydrogen peroxide, leading to parasite death [74]. A theory for a possible mode of action of MQ is that it acts as a protein synthesis inhibitor through direct binding to a cytoplasmic ribosome [75].

Amodiaquine (AQ) and Piperaquine (PIP):

AQ was discovered as a potential antimalarial around the same time as CQ [76]. AQ has a similar mode of action as CQ and is metabolized by the primary metabolite monodesethylamodiaquine. It is effective against some CQ resistant strain of *Plasmodium falciparum*, although cross-resistance does exist. It is most commonly used as in combination with artesunate (ACT) or in combination with sulfadoxine-

pyrimethamine [36]. PIP was originally made in the 1960s and is approved in combination with dihydroartemisinin. It was used in China and Indochina as prophylaxis and treatment for two decades post establishment. It is thought to act on mature trophozoites and target heme detoxification [77]. The widespread use of PIP contributed to the formation of high levels of resistance. It was soon however discovered to be suitable in combination with artemisinin derivatives. This provided an inexpensive, short-course treatment with a high cure rate. After extensive use in China, resistance to the drug emerged. However, CQ-resistant parasites in Africa are susceptible to PIP, making it possible to use PIP and DHA in combination in African countries [78].

Tafenoquine (TQ):

Recently approved in 2018 by the FDA, TQ was created as an alternative antimalarial for the radical cure of *P. vivax* malaria and for prophylaxis use for malaria. Work with TQ began in the 1970s by scientists at the Walter Reed Army Institute of Research (WRAIR). TQ is an alternative to PQ due to its longer half-life and it solves issues regarding poor adherence compared to PQ, as it provides a radical cure in a single dose [79]. However, TQ remains contraindicated for patients with a G6PD deficiency or patients who are pregnant [80]. The exact mechanism of action of TQ is currently unknown. Since TQ is active against both liver and blood stages of both *P. falciparum* and *P. vivax*, it is hypothesized that it contains different modes of action for the different parasite stages. As in PQ, the cytochrome P450 metabolism of TQ plays a role in *P. vivax* liver stage activity, but that doesn't seem to be the case for its blood shizonticidal activity [81, 82].

Antifolates

During World War II, antifolates were developed as part of an antimalarial research program. Most antifolates used today were developed in the 1940s and 1950s [83]. Antifolates work by decreasing pyrimidine synthesis, leading to reduced DNA, serine, and methionine formation. Type I antifolates compete for the active site of dihydropteroate synthase (DHPS). Type II antifolates inhibit parasite dihydrofolate reductase (DHFR) [84]. When used alone, resistance developed. However, when used in combination, a synergy is observed and a pair are very effective in treating all types of malaria. Often the combination involves sulfadoxine and pyrimethamine (SP). Pyrimethamine is reported to be active against all parasite stages except sporozoites, and sulfadoxine is reported to display blood stage activity and activity against sexual stages [72]. SP is cheap and provides protection from new infections for a prolonged period due to is long half-life. However, resistance to SP emerged almost immediately and it is no longer effective for *Plasmodium falciparum* in areas of Asia, Africa, and South America. It is also ineffective for *Plasmodium vivax* in many areas. Now, it is used in combination with artesunate as an effective ACT wherever SP resistance is low [36].

Atovaquone (ATOV)

In 1996, ATOV, a naphthoquinone, was first introduced for treatment. It displays activity against *Plasmodium spp*., and it works by collapsing the mitochondrial membrane potential and inhibiting the electron transport chain [85]. ATOV can inhibit pre-erythrocytic development in the liver, oocyst development in the mosquito, and it has blood stage activity [72]. Since resistance to atovaquone develops rapidly, it is not

effective when used alone. However, when paired with proguanil or cycloguanil, there is a synergistic effect. The issue with atovaquone is that it is expensive to manufacture, and thus its use isn't practical for malaria-endemic countries. It is useful as an alternative to MQ for travelers from non-endemic countries that are traveling to endemic countries [36].

Antibiotics

Apicomplexan organisms are defined by the presence of, an apicoplast- a non-photosynthetic organelle evolutionarily obtained following a secondary endosymbiotic relationship between a eukaryote and red algae. Antibiotics have antimalarial activity because they can inhibit prokaryote-like processes in *Plasmodium*, such as protein synthesis and DNA/RNA replication. Antibiotics do not immediately kill the parasites, but rather cause a delayed death in the second cycle [78]. Because of this, there is a longer parasite clearance time and a prolonging of symptoms. Thus, antibiotics are used in combination treatment. Doxycycline (DOX), clindamycin (CLIN), and tetracycline (TET) inhibit protein synthesis. These drugs affect liver and blood stage parasites and are prescribed for malaria treatment [36].

Artemisinins (ART)

In 1971, Chinese scientists found that extracts of *A. annua* killed *Plasmodium berghei* in mice and a year later they identified the active ingredient. This is now known as artemisinin [86]. In 1985, artemisinin was extracted in the US at WRAIR [87]. Today, it is still obtained by extraction but there is a growing demand for high yields of the drug.

Methods are being developed for synthetic production of artemisinin precursors. Commercial production of a semi-synthetic artemisinin began in 2013. Briefly, artemisinin acid is produced by yeast fermentation and then followed by chemical conversion to artemisinin [88]. Currently, Artemisinins are the most important class of antimalarials and are used in combination for routine treatment as well as for treatment of severe malaria.

ART is a sesquiterpene trioxane lactone that contains an endoperoxide ring which is critical for ART's antimalarial activity [87]. Since ART is not soluble in oil or water, it must be given orally. Thus, chemical modifications were introduced to improve solubility. The most common derivatives of ART are dihydroartemisinin (DHA), artemether (AM), artesunate (AS), and artilenate (AL) [89, 90]. These derivatives offer different routes of administration. Most useful clinical ARTs are metabolized to DHA after absorption, and then to inactive metabolites via hepatic cytochrome P-450 and other enzyme systems [91]. Artemisinin itself isn't converted to DHA but is converted to inactive metabolites.

This class of compounds is the most rapidly acting and effective antimalarial to date. The parent drug and the derivatives all exhibit extraordinary activity against *Plasmodium falciparum* isolates, even those that are resistant to other antimalarial drugs. These drugs are active at low nanomolar concentrations and produce faster parasite and fever clearance times than any other antimalarial drug and reduce gametocyte carriage, effectively reducing transmission of malaria [92]. Furthermore, it has broad stage specificity and can eradicate young ring stages, which reduces the number of parasites that can mature and sequester in capillaries [93]. This is critical in treating severe malaria

as preventing the sequestration can prevent cerebral malaria. Thus, ART in this sense is better than QN, as QN does not prevent sequestration.

One of the biggest benefits of ART drugs is that they are effective in treating severe malaria that is resistant to CQ. Furthermore, ARTs can be used in combination with other drugs for high cure rates. Artemisinin combination treatments (ACTs) pair ARTs, which have a short elimination time, with a longer elimination time drug. ACTs work by having ART rapidly reduce the number of parasites that the partner drug then has to remove. They also prevent parasites that are resistant to the partner drug from emerging [93]. The combination of AS+MQ is a good example that has these properties and is very effective, even in areas of MQ resistance. However, the short half-life of ART drugs may be responsible for frequent recrudescence that has been observed in patients after treatment with a single ART drug (about 10%) [94, 95].

Mechanism of action

Although much research has been and is being devoted to ARTs and their effect on *Plasmodium* spp., the mechanism of action remains debated. It has been suggested that ARTs generate reactive oxygen species, which can be enhanced in the presence of free iron (heme) that is found in parasites after hemoglobin digestion. The iron-catalyzed reductive cleavage of the endoperoxide may give rise to O-centered, or alkoxyl, radicals and then C-centered radicals and neutral products become activated by free ferrous or heme iron. These radicals may cause effects by overwhelming the parasite oxidative stress mechanisms [96]. Alternatively, the peroxide could also undergo ring-opening via protonation, or formation of a complex with a metal ion to generate an open

hydroperoxide or metal peroxide. This can lead to conversion into a peroxy radical, or the transfer of oxygen to oxidizable substrates [84]. Other studies have found that artemisinin can interfere with heme detoxification in the digestive vacuole or that it localizes to neutral lipid bodies in the digestive vacuole where they damage parasite membranes after oxidation reactions [97]. Since more hemoglobin is metabolized by later stages, using the above hypothesized mechanisms, it makes sense to think that more mature stages are the main target of ARTs. This is how it appears that CQ works on older trophozoites, where large amounts of ferrous heme are produced and are prevented from detoxification by CQ.

ARTs have also been found to target mitochondrial function by interfering with the electron transport chain and by inducing reactive oxygen species that cause mitochondrial membrane potential loss and disrupt the normal function of mitochondria [98, 99]. Another recent hypothesis is the inhibition of a calcium-dependent ATPase [100]. Evidence suggests that ARTs inhibit PfATP6 outside the digestive vacuole after iron-activation. Further evidence also suggests that mutations within *pfatp6* are responsible for ART resistance in *Plasmodium falciparum* [101, 102].

Another recent study claims that ART kills parasites by a two-step mechanism which causes protein damage and compromises parasite proteasome function. The accumulation of proteasome substrates then activates the ER stress response which causes the DHA-mediated killing of *Plasmodium* [103].

All these studies point to plausible mechanisms of action, but none are largely accepted so far. The question of the true mechanism of action of ART is becoming more demanding as reports of emerging resistance to ART have been published [104-108].

Artemisinin resistance

Reports of ACT failure and resistance to artemisinin are emerging. Resistance was first reported in western Cambodia and has since spread or emerged independently in other areas of Southeast Asia, and now in Africa as well [108]. Partial artemisinin resistance is seen in delayed parasite clearance following ART therapy. Full resistance is represented by strains that survive and multiply despite exposure to high drug doses. Point mutations in the *PF3D7_1343700* kelch propeller domain on chromosome 13 (K13) have been identified as a molecular marker for *in vitro* and *in vivo* artemisinin resistance [109]. K13 mutations in artemisinin resistant parasites are less susceptible to oxidative damage created by the drug. While there isn't a clear understanding of the mechanism of ART resistance in the parasite yet, many studies have connected resistance to increased cellular stress, altered DNA replication, activated unfolded protein responses, reduced protein translation, and increased levels of phosphatidylinositol 3-phosphate [110-114].

The cellular function of K13 and its involvement in artemisinin resistance remained unknown, until recently. A recent study identified interactors of K13 using a novel BioID approach. Inactivating these proteins rendered parasites resistant to ART, which revealed a critical pathway for ART resistance. These proteins were shown to be important for hemoglobin endocytosis and that ART resistant parasites had reduced hemoglobin endocytosis. By reducing endocytosis, the concentration of active drug inside the parasite is reduced, thus allowing the parasite to avoid death [115].

Dormancy

Liver stage dormancy (Hypnozoites)

In some *Plasmodium* species, such as *P. vivax, P. ovale,* and *P. cynomolgi,* a subset of parasites can arrest growth in the liver at an early asymptomatic stage known as hypnozoites [116]. These dormant parasites can remain quiescent anywhere from days to years. Hypnozoites have been shown to relapse *in vitro* using a *P. cynomolgi* reporter line that expresses GFP and shows mCherry expression when schizogony occurs, and clinically hypnozoites have led to relapses in infection when the parasite resumes growth [117]. However, the mechanisms behind hypnozoite formation and subsequent relapses are currently unknown [118]. *Plasmodium* hypnozoites are insensitive to most marketed antimalarials except primaquine and tafenoquine, which are contraindicated in populations with a G6PD deficiency, highlighting the need for new antimalarials with hypnozoite killing activity [119].

Unfortunately, attempts to identify such compounds are hindered by our limited understanding of the molecular pathways that enable hypnozoite survival and reactivation. Furthermore, until recently, study of these forms proved difficult due to a lack of suitable *in vitro* culture systems. However, progress has been made towards the development of *P. vivax* model systems that can support liver stage hypnozoite and schizont development and these have proven to be instrumental for molecular studies to understand parasite biology and for anti-hypnozoite drug screens [118, 120, 121]. This has led to studies which have shown that the hypnozoite recruits various host cell proteins in order to sustain its development. These include GLUT1, aquaporin-3, and protein traffic modulators such as COPB2 and GGA1 [122-125]. Many of these host factors are

recruited to the parasitophorous vacuole membrane. This has helped us to begin to understand parasite development in the liver, but questions remain regarding hypnozoite dormancy and reactivation.

Currently, there is no molecular marker for hypnozoites, making it difficult to study these elusive forms. A schizont specific marker, Liver Stage Protein-2 (LISP2) was found to be expressed very early on during schizogony, but it is downstream of DNA synthesis and replication, therefore it does not indicate the time at which DNA synthesis is initiated [126]. A marker for the initiation of DNA synthesis would ideally identify relapsing parasites coming out of dormancy. The trigger for hypnozoite activation has remained perplexing. Hypnozoite activation has been proposed to be epigenetically controlled, activated by subsequent mosquito bites, activated by other present infectious diseases, or chemically activated [127-131].

Drug Induced Dormancy in Plasmodium falciparum

A form of dormancy is also found in a drug induced manner in the asexual blood stages. Recrudescent infections have been observed in patients after treatment with a single ART drug (about 10%) [95]. Furthermore, up to 50% of patients that are given artemisinin monotherapy have shown recrudescent infections [132, 133]. When parasite clearance is observed in peripheral blood smears, patients are considered to have recovered from malaria. However, some persistent parasites can fall below the level of microscopic detection and lead to recrudescence when parasetimia increases and clinical symptoms of malaria return [93].

Drug induced dormancy has been theorized as a means by which *P. falciparum*

blood stage parasites evade drug stress and eventually recrudesce following exposure to drugs. A growth arrest of *in vitro* ring stage parasites exposed to DHA has been reported and is now accepted. These induced dormant parasites then resume growth after 3-8 days post DHA treatment. When early ring stages are exposed to DHA, a morphological change is induced which transforms the ring into a small form that has condensed nuclei and cytoplasm and an absence of the vacuole [134, 135]. Exposure to 700 nM DHA for just 6 hours is enough to induce this phenotype *in vitro*. These conditions are characteristic of the conditions in patients treated with artemisinin. Once drug pressure is removed, growth commences as the parasite continues to develop.

Furthermore, Hott et. al. have demonstrated that pyrimethamine is capable of inducing a second cycle ring stage dormancy [136]. When exposed to drug, rings are not immediately arrested, rather development continues through schizogony and it is the daughter rings of the subsequent cycle that undergo arrested development. These parasites appear similar to those exposed to DHA, morphologically, and recrudescence is seen 5-11 days later [136].

While the mechanism the parasite utilizes to enter and exit dormancy to evade ART drug pressure remains elusive, recent data suggests that phytohormones (plant hormones), including gibberellic acid (GA) and abscisic acid (ABA), activate DHA-induced dormant rings up to 48 hours earlier than control parasites treated with DHA alone [137]. In plants, these signaling phytohormones promote initiation or release from dormancy, respectively [138, 139].

Purine Transport & Metabolism

Plasmodium parasites are purine auxotrophs meaning they cannot synthesize their own purines de novo but instead rely on salvaging them from the host [140]. Work by Bungener and Neilsen showed that *P. berghei* incorporates [3H]adenosine and [3H]hypoxanthine purines, but not [3H]uridine and [3H]thymidine pyrimidines, into nucleic acids [141, 142]. Studies have demonstrated that *Plasmodium* relies on de novo synthesis of pyrimidine and lacks the ability to metabolize exogenous pyrimidines [143], which is contrary to the parasites being entirely dependent on the salvage of extracellular purines [140]. In fact, the continuous culture of *P. falciparum* blood stage parasites in serum-free media is reliant on the supply of exogenous purines, such as hypoxanthine [144].

The mechanisms which *Plasmodium* uses to salvage purines are complex with regard to primary sources and to the routes of conversion. Purines are transported across several membranes to reach the parasite and extensive research has identified equilibrative nucleoside transporters (ENT) which aid in this process. To enter the host cell in humans, purines are transported via hENT1 and hENT2 [145]. The hENT1 and hENT2 transport both purines and pyrimidines. The imported purines can then be either modified within the host cell through a subset of purine salvage pathway enzymes. In the red blood cell, imported adenosine is either phosphorylated by adenosine kinase (hAK) and converted to AMP or it is deaminated by adenosine deaminase (hADA) into inosine. The inosine is subsequently converted to hypoxanthine by purine nucleoside phosphorylase (hPNP). The hypoxanthine can then be modified to IMP by hypoxanthine guanine phosphoribosyl transferase (hHGPRT). The hypoxanthine can also be released back into the plasma and either oxidized to xanthine or uric acic [146-148].

Early work shows that adenosine is rapidly transported across the parasite plasma membrane [149] and subsequent studies using sequence analysis found that the *P. falciparum* genome contains four putative ENT homologues termed PfENT1-4 [150]. The primary transporter for purine import is via PfENT1. Knockout studies of PfENT1 gene have shown to be conditionally lethal as parasites with a PfENT1 gene deletion were only viable when the purines adenosine, inosine, or hypoxanthine were present at super-physiological concentrations [151]. Furthermore, it has been shown that the parasite contains a sequential pathway for exogenous purine conversion into hypoxanthine [151]. Hypoxanthine is a key precursor for all purine synthesis in *Plasmodium* metabolism. *Plasmodium* does not have any adenosine kinase activity. Instead, *P. falciparum* salvages adenosine by conversion to hypoxanthine using the sequential steps of converting adenosine to inosine by the adenosine deaminase (PfADA) and inosine to hypoxanthine by the purine nucleoside phosphorylase (PfPNP). Hypoxanthine is then converted to IMP by hypoxanthine-guanine-xanthine phosphoribosyltransferase (PfHGXPRT). IMP is then the metabolic precursor for all purine nucleotides and deoxynucleotides [148].

Scope of Study

Malaria is caused by protozoa of the genus *Plasmodium*, and it remains a major global health problem that affects 3.2 billion people. The current antimalarial treatments target asexual blood and liver stages, as well as transmission between the mosquito vector and human host. However, the current gold standard treatment, artemisinin, which targets the asexual blood stages, is ineffective in addressing significant problems when the parasite

enters an artemisinin-induced dormant period and evades the antimalarial. Furthermore, the only two current drugs available to target both the dormant liver stage hypnozoite and active liver stage schizonts are less than ideal since they are not indicated for patients with a G6PD deficiency and pregnant women. Other drugs fail to treat dormant liver stage hypnozoites, which are common in *P. vivax* infections, and lead to reoccurring infections months to years after primary infection. Currently, the mechanisms by which *Plasmodium* parasites enter either asexual blood stage artemisinin-induced dormancy and later recrudesce to continue development, or liver stage hypnozoites enter dormancy and later activate and lead to recurring infections, are unknown. In addition, parasites do not recrudesce or activate in a synchronous manner. The malaria field not only lacks the information in understanding these mechanisms, but also lacks the tools to answer these questions to then develop better therapeutics. We believe that developing tools to study these phenomena, as well as elucidating the mechanisms and identifying the targets which regulate these dormant parasites, is essential for the development of novel antimalarial treatments that will truly target and prevent parasite drug evasion.

We hypothesized that we could take advantage of the parasite's DNA replication and cell cycling to help in developing a tool to study *Plasmodium* dormancy, as dormant parasites do not undergo DNA synthesis and replication until they reactivate. We postulated that since *Plasmodium* is a purine auxotroph, we could utilize a combination of alkyne-labeled purines and click chemistry to study and differentiate between dormant and active parasites. A common tool already in use to study DNA replication and cell cycle in human cells is the labelling of newly replicated DNA with the modified nucleotide bromodeoxyuridine (BrdU), followed by immunofluorescent detection. However,

Plasmodium does not incorporate BrdU because it does not salvage thymidine analogues, like BrdU, for conversion into nucleotides. Thus, in this study, we aimed to design a novel tool using the modified nucleotides: 7-deaza-7-ethynyl-2'-deoxyadenosine (EdA), 7-deaza-7-ethynyl-2'-deoxyinosine (EdI), 7-deaza-7-ethynylhypoxanthine (EdH), and 8-ethynylhypoxanthine (8eH), to study DNA synthesis and replication to differentiate between dormant and active parasites.

Next, we wanted to elucidate the mechanism which regulates dormancy. Previously, the effects of the addition of exogenous phytohormone Gibberellic Acid (GA) to dihydroartemisinin (DHA)-induced dormant asexual blood stage ring *P. falciparum* parasites had been explored in the lab. We found that dormant ring stages exposed to GA recovered from dormancy up to 48 hours earlier than parasites exposed to DHA alone. We hypothesized that we could utilize this phenomenon and GA as a tool to understand how parasites enter and exit dormancy. We proposed to localize and identify the interacting partners of GA in asexual blood stage *P. falciparum* using chemical biology approaches. Our research strategy, which involved taking a novel approach by utilizing click chemistry with modified purines and/or plant hormones, should help us broaden our understanding of parasite dormancy mechanisms. Ultimately, this will have a broad impact on global health in terms of the development of antimalarials and the aim to eradicate this disease.

CHAPTER 2

ALKYNE MODIFIED PURINES FOR ASSESSING ACTIVATION OF *PLASMODIUM VIVAX* HYPNOZOITES AND GROWTH OF PRE-ERYTHROCYTIC AND ERYTHROCYTIC STAGES IN *PLASMODIUM SPP.*[1]

[1]Botnar, A., Lawrence, G., Maher, S.P., Vantaux, A., Witkowski, B., Shiau, J.C., Merino, E.F., De Vore, D., Yang, C., Murray, C., Cassera, M.B., Leahy, J.W., and Kyle, D.E. Submitted to *International Journal of Parasitology*, 10/12/2021.

Abstract

Malaria is a major global health problem which predominantly afflicts developing countries. Although many antimalarial therapies are currently available, the protozoan parasite causing this disease, *Plasmodium spp.,* continues to evade eradication efforts. One biological phenomenon hampering eradication efforts is the parasite's ability to arrest development, transform into a drug-insensitive form, and then resume growth post-therapy. Currently, the mechanisms by which the parasite enters arrested development, or dormancy, and later recrudesces or reactivates to continue development, are unknown and the malaria field lacks techniques to study these elusive mechanisms. Since *Plasmodium spp.* salvage purines for DNA synthesis, we hypothesized that alkyne-containing purine nucleosides could be used to develop a DNA synthesis marker which could be used to investigate mechanisms behind dormancy. Using copper-catalyzed click chemistry methods, we observe incorporation of alkyne modified adenosine, inosine, and hypoxanthine in actively replicating asexual blood stages of *P. falciparum* and incorporation of modified adenosine in actively replicating liver stage schizonts of *P. vivax*. Notably, these modified purines were not incorporated in dormant liver stage hypnozoites, suggesting this marker could be used as a tool to differentiate replicating and non-replicating liver forms and, more broadly, as a tool for advancing our understanding of *Plasmodium* dormancy mechanisms.

Introduction

Malaria is caused by parasites of the genus *Plasmodium* and remains a major global health problem that infects 218 million and kills about 409,000 people a year, mostly children under the age of five. While much effort has been made over the years towards the control and elimination of this disease, recent progress has plateaued [1]. Of the five species affecting humans, *P. falciparum* and *P. vivax* account for most cases and pose the greatest threat. Though *P. falciparum* is the deadliest of the human infecting species, *P. vivax* is more widespread geographically and produces a dormant uninucleate liver stage. Termed hypnozoites, these forms can persist for days, months, and even years, before an unknown mechanism stimulates their reactivation and causes relapsing infections [116]. *P. falciparum* does not produce hypnozoites, however, a stress-induced growth arrest in the asexual blood stage ring stage parasites has been observed when treated with artemisinin (ART) monotherapy [135, 152, 153]. This mechanism of induced quiescence is theorized to be a means for drug evasion and subsequent parasite recrudescence once drug pressure is removed. Both species present their own unique obstacles, in terms of eradicating malaria, with quiescence being a common thread.

Hypnozoites are insensitive to all currently marketed antimalarials except the 8-aminoquinolones primaquine and tafenoquine. However, these drugs are contraindicated in patients with a glucose-6-phosphate dehydrogenase (G6PD) deficiency or who are pregnant [46, 47]. This highlights the urgency of discovering novel anti-malarial drugs, the development of which would be aided by a better understanding of the hypnozoite's basic biology. Whilst suitable high-throughput *in vitro* assays for screening compounds against *P. vivax* liver stage parasites have been recently developed, to date there are no

specific markers to exclusively distinguish hypnozoites from liver schizonts [120]. A recombinant antibody reactive to the parasitophorous vacuole membrane (PVM) resident protein upregulated in Infectious Sporozoites 4 (PvUIS4) has been generated, but it immunofluorescently stains both hypnozoites and liver schizonts [154]. Differentiation between the two forms relies on parasite size and specific chemosensitivity, thus much care must be taken to morphologically distinguish hypnozoites from early schizonts [155]. In addition to hypnozoite-specific markers, the identification of markers indicating reactivation of DNA synthesis in hypnozoites would aid in characterizing the mechanism of dormancy and resumption of growth. Two examples of similar markers are Liver-Specific Protein 2 (LISP2), and acetylated lysine 9 of histone H3 (H3K9Ac) [118, 126]. LISP2 has been found to express in early developing liver stage parasites; however, it is limited in that staining is not observed until three days post-infection in *P. vivax.* Furthermore, while LISP2 expression is an early event in liver stage schizont development, the timing of increased expression of LISP2 versus DNA replication has not yet been characterized [156]. H3K9Ac has been elegantly used to accurately count individual nuclei in hypnozoites versus liver stage schizonts [118]. However, this marker indicates only nuclear division and not necessarily active DNA synthesis. In order to differentiate and capture hypnozoites at the moment of reactivation, a marker for DNA synthesis is needed.

Acute, uncomplicated *P. falciparum* infections are most often treated with ART combination therapies (ACT) that are active against blood stages [36]. ART derivatives are fast acting drugs and are extremely effective in reducing parasite biomass. While this treatment has been extremely effective in reducing malaria burden, slower parasite

clearance times have been reported as resistance to ART treatment begins to rise [107, 157-159]. Failure rates of ART monotherapy vary widely, anywhere from 2%-50%, and these have been shown to associate with developmentally arrested ring stages [132-134]. While *in vitro* culturing of *P. falciparum* asexual blood stage and induction of dormancy is possible, elucidating the underlying mechanisms of the induced dormant rings remains technically challenging. These dormant parasites present phenotypically with condescend nuclei and reduced cytoplasm, and thus are difficult to differentiate from dead parasites. Although much work has been done to provide insights into DHA-induced dormancy, the molecular mechanism that allows some parasites to enter this stage and later recrudesce is unknown. Furthermore, these dormant stages recrudesce asynchronously, and we currently lack the tools to differentiate between early versus late activators. The development of a DNA synthesis marker that differentiates latent versus active parasites would aid in studying how DHA-induced dormant parasites recover from quiescence.

In many organisms, 5-bromo-2'-deoxyuridine (BrdU) and ethynyl-2'-deoxyuridine (EdU), analogs of the nucleoside thymidine, have been used to identify proliferating cells versus non-proliferating cells [160, 161]. Previous attempts have been made to adapt these labelling techniques for *Plasmodium*, however they failed due to the parasite's requirement for de novo synthesis of pyrimidines [162]. Although *P. falciparum* expresses transporters that should allow BrdU to be taken up [163, 164], the parasite lacks a thymidine kinase (TK) and thus cannot convert thymidine from a deoxynucleoside into a deoxynucleotide. Recently, studies showed that transfection with TK can allow BrdU labelling in *P. falciparum*, however parasites became much more sensitive to BrdU toxicity [165]. Furthermore, this technique is not suitable for

Plasmodium species that cannot be easily cultured for transfection, such as *P. vivax*. *Plasmodium* is a purine auxotroph however, and thus salvages host cell purines [148]. Therefore, similar labeling techniques using alkyne modified purines instead of pyrimidines should be amenable to study *Plasmodium* biology. Mammalian cells incorporate alkyne modified purine versions of adenosine, 7-deaza-7-ethynyl-2'-deoxyadenosine (EdA) and guanosine, 7-deaza-2'-deoxyguanosine (EdG), and recent work with a related apicomplexan, *Cryptosporidium parvum,* showed incorporation of EdA [166, 167]. Thus, we hypothesized alkyne modified purines present a potential DNA synthesis marker that can be designed to differentiate between active versus proliferating and dormant versus nonviable parasites. The sequential steps of *P. falciparum*'s metabolism of adenosine to inosine via an adenosine kinase (AK) followed by the conversion from inosine to hypoxanthine via the purine nucleoside phosphorylase (PNP) presents an additional benefit to synthesize and investigate incorporation of alkyne labeled inosine and hypoxanthine. Hypoxanthine is the closest precursor in the parasite's metabolic pathway for all purine nucleotides and deoxynucleotides which are used for nucleic acid synthesis.

We hypothesized a modified purine could serve as a marker for reactivation from dormancy as an indicator of DNA synthesis and we designed several purine analogs to be labelled using fluorescent chemo-labelling ("click chemistry"). Click chemistry provides the advantage of no animal reactivity, no potential for cross-reactivity, easier production and storage, and increased flexibility in multicolor experiments. In this study, the development and application of clickable nucleoside analogs EdA, 7-deaza-7-ethynyl-2'-deoxyinosine (EdI), 7-deaza-7-ethynylhypoxanthine (EdH), and 8-ethynylhypoxanthine

(8eH), collectively termed EdX, as DNA synthesis markers of proliferating parasites is described. Furthermore, we use EdA staining to help differentiate between dormant and developing liver stage parasites.

Materials and Methods

Alkyne modified purine derivatives (EdX)

Given the ability of *P. falciparum* to salvage purines, we hypothesized that alkyne modified derivatives of purine precursors could be developed as tools for the study of DNA synthesis in blood and liver stages of the life cycle. Previous studies with mammalian cells demonstrated that EdA and EdG can be used for cell proliferation studies. For our studies with malaria parasites, we obtained EdA from a commercial source (Carbosynth, United Kingdom), yet similar derivatives of inosine and hypoxanthine required novel synthetic methods. Therefore, we collaborated with Dr. James W. Leahy and Grant Lawrence at the University of South Florida to synthesize EdI, EdH, and 8eH.

In vitro culture of intraerythrocytic P. falciparum parasites

Plasmodium falciparum clone W2 (Indochina II) was cultured using standard techniques [9]. Briefly, parasites were maintained at 37°C in hypoxic conditions (90% N_2, 5% CO_2, 5% O_2) at a hematocrit of 2% A+ human red blood cells (RBCs; Interstate Blood Bank, Memphis, TN). Parasites were cultured in complete medium consisting of RPMI1640 supplemented with 25 mM HEPES, 0.24% (w/v) sodium bicarbonate and either a) 10% heat-inactivated A+ human plasma (Interstate Blood Bank, Memphis, TN)

or b) 1% (w/v) AlbuMAX II (Thermo Fisher) and 320 μM hypoxanthine (Sigma). Parasite development was monitored with light microscopy of Giemsa-stained blood smears.

EdX incorporation and Cu-catalyzed azide-alkyne staining of active intraerythrocytic parasites

Asynchronous *P. falciparum* was split to 2% parasitemia in a 2% hematocrit and supplemented with 10 μM EdA (Carbosynth, United Kingdom), EdI, EdH, and/or 8eH, and incubated for 48 hours at 37 °C in hypoxic conditions. After incubation, parasites were briefly centrifuged, and the supernatant removed. Infected cells were then fixed in a solution containing 4% paraformaldehyde and 0.05% glutaraldehyde for 15 minutes at room temperature, adapted from Balu et. al. 2010 [168]. Following a wash with phosphate buffer saline (PBS, pH 7.4), parasites were prepared for the azide-alkyne click reaction by permeabilization with 0.1% Triton X-100 for 10 minutes, followed by a 1-hour incubation with 3% BSA at room temperature. Cells were then stained with a freshly prepared staining mix containing 2 mM $CuSO_4$ (Sigma), 12 μM Alexa Fluor Azide 488 (Thermo Fisher), and 10 mM sodium ascorbate (Sigma) for 1 hour in the dark at room temperature. Parasites were then washed once with PBS and co-stained with 10 μg/mL Hoechst 33342 (Thermo Fisher) for 15 minutes followed by three washes with PBS. They were then mounted onto poly-L-lysine coated slides and imaged with a Zeiss Axio Observer.Z1/7. The FIJI plugin, JACoP, was used to generate Mander's coefficients and Pearson's coefficient, *r*, for co-localization of Hoechst 33342 nuclear staining and EdX incorporation.

Cytotoxicity assays of EdX on intraerythrocytic parasites

Cytotoxicity of the modified purines was assessed by measuring the increase in parasitemia over time. Briefly, asynchronous *P. falciparum* was split to 0.5% parasitemia in a 2% hematocrit and supplemented with 10 µM EdX. Parasites were incubated at 37 °C in hypoxic conditions and allowed to grow for 5 days. On day 3 parasites were split 1:5 to avoid parasite death due to overgrowth. Samples were taken daily and fixed with 4% paraformaldehyde and 0.05% glutaraldehyde and stored at 4 °C until all samples were collected. Parasites were then stained with 10 µM Hoechst 33342 for 15 minutes followed by three washes with PBS and percent parasitemia was analyzed via flow cytometry using a Beckman Coulter CytoFLEX. Primary gating was performed based on background fluorescence from uninfected red blood cells to obtain parasite-infected red blood cells as an indication of parasitemia. Results were then visualized via a GraphPad Prism 9 plot where statistical analysis was also conducted using a 2-way ANOVA multiple comparisons test.

Human Subjects Consideration

P. vivax isolates were collected into a heparin tube (BD) via venipuncture from human volunteers following approval by the Cambodian National Ethics Committee for Health Research (113NHECR). Protocols conformed to the Helsinki Declaration on ethical principles for medical research involving human subjects (version 2002) and informed written consent was obtained from all volunteers or legal guardians.

In vitro liver stage P. vivax incorporation of EdX

Primary human hepatocytes (PHH) were infected with *P. vivax* sporozoites as previously described [120]. Briefly, *Anopheles dirus* mosquitos were fed a bloodmeal containing *P. vivax* gametocytes and maintained on a 12:12 L:D cycle and 10% sucrose in water. Two days prior to infection, PHHs (lot BGW, BioIVT) were seeded into collagen-coated 384-well plates (Greiner Bio-One) at a density of 18,000 cells per well. Mosquito salivary glands were aseptically dissected on days 16-21 post feeding to obtain *P. vivax* sporozoites. PHH seeded plates were then infected with 5,000-18,000 sporozoites per well. Infected PHHs were then exposed to EdX on days 5, 6, 7, and 8 post infection (dpi) with 2 μM EdX, 10 μM EdX, and DMSO vehicle control. Media was exchanged daily immediately before EdX exposure. At 9 dpi, cultures were fixed with 4% paraformaldehyde in PBS. Fixed cultures were stained with recombinant mouse anti-*P. vivax* upregulated in infectious sporozoites-4 antibody (rPvUIS4) [154] diluted 25,000-fold in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) overnight at 4 °C in the dark. Following three washes with PBS, cells were then stained with rabbit anti-mouse Alexafluor488 (Abcam) diluted 1:1000 diluted in staining buffer for 1 hour at room temperature in the dark. Cultures were then washed three times with PBS followed by staining with a freshly prepared staining mix containing 2 mM $CuSO_4$, 12 μM Alexa Fluor Azide 594 (Thermo Fisher), and 10 mM sodium ascorbate for 1 hour in the dark at room temperature. The cells were then washed three times with PBS and counterstained with 10 μg/mL Hoechst 33342 for 15 minutes at 37 °C. Cultures were washed once with PBS and stored in PBS prior to automated high content imaging with a 20x objective on an ImageXpress confocal microscope (Molecular Devices, San Jose, CA). Liver stage

parasites and host cell hepatocytes were quantified using the MetaXpress software version 6.6.1.42 for ImageXpress. Individual images were also obtained with a 100x objective on a DeltaVision II deconvolution microscope (Applied Precision Inc., Currently Leica Microsystems, Buffalo Grove, IL). Analysis was conducted using GraphPad Prism 9 with an ordinary one-way ANOVA multiple comparisons test.

HepG2 EdX Staining

HepG2 hepatoma cells were cultured in collagen-coated T75 flasks in minimum essential medium eagle with Earle's BSS (MEM Eagle EBSS) from Lonza (Walkersville, MD) supplemented with 10% FBS and 4.4 mM sodium pyruvate at 37 °C with 5% CO_2. Cells were seeded at 5,000 cells per well in a collagen coated 384 well plate (Greiner Bio-One). EdX (2 μM or 10 μM) and 0.1% DMSO vehicle control were added 24 hours post seed and allowed to incorporate for 48 hours at 37 °C and 5% CO_2. Cells were then fixed with 4% paraformaldehyde for 15 minutes at room temperature. Fixed cells were stained with freshly prepared staining mix containing 2 mM $CuSO_4$, 12 μM Alexa Fluor Azide 488, and 10 mM sodium ascorbate in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) overnight at 4 °C in the dark. Following three washes with PBS, cells were counterstained with 10 μg/mL Hoechst 33342 for 15 minutes at 37 °C. Cultures were washed once with PBS and stored in PBS prior to automated high content imaging with a 4x objective on a Lionheart imaging system (Biotek). Viability was measured by counting cell nuclei using Gen5 software (Biotek) and statistical analysis was conducted using GraphPad Prism 9 with an ordinary one-way ANOVA multiple comparisons test and unpaired t-test.

In vitro liver stage P. berghei incorporation of EdA

An. stephensei mosquitos were fed a *P. berghei* gametocyte-infected bloodmeal and maintained on 12:12 L:D cycle and 10% sucrose in water. One day prior to infection with sporozoites, HepG2 cells were seeded at 17,500 cells per well in a collagen-coated 384 well plate (Greiner Bio-One). Mosquito salivary glands were aseptically dissected on day 20-22 post feeding to obtain *P. berghei* sporozoites. HepG2 seeded plates were then infected with 6,000 sporozoites per well. Infected and uninfected cultures were then treated 24 hours post infection (hpi) with 2 μM EdA, 10 μM EdA, and 0.1% DMSO vehicle control. Media was exchanged before EdA treatment. At 48 hpi, cultures were fixed with 4% paraformaldehyde in PBS. Fixed cultures were stained with mouse monoclonal antibody 13.3 anti- Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) diluted 10,000-fold in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) overnight at 4°C in the dark [120]. Anti-GAPDH was obtained from The European Malaria Reagent Repository. Following three washes with PBS, cells were then stained with rabbit anti-mouse Alexafluor488 diluted 1:1000 diluted in staining buffer (0.03% Triton X-100, 1% (w/v) BSA in PBS) for 1 hour at room temperature in the dark. Cells were then washed three times with PBS followed by staining with a freshly prepared staining mix containing 2 mM $CuSO_4$, 12 μM Alexa Fluor Azide 594, and 10 mM sodium ascorbate for 1 hour in the dark at room temperature. Cells were then washed three times with PBS and counterstained with 10 μg/mL Hoechst 33342 for 15 minutes at 37 °C. Cells were washed once with PBS and stored in PBS prior to imaging at 40x on a Zeiss Axio Observer.Z1/7 (Pleasanton, CA).

In vitro P. falciparum EdA, ^{3}H-hypoxanthine, and ^{3}H-adenosine incorporation in dihydroartemisinin (DHA)-induced dormant asexual blood stage parasites

P. falciparum (W2) asexual blood stage parasites were synchronized to ring stages using 5% D-sorbitol and were then split to 2% parasitemia in a 2% hematocrit. Dormancy was then induced 48 hours post-synchronization with 700nM DHA for 6 hours while parasites were incubated at 37°C in hypoxic conditions. Following three washes with RPMI1640, parasites were re-suspended and cultured in complete media containing 10% heat-inactivated A+ human plasma. Parasite recrudescence and development was monitored daily with light microscopy of Giemsa-stained blood smears and media was changed every 48 hours. Aliquots of 100 µL were transferred daily (up to 10 days post dormancy induction) to a 96 well plate where 5 µCi ^{3}H-hypoxanthine (Perkin Elmer), 5 µCi ^{3}H-adenosine (Perkin Elmer), or 10 µM EdA was supplemented. These aliquots were incubated for 24 hours at 37 °C in hypoxic conditions after which they were either frozen at -80 °C (for tritiated samples) or fixed with 4% paraformaldehyde and 0.05% glutaraldehyde and stored in 4 °C until all samples were collected (for EdA-labeled samples). Uninfected red blood cells (uRBC) and active asynchronous parasites at a starting 2% parasitemia were used as controls. Once all samples were collected, click chemistry was performed on EdA labelled samples as described above and incorporation was measured via flow cytometry on a Beckman Coulter CytoFLEX (Indianapolis, IN). Tritiated samples were harvested and counted on a Perkin Elmer Microbeta^{2} scintillation counter (Waltham, MA). Data was graphed and analyzed using GraphPad Prism 9.

Results

Synthesis of EdI, EdH, and 8eH

The synthesis of EdI and EdH was initiated from commercially available 6-chloro-7-deazapurine. From a cost-effective perspective, it was most pragmatic to prepare 8eH from inosine. Structures of the EdX compounds used in this study, including EdA (purchased from Carbosynth, United Kingdom) are shown in Figure 2.1.

Incorporation of EdX analogues into asexual blood stages of P. falciparum.

To evaluate if EdA, EdI, EdH, and 8eH were incorporated into newly synthesized DNA of *Plasmodium*, we conducted labeling studies using a copper-catalyzed azide-alkyne cycloaddition chemical reaction with a fluorescent azide probe. Using an *in vitro* asynchronous asexual blood stage culture of *P. falciparum*, we observed that the addition of 10 μM of each of the alkyne modified purines resulted in nuclear staining after a 48-hour incubation period, equivalent to a full asexual blood stage life cycle (Figure 2.2). The observed staining co-localized with nuclear Hoechst 33342 staining, which preferentially binds to the A-T regions of DNA, and a Fiji JACoP Pearson's coefficient for EdA, EdI, EdH, 8eH was calculated to range from 0.991 to 0.997, thus indicating that the modified purines were incorporated into parasite DNA. Manders' coefficients for the four images shown in Figure 2.2 were also calculated and ranged from 0.925 to 0.999.

Considering the potential cytotoxicity of the modified purines, we assessed parasite growth in the presence of these compounds to quantify their effects on parasite proliferation. Furthermore, because serum supplements contain different levels of purines, we evaluated the cytotoxic effects of EdA, EdI, EdH, and 8eH over a 5-day

incubation period in complete media containing albumax II alone (1% w/v), complete media containing albumax II and supplemented with hypoxanthine (10 μM), and complete media containing 5% A+ human plasma. The media containing albumax II alone lacks any purines for the parasites to salvage whereas media supplemented with hypoxanthine or human plasma contains purines, although at different levels. In media without hypoxanthine supplementation, all modified purine compounds showed a deleterious effect ($p < 0.0001$) on parasite proliferation as compared to the unmodified hypoxanthine media control (Figure 2.3a). Cultures with media containing either supplemented hypoxanthine or human plasma were unaffected by the addition of alkyne modified purines (Figure 2.3b-c). These results indicate that while the EdX analogues incorporated into asexual blood stage *P. falciparum* parasite DNA, unmodified purines are necessary for continued parasite growth over time.

P. vivax actively replicating liver stage schizonts incorporate EdA and can be differentiated from dormant hypnozoites via high-throughput content imaging

After invading a hepatocyte, a *P. vivax* sporozoite develops into either an actively replicating liver schizont or a dormant hypnozoite with a single nucleus [169]. Over the first 3-5 days of liver stage culture, hypnozoites and schizonts are of similar sizes and are indistinguishable [155]. Therefore, to verify that EdX analogs incorporate into actively replicating parasite DNA in the liver stage, cultures were supplemented with 2 μM or 10 μM of EdA, EdI, EdH, and 8eH at 5, 6, 7, and 8 dpi and then cultures were fixed at 9 dpi. By using fluorescent microscopy, we observed that the addition of EdA resulted in nuclear staining of only actively replicating liver stage schizonts, whereas dormant

hypnozoites did not incorporate EdA. The EdA fluorescence co-localized with nuclear Hoechst 33342 staining, similar to our *P. falciparum* asexual blood stage staining (Figure 2.4). However, unlike in *P. falciparum* asexual blood stage parasites, *P. vivax* liver stage parasites did not incorporate EdI, EdH, or 8eH (data not shown).

We next evaluated if the EdA staining in *P. vivax* liver stage schizonts could be identified in a high-throughput manner using an automated high content imaging system. We showed that EdA incorporation can be identified and imaged at 20x with a high content imaging system (Figure 2.5a). We tested two concentrations of EdA and observed that separation of liver stage schizonts and dormant hypnozoites can be accomplished using 2 or 10 μM EdA (Figure 2.5b-c). Incorporation of EdA was quantified using maximum fluorescence intensity (of EdA) for each parasite in culture compared to parasite net growth area (PvUIS4). Typically, 150 μm^2 is used to demarcate hypnozoites versus liver schizonts based on parasite size alone. Separate DMSO-treated cultures were used as a negative control for click labeling. This analysis revealed that, while most hypnozoite forms were negative for EdA, some forms of similar size and morphology were positive for EdA and were therefore synthesizing DNA at some point between days 5-8 dpi and these forms are likely reactivating. We also assessed the effect of EdA and its cytotoxicity on PHH, which are non-replicative, and were not found to incorporate EdA. However, we noticed a slight cytotoxic effect of EdA on PHH count as compared to DMSO vehicle control. Cultures treated with 2 μM and 10 μM for three consecutive days had a slight statistically significant decrease in hepatocyte nuclei count, yet the toxic effect did not appear to hinder parasite growth (Figure 2.5d-e).

P. berghei liver stage schizonts incorporate EdA

P. berghei does not relapse *in vivo* and produces only liver stage schizonts. To confirm that all *P. berghei* liver stage parasites are actively replicating, and also confirm the incorporation of EdX in liver stages, we added EdA to *P. berghei* infected hepatocyte cultures for 24 hours prior to fixation. As shown in figure 2.6, all parasites were found to be positive for EdA staining. *P. berghei* liver stage assays are routinely performed by using the human hepatocarcinoma cell line (HepG2) as host cells [170]. Since HepG2 cells are actively replicating, we assessed HepG2 incorporation of EdA at 2 μM and 10 μM alone in the absence of infection for up to 72 hours (Figure 2.7). We noted that while HepG2 cells successfully incorporate EdA, this modified purine is noticeably cytotoxic to the hepatocytes. While 60% of HepG2 cells incorporate EdA at the lower concentration of 2 μM, only 20% of HepG2 cells incorporate EdA at 10 μM (Figure 2.8). HepG2 nuclei counts were significantly reduced at both concentrations as compared to DMSO control. Altogether, our results suggest that EdA can be used for shorter times at lower concentrations with HepG2 cells and can be used for longer durations with PHH.

P. falciparum asexual blood stage parasites recrudescing from dihydroartemisinin (DHA) – induced dormancy do not incorporate EdA, but do incorporate $^{[3H]}$ hypoxanthine and $^{[3H]}$ adenosine

Previous studies have confirmed that exposure of ring stages to DHA induces a dormant phenotype that is both time of exposure and DHA concentration dependent [135, 153]. Due to the successful incorporation of EdA in *P. vivax* liver stage schizonts, we assessed if EdA incorporation could be used to differentiate DHA-induced dormant *P.*

falciparum rings from recrudescing parasites. Following exposure to 700 nM DHA, parasites were sampled daily and pulsed with 10 µM EdA for 24 hours. We observed that as parasites recrudesced and the number of infected red blood cells (parasitemia) increased, incorporation of EdA did not increase concomitantly (Figure 2.9a). Thus, we assessed if this lack of correlation was due to potential purine storage in the parasite as a response to DHA treatment, or if DHA treated parasites became more sensitive to the alkyne modification to adenosine and thus were unable to uptake and incorporate EdA. Parasites were treated with DHA and then sampled daily and pulsed with either ^{3}H-hypoxanthine or ^{3}H-adenosine for 24 hours. Daily Giemsa smears were also collected to assess morphology of recrudescing parasites. To compare the incorporation of radiolabeled purines in recrudescing parasites, a control sample of parasites that did not undergo DHA treatment was included. The control sample of parasites received 24-hour pulses of radiolabeled hypoxanthine or adenosine. Control samples replicated and reached high parasitemia by day 2 and sampling was stopped. We observed that as parasites recrudesced and parasitemia increased, incorporation of radiolabeled purines occurred at the same rate as parasitemia measured by microscopic analysis of Giemsa-stained blood smears (Figure 2.9b). Altogether, our data suggest that either DHA exposed parasites do not store purines or that the alkyne modification affects incorporation into DNA of DHA treated parasites.

Discussion

The ability of *Plasmodium* to convert into a dormant phenotype and later reactivate causing recrudescent or relapse infections remains a serious barrier towards

malaria eradication. Reactivation occurs both naturally in the liver stages and following a drug-induced growth arrest during the intraerythrocytic life cycle [171]. The mechanisms by which the parasite enters dormancy and later recrudesces or reactivates to continue development is not fully understood and we lack tools to study these mechanisms. For *P. vivax* liver stages, a H3K9Ac marker for nuclear division and a LISP2 marker have been developed and associated with reactivation [118, 126]. However, these markers are downstream of DNA synthesis and replication, and do not indicate the time at which exact DNA synthesis is initiated in activated hypnozoites. Since purine and pyrimidine nucleotides are the building blocks of nucleic acids, biosynthesis or incorporation of these building blocks marks initiation of DNA synthesis [172]. Since *Plasmodium* salvages purines from the host [164], we hypothesized that alkyne-containing purine nucleosides could be developed as makers for DNA synthesis markers to differentiate between active and dormant parasites. Our study provides the first report of a clickable DNA synthesis marker for *Plasmodium* which can easily be integrated into a staining panel design due to its flexibility. We show that EdA, EdI, 8eH, and EdH are incorporated into DNA in *P. falciparum* asexual blood stages, and that EdA is incorporated into actively replicating liver stage schizonts in *P. vivax* and *P. berghei*, but it does not incorporate into dormant *P. vivax* liver stage hypnozoites.

The methodology described in this study represents a valuable tool for *P. vivax* liver stage studies as it is the first to describe a DNA synthesis marker which can be used to distinguish actively replicating and dormant liver stages of the parasite. Interestingly, we noted that some liver forms of similar size and morphology to that of PI4K-insensitive hypnozoites do incorporate DNA, indicating that these forms could be newly reactivating

parasites (Figure 2.4 and 2.5). Alternatively, recent reports describe how liver stage parasites must constantly buffer themselves against host cell lysosomes [173]. Thus, it is also possible that these small, EdA-positive liver stage forms are schizonts which failed to develop due to factors such as the host response to infection. Our data suggest EdA could be used in future studies to identify and characterize newly reactivating parasites and other host-parasite interactions. Interestingly, only EdA was found to incorporate in *P. vivax* and *P. berghei* liver stage parasites. Previous work has shown rapid metabolism of inosine and hypoxanthine into allantoin by rat hepatocytes, which could explain why parasite incorporation of EdI, 8eH, or EdH was not achieved in the liver stage [174].

On the other hand, we observed that active blood stage *P. falciparum* parasites incorporated all the alkyne-modified purines (Figure 2.2). This creates a novel opportunity to study DNA synthesis pathways and cell cycling. Unexpectedly, *P. falciparum* recrudescing blood stage parasites coming out of DHA-induced dormancy did not incorporate any of the modified purines (Figure 2.9a). Moreover, when comparing incorporation of radiolabeled hypoxanthine and adenosine, to EdA incorporation in recrudescing parasites, we found that EdA was not incorporated while the radiolabeled purines were metabolized and incorporated into DNA. Previous work has shown that artemisinin-resistant *P. falciparum* has altered metabolic programming [175]. Blood stage parasites showed decreased levels of adenosine deaminase and hypoxanthine-guanine phosphoribosyltransferase (HGXPRT) in Kelch13 (K13) mutant parasites, suggesting a prior pooling of purines in response to ART drug exposure [175]. While the work reported here was conducted utilizing a K13-wild type *P. falciparum* strain sensitive to ART, it is possible that in response to DHA exposure, even K13-wild type

parasites may accumulate internal stores of purines after exposure to DHA. Alternatively, structural modifications to purine precursors may cause steric hinderance of the purine salvage enzymes (e.g., purine nucleoside phosphorylase). Although the EdX may not be useful for DHA-induced dormancy studies in *P. falciparum*, they remain valuable as an alternative to radiolabeled purines, as these alkyne-modified purines can potentially be used as an inexpensive tool for investigating the parasite's scavenging mechanisms.

In *P. falciparum* blood stage *in vitro* cultures, we observed that in the presence of hypoxanthine, the modified purines were not cytotoxic. However, parasites lacking supplementation of hypoxanthine for purine salvage were not able to sustain growth when supplemented with the alkyne modified purines alone. Nevertheless, incorporation of EdA, EdI, 8eH, and EdH was observed in media supplemented with hypoxanthine. These data suggest that parasite DNA replication enzymes sense a difference between modified and unmodified purines; however, incorporation still occurs at a sufficient low enough rate that it cannot replace hypoxanthine to sustain replication and parasite growth, but it can be visualized via fluorescence microscopy. In our *P. vivax* and *P. berghei* liver stage experiments, it is important to note that a slight hepatocyte toxicity from EdA was observed which could confound our studies. Nevertheless, parasite growth remained unaffected (figure 2.5). In the present study, long incubations times such as 48-72 hours were used. However, to assess reactivation from dormancy, shorter incubation times could be used in future studies to obtain narrower time windows of reactivation and to limit negative impacts on the assay due to hepatocyte toxicity.

This methodology provides many opportunities for the study of malaria parasite biology. While most preliminary *in vitro* drug screening for liver stage activity is

conducted using *P. berghei*, medium throughput platforms using high-content imaging of *P. vivax* liver stages are now being used to confirm and optimize anti-hypnozoite hits [120]. By incorporating EdA into the high-content pipeline, we can further characterize both the parasite forms quantified during analysis, as well as gain a better understanding of the effect of agonists or antagonists of hypnozoite reactivation. Ideally, future experimentation will involve a *P. vivax* time course with combination of LISP2, EdA, and H3K9Ac staining to better define the timing of DNA replication, nuclear division, and membrane synthesis in reactivating parasites. Furthermore, EdA staining approach could aid in elucidating the cause of hypnozoite reactivation, which has been hypothesized to include fever, hemolysis, malaria reinfection, and chemical reactivation [129, 131]. Recently we reported the recruitment of host aquaporin 3 to the parasite PVM, as well as the formation of a tubulovesicular network around *P. vivax* liver forms; these mechanisms have been hypothesized to be part of the parasite's nutrient-scavenging pathways [124, 155]. EdA staining could be used as a bait to further elucidate pathways responsible for purine scavenging. Additionally, we can postulate that EdA would incorporate into *P. cynomolgi* liver stages and could therefore be useful for *P. cynomolgi* drug discovery platforms. *P. cynomolgi* produces hypnozoites, although *P. cynomolgi* hypnozoites and liver stage schizonts have been reported to be much smaller than their *P. vivax* counterparts [127, 176, 177]. Yet, size alone is often used as the defining attribute of hypnozoites versus schizonts during high-content analysis. Given our finding that some hypnozoite-like forms are synthesizing DNA, and *P. cynomolgi* liver forms are both relatively smaller than those of *P. vivax*, misclassification of hypnozoites and schizonts is

very possible in this model as well, but it could be better characterized using a marker such as EdA.

Herein, we detail the first reported use of alkyne modified purines to study the biology of *Plasmodium*. The use of alkyne-modified purines enables Chemo-labelling ("Click chemistry"), providing advantages over traditional antibody staining in that researchers do not have to consider animal/species cross-reactivity. Furthermore, it offers increased flexibility since the alkyne modified purines can be "clicked" to any azide-linked molecule of choice and can also be leveraged in a high-throughput manner using high-content imaging systems. In addition, the novel purine analogues reported here may have potential uses in other organisms that have yet to be tested and validated. This new tool is inexpensive, easy to incorporate into current workflows, and provides flexibility, making it an ideal tool as a DNA synthesis marker.

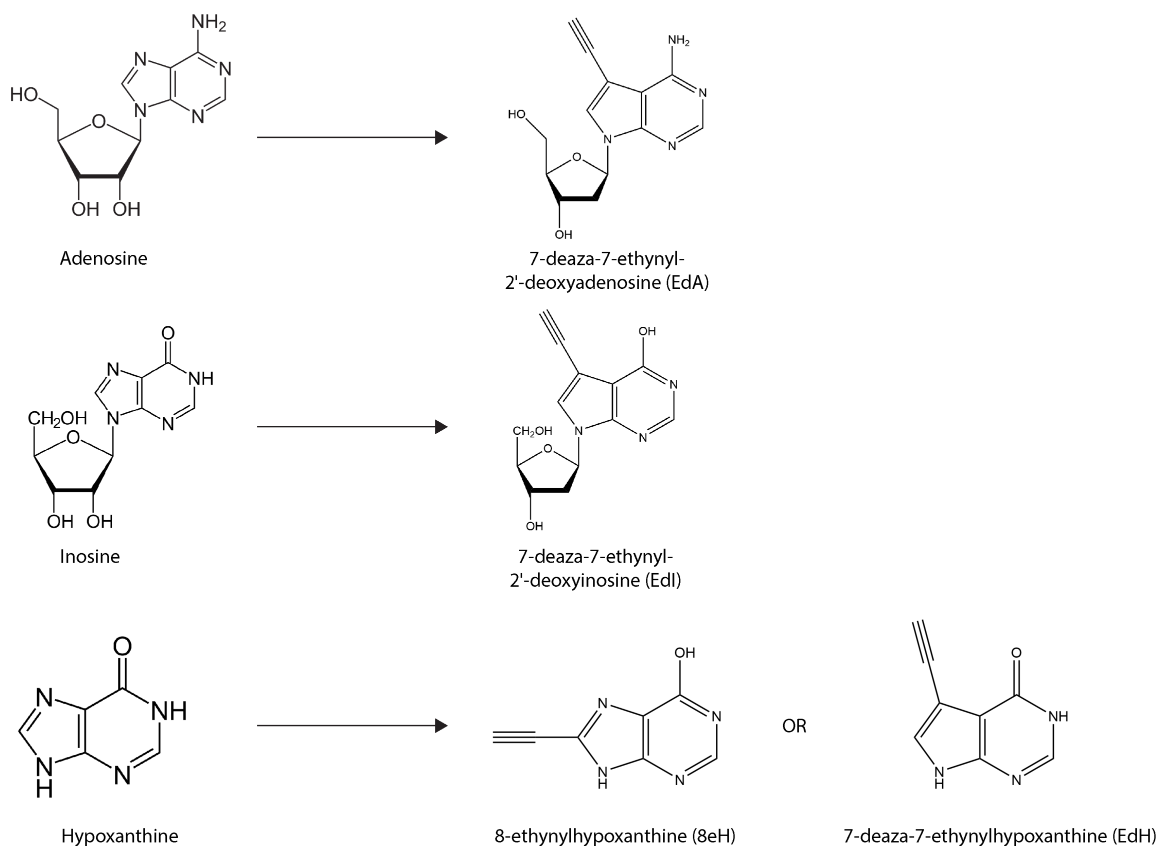


Figure 2.1. Structures of the alkyne modified purines (EdX). Modified adenosine (EdA) is commercially available and was purchased from Carbosynth (United Kingdom). Modified Inosine (EdI) and modified hypoxanthine (EdH and 8eH) were synthesized by Dr. James W. Leahy and Grant Lawrence (University of South Florida).

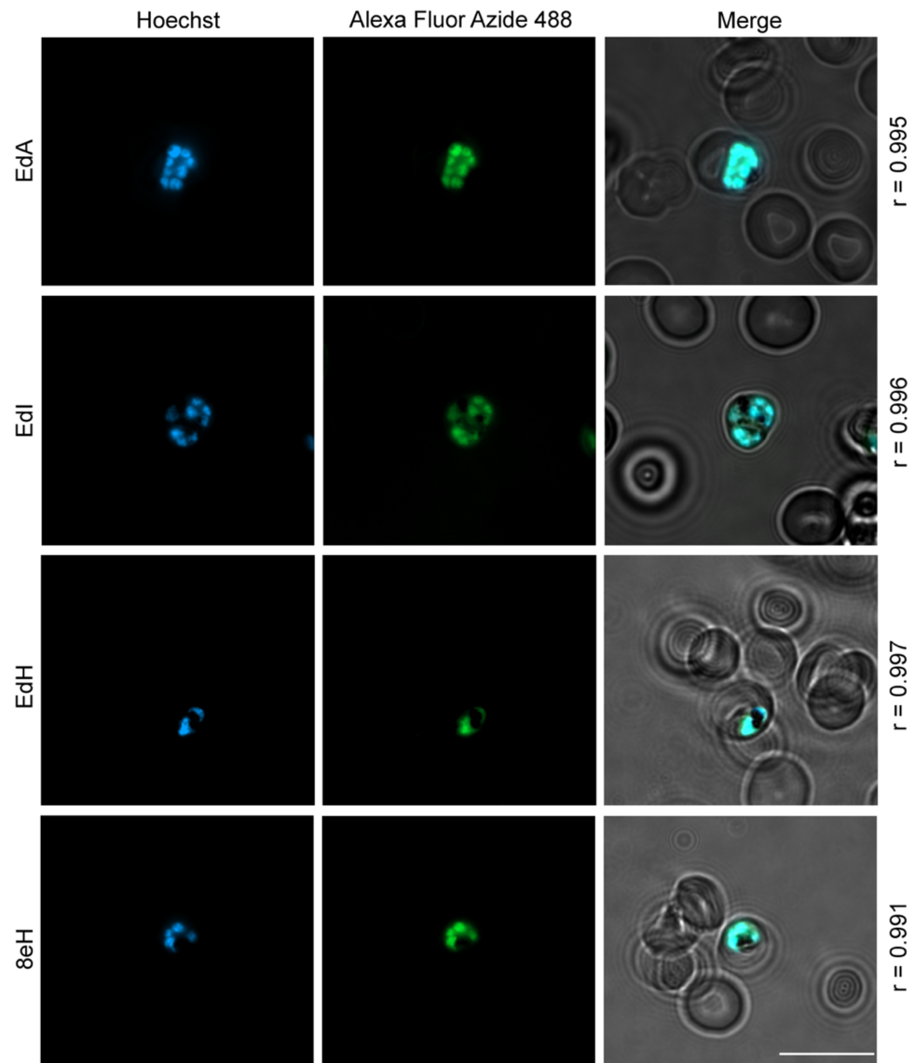


Figure 2.2. Alkyne modified purines incorporate into the replicating asexual blood stage *P. falciparum*. Detection of modified purines in *P. falciparum* after a 48-hour incubation with 10 µM of EdA, EdI, EdH, and 8eH (green). Parasites were co-stained with 10 µg/mL Hoechst 33342 (blue). Images were obtained on a Zeiss Axio Observer.Z1/7 microscope with a 100x objective. Colocalization was assessed and Pearson's coefficient (r) was calculated using Fiji JACoP plugin. Scalebar = 10 µm.

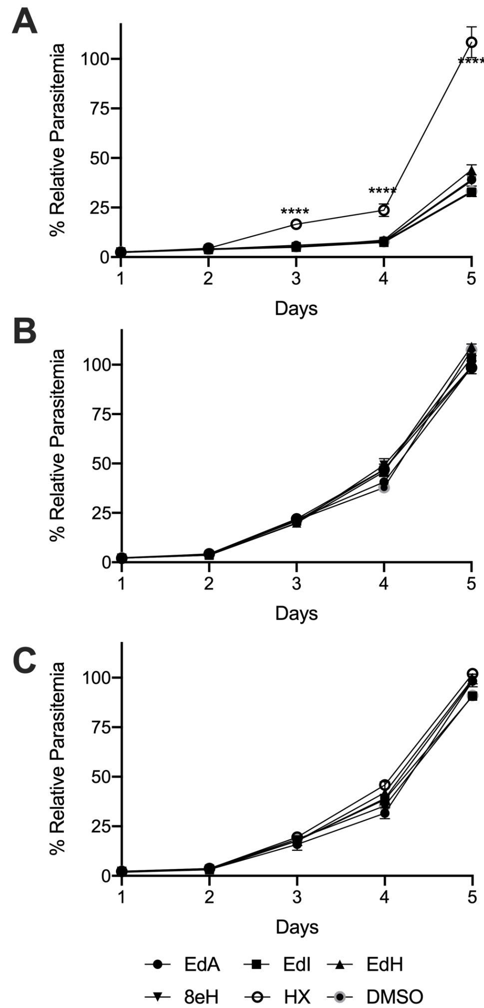


Figure 2.3. *P. falciparum* asexual blood stage growth depends on unmodified purines in culture media. *P. falciparum* asexual blood stages were grown in media supplemented with 10 μM EdA, EdI, EdH, 8eH, hypoxanthine (HX), or 0.1% DMSO vehicle control in **(A)** complete media supplemented with Albumax II, **(B)** complete media supplemented with Albumax II and hypoxanthine, or **(C)** complete media supplemented with A+ human plasma. Growth was assessed by flow cytometry using a Beckman Coulter CytoFLEX. Data shown are one representative experiment of three independent experiments. Errors (SD) were omitted when smaller than the marker. Significance assessed by 2-way ANOVA and Dunnett's multiple comparisons test, **** $p < 0.0001$.

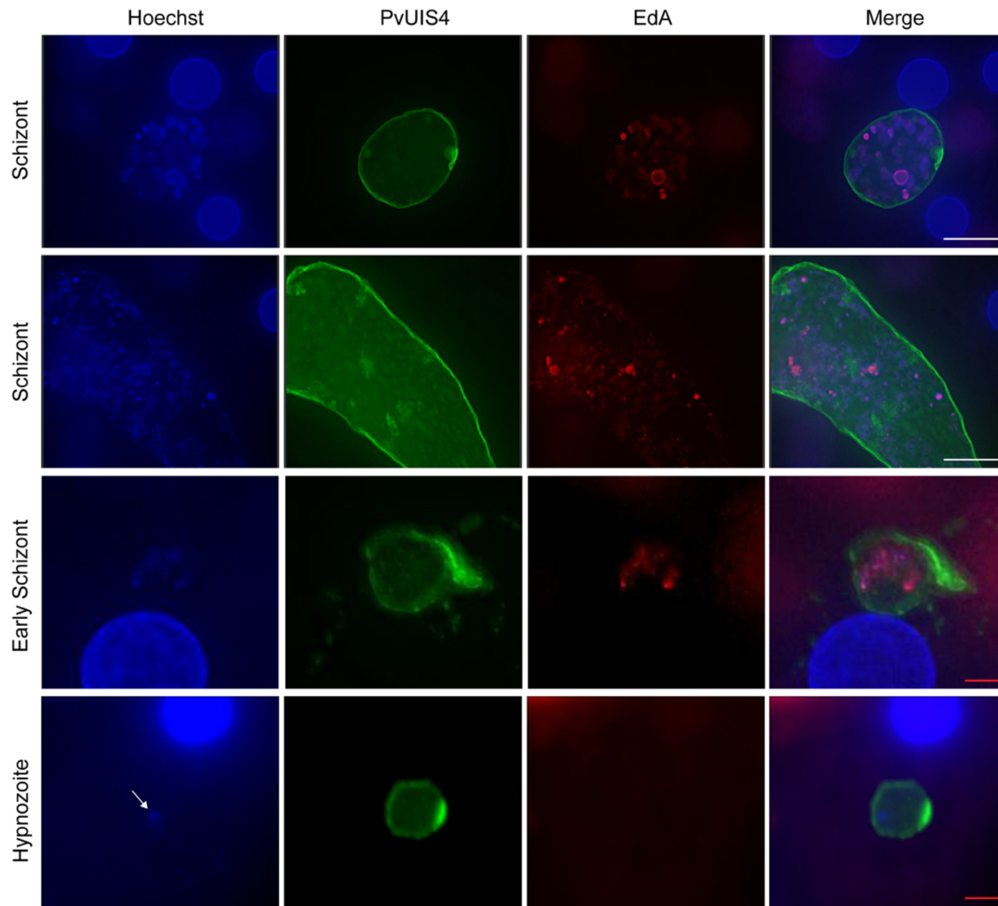


Figure 2.4. Alkyne modified adenosine (EdA) incorporates in replicating *P. vivax* liver stage parasites, but not in hypnozoites. Primary human hepatocytes were infected with *P. vivax* sporozoites and incubated with 10 µM EdA on days 5-8 post infection. Detection of EdA was assessed via a copper-catalyzed click reaction (red). Parasites were co-stained with 1:25,000 PvUIS4, a parasitophorous vacuole membrane stain (green), and 10 µg/mL Hoechst 33342 (blue). Arrow points to single nucleus in *P. vivax* liver stage hypnozoite. Images were obtained on a DeltaVision II deconvolution microscope at 100x objective. White scalebar = 15 µm. Red scalebar = 5 µm.

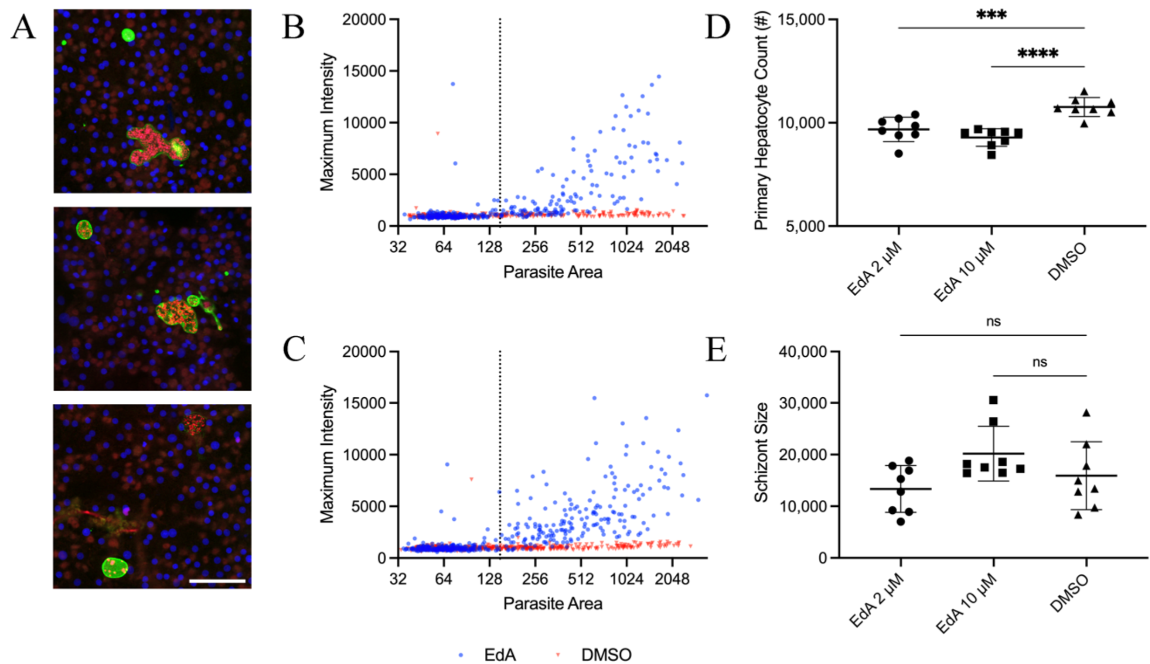


Figure 2.5. EdA incorporated into *P. vivax* hypnozoites and liver stage schizonts can be identified and quantified via high content imaging. *P. vivax* infected primary human hepatocytes (PHH) were cultured in media supplemented with EdA and fixed at day 9 post-infection prior to immunofluorescent staining and click labeling. **(A)** Images acquired of *P. vivax* liver stage parasites (UIS4, green) with incorporated EdA (red) imaged at 20x with an ImageXpress confocal high content imaging system. Bar represents 100 µm. Incorporation of either **(B)** 2 µM or **(C)** 10 µM EdA was quantified using maximum fluorescence intensity for each parasite in culture with a net growth area shown on the x-axis. Dotted lines represent the typical cutoff of 150 µm^2 used to demarcate hypnozoites versus liver schizonts based on parasite size alone. Separate DMSO-treated cultures represent a negative control for click labeling. Notably, EdA at both concentrations is incorporated in several hypnozoites, indicating DNA synthesis is occurring and these forms are likely reactivating. **(D)** EdA cytotoxicity was assessed on

PHH using nuclei count as an indicator of toxicity. **(E)** Liver stage schizont size was assessed for EdA vs. DMSO treated parasites. Figure is a representative of one independent replicate out of two ($n = 2$). An ordinary one-way ANOVA Dunnett's multiple comparisons test was used for **D** and **E**. *** $p = 0.0005$, **** $p < 0.0001$.

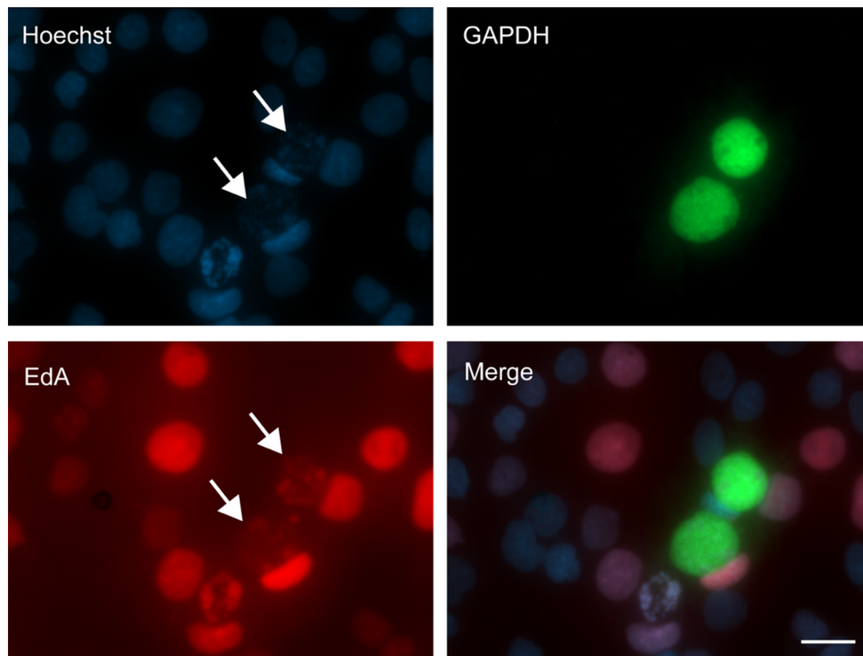


Figure 2.6. Alkyne modified adenosine (EdA) incorporates in replicating *P. berghei* liver stage parasites. HepG2 cells were infected with *P. berghei* sporozoites and 10µM EdA was supplemented at 24 hours post infection. Cells were then fixed 48 hours post infection. Detection of EdA was assessed via a copper-catalyzed click reaction (red). Parasites were co-stained with 1:10,000 GAPDH (green) and 10 µg/mL Hoechst 33342 (blue). Images were obtained on a Zeiss Axio Observer.Z1/7 microscope at 40x objective. Scalebar = 20 µm.

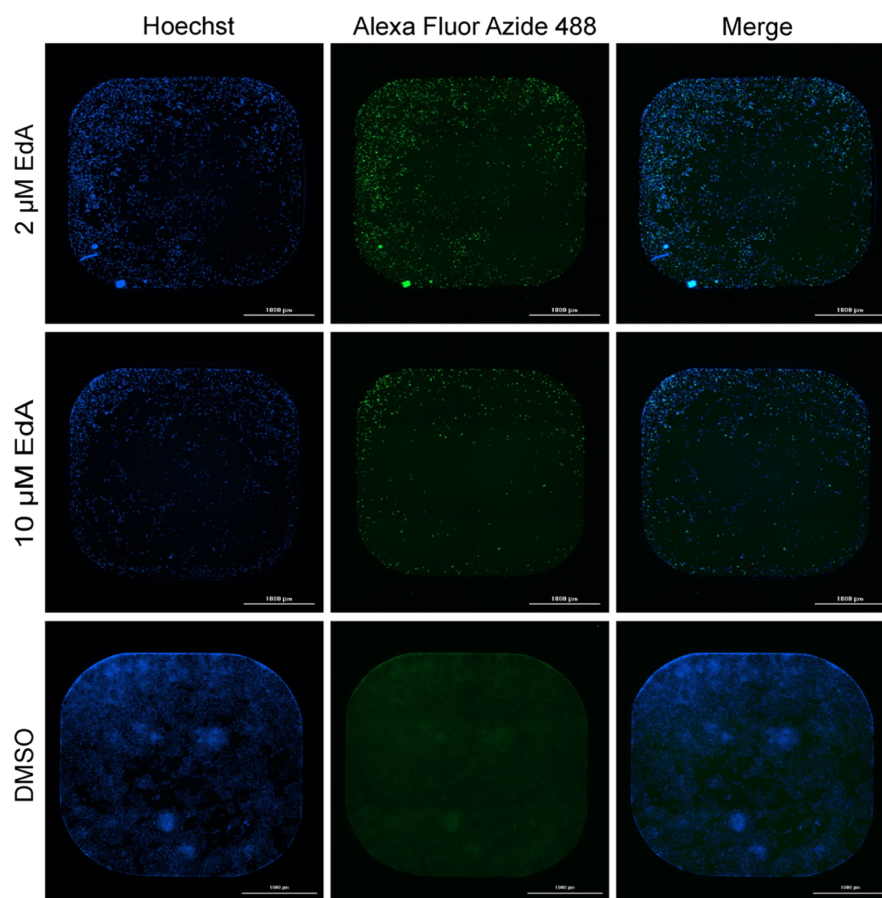


Figure 2.7. Alkyne modified adenosine (EdA) incorporates in replicating HepG2 mammalian cells. HepG2 cells were seeded at 5,000 cells/well and 2 μM or 10 μM EdA was then supplemented at 24 hours post-seed. Cells were then fixed 72 hours post-seed. Detection of EdA was assessed via a copper-catalyzed click reaction (green). HepG2 nuclei were co-stained with 10 μg/mL Hoechst 33342 (blue). Images were obtained on a Lionheart FX automated microscope at 10x objective.

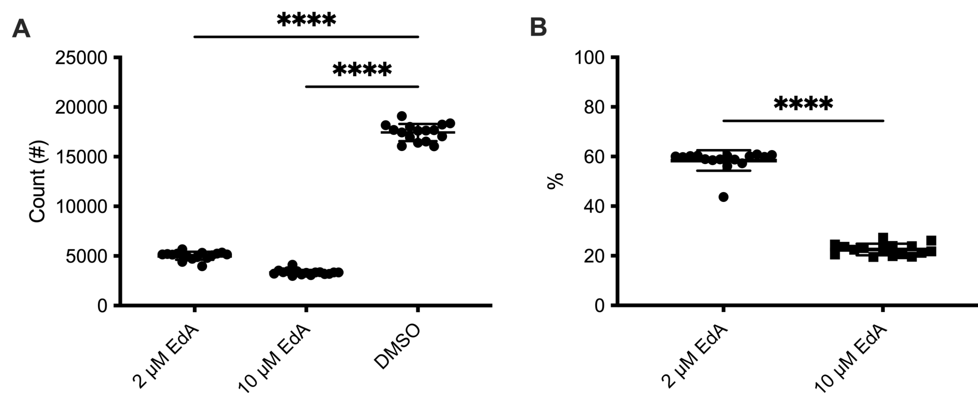


Figure 2.8. EdA labels replicating HepG2 mammalian cells and is cytotoxic. HepG2 cells were seeded at 5,000 cells/well and 2 µM or 10 µM EdA was supplemented at 24 hours post-seed. Cells were fixed 72 hours post-seed. EdA incorporation was assessed via a copper-catalyzed click reaction and HepG2 nuclei were co-stained with 10 µg/mL Hoechst 33342. Analysis was then conducted using Gen5 software. **A)** Nuclei count was assessed by Hoechst nuclear staining and **B)** EdA incorporation was assessed as percentage of EdA positive nuclei. Data shown are one representative experiment of two independent experiments (average ± SD). Significance was assessed using an ordinary one-way ANOVA with Dunnett's multiple comparisons test (**A**) or an unpaired t-test (**B**), **** $p < 0.0001$.

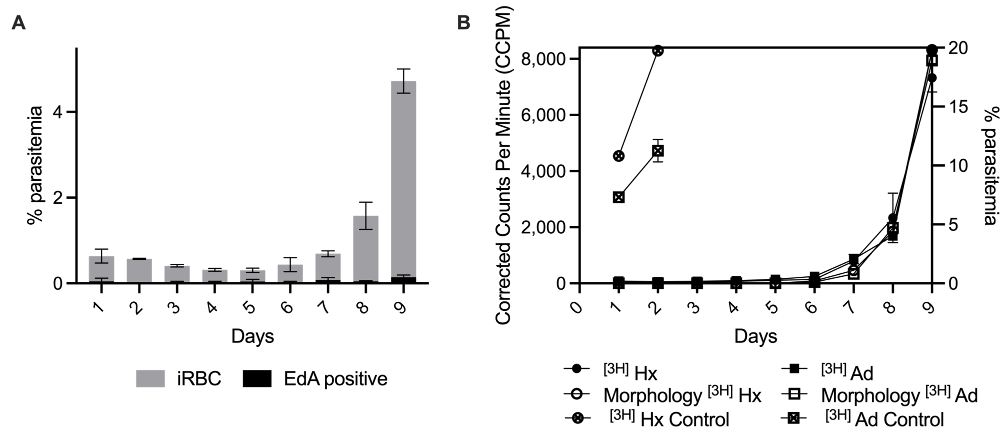


Figure 2.9. Asexual blood stage parasites recrudescing from DHA-induced dormancy incorporate [^{3}H]hypoxanthine and/or [^{3}H]adenosine but do not incorporate EdA. A) DHA dormancy was induced in *P. falciparum* asexual blood stage parasites and daily samples were acquired and pulsed with 10 µM EdA for 24 hours. Pulsed samples were analyzed via flow cytometry on a Beckman Coulter CytoFLEX. **B)** DHA dormancy was induced in *P. falciparum* asexual blood stage parasites. Daily samples were acquired and pulsed with [^{3}H]-hypoxanthine (Hx) or [^{3}H]-adenosine (Ad) for 24 hours. Daily Giemsa-stained smears were performed to assess recrudescence (% parasitemia). Control parasites were not treated with DHA. Radiolabeled samples were analyzed by a Perkin Elmer Microbeta^2 scintillation counter. Date shown are one representative experiment of two independent experiments, (average ± SD).

CHAPTER 3

INVESTIGATING DIHYDROARTEMISININ-INDUCED DORMANCY IN *PLASMODIUM FALCIPARUM* USING A CHEMICAL BIOLOGY APPROACH AND GIBBERELLIC ACID [2]

[2] Botnar, A., Huang, L., Kim, C., Manetsch, R., and Kyle, D.E. To be submitted to *ACS Infectious Diseases.*

Abstract

Dormancy has been broadly described as an adaptive stress response through which cells exit the cell cycle and enter a state of quiescence. In *Plasmodium falciparum,* dormancy has been proposed as a mechanism of drug evasion and resistance formation. Artemisinin, the current gold standard of all drugs used to control malaria, has been shown to cause drug-induced dormancy in early blood stage parasites, which later recrudesce and continue growth. Previous efforts have explored phytohormones abscisic acid and gibberellic acid and their effects on dihydroartemisinin-induced dormant parasites. These studies found both phytohormones induced an early recrudescence phenotype, but gibberellic acid was much more potent at inducing this phenotype than abscisic acid. The mechanisms by which *Plasmodium* enters and exits dormancy remain elusive. Therefore, we hypothesized that gibberellic acid could be developed as a tool to explore these unknown mechanisms. Gibberellic acid (GA) analogs with clickable alkyne moieties were synthesized and the GA derivatives were found to localize in the cytoplasm. Additionally, we conducted pulldown studies with GA derivatives and obtained 50 potential interacting proteins of GA. Many of these proteins are annotated as transcription, translation, post translational modification, or transport proteins. We also identified a putative AP2 transcription factor via the pulldown experiments as well as some 26S protease and proteasome subunit proteins. The dormancy and recrudescence mechanisms remain unknown, however this study identifies potential players of these processes and presents a novel approach utilizing phytohormones to understand *P. falciparum* biology.

Introduction

An estimated 218 million cases of malaria and 409,000 deaths occurred worldwide in 2019 with children under the age of 5 accounting for a majority of the deaths [1]. Malaria is caused by protozoan parasites of the *Plasmodium* genus, of which *Plasmodium falciparum* is responsible for the most lethal form of the disease. Currently, Artemisinin-based combination therapy (ACT) remains the gold standard treatment recommended by the World Health Organization due to its potency and rapid parasite clearance time [36]. ACTs are the most effective treatment drugs and are recommended because there is a risk of recrudescence and resistance following artemisinin monotherapy. Despite the use of ACTs, artemisinin (ART) resistance is developing and was first reported in regions across Southeast Asia [178, 179]. Recently however, ART resistance has been reported in India and Africa, which highlights the expansion of resistance westward and/or its independent emergence [108, 180]. This poses a potential risk for the control of malaria and in Africa, were 90% of the deaths occur [181], there could be a devastating impact on malaria-related morbidity and mortality if ART resistance continues to spread. It is estimated that if ACT resistance reaches similar levels in Africa as is currently observed in Cambodia, that there would be an additional 78 million clinical malaria cases over a 5 year period [182]. In order to address this issue, it is vital that we understand biological mechanisms which the parasite utilizes to evade drug pressure.

One such mechanism, drug-induced dormancy, is characterized by a growth arrest in the asexual ring stages of *P. falciparum* and has been proposed as a mechanism for drug evasion and subsequent recrudescence following the removal of drug pressure [183,

184]. Upon exposure to an ART derivative, early ring stage parasites are arrested as dormant forms and morphologically have condensed nuclei and reduced cytoplasm. This phenotype has been demonstrated both *in vitro* and *in vivo,* and following removal of the drug, parasites recrudesce and parasitemia increases to clinically detectable levels [134, 153, 185]. ART derivatives have not been the only drugs shown to induce dormancy; the antifolate pyrimethamine was found to induce second cycle dormancy in daughter progeny following drug exposure in the previous cycle [186]. ART derivative drug-induced dormant parasites have been shown to maintain low levels of metabolic activity as fatty acid synthesis, pyruvate metabolism, and the isoprenoid pathway remain upregulated processes through the quiescent state [137, 187]. Recently, airyscan microscopy (ASM) showed that the mitochondria of dihydroartemisinin (DHA)-exposed parasites are altered and enlarged relative to the mitochondria of actively replicating ring stage parasites [188]. This suggests a possible mito-nuclear interaction which could support communication pathways and result in a survival response. Furthermore, cyclin and cyclin dependent kinase (CDK) genes have been correlated with dormancy and recrudescence [189]. Interestingly, recrudescence times vary between *P. falciparum* parasites that have distinct genetic backgrounds, but the differences cannot be attributed to Kelch 13 (K13) polymorphisms [136, 190]. Therefore, PfK13 status and ART resistance is independent from the parasite's ability to become dormant and survive ART treatment [191]. Nevertheless, with many efforts to characterize this drug-induced dormancy phenotype, the mechanism by which the parasite enters this state and later recrudesces to continue growth remains a mystery.

Dormancy is not an exclusive phenomenon to *Plasmodium*. In fact it is widely observed in plants and has been extensively studied [192]. In plants, dormancy is a highly regulated process that is triggered by abiotic stress and results in developmental arrest which protects the plant against unfavorable conditions. These processes are controlled by signaling plant hormones (phytohormones), abscisic acid (ABA) and gibberellic acid (GA_3), which promote the induction or release of the seed from dormancy, respectively [193]. Apicomplexa share an ancestral origin with plants in the form of the relict, non-photosynthetic plastid apicoplast. The apicoplast is believed to have been evolutionarily obtained following a secondary endosymbiotic event between a eukaryote and red algae [194], and the sole known essential function of the apicoplast is to synthesize isoprenoids [195]. Prior work in another Apicomplexa, *Toxoplasma gondii,* showed that ABA blocks egress of the parasite from host cells [196]. Recently, the effects of ABA and GA_3 on DHA-induced dormant *P. falciparum* were explored and an early recrudescence phenotype was observed in response to exogenous addition of these phytohormones [137]. Intriguingly, dormant ring stages supplemented with GA_3 recovered up to 48 hours earlier than parasites exposed to DHA alone, which is equivalent to a whole asexual blood stage lifecycle.

The early recrudescence phenotype produced by the phytohormone GA_3 provides an interesting means to explore the mechanisms behind dormancy and the regulatory network that stimulates developmental arrest in *P. falciparum* as a response to DHA treatment. In this study, we utilized the dormancy-regulating phytohormone GA_3 as a tool to investigate the parasite's dormancy mechanisms. Using alkyne modified GA_3 derivatives, we localized the phytohormone to the cytoplasm of the asexual stages of *P.*

falciparum. We also utilized the modified GA_3 derivatives to pulldown interacting proteins of the phytohormone and identified potential players of the early recrudescence phenotype. These findings help shed light on a previously uncharacterized mechanism and will aid in our understanding of growth regulation in *P. falciparum* in response to DHA treatment.

Materials and methods

GA_3 Derivatives

GA_3 derivatives were synthesized by our collaborators, Dr. Roman Manetsch and Lili Huang, at Northeastern University in Boston, Massachusetts.

In vitro culture of intraerythrocytic P. falciparum parasites

P. falciparum clones W2 (Indochina II) and D10-ACP_{LEADER}-GFP (MRA-568) were maintained in culture using previously described methods [197]. Briefly, cultures were maintained at a hematocrit of 2% A+ human red blood cells (RBCs; Interstate Blood Bank, Memphis, TN) in complete culture medium consisting of RPMI 1640 supplemented with 25 mM HEPES, 0.24% (w/v) sodium bicarbonate and 10% heat-inactivated human A+ plasma (Interstate Blood Bank, Memphis, TN). D10-ACP_{LEADER}-GFP was cultured in the presence of 100 nM pyrimethamine. Parasite development was monitored with light microscopy of Giemsa-stained blood smears. Prior to dormancy and recrudescence assays, cultures were synchronized to ring stage using 5% (wt/vol) D-sorbitol (Sigma-Aldrich) and washed three times with RPMI 1640 medium [198].

In vitro antimalarial activity of GA_3 derivatives against blood stages of P. falciparum

GA$_3$ derivatives at 10 mM were diluted 1:10 and then serially diluted in duplicate over 11 concentrations. *P. falciparum* (W2) asexual blood stage parasites were synchronized to ring stages using 5% D-sorbitol and were then split to 0.5% parasitemia in a 2% hematocrit. In 96-well plates, a volume of 90 µL/well of parasitized RBCs was added on top of 10 µL/well of GA$_3$ derivative. Positive and negative controls were included in each plate. Positive controls consisted of drug-free parasitized RBCs and negative controls consisted of uninfected RBCs. Assay plates were incubated for 24 hours at 37 °C in 90% N_2, 5% CO_2, 5% O_2, then 1 µCi/well ^{3}H-hypoxanthine (Perkin Elmer) was added, and plates were incubated another 24 hours after which assay plates were frozen at –80 °C to lyse RBCs. Assay plates were later removed from –80 °C and allowed to thaw at room temperature before harvesting and counting samples on a Perkin Elmer Microbeta^2 scintillation counter (Waltham, MA). Data were imported into CDD Vault software for calculation of the half-maximal inhibitory concentrations (IC_{50}) and curve fitting analysis.

Structure-activity relationship (SAR) of GA_3 derivatives and their early recrudescence activity in DHA-induced dormant P. falciparum

Previously described methods were followed in order to assess the early recrudescence phenotype of exogenous addition of GA$_3$ derivatives to DHA-induced dormant asexual blood stage parasites (W2) [199]. Briefly, *P. falciparum* (W2) asexual blood stage parasites were synchronized to ring stages using 5% D-sorbitol and were then split to 2% parasitemia at a 2% hematocrit. Dormancy was then induced by adding 700

nM DHA (Sigma) for 6 hours. DHA was washed out three times with RPMI 1640 and cultures were then resuspended in fresh culture medium and supplemented with 10 µM GA_3 (Sigma-Aldrich), 10 µM of GA_3 derivative, or 0.1% vehicle (dimethyl sulfoxide [DMSO]) control. Complete medium was changed every 48 hours. Giemsa-stained blood smears were obtained daily and used to monitor parasite recrudescence. Recrudescence was analyzed as percent normal to total (dead and dormant) parasites and graphed via GraphPad Prism 9.

In vitro localization of GA_3 derivatives via Cu-catalyzed azide-alkyne click chemistry in P. falciparum intraerythrocytic parasites

Copper catalyzed azide-alkyne click methods were utilized following similar methods to those previously reported [166]. Briefly, 10 µM GAD1566 or GAD1569 was added to an asynchronous D10-ACP_{LEADER}-GFP (MRA-568) culture at 2% parasitemia and 4% hematocrit and incubated for 48 hours. A sham control with 0.1% DMSO was also grown in parallel. After incubation, parasites were briefly centrifuged, and the supernatant removed. Cells were then fixed in a solution containing 0.05% glutaraldehyde for 15 minutes at room temperature. Following a wash with phosphate buffered saline (PBS, pH 7.4), parasites were prepared for the azide-alkyne click reaction by permeabilization with 0.1% Triton X-100 for 10 minutes, followed by a 1-hour incubation with 3% BSA at room temperature. Cells were then stained with a freshly prepared staining mix containing 2 mM $CuSO_4$ (Sigma), 12 µM Alexa Fluor Azide 594 (Thermo Fisher), and 10 mM sodium ascorbate (Sigma) for 1 hour in the dark at room temperature. Parasites were then washed once with PBS and co-stained with 10 µg/mL

Hoechst 33342 (Thermo Fisher) for 15 minutes followed by three washes with PBS. They were then mounted onto poly-L-lysine coated slides and imaged with a Zeiss Elyra S1 (SR-SIM) super resolution microscope.

In vitro competition of GAD1566 and GA_3 click staining in P. falciparum intraerythrocytic parasites

Asynchronous W2 *P. falciparum* was split to 0.5% parasitemia in a 2% hematocrit into a 6-well culture plate. GAD1566 and GA_3 were then added in various ratios as follows for GAD1566:GA_3 per well; 1:0, 1:1, 1:2, and 0:1. Cultures were then incubated for 48 hours at 37 °C in 90% N_2, 5% CO_2, 5% O_2. After incubation, parasites were briefly centrifuged, and the supernatant removed. Cells were then fixed and stained as described for *in vitro* localization of GA_3 derivatives except for staining was conducted with an Alexa Fluor Azide 488 instead of an Alexa Fluor Azide 594. Once mounted onto poly-L-lysine coated slides, cells were imaged with a Zeiss Elyra S1 (SR-SIM) super resolution microscope.

Pulldown of GA_3 interacting partners and proteins identification via liquid chromatography-mass spectrometry (LC-MS)

Asynchronous W2 *P. falciparum* was grown to a high parasitemia (>10%) in a 2% hematocrit culture (25 mL). RBCs were lysed with 0.5% saponin for 5 minutes on ice followed by three washes with PBS. The parasite pellets were then freeze-thawed in –80 °C to release proteins. Parasite lysate was then incubated overnight at 4 °C with 10 µg LH-129, a biotinylated version of GAD1566 (synthesized by collaborators in

Northeastern University, Dr. Roman Manetsch and Lili Huang), or with 10 µg GA_3 as control. Streptavidin MagneSphere Paramagnetic® beads (Promega) were then prepared by washing with 1 mL binding/wash buffer (Tris buffered saline with 0.1% tween 20). Beads were then added to the parasite lysate mixture and allowed to incubate overnight at 4 °C. The following day, beads/lysate were washed twice with binding/wash buffer. Protein was then eluted with 8 M Guanidine Hydrochloride (GuHCl). Eluted proteins were then separated on a NuPAGE 4-12% Bis-Tris SDS-PAGE gel (Invitrogen) and visualized via silver stain. Proteins pulled-down with LH-129 were cut from the silver stained SDS-PAGE gel and sent to the Northeastern University proteomics core for digestion into peptides and identification via LC-MS. Data analysis of proteins was conducted using PlasmoDB release 54. An interactome network of proteins identified via LC-MS was created via clustering using the Markov Cluster Algorithm in STRING.

Results

Structure Activity Relationship (SAR) study and the early recrudescence phenotype of modified GA_3 derivatives on DHA-induced dormant P. falciparum asexual blood stage parasites

Previous work showed an early recrudescence phenotype when 10 µM GA_3 was exogenously supplemented to DHA-induced dormant *P. falciparum* asexual blood stage parasites [137]. Therefore, we synthesized GA_3 derivatives in which functional groups were modified with either a clickable azide (GAD1566, GAD1567, and GAD1569) or a novel sulfo-click handle on GAD1570 (Figure 3.1). In order to avoid confounding our recrudescence assay due to potential antimalarial activity, first we assessed the activity of

the GA_3 derivatives and calculated their half-maximal inhibitory concentrations (IC_{50}). The calculated IC_{50} values for our GA_3 derivatives ranged from 25.6 (± 3.99) μM to greater than 100 μM (Figure 3.1). Since the recrudescence phenotype assay is conducted at 10 μM, we were confident that any phenotype, such as a loss of early recrudescence, observed in response to GA_3 derivatives was not due to direct antimalarial activity of the GA_3 derivatives. We next evaluated the GA_3 derivatives in the recrudescence assay and compared their activity to the recrudescence phenotype of GA_3 supplemented parasites and DMSO vehicle control supplemented parasites. DHA-induced dormant asexual blood stage parasites were supplemented with 10 μM GA_3 derivative, 10 μM GA_3, or 0.1% DMSO and recrudescence was monitored over time via Giemsa smears. Recrudescence was monitored as percent of morphologically normal rings, trophozoites, and schizonts over total (normal plus dead or dormant) parasites. We found that parasites supplemented with GAD1566 and GAD1569 responded similarly to GA_3 in terms of early recrudescence and were statistically significantly different as compared to DMSO treated parasites. In contrast, parasites supplemented with GAD1567 had a partial early recrudescence phenotype and parasites supplemented with GAD1570 had no early recrudescence phenotype (Figure 3.2). GAD1570 is a large molecule containing aromatic rings, and therefore it is possible that it could not enter the parasite in order to activate parasites and cause early recrudescence.

Localization of GA_3 derivatives (GAD1566 and GAD1569) in P. falciparum asexual blood stage parasites

Since GAD1566 and GAD1569 contain alkyne modifications, are similar to GA_3, and they produced an early recrudescence phenotype when supplemented to DHA-induced dormant parasites, we performed cellular localization studies using a copper-catalyzed click chemistry approach. For these studies,10 μM of GAD1566 and 10 μM of GAD1569 were supplemented to an asynchronous culture of asexual blood stage parasites containing a green fluorescent protein (GFP) labeled acyl carrier protein (ACP) and allowed to incorporate for 48 hours. A 0.1% DMSO vehicle control was conducted in parallel. Localization of GA_3 derivatives was then conducted with a copper-catalyzed azide-alkyne cycloaddition chemical reaction with a fluorescent azide probe. We then observed localization of the GA_3 derivatives via high resolution microscopy and found them to localize to the cytoplasm in all three asexual blood stages of *P. falciparum* (Figure 3.3). Since GA_3 is a phytohormone, we had originally hypothesized its localization to the plastid apicoplast. However, since it is a signaling hormone, it is not surprising to find it localized to the cytoplasm. To confirm that the alkyne modification did not change the localization of the phytohormone, we conducted a competition study of GAD1566 with GA_3. GAD1566 localization was competed with increasing GA_3 concentrations. We found that as GA_3 concentration increased, GAD1566 mean fluorescence intensity decreased (Figure 3.4). These data suggest that chemical modification to make GAD1566 did not affect cellular localization and that GA_3 and GAD1566 interact with the same proteins/receptors in asexual blood stage *P. falciparum* parasites.

Pulldown of GA_3 interacting partners and identification via LC-MS

Since GAD1566 and GAD1569 both produced an early recrudescence phenotype and both localized to the cytoplasm, we biotinylated these compounds (Figure 3.5) in order to perform pulldown assays in an attempt to identify their interacting partners. A schematic of our pulldown approach is shown in Figure 3.6a. Briefly, the pulldown was performed by obtaining parasite lysate and incubating it with biotinylated versions of GAD1566 and GAD1569 (termed LH129 and LH130, respectively) and GA_3 (as a control), followed by binding to magnetic streptavidin beads. Interacting partners of the compounds were then eluted and visualized on a silver stained SDS-PAGE gel (Figure 3.6b). We observed bands present in the pulldown with LH129 that were not present in the pulldown with native GA_3. These bands were extracted, and proteins were subsequently digested into peptides and analyzed via LC-MS. Protein identification was conducted using annotated data of *P. falciparum* 3D7 available through PlasmoDB release 54. We identified 50 proteins in total from our pulldown with LH129. Biological process and cellular component gene ontology (GO) terms for the 50 proteins of interest were evaluated via PlasmoDBv54 (Figure 3.6c). For biological process, a majority of the pulled-down LH129 interacting proteins were involved in cellular processes, regulation of transcription, and metabolic processes. For cellular component, a majority of the pulled-down LH129 interacting proteins were in vesicles. In terms of function, the extracted proteins were involved in either carbohydrate metabolism, histone modification, invasion and motility, post translational modifications, protein trafficking, transcription, translation, or they had unknown functions.

Recent work from the David A. Fidock lab at Columbia University in New York investigated ART-resistant K13 mutations and their effect on the parasite proteome. They found that K13 mutations alter the parasite proteome and proteins are differentially expressed (DE) between K13 mutant and wildtype parasites [175]. Considering that we are looking for proteins involved in the early recrudescence phenotype in response to DHA-induced dormancy and GA_3 supplementation, we evaluated the DE profile of our 50 proteins based off of the data from Mok et. al. 2021 [200]. Of our 50 pulled-down proteins, 45 were detected in the ring stage parasites in the isobaric labeling-based quantitative proteomics experiments conducted by Mok et. al. (Figure 3.7a). These proteins did not differ in terms of DE when comparing K13 mutant to wildtype parasites, which was not surprising as the early recrudescence phenotype observed by the exogenous addition of GA_3 to DHA-induced dormant parasites is observed in both wildtype and K13 mutant parasites [137]. Furthermore, we evaluated the essentiality of these proteins using data from previous work in which transposon mutagenesis was used to identify dispensable or essential proteins [201]. Using a mutagenesis index score (MIS) from 0 to 1, with 0 being essential and 1 being dispensable, we found that proteins from the GA_3 derivative pulldowns varied across the board in terms of their MIS (Figure 3.7a). It is important to note that these data only identify the MIS for proteins involved in *in vitro* active growth of asexual blood stages. Therefore, the essentiality of these proteins in terms of recrudescence is yet to be explored.

Next we created an interactome network of the 50 pulled-down proteins and clustered proteins based on the Markov Cluster Algorithm in STRING (Figure 3.7b). The interactome shows an enrichment, indicating that these proteins are at least partially

biologically connected as a group. Clustering of ribosomal, elongation, and other translation proteins is observed in the red nodes. Clustering of 26S protease regulatory subunits, proteasome subunits, and other post translational modification proteins is observed in the tan nodes. Epigenetic regulatory histone proteins are also seen to cluster together in the light green nodes found below the tan cluster. Excitingly, a putative AP2 domain transcription factor was one of our pulled-down proteins (PF3D7_1139300), but it is not in a cluster with other proteins. Nevertheless, ApiAP2 family members have been suggested to be involved in life cycle progression and differentiation processes, suggesting an interesting protein to investigate further.

Discussion

The significant decrease in malaria-related deaths worldwide over the past two decades has been attributed largely to the administration of effective antimalarials against *P. falciparum*, in particular, ACTs [1]. However, progress towards malaria control and elimination has stalled and the global estimate of malaria cases and deaths has remained virtually unchanged over the past few years [1]. The African region, where *P. falciparum* is present, shoulders a majority of the overall disease burden. Unfortunately, resistance to these frontline artemisinins and partner drugs is spreading, creating it more difficult to control this disease [108, 178-180]. Furthermore, small populations of early blood stage parasites can evade ART by entering a dormant state, thus surviving drug pressure [134]. This dormancy phenomenon is independent of resistance as both ART sensitive and resistant parasites produce this phenotype. The mechanisms by which the parasite enters and exits this state of dormancy remain elusive. Understanding the nature of these

dormant parasites and their ability to enter dormancy followed by their ability to recrudesce and reestablish actively propagating parasites after ART exposure is vital towards the goal of malaria control and elimination.

In the present study, we have utilized the early recrudescence phenotype observed with exogenous addition of GA_3 to investigate DHA-induced dormancy and to identify some potential key players in the reactivation of dormant parasites. GA_3 is a signaling phytohormone which in plants promotes the release of the seed from dormancy [193]. Traditionally, hormones are chemical messengers that coordinate different processes such as growth, metabolism, and fertility. In apicomplexans, plant hormone cytokinins have been found to control cell cycle progression and plastid replication [202], and the plant hormone salicylic acid is produced by malaria parasites and found to affect host immunity and cerebral malaria outcome [203]. Due to the significant effects GA_3 has been previously shown to have on DHA-induced dormant parasites [137], we used this phytohormone and derivatives thereof as tools to explore the mechanisms underlying dormancy and the regulatory network that potentially promotes cell cycle arrest and subsequent recrudescence in *P. falciparum*.

We synthesized modified GA_3 derivatives which were equipped for localization and pull-down studies via an azide-alkyne copper facilitated cycloaddition (Figures 3.1 and 3.5). The modified derivatives, GAD1566 and GAD1569, produced the early recrudescence phenotype as compared to the unmodified GA_3 when supplemented to DHA-induced dormant *P. falciparum* asexual blood stage parasites (Figure 3.2). Since GAD1566 and GAD1569 contained alkyne modifications, we utilized a click chemistry approach to localize the phytohormone within the *P. falciparum* ring, trophozoite, and

schizont blood stages. Since these are plant hormones, we had hypothesized their localization to the relict, non-photosynthetic plastid apicoplast. Therefore, we conducted localization studies with a parasite strain which contained a GFP tagged acyl carrier protein, which is located in the apicoplast. However, we found that GAD1566 and GAD1569 both localized to the cytoplasm (Figure 3.3). This is not altogether surprising as receptors for traditional hormones can be found on cell membranes, within the cytoplasm, or in the nucleus.

In plant biology, the GA signaling pathway has been identified and well described. Briefly, GA binds to a soluble GID1 receptor which then interacts with a DELLA repressor protein in a GA-dependent manner, thereby inducing DELLA protein degradation via the E3 ubiquitin ligase [204]. Searches of the available genome data have failed to identify GID1 or DELLA proteins in *P. falciparum*. Therefore, to identify proteins involved in the recrudescence mechanism, we used biotin modified GA_3 derivatives to perform pull down experiments to extract potential interacting proteins of the phytohormone. Our proteomics analysis identified 50 proteins of interest (Figure 3.7). Functionally, the proteins of interest are involved in histone modification, transcription, translation, post-translational modification, protein trafficking, transport, metabolism, and invasion and motility. We assessed the differential expression of these proteins using previously reported data of ART resistant versus wildtype parasites and we found that the proteins of interest were not differentially expressed. This was not unexpected, as the parasite's ability to recrudesce is independent of the PfK13 status [191].

One of the proteins of interest identified in our pull-down studies is a putative AP2 domain transcription factor (PF3D7_1139300). ApiAP2 transcription factor family

members have been described to have important roles in regulating life cycle transitions. They were first described in 2005 as a class of putative transcription factors that carried a domain similar to the Apetala2/ERF (ethylene response factor) (AP2/ERF) integrase DNA binding domain, which is present in many plant transcription factors [205]. Furthermore, gene expression data has shown that ApiAP2 family members are expressed in different stages throughout apicomplexan parasite development, suggesting that ApiAP2 transcription factors are involved in life cycle progression and differentiation processes, like their plant homologues [206]. PF3D7_1139300 has not been extensively characterized, however it has been found to associate with PfAP2-G2 [207]. The ApiAP2 transcription factor, PfAP2-G2 has been shown to play a role in the maturation of *P. falciparum* gametocytes, and disruption of it prevents sexual parasites from maturing beyond stage III gametocytes. Furthermore, single cell RNA-seq has shown upregulation of the gene encoding PF3D7_1139300 when *ap2*-g expression peaks, just before egress in committed schizonts [208]. However while the transcriptomic studies looking at mRNA expression levels of *pfap2-g2* and *pf3d7_1139300* showed expression at similar times through asexual development for wildtype parasites, a knockout of *pf3d7_1139300* did not make a difference compared to the wildtype whereas a knockout of *pfap2-g2* affected the mRNA expression levels [207]. Therefore, we cannot preclude the possibility of PF3D7_1139300 as having a role in *P. falciparum* recrudescence from DHA-induced dormancy. Intriguingly the gene encoding this AP2 protein was deemed as dispensable in *P. falciparum* saturation mutagenesis studies [201]. However, the mutagenesis study was conducted on actively propagating asexual blood stage parasites. Therefore, future studies

will need to evaluate the relative fitness cost of the disruption of this gene for recrudescing parasites.

We also identified multiple members involved in translation, including protein components of the 40S and 60S ribosomes and several translation initiation and elongation factors. Multiple 26S proteasome subunits were also identified. The 26S proteasome has been described to likely play a role in degrading ART-damaged proteins and DHA has been shown to kill parasites by causing protein damage and compromising parasite proteasome function [103]. Therefore, it has been suggested that a role for K13 is as a ubiquitin ligase adaptor protein that could help deliver proteins for proteasome-mediated degradation. Since our proteins were identified via a pull down with GAD1566, a phytohormone shown to induce early recrudescence, we can suppose a similar role for the 26S proteasome for parasites exiting DHA-induced dormancy. Our pull-down experiments produced several proteins of interest which could potentially play a role in *P. falciparum* recrudescence from DHA-induced dormancy. Further work is required to investigate these proteins, such as creating mutant strains which lack the ability to express these proteins and evaluating the effect on parasite recrudescence. This will aid in further improving our understanding of the dormancy and recrudescence phenomena.

Herein, we detail the localization of GA_3 derivatives, GAD1566 and GAD1569, in asexual blood stage *P. falciparum* using an alkyne-azide copper facilitated cycloaddition. GAD1566 and GAD1569 both localize to the cytoplasm of rings, trophozoites, and schizonts in *P. falciparum.* We further detail the pull down and identification of potential interacting partners of GA_3. The methodology used in this study not only assisted in our efforts to localize the phytohormone GA_3 and identify its

interacting partners, but it can also potentially aid in mechanism of action studies for novel antimalarials. Our results demonstrate that novel drugs can be modified to contain an alkyne functional group and the derivative(s) can be used to conduct alkyne-azide cycloaddition reactions with a fluorophore to aid cellular localization studies. These same methods can then be employed to conduct pulldown experiments and combine the acquired data to identify potential mechanisms of action, highlighting the utility of our methodology beyond just investigating dormancy mechanisms. However, in terms of dormancy and recrudescence mechanisms, we have identified proteins of interest to be further investigated. This information will aid in our understanding of how the parasite evades drug pressure and assist in the overall goal to control and eliminate malaria disease.

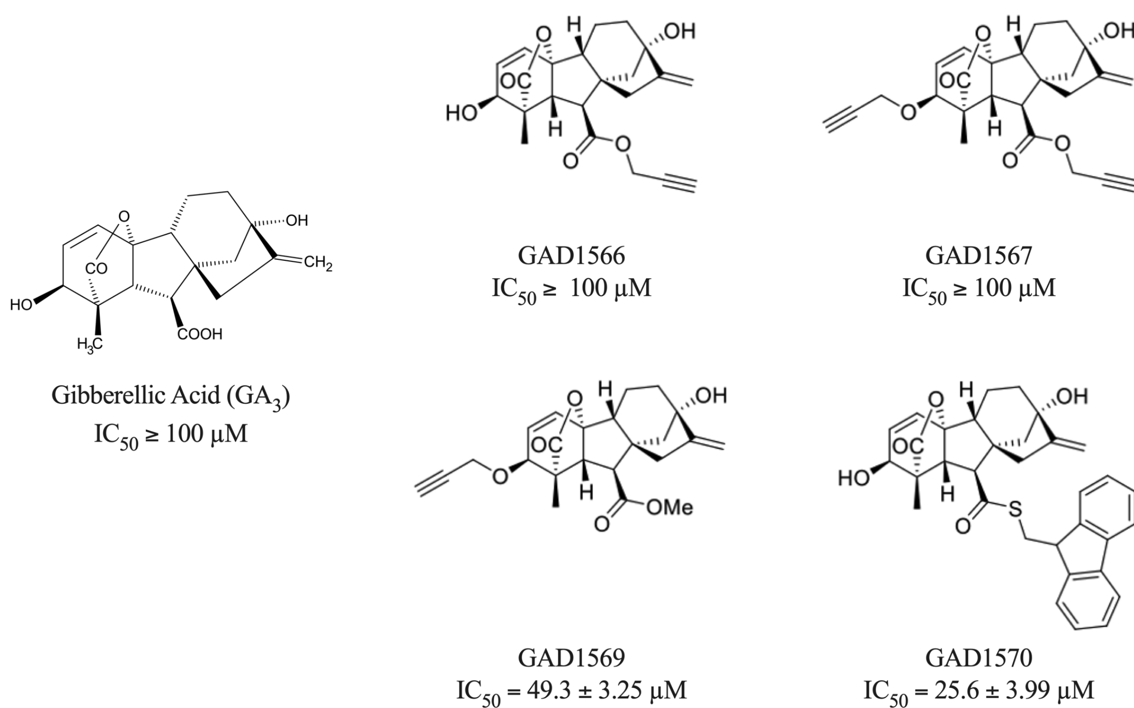


Figure 3.1. Structures of GA_3 derivatives. Gibberellic Acid (GA_3), a commercially available phytohormone, was modified to produce GAD1566, GAD1567, GAD1569, and GAD1570 by Lili Huang and Roman Manetsch, Northeastern University. The carboxylic acid in GA_3 was modified to contain an alkyne in GAD1566. The carboxylic acid and hydroxyl in GA_3 were both modified to contain alkynes in GAD1567. The carboxylic acid was methylated, and the hydroxyl was modified to contain an alkyne in GA_3 to produce GAD1569. The carboxylic acid in GA_3 was replaced to contain a thioester and an aromatic ring in GAD1570. The half-maximal inhibitory concentration (IC_{50}) of GA_3 and the GA_3 derivatives was obtained via a $^{[3H]}$ Hypoxanthine incorporation assay.

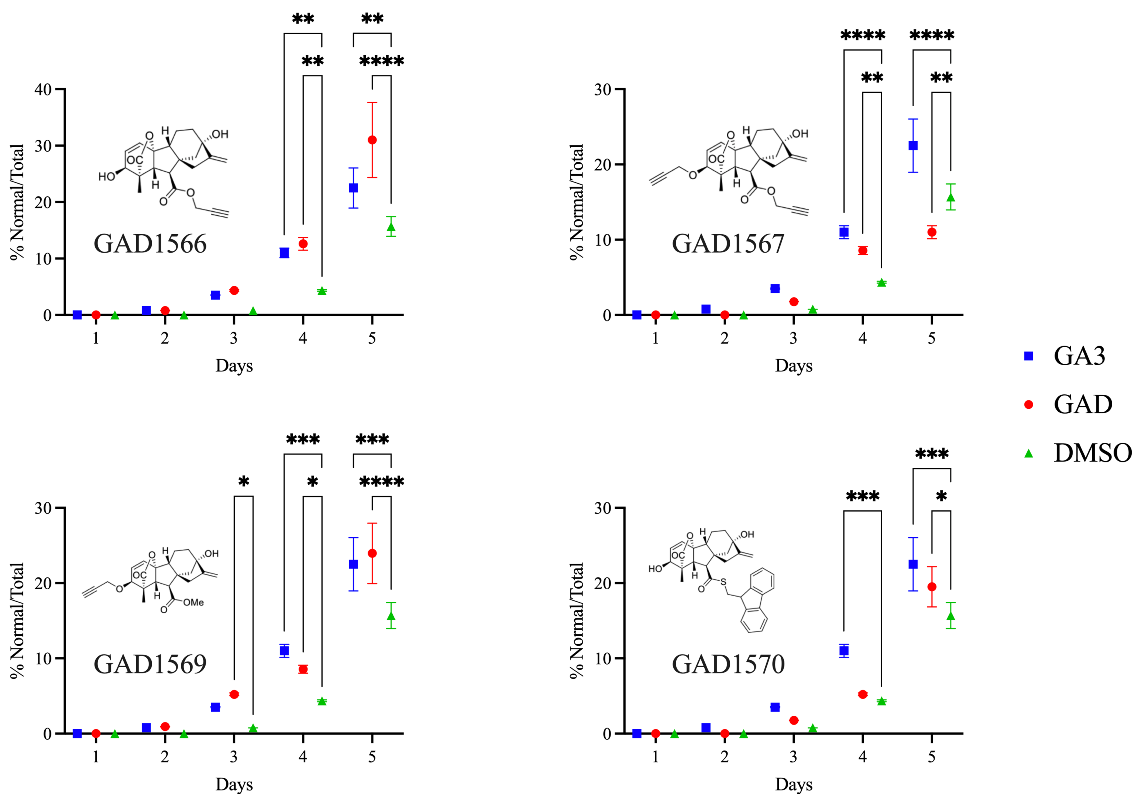


Figure 3.2. GAD1566 and GAD1569 contain the same early recrudescence phenotype as GA_3 when supplemented to DHA-induced dormant *P. falciparum* asexual blood stage parasites. D-sorbitol synchronized *P. falciparum* rings were DHA treated to induce dormancy and then supplemented with 10 µM GA_3, 10 µM GA_3 derivative, or 0.1% DMSO vehicle control. Recrudescence was monitored via daily Giemsa smears and recorded as percent normal (ring, trophozoite, or schizont) parasites to total (normal plus dead or dormant) parasites. Data shown are one representative experiment of two independent experiments. Error bars (SD) were omitted when smaller than the marker. Significance assessed by 2-way ANOVA and Dunnett's multiple comparisons test, * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

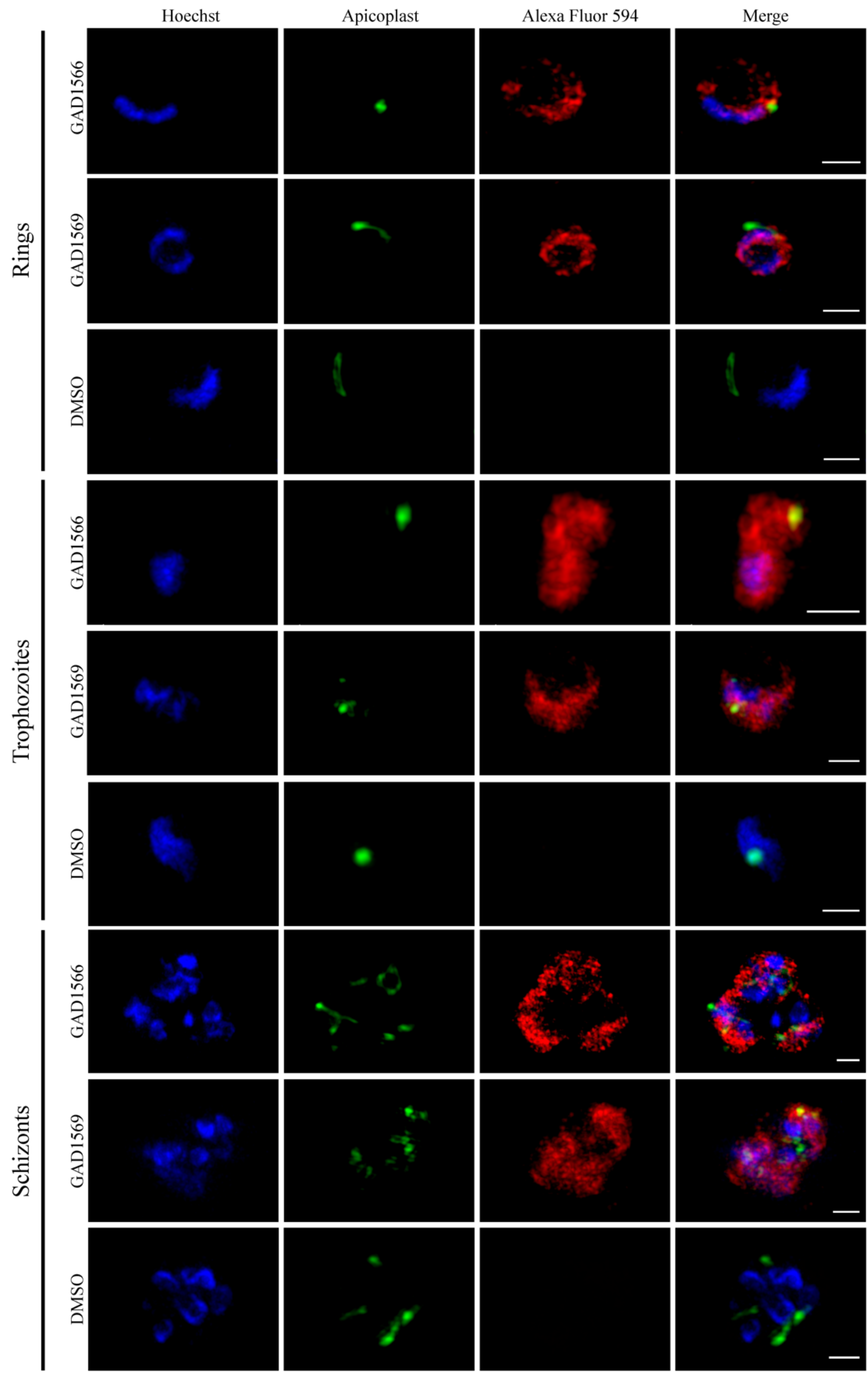


Figure 3.3. GA_3 derivatives GAD1566 and GAD1569 localize to the cytoplasm in all three asexual blood stages of *P. falciparum*. GA_3 derivatives, GAD1566 and

GAD1569, were incubated in an asynchronous culture of D10-ACP_L-GFP *P. falciparum*. A 0.1% DMSO vehicle control was incubated in parallel. GA_3 derivatives were then localized via a copper-catalyzed click reaction (red) and found to localize to the cytoplasm in all three asexual blood stages (rings, trophozoites, and schizonts). Parasites were co-stained with 10 µg/mL Hoechst 33342 (blue). The acyl carrier protein is GFP tagged and thus the apicoplast was visualized in green. Images were obtained on a Zeiss Elyra S1 (SR-SIM) super resolution microscope with a 100x objective. Scalebar = 1 µm.

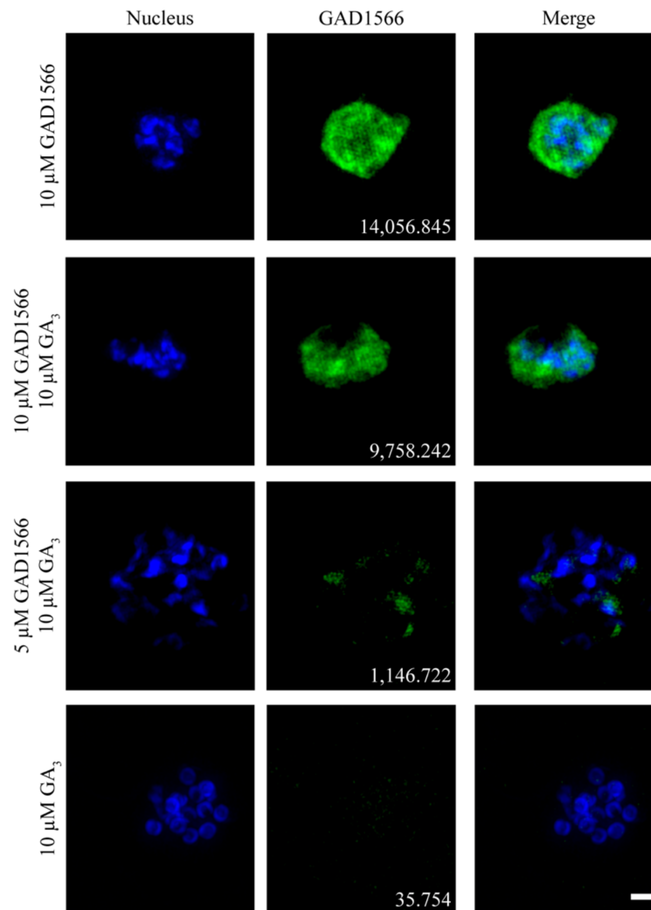


Figure 3.4. GA_3 and GAD1566 interact with the same partners as increasing concentrations of GA_3 compete out the GAD1566 signal. Ratios of 1:0, 1:1, 1:2, and 0:1 of GAD1566:GA_3 were incubated with an asynchronous culture of W2 *P. falciparum* and localized via a copper-catalyzed click reaction (green). Parasites were co-stained with 10 µg/mL Hoechst 33342 (blue). As increasing amounts of unmodified GA_3 were added, localization signal of the click reaction decreased, indicating that the alkyne modification to make GAD1566 did not affect cellular localization of the phytohormone. Images were obtained on a Zeiss Elyra S1 (SR-SIM) super resolution microscope with a 100x objective. In order to compare mean intensities across samples, the same acquisition settings were used for all samples. Mean intensities were then calculated with Image J and are reported here in white text. Scalebar = 1 µm.

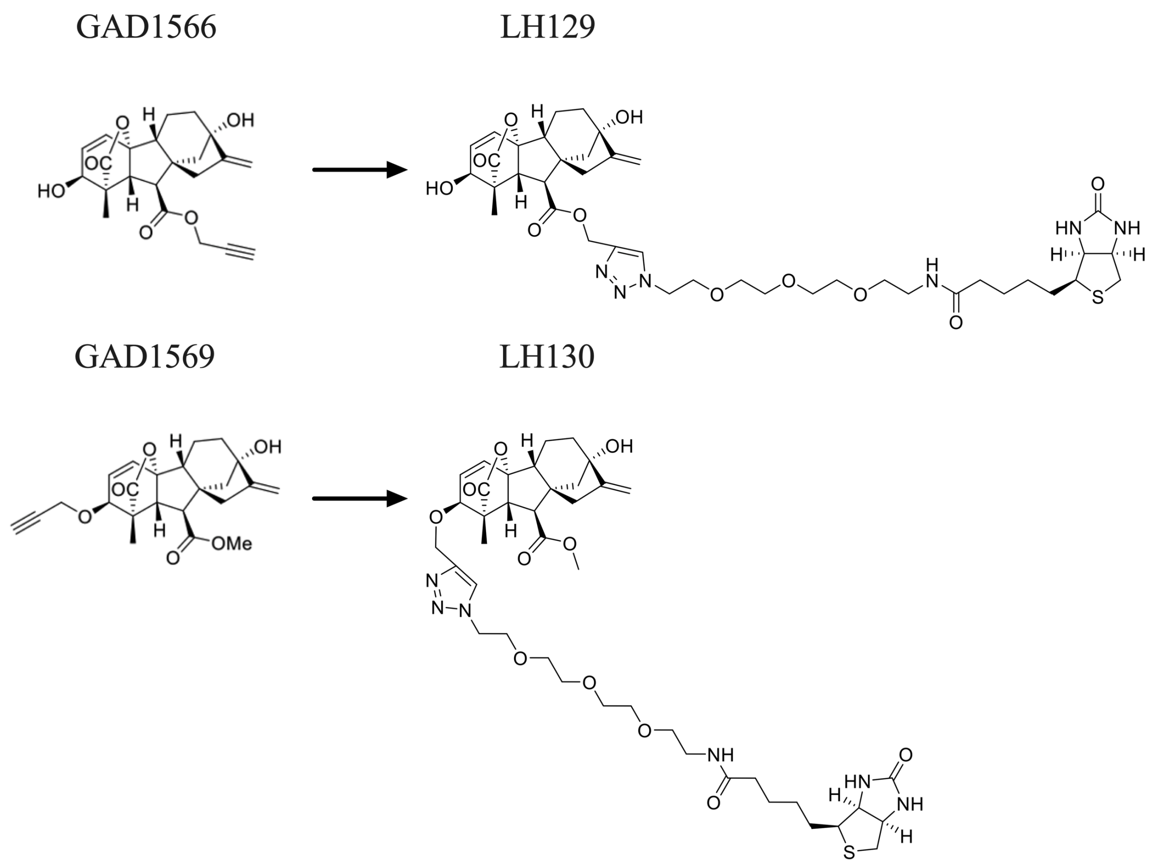
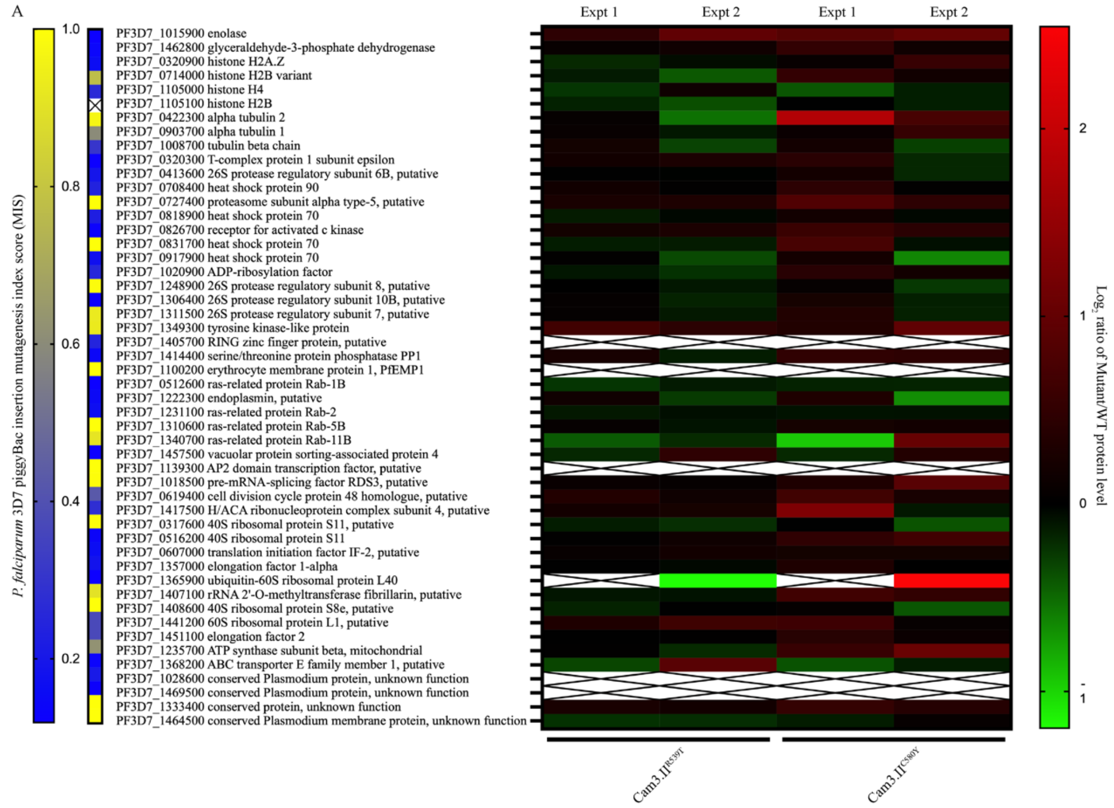


Figure 3.5. Structures of biotinylated GA_3 derivatives. A biotin PEG was added to GAD1566 and GAD1569 via an azide-alkyne cycloaddition by Lili Huang and Roman Manetsch (Northeastern University). New biotinylated derivatives were termed LH129 and LH130, respectively.

Figure 3.6. **Interacting proteins of GA_3 are involved in cellular processes, regulation of transcription and translation, and cellular protein metabolic processes, and are found in vesicles**. **A)** A schematic of the protein pulldown process (created with BioRender.com). Briefly, a culture of *P. falciparum* was grown to high parasitemia and then protein was released via saponin lysis and freeze-thaw. Parasite lysate was then incubated with 10 µg of LH129, LH130, or GA_3 as control. This was followed by binding to magnetic streptavidin beads. The bead/lysate/protein complex was then washed, and proteins were subsequently eluted and digested into peptides for LC-MS analysis. **B)** Protein pulldown was confirmed prior to LC-MS via a silver stained SDS-PAGE gel. LH127 is an intermediate that lacks biotin but contains the cycloaddition and was run as an additional control. Bands present in LH129 that were absent in LH127 and GA_3 control were extracted and analyzed via LC-MS. **C)** Word cloud representation of

functional analysis of significant biological process and cellular components (Gene Ontology database via PlasmoDBv54) for the 50 pulled-down proteins identified via LC-MS along with their corresponding *P* values calculated via Fisher's exact test.

A



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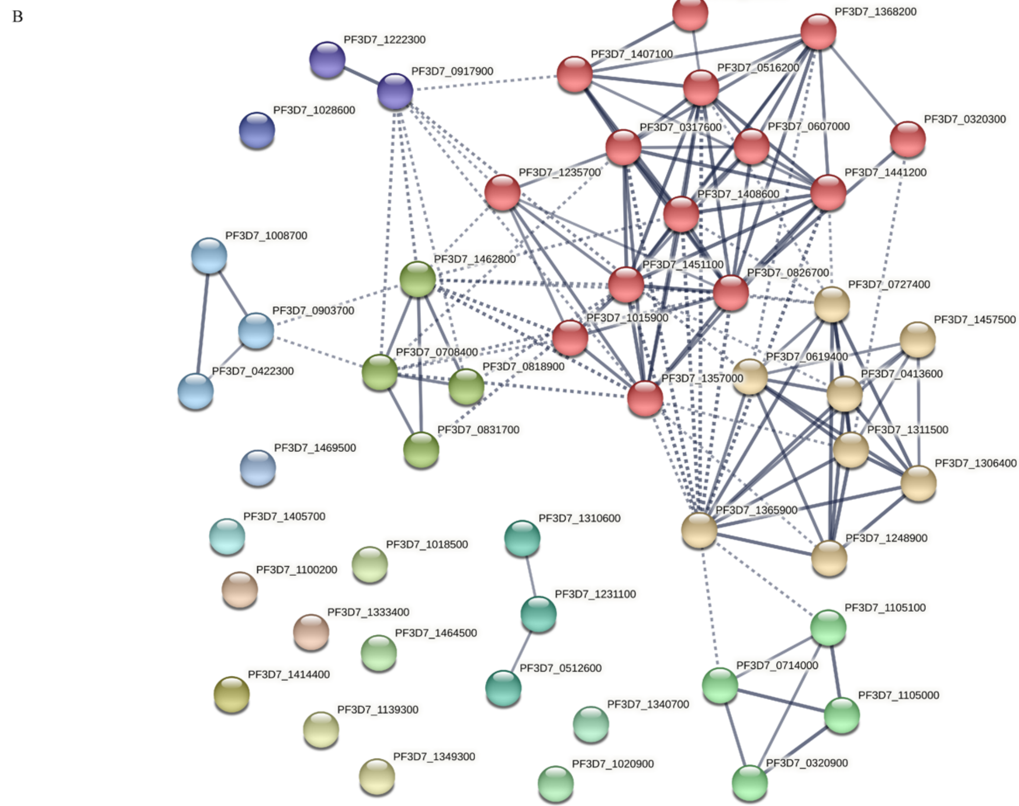

Figure 3.7. Interacting partners of GA_3 do not present a differential expression profile in K13 mutant parasites versus wildtype parasites but do contain an enriched interactome with clustering for translation & post-translational modifications. A) Heat map of the *P. falciparum* 3D7 piggyBac insertion mutagenesis index score (MIS)[1] for the 50 proteins followed by gene ID and description. Proteins are organized by function and are followed by a heat map of the log_2 fold change of significantly different protein levels in Cam3.II^{R539T} or Cam3.II^{C580Y} mutants relative to the isogenic Cam3.II^{WT} parasites for the differential expression of the 50 proteins[2]. Proteins with no differential expression data are marked with white boxes with an X through them. **B)** Interactome network of the 50 proteins identified via pull down with biotinylated GA_3 derivatives. Proteins were clustered using the Markov Cluster Algorithm in STRING. Line thickness depicts the confidence/strength of the relationship between proteins (nodes). Solid lines represent intra-cluster interactions while dashed lines represent the interaction between clusters.

[1]Min Zhang et. al. 2018 *Science*

[2]Sachel Mok et. al. 2021 *Nature Communications*

CHAPTER 4

SUMMARY

Despite tremendous efforts to eliminate malaria, this disease continues to be a leading cause of illness and death in many countries around the globe. Two human infecting species of *Plasmodium, P. falciparum* and *P. vivax*, each present their own obstacles towards malaria control and elimination. *P. falciparum* remains associated with the greatest mortality and morbidity rates; however, *P. vivax* is more widespread and potentially represents a greater burden on human health due to its naturally occurring dormant liver stage, the hypnozoite. While dormancy was originally thought to only be an issue in the naturally occurring liver stage of *P. vivax*, recent evidence of drug-induced dormancy in the blood stages of *Plasmodium spp.* is now posing as an additional threat. Artemisinin-based combination therapies are currently the recommended first line treatment for malaria, but unfortunately, they have been found to induce dormancy in early blood stage parasites. Furthermore, resistance to artemisinin (ART) has emerged independently, and begun to spread in southeast Asia, India, and Africa.

The mechanisms of dormancy, both in a naturally occurring and a drug-induced state, remain a mystery. Most antimalarials function by interfering with essential metabolic processes of the parasite, or by introducing toxins which are harmful to *Plasmodium*. A few examples include chloroquine, which interferes with the breakdown of toxic heme, and primaquine, which is thought to inhibit membrane trafficking events

involved in uptake of metabolites. Therefore, actively replicating and growing parasites are susceptible to damage by these antimalarials. However, dormant parasites are able to exist and evade drug pressure as they arrest growth and shut or slow down certain metabolic activities. *P. vivax* hypnozoites can be effectively killed with primaquine or tafenoquine however, these drugs are not recommended for pregnant women, children, or patients with a glucose-6-phosphate dehydrogenase (G6PD) deficiency. The other antimalarials remain ineffective against hypnozoites and without effective treatment, vivax malaria is able to relapse following activation of the liver stage hypnozoites. The same phenomenon occurs in the blood stage with drug-induced dormancy. Parasites arrest growth and are able to exist in the presence of drug. Once drug pressure is removed, the parasites exit this arrested state and resume their growth. Our lack of understanding of the mechanisms by which parasites enter and exit dormancy, in both the naturally occurring and drug-induced states, is only exacerbated further by the lack of techniques to study this phenomenon.

In this study, we exploited parasite biology to develop a novel technique to study dormancy. Since *Plasmodium* is a purine auxotroph, we explored the use of alkyne-modified purine analogues as DNA synthesis markers. We hypothesized that dormant parasites would not incorporate the modified purines, as they are not replicating and thus do not synthesize DNA, but that active and/or reactivating parasites would begin to synthesize DNA and thus incorporate the modified purines. We observed that actively replicating *P. falciparum* asexual blood stage parasites incorporated alkyne-modified adenosine, inosine, and hypoxanthine. However, *P. falciparum* dihydroartemisinin (DHA)-induced dormant parasites did not incorporate any of the modified purines, even

when recrudescing and resuming normal growth. Some potential theories could explain why recrudescing parasites did not incorporate the modified purines, but further research is warranted. ART resistance due to kelch 13 (K13) mutations has been shown to rewire *P. falciparum's* metabolic programming to enhance survival. Perhaps since DHA is an active metabolite of artemisinin, it affects the metabolic program of recrudescing parasites. To date, transcriptomic and metabolomic studies have only compared ART resistant to ART sensitive parasites. Ideally, investigating the transcriptomic and metabolomic profiles of the same parasite strain pre-, during, and post- DHA induced dormancy in a single cell approach could reveal why the modified purines were not incorporated in recrudescing parasites. Furthermore, this approach would aid in elucidating the regulatory network which controls cell cycle arrest in response to DHA and subsequently, recrudescence.

While the mechanisms behind drug-induced dormancy remain elusive, studying them is less difficult compared to naturally occurring liver stage dormancy due to the ability for *in vitro* culture of blood stage *P. falciparum*. Naturally occurring dormancy in the form of the liver stage hypnozoite in *P. vivax* remains difficult to study, highlighting the importance to develop novel tools and techniques in order to investigate hypnozoite biology. We studied the incorporation of our alkyne-modified purines in a *P. vivax* liver stage assay and demonstrated that actively replicating liver stage schizonts incorporate the alkyne modified adenosine (EdA) while hypnozoites did not. Furthermore, EdA was able to detect reactivating hypnozoites. To date, there is only one potential marker for *P. vivax* hypnozoite reactivation, liver-specific protein 2 (LISP2), however this marker produces a weak signal, and therefore it cannot be utilized for high-throughput analysis.

We confirmed that EdA can be applied in a high-throughput, high content imaging approach. Thus, we developed a novel technique to study dormant liver stage parasites. Many theories exist as to how hypnozoites reactivate. Ideally, moving forward we will test these theories using EdA. Understanding how hypnozoites reactivate and cause relapsing infections could aid in developing better antimalarial therapeutics that would eliminate these forms and be appropriate for use in all patients.

Alternatively, we examined dormancy using the phytohormone gibberellic acid (GA). Previous work showed that parasites supplemented with GA recovered out of DHA-induced dormancy earlier than non-supplemented parasites. This early recrudescence occurred via an unknown mechanism and thus we hypothesized that we could utilize GA to identify key players in parasite recovery from drug induced dormancy. Since drug resistance can form due to dormancy and treatment incompliance, finding the mechanism which releases parasites from dormancy is a potential way to circumvent drug evasion. If we understood the mechanism of recrudescence and utilized it to our advantage, we could push parasites out of dormancy, making them more susceptible to antimalarial treatments. In this study, we developed modified GA derivatives (GAD) to localize GA within *P. falciparum* blood stage parasites and to pulldown potential interacting partners. GAD localized to the cytoplasm, which is not unexpected as typically, many hormone receptors are found within the cytoplasm and are referred to as intracellular or nuclear receptors. Once a hormone binds to its receptor, a signaling pathway is initiated which ultimately leads to a change of some sort in the targeted cell. GA likely binds to a receptor in *Plasmodium* which initiates a signaling cascade leading to recrudescence.

While the GA signaling pathway has been extensively described in plant biology literature, we were unable to find orthologous genes in *P. falciparum*. Therefore, we conducted pulldown studies to identify proteins of interest which may play a role in the early recrudescence phenotype observed with GA. We identified several proteins of interest involved in transcription, translation, post translational modification, and protein trafficking. Additional replicate pulldown studies will need to be conducted to confirm our results and improve confidence in selecting proteins for further characterization. In order to characterize these proteins and identify which play a direct role in recrudescence, moving forward we will create strains which lack the ability to express these proteins via conditional knockdown and test for their recrudescence phenotypes. This approach will be useful in further characterizing the GA signaling pathway within *Plasmodium* and aid in identifying the mechanisms which control parasite recrudescence.

In summary, we have developed a novel technique to study dormancy mechanisms using alkyne modified purines and the phytohormone GA. We have identified EdA as a novel DNA synthesis marker which captures reactivating hypnozoites in a high throughput manner and we have shown that alkyne modified purines are incorporated in actively replicating *Plasmodium* parasites, presenting novel techniques for cell cycle studies. Furthermore, we have extracted proteins using GA to identify potential players of recrudescence from drug-induced blood stage dormancy. Dormancy remains one of the biggest obstacles to eradicating malaria and our techniques can provide insights into how dormancy is regulated and manipulated to provide the parasite protection and drug evasion. Conducting studies using EdA to investigate theories of

hypnozoite reactivation and further characterizing the proteins from GA pulldown will be important future work towards understanding the elusive dormancy phenomenon.

CHAPTER 5

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