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MMV Malaria Box Activity Screening in Dormant Plasmodium falciparum Phenotypes

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MMV Malaria Box Activity Screening in Dormant *Plasmodium falciparum* Phenotypes

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Public Health
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ABSTRACT

The causative agent of malignant tertian malaria, *Plasmodium falciparum* undergoes an arrested growth phenotype of its erythrocytic stage when under drug-stress. Recent artemisinin treatment failures seem to be indicative of such induction followed by recrudescence rather than actual therapeutic failure. Likewise, *P. vivax* hypnozoites are the prototypic dormants and the latent infections for which they are responsible prove most difficult to treat. Dihydroartemisinin, an artemisinin-derivative, can be used to exploit this mechanism by inducing a dormant state in ring-stage *P. falciparum* parasites and in turn, their recovery may be used as a screening period for compounds that inhibit or foster growth.

Specifically, parasites stably transfected with luciferase were used to quantitatively observe growth (or lack thereof) response of parasites to the phytohormone gibberellic acid and the herbicide, fluridone. Using their behavior as comparative controls, the Medicines for Malaria Venture (MMV) Malaria Box was screened for similar activity. The most active compound, 1,2,3,4-tetrahydroacridin-9-ol a quinoline-derivate caused cells to wake even earlier than expected. Since quinine and other such drugs have historically been most effective in treating malaria, it seems appropriate that such a finding was made. Following this the MMV Box was screened again against uninduced 3D7 parasites to determine if any were capable of causing a dormant response under the hypothesis that such a reaction is a defensive adaptation of *P. falciparum*.
Four compounds were found to be active of which two appear to be inducing dormancy in the second cycle rather than the first akin to DHA. These quiescent periods also appear to be shorter indicating that the latter is more efficient. It is possible that given the length of interaction with artemisinin, *P. falciparum* is more adept to respond to its derivatives likewise the mechanism of action may be different enough to change the nature of the response.
INTRODUCTION

Plasmodium

As an obligate intracellular parasite, the apicomplexan *Plasmodium falciparum* is one of the causative agents of malaria. It is not the only cause however, as this disease may be initiated by five additional species including *P. vivax*, *P. knowlesi*, *P. malariae*, *P. ovale* and most recently discovered, *P. cyanomolgi*. Its definitive host is its mosquito vector whereas humans serve as both reservoir and intermediate host. The disease, for which it is responsible, is thus a result of its journey through a fairly elaborate life cycle (White et al., 2014).

Malaria Epidemiology

An ancient disease, malaria can trace its origins to medieval Italian as *mal aria* or simply ‘bad air’ in reference to an area of Rome known as the Pontina which was in turn characterized by its surrounding swamp and as one would expect, high rates of malaria transmission (Beltz, 2011). To date, it continues to be one of the most prevalent parasitic infections of critical need for sustained attention. According to the World Health Organization, on a global scale 3.4 billion people may be considered at risk. In 2012 they estimated 207 million cases resulted 627,000 deaths with more than 85% of these cases and 90% of the deaths occurring on the African continent (WHO, 2013). Of note is the fact that although a majority of individuals may be parasitized at any given time, only a minority will experience severe symptoms. This is especially true in areas of high endemicity where repeat exposure results in some development of
natural immunity. In this way, such places see malaria as more of an issue of morbidity rather than mortality. Furthermore, these nations are left at a stark economic disadvantage due to an ill workforce, as well as lack of tourism and company investments and the educational loss future generations may incur effectively keeping their society underdeveloped (Marsh & Snow, 1997) (Lopez Del Prado et al., 2014).

**Life Cycle**

The life cycle may be divided into two forms – sexual reproduction/sporogony, which occurs in the mosquito, and asexual reproduction/schizogony, which occurs in the human. A human may only gain infection through the bite of an infected *Anopheles* mosquito specifically through the entry of sporozoites into the wound, as she takes a blood meal. Sporozoites are motile and travel from the dermis to the parenchyma of the liver within the first 15 minutes to a few hours, where they infect hepatocytes. Over 2 – 7 days, the sporozoites develop/differentiate turning the infected liver cell into a schizont, which in turn bursts releasing on average 10,000 – 30,000 merozoites (White et al., 2014). These are motile as well and travel to the bloodstream to infect erythrocytes. Blood stage parasites follow a cycle of invasion, growth, and emergence by red blood cell (RBC) lysis that takes 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale*, 72 hours for *P. malariae*, and 24 hours for *P. knowlesi*. It is this cyclic release that causes malarial pathology (White et al., 2014).

Rather than continuing with this sequence, some merozoites instead commit to differentiate into gametocytes, a gamete precursor. Development is divided into 5 morphologically distinct stages with the final circulating in the peripheral blood until it is picked up by another *Anopheles* mosquito to continue transmission. Within the mosquito, the macro-
and microgametocytes combine to form a zygote. This zygote passes through the insect’s midgut via yet another motile stage, the ookinete, before encysting on the outside of the gut as an oocyst. The latter subsequently bursts and release sporozoites, which travel to the salivary gland to render the host vector competent in passing infection forward.

A distinctive feature that distinguishes *P. falciparum* from *P. vivax/P. ovale* malaria is the presence of a latent liver form in the latter (Markus, 2011). Hypnozoites are a dormant stage that may persist for weeks or even years in affected individuals. Elimination is therefore much more difficult as will be discussed in greater detail subsequently.

**Disease Signs & Symptoms**

Malaria manifests itself most distinctively in the marked paroxysms attributed to the cyclic lysis of infected RBCs (Heymann, 2008). Symptoms may include fever/chills, nausea, headache, fatigue and mild anemia at this initial stage. It is a recommendation of the WHO that any individual in an endemic area presenting with fever be screened through microscopy or a rapid diagnostic test where available. Real-time polymerase chain reaction (qPCR) remains the gold standard but may not be available in many regions. Microscopy can be just as effective though it would be necessary to take repeat samples over 12 to 24 hours as the parasitemia may vary depending on which point of the life cycle the protozoan is in. An undetected and likewise untreated infection of *P. falciparum* malaria can have severe complications such as enlargement of the spleen and/or liver, often presented with jaundice in children (Heymann, 2008). Age seems to be a determining factor regarding which symptoms manifest. Adults may experience pulmonary edema and kidney injury while children may more commonly see severe anemia, and
hypoglycemia. Across all age groups, coma as a result of cerebral malaria can occur contributing to over 1 million malaria deaths every year.

The pathogenesis of this complication is not exactly understood. Mechanistically, it seems to involve microvascular sequestration by infected red blood cells (RBCs), overproduction of pro-inflammatory cytokines and dysfunctional coagulation (Mohanty et al., 2014). A known method of immune evasion, cytoadherence allows parasites to remain undetected by binding to endothelial tissue through modified RBC surface ligands. It is the reason that peripheral blood samples are only able to detect the earliest parasite stages and why screening must be done over a period of 24 hours. As the infection progresses, erythrocytes adhere to each other in a process called rosetting as well as to the lining of tissues, most detrimentally that of blood vessels (Wright, 2014). If this sequestration occurs in the brain, blood flow may be obstructed leading to symptoms like ataxia, swelling/edema and hemorrhage all of which contribute to the patient falling into a malaria-induced coma. There appears to be evidence that upregulation of proinflammatory cytokines like tumor necrotic factors (TNF) and interferon-γ (INF-γ) at this site correlates with higher levels of parasite density. Cerebral malaria is survivable though not without some consequences, 3 – 15% of children incur permanent neurological damage like cortical blindness, cerebral palsy, deafness, and learning impairments (Ioannidis, Nie, & Hansen, 2014).

**Prevention & Treatment**

The best course of action regarding treatment is thus early detection with the screening and diagnostic methods already described being applied to at-risk populations. Hence control methods can be aimed at three distinct groups: patients, the exposed and the mosquito vector
itself (Hafalla, Silvie, & Matuschewski, 2011). The latter two may be the most essential. Curbing mosquito populations through source reduction of their habitats, draining and clearing of breeding areas has had some effect in decreasing incidence. However, indiscriminate control programs are hardly cost-effective and the Anopheles mosquito is highly anthropophilic; consequently an appropriate strategy would need to target it indoors and out (Beier et al., 2008).

In other words, the management of the vector needs to be integrated with the needs of the community and the ecological circumstances of the area. The use of long-lasting insecticide treated bed nets can reduce incidence by 50% in high-transmission areas and 62% in those of low (Ulrich et al., 2013). Likewise, indoor residual spraying has shown to be similarly effective if combined with robust surveillance to ensure that control correlates to risk. Several compounds have environmental implications and it is imperative that they be used with care. It should be noted that the goal of vector control is not annihilation of the insect population but rather the sufficient reduction of malaria transmission in an effort to reduce the entomological inoculation rate. This is the case for most temperate countries, the US included, where the mosquito lives on but the malarial parasite is no longer endemic.

In terms of preventative treatment, malarial prophylaxis may be suppressive or causal with the primary distinction being the former affects blood stage infections only while the latter can affect parasites in both the liver and blood (Lopez Del Prado et al., 2014). Some examples of suppressive prophylaxis include doxycycline, mefloquine and chloroquine while causal prophylaxis may be drugs like malarone and primaquine. Chloroquine is typically used in the treatment of non-falciparum malaria as resistance has been spreading especially in endemic areas with previously high use. The specific regimen used and its dosage depends on the individual taking said medication and the region to which they travel (Lopez Del Prado et al., 2014).
Mechanisms of action vary but all in some manner interfere with an essential functional component of the cell be it in terms of metabolism or replication. To start, the tetracyclines interfere with apicoplast replication. The quinolones disrupt hemoglobin digestion allowing heme to accumulate to toxic levels as a waste product. Malarone consists of two compounds atovaquone and proguanil hydrochloride, and together they obstruct pyrimidine biosynthesis and thereby DNA replication (Flannery, Chatterjee, & Winzeler, 2013). Lastly primaquine has been shown to inhibit the proper formation of transport vesicles and severely disrupt function of mitochondria. It is important in treatment of *P. vivax* malaria since it is one of the only drugs capable of killing hypnozoites and has recently been show to have rapid and potent gametocytocidal activity in *P. falciparum* (Ashley, Recht, & White, 2014).

The use of primaquine is problematic however due to concerns of hemolysis developing in individuals with glucose-6-phosphate dehydrogenase deficiency (G6PD), an X-linked recessive condition that is characterized by incredibly low levels of the titular enzyme (Cappellini, 2008). G6PD is involved in the pentose-phosphate pathway an essential process for RBCs, which serves as a source of reduced glutathione, an incredibly important antioxidant. Since erythrocytes are the primary oxygen carriers in the body, they are at significant risk of oxidative damage. Without enough glutathione to offset this damage, the RBC is left weakened and is usually cleared from circulation by the spleen. This defect confers some protection against *P. falciparum* malaria possibly because infected cells are more likely to be cleared in this manner (Guindo et al., 2007).

Nonetheless, though these drugs may be used to treat malaria and indeed, in combination some are, they are primarily chemoprophylactics that are given to transiently exposed individuals. People living in endemic regions are consistently re-infected and giving these drugs
to them in monotherapy would only encourage the development and rapid spread of resistance.
The WHO recommended first-line of treatment worldwide is artemisinin-combination therapy, hereafter referred to as ACT.

**Artemisinin Combination Therapy**

Artemisinin is a sesquiterpene lactone first isolated from Chinese wormwood in the 1960s when it was evident that mosquitoes had developed a resistance to DDT and chloroquine was beginning to lose its effectiveness in much of the world ("Etymologia: Artemisinin," 2014). The Chinese government began searching for a new drug by undertaking a systematic inquiry into various plants used in traditional herbal medicines to find some new antimalarial. The herb *Artemisia annua* or *qinghao* was one of these hundreds of species examined. In the pharmacopeia consulted, it was listed as being cool in nature, *yin*, so as to be useful in treating internal heat, *yang*. Indeed historical text points to it being implicated in the treatment of an “intermittent fever illness” in 1596 AD (Cumming, Ploypradith, & Posner, 1996).

Based on the information in these texts, scientists successfully extracted the crystallized artemisinin compound. Over the next few decades, they demonstrated its potent ability to clear infection in mice and even in military volunteers (Faurant, 2011). They found no obvious side effects and that it was much more effective than any other drug at clearing both blood-stage infection as well as more complicated cases of cerebral malaria. Politics at the time kept this information from western organization like the WHO until the 1980s, when much progress was made in a short time.

Its unique structure is the basis of its antimalarial property. The endoperoxide linkage contained in the 1,2,4-trioxane ring acts as a trigger for artemisinin to release a cascade of
reactive oxygen radicals in the vicinity of the parasite. Specifically, this process occurs in response to the plasmodium’s obligatory digestion of hemoglobin and generation of hemozoin. Hemozoin is formed through biocrystallization of the insoluble iron complex released by hemoglobin consumption. Often called malaria pigment, the current theory suggests its interaction with artemisinin to form cytotoxic iron-oxo intermediates as well as electrophilic alkylation agents that together cause fatal damage to the protozoan (Meshnick, 2002).

Artemisinin combination therapy is presently the most effective treatment option for uncomplicated *P. falciparum* malaria. The combination consists of an artemisinin compound such as artesunate, dihydroartemisinin or artemether being accompanied by an often-slower acting and different class drug. Currently there are five such combinations recommended by the WHO: artesunate-mefloquine, artesunate-amodiaquine, dihydroartemisinin-piperaquine, artemether-lumefantrine and artesunate-mefloquine (WHO, 2014). These drugs are normally administered as fixed-dose formulations together in one tablet, except for artesunate-mefloquine, which is given as loose dose i.e. two separate tablets that are packaged together. There are several advantages to the use of these drugs in this manner, namely their ability to act on all asexual stages, reducing parasite biomass by approximately 10,000 fold per life cycle rapidly alleviating symptoms as well as the reduction of gametocyte production and curbing of further transmission. Additionally, these drugs can affect parasites with multiple drug resistances and overall have better potency, faster efficacy and delay the development of resistance (WHO, 2014).

In recent years the latter has not proven to be enough. Anywhere from 3% to 50% of patients fail the treatment and experience parasite recrudescence in monotherapy (Cheng, Kyle, & Gatton, 2012). Such a wide range of failures correlates with both duration of the treatment,
with the highest rates corresponding to the shortest treatment times, and size of the parasite burden. Essentially, individuals with high parasitemia who are not treated for a long enough period to overcome ART’s short half-life will have parasites survive the drug administration and persist in their infection (White et al., 2009). Although increasing the duration of treatment reduces these instances, it does not eliminate them and furthermore isolates of parasites collected from these patients remain susceptible to the drug in vitro (Teuscher et al., 2010) indicating that the cause of these outcomes is not outright resistance. Although combination therapy seems to be assuaging these events, reports of decreased sensitivity has had many fearing recrudescence as a mechanism toward the establishment of resistance.
BACKGROUND

Recrudescence & Dormancy

Recrudescence is a phenomenon common to *Plasmodium* parasites and is often considered a sign of treatment failure although that many other factors can also contribute including improper dosing, poor compliance among patients, and poor quality of drugs administered (Cheng et al., 2012). The term itself is defined as a situation in which a patient’s parasitemia drops below detectable levels before increasing at a later time. This phenomenon differs from relapse, which specifically refers to the re-activation of the dormant hypnozoites in the liver of individuals infected with *P. vivax* or *P. ovale* (Hulden & Hulden, 2011). However there is evidence indicating that a similar quiescent stage is the causal mechanism behind what is hypothesized to be an adaptive stress response (Cheng et al., 2012).

Such a failure of treatment and subsequent recrudescence is required in nearly all cases of newly developed drug resistance. In simplest ecological terms, administration of antimalarials bottlenecks the parasite population toward the least susceptible subjects, which go on to produce gametocytes that may possess some adaptation against the drug of choice. The risk is exacerbated in individuals with high parasite burdens. It is logical to assume that more severely ill patients will have a higher chance of being treated and thereby a higher chance of their treatment failing. By extension, treatment failure often entails further treatment and thus a second opportunity to bottleneck the parasite population and once again, select for the least susceptible protozoa. In fact, observations of resistance development have been consistently
preceded by recrudescence events. A pilot study performed in Ghana in 1962 first noted this association where an abundance of treatment failures eventually led to pyrimethamine resistance resulting from improper self-medication (Charles, 1962). The patients who experienced recrudescence had parasites that remained less susceptible to pyrimethamine over the observation period.

To determine whether dormancy could be a plausible explanation for some failures of ART treatment, Codd et al created an infection model to simulate *P. falciparum* interaction with host immune response incorporating both antigenic variation of the parasite and the parameter of dormancy induction (Codd et al., 2011). The latter was expressed via a daily dose of artemisinin (ART) for 1, 3 or 7 days, which killed all but a small percentage of parasites. These parasites would later re-emerge at the same stage that they went dormant, expressing the same surface receptors under the assumption that ART targets immature stages more readily.

All simulated infections had some level of recrudescence due to the presence of dormant stages, however just a small proportion of these could be measured clinically due to the intervention of the immune system. This observation makes sense as it has been demonstrated in numerous studies that natural immunity often develops following repeat exposures. Furthermore, their simulated failure rates were comparable to that observed in clinical field trials thus providing legitimacy to the hypothesis that dormancy and treatment failure are linked (2011).

**Drug-Induced Dormancy**

Previously published *in vitro* data have demonstrated that the short half-life of artemisinin drugs results in a plasma concentration of the drug above the minimum inhibitory concentration (MIC) (Teuscher et al., 2010). This period allows some parasites to escape
exposure and thereby maintain a minute yet significant population for the infection to persist. These explanations do not hold up to scrutiny, however, in cases where the treatment length is increased and recrudescence still occurs.

The alternative supposition holds that surviving parasites enter a quiescent stage of little to no growth similar to that occurring in *P. vivax* hypnozoites. These so-called dormants endure the drug treatment and recover once it is discontinued. Laboratory studies demonstrate that parasites at the ring stage when exposed to an artemisinin derivative such as artesunate or as is used in this thesis dihydroartemisinin (DHA) will tolerate said treatment by rapidly arresting their growth in a dose-dependent manner.

These drug-induced dormant parasites are morphologically distinct from their ring-stage asexual counterparts with a condensed cytoplasm and an overall much smaller size. In this stage, the parasites exhibit a reduced metabolism, which may be measured through the stage-specific expression of luciferase-transfected parasites. Bioluminescence reporters are common tools in ascertaining various biological functions in a quantitative manner (Khan et al., 2012). It is an enzyme-catalyzed process where photon emission is derived through the completion of an exothermic reaction namely between the luciferase enzyme and its substrate, D-luciferin. Firefly luciferase from *Photinus pyralis* is an enzyme requiring no post-translational modification meaning it can be successfully transfected into malaria parasites and has, by members of the Kyle lab with the laboratory strain 3D7 and patient isolate 4G.

Luciferase acts by first combining its substrate with ATP to form the intermediate luciferyl-AMP which promptly reacts with oxygen to create another, oxyluciferin. The latter being in a high-energy state allows its transition back to ground to yield a yellow-green light, which can be detected by a luminometer. As ATP content is an indicator of metabolic status, it
can conceivably be used to detect abnormal metabolic activity imposed by drug action (Azevedo et al., 2014). Transfected parasites produce the luciferase enzyme and produce more of it depending on the stage that they are in. In this way, higher relative light units (RLUs) are indicative of the presence of more mature stages and signs of their growth and recovery. Dormant parasites do not exhibit high levels of ATP by the very nature of their quiet state; they are not proliferating.

**Dormancy in Exoerythrocytic Stages**

Dormancy is not a new concept. As was mentioned in brief, there exists a point in the life cycle of *P. vivax* and *P. ovale* where quiescence occurs (Mueller, Zimmerman, & Reeder, 2007). During the liver stage while the majority of infective sporozoites are developing into schizonts and bursting from their host hepatocytes to release merozoites into the bloodstream, a small population remains. The precise mechanism behind dormancy induction and reactivation is unknown, although there has been informed speculation regarding why *P. vivax* parasites hibernate in this manner.

The length of dormancy seems to vary across strains with the longest being seen in places like Finland and northern Moscow where relapse can last 9 years and the shortest in New Guinea, only 17 days (Hulden & Hulden, 2011). While the differences in dormancy of hypnozoites may be explained by polymorphic differences among *P. vivax* strains, variation among relapse times seems to directly correlate with the seasonal variation of the mosquito. An unpredictable vector population appears to be a key ingredient for a long dormancy period and furthermore, relapse may be triggered by the bites of new vectors. A study in the UK demonstrated that onset of vivax malaria was restricted to the summer months regardless of
when the infection was contracted, and despite the vast dissimilarity in patient incubation periods (Venkatesan et al., 2003)

Several advantages exist for hypnozoite development with the foremost being the amplification of the likelihood of a parasite reaching its vector, as the host may be infective on multiple occasions. This advantage is maximized if the awakening of hypnozoites is not random but in response to some trigger by the mosquito vector. Since sporozoites only infect the salivary gland, it may be supposed that the hypnozoites hiding in their hepatocyte hosts may be able to recognize something within the mosquito saliva (Hulden & Hulden, 2011). More research is required to determine if such a compound exists but exploiting it could potentially induce a relapse and allow parasites to be targeted by other medications.

Both of these stages represent an arrested developmental state with the key difference being causality – one is induced by drug stress in the blood while the other by the parasite’s metabolism as it waits to be transmitted. On a related note, although not referred to as dormant, stage IV and V gametocytes also appear to have a quiescent component to their developmental track. While most drugs targeting the blood stages also affect young gametocytes (stages I – III), the mature stage V is not sensitive to their effect (Miguel-Blanco et al., 2015). It is believed that they instead remain in a state of relative inactivity for up to 22 days in the peripheral bloodstream until either being picked up by a feeding mosquito or dying. Since the mechanism of dormancy induction is not yet understood, there exists the possibility that the contributory method is similar among *P. vivax* hypnozoites and *P. falciparum* quiescent stages. Thus testing of compounds against the induced-dormant phenotype could be informative for more than one circumstance and encourage further study.
Dormancy in Other Organisms

A common feature to many microorganisms, dormancy is a trait that allows a species to respond to the temporal irregularity of its environment. It is thought to be important in natural systems, some 20 – 80% of microbial samples appear metabolically inactive and many can be resuscitated with supplements of required resources (Jones & Lennon, 2010). It is regulated by a multitude of environmental cues and many microorganisms that resist temperature changes, desiccation, and antibiotics do so by entering a non-proliferating dormant stage. The ubiquity of this characteristic behavior comes as no surprise, mathematical models have demonstrated that it influences species richness and microbial diversity by ensuring survivability during times that would be inhospitable otherwise (Jones & Lennon, 2010).

In baker’s yeast, *Saccharomyces cerevisiae*, quiescence is often styled as a “Sleeping Beauty” state (Gray et al., 2004) and can be induced by a limitation of nutrients. In this way, dormancy induction can be described as a survival mechanism (Markus, 2012). In humans, tissue homeostasis and post-injury regenerations are facilitated by adult, sometimes tissue-specific, stem cells (ASCs) (Rumman, Dhawan, & Kassem, 2015). These cells are sustained inside normal tissues in an undifferentiated and dormant state. They are activated following the advent of an injury through the production of mechanical signals at the site of injury. These signals lead to an amplification of the ASCs, which eventually differentiate into the appropriate cell types.

Cellular quiescence in this sense is a transient stage wherein the cells have exited the cell cycle. Commonly called G0, the cells contain an unreplicated genome, altered and slowed metabolism, an increase in autophagocytosis and a markedly distinct morphology – one that has already been described among *P. falciparum* dormants. Cell cycle arrest is reversible however, making it different from that seen in terminally differentiated, dying cells (Rumman et al., 2015).
Tuberculosis

Recurrence in tuberculosis infections has been a distinguishing feature for years, slightly less so in modernity, which saw the advent of combination therapy in the usage of isoniazid (INH), rifampin (RIF), and pyrazinamide (PZA). Statistically, reactivation risk was reduced from 20% in the 1960s to today’s rates of 1 – 2% (Gomez & McKinney, 2004). These numbers may not be wholly representative as historically no note was made concerning reactivation versus secondary infection. On a global scale, this continues to be the case and geographical constraints are used to provide some distinction. Areas of high transmission are typically considered to have more re-infections while the opposite is true of areas of low transmission, where more recurrences are to be expected.

When it comes to the disease itself, it is important to define the key terms utilized in describing pathogenesis (Gomez & McKinney, 2004). Latency is the presence of a TB lesion that does not produce any discernable symptoms. An individual may be in a latent infection prior to the onset of overt disease or after a spontaneous resolution. Without treatment, chronic latency is considered the typical TB outcome and the infection may reoccur even after years of no clinical signs. Latent infections are also transmissible. Persistence specifically refers to continuous infection in times of adversity such as under drug pressure. The mycobacterium can be incredibly resilient, the mere fact that it uses macrophages as its cellular host is a demonstration of its ability to persist despite its environment. Another example is the incredibly difficult and the slow rate at which anti-TB drugs clear the bacteria. This feature has made its treatment a challenging one especially in the developing world.

Lastly, dormancy in TB may describe the nature of the disease in addition to the metabolic conditions inside the characteristics tubercle. It was first used to explain the state of
bacilli that survive in closed and necrotic lesions during clinical latency. Though this model was quite speculative, it brought the idea of dormancy to the forefront. In the 1940s surgical removal of lesions was common and allowed scientists to study the bacteria therein. By the 1950s, there were several reports showing the presence of acid-fast bacilli within lesions removed from patients that have been undergoing treatment and testing sputum-negative. Some of these bacilli were culturable but those taken from closed lesions were not. The debate began regarding whether unculturable bacteria were truly dead or dormant. Through the work of several teams of scientists and over the past two decades, it was postulated that the aforementioned conditions within these particular lesions contributed to their dormant response. Specifically lowered oxygen stress, as well as the presence of substances such as long-chain fatty acids, lactic acids and others with bacteriostatic properties (Gomez & McKinney, 2004). Together they reduce bacterial metabolism and render the tubercle bacilli defiant to drug pressure. Since all present medicines are aimed at targeting cell division and growth, it makes sense why they exhibit such poor activity against these lesions.

**Cancer Cells**

Dormancy among tumor cells is an occurrence that has been known to clinicians for many years. It was first identified in the 1940s and redefined in the 1950s as a temporary mitotic growth arrest. Arresting of growth occurs naturally due to cell aging or senescence; it is triggered by numerous signals like oncogene-induced stress (Sosa, Bragado, & Aguirre-Ghiso, 2014). Unfortunately, as was already mentioned this is an irreversible process and could not be the mechanism behind dormancy. However, since the cells are no longer proliferating, it stands to reason that they are in a G0/G1 arrest.
It is just as poorly understood as that which occurs in microorganisms though somewhat more complicated. The term has been used loosely to refer to cells “lying in wait” but abundant evidence exists to point to its involvement in numerous malignancies specifically in the phenomenon of metastasis. Metastasis is a rather inefficient process. Although metastases may form from just a subset of cells, the cancerous progeny that develop exist in three distinct states, only one of which is clinically detectable (Goss & Chambers, 2010). The other two, micrometastases, somewhat dormant cells whose growth is balanced through apoptosis, and solitary, true dormant cells may only be detected through histology. In other words, the actual extent of dormancy in affected cells is not well understood.

Persisting in a quiescent state may allow the disseminated tumor cells (DTCs) to avoid treatment in a number of ways, the most obvious being their dormant nature but also the fact that the cells disseminate at early enough stages to allow themselves to diverge genetically from the primary tumor (Sosa et al., 2014). The crux of the issue is then targeting as the therapies devised according to the characteristics of the primary tumor no longer apply. There is also some evidence of the existence of pre-metastatic niches or areas that contain different host cell types capable of “cross-talking” with the DTCs. These niches thenceforth support the dormants as well as their growth and evolution.

Whereas with dormant malarial infections, there could be some advantage to finding a compound to wake the parasites in combination with drugs like DHA and piperaquine to ensure as high a clearance as possible. In the case of tumor cells, this is not recommended due to the lack of available effective and specific cytotoxic treatments (Sosa et al., 2014). Additionally because DTCs are genetically heterogeneous, awakening dormant ones would only expand the genetic and possibly epigenetic diversity therein, potentially spreading mechanisms of resistance.
Instead present research is concentrated in either keeping DTCs dormant or determining a method for eradicating them despite their dormancy.

**Dormancy in Plants**

Unlike most other organisms, plants are bound to their locations and truly at the whims of their environments. It is for this reason that they have established a cyclic pattern of developmental states, alternating between growth and non-growth. Transitions between these phases are tightly regulated to ensure that the most vulnerable stages occur during the most favorable of times. These two stages are bud dormancy and seed dormancy (Graeber et al., 2012). A decent example of the former would be prevention of bud growth in perennial plants during winter months (Rohde & Bhalerao, 2007). Both intervals are depicted as being times of very low metabolic activity and insensitivity, albeit temporary, to signals promoting growth.

An innate property, seed dormancy is a key determining factor in germination times. It is essentially a plant’s adaption to the diversity of its habitat, with a genetic basis and heavy environmental influence. As a quantitative trait, its intensity can vary over time. In fact, seeds typically experience a primary and a secondary dormant state (Graeber et al., 2012). Primary dormancy occurs immediately following maturation of seeds and grows more pronounced during drying and storage. It can however wane depending on the conditions for germination being met. Conversely, secondary dormancy occurs when seeds on the cusp of activation are re-induced typically in stints of high or low temperature. In this way, plants and their cultivators can control both when germination occurs and how many seeds sprout.

Dormancy induction is coordinated by a diverse group of regulators at different times with different levels of specificity. The two main and antagonistic hormones are abscisic acid
(ABA) and the gibberellin group of hormones, of which gibberellic acid (GA) is the most well-known (Graeber et al., 2012). Their balance within a plant is vital for controlling both induction and maintenance of dormancy. As will be elaborated in subsequent sections, they also appear to have an effect on the drug-induced dormant state of *P. falciparum*, indicating that the latter must possess similar pathways and possibly a similar mechanism for its own quiescent response.

**Plasmodium Plant Origins**

The *Plasmodium* genus, consisting of some 200 species of parasitic protozoa, is a member of the Apicomplexa phylum, so named for its unique possession of an apical complex – a collection of anterior structures that play a key role in cell invasion and the establishment of infection. This apicoplast is actually a relict plastid whose origin and function have been extensively studied since its discovery in the 1990s (Lim & McFadden, 2010).

All plastids, from chloroplasts to leucoplasts, are the results of an endosymbiotic relationship between an ancestral prokaryote and its eukaryotic host. Evidence exists to support the notion that all plastids are resultant from a single such event with a cyanobacterial organism. The apicoplast falls within this group of exogenous organelles and is distinguished as a secondary plastid being the product of one eukaryote engulfing another, this other containing a primary plastid of the aforementioned cyanobacterial variety (Waller, 2004). Over time the latter eukaryote was stripped of its superfluous features and most of its genes assimilated with those of the host. The only remaining evidence of its previous existence is the continued presence of three or four membranes. This is in contrast to a primary plastid, which would only have two; these being homologous to the membranes of the Gram-negative bacterium from which they were derived.
As far as *P. falciparum* is concerned, each parasite contains just one apicoplast, which is intimately linked to the mitochondrion throughout the asexual stage of its life cycle. The exact reason for this association is presently unknown. Although, a recent study by Kobayashi *et al* found the first experimental evidence that there exists an actual physical bond between the two organelles. Their hypothesis as to why this association occurs involves heme biosynthesis in which both the mitochondrion and the apicoplast are known to be involved. The production of one component (5-aminolevulinic acid) seems to begin in the former and continue in the latter. Their proximity may be a requirement for successful transportation across the multitude of membranes (Kobayashi *et al.*, 2007).

Despite lacking the photosynthetic capabilities that better-known plastids are characterized by, the apicoplast has proven itself to be essential to the malaria parasite and indeed to most Apicomplexans. Drugs that target its transcription ultimately lead to the death of the protozoan. Parasites engineered to lack the organelle are able to invade tissues and multiply a few times but eventually decline and die implying its importance in maintaining metabolism (Lim & McFadden, 2010). Furthermore, proteome analysis has shown apicoplast involvement in the biosynthesis of fatty acids, heme, and isoprenoids. These functions are analogous to those occurring in plant plastids.

Isoprenoids, also sometimes called terpenoids, are a vastly diverse group of natural products that are found in all organisms and have important functions therein. They are made up of repeated units of isopentenyl pyrophosphate (IPP) or its isomer, dimethylallyl diphosphate (DMAPP) (Jordão, Kimura, & Katzin, 2011). The former is synthesized by one of two pathways distinguished by the presence or absence of mevalonate. The classic pathway supplies IPP to most if not all eukaryotic animals including all mammals, some bacteria, algae, and higher plants.
as well as the Trypanosomes, namely *Trypanosoma* and *Leishmania* parasites. In humans IPP is required to initiate the biosynthesis of cholesterol and indeed, statins target HMG-CoA reductase, a key enzyme at the start of this pathway. Alternatively, the mevalonate-independent pathway also called the methylerthyritol phosphate (MEP) pathway is specific to photosynthetic organisms like cyanobacteria, algaes, plants, and most fittingly, apicomplexan parasites and *M. tuberculosis* (Jordão et al., 2011).

In *Plasmodium*, isoprenoids serve as precursors to compounds involved in electron transport and glycoprotein formation and also act as prosthetic groups on various enzymes (Waller, 2004). The apicoplast synthesizes these compounds via the MEP pathway and is the only site to do so. This fact has opened new avenues of research since several enzymes from this pathway are wholly absent in humans and could serve as excellent targets for drug development.

**Phytohormones and Dormancy**

As was stated in a preceding section, plants utilize two distinct hormones for regulation of growth. These work antagonistically in intricately linked pathways to both induce and release a seed from dormancy. ABA, a carotenoid catabolite, produced during later stages of maturation and whose levels appear to correlate with day-length, inhibits division, and lowers overall metabolism (Zheng et al., 2015). The specific mechanism for ABA’s influence is beyond the scope of this thesis but it requires mentioning that its presence appears to directly influence dormancy induction. In light of the function of the apicoplast and the synthesis of IPP, a precursor for carotenoid biosynthesis among many other processes, the Kyle lab is currently researching ABA’s role in drug-induced dormancy. Having detected its presence in the erythrocytic stage and further quantified its levels in response to carotenoid inhibitors and
apicoplast-targeting substances alike, the present hypothesis poses that ABA may act a stress sensor in *P. falciparum*, regulating the dormant response (Duvalsaint, unpublished data).

Conversely gibberellic acid controls growth in opposition to ABA by stimulating mitotic division and increasing the germination of seeds (Toyama et al., 2012). It too may have an effect on the aforementioned induced-dormant state and warrants investigating, as it is possible and even, probable that it may behave similarly. Along the same line of thinking the herbicide fluridone (FLD) specifically works by inhibiting phytoene desaturase, an enzyme involved in carotenoid synthesis (Tonhosolo et al., 2009). Preliminary data has demonstrated that *in vitro* dormants when exposed to FLD experience a growth delay, effectively keeping the parasites in their quiescent state longer.

**MMV Malaria Box**

Medicines for Malaria Venture (MMV) is non-profit organization established in Switzerland in 1999 who’s mission is to reduce the burden of malaria by discovering, developing and facilitating new and affordable antimalarial drugs (Ridley, 1999). As a part of this facilitation, they provide the appropriately named Malaria Box to researchers in malaria and other neglected disease fields. The box contains 400 diverse compounds generated from 20,000 hits, which were gathered in an extensive screening campaign of almost 6 million compounds from the libraries of St. Jude’s Children’s Research Hospital, Novartis, and GlaxoSmithKline (Spangenberg et al., 2013). Originally screened for efficacy against asexual blood stages of *P. falciparum*, it was also tested for cytotoxicity and all compounds included are those with 10-fold less potency against human cell line than the *P. falciparum* target. To date, these boxes have been dispatched to 27 countries and tested against a wide variety of targets including malaria but
also other neglected disease such as trypanosomiasis, leishmaniasis, schistosomiasis, TB, and *Naegleria fowleri*.

The 400 compounds are provided as five 96-well plates of 10 mM stock solutions in 100% DMSO (Lucantoni et al., 2013). Plates are divided by drug- and probe-like characteristics by which the compounds are grouped with the first plate consisting of the 80 most active of both categories and the other four comprising two plates of drug-like and probe-like, respectively. Prior to this distinction, compounds were chosen to achieve maximum diversity and cross-referenced with commercial availability. Half of the selected 400 were qualified as drug-like for being rule of 5 compliant (Spangenberg et al., 2013).

Prior to Chris Lipinski establishing the now widely used rule of thumb for determining the physiochemical features of a potential drug, many clinical candidates would fail development. The rule covers the ADME criteria regarding the outlook of a pharmacological compound within an organism. Specifically absorption, distribution, metabolism and excretion are purportedly managed by certain molecular properties the rule encompasses. The rule thusly states that an orally active compound must have a molecular weight below 500 D, a partition coefficient log P no greater than 5, no more than 5 hydrogen bond donors and no more than 5 hydrogen bond acceptors (Lipinski, 2004). Meeting these guidelines does not guarantee that a compound will be effective, it simply uses information from previously identified drug-like compounds to determine the probability that a compound of interest will not encounter any issues in its oral activity. The REOS (Rapid Elimination of Swill) and PAINS (Pan Assay Interference Compounds) filters further analyzed MMV drugs that did not meet one of these criteria and following this second review; the second category of probe-like was established (Spangenberg et al., 2013).
AIMS & OBJECTIVES

The first objective of this investigation was to confirm the effect of gibberellic acid and fluridone on drug-induced dormant *P. falciparum* and to optimize such an assay for screening. Secondly with the prior two compounds as comparative controls, the goal of the initial MMV screen was to find those compounds, if any, that behave similarly and are able to either wake a DHA-induced dormant as GA or conversely, to induce the parasite to remain dormant beyond the typical recovery period as FLD.

The third objective was to screen *P. falciparum* in absence of DHA exposure and determine whether any MMV Malaria Box inhibitors triggered a dormant phenotype response. The hypothesis being that a response to stress in the parasite is to become dormant, it stands to reason that drugs other than artemisinin and its derivatives are capable of inducing its initiation.
METHODS

*Plasmodium falciparum* Culturing

*Plasmodium falciparum* parasites were routinely maintained with 10% complete media (CM) and O+ blood. Specifically 3D7 a chloroquine-sensitive laboratory strain and 4G, a patient-isolate clone, both having been previously successfully transfected with the luciferase reporter. Slides were taken daily and media changed on alternating days. If parasitemia was determined to be greater than 3%, cultures were split down and red blood cells (RBCs) were supplanted. On days that an assay was to be performed, cultures were synchronized at the ring stage with 5% D-sorbitol. Samples were frozen when parasitemia was high for maintenance of stock and thawed when needed for experimentation.

**Freezing and Thawing Protocols**

Cultures at high percent parasitemia with a predominance of rings were centrifuged at 9,660 xg for 5 minutes at 4°C. The resultant supernatant was then removed with a 5 ¾” unplugged Pasteur pipette attached to the intake line of a standard laboratory vacuum. The volume of the packet cells was then estimated, dubbed V₁ and glycerolyte was added drop-wise at 0.33x of this volume. Obtained from Baxter Blood Products, glycerolyte solution consists of 57% glycerol buffered with sodium lactate, and potassium chloride filtered through a sterile 0.22 μ vacuum filter before use.
The new volume of packed cells and glycerolyte, $V_2$, was allowed to stand at room temperature for 5 minutes before more glycerolyte was added at 1.33x of this new volume. Samples were finally divided into aliquots of 400 µl into sterile cryotubes labeled with the name of the clone, its present parasitemia and the date of its freezing. Tubes were then placed and stored in a – 80º freezer.

Thawing samples involved essentially reversing the above steps. Cryotubes were immediately placed in a 37º water bath for 1 – 2 minutes and then transferred into a 50 mL sterile centrifuge tube; this volume was dubbed $V_1$. 12% NaCl was then added drop-wise at 0.1x volume of $V_1$ while the tube was swirled. After allowing the new volume, $V_2$, to stand for 5 minutes 1.6% NaCl was added at 10x said volume. The tube was then centrifuged at 9,660 xg for 5 minutes. The supernatant was removed, 0.9% NaCl was added and also removed in the same manner. The resultant pellet was then re-suspended in 15% CM and washed, spun and re-suspended again with 150 µl of fresh red blood cells in a sterile, vented culturing flask which in turn was placed in a mixed gassed incubator for 24 hours.

**Plasma, Media and Blood Preparation**

Plasma was obtained from Interstate Blood Bank and promptly heated in a water bath for 60 minutes at 60º then refrigerated at – 4º for 15 to 20 minutes before being steriley transferred to 500 mL conical centrifuge bottles, which were thereafter centrifuged at 842 xg for 15 minutes at 4º C. Care was taken to avoid the lipid/protein layer that precipitated out when pooling plasma pots before aliquoting into 15 mL centrifuge tubes which were frozen and stored at – 80º.

Blood, a crucial ingredient for culturing and experimenting with *P. falciparum*, was processed on a weekly basis when received, likewise from Interstate Blood Bank. Bags were
thoroughly sterilized before being aliquoted into 50 mL centrifuge tubes in a suspension of RPMI 1640, HEPES and sodium bicarbonate then spun at max speed in four successive increments of 15, 5, 5 and 15 minutes. After each spin, the supernatant and buffy coat were removed until eventually the final pellet was matched with an equal volume of this washing media for a working suspension of 50% hematocrit. RBCs were stored at –4°C for approximately one week at a time.

The CM that has already been mentioned in previous paragraphs is similar to the blood wash media in that it consists primarily of RPMI 1640. When HEPES and sodium bicarbonate are also added, the media simulates the environment found in human blood via a bicarbonate buffer system. For the purposes of routine culturing 100 mL of CM was made with 10% plasma consisting of 86.8 g RPMI, 3.2 g NaHCO₃ and 10 mL pre-warmed plasma. Phenol red is a pH indicator that was included with the standard culturing media but left out during assays primarily because the luminescence signal proved stronger without its presence.

**Slide Preparation**

Slides were taken daily to determine parasitemia and gain a snapshot look into the parasite culture’s health. Spent media was aspirated and the flask set on its end so as to allow cells to settle. A sample of 2 µl was removed using a 10 µL pipette. One drop was placed on the front of the clean glass slide, this being the thick smear, while the remainder of the aliquot was dispensed towards the middle, the thin smear. Using another clean slide this second droplet was spread along the surface smoothly and the whole slide was allowed to dry before being fixed with methanol and stained with 20% Giemsa for 15 minutes, after which it was promptly rinsed with cold water and allowed to air dry.
Parasitemia counts were obtained by examining prepared slides under 100x magnification with immersion oil. Areas of 100 – 200 red blood cells were identified and quantified as fields of which 5 total were used to form an average percentage. If parasitemia was greater than 3% the culture was “split” meaning a sample of the infected red blood cells was transferred to a new culture flask with an appropriate amount of fresh blood and fresh media according to the simple calculation: $C_1V_1 = C_2V_2$ with the new concentration corresponding to a lower, desired parasitemia.

**Dormancy Induction**

Slides were taken as described in the previous section and if found to be predominantly ring stages, the culture was transferred with a 10 or 25 mL pipette from its flask to a 50 mL centrifuge tube, then spun for 5 minutes at 9,660 xg. The supernatant was removed via aspiration and replaced with pre-warmed 5% D-sorbitol. The tube was then placed on a shaker for ten minutes and centrifuged again at the same settings. The supernatant was removed and the culture was washed 3 times with RPMI 1640 without phenol red, the final washing step included the addition of an appropriate amount, typically 10 or 20 mL of CM, also without phenol red. The new culture was left to stand at room temperature for ten minutes and a second slide was taken to determine new parasitemia.

Volume varied depending on the requirements of the particular assay being performed but DHA’s concentration was kept constant at 700 nM. After adding the latter to the culture flask, it was allowed to incubate for 6 hours with DMSO-exposed infected RBCs and unexposed RBCs as positive and negative controls, respectively. Following this period, each culture was transferred from its flask to a 15 mL tube and centrifuged for 5 minutes at 9,660 xg. The
supernatant was removed and fresh RPMI 1640 was used to wash out the drugs three times with repeated spins and subsequent removal. A multichannel pipette was then used to plate and set up the particular assay being performed while 2 µl of DHA-exposed, DMSO-exposed and unexposed RBC’s were removed for preparing slides to confirm dormancy induction.

**Drug Assays**

The ring stages of *P. falciparum* were exposed to DHA as described above in order to induce dormancy and use the recovery period as the screening period for potential inhibitors. The *P. falciparum* strains utilized were stably transfected with luciferase enzyme meaning that each assay used bioluminescence measured in RLUs as basis for measuring activity.

**Optimization**

The goal of optimization was to determine which percent parasitemia was most effective for observing recovery and to determine whether the positive and negative controls were appropriate comparative measures of activity. Parasitemia and predominating life stage was determined via microscopy after which the culture was synchronized with 10 mL of 5% D-sorbitol for ten minutes, washed 3 times with RPMI 1640 and new parasitemia counts were made. Based on this information, three centrifuge tubes were used to make 4 mL aliquots each at 2% hematocrit with 0.5%, 1.0% and 2.0% parasitemia as the main testing variable, in a separate tube a 2% hematocrit suspension of red blood cells was made at 1 mL. Using a 5 mL pipette these cultures were transferred to a 6-well plate with the top row containing 2 mL of each parasitemia and 16 µl of DHA whereas the bottom row, the control, contained 1 mL of each parasitemia and 8 µl of DMSO, a separate plate contained the RBC suspension.
All were incubated for 6 hours to induce dormancy, transferred to falcon tubes and washed 3 times with RPMI 1640. A multichannel pipette was used to transfer cultures to a 96-well plate and 10 µl of D-luciferin was added to each well. Luciferin stock consists of 30 mg/mL of D-luciferin in 1 mL of PBS at pH of 6.1 – 6.5, this is then diluted 1:20 prior to use or 300 µl of stock in 5.7 mL CM. Plates were sealed and read by a TopCount Luminometer at the 0 hour and 6 hour time points then each subsequent day at the original incubation time until recovery of dormancy could be definitely observed.

**Dormancy Recovery Test**

To determine the typical period of recovery, dormancy was induced as previously described and a 12-well plate was set up with 1 mL of parasite suspension per well as well as 8 µl of DMSO in the first row and 8 µl of DHA in the second, the third was left empty. Half the tested culture was kept at 1% parasitemia and 2% hematocrit while the other half at 2% parasitemia and 4% hematocrit. 90 µl were transferred daily to a column of a 96-well plate complete with 10 µl of D-luciferin. Additionally 2 µl were taken for slide preparation and the cultures were fed every two days to maintain both volume and concentration being tested.

This recovery test was performed for 3D7 and 4G with recovery being monitored by both luminescence values and by the microscopic detection of phenotypic changes. Feeding calculations hinged on the assumption that 180 µl of culture was removed every two days and that the numbers of infected cells could be quantified based on the percent hematocrit used. Media was thus replaced according to this estimated displacement.
Dormancy Recovery Assay with GA/FLD

In order to determine what qualifies as a hit when screening further inhibitors, it was necessary to test and quantify active compounds for such comparison. Gibberellic acid has a molecular weight of 346.37 g/mol and accordingly 3.46 mg was dissolved in 1 mL of a solution consisting of 30% H₂O and 70% ethanol, this made a stock concentration of 10 mM. This was further diluted to two concentrations 10 µM and 100 µM of which 10 µl was used in the assay for final concentrations of 1 µM and 10 µM, respectively.

Fluridone has a molecular weight of 329.32 g/mol and likewise dissolving 3.29 mg in 1 mL DMSO made a stock solution of 10 mM. These were further diluted to 500 µM and 100 µM. As 10 µl was the volume used in the assay final concentrations were thusly 50 µM and 10 µM, respectively. Mother plates were made containing 600 µl of each of both drugs at both concentrations with CM for control.

Following dormancy induction as previously described, 80 µl of 3D7 parasite was transferred to a 96-well plate with 10 µl of both drug and D-luciferin. Readings were taken at 0 hour and 6 hour time points and then each subsequent day at time of first incubation. On day 2, the plates were sealed, their lids secured with paraffin and spun at 2,000 rpm for 2 minutes. Using a multichannel pipette, 80 µl was removed being careful as to not disturb the pellet, 80 µl of RPMI was added and plates were sealed and spun again after which another 80 µl of this resultant waste was removed. This was repeated a third time after which the plate had CM only added to the first four rows and CM with drug as per the mother plate, added to the second four rows. In this manner, the recovery could be monitored with parasites that were exposed to drug for 48 hours and parasites being continually exposed.
MMV Box Screening

As there were 5 plates to process, each with 80 drug or probe-like compounds to screen, culture was brought to a high parasitemia at 4% hematocrit with estimated need of at least 800 µl of infected RBCs. Parasitemia was assessed, sorbitol-synchronized and dormancy induced as previously described. The MMV original plates were diluted from 0.1 mM in 100% DMSO down to 1 µM in 1% DMSO. Both mother and daughter plates were made with the Biomek robot and in addition to the drugs to be screened, controls of 1 µM and 10 µM Gibberellic acid, 10 µM and 50 µM Fluridone, 80 µl of uninfected and untreated RBCs, 80 µl DMSO-treated iRBCs and 80 µl DHA-treated iRBCs.

80 µl of parasite was added to each well containing the MMV compounds with 10 µl of D-luciferin as well. Plates were read at the 0 and 6-hour time points then incubated at 37° and read each day at the time of original set-up. Following 48 hours after initial exposure, plates were spun and washed three times with RPMI 1640 after which CM was replaced and plates re-incubated and continued to be monitored daily for RLU values indicative of recovery.

This experiment ultimately yielded very few hit compounds so that the experiment was repeated at 2 µM concentration, 2% DMSO. The controls were adjusted to the latter parameter and likewise plates were monitored until recovery was evidenced in the luciferase readings.

Dormancy Induction Screen

To determine if any of the MMV compounds are capable of inducing a dormant response, the plates were processed in the manner described above at 2 µM and 3D7 alone was added. Controls remained the same. The plates were incubated at 37° C for 48 hours, after which
they were washed 3x with RPMI and from each well a slide was made. These slides were then analyzed for activity and categorized as being “hits” if any dormant parasites were present.

**Confirmation Assay**

The 32 compounds that qualified as hits in the aforementioned screen were extracted from the mother plate, diluted to 2 µM once again and added to a 24-well plate containing 500 µl of 3D7 at 2% parasitemia, 4% hematocrit. Plates were allowed to incubate at 37° for 48 hours just as in the previous experiment; upon which they were washed 3x with RPMI and media was replaced. A 90 µl sample was taken at this time for luciferase readings and slides were made daily. At the first sign of recovery, the plates were placed at a tilt, the cells allowed time to settle and the media promptly aspirated and replenished to maintain the 4% hematocrit. Culture and slides were maintained until either recovery or lysis was observed first. The figure below demonstrates the morphology present at the start of the experiment i.e. predominately ring stages at a relatively high parasitemia of 10%, for comparison see Table 2 of the dormant phenotype.

![Figure 1. Morphology of 3D7 Plasmodium falciparum prior to DHA-exposure](image)
RESULTS

Dormancy Induction & Recovery

First it was important to determine at what parasitemia to perform further assays. The surface area of a 96-well plate is less than half a milliliter and can consequently become overwhelmed if parasitemia and/or hematocrit become too high. Tightly synchronized 3D7 parasites were used for each experiment, typically at a 10% or higher parasitemia of exclusively rings. Following exposure to 700 nM DHA for 6 hours, parasites were transferred to a 96-well plate and monitored until recovery.

The optimization assay depicted in Figure 1 illustrates the rapid drop-off of activity that occurs within the first few hours, and continues until the 3rd day where a slight increase was measured via the luciferase reporter. The parasites seem to recover around the 5th day of incubation, and would have continued to grow had the experiment not been stopped on day 8. Background levels, corresponding to that of the negative control uninfected RBCs, were low never much higher than 50 RLU. The parasitemia with the peak rate of recovery was 2% and this is the one chosen for the remainder of assays attempted.

Figure 2 illustrates the second objective of optimization that is ensuring that the controls used are appropriate for observing normal growth and activity with relative luciferase units (RLUs). The growth curve of DMSO demonstrates the stage-specific expression the transfected enzyme is known by. Khan et al validated this association by culturing parasites at the
trophozoite stage, measuring their ATP values narrowly and accompanying this data with hematocytometer counts of parasitemia every two hours during a 10-hour exposure to drugs of varying efficacy (Khan et al., 2012). Changes in ATP recorded in RLUs correlated closely with the morphological changes observed and the time required for recovery. Based on this evidence, one can be confident in the snapshot luciferase activity provides.

Confirmation of 2% parasitemia and 4% hematocrit as being the parameters to use can be seen in Figure 3. The inclusion of the patient isolate 4G was to test its recent luciferase transfection as being stable enough for use and eventually perform all screens on it in addition to the 3D7 laboratory strain. However this did not ultimately prove to be the case so this data is provided solely for the former purpose. It is important to note that 4G recovered a day prior to 3D7 and exhibited a much more consistent expression curve.

**Effect of Gibberellic acid and Fluridone**

Both GA and FLD were used at two different concentrations based on previously performed studies and kept incubated with the previously DHA-induced dormants for 48 hours before being washed out of the plate and replaced with media. Since FLD has inhibitory properties, we would expect to see a delay in 3D7’s emergence from its inactive state while GA being a phytohormone is expected to instead cause the cells to wake before the typical recovery period (Duvalsaint, unpublished).

With both compounds, the 10 µM concentration proved to be the more influential, see figures 4 and 5 respectively. These graphs suggest at these concentrations, GA- and FLD-exposed dormant parasites saw comparably higher rates of recovery and more clearly demonstrated luciferase stage-specific expression. In terms of activity against the DHA-induced
dormant, when compared to a dormant with no other drugs for intervention both acted as expected. Figure 6 clearly shows with respect to normally recovering cells, FLD produced a distinct delay and kept parasites dormant until day 6 while GA caused cells to recrudesce by the second day following removal of drug pressure.

**MMV Malaria Box Inhibitory Screen**

MMV plates were obtained approximately a month prior to use against dormant 3D7. The mother plate of 10 mM in 100% DMSO was shared among members of the Kyle lab and an initial dilution to 100 μM was made so that the plates could be distributed. As is described in the methodology, a primary screen was done at 1 µM but this ultimately did not warrant any worthwhile results. It is possible that this concentration was too low to have any pronounced effect on the non-proliferating cells.

In any case, the 100 μM mother plates were thawed and a sample of each compound was diluted to 2 μM instead. Since the concentration is in a 1:1 association with DMSO, a higher concentration of MMV drug meant higher presence of DMSO at 2%. To offset any potential interference this may cause, the control DMSO was also brought to 2% and monitored closely for any signs indicating that the cells were not growing optimally.

Of the 400 compounds screened, about 20 exhibited activity worthy of scrutiny and of these 3 compounds found to have activity similar to GA and can be seen in Figure 7 and Table 1. All three caused the DHA-induced dormant parasites to wake before the characteristic recovery period and to continue growing normally after the period would typically end. MMV665843, a member of the drug-like set from GlaxoSmithKline had the most powerful response. As can be
seen in Figure 8, it caused dormants to wake much sooner than the DHA average of 4 days and sooner even than the comparatively positive control of GA.

It was difficult to distinguish what parasite sample died in exposure to the compounds versus simply never recovered and in the future, the assay would need redesigning to accommodate this distinction and detect for FLD-like activity.

**Dormancy Screen**

Under the hypothesis that dormancy is an innate response to stress from drug pressure, it stands to reason that compounds other than artemisinin and its derivatives are capable of triggering it. To test this notion, the MMV plates were once again diluted to 2 µM and added to 96-well plates in the same manner previously described. The primary modification was the use of uninduced 3D7 and a 48-hour incubation period. After which, the plates were washed free of all drug and slides were made per each well. These were then categorized morphologically for the presence of dormancy-inducing compounds. Figure 9 shows the distribution of these categorized stages across the 5 plates.

Morphological cues were used to identify which compounds could be inducing dormancy, specifically by comparing its appearance to that found within published data (Grobler et al., 2014). Of the 400 compounds screened, 32 (Appendix, Table 2) were found to have induced what appeared to be dormancy based solely on identification through microscopy and accompanying luciferase data for the first 48 hours. These compounds were then selected for confirmatory testing.
**Confirmation**

In order to validate the previously described results, a confirmation assay was designed to expose ring-stage 3D7 for 48 hours, just as is in the preliminary screen and to monitor their decline and eventual recovery over time. Two 24-well plates were used to assess 32 test compounds, a negative control of uninfected RBCs, a positive control of unexposed iRBCs with 2% DMSO as well as DHA and pyrimethamine (PYR) serving as comparative controls of dormancy induction.

Although the latter has been known to induce a dormant response (Nakazawa, Kanbara, & Aikawa, 1995), it was only recently observed by members of the Kyle lab (Hott, 2015) that PYR causes dormancy in the second cycle of development. In other words, unlike DHA, which affects the ring stage and immediately causes an arrest of growth, PYR does not do so until schizogony has been initiated. A few merozoites may escape this fate but then arrest as rings upon invasion of new RBCs. Its inclusion in this assay is to determine whether the MMV compounds induce dormancy in the first or second stage. However, this did not prove to be valuable information as the parasite used (luciferase-transfected 3D7) by the nature of its transfection is resistant to PYR. For that reason, any speculation regarding at which stage dormancy occurred is just that and may not hold up if the experiment were repeated with another such inducer, such as mefloquine.

Of the 32 compounds, 4 were found to exhibit the highest activity in inducing a dormant response (Table 3). All compounds exhibited the expected growth curves as can be seen in Figure 10. MMV666116, MMV665969 and MMV000787 appear to be causing growth reduction at the 96 HR time point rather than the 48 HR, indicative of a PYR-type targeting. MMV019918 on the other hand behaves more like DHA, inducing 3D7 arrest at the 48 HR mark.
Counting of slides concurrent to the daily plate reading ensured that there were two
different means of discerning the effect of drug on parasite growth and development (See Table
2). The former is additionally conveyed in Figure 11 as a graph of the ratio of normal parasites
(that is ring-stage, trophozoites/schizonts) out of a total of all parasites present in a respective
field. For the sake of simplicity, dead and dormant parasites were pooled together as it is difficult
to distinguish between them on a microscope slide. These values correlated nicely with the
TopCount readings. Of note is MMV019918, which seems to influence parasites to behave
similarly to DHA. Slide counts appear to detect a decline in overall healthy parasites around the
48-hour mark while the other 3 compounds saw this drop around 72 and 96 hours.
Tables and Figures

Figure 2. Optimization Assay: DHA. Exposed 3D7 entered dormant state within 6 hours of initial contact and maintained low metabolic rate until 96 hours post-exposure.
Figure 3. Optimization Assay: DMSO. Exposed 3D7 serves as the positive control and exhibits normal growth as well as stage-specific expression of luciferase enzyme.
Figure 4. Dormancy Recovery Assay: 3D7 and 4G. Both parasites were exposed to 700 nM DHA for 6 hours, 4G was included to test its recent luciferase transfection.
Figure 5. The effect of fluridone (FLD) on DHA-induced dormant ring stages of *P. falciparum* strain 3D7 was examined at 10 µM and 50 µM concentrations.
Figure 6. The effect of gibberellic acid (GA) on DHA-induced dormant ring stages of *P. falciparum* strain 3D7 was examined at 1 µM and 10 µM concentrations.
**Figure 7. Dormancy Recovery: FLD/GA versus DHA-induced dormant** – comparison between GA and FLD at 10 μM exposed to induced dormant 3D7 parasites for 48 hours.
Figure 8. MMV Screen at 2 µM Active Compounds – all three compounds are from the same plate consisting of the drug-like set. All three hail from different libraries, that is St. Jude’s (orange), GSK (black), and Novartis (teal) respectively.
<table>
<thead>
<tr>
<th>HEOS Compound ID</th>
<th>Source</th>
<th>Name</th>
<th>MW</th>
<th>Inhibition @ 5 µM</th>
<th>EC_{50} ChEMBL (µM)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMV665806</td>
<td>St. Jude</td>
<td>1-(2,3-dimethylindol-1-yl)-3-[(2-methylecyclohexyl)amino]propan-2-ol</td>
<td>314.5</td>
<td>96%</td>
<td>0.5273</td>
<td>C_{20}H_{30}N_{2}O</td>
</tr>
<tr>
<td>MMV665843</td>
<td>GNF</td>
<td>1,2,3,4-tetrahydroacridin-9-ol</td>
<td>199.3</td>
<td>82%</td>
<td>0.628</td>
<td>C_{13}H_{13}NO</td>
</tr>
<tr>
<td>MMV665897</td>
<td>GSK</td>
<td>6-methoxy-4-methylquinazolin-2-amine</td>
<td>189.2</td>
<td>63%</td>
<td>0.927</td>
<td>C_{10}H_{11}N_{3}O</td>
</tr>
</tbody>
</table>
Figure 9. MMV Malaria Box Screen Most Active Compound – induction of growth in DHA-induced dormants occurred 24 hours before GA and 48 hours before 3D7 exposed to DHA alone
Figure 10. Morphologically Categorized Results of MMV Phenotype Screen – uninduced parasites were exposed to 2 \( \mu \text{M} \) of each compound of which there were 80 per provided plate for 48 hours then categorized morphologically with microscopy. Categories were unhealthy but growing (red), distinctly normal life stages (green) and distinctly dormant or dead (blue).
Figure 11. MMV Dormancy Screening: Active Compounds with positive control (DHA, blue) and negative (DMSO, orange). All were exposed for 48 hours, MMV compounds at 2 µM, DHA at 700 nM and DMSO at 2%, respectively.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
</tr>
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<td>MMV666116</td>
<td><img src="image1.png" alt="Image" /></td>
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<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>MMV019918</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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<tr>
<td>MMV665969</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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<tr>
<td>MMV000787</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
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</table>

Table 2. Confirmation Assay: MMV compound morphology

3D7 susceptibility to MMV compounds

<table>
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<th>Time (hours)</th>
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<tbody>
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<td>48</td>
</tr>
</tbody>
</table>
Figure 12. Confirmation Assay: Ratio of normal to total parasites with dead and dormants counted together for the sake of simplicity. Parasitemia values could not be calculated as these counts were obtained from thick smears. All were 100% normal at 0 hour, slides taken following 48 hour incubation.
Table 3. MMV Confirmation Assay Active Compounds Provided Information

<table>
<thead>
<tr>
<th>HEOS Compound ID</th>
<th>Source</th>
<th>Name</th>
<th>MW</th>
<th>Inhibition @ 5 µM</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; ChEMBL</th>
<th>Structure</th>
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</thead>
<tbody>
<tr>
<td>MMV666116</td>
<td>GSK</td>
<td>2-[[9-[(2-fluorophenyl)methyl]-2,9-diazaspiro[4,5]decan-2-yl]methyl]phenol</td>
<td>354.5</td>
<td>98</td>
<td>0.679</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>MMV019918</td>
<td>GSK</td>
<td>N-[[5-(4-bromo-2-chlorophenyl)furan-2-yl]methyl]-1-piperidin-4-ylmethanalmine</td>
<td>383.7</td>
<td>96</td>
<td>1.505</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>MMV665969</td>
<td>St. Jude</td>
<td>N-[(5-chloro-8-hydroxyquinolin-7-yl)-(4-methylphenyl)methyl]-3-methoxybenzamide</td>
<td>432.9</td>
<td>97</td>
<td>15</td>
<td>![Structure Image]</td>
</tr>
<tr>
<td>MMV000787</td>
<td>St. Jude</td>
<td>7-[[4-(3-chlorophenyl)piperazin-1-yl]methyl]-5-(propoxymethyl)quinolin-8-ol</td>
<td>425.9</td>
<td>97</td>
<td>1.096</td>
<td>![Structure Image]</td>
</tr>
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</table>
DISCUSSION

Effective malaria treatment is fraught with challenges. Resistance is seemingly around the corner for every previously superlative drug, while being deeply entrenched among those medications that a few decades ago were vital to chemotherapy. For artemisinin, the first signs of resistance were observed in a 2008 study of parasite clearance times following monotherapy with artemesunate. Clearance was slower among patients from the Eastern Thai-Cambodian border than that of patients from the Thai-Myanmar border to the West (Dondorp et al., 2009). Though an alarming fact on its own, when coupled with the proportional increase of such slow-clearing infections from 0.6% in 2001 to 20% in 2010 with rates approaching 42% in Cambodia one must conclude that there is cause for alarm (Flannery et al., 2013). Resistance is not only emerging but also spreading.

Now more than ever we have need of new medicines as well as new approaches for producing those medicines. The link to plant physiology among Plasmodium spp. is basically irrefutable and imperatively needs further investigating. By elucidating essential biosynthetic pathways behind such compounds like abscisic acid, we can find hitherto unknown targets as well as broaden our knowledge of the evasion mechanisms that are at the disposal of our protozoan foes.

Evasion is the primary response of malaria in response to the immune system’s efforts and this tactic appears to have been extended to one of the most powerful drugs we have. Dormancy under drug pressure is technically not treatment failure but evidence of adaptation. If
this is a stepping-stone to something more advanced, only time and future studies will tell. In any case, understanding is needed regarding what compounds can affect parasites in this state and furthermore which compounds may be expected to induce such a state themselves so that their use in monotherapy should be discouraged.

Much of what was covered and tested in this thesis has already been affirmed and established by the work of others. *Plasmodium falciparum* when grown in culture to a high parasitemia consisting exclusively of the early ring stage will enter into a non-proliferating state when exposed to DHA, the timing of which will vary depending on the concentration used. It will wake from this state if unperturbed typically in 96 hours. The addition of gibberellic acid will cause activation a day before the recovery window and addition of the herbicide fluridone will delay activation by usually another day. Together they provide evidence that isoprenoids synthesis occurs within the protozoan and it is intrinsically tied to metabolism and growth.

Hence why these two compounds are under such investigation and why they were used as comparable controls for the screening of potential MMV inhibitors or activators as would be the case. The treatment implications of finding a compound to behave like GA are substantial, though all of this work is preliminary and it would take years to properly adapt and develop a formulation of ACT containing this extra substance to target those parasites which may have escaped the traditional round of treatment by entering dormancy. Pulling these protozoa back into proliferation would allow the combination drug to clear them from the blood before the overall treatment could be called a failure.

In terms of findings, the MMV Malaria Box screen against induced-dormant parasites was fruitful in the discovery of the quite active and most enigmatically titled MMV665843. By another name, 1,2,3,4-tetrahydroacridin-9-ol is a quinolone-derivative that has been screened in
over 800 different assays against a plethora of pathogens including malaria, various different cancer cells, HIV, Trypanosoma brucei and Leishmania spp. to name a few ("PubChem Compound Database; CID=95996," 2005). According to the supporting data given to researchers by MMV, it is a drug-like compound provided by Novartis that meets all criteria of Lipinski’s rule in that it has a low molecular weight, few hydrogen bond acceptors or donors, and an ALogP of 3.29. Making it an orally active compound that should encounter few problems in the pharmacological sense. MMV also reports that 1,2,3,4-tetrahydroacridin-9-ol had an 82% inhibition at 5 μM of the asexual stages of 3D7 in their preliminary activity screening as well as an EC$_{50}$ of 0.628 μM.

The EC$_{50}$ is the representative measure of the concentration that is 50% of the effective dose for the particular drug for which it was calculated. In this case, 0.628 μM is the concentration at which half of the targeted population (P. falciparum infected RBCs) would be expected to have some response following exposure. Since this assay tested the compounds at 2 μM, a concentration 1.5x larger than its EC$_{50}$, it is not altogether surprising as to why it exhibited a strong effect (see Fig. 8).

It should be noted that both 1,2,3,4-tetrahydroacridin-9-ol and its runner-up in terms of activity, MMV665897 had the two lowest percent inhibition values of all of the 20-something compounds from which they were selected. Conversely, all four of the active compounds in the confirmatory assay regarding the induction of dormancy rather than waking from it, had comparable percent inhibitions, which averaged at 97%. They all also had generally much larger molecular weights and two were members of the probe-like set from St. Jude’s, meaning that they did not meet Lipinski’s criteria. The higher inhibition could account for its dormancy induction, 3% of parasites may be surviving the treatment by slowing their growth and then
recrudescent a few days later. In the inhibitory screen against DHA-induced dormants, these compounds did not exhibit activity similar to GA or FLD. Exposed parasites seemed to recover around the same time as unexposed though to a lesser extent (Figure 12).

The same screen was not, however, successful in identifying an MMV compound with an effect similar to that of FLD. Although there were some samples tested that seemed to show such activity, for example MMV396665 (Fig. 13) clearly recovers 24 hours after DHA-induced control parasites but its magnitude could be attributed to background noise so the decision was made to leave it out of the hit compound group. The possible explanation for this outcome would be speculative. It is possible that the FLD dilutions were made improperly or that there was some sort of mistake made in the plating. Mycoplasma contamination could also be a culprit as it was a frequent issue throughout the study but this would not explain why GA-exposed samples behaved as expected. In any case, the RLU values were too low for this to be deemed significant.

Structurally the induction screen hits are incredibly diverse and complex. One is a phenol, one an amine, another an amide and the last a quinoline derivative such as 1,2,3,4-tetrahydroacridin-9-ol. Quinolines are historically among the most significant antimalarial agents having played a central role in shaping the early years of chemotherapy. Drugs belonging to this family are found in the medicine cabinets of tropical clinicians throughout the affected world (Kaur et al., 2015). Due to the widespread resistance to chloroquine, the mechanism of action by which quinolines affect plasmodium, namely by inhibiting hemozoin production so that hemoglobin accumulates in the food vacuole and it being toxic to the parasite leads to its death, has led to sufficient mutations in the former to reduce the efficacy of this process. The solution to this dilemma is the synthesis of hybrids (2015) to which these two MMV drugs may also belong.
The overall implications for the results of this thesis are that compounds can be used to affect parasites in the currently oft-mentioned dormant state. Some, along the lines of GA and 1,2,3,4-tetrahydroacridin-9-ol may be incorporated into the combination therapy so as to ensure as many parasites are cleared as possible. On the other hand, if the mechanism of dormancy is one day elucidated to be similar across *Plasmodium* species these compounds may be used to induce a relapse so as to permanently rid parasites from the liver in *P. vivax* infections. Moreover, the MMV screen proved that compounds other than artemisinin and its derivatives are capable of inducing dormancy despite their vast differences implying that this quiescent state is most likely an adaptive stress-response utilized by the parasites.

Furthermore some of these compounds seem to have induced a dormant phenotype in the second cycle of growth. Despite the lack of comparative control due to parasites being resistant to PYR, the data does seem to indicate as much to be the case. MMV666116 and MMV665969 cause a precipitous drop in ratio of normal to total parasites at the 96-hour mark, with the decline beginning at 72 hours (Fig. 11). This seems to correlate with the activity of PYR found by the Kyle lab (Hott, 2015), arrest of growth occurs among the trophozoites and not rings as with DHA, moreover the second decline at 96 HR could represent merozoites that made it through a complete schizogony only to go dormant once inside new RBCs. Interestingly, the seemingly 2nd-cycle induced dormant state was much shorter than that of the more traditional manifestation exhibited by MMV019918. It is possible that since these compounds follow a different mechanism or at least target a different life stage, *P. falciparum* possess a different method of avoiding them to that used in DHA. Nevertheless, one thing is certain: further study is needed to determine the nature of this group of inhibitors, as well as the screening of larger compound libraries for similar forms of activity.
Figure 13. Results from Inhibitory Screen for Dormancy-inducing Compounds – DHA-induced dormants exposed to 2 µM of each compound for 48 hours did not seem to recover similar to GA or FLD but more like DHA-only exposed parasites.
Figure 14. MMV Screen: Fluridone (FLD)-esque compound. Sample with activity similar to that of FLD, namely delaying recovery by 24 hours, was left out of “hits” category due to RLU values that may not be significant. RBCs served as a negative contro
REFERENCES


BIBLIOGRAPHY


APPENDIX

Table 4. MMV Dormancy Screen Morphology

<table>
<thead>
<tr>
<th>HEOS Compound ID</th>
<th>Source</th>
<th>Set</th>
<th>Morphology</th>
<th>Stage</th>
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<tbody>
<tr>
<td>MMV019406</td>
<td>GSK</td>
<td>probe</td>
<td>difficult to tell, could be ring or dormant</td>
<td></td>
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<tr>
<td>MMV006427</td>
<td>GNF</td>
<td>drug</td>
<td>dormant</td>
<td></td>
</tr>
<tr>
<td>MMV006203</td>
<td>GNF</td>
<td>probe</td>
<td>some dormant, some may be rings</td>
<td></td>
</tr>
</tbody>
</table>

1 Morphology following incubation with each MMV Malaria Box compound at 2 µM concentration for 48 hours. Prototypic dormant phenotype is much smaller than normal ring stages with a shrunken nucleus, almost pyknotic in shape and a tail of condensed cytoplasm.
<p>| MMV665915 | GNF; GSK | drug | rings, top left may be dormant |
| MMV020500 | GSK      | drug | dormants |
| MMV665831 | GNF; GSK | probe| one may be dormant, the other either ring or troph |
| MMV001038 | GNF; St. Jude | drug | may ring or dormant |
| MMV006861 | GNF      | probe | may be ring or dormant |
| MMV000634 | St. Jude | drug | dormant |
| MMV665928 | GNF; GSK | drug | dormant |
| MMV665946 | GSK; St. Jude | drug | dormant |
| MMV665888 | GSK | drug | dormant |
| MMV666067 | GSK | drug | dormant |
| MMV665789 | GNF | drug | may be ring or dormant |
| MMV011944 | GNF; GSK | drug | dormant, some may be rings |
| MMV019780 | GSK      | drug | dormants                  |
| MMV665906 | GSK      | drug | dormant                   |
| MMV666116 | GSK      | drug | may be ring or dormant    |
| MMV006545 | GNF      | drug | dormant                   |
| MMV011795 | GNF; GSK | drug | may be dormant or dead    |</p>
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<tr>
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<th>drug</th>
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