DEVELOPMENT OF A LUCIFERASE-BASED ASSAY TO SCREEN FOR GAMETOCYTE-SPECIFIC ANTIMALARIAL DRUGS

by

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For centuries, Malaria has continued to be one of the most deadly infectious diseases in the world. Almost all of the current antimalarial drugs target the asexual blood stages of the *Plasmodium* parasite responsible for the clinical pathology of malaria, but nearly all have no activity against the mature gametocyte or sexual stage that is responsible for the transmission of the parasite through the mosquito vector. Renewed interest in global eradication of malaria has turned some of the focus on blocking transmission. We have developed transgenic *Plasmodium berghei* that expresses luciferase under the control of gametocyte-specific promoters. Pb920Lux is a transgenic parasite that expresses luciferase in both male and female gametocytes, while Pb610Lux is a transgenic parasite that expresses luciferase in the male gametocyte. Immunofluorescence assay (IFA) shows luciferase is expressed in some parasites and in accordance with a previous study, suggest these may be gametocytes. These transgenic parasites were then used in a luciferase-based drug assay. Seven known antimalarial drugs were used to confirm the validity of the assay. Four of the known drugs had gametocidal activity, while three of the drugs have no gametocidal activity. We first created a dose response using the Pb920Lux and Pb610Lux along with PbGFPLuxcon as our control. From there we calculate the IC_{50} values of these drugs and compared them to the IC_{50} values calculated using PbGFPLuxcon (control).

As expected, the transgenic parasites showed significant gametocidal activity in the four known drugs and no significant activity in the three non-gametocidal drugs. We confirmed this finding by calculating the percentage of parasites and gametocyte by light microscope and compared these to our findings with the luciferase-based assay. Next we analyzed four unknown drugs and found that they contain no gametocidal activity. The public health importance of developing a luciferase-based assay specific for gametocytes is to provide a simple and efficient method of detecting gametocidal drugs in order to prevent the transmission of malaria.

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LIST OF ABBREVIATIONS

Ato, Atovaquone

DAPI, 4',6-diamidino-2-phenylindole

Dhfr-ts, dihydrofolate reductase-thymidylate synthase

DMSO, Dimethyl sulfoxide

DNA, deoxyribonucleic acid

FACS, Fluorescence Activated Cell Sorting

FBS, Fetal Bovine Serum

FDA, Food and Drug Administration

GFP, Green Fluorescent Protein

GP, glycoprotein

HPI, Hours Post Infection

HTS, High Throughput Screening

IACUC, Institutional Animal Care and Use Committee

IC_{50}, Inhibitory Concentration of 50%

IFA, Immunofluorescence Assay

Luc-IAV, Firefly luciferase

Lux, Luciferase

PbHSP70, *Plasmodium berghei* Heat Shock Protein 70

PBS, Phosphate Buffer Solution

PCR, Polymerase Chain Reaction

PyrR, Pyrimethamine resistance gene

RBC, Red Blood Cells

RLU, Relative Light Units

SEM, Standard error of mean

Tet, Tetracycline

1.0 INTRODUCTION

1.1 DESCRIPTION OF THE PROBLEM

Despite major progress to control and prevent malaria through insecticide-impregnated bed nets and combinational drug therapies, an estimated 3.3 billion people remain at risk for contracting malaria [1]. Malaria accounts for an estimated 0.5-2.5 million deaths each year the majority of cases are children in sub-Saharan Africa [2]. Most malaria endemic countries are located along the equator in tropical regions (Fig. 1). The emergence and the spread of resistance to affordable antimalarial drugs have contributed to the sharp increase in malaria-caused mortality [2]. In recent years there has been a renewed interest in finding new and improved ways to eradicate malaria with an increased interest on interrupting the transmission from human host to mosquito vector. This is due in part to a significant increase in the prevalence of gametocytes after the widespread partial use of some antimalarial therapies, long duration of infection, anemia and partially effective immune responses [3]. There is evidence that antimalarial drug resistance spreads because of the greater transmission potential of resistant parasites in the presence of the drug [4]. Although, scientific rationale for targeting the sexual stage of *Plasmodium* is becoming clear, most of the current drugs available targets the asexual blood stage and are eliminated slowly from the body. Therefore, if they are used intensely in malaria endemic areas, a significant proportion of the population will have variable amounts of antimalarial drugs in their

systems making these concentrations act as a selective filter for favoring transmission of drug resistant gametocytes.

1.2 MALARIA

Malaria is a mosquito-borne infectious disease of both humans and animals. The disease is a result of the parasite multiplying within the red blood cells. Symptoms of malaria include chills, fever, headache, malaise, fatigue, and muscular pain. In humans, symptoms can range from mild to severe which includes coma or death. The symptoms can fist appear 10-16 days after an infectious mosquito bite [5]. There are five known species that can infect and be transmitted to humans. The most severe is *Plasmodium falciparum*, while other malarial diseases caused by *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* are generally milder. Unlike *P. falciparum*, *P. vivax* and *P. ovale* can develop into dormant liver stages that can be reactivated after two years (*P. vivax*) and four years (*P. ovale*) of being symptomless [6]. The fifth species called *Plasmodium knowlesi* is a zoonotic disease caused by malaria in macaques. *P. knowlesi* can resemble either *P. falciparum* or *P. malariae* under light microscope and therefore polymerase chain reaction (PCR) is required for confirmation [6].

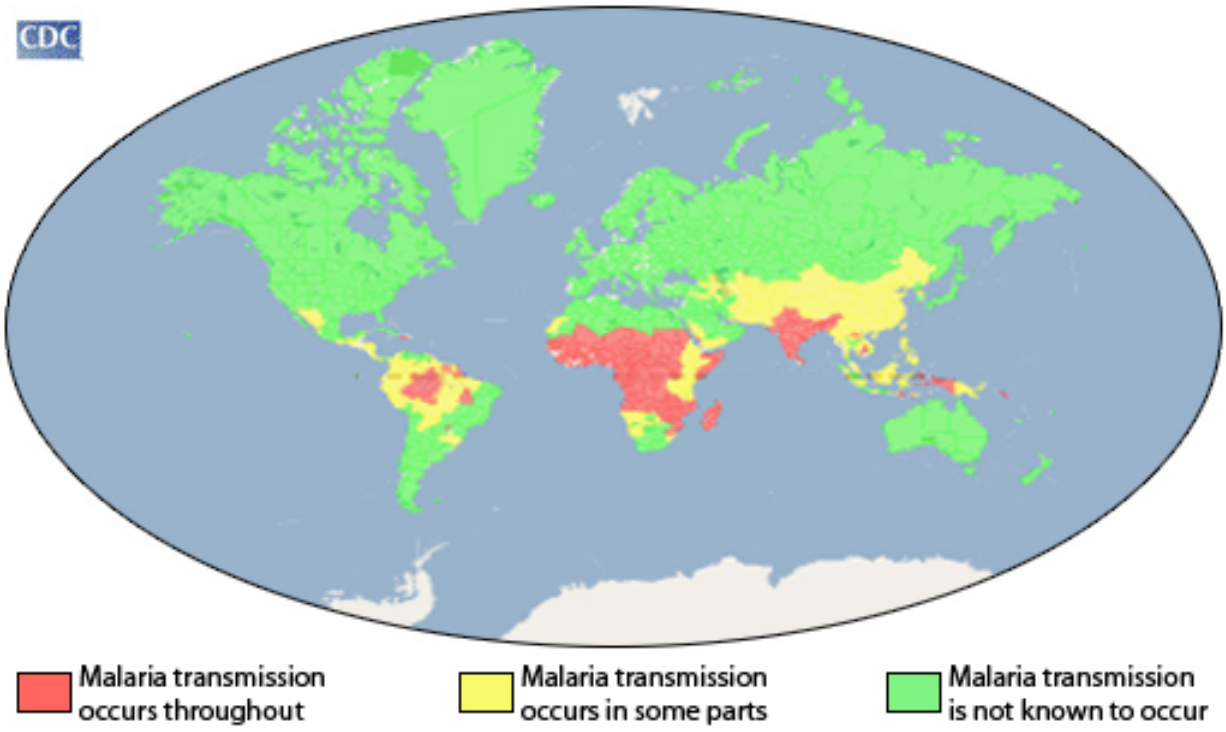


Figure 1. Spatial Distribution of *P. falciparum* Endemicity.

Map of malaria endemic countries with the heaviest rate of infection located mostly in sub-Saharan Africa [7].

1.2.1 *Plasmodium* Lifecycle

Plasmodium spp. has a very complex lifecycle, one in the host and the other in the mosquito vector (Fig. 2). The lifecycle of malaria begins when the mosquito takes a blood meal from the vertebrate host that contains mature gametocytes. The gametocytes then migrate to the mosquito's midgut and become male and female gametes. The gametes then undergo fertilization to create zygotes and ookinetes. The ookinetes migrate to the exterior of the midgut and develop into oocyst and oocysts that contain sporozoites. Once the mosquito takes a blood meal, the sporozoites leave the salivary glands and enter into the blood stream to invade liver cells. Within the liver cells, the parasite undergoes development into trophozoites and then schizonts. This is the liver stage of the parasite. Once the schizonts mature they are released back into the blood stream as merozoites to become blood stage parasites in order to infect red blood cells (RBC). Once they infect RBCs, they go through the process of forming rings, trophozoites, and schizonts. This stage of the lifecycle is also known as the asexual blood stage. Once they develop into schizonts, the parasites can either become merozoites that are released to reinfect more RBCs or enter into the gametocyte stage. This part of the blood stage is known as the sexual blood stage. The gametocytes circulate in the blood in a state of developmental arrest until another mosquito takes a blood meal from the infected person and the cycle begins again.

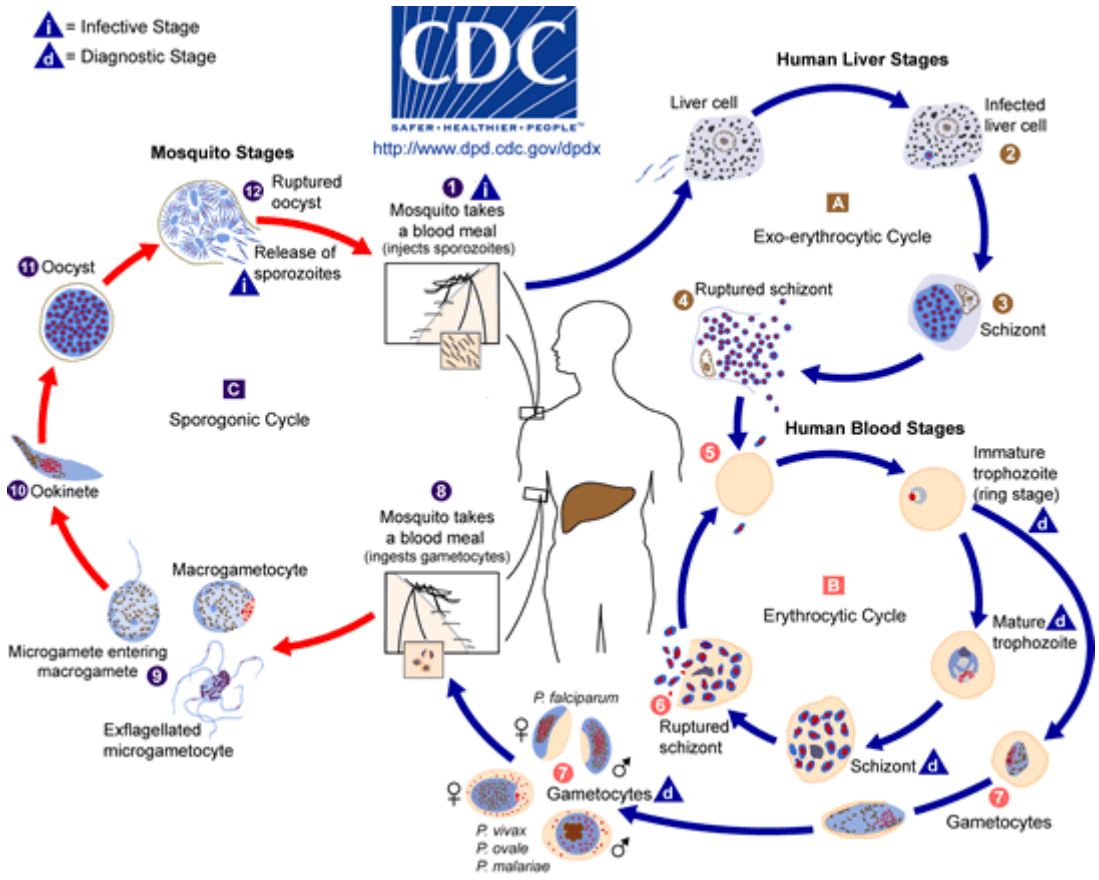


Figure 2. *Plasmodium spp*. lifecycle in human host.

All *Plasmodium spp*. have very similar life cycle, where they both require two stages in order to complete their life cycle. Plasmodium spp. has three main cycles: (A) Exo-erythrocytic and (B) erythrocytic, (C) Sporogonic [8].

1.2.2 Comparison of Rodent and Human Plasmodia

Rodent and human *Plasmodium* has been shown to be analogous in most essential aspects of structure, physiology and lifecycle [9]. The basic biology of *P. berghei* (rodent) and *P. falciparum* (human) is very similar. Rodent parasites have well characterized clones and genetically modified mutant lines, including transgenic parasites expressing reporter genes like luciferase. The manipulation of the whole lifecycle of the rodent parasite is simpler and safer than the human parasite. In addition, their molecular basis of drug-sensitivity and resistance is similar [10]. There are some major advantages to working with rodent parasites. For one, the gametocyte development takes less time from 48 hours - 12 days with *P. falciparum* to 26 - 30 hours with *P. berghei* [10]. In addition to the difference in gametocyte developmental time, the two morphologies are distinct. *P. falciparum* gametocytes take on a banana shape, while in *P. berghei* gametocytes are oval shaped (Fig. 3).

1.2.3 Gametocytogenesis of *P. berghei* and *P. falciparum*

During each asexual cycle of *P. berghei*, a small portion of the parasites halt asexual multiplication and start to differentiate into sexual cells. The haploid macrogametocytes (female) and the microgametocytes (male) are the precursor cells of the female and male gametes. There seems to be evidence that approximately 5-25% of the *P. berghei* parasites commit to sexual differentiation [11]. This somewhat fixed percentage of sexual development is different from *P. falciparum*, where there are periods of purely asexual multiplication that are alternated with periods of gametocyte production [10]. In comparison *P. berghei* has a much shorter period for gametocytes development. During the first 16 hours of development it is

difficult to distinguish gametocytes from asexual trophozoites using a light-microscope or electron-microscope [12]. After 18-22 hours sex-specific gametocytes fill the erythrocyte [13]. Only after 24 hours do the differentiating features of the female and male gametocytes become apparent [13]. There has been some evidence that *P. berghei* commit to sexual development in the trophozoite stage, while *P. falciparum* commitment is during the previous trophozoite cycle [9]. In *P. berghei*, there is no known molecular mechanism that induces and regulates the switch from asexual to sexual, although there seems to be evidence that in both *P. berghei* and *P. falciparum*, environmental factors have an influence [12] Gametocytogenesis has been observed, depending on clinical symptoms [14]. Several studies have identified different potential mechanisms, such as immune stress response, acting on gametocytogenesis through the effects of lymphocytes [15], and hematological disruptions like anemia and lysis of RBC [16-18], all within the dynamics of a *Plasmodium* infection.

Figure 3. *P. berghei* morphology at different blood stages.

Stages shown are from synchronized blood stage infections of the ANKA strain of *P. berghei* at different hours post infection (hpi) [13].

1.3 ANTIMALARIAL DRUGS

Antimalarial therapies play a critical role in the control, prevention and ultimately elimination of malaria. The major problem is that if the current class of drugs loses their effectiveness then control, prevention and elimination may no longer be possible. Most drugs that are used as antimalarial therapies affect the blood stage or asexual stages of *Plasmodium*, while only a few are effective against gametocytes. Although, if treated early they can be effective against the transmission of malaria, but this is not straightforward. For *P. falciparum*, all anti-malarial drugs which kill the asexual stages also kill the early stages of the gametocytes, but are not as effective in the mature gametocytes [4]. Even a 95% reduction in transmission from 500 to 25 infective bites per year won't yield noticeable change in the incidence or prevalence rates; this is also in part due to asymptomatic gametocyte carriers [4]. Asymptomatic gametocyte carriers are people who are infected with gametocytes, but they don't show any malarial symptoms. In addition, a partially effective antimalarial drug not only has decreased activity against the asexual blood stage, it also adds a sufficient amount of stress for the parasite to form drug resistant gametocytes. At least one male gametocyte's progeny (eight microgametes) and one female gametocyte are required in a mosquito blood meal for an infection to occur (approx. 2-3 μL) [4]. Therefore, the gametocyte density of one per μL is well below what can be detected by routine microscopy [19]. Thus the combination of reducing the asexual stage as well as the sexual stage is the best way to reduce transmissibility, but this is not straightforward, due to variations in immunity, pharmacokinetics and pharmacodynamics causing' noticeable capriciousness in transmissibility as it relates to the activity of antimalarial drugs [14]. Three components need to be considered when looking at the effects of antimalarial drugs on transmissibility; a) an activity against asexual stages and early gametocytes, b) activity against mature gametocytes and c)

sporontocidal effects in the mosquito [4]. Table 1 shows a summary of the drugs used in this study that reflects most of the commonly used drugs to treat malaria.

1.3.1 Pyrimethamine

A 4-aminoquinoline folic acid antagonist used to treat acute malaria [20]. It is most commonly used to treat uncomplicated, chloroquine resistant, *P. falciparum*. Pyrimethamine is most effective against erythrocytic schizonts and contains no gametocidal activity [20]. It inhibits the *dihydrofolate reductase* of plasmodia by blocking the biosynthesis of purines and pyrimidines needed for DNA synthesis and cellular multiplication resulting in the failure of nuclear division [20]. Pyrimethamine has been widely used as a monotherapy in mass drug administrations in Asia and South America, which may have been a contributing factor in the spread of pyrimethamine resistance. Mutation in the binding affinity of pyrimethamine to *dihydrofolate reductase* contributes to the widespread resistance. Pyrimethamine is most active by hepatic metabolism and has a half-life of 96 hours. It is considered a slow-acting drug.

1.3.2 Primaquine

This drug is an 8-aminoquinoline that is orally given as a radical cure and preventive for the relapse of *P. vivax* and *P. ovale* [21]. It is one of the oldest of the currently used antimalarial drugs, but also the least well understood. It was first developed to prevent *P. vivax* relapse in U.S. soldiers returning to the United States from World War II and the Korean War [22]. It has also been used to prevent the transmission of *P. falciparum*. Its adverse effects include anemia and gastric intestinal (GI) disturbances [20]. Primaquine is used in combination with

chloroquine for the treatment of all types of malaria. It works by interfering with the parasite's mitochondria, the part responsible for supplying energy [20]. Primaquine not only kills the hepatic form of *P. vivax* and *P. ovale*, it also kills the gametocytes of all types of plasmodia. It is least effective against the asexual blood stage and therefore is always used in conjunction with a schizonticide [23]. Although, its mechanism of action is still not well understood, it is thought to bind or alter the properties of protozoal DNA [23]. Its active metabolites (several have been reported) have been shown to be more potent than primaquine [21]. It has a half-life of 3.7 - 7.4 hours. Primaquine is the only known drug with fast and direct activity against *P. falciparum* gametocytes and high gametocyte activity in all other species [23].

1.3.3 Quinine

It is an alkaloid and active ingredient in extracts derived from the bark of the chinchona tree. It has been in use since 1633 as an antimalarial drug [24]. It is primarily used to treat life-threatening chloroquine-resistant *P. falciparum* malaria. It acts as a blood schizonticide in *P. vivax* and *P. ovale* and a poor gametocytocidal prophylaxis [24]. Due to being a weak base, quinine is concentrated in the food vacuoles of *Plasmodium* [24]. As a schizonticidal therapy, it is less effective and more toxic than chloroquine, making it more effective in the management of severe *P. falciparum* malaria in areas with known chloroquine resistance [24]. The suspected mechanism of action for quinine and other related antimalarial drugs is that they interfere with the parasite's ability to break down and digest hemoglobin [24], ultimately starving as well as building up toxic levels of partially digested hemoglobin within the parasite [24]. Over 80% of quinine is metabolized by the liver into 3-hydroxyquinine. It has a half-life of 18 hours and it is known to cause drug induced thrombocytopenia (DIT). Quinine induces the production of

antibodies against glycoprotein (GP) Ib-IX complex or more rarely the platelet-glycoprotein complex (GP) IIb-IIIa, resulting in the increase in platelet clearance leading to thrombocytopenia [25].

1.3.4 Atovaquone

Atovaquone is a relatively new antimalarial drug that is able to block the cytochrome in the parasite. It acts by affecting mitochondrial electron transport and parallels processes like ATP and pyrimidine biosynthesis [26]. Unfortunately, a single nucleotide mutation in the parasite's *cytochrome b* gene causes drug-resistance [27]. Atovaquone is only used in combination with proguanil (an antimalarial drug that is effective against the asexual blood stage) resulting in a very high cure rate in uncomplicated *P. falciparum* [26]. There has been some evidence of limited metabolism, but no metabolites have been identified [26]. Atovaquone has antimalarial activity against the asexual and sexual blood stages of *Plasmodium*. In addition, this drug prevents the formation of sporozoites by interfering with oocyst development in the mosquito [4]. The unaffordability of this drug to third world countries that need it the most may be one of the reasons for the delay in drug-resistance [27, 28]. Atovaquone has a half-life of 2.2 – 3.2 days due to a presumed entrohepatic cyclin and eventual fecal elimination [26].

1.3.5 Tetracycline

This is a broad spectrum polyketide antibiotic produced by *Streptomyces* genus of Actinobacteria. It is a cyclin antibiotic that targets the apicoplast ribosome, resulting in abnormal cell division [22]. It is normally used as a prophylaxis because of delayed antimalarial

effects on the *apicoplast* gene. If used as a treatment it must be combined with other antimalarial drugs like quinine. Tetracycline has been reported to cause hemolytic anemia, a condition in which red blood cells are prematurely destroyed [29, 30]. Tetracycline is a slow-acting drug that has antimalarial activity against the asexual stage of *Plasmodium*.

1.3.6 Artesunate

Artesunate is a part of the artemisinin group of antimalarial treatments. Artemisinin, also known as Qinghaosu in Chinese, is found in the leaves of *Artemisia annua*. It was one of nearly 5,000 traditional Chinese medicines used to treat malaria and the only one found to be effective. Artesunate is a semi-synthetic derivative of artemisinin. It is used to treat severe malaria in areas where transmission is low. Artesunate is a sesquiterpene lactone containing an unusual peroxide bridge, which is believed to be the mechanism of action [29]. Dihydroartemisinin is the active metabolite of all artemisinin compounds. This category of drugs is known for their fast action in clearing the blood of asexual and sexual parasites. Both of these characteristics allow for the minimization of drug-resistance, but combinational treatment is still highly recommended.

1.3.7 Amodiaquine

Amodiaquine is used for acute malarial attacks in non-immune subjects [31]. It is a 4-amioquinoline that is similar to chloroquine in structure and activity. It has been in use for over 40 years [31]. Amodiaquine has been shown to be just as effective as chloroquine, as well as effective against some chloroquine-resistant strains. The mechanism of action for amodiaquine has yet to be determined. The 4-aminoquinolines can depress cardiac muscle, impair cardiac

conductivity and produce vasodilation resulting in hypotension [32]. Amodiaquine is thought to inhibit heme polymerase activity causing the accumulation of free heme, which is toxic to the parasite [31]. It has been found to be effective against immature gametocytes, but ineffective against mature gametocytes of *P. falciparum* [25]. Hepatic biotransformation of amodiaquine to desethylamodiaquine (principal biologically active metabolite) is the main route of clearance with little escaping untransformed. It has a half-life of 5.2 ± 1.7 minutes and therefore a fast acting drug [31].

Table 1. Summary of Antimalarial Drugs Used in this Study

Drug	Blood Stage Specificity		Use	Resistance	Liver Metabolize
	Asexual	Gametocytes			
Pyrimethamine*	✓	✓	Radical cure and casual prophylaxis	Y	Y
Primaquine	✓	✓	Radical cure and casual prophylaxis	Y	N
Quinine	✓		Radical cure	N	Y
Atovaquone	✓	✓	Radical cure and casual prophylaxis	Y	N
Tetracycline	✓		Radical cure	N	N
Artesusnate	✓	✓	Radical cure	N	Y
Amodiaquine	✓		Radical cure and suppressive prophylaxis	Y	Y

1.3.8 Need for Gametocidal drugs

The need to develop and discover new antimalarial therapies can be overwhelming. The cost of a new drug discovery exceeds $750 million per new chemical compound which adds to the burden that third world countries already face [33]. In addition, the success rate for new chemotherapies to move into clinical trials is extremely low. This is true for all new drugs, but it

is significantly worse for antimalarial therapies with Malarone (GlaxoSmithKline, UK) the newest drug to be approved by the FDA in the last decade [33]. Malarone is a combinational therapy consisting of atovaquone and proguanil hydrochloride [26]. Furthermore, most of the commercially available antimalarial drugs target the asexual stage with very few drugs having gametocidal activity. Currently, primaquine is the only drug that has a direct activity against gametocytes. In order to prevent the transmission of malaria to new hosts, additional antimalarial drugs with gametocidal activity need to be discovered. In order to improve the search for new antimalarial therapies, simple and efficient assays need to be developed.

1.4 DRUG SCREENING ASSAYS

For decades the effectiveness of antimalarial therapies has been measure in vitro by quantifying the parasites uptake of a radioactive substrate as a measure of viability in the presence of the test drug [34]. [^{3}H] Hypoxanthine was the most widely used radiolabel, but in more recent years the switch has been toward fluorescence or luminescence-based assays. While the previous method was both reliable and accurate, the more recent methods have proven to not only be effective, but also simple, efficient and inexpensive. Recent advances in chemical synthesis and identification of potential drug compounds has increased the demand for high-throughput screening (HTS) assays. The trend for HTS assays thus far are based on fluorescence or luminescence detection methods. Although both methods are effective, fluorescent assays can have a higher background because of the strong light emissions, in contrast, luminescent assays are virtually absent of any background noise [2]. In addition, luminescent assays have greater sensitivity in signal detection, making miniaturization of the assay possible [2]. Another way to measure the effects

of antimalarial drugs on asexual and sexual *Plasmodium* is fluorescence activated cell sorting (FACS). FACS is a technique for counting and analyzing cell populations. Although this technique is highly accurate, it can be costly and time consuming. Therefore, developing a luciferase-based assay to screen for antimalarial drugs with gametocidal activity would allow for quick and easy analysis of the drug's effectiveness.

1.4.1 Luciferase-based Antimalarial Assay

Firefly luciferase is the most widely used bioluminescent reporter. This enzyme catalyzes a two-step oxidation reaction to produce light in the green to yellow region or 550 – 570 nm [35]. According to Promega's Bioluminescence Reporter manual, the first step involves the activation of the luciferyl carboxylate by ATP, which yields a reactive mix anhydride. The second step is the activation of the intermediate with oxygen to create a transient dioetane. The dioxetene breaks down to the oxidized products, oxyluciferin and CO2 [35]. Several studies have used luciferase as a reporter to analyze different drug effects on mixed blood stages of *Plasmodium* using both *P. berghei* and *P. falciparum* respectively [36, 37]. There have been no reported studies that were able to analyze the effects of drug compounds throughout the early and late stages of gametocyte development.

2.0 STATEMENT OF THE PROJECT

Antimalarial drug screenings can be time-consuming and complicated. Screenings usually involve the use of whole cell assays to determine the sensitivity of drugs on in vitro growth using *P. falciparum* and/or testing the in vivo sensitivity of selected drugs in small animal models using *P. berghei* [37]. Previous studies mostly focused on the mixed blood stage (gametocytes plus asexual) of *Plasmodium* or just on the asexual stages. We have not found any drug studies that focus on *P. berghei* gametocytes. Our goal is to find a simple and effective way to screen for potentially new drugs that decrease the presence of gametocytes as well as possibly preventing gametocytemia. Gametocytemia is a sensitive indicator of emerging drug resistance due to measurable increase in transmission, and more specifically resistance transmission is seen before there are detectable changes in treatment failure rates [4]. In this study, we created a transgenic *P. berghei* that contains a gametocyte specific promoter attached to a luciferase reporter. We tested the expression of this reporter in comparison with PbGFPLuxcon, a wild-type strain of *P. berghei* that expresses luciferase constitutively throughout its lifecycle, to known drugs with and without gametocidal activity. We hypothesized that antimalarial drugs that have gametocidal activity will have similar IC_{50} values in the transgenic parasites as they do in wild-type, while the IC_{50} value of antimalarial drugs with non-gametocidal activity will be higher in the transgenic parasite as compared to the wild-type. Lastly, we believe that novel

antimalarial drugs can be identified using these transgenic parasites in a larger scale luciferase-based screening. To test these hypotheses, our objectives were:

Specific Aim 1: Construct gametocyte specific luciferase expressing transgenic parasite

Specific Aim 2: Develop and test the sensitivity of a standardized luciferase-based drug assay using the transgenic parasites from AIM 1.

Although there has been great advancement in the study of asexual parasites in *P. falciparum*, the sexual stage has not had such advancement. The slow progression in the study of *P. falciparum* gametocytes is predominately due to the difficulties of differentiating the early stage gametocytes from the asexual as well as obtaining an adequate number of gametocytes [38]. There are a few advanced techniques for developing *P. falciparum* gametocytes, but unfortunately they are difficult to set up, require costly equipment and have a high rate of contamination with bacteria and fungus [39]. Whereas, *P. berghei* has shown a fixed range of gametocytes production, it can be grown in vitro as well as in vivo and it reacts similarly to antimalarial drugs. We believe that this simple and efficient luciferase-based screening will contribute to the finding of new drug compounds that can interfere with the transmission of *Plasmodium*.

3.0 MATERIALS AND METHODS

3.1 CONSTRUCTION OF TRANSGENIC PARASITES

3.1.1 Identify conserved Plasmodia gene promoters

A literature review was performed to find promoters that were exclusively expressed in both gametocyte sexes, as well as expressed in only male and only female gametocytes. It was shown through proteome analysis and confirmed with GFP expression that promoters PBANKA_061920 (PB000198.00.0), PBANKA_130070 (PB000652.01.0) and PBANKA_041610 (PB000791.03.0) are exclusively expressed in both gametocytes, female gametocytes and male gametocytes respectively [40]. PBANKA_061920 is a conserved hypothetical protein gene, PBANKA_130070 is a LCCL domain-containing protein gene, and PBANKA_041610 is a putative chain dynein-phosphatase gene. All three genes are syntenic and have orthologs that are found in *P. falciparum*

3.1.2 Transgenic Parasites

Two different transgenic parasite lines of *P. berghei* are being used in this study, Pb920Lux and Pb610Lux. Both lines express firefly luciferase (Luc-IAV) and have been generated in reference clone of the ANKA strain. The constructs express luciferase under the control of a conserved

hypothetical protein promoter (Pb920Lux) and the putative heavy chain dynein-phosphatase promoter (Pb610Lux). A plasmid construct pL0027 was used, which contain a pyrimethamine-resistant form of the *dihydrofolate reductase* (dhfr-ts) gene of *Toxoplasma gondii* as a drug-selectable marker. The *eef1αa* promoter found in pL0027, which is a constitutive promoter that is active throughout all the blood stages, was exchanged for the promoters PBANKA_06920 and PBANKA_071610. The insert containing the PBANKA_130070 promoter was unstable and therefore was unable to produce a plasmid construct.

The promoters were amplified by PCR using primers for PBANKA_061920 (5' forward: CCACATGTCCCGGGGATATCAATTTTTATAGTTGTTGCAC and 5' Reverse: CGCGGATCCTTTTATATCTGTCTTATTAAGATTC) that produces a 1,067 base pairs (bp) fragment and PBANKA_071610 (5' forward: CCACATGTGATATCTAGTGGAAGTAAAACCGAGC and 5' Reverse: CGCGGATCCTTTTTATCATTTGGATAATTAATTC) that produces a 1,992 bp fragment. The promoter fragments were ligated at the *BamHI* and *EcoRV/Sm*aI site. The constructs were briefly transformed into Hβ10 competent cells (NEB) and identified using the ampicillin resistance gene found on the pL0027. The appropriate plasmids were linearized using the unique *ApaI* restriction site and transfected into PBANKA schizonts using electroporation using Amaxa nucleofactor [41].

The parasites were cultured using Swiss mice under a four-day treatment of pyrimethamine drinking water until parasitemia reached approximately 5%. Afterward, a luciferase assay was performed to check luciferase expression. In addition, the transgenic parasites were sequenced to confirm the insertion of the promoter. Two transgenic lines of Pb610Lux and one of Pb920Lux were further characterized. PbGFPLuxcon (MRA-868) is a

transgenic parasite that constitutively expresses a GFP-luciferase fusion protein under the control of an ef-1αa promoter [42]. This transgenic parasite doesn't contain a pyrimethamine resistance (*pyrR*) gene and was obtained from MR4 (www.MR4.org), a depository for transgenic malaria parasites.

3.1.3 Characterization

The vector was introduced into the blood stage genome by targeted integration of the fragment into the d-ssu-rrna of P. berghei, which is present in the vector. The c-ssu-rrna and d-ssu-rrna are 95% identical [43] and therefore this vector can integrate either into the c- or d-rrna gene unit (Fig. 2). Three predicted integration events in the transgenic parasite line were confirmed by PCR analysis using integration primers: L665, L740, L635, and L739 [43]. L635 (5'-TTTCCCAGTCACGACGTTG) and L739 (5'-TTTGGATATTTTCATATATG) verifies 5' insertion of the vector and L665 (5'-GTTGAAAAATTAAAAAAAAAC) and L740 (5'-CTAAGGTACGCATATCATGG) verifies 3' insertion of the construct. To measure the amount of wild-type, any parasite without the insertion of the construct, primers L739 and L740 of wt c- and d-ssu-rrna were used.

3.1.4 IFA

To confirm that luciferase is being expressed in gametocytes an IFA was performed. After the blood is harvested from the mice, 25 µL in 75uL of media was used and washed twice with

500uL of PBS. The cells are fixed with 0.1% triton X-100 in PBS, then washed twice and blocked with 5% rabbit serum in PBS. RBCs were incubated in PbHSP70 mAb 1:40 and luciferase mAb 1:2000 for 4°C overnight. The cells were then washed twice in PBS and incubated in secondary antibody at 1:400 for 30 minutes. The cells were washed twice and incubated for 5 minutes in 1:2000 DAPI. Lastly, the cells were washed twice and suspended in 500 µL of PBS. Cells were fixed with either ProLong Anti-Fade mounting media (Molecular Probes, OR) or mounting media provided by Center for Biological Imaging (U. of Pittsburgh, PA). Images were taken with Magnifire software.

3.2 LUCIFERASE-BASED DRUG SCREEN

3.2.1 Mice

Swiss Webster female mice, 6 to 8 weeks old at the time of primary infection were used throughout the study. The room temperatures were kept between 22 - 25°C and the mice were fed mouse pellets. During transgenic parasite development, day 2 - 6 post-infection, the mice were given pyrimethamine supplemented drinking water. The drinking water contains 7 mg/mL of pyrimethamine dissolved in DMSO and diluted 100 times with milliQ water. For assay testing, the infected mice were give pyrimethamine supplemented drinking water on days 2 and 3 post-infection. All studies involving laboratory mice were performed in accordance with the IACUC guidelines on animal use and protocol.

3.2.2 Parasitological and Hematological Parameters

Parasitic infections were monitored by thin smears of tail blood. They were methanol-fixed and Giemsa-stained. Parasitemia (P = % of infected erythrocytes) was determined by microscopy of the thin smears. Luciferase activity and assay blood concentrations were kept at 5% hematocrit.

3.2.3 Drugs

Seven known antimalarial drugs were used to determine the sensitivity of the drug assay: pyrimethamine (MP Biomedicals, OH), primaquine (Sigma-Aldrich, MO), quinine (Sigma-Aldrich, MO), atovaquone (Sigma-Aldrich, MO), tetracycline (Sigma-Aldrich, MO), artesunate (Sigma-Aldrich, MO), and amodiaquine (Sigma-Aldrich, MO). Pyrimethamine was dissolved in DMSO to a final stock solution of 10 x 10^3 µM and serial dilutions with complete culture medium were prepared, ranging from 1 - 100µM. Primaquine was dissolved in water to a final concentration of 1 x 10^3 µM and serial dilutions with complete culture medium were prepared, ranging from 1 - 100µM. Quinine was dissolved with 100% ethanol to a final concentration of 100 µM and serial dilutions with complete culture medium were prepared, ranging from 0.1 – 10µM. Atovaquone was dissolved in DMSO to a final stock solution of 40 µM and serial dilutions with complete culture medium were prepared ranging from 5 x 10^{-4} - 5 x 10^{-2}µM. Tetracycline and amodiaquine were dissolved in DMSO and ethanol respectively to a final concentration of 1 x10^4 µM. They were serial diluted with complete culture medium ranging

from 10 – 1 x 10^3 μM. Artesunate was dissolved with DMSO to a final solution of 20 μM and serial dilutions with complete culture medium were prepared, ranging from 0.01 - 1 μM. Unknown drugs L1, L3, and L4 were dissolved with DMSO to a final concentration of 200 μM and L2 to a final concentration of 100 μM. All unknowns were serial diluted with complete culture medium ranging from 0.5 - 50 μM. Complete media was 500 mL of RPMI 1640 with 0.2% sodium bicarbonate, 2 mM L-glutamine, 10% (vol/vol) fetal bovine serum (FBS) and 2.5 mg/mL gentamicin.

Controls for the assay were as follows: complete culture media; 1% DMSO in complete culture media; 10% ethanol in complete culture medium; uninfected erythrocytes; and *P. berghei* infected erythrocytes.

3.2.4 Optimization

To establish the best conditions for the luciferase assay, the following parameters were optimized: parasitemia, hematocrit and temperature. Once parasitemia reached 5%, the blood was harvested using 100 uL of 200 U/ml heparin solution through cardiac puncture. The blood is transported at a temperature around 37°C and kept warm on a slide warmer. Parasitemia at 5% was used with various hematocrits in a total of 100 μl medium and incubated in a 96-well plate. They were incubated at 37°C in 5% CO_2, 5% O_2 and 90% NO_2. After 24 hours, Steady-Glo Luciferase Assay System (Promega, WI) was performed according to the manufacturer's instruction. Briefly, 100 μl of the reagent was added in each well and incubated for five minutes to allow for lysis of erythrocytes, and then read with a luminometer (Veritas). To validate the sensitivity of the assay, the 50% inhibitory concentration (IC_{50}) values were calculated using

SigmaPlot software, version 12.0. The IC_{50} values for each of the drugs from the transgenic parasites were compared to PbGFPLuxcon. Each 96-well plate contained three wells of each control and antimalarial drug dilution at the highest concentration. Images of infected blood with and without drug treatment were taken after 24 and 48 hours incubation under drug pressure. The smears are stained with Giemsa stain and images are taken under a light microscopy using Magnifire software.

3.2.5 Data Analysis

IC_{50} values were calculated using pharmacology in SigmaPlot. Standard curves analysis was done by using a four parameter logistics and a curve fit tolerance of 1×10^{-10}. The IC_{50} was calculated in three independent experiments from each construct. The drugs IC_{50} values from PbGFPLuxcon were used as a control to compare their significance with Pb920Lux and Pb610Lux using GraphPad InStat software. All comparisons had a sample size of three IC_{50} values. Gametocidal drugs IC_{50} values were calculated for significance using a one-sided t-test, while non-gametocidal drugs IC_{50} values were calculated for significance using a two-sided t-test.

4.0 RESULTS

4.1 SPECIFIC AIM 1 RESULTS

4.1.1 Transgenic Parasite line that expresses luciferase

Two new DNA vectors (Pb920Lux and Pb610Lux) were created to integrate the luciferase gene into the *P. berghei* genome. Fig. 4 shows a general schematic representation of pL0027 and the general promoter regions for PBANKA_06920 and PBANKA_041610. This vector contains previously described *dhfr-ts* gene of *T gondii* under the control of the *dhfr-ts* promoter of *P. berghei*. Additionally, transfection of these plasmids would result in transgenic parasites that would express luciferase under the control of PBANKA_06920 and PBANKA_041610 promoters. PBANKA_06920 was chosen since this promoter permits the expression of luciferase in both male and female gametocytes, while PBANKA_041610 permits expression of luciferase only in male gametocytes [40]. Both promoters showed high levels of GFP expression during their previously stated phases [40] and therefore have proven to be great candidates for this luciferase vector. The expression cassettes were cloned in competent cells and selected using their *dhfr-ts* drug selectable marker. Both vectors were sequenced to confirm the ligation of the specific promoters into the luciferase expressing cassette.

They were linearized at a unique *ApaI* site within the *dssurrna* region of the vector (Fig. 4C). The linearized vectors (Pb920Lux and Pb610Lux) were introduced into purified schizonts by electroporation. The *dssurrna* region of the plasmid is complementary to the *c-rrna* or *d-rrna* unit found in the parasites genome. Pyrimethamine resistant parasites were selected and partially cloned. Partially cloned transgenic parasite contains untransfected PBANKA. There was one partial clone for Pb920Lux and six partial clones for Pb610Lux. Out of the six partial clones for Pb610Lux, two were selected to be characterized (Pb610.1Lux and Pb610.2Lux).

The correct insertion of the vectors into the *c/ d-rrna* unit was confirmed by PCR (Fig. 5). All three transgenic parasites were confirmed to be completely integrated into *P. berghei* (Fig. 5A-C).

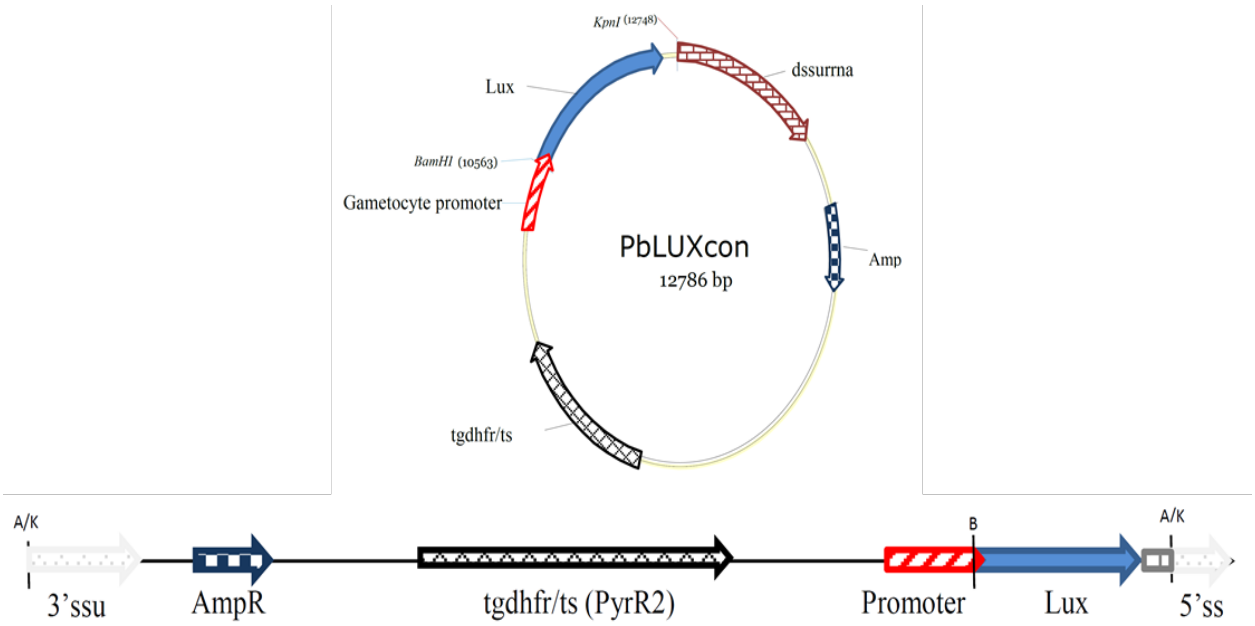


Figure 4. General Schematic of the Structure of PbLuxcon parasite.

A schematic representation of PbLuxcon containing a general promoter site in the location of the desired promoter region for Pb920Lux and Pb610Lux.

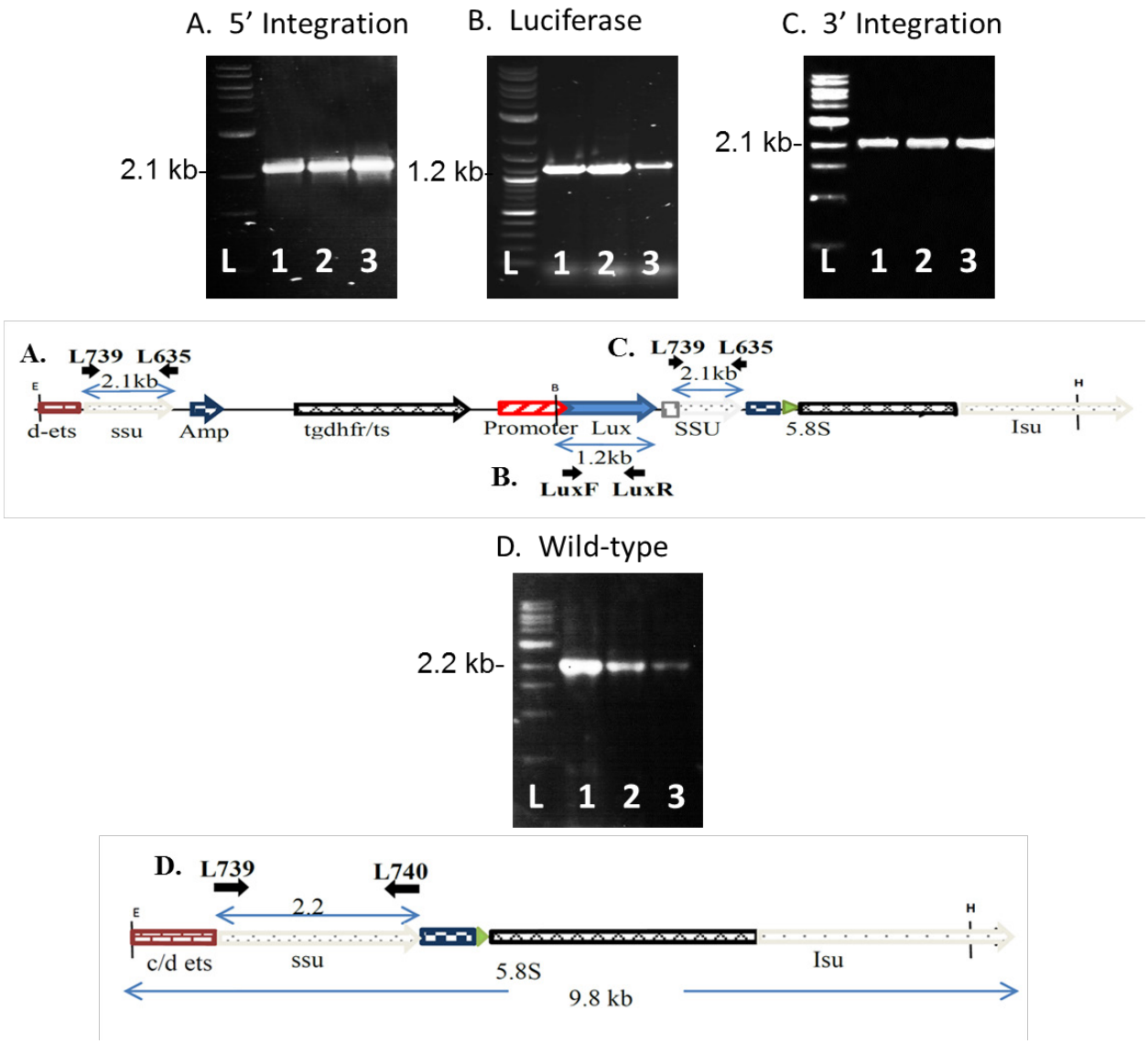


Figure 5. Characterization of Transgenic Parasites.

(A) Verification of the 5' integration using primers L635 and L739. (B) Verification of luciferase gene using LuxF and LuxR primers. (C) Verification of 3' integration using primers L665 and L740. (D) Presence of wild-type PBANKA using primers L739 and L740. Primer size and location (black arrows) are indicated on the vectors. Each lane is as follows: Lane 1- 920Lux; Lane 2- 610.1Lux; Lane 3- 610.2Lux

4.1.2 IFA of Transgenic Parasites

To confirm that luciferase is only present during the gametocyte stage, we performed an Immunofluorescence assay (IFA) (Fig. 6). In figure 6, PbGFPLuxcon is a transgenic parasite that constitutively expresses luciferase throughout the whole lifecycle. It was used as a control to determine luciferase expression within the parasite. All three shows the presence of the nucleus using DAPI (blue) and the presence of the parasites using PbHSP70 (green). Since each parasite contains a single nucleus, the number of parasites within the cell can be enumerated. Next, we confirmed the presence of luciferase. PbGFPLuxcon show the presence of luciferase in multinucleated RBCs that are infected with *P. berghei*, whereas both Pb920Lux and Pb610Lux show the presence of luciferase in a single nucleated RBC. This suggests that Pb920Lux and Pb610Lux may express luciferase in gametocytes. Bright Field images show the number of RBCs within the frame. The limitation of this IFA is that we cannot completely confirm luciferase expression in gametocytes because we are not in possession of gametocyte specific antibody markers.

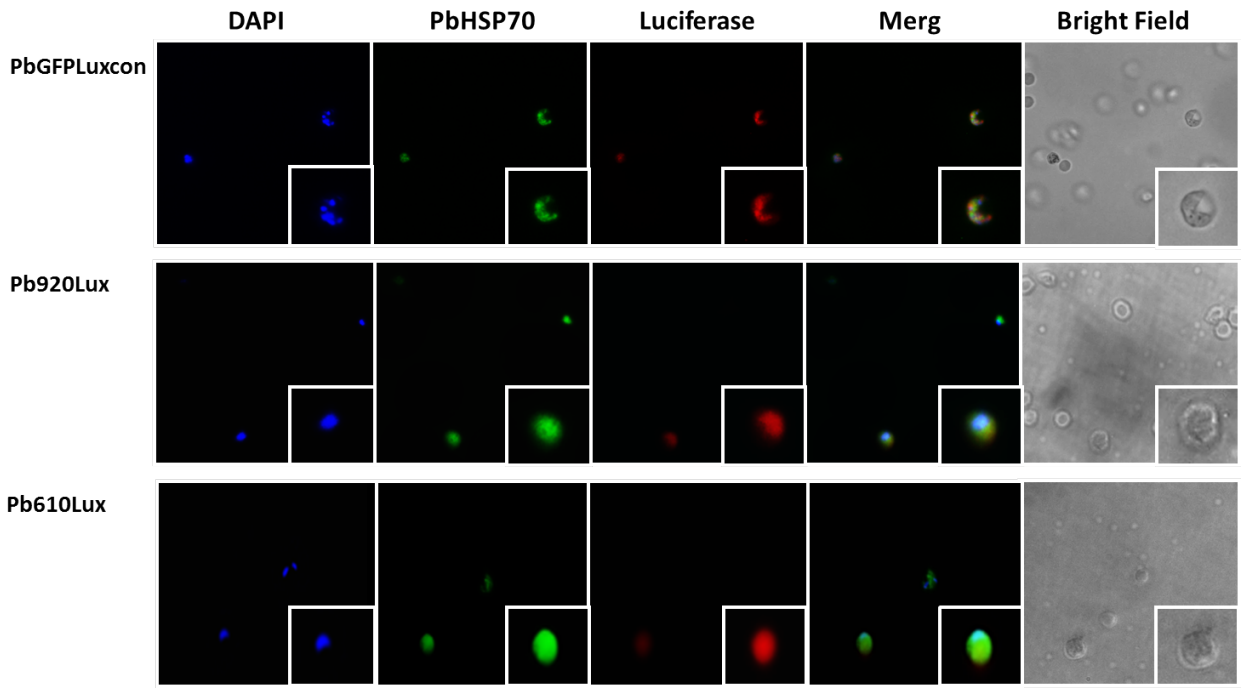


Figure 6. Luciferase Expression in Gametocytes.

(A) PbGFPLuxcon is a transgenic parasite that constitutively expresses luciferase throughout whole lifecycle. (B) Pb920Lux expresses luciferase during the sexual (gametocyte) stage. (C) Pb610Lux expresses luciferase only in male gametocytes. All images were taken at 100X magnification.

4.2 SPECIFIC AIM 2 RESULTS

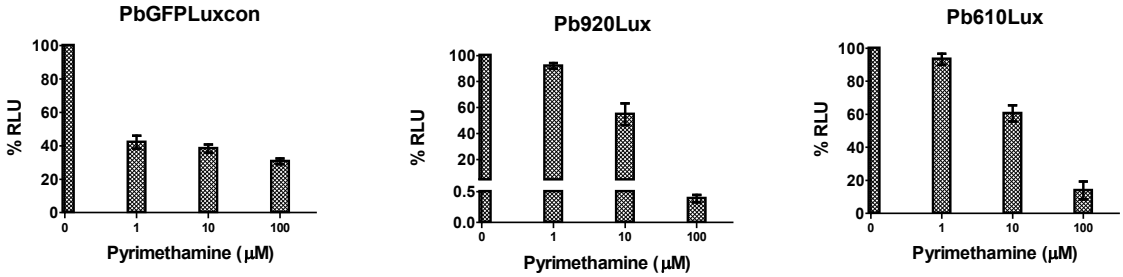
4.2.1 Drug Dose Response and IC_{50} Comparison

Drug response was monitored by measuring luciferase activity of the transgenic parasites after 24 hours in the presence of three different concentrations of seven drugs. We first set out to create an appropriate dose response for each transgenic parasite using the seven selected drugs (Fig. 7A-G). Each transgenic parasite was injected into Swiss mice and allowed to reach a parasitemia of approximately 5%. Once 5% parasitemia was reached, the blood was harvested, plated in a 96-well plate containing all seven drugs at their selected concentrations and incubated for 24 hours. After 24 hours, luciferase activity measurements were taken and a dose response was calculated for each drug.

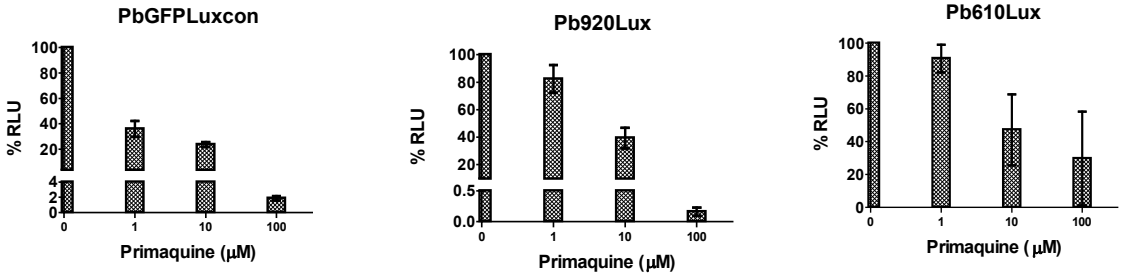
We compared the positive control (PbGFPLuxcon) to what has been previously reported (Table 2). We found that PbGFPLuxcon produced similar IC_{50} drug values as previously reported values. This confirms that our assay is sensitive enough to measure drug effects on mixed blood stages. Next, we compared the IC_{50} drug values from PbGFPLuxcon and compared them to the IC_{50} values from Pb920Lux and Pb610Lux to PbGFPLuxcon (Table 3). **We hypothesized that any known drugs that have an effect on gametocytes will not have a significant different IC_{50} value from our transgenic parasites when compared to PbGFPLuxcon, while known drugs that do not have an effect on gametocytes will have a significant difference in IC_{50} values.** PbGFPLuxcon is a transgenic parasite that constitutively expresses luciferase, but does not contain a pyrimethamine resistant drug marker and is sensitive

to this drug. Whereas, our transgenic parasites do contain a pyrimethamine resistance gene and therefore we expect the difference in IC_{50} values to be significantly different for pyrimethamine. As expected, there was a significant difference of pyrimethamine IC_{50} values between PbGFPLuxcon to Pb920Lux and Pb610Lux (Fig. 8A). Furthermore, we expected to find a significant difference of IC_{50} values of quinine, tetracycline and amodiaquine between our transgenic parasites and PbGFPLuxcon. Although this was proven to be true for tetracycline (Fig. 8E), we found no significant difference of IC_{50} values for quinine and amodiaquine between PbGFPLuxcon and Pb610Lux (Fig. 8C and G). As for antimalarial drugs (primaquine, atovaquone, and artesunate) that do have an effect on gametocytes, as expected there was no significant different in IC_{50} values (Fig. 8B, D and F).

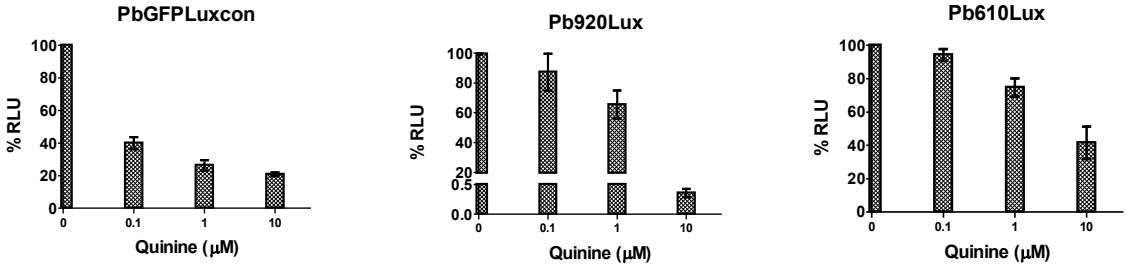
A.


PbGFPLuxcon
Pb920Lux
Pb610Lux
% RLU
Pyrimethamine (μM)

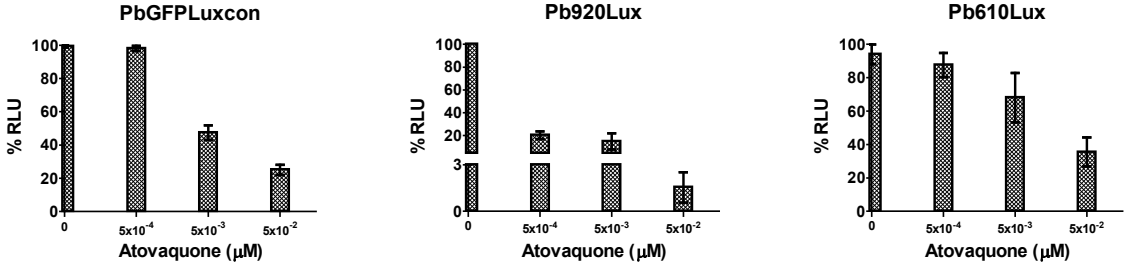
B.


PbGFPLuxcon
Pb920Lux
Pb610Lux
% RLU
Primaquine (μM)

C.


PbGFPLuxcon
Pb920Lux
Pb610Lux
% RLU
Quinine (μM)

D.


PbGFPLuxcon
Pb920Lux
Pb610Lux
% RLU
Atovaquone (μM)

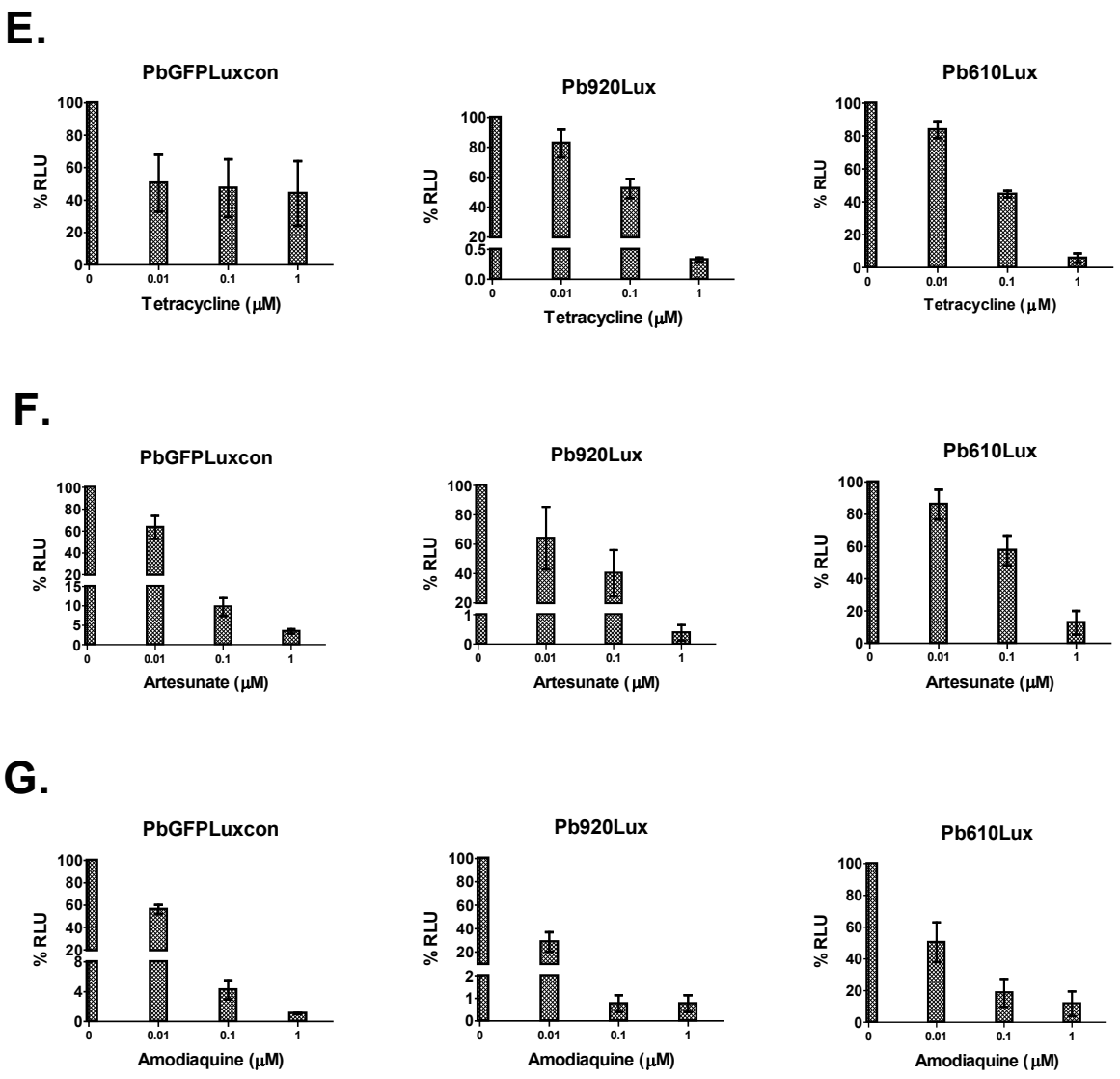


Figure 7. Dose Response of Known Drugs.

(A-G) Known antimalarial drug dose response of PbGFPLuxcon, Pb920Lux and. Error bars are SEM. Each assay was done in triplicates and averaged to create the percentage of RLU.

Table 2. Summary of IC_{50} Values Calculated and Previously Reported

Drug	Calculated IC50 PbGFPLuxcon (μM)	Reported IC50 (μM)	Assay type/ Source (ref. #)
Pyrimethamine*	0.425	0.006	48hr Microtest/ Petersen, 1987 **(ref. #45)**
Primaquine	14.735	15	FACS/ Peaty, 2009 **(ref. #3)**
Quinine	0.03	0.05	FACS/ Peaty, 2009 **(ref. #3)**
Atovaquone	0.004	0.003	FACS/ Peaty, 2009 **(ref. #3)**
Tetracycline	0.17	0.16	In vitro resistance test/ Jelinek, 1995 **(ref. #47)**
Artesusnate	0.014	0.018 +/- 0.002	Luciferase Assay/ Cui, 1995 **(ref. #3)**
Amodiaquine	0.011	0.012	In vitro resistance test/ Randrianairvelojosia, 2002 **(ref. #46)**

Table 3. Summary of IC_{50} Values

Drug	Expected Results	Calclulated IC50 PbGFPLuxcon (µM)	Calculated IC50 PB920Lux (µM)	Calculated IC50 PB610Lux (µM)
Pyrimethamine*	↑	0.425	11.796	15.06
Primaquine	≈	14.735	15.938	11.061
Quinine	↑	0.03	1.5	5.47
Atovaquone	≈	0.004	0.002	0.012
Tetracycline	↑	0.166	172.36	138
Artesunate	≈	0.014	0.087	0.378
Amodiaquine	↑	0.011	5.67	9.3

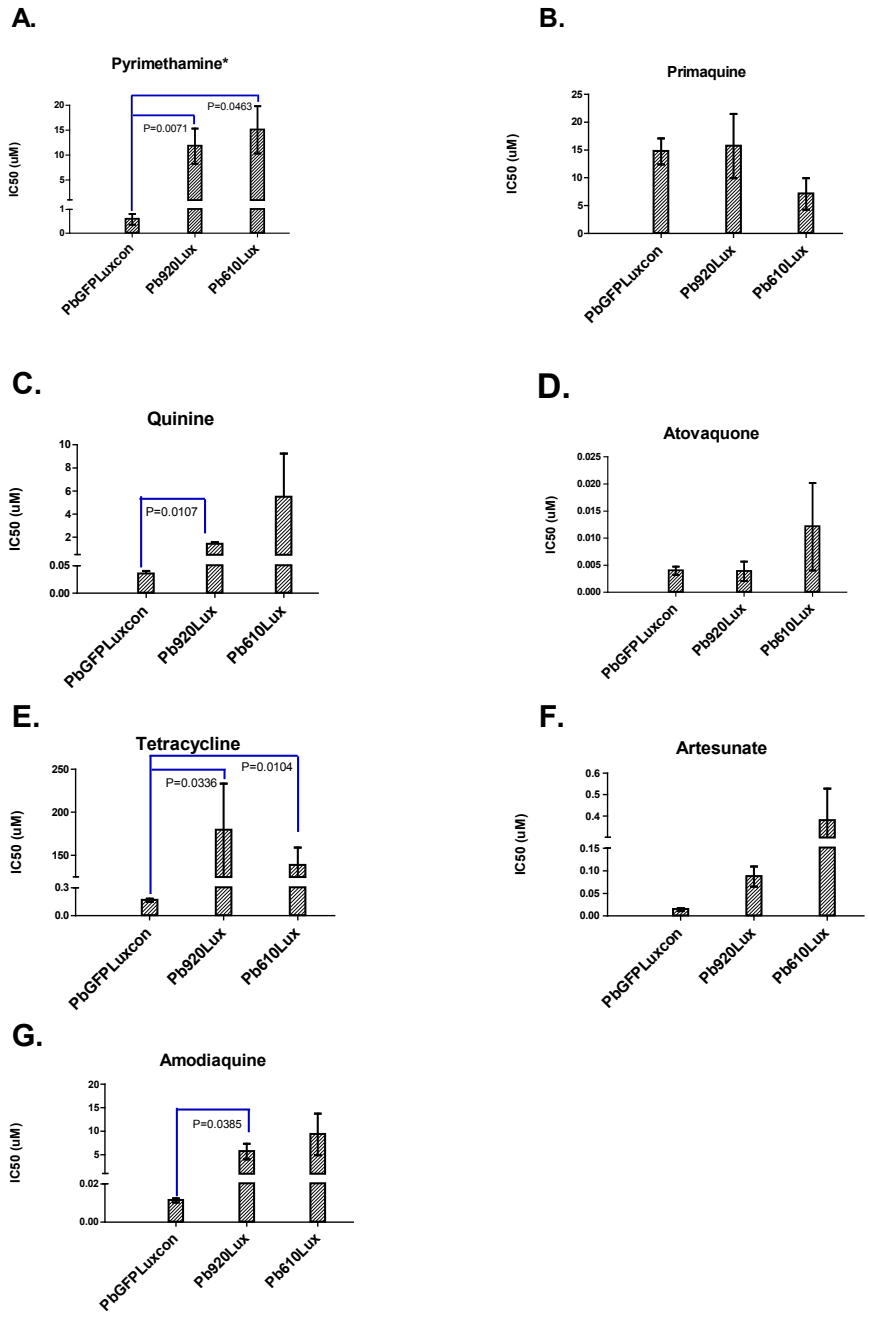


Figure 8. IC_{50} Comparisons of Transgenic Parasites.

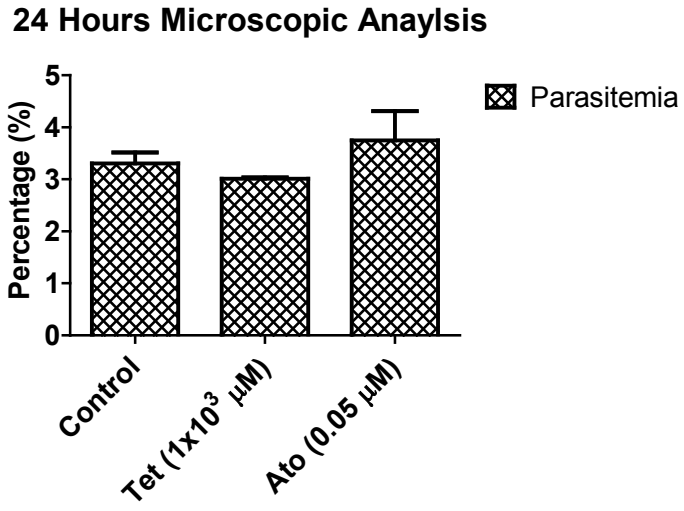
(A-G) IC_{50} values of known antimalarial drugs from PbGFPLuxcon, Pb920Lux and Pb610Lux in. Error bars are SEM. Each IC_{50} was averaged. Pb920Lux and Pb610Lux IC_{50} values compared to PbGFPLuxcon using a t-test.

4.2.2 Microscopic Examination of Known Drug Compounds

In order to further validate the accuracy of this luciferase assay, we calculated the percentage of parasites and gametocytes from infected blood under no drug treatment, tetracycline treatment and atovaquone treatment after 24 hours (Fig. 9). Tetracycline and atovaquone smears were taken at the highest serial concentration of $1x10^3$ μM and 0.05 μM, respectively. After 24 hours, infected blood had approximately 4% parasitemia with approximately 9% of the mixed parasite population being gametocytes. Tetracycline treated infected blood had approximately 3% parasitemia with 9% of those being gametocytes. Atovaquone had 4% parasitemia with approximately 4% of those being gametocytes.

Blood smears were taken at 24 hours and 48 hours (Fig. 10). Infected blood (gametocytes) with no drug treatment at 24 hours looks approximately the same as non-treated infected blood at 48 hours, while infected blood treated with $1x10^3$ μM of tetracycline at 24 and 48 hours shows slightly abnormal gametocytes. The gametocytes have caused the usual enlargement of erythrocytes, but the purplish color of the gametocytes and their nuclei are slightly off color. In addition, there seems to be an abundance of brown granules accumulating within the parasite at both time frames. Infected blood treated with 0.5 μM of atovaquone at 24 and 48 hours shows abnormal gametocytes. At both time frames, the gametocytes has caused the usual enlargement of erythrocytes, but the parasite no longer has the purplish stain shown in the untreated gametocytes. Furthermore, they show the same accumulation of brown granules that were shown the tetracycline treated gametocytes. The nuclei in the atovaquone treated gametocytes were still well defined.

A.



B.

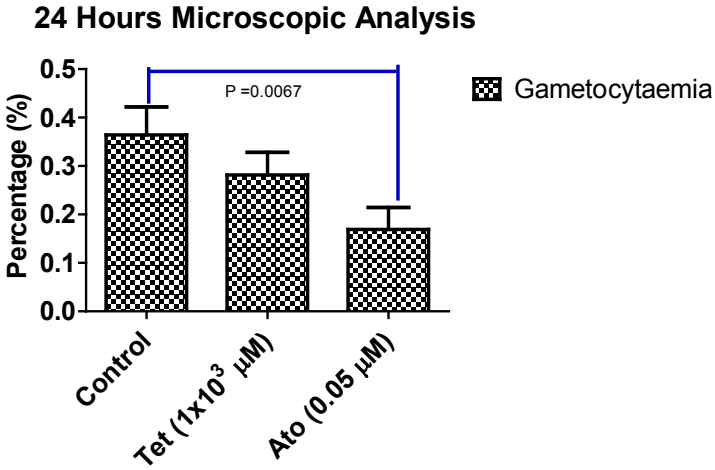


Figure 9. Percentage of Parasites and Gametocytes.

(A and B) Blood smears were taken after 24 hours. (A) The percentage of parasites and (B) gametocytes is their amount over the number of RBC. Both parasitemia and gametocytaemia was microscopically counted. This experiment was done in triplicate and error bars are SEM.

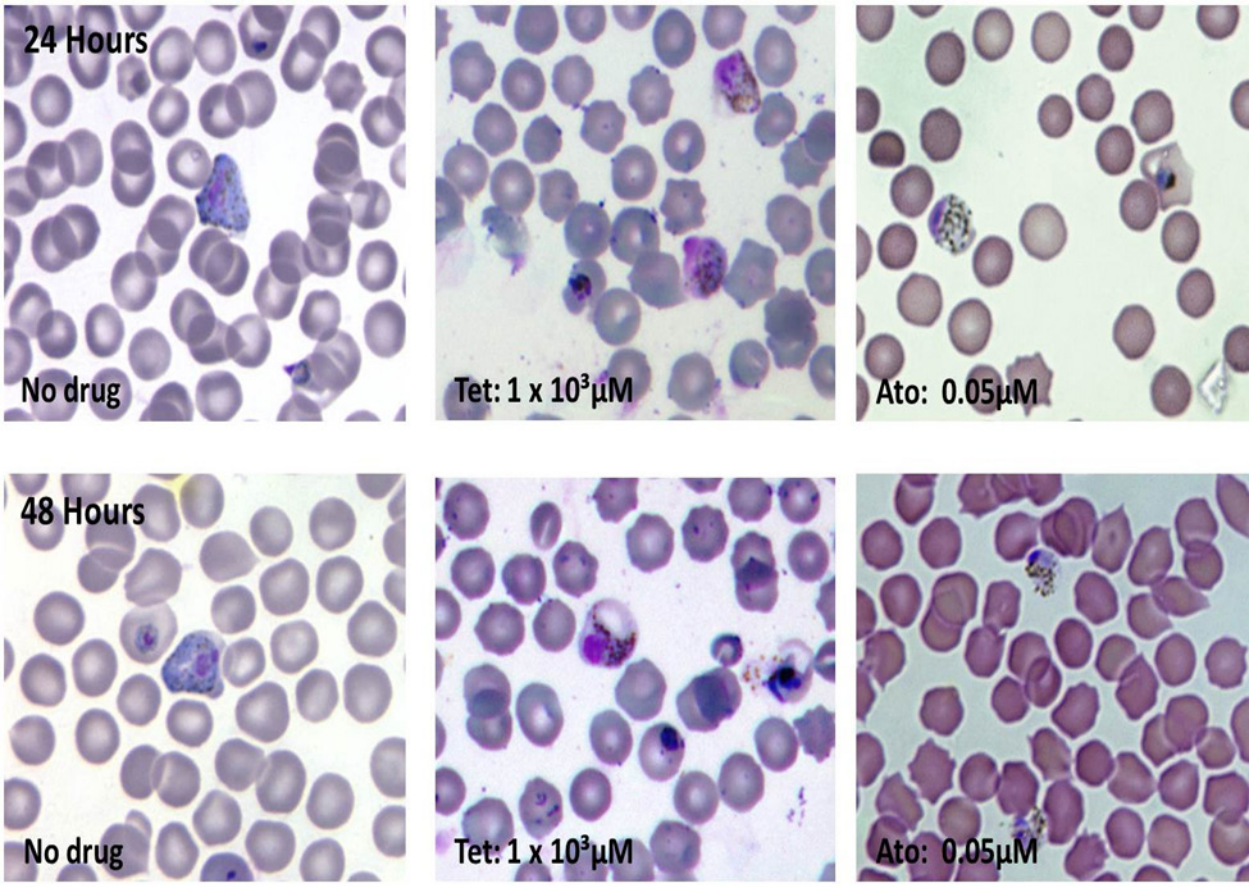


Figure 10. Microscopic Images after 24 hours and 48 hours.

(Left) Microscopic images of infected RBCs after 24 and 48 hours. (Middle) Microscopic images of infected RBCs treated with $1x10^3$ µM of tetracycline after 24 and 48 hours. (Right) Microscopic images of infected RBCs treated with 0.05 µM of atovaquone for 24 and 48 hours. All images were taken at 100x magnification.

4.2.3 Effects of Unknown Drugs Compounds

Once the luciferase assay was validated, we then wanted to do a sample assay on a couple of unknown drugs to see their affect against gametocytes (Fig. 11). The effect of L1 on PbGFPLuxcon, Pb920Lux and Pb610Lux doesn't show a dose response (Fig. 11A). L1 showed a 40 - 50% decrease in PbGFPLuxcon and Pb920Lux, while Pb610Lux had no effect. L2 did show a dose response with PbGFPLuxcon, but no dose response was seen for Pb920Lux and Pb610Lux (Fig. 11B). This drug did cause a 40 - 60% decrease of Pb920Lux, but had no effect on Pb610Lux. L3 showed a minor dose response for PbGFPLuxcon, but there was no dose response for Pb920Lux or Pb610Lux (Fig. 11C). This drug also had no effect on Pb610Lux. L4 on had no effect on any of the transgenic parasites. Interestingly, at 5μM the inhibitory effect of the drug seems to weaken before returning to approximately 40 - 50% inhibition. Furthermore, this drug along with the others had no effect of Pb610Lux.

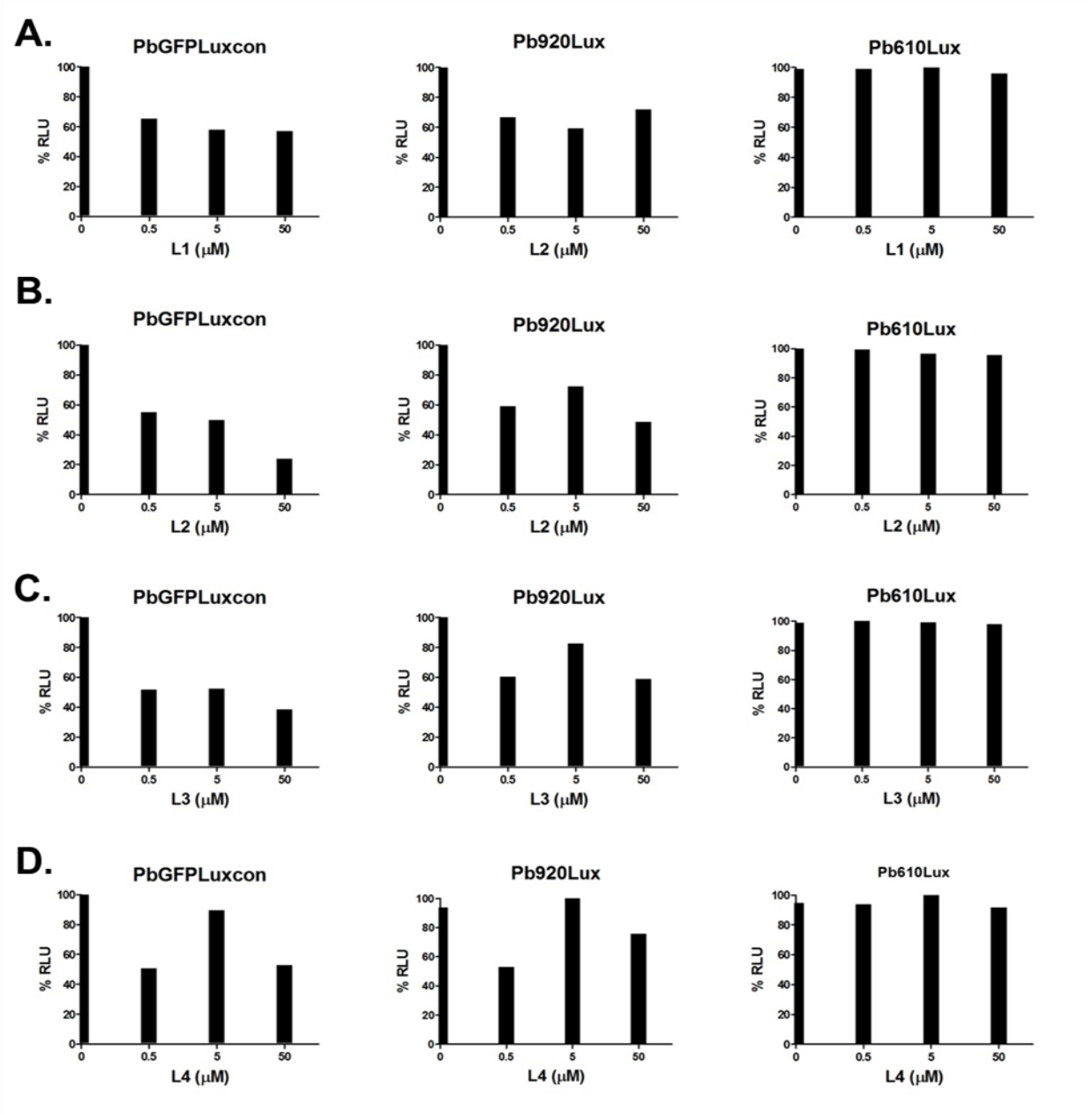


Figure 11. Dose Response of Unknown Drugs.

(A-D) PbGFPLuxcon, Pb920Lux and dose response of unknown drugs (L1-L4). Each assay was done in once.

5.0 DISCUSSION

To our knowledge, luciferase based drug assays are the simple and most efficient way to study the effect of drug compounds on *Plasmodium*, but as drug assay techniques have advanced over the last couple of years the way to analyze the drug effects on gametocytes has not advanced as quickly. The best way to study a drug's effect on gametocytes would be to use the human strain *P. falciparum*; this would come with its own challenges. As previously stated, *P. falciparum* gametocytes take longer to develop as compared to *P. berghei* and over time *in vitro* cultured *P. falciparum* can lose their ability to produce gametocytes [39]. In addition, cultivation systems may be effective at producing viable *P. falciparum* gametocytes, but at a cost of expensive equipment and high rates of contamination. Therefore, the best alternative has been working with *P. berghei* to screen for possible antimalarial drugs. *P. berghei* allows for the study of antimalarial drugs *in vitro* as well as *in vivo* and tends to have the same drug susceptibility as *P. falciparum*.

Transgenic *P. berghei* parasites expressing GFP-Luciferase have been generated before [37]. However, the disadvantage of these transgenic parasites is the inability to quickly and efficiently study the effect of drug candidates on the gametocyte stage of the parasite since both asexual and sexual stages express luciferase. In our study we were able to identify and create two transgenic parasites that sufficiently express luciferase, one in gender mixed gametocytes and the other in the male gametocyte. One advantage is that unlike previous studies using

PbGFPLuxcon [42], we were able to reduce the amount of labor required to do an assay by eliminating the process of separating gametocytes from the asexual blood stage. This allowed us to use these transgenic parasites for a simple and efficient luciferase-based drug assay. Another advantage of our transgenic parasites is that not only can we study the overall effects of drugs on gametocytes, but we can also distinguish their effect on the male gametocyte.

Creating transgenic parasites that expresses luciferase in gametocytes has allowed us to develop and standardize a luciferase-based drug assay using seven known antimalarial drugs. Four of the seven drugs had a known effect on gametocytes, while the other three had no known effect. In order to confirm the optimization of our assay, we tested the antimalarial drugs IC_{50} values from PbGFPLuxcon to those previously reported. We found that our calculated drug IC_{50} values were similar to those previously reported and therefore confirmed the optimization of our assay (Table 2). Next, we tested the effects of the seven known antimalarial drugs using our transgenic parasites. We hypothesized that using our transgenic parasites; gametocidal drugs would show a similar IC_{50} when used with PbGFPLuxcon (control), while those with no known gametocidal activity would show a significantly higher IC_{50} (Table 3). We found that pyrimethamine and tetracycline IC_{50} values were both significant for Pb920Lux and Pb610Lux, while quinine and amodiaquine IC_{50} values were only significant for Pb920Lux (Table 4). This may be explained by many different factors which include the possibility that Pb610Lux may be more sensitive to these drugs. This cannot be confirmed without creating a transgenic parasite that expresses luciferase in the female gametocyte. As expected for the antimalarial drugs that have gametocidal activity (primaquine, atovaquone, and artesunate), there was no significant difference in their IC_{50} values (Table 4). Table 4 is the comparative summary of Pb920Lux and Pb610Lux to PbGFPLuxcon.

Table 4. Comparative Summary of Transgenic Parasites

Drug	Expected Results	PbGFPLuxcon/ Pb920Lux (P-value)	PbGFPLuxcon/ Pb610Lux (P-value)	Significance
Pyrimethamine*		0.0072	0.0463	**Y**
Primaquine		0.853	0.1057	**N**
Quinine		0.0107	0.143	**Y/N**
Atovaquone		0.395	0.4964	**N**
Tetracycline		0.0336	0.0104	**Y**
Artesunate		0.0836	0.0721	**N**
Amodiaquine		0.0385	0.0852	**Y/N**

Next, we wanted to test the sensitivity of our luciferase-based drug assay microscopically. Microscopic analysis of the effect of a drug on *Plasmodium* has been the gold standard and therefore we wanted to confirm our results with what we can see microscopically. Our results gave us approximately 4% parasitemia for non-treated infected blood, which was similar to that of tetracycline and atovaquone. While the percentage of gametocytes in the infected blood and tetracycline were similar, there was a marked difference with atovaquone. In addition, we noticed that the gametocytes in the non-treated infected blood looked significantly different from those treated with tetracycline and atovaquone. Both drugs show transgenic parasites had an accumulation of brown granules; in addition atovaquone treated gametocytes completely lost their purplish stain. Therefore, we extended the drug treatment for 48 hours to see if there was significant affect. Although we did not see any difference in the blood smears that were taken at 24 and 48 hours, we did see the same differences of the drug treated gametocytes from the untreated. This may be explained by the fact that tetracycline causes abnormal cell division [30] and although tetracycline doesn't have a significant effect on gametocytes it is still causing an effect that may be a stress response. Whereas, atovaquone does have an effect on gametocytes electron transport and therefore the parasite may look relatively normal, it is no longer functioning properly. Thus, the gold standard may be counting parasites by light microscopy; a potential problem with this is the inability of distinguishing dead or dying parasites from healthy one as well as distinguishing immature gametocytes from mature ones.

After we validated our luciferase-based assay, we then wanted to test it on a set of unknown drugs. These drugs were used in a previous study to analyze their effects on different *Plasmodium spp.* [44]. Although the identities of the drug compounds are unknown, we do know that they are fatty acids derivatives that are extremely unstable and were in limited supply

within our laboratory. There have been a few reports that have shown fatty acids displaying antimalarial activity [44-47]. These particular unknown are isometric C_{16} acetylenic fatty acids, whose mechanism of action is suspected to be related to the position of the triple bond in a C_{16} acyl chain [44]. We wanted to see if there was any gametocidal activity among these four unknown drugs and therefore we set up serial dilutions to get a dose response. Due to their instability, we were only able to set on a single assay for all three parasites. Our conclusion is that none of these drugs show any gametocidal activity, although to truly confirm, these experiments would have to be done at least two more times.

The renewed interest in stopping the transmission of malaria is not only a noble goal, it is probable, but until a vaccine is created, the next best thing is to find additional antimalarial drugs. In order to do that, we needed to develop simple and efficient methods for screening large library of drug compounds. In this study we used firefly luciferase; the advantage of our luciferase-based assay is the low background noise, which allows for easy analysis of the samples. The protocol was designed to involve the least amount a steps while giving optimal results. By creating a transgenic parasite line that expresses luciferase in gametocytes, we have reduced the amount of labor it takes to purify gametocytes for antimalarial testing. Additionally, by creating a luciferase-based assay there was no need to filter out any background noise because unlike fluorescence, photons are not required to be in an excited state and therefore does not produce an inherent background when measurements are taken.

A limitation of working with *P. berghei* parasites is the inability to synchronize the infection; although we did not detect many discrepancies when comparing the known antimalarial drugs' IC_{50} values from our transgenic parasites to PbGFPLuxcon, this may explain the various degrees of significance (Table 4). Another limitation of our luciferase-based assay is

that were not able to test its sensitivity with a transgenic parasite that expresses luciferase in female gametocytes. This would have allowed us to compare our results with the luciferase expressed in male gametocyte to those in female gametocytes. This would allow us to confirm or deny the sensitivity of male gametocytes to the known drugs. The importance is one gender may be more sensitive to a drug and therefore, even if you don't see a significant difference in gametocytes drug sensitivity overall, transmission can still effectively be inhibited. Although, a previous study affirms that the promoters we attached to the luciferase reporter is expressed in mixed gametocytes and male gametocytes [40], we would have to confirm those findings with a mixed gametocyte and a male gametocyte antibody marker. An additional limitation of our assay is most antimalarial drugs must be metabolized in the liver to allow for maximal effect, while our *in vitro* model only results in limited drug effects. Even with those disadvantages we believe, our luciferase-based drug assays is one of the best and easy methods to screen for antimalarial drugs. Luciferase-based assays are not only quick and easy, they also produce virtually no background noise, this allows for a sample to be using in a high-throughput screening, which is normally done in a 384-well plate.

We have shown that these transgenic parasites that express luciferase in gametocytes can be using in a gametocyte-specific drug-screening assay. Although, we only tested this with a small sample of drug compounds, future studies would include decreasing the sample volume to allow for use in a 384-well plates and testing substantially more drug compounds. These transgenic parasites can also be used for screening drugs with gametocidal activity in in vivo infections in rodents. In addition, creating a transgenic parasite that expresses luciferase in female gametocytes would allow for better comparison of different drugs effects on sex-specific gametocytes. Furthermore, future studies would include fusing GFP to luciferase, to aid in the

analysis of gametocytes. The significance of this luciferase-based assay is that this is a quick and easy method to detect compounds that have an antimalarial effect not only in gametocytes, but more specifically in male gametocytes. In this study we used unknown fatty acid compounds and although we were unable to detect any gametocidal activity, this assay can be used to screen various unknown compounds. Lastly, this assay seems to detect gametocytes in the early as well as the late stages and therefore can detect the effects of gametocidal drugs in earlier stages, which is something that has not been done before using a luciferase assay. By providing a simple and sensitive method for screening gametocidal drugs, this can add to the broad efforts of the eradication of malarial worldwide.

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