OPTIMIZATION OF ANTIMALARIAL DRUG REGIMENS IN A CYTOCDIAL MURINE MALARIA MODEL

by

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ABSTRACT

Statement of Problem: Although malaria is a preventable and curable disease, over 200 million cases and 400,000 deaths were reported in 2016, with the burden of disease borne predominantly by young African children. Substantial headway has been made against this deadly disease in the last decade; however, drug resistant parasites continue to pose a challenge to the progress. We hypothesized that the efficacy of antimalarial drugs, both current and pipeline, could be improved by optimizing the treatment regimen including factors such as dose, dosing schedule, and treatment duration.

Methods: We adapted a transgenic *Plasmodium berghei* GFP-luciferase reporter parasite to develop a cytocidal murine model of malaria with quantification of parasites per μL in the range of 1,200,000 – 600. This cytocidal model was used to study the efficacy of antimalarial drug regimens. This model is superior to the prevalent suppression assays as it more accurately mimics treatment of human malaria in the field. We also evaluated the utility of an artesunate-resistant *P. berghei SANA* as a model for evaluating the efficacy of antimalarials against *P. falciparum* isolates exhibiting delayed clearance to the artemisinins.

Results: Extending the duration of the artemisinins from 3 days to 6 days enhances the efficacy of artemisinin combination therapies suggesting multiple lifecycle drug exposure performs better than single cell cycle exposure. Increasing the dosing frequency of fosmidomycin and clindamycin from twice daily to three or four times daily enhances their efficacy, which is indicative of a time above minimum inhibition concentration

killing pattern. *P. berghei* SANA shows promise as a model of the drug sensitivity of artemisinin tolerant parasites.

Conclusions: Successful human drug regimens require three key considerations: the dose of the drug, the schedule or frequency with which the drug is dosed each day, and the duration of daily treatment. This work demonstrates that there is substantial room to improve the efficacy of antimalarial drug regimens. Developing a novel murine model of artemisinin-tolerant *Plasmodium* parasites will further accelerate the development of next-generation antimalarials and serve as a platform on which currently approved antimalarials can be evaluated and improved.

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INTRODUCTION

This thesis describes several studies of antimalarial drugs in a rodent malaria model and efforts to understand how drug efficacy may be enhanced or more clearly understood. Critical to understanding the experimental rationale are five aspects of malaria. The epidemiology provides a scope of the public health problem caused by the malaria parasite. The life-cycle details the morphological and metabolic changes that the parasite undergoes, which are crucial factors in drug susceptibility. A brief mention of the progress to date on malaria vaccine development contextualizes the role of antimalarial drugs as prevention and treatment strategies. An overview of the antimalarial drugs classes and their targets provides an understanding of the current treatment landscape. Lastly, factors affecting drug efficacy are discussed to broaden the understanding of requirements for successful treatment and resistance prevention. Experimental objectives and key questions for the thesis are then outlined.

EPIDEMIOLOGY

Human malaria is caused by five different species in the genus *Plasmodium*: *falciparum, ovale, vivax, malariae,* and *knowlesi. P. falciparum* and *P. vivax* are the most important species of human malaria parasites. *P. falciparum* is responsible for 96% of all malaria cases while *P. vivax* is the most geographically widespread and is the prominent *Plasmodium* species in the Americas (1). Each species has unique morphological and clinical characteristics, but it is important to recognize the presence or absence of the hypnozoite, the dormant liver stage that is responsible for the relapsing infections that can occur up to several months following treatment. The hypnozoite stage is only present in

P. vivax and *P. ovale*. This has implications for the selection of an appropriate drug treatment as infections with *P. vivax* or *P. ovale* require additional or alternative treatment to kill the hypnozoite. This thesis is primarily concerned with *P. falciparum* infections and using the rodent malaria parasite, *P. berghei*, as a model.

Humans have a longstanding history with malaria, the most significant protozoan parasitic disease worldwide. The intermittent fevers of malaria have been described in ancient texts by Hippocrates c. 500 B.C and in Chinese texts c. 1000 B.C. (2). DNA from the most lethal malaria parasite, *P. falciparum,* was isolated from bone tissue of ancient Egyptian mummies (c. 2000 B.C.) (3). Evidence for the co-evolution of humans and malaria and the strong selective force exerted by this parasite is found, most strikingly, in the genetic makeup of those with hemoglobinopathies (4). Individuals heterozygous for hemoglobin S (HbS) do not suffer from sickle cell anemia and have a substantially reduced risk of experiencing severe malaria (5). In Sub-Saharan Africa, where most malaria cases occur, the HbS allele has been reported in up to 15% of individuals. By contrast, the HbS allele frequency in Western Europe and North America was found to be less than 1% (6). Similarly, reduced expression of the α- or β-globin alleles of hemoglobin are known to protect against severe malaria and co-localize with malaria endemic regions. The thalassemias have a 10-20% prevalence in Sub-Saharan African and the Mediterranean versus 1.5% worldwide (7, 8).

Recently, great strides have been made in controlling malaria-induced mortality worldwide. In 2000, there were approximately 223 million cases of malaria resulting in 985,000 deaths (9). The most recent data from the World Health Organization indicated there were 216 million cases of malaria resulting in 445,000 deaths in 2016 (1). The

number of cases has largely remained constant despite significant population growth, but the number of deaths has been reduced by approximately 50%. Accounting forthis downward trend in deaths are the increased distribution of insecticide treated bed nets, implementation of rapid diagnostic tests, and provision of the highly efficacious artemisinin combination therapy (ACT), which replaced chloroquine, which was rendered ineffective due to parasite drug resistance. There are three key terms necessary for understanding the progress made against malaria: control, elimination, and eradication. Control aims to reduce the malaria case incidence and associated mortality, but requires continued efforts to maintain such advances. Elimination refers to interruption of local transmission in a defined geographic area. Eradication requires the incidence of malaria to be permanently reduced to zero, therefore eliminating the need for further interventions (10). Over 100 countries have already eliminated malaria and 35 countries are currently classified as "eliminating malaria" by the World Health Organization. Once a country has experienced zero locally acquired cases of malaria, it is certified as malaria-free (10). There are 91 countries with ongoing malaria transmission and, as can be seen in Figure 1.1, the majority of these countries are in Africa or lie along the equator (1, 11). The geographic distribution of malaria is largely driven by the suitability of the habitat for the *Anopheles* mosquito vector. Over 400 species of *Anopheles* mosquitoes exist, but only 41 are thought to be the dominant vector species that contribute significantly to the public health problem of malaria (10). Distribution of bed nets treated with long-lasting insecticides and indoor residual spraying of insecticides are key components of malaria control efforts. However, the spread of insecticide

resistance and changes in mosquito foraging behavior continue to present challenges for entomologists and public health officials (12).

Important to highlight is that the majority of malaria associated morbidity and mortality occur in low and middle income countries (LMIC). Beyond the direct costs associated with malaria illness, diagnosis, and treatment, economic development is hindered by decreasing the productivity of human capital and placing strain on already burdened healthcare systems.

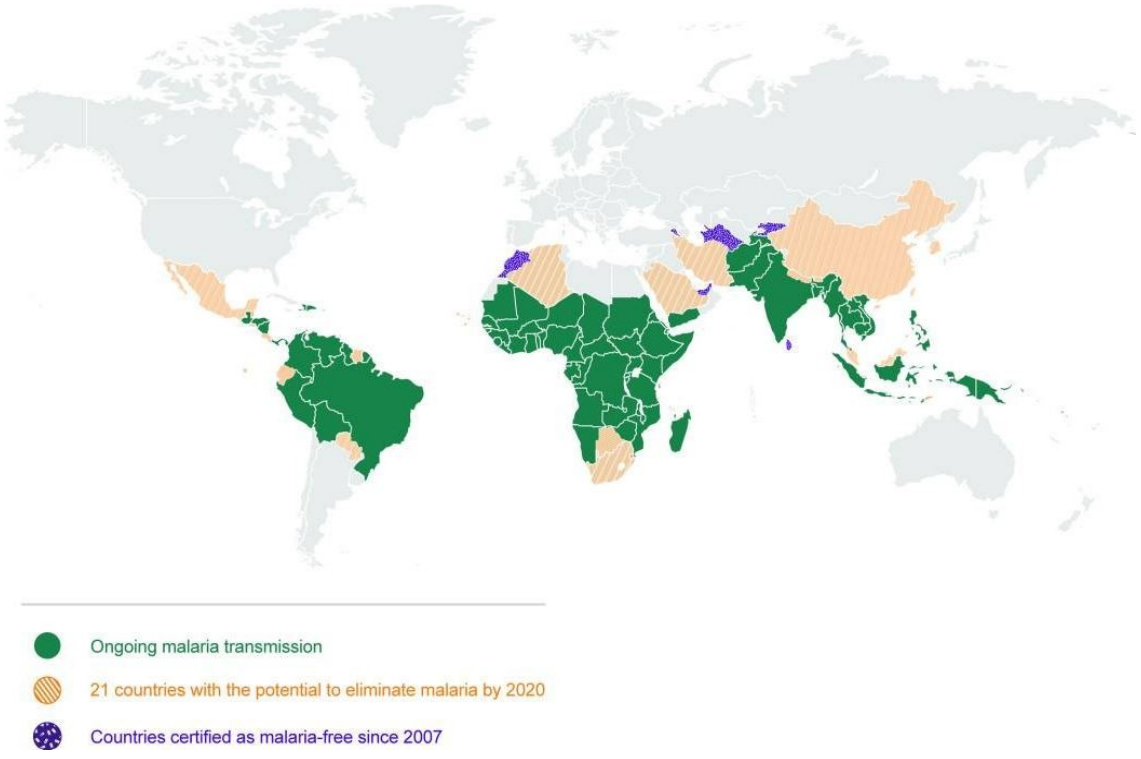

FIG 1.1. Global Malaria Transmission. Figure selected from Rabinocih, RN. *et al.* 2017. malERA: An updated research agenda for malaria elimination and eradication. PLOS Med.

LIFE CYCLE OF THE MALARIA PARASITE

Part of the difficulty in decreasing the worldwide prevalence of *Plasmodium* infections is due to the complexity of the parasite's life cycle, which is represented in Figure 1.2 (13). Humans are the intermediate host for the parasite, where asexual replication occurs. Female *Anopheles* mosquitoes are the definitive host, where sexual replication occurs. This thesis is primarily concerned with the human stages of *Plasmodium* development. Human infection begins with the bite from a female *Anopheles* mosquito, where the infective sporozoite stage is injected into the skin (14). The sporozoite then uses gliding motility to locate a blood vessel and proceeds to the liver to develop into a schizont over approximately seven days. Merosomes then bud from a hepatocyte to release thousands of extracellular merozoites into the blood stream, which must quickly infect an erythrocyte (15). This begins the parasite's asexual blood replication cycle, which is responsible for the clinical symptoms classically associated with malaria infections. Following invasion of the erythrocyte, *P. falciparum* spends ~24 hours in the ring stage, which is characterized by low metabolic activity. During hours 24-36, the parasite develops into a trophozoite, which is characterized by an increase in hemoglobin digestion and remodeling of the host-cell surface with parasite proteins. During hours 36-48, the parasite undergoes DNA synthesis, mitosis, and nuclear division to develop into a schizont containing ~10-20 daughter merozoites (16). The schizont ruptures and releases the merozoites into the blood, continuing the asexual cycle.

Beyond the asexual erythrocytic cycle, a small percentage of merozoites are committed to the sexual mode of reproduction and develop into male and female gametocytes, the forms of the parasite that are infective to the mosquito. Although not

completely understood, it is thought that a stressful environment induced by factors such as the release of *Plasmodium* hemozoin, the induction of the host immune response, erythrocyte lysis/anemia/reticuloyctosis, and exposure to antimalarial drugs, such as chloroquine, result in an increase in gametocytogenesis (17). Importantly, antimalarial drugs, including our current gold standard artemisinin combination therapies, have varying efficacies with which they are able to kill different developmental stages of gametocytes (18). Generally, the later stage gametocytes (V) are less susceptible to antimalarials than are the earlier stage gametocytes (I), suggesting decreased metabolic activity including reduced hemoglobin digestion, DNA synthesis, and apicoplast function (19). As a result of their decreased susceptibility to our first line antimalarials, propagation of the parasite may continue within a population despite the successful treatment of a blood stage infection within an individual.

Once an *Anopheles* mosquito takes a gametocyte-containing blood meal from an infective human host, temperature changes in the mosquito midgut trigger the male gametocytes to undergo exflagellation and fertilize the female gametocytes. This results in a diploid zygote which develops into a motile ookinete that traverses the mosquito midgut. In the haemocoel, the ookinete differentiates into an oocyst, which undergoes sporogony and fills with thousands of sporozoites. Upon rupture, the sporozoites migrate via the hemolymph to the salivary glands of the mosquito, where they mature and become poised to infect another human host (20).

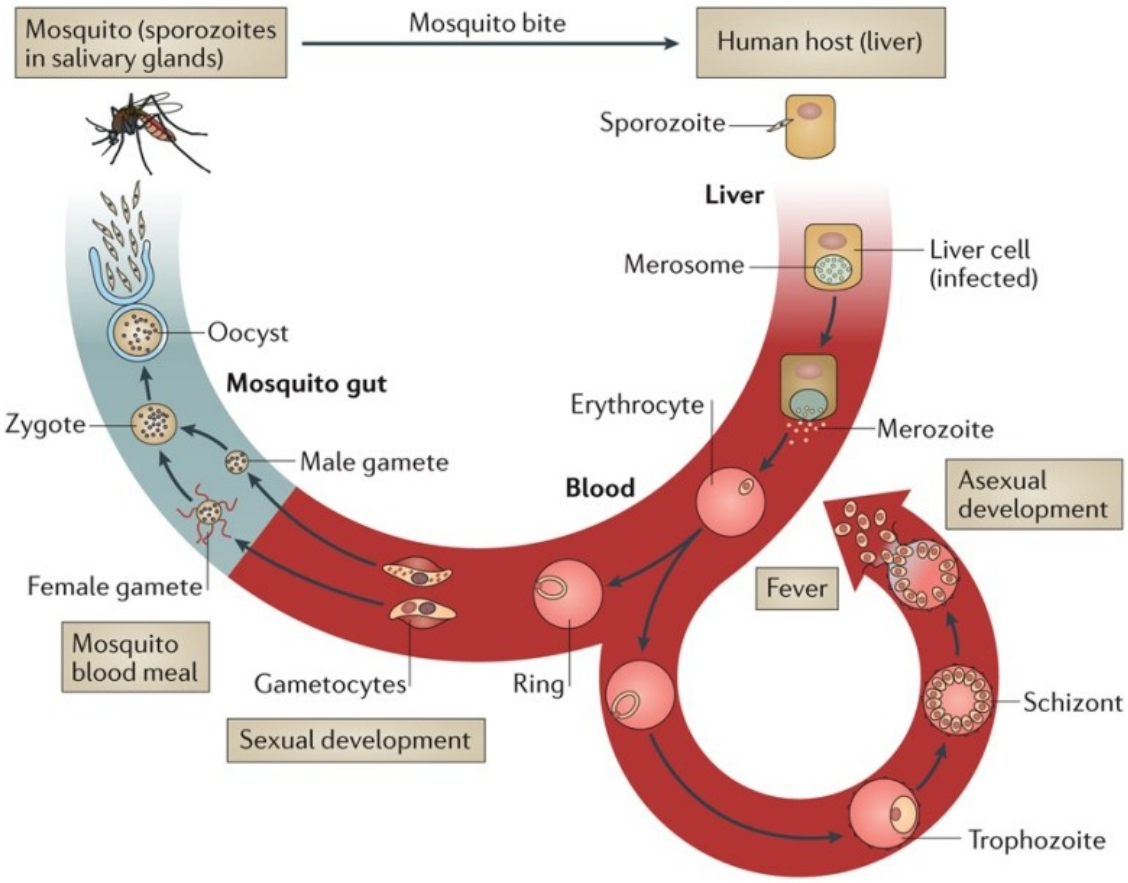

FIG 1.2. Life Cycle of the Malaria Parasite. Figure selected from Haldar, K. *et al.* 2018 Drug resistance in *Plasmodium*. Nat Rev Microbiol.

VACCINE DEVELOPMENT

Understanding the role of antimalarial drugs as preventative and curative agents requires knowledge of the current state of an antimalarial vaccine. Highlighted here are salient points regarding *P. falciparum* vaccine development. The first malaria vaccine to gain approval from European Medicines Agency and to be recommended by the World Health Organization (WHO), RTS,S/AS01 (Mosquirix™), is to undergo three large scale pilot studies that are scheduled to begin in 2018 in Ghana, Kenya, and Malawi (21). A phase III clinical trial of RTS,S/AS01 determined the efficacy of three doses against an

episode of clinical malaria within 48 months to be 26% among children aged 5-17 months and 18% in infants (22). The large scale pilot studies are intended to assess the operational feasibility and impact on child mortality and will conclude by 2022 (21). The approval and planned pilot studies of this pre-erythrocytic stage vaccine represent significant progress; however, a key limitation of this vaccine is the lack of durability of the protection.

Another vaccine, a live, attenuated *P. falciparum* whole sporozoite vaccine (Sanaria® PfSPZ) is currently under investigation in clinical trials across the United States, Europe, and Africa. Recently, a phase I clinical trial including three intravenous injections of 2.7×10^5 irradiated *P. falciparum* sporozoites indicated a good safety profile and was found to have induced antibodies against *P. falciparum* circumsporozoite protein in 14 of 20 adult males in Equatorial Guinea (23). As vaccine development continues, several questions remain to be answered. How will a vaccine with limited durability affect the development of the adaptive immune response and the ability of an individual to cope with disease at older ages in endemic areas? What is the cost-effectiveness of a vaccine intervention relative to other malaria interventions? What are the appropriate regions for vaccine deployment (24)? Of particular relevance for this thesis will be to see if the deployment of a limited efficacy vaccine will divert funds from other more effective malaria control interventions such as antimalarial drugs.

CURRENT ANTIMALARIAL DRUGS

Antimalarial drugs are an essential component of successful malaria control. The earliest known antimalarial, the bark of the cinchona tree, was discovered to resolve fevers in the 17th century by native populations and Jesuit missionaries in Peru. In 1820, quinine was isolated and found to be the active ingredient in the bark, precluding the need to grind up the bitter bark and consume it with wine (25). For approximately the next 100 years, quinine was the only available antimalarial. Interestingly, the greatest progress in antimalarial drug development followed some of the world's greatest conflicts: World War I, World War II, and the Vietnam War. As a result of non-immune military members traveling to malaria-endemic areas, there was a push to develop novel antimalarials. The Walter Reed Army Institute of Research led an antimalarial development campaign during World War II, which ultimately resulted in the discovery of chloroquine. A subsequent push for malaria eradication led to widespread deployment of chloroquine with the insecticide DTT. However, *P. falciparum* and *P. vivax* parasites developed chloroquine resistance in the 1960s, slowing progress in the fight against malaria and increasing mortality rates among young African children (26). During the Vietnam War, military members on both sides suffered devastating losses from malaria. China spearheaded new drug development efforts, which ultimately led to the discovery of artemisinin from the sweet wormwood plant, *Artemisia annua*. This discovery ultimately led to Professor Youyou Tu receiving the 2015 Nobel Prize in Physiology or Medicine and has laid the foundation of our current first-line therapies against malaria (27).

Artemisinin Combination Therapies (ACTs) couple the potent killing of the artemisinin compounds with partner compounds that have longer half-lives in order to

clear residual parasites (1). The artemisinin compounds – artesunate, dihydroartemisinin, artemether, and artemisone – are endoperoxide compounds that rapidly kill parasites after iron activation, free radical formation, and bystander protein alkylation (28). Partner compounds (amodiaquine, piperaquine, sulfadoxine-pyrimethamine, pyronaridine, lumefantrine, and mefloquine) fall into several classes. The 4-aminoquinolines - amodiaquine, piperaquine, pyronaridine, and chloroquine - are known to inhibit hemozoin crystal formation, resulting in the accumulation of toxic heme in the parasite during hemoglobin digestion (29). The amino alcohols - lumefantrine, mefloquine, and quinine - are structurally similar to the quinoline compounds and also interfere with heme crystallization.

Antifolates such as pyrimethamine, proguanil, dapsone, and sulfadoxine interfere with successive steps in folate metabolism, which is necessary for the synthesis of purine, pyrimidine, and amino acids (30). Sulfadoxine-pyrimethamine alone is also used to prevent malaria in pregnancy though intermittent preventative treatment in the 2nd and 3rd trimester (31)

Beyond ACTs, there are two classes of drugs that offer distinct advantages: the 8-aminoquinolines and antibiotics. The 8-aminoquinolines- primaquine and tafenoquine - are unique in that they have parasiticidal activity that targets the *P. falciparum* sexual stage gametocytes and *P. vivax* dormant liver hypnozoites (32–34). Several antibiotics including doxycycline, azithromycin, clindamycin, and fosmidomycin target prokaryotic metabolic pathways in the apicoplast (35). Doxycycline is a commonly prescribed malaria prophylaxis for travelers (36). Antibiotics are attractive antimalarial agents as the

drug targets are lacking in mammalian cells and there is often an abundance of data supporting their lack of toxicity in humans as some have been used for other indications.

Lastly, it is worth mentioning atovaquone, as it is the most commonly prescribed malaria prophylaxis in combination with proguanil (36). Atovaquone inhibits the electron transport chain in the parasite mitochondria and, while resistance has been reported due to a mutation in cytochrome b, mutant parasites are not thought to be transmissible by the mosquito host (37, 38).

FACTORS AFFECTING DRUG EFFICACY

Implementing successful drug treatment regimens requires a comprehensive understanding of factors that affect drug efficacy beyond the identification of a drug target. Such factors can be divided into three groups: the patient, the drug, and the parasite.

Patient relevant considerations are adherence to the treatment regimen, sex, age, pharmacogenetics, and immune status. Adherence to a drug regimen may be influenced by efficacy, side effects, convenience, or knowledge of malaria (39, 40). For antimalarial drug regimens, the WHO recommends 3 day courses of treatment, in part to increase patient adherence (31). If patients are noncompliant in completing their prescribed drug regimen, pathogens may be exposed to sub-therapeutic drug concentrations, increasing the likelihood of selection of drug resistant populations. In addition to behavioral considerations, the sex of the patient is known to influence pharmacokinetic and pharmacodynamic factors. It has been suggested that women, on average, experience

more serious adverse events, which may be related to differences in receptor number or binding affinity, lower volume of distribution, or slower drug clearance (41). Additionally, alterations in estrogen and progesterone level are known to affect drug accumulation and autoimmunity (41). Age is also a critical factor that alters the pharmacokinetic parameters of volume of distribution, bioavailability, and clearance. Elderly patients tend to have reduced renal function, leading to prolongation of drug clearance time and increasing the likelihood of toxicity (42). Of particular relevance for antimalarial drug regimens are pediatric populations. Generally, drug absorption, metabolism, and excretion are reduced in pediatric populations, while the volume of distribution and unbound fraction are increased (43). Thus, when antimalarial drugs undergo pharmacokinetic evaluation, it is necessary to consider the key populations that are at risk or this disease: children in endemic areas, especially those under 10 years of age; semi-immune adults in endemic areas; non-immune travelers; and non-immune military members travelling to endemic areas. Another patient characteristic that can effect drug efficacy is the patient's genetic makeup. Pharmacogenetics explores the changes in drug metabolism resulting from genetic changes such as in cytochrome P450 enzymes, or drug transport proteins such as P-glycoprotein (44). The clearest example of malaria drugs with altered pharmacokinetics are primaquine and dapsone (diaminodiphenyl sulfone, DDS). Individuals with a glucose 6-phosphate dehydrogenase (G6PD) deficiency, the most common inherited enzyme deficiency worldwide, are at a heightened risk of acute hemolytic anemia when the enzyme activity is below 10% of normal levels (45) The hypersensitivity of G6PD deficient patients' erythrocytes to oxidative stress is dose dependent, indicating that toxicity may be reduced with lower

drug doses (45, 46) Dapsone is no longer used clinically for malaria and the addition of single low doses of primaquine to ACT regimens for *P. falciparum* gametocyte clearance is currently under investigation (47, 48) The final patient consideration that is relevant for this thesis is the level of immunity that the individual already possesses against the pathogen (49). Varying levels of endemicity are accompanied by varying levels of adaptive immunity. In geographic areas that experience stable, year-round malaria transmission, young children are often non-immune whereas the adults have built up immunity over their lifetimes. In a murine model of malaria, it was recently shown that mice deficient in their adaptive immune system were unable to cure a *P. yoelii* following 15 days of artesunate treatment whereas wild-type mice were cured (50). Moreover, in Southeast Asia, it has been reported that seropositivity to malaria antigens was inversely correlated with parasite clearance time, especially of parasites with kelch13 mutations (51). Indicative of increasing immunity, age-dependent clearance of chloroquine resistant parasites has also been observed (52). Taken together, these data highlight the important patient-specific characteristics that may affect the outcome of a drug treatment regimen.

Understanding a compound's pharmacokinetics and ensuring the quality of the compound is essential for the successful implementation of antimalarial treatment regimens. Pharmacokinetic parameters include the drug absorption, volume of distribution, metabolism, and excretion. Drug absorption and volume of distribution are necessary considerations for the common ACT partner compound, lumefantrine, as it is lipophilic and hydrophobic. Due to variations in the level of absorption and increased volume of distribution, the plasma concentration of lumefantrine can vary widely

between patients. Consuming a small amount of fat in the form of soy milk was found to increase the lumefantrine plasma area under the curve (AUC), a relationship of the drug plasma concentration over time, by five-fold (53). Thus, lipophilic antimalarials may require the additional consideration of providing the patient with a small amount of food in order to achieve maximal drug efficacy. Additionally, the pharmacokinetic parameters of metabolism and excretion factor into a compound's plasma half-life. As *P. falciparum* has a 48-hour life cycle with varying stage-specific drug susceptibilities, the half-life of an antimalarial drug should be taken into consideration when designing treatment strategies. Artemisinin compounds have short half lives of less than one hour, while the quinoline compounds have much longer half lives of several days. Thus, if an artemisinin compound is administered when most of the parasites are in the ring stage, during which they exhibit reduced metabolism and decreased drug susceptibility, therapeutic efficacy may be diminished. Furthermore, different patient populations, such as children or pregnant women, may experience altered pharmacokinetics that may lead to an insufficient therapeutic response (54). Beyond the pharmacokinetics, the quality of the drug is, obviously, a significant factor for drug efficacy. Counterfeit drugs and non quality-assured drugs pose a threat to both the individual patient through treatment failures and/or toxicity as well as the population, as they may facilitate the development of drug resistance and decrease confidence in the healthcare system (55). A survey of over 200,000 outlets of antimalarial drugs in eight African countries revealed non quality-assured drugs were available in 42% and 48% of private sector outlets in Keyna and Nigeria, respectively (56). In seven countries in Southeast Asia, it was reported that 35% of the 1,437 drugs tested failed chemical analysis (57). In summary, the varying

pharmacokinetics of antimalarial drugs and the availability of counterfeit drugs represent additional challenges for malaria control efforts.

Parasite relevant considerations are the stage of life cycle and the presence or absence of drug resistance mutations. The susceptibility of *P. falciparum* to the antimalarials changes as it progresses through the asexual life cycle. It has been shown that rings are less susceptible to the artemisinins and chloroquine than are schizonts and trophozoites. (58, 59). This is, in part, due to the increased digestion of hemoglobin that occurs at the trophozoite stage, as many antimalarials inhibit the process of heme crystal formation, which is responsible for detoxifying heme, the byproduct of hemoglobin degradation. Acquired resistance is a much more challenging issue as antimalarial drugs can be rendered almost entirely useless. The development of resistance to antimalarial drugs has been a continual challenge throughout the 20th century. Quinine resistance was first identified in 1910 and has been followed by resistance to chloroquine, sufadoxine-pyramethamine, mefloquine, and now delayed clearance to the artemisinins (Figure 1.3) (60). The recent development and spread of delayed clearance to the artemisinin compounds, as well as resistance to ACT partner compounds, throughout Southeast Asia represents the most immediate challenge for malaria control efforts (61). While it is crucial that novel antimalarials are developed, an alternative strategy is to ensure that our currently approved antimalarials are deployed more effectively in regards to dose, schedule and duration.

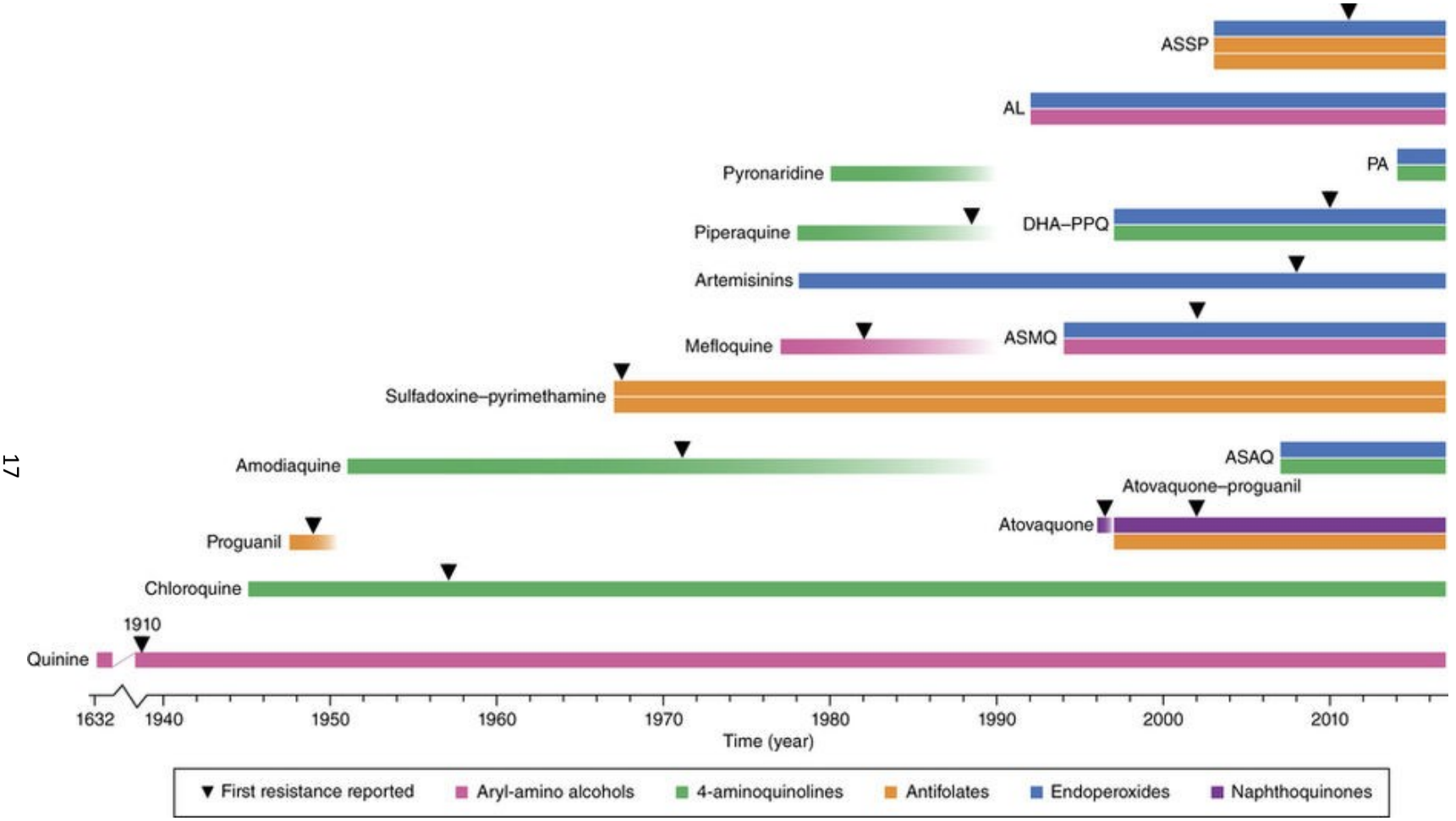

FIG 1.3. Historical Overview of the Introduction of Antimalarial Drug Resistance. Figure selected from Blasco, B. *et al.* 2017 Antimalarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic. Nat Med.

THESIS OBJECTIVES

Broadly, this work was focused on understanding how antimalarial drug efficacies can be improved. In humans, drug efficacy is measured as the reduction in a high density parasitemia. Metrics used to evaluate the clinical efficacy of antimalarials are the parasite clearance rate and the parasite clearance time. However, laboratory drug assays commonly measure cytostatic drug efficacy as percent of parasitemia growth compared to untreated drug control over a 3-day time period. For example, if the untreated control parasite multiplies by 100 and the drug-treated test parasite is essentially static at 1% growth, this is regarded as 99% inhibition. This cytostatic assay does not accurately assess parasite death. For example, if drug is applied for a short period of time, upon removal of the drug, the parasite growth may recur. Thus, this thesis sought to identify an *in vivo* model with greater clinical relevance. A schematic of the relationship between parasite growth, drug treatment, and time in a cytostatic and cytocidal *in vivo* model is displayed in Figure 1.4. Additionally, three main limitations of *in vitro P. falciparum* models led us to work mostly in the rodent model, *P. berghei*. Drug inhibition assays *in vitro* are largely unable to account for the unique pharmacokinetic parameters of each drug. Low parasitemia is necessary for *in vitro* inhibition of *P. falciparum* compared to untreated drug controls and thus it is challenging to assess cytocidal pharmacodynamic activity of drugs. Maintenance of *P. falciparum* cultures *in vitro* for an extended period of time is challenging and laborious, thereby making it difficult to monitor parasite recrudescence over multiple life cycles. The four experimental objectives of this thesis and the primary questions this work attempted to address are as follows.

(1) Adapt a transgenic *P. berghei* expressing GFP-luciferase for use in a cytocidal model of antimalarial drug efficacy. Can we improve upon our existing models to better evaluate the anti-*Plasmodium* activity of our current and future antimalarials?

(2) Evaluate the impact of extending the treatment duration of the artesunate component of artemisinin combination therapies from spanning a single asexual cycle to spanning three asexual cycles. Can the efficacy of our front-line therapies be improved by targeting the parasite over multiple life cycles, as so many other anti-infectives are already administered in this way?

(3) Evaluate the impact of increasing the dosing frequency of the apicoplast inhibitors fosmidomycin and clindamycin. Currently being tested in human clinical trials, is the efficacy of fosmidomycin and clindamycin being limited by the treatment regimen stipulating twice daily dosing?

(4) Analyze the phenotype and genotype of artemisinin-tolerant *P. berghei* SANA. Can this strain of *P. berghei* be used as an *in vivo* model for the *P. falciparum* isolates that exhibit delayed clearance to the artemisinin compounds in Southeast Asia?

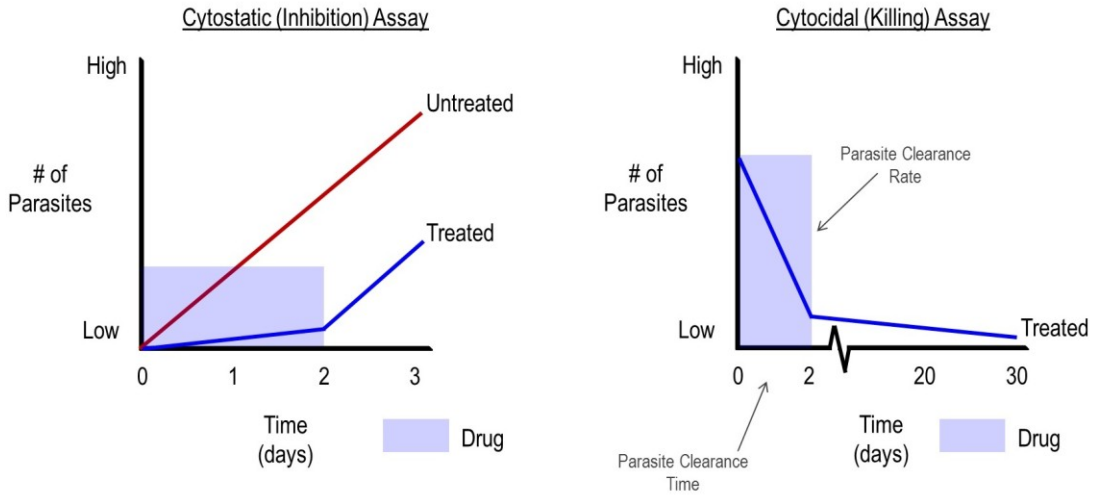

FIG 1.4. Comparison of Cytostatic and Cytocidal *In Vivo* Models

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CHAPTER 2

Impact of Extended Duration of Artesunate Treatment on Parasitological Outcome in a Cytocidal Murine Malaria Model

Adapted from the following manuscript:

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ABSTRACT

Artemisinin-based combination therapies are a key pillar in global malaria control and are recommended as a first-line *Plasmodium falciparum* treatment. They rely upon a rapid, 4-log reduction in parasitemia by artemisinin compounds with a short half-life and the killing of remaining parasites by a partner compound with a longer half-life. Current treatment guidelines stipulate giving three doses at 24 hour intervals or six doses at 12 hour intervals over a 3-day period. Due to the short half-life of artesunate and artemether, almost all of the resulting cytocidal activity is confined within a single 48 hour asexual *P. falciparum* cycle. Here we utilized a luciferase-reporter *P. berghei ANKA* in a cytocidal model in which treatment was initiated at a high parasitemia, allowing us to monitor greater than a 3-log reduction in parasite density as well as 30-day survival. In this study, we demonstrated that increasing the artesunate duration from spanning one asexual cycle to spanning three asexual cycles, while keeping the total dose constant results in enhanced cytocidal activity. Single daily artesunate doses at 50 mg/kg over seven days were the minimum necessary for curative monotherapy. In combination with a single sub-human equivalent dose of partner drug amodiaquine or piperaquine, the duration of artesunate spanning three asexual cycles is able to cure 75% and 100% of mice, respectively, whereas 0% and 33% cure is achieved with the single asexual cycle artesunate duration. In summary, cytocidal activity of the artemisinin compounds, such as artesunate, can be improved by solely altering the dosing duration.

INTRODUCTION

Infection with the malaria parasite resulted in approximately 438,000 deaths in 2015, with 99% of the cases attributable to the most lethal species, *Plasmodium falciparum* (1). As there is no available vaccine, antimalarial drugs remain the primary defense against this deadly disease. Current guidelines from the World Health Organization recommend the use of artemisinin-based combination therapy (ACT) for cases of uncomplicated malaria caused by *P. falciparum* (2). Naturally occurring artemisinin is not used clinically, rather it has been replaced by its semisynthetic derivatives artesunate and artemether, or by the metabolite of the semisynthetic derivatives, dihydroartemisinin (3). Dihydroartemisinin is activated by iron and or heme to cause radical damage of surrounding proteins, resulting in the reduction of the total parasite burden more rapidly than other antimalarials (4). However, both dihydroartemisinin and the parent drugs have a short plasma half-life of less than a few hours, thereby necessitating the need for a partner drug with a longer plasma half-life to clear residual parasites (5, 6). Over the past decade, the adoption of ACT as a first-line treatment has been integral in a promising 30% decrease in malaria associated mortality rates (1).

Malaria control efforts now face new challenges with the increasing spread of *P. falciparum* isolates with a delayed clearance phenotype throughout the Greater Mekong subregion of Southeast Asia (7, 8). This phenotype is genetically associated with mutations in the *P. falciparum* Kelch-13 gene. Delayed clearance is defined as the presence of parasites in the blood 72 hours following initiation of chemotherapy, which corresponds to a parasite clearance half-life of greater than 5 hours (8). Importantly, there

is no correlation between parasite clearance half-life and increased standard 72-hour constant drug IC_{50} to artesunate or artemether (9–11). However, a ring-stage survival assay, which involves exposing early ring stage parasites to short, 6 hour drug pulses of pharmacologically relevant concentrations of DHA, can discriminate between the tolerant and sensitive parasites (12). Early ring stages of *P. falciparum* are less metabolically active and therefore less susceptible to drugs than are the more metabolically active trophozoites and schizonts (13, 14). *P. falciparum* isolates with the Kelch-13 mutations maintain a 48-hour asexual erythrocytic cycle, but have a prolonged ring stage and compensatory shorter trophozoite stage (15). Changes in antimalarial drug sensitivity in accordance with the stage of the parasite life cycle underscore the need to understand the effect of artemisinin treatment duration on parasitological outcome (16). The consequences of decreasing efficacy of ACT are twofold: a) individuals fail to clear the infection within a standard course of treatment, resulting in significant human and economic costs, and b) placing increased selective pressures on the partner drug, thereby compromising future efficacy of the quinolines or other partner compounds (17).

Considerations for antimicrobial treatment regimens include the amount of drug administered (dose), the frequency of dosing (schedule), and the length of treatment (duration). Current ACT regimens in Southeast Asia involve 3 doses of 4 mg/kg artesunate given at the time of clinic visit (day 1) and the following two days (9). Although this treatment regimen is designed to target the parasite over three days and two life cycles, this is often not the case (18). Most patients are diagnosed between 2 pm and 5pm (19). The second and third doses of artemisinin are typically consumed the next morning (16 hours) and subsequent morning (40 hours). The artemisinin derivatives,

artesunate and artemether and their metabolite dihydroartemisinin, have elimination half-lives ($t_{1/2}$) of approximately 0.5, 2, and 0.5 hours, respectively (20, 21). Due to the short plasma half-life of artesunate, artemether, and dihydroartemisinin, patients clear most all of the drug within a single 48-hour life cycle of *P. falciparum*, as the drug is found at sub-nanomolar concentrations 12 hours after the last dose (22).

We hypothesize that spacing three identical artesunate doses over three separate asexual cycles, rather than confining them to a single asexual cycle, results in a greater log reduction in *P. berghei* parasites, making a difference between cure and no cure when combined with partner compounds. The *P. berghei* model has previously been utilized to characterize single dose pharmacokinetics and pharmacodynamics of dihydroartemisinin and piperaquine (23–25). Additionally, a 14-day duration of 100 mg/kg artesunate was found to be curative in C57BL/6 mice in a suppressive test, whereas a 14-day duration of 10 mg/kg or 7-day duration of 100 mg/kg were not curative (26). Also, in a *P. vinckei* suppression test in C57BL/6 mice, 4 consecutive daily doses of artesunate were curative when given intraperitoneally at 30 mg/kg or orally at 80 mg/kg (27).

To investigate the optimization of antimalarial dosing regimens, we have adapted a transgenic, luciferase reporter *P. berghei* to study cytocidal drug activity in a murine model in which drug treatment is initiated at a high parasitemia (~10%) and daily parasitemia and survival are monitored for at least 30 days. The transgenic parasites have previously been used to study parasite sequestration and parasite inhibition *in vivo* (28, 29). Our parasiticidal model more accurately mimics infections and treatment outcomes in the field and is superior to currently used *in vitro* drug suppression assays, which often investigate parasite inhibition at a low parasitemia compared to untreated controls. In the

present study, we compared the efficacy of a single life cycle duration of artesunate with a triple life cycle duration of artesunate in our cytocidal *P. berghei* model. We also assessed the cytocidal activity of the two durations in combination with the 4-aminoquinolines-piperaquine and amodiaquine.

MATERIALS AND METHODS

Ethics statement. All experimentation was carried out under an approved protocol by the Animal Care and Use Committee of the Johns Hopkins University (MO15H319)

Drug preparation and dosing Artesunate (Sigma Aldrich) was dissolved in 5% $NaHCO_3$. Amodiaquine dihydrochloride dehydrate (Sigma Aldrich) was dissolved in nuclease-free water. Piperaquine tetraphosphate tetrahydrate (AvaChem Scientific) was dissolved in 75 mM HCl in nuclease-free water. Artesunate was administered via intraperitoneal injection in 200 µL; amodiaquine and piperaquine were administered via oral gavage in 200 µL. The human equivalent doses of each drug in mice was calculated using the K_m factor of 12, a ratio of body weight (kg) to surface area (m^2) (30). The quinolines were dosed on salt weight.

***In vivo* cytocidal model of murine malaria.** Female BALB/cJ mice (Jackson Labs) weighing 20 grams ± 20% were used for all experimentation. BALB/cJ mice were chosen as the inbred mouse strain for our experiments because, unlike C57Bl/6, BALB/cJ are much less susceptible to early death on day 7-10 by experimental cerebral malaria with *P. berghei* ANKA infection. For malaria blood stage infections and drug responses, there are no sex differences in BALB/cJ mice. As male and female BALB/cJ mice display differences in tissue iron levels, female mice were used for consistency throughout all experiments (31). Mice were infected with 500,000 erythrocytes infected with *P. berghei ANKA GFP-luciferase* from a donor mouse. For all experiments, *P. berghei* were passaged through 3 or fewer mice. We obtained original stock from BEI Resources American Type Culture Collection (ATCC) and transmitted the parasite

through *Anopheles stephensi* mosquitoes to generate a fresh stock in 5 mice, for which blood was aliquoted for frozen stocks. A frozen stock was injected into a donor mouse, which was then passaged through no more than two mice before starting from a new fresh frozen stock for a new set of experiments. For all experiments, parasitemia was monitored by Giemsa-stained thin blood film and luciferase analysis until approximately 10% parasitemia was reached. There were no significant differences in starting parasitemia between treatment groups. Mice were grouped by initial weight and with no significant differences between treatment groups (unpaired t-test). After drug treatment, mice were followed up for 30 days with regular blood sampling for the luciferase assay. Mice were euthanized when recrudescent parasitemia exceeded the original parasitemia or until >20% weight loss occurred.

Luciferase assay and analysis. A dilution series was performed using *P. berghei* infected blood to establish a standard curve for the translation of luciferase signal (total flux) into number of parasites per well. Blood (5 µL) was drawn from the tail vein of each of 24 mice that harbored parasitemias ranging from approximately 6-12% and added to 45 µL of lysis buffer. Two-fold dilutions in lysis buffer (20 mM Tris (pH 7.5), 5 mM EDTA, 0.008% (wt./vol) saponin, and 0.08% (vol/vol) Triton X-100) were performed 15 times. The impact of decreasing hematocrit was found to be insignificant. Samples were then processed for the luciferase assay with the number of parasites per well ranging from 300 to 600,000. A total of 0.5 µL of whole blood is analyzed per well in the luciferase assay. During the drug treatment, 5 µL of blood was collected from the tail of each mouse at regular intervals and deposited into 45 µL of lysis buffer in a 96 well plate (29). Samples were stored at -80°C until processed. A total of 5 µL of blood/lysis buffer

(whole blood equivalent of 0.5 µL) was transferred to a black, opaque 96-well plate and 95 µL of luciferase buffer (20 mM Tricine, 100 µM EDTA, 1.07 mM K_2CO_3, 2.67 mM $MgSO_4$, 17 mM DTT, 250 µM ATP, 250 µM D-Luciferin) was added. Luciferase activity was measured in the IVIS Spectrum In Vivo Imaging System and analyzed using Living Image v. 4.4 software. The raw luciferase activity is reported as radiant flux in photons/second. Less than 1,000 photons/second was below the limit of detection. Total radiant flux was compared to parasites per well using GraphPad Prism 5 software and the standard curve equation derived in Figure 2.1. Blood film parasitemia was compared to the luciferase assay when parasitemia was detectable by microscopy. Radiant flux above 52,000 corresponding to 300 parasites per well or 600 parasites per µL was the robust limit of quantification.

Increased duration of artesunate Mice were administered 50 mg/kg of artesunate at three separate time points to best replicate the human ACT regimen as well as to test an extended duration of the ACT regimen. Five days following infection, the first group of mice were given three artesunate doses within a single asexual life cycle of *P. berghei*, at time 0, 8, and 20 hours. The second group of mice were given three artesunate doses within three asexual life cycles of *P. berghei*, at time 0, 24, and 48 hours. We performed four independent trials with >12 mice total in each group.

Real-time PCR quantification of infection. The level of *P. berghei* infection was quantified during the *in vivo* artesunate duration experiments with three mice in each treatment group. At regular intervals, 50 µL of blood was collected from the tail of each mouse. It was deposited into 150 µL of 1 mg/ml Heparin sodium salt in PBS and DNA extraction was performed with the QIAamp DNA Blood Mini Kit. The level of infection

was measured using oligonucleotide primers for *P. berghei ANKA 18S* and *M. musculus β-actin* (Table 1). Amplification was performed using BioRad IQ Multiplex Powermix with the BioRad CFX384 Real-Time PCR Detection System. The standard curves for 18S and β-actin were generated using a cDNA plasmid and known concentrations of mouse genomic DNA, respectively. The qPCR was carried out concurrently with the luciferase assay (represented in Figure 2.2A) and thin blood film analysis. Data analysis was carried out using GraphPad Prism 5.

Target		Sequence (5' – 3')
Plasmodium berghei 18S rRNA	Forward	AAG CAT TAA ATA AAG CGA ATA CAT CCT TA
	Reverse	GGA GAT TGG TTT TGA CGT TTA TGC G
	Probe	6-FAM CAA TTG GTT TAC CTT TTG CTC TTT
Mus musculus β-actin	Forward	GTA TCC CGG GTA ACC CTT CT
	Reverse	GCA GAA ACT GCA AAG ATC CA
	Probe	Cy5 TGG CCA GCT TCT CAG CCA CG

Table 2.1. Sequences of primers and probes used to quantify *P. berghei* infection

Increasing daily doses of 50 mg/kg artesunate. We administered 50 mg/kg of artesunate to groups of three mice for durations spanning 3 to 13 days. We then monitored parasite recrudescence and the number of days until initial parasitemia was reached, at which point the mice were euthanized. A single dose of 350 mg/kg of artesunate was administered to a single group of three mice to identify any differences in cytocidal activity when compared to the 7-day duration of 50 mg/kg.

Increased duration of artesunate in combination with 4-aminoquinolines. First, we sought to identify a subcurative dose of amodiaquine and piperaquine that

would result in cytocidal activity when administered alone, but that would not be curative in combination with artesunate administered within a single asexual life cycle. For this, we administered single doses of each drug at 0 hours and monitored parasitemia using the luciferase assay. Amodiaquine was administered at 6 and 12 mg/kg and piperaquine was administered at 9 and 18 mg/kg which are approximately $1/10^{th}$ and $1/20^{th}$ of the human equivalent dose, respectively. Second, we combined a single dose of the quinoline compound with both durations of the three dose artesunate regimens. The 4-aminoquinoline compounds have half-lives far exceeding that of artemisinin compounds and thus we administered only a single dose of both amodiaquine and piperaquine at time 0, coinciding with a 10% parasitemia and the first dose of artesunate.

RESULTS

Luciferase assay and analysis. Based on the standard curve generated from the dilution series, we identified the limit of quantification of the luciferase assay at 52,000 photons/second corresponding to 300 parasites per well or 600 parasites per µL (Figure 1.1). A limit of detection at 1,000 photons per second corresponds to 0.1 parasites per mL using the equation derived in Figure 2.1. We were able to compare blood films to luciferase robustly above 600 parasites per µL, which with approximately 10 million mouse erythrocytes per µL is 0.006 percent parasitemia. We graphed the radiant flux transformed to parasites per µL between the limit of detection and limit of quantification.

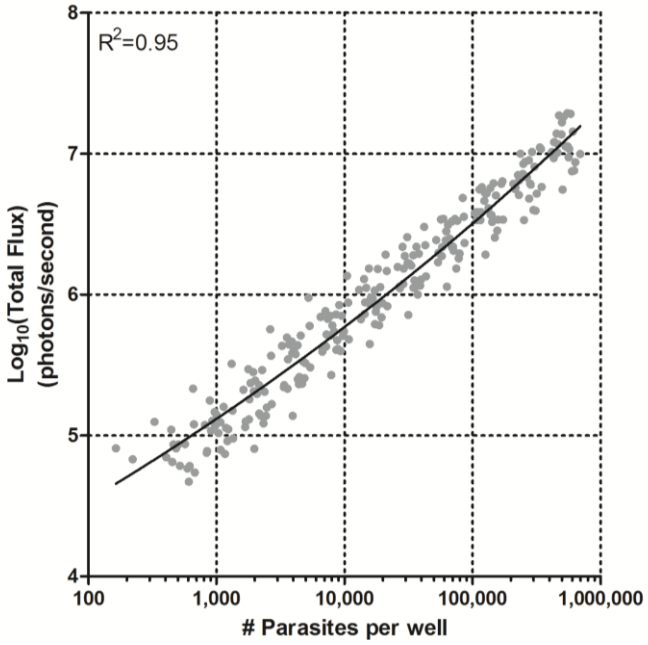

FIG 2.1 Relationship between parasite density and total flux (TF). A dilution series was performed on blood samples from 24 mice ranging in parasitemia from 6-12%. The data was fit with a nonlinear regression of a log-log line (R^2 = .95) giving the equation $x=10^{\wedge(\log(y)-.55)/.05)}$ where y=log (TF). From this equation, the number of parasites in a given well can be quantitatively estimated within a range of 300-600,000 parasites/well.

Increased duration of artesunate. In order to determine the effect of an increase in artesunate duration on parasitological outcome, we evaluated the cytocidal activity of two different regimens of artesunate. We varied the dosing interval of artesunate, but held several variables constant: starting parasitemia (~10%), total dose of artesunate, and route of administration. As the three 50 mg/kg doses of artesunate are noncurative by themselves, recrudescence can be used as a surrogate measure of total parasite killing. That is, a longer delay in parasite recrudescence indicates greater overall cytocidal activity. The mice that received the single asexual cycle duration of artesunate (0-8-20 hours) all had a quantifiable recrudescent infection by day 4, while those that received the triple asexual cycle duration of artesunate (0-24-48 hours) had a quantifiable recrudescent infection by days 6 or 7 (Figure 2.2). An observed 4 log kill was seen over 48 hours (2 log kill in the single 24 hour lifecycle) for both regimens with a similar rate of initial parasite reduction. There was a difference of three days in return to initial parasitemia. Two way ANOVA was performed to determine any statistically significant differences in time to return of initial parasitemia and, in all four trials, the time to return of initial parasitemia was significantly less in those mice receiving all of the artesunate within a 20 hour period.

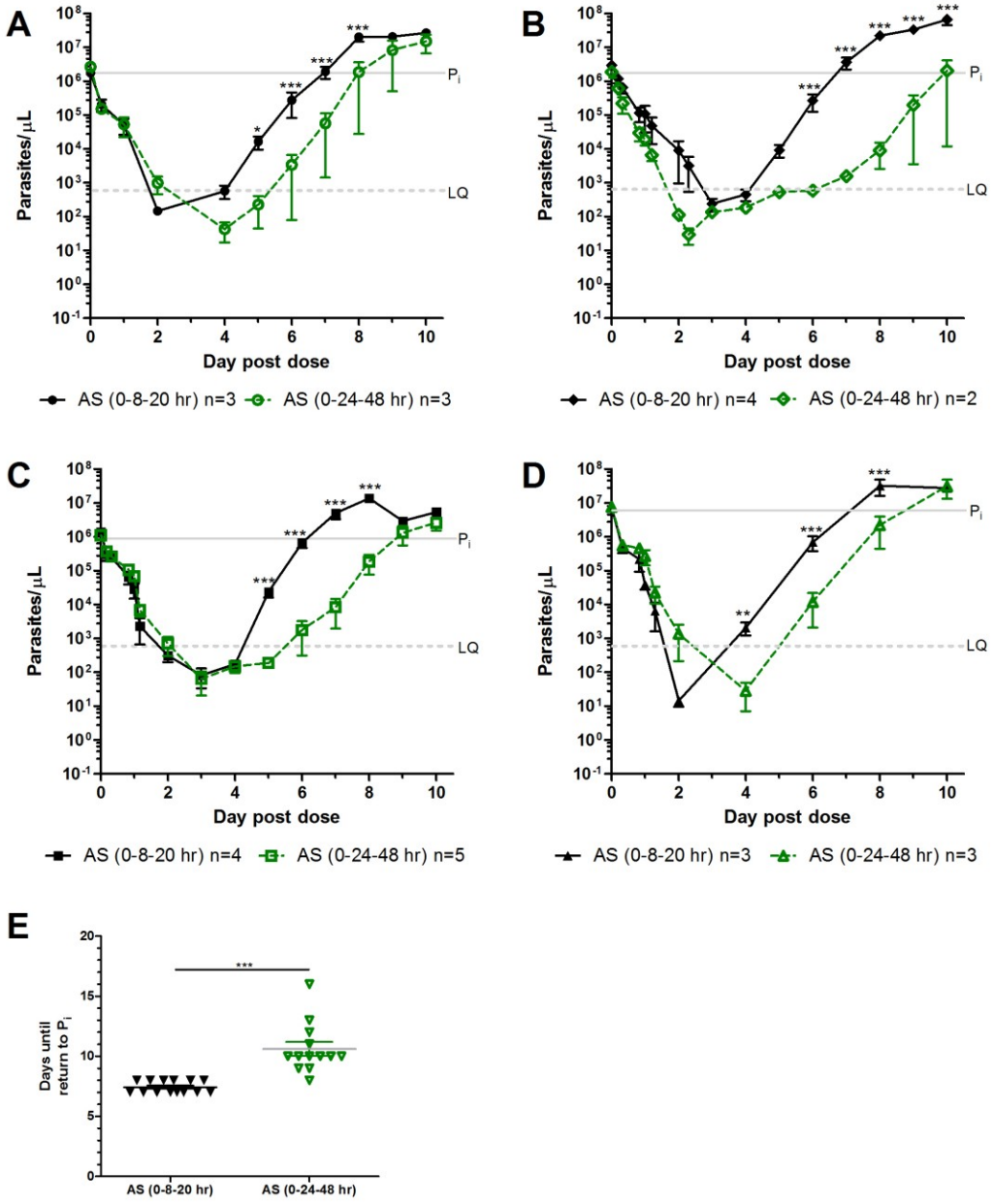

FIG 2.2 Effect of increased artesunate duration on *P. berghei ANKA* infection. Three doses of 50 mg/kg of artesunate were administered at either 0, 8, and 20 hours (black) or at 0, 24, and 48 hours (green) to female BALB/cJ mice. Time 0 corresponds to a parasitemia of ~10%, as determined by counting the percentage of infected erythrocytes in Giemsa-stained blood films. Four independent trials are represented as mean ± SEM and analyzed using two-way ANOVA (A-D). Each graph is annotated with initial parasitemia (P_i) and luciferase assay limit of quantitation (LQ). Time of recrudescence data is summarized for all four trials and analyzed using unpaired t-test (E) ***p<0.001; **p<0.01; *p<0.05

Real-time PCR quantification of infection In order to demonstrate in another manner the enhanced cytocidal activity of the increased artesunate interval observed by the luciferase assay, we performed real-time PCR concurrently with the luciferase assay trial depicted in Figure 2.2A. At the same time blood was collected for the luciferase assay, 50 µL of blood was collected for PCR. The PCR results confirm what was observed by luciferase assay as recrudescence occurs 2-3 days earlier in the single asexual cycle duration group relative to the extended duration group (Figure 2.3).

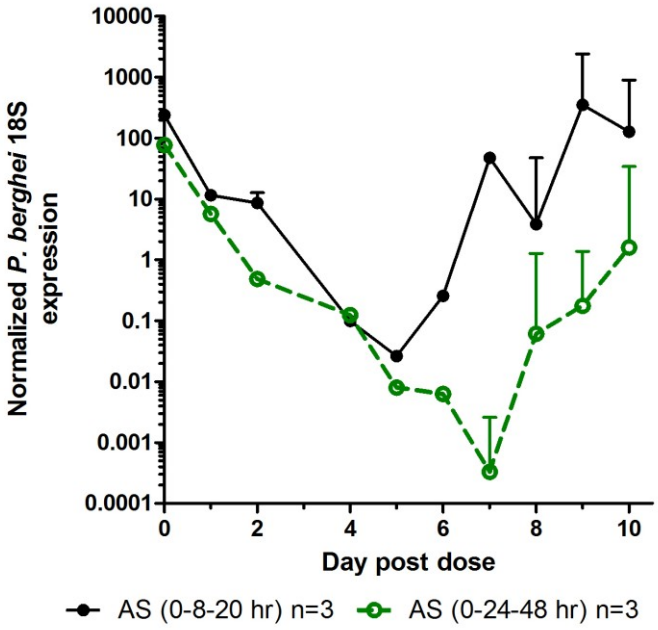

FIG 2.3 qPCR verification of effect of increased artesunate duration on *P. berghei ANKA* infection. At the time of blood sampling for the luciferase assay, DNA was extracted and infection was quantified using TaqMan primers and probes for *P. berghei ANKA 18S* and normalized to *M. musculus β-actin*. Data is represented as the mean ± SEM for the normalized *P. berghei* 18S expression.

Increasing daily doses of 50 mg/kg artesunate. Additionally, we wanted to identify in this model a curative duration of human equivalent artesunate when dosed daily (Table 2.2). Administering artesunate only for 4, 5, or 6 days resulted in 66%, 50%, and 33% recrudescence and return to initial parasitemia by day 13, 11-13, and 14, respectively. Administering artesunate for 7, 9, 11, or 13 days all resulted in 0% recrudescent infections returning to the level of initial parasitemia by 30 days. With an initial ~10^9 parasites/2 mLs of blood in a single mouse, we had slightly less than a 2 log kill every 24 hours in this model. A single mouse in the 7 day duration group did experience transient recrudescent parasitemia after day 30, but later cleared the infection. To detect any alteration in drug susceptibility for this recrudescent parasitemia, infected blood was transferred to a naïve mouse at day 45. Subsequently, the parasitemia multiplied at a log each 24 hours (the normal rate) and was fully susceptible to treatment with artesunate. We then further explored the impact of duration of drug treatment versus total dose of drug administered. We summed the total dose of drug received over 7 days (350 mg/kg*.02 kg = 7 mg), and administered this as a single bolus. Although the same total amount of drug was given, recrudescence occurred in all 3 mice and return to initial parasitemia occurred by day 10 or 11.

Duration of artesunate treatment (days)	Total dose of artesunate (mg/kg)	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
3	150	5/5	9, 10, 11, 11, 6
4	200	2/3	13, 13
5	250	3/6	13, 13, 11
6	300	1/3	14
7	350	0/3	n/a
1	350	3/3	10, 11, 10
9	450	0/3	n/a
11	550	0/3	n/a
13	650	0/3	n/a

Table 2.2. Identifying a curative, cytocidal dose of artesunate on *P. berghei ANKA* infection. 50 mg/kg of artesunate was administered in 24-hour intervals to female BALB/cJ mice for durations of 3, 4, 5, 6, 7, 9, 11, or 13 consecutive days. 350 mg/kg of artesunate was administered once to one group of mice. Starting parasitemia was ~10% as determined by counting the percentage of infected erythrocytes in Giemsa-stained blood films.

Increased duration of artesunate in combination with 4-aminoquinolines. To determine the effect of an increase in artesunate duration in combination therapy, we added the 4-aminoquinolines amodiaquine and piperaquine to each of the artesunate intervals. Single doses of amodiaquine at 6 and 12 mg/kg resulted in recrudescent parasitemia exceeding that of initial infection by days 4 and 6, respectively (Figure 2.4A). When combinations of 6 mg/kg or 12 mg/kg amodiaquine with both durations of artesunate were administered, enhanced cytocidal activity was observed with the extended duration of artesunate (Figure 2.4A-B). This is evident by the delay in parasite recrudescence that occurs with the enhanced duration of artesunate as analyzed by two-

way ANOVA. Enhanced cytocidal activity was also observed with amodiaquine 18 mg/kg in combination with the extended duration of artesunate (Figure 2.4C). All of the mice that received either artesunate duration in combination with amodiaquine 6, 12, or 18 mg/kg eventually experienced recrudescent parasitemia exceeding that of pre-treatment levels and were euthanized. However, when combination therapy included 24 mg/kg amodiaquine, 66% of mice receiving the extended duration of artesunate cleared the infection and survived to day 30. By contrast, 100% of the mice receiving the single asexual cycle duration of artesunate with amodiaquine 24 mg/kg experienced recrudescent parasitemia exceeding pre-treatment levels by day 11 (Figure 2.4D, 2.5A). When administered combination therapy including 42 mg/kg amodiaquine, 75% of mice receiving the extended duration of artesunate cleared the infection and survived to day 30 while 100% of the mice receiving the single asexual cycle duration of artesunate and amodiaquine 42 mg/kg experienced recrudescent parasitemia exceeding pre-treatment levels by day 11 (Figure 2.4E, 2.5B). All of the mice that survived until day 30 were parasite negative by Giemsa-stained blood films on day 14, counting at least 1000 erythrocytes for a limit of detection of 0.1% parasitemia.

FIG 2.4 Effect of increased artesunate duration in combination with amodiaquine. In combination with a single dose of amodiaquine (Amq), three doses of 50 mg/kg of artesunate (AS) were administered at either 0, 8, and 20 hours (solid line, grayscale) or 0, 24, and 48 hours (dashed line, green) to female BALB/cJ mice. Time 0 corresponds to a parasitemia of ~10%, as determined by counting the percentage of infected erythrocytes in Giemsa-stained blood films. Single doses of amq at 6 mg/kg (A) or 12 mg/kg (B) were administered alone and in combination with AS. Single doses of amq at 18 mg/kg (C), 24 mg/kg (D), or 42 mg/kg (E) were administered in combination with AS. Data is represented as mean ± SEM (or individual mice when group survival varied) and analyzed by two-way ANOVA. Each graph is annotated with initial parasitemia (P_i) and luciferase assay limit of quantitation (LQ). Time of recrudescence data is summarized for all five trials and analyzed using unpaired t-test (F) ***$p<0.001$; **$p<0.01$; *$p<0.05$

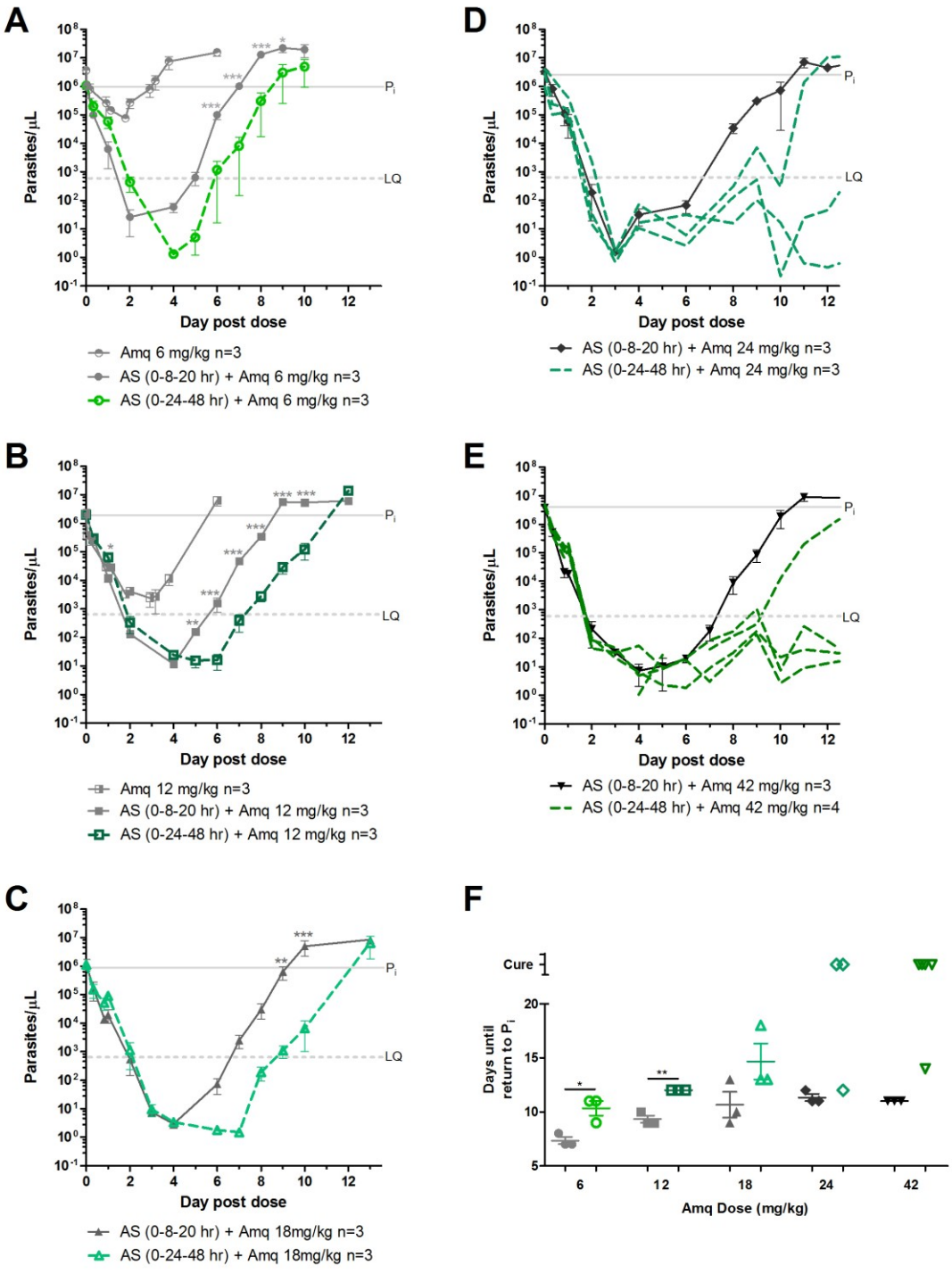

FIG 2.4 Effect of increased artesunate duration in combination with amodiaquine.

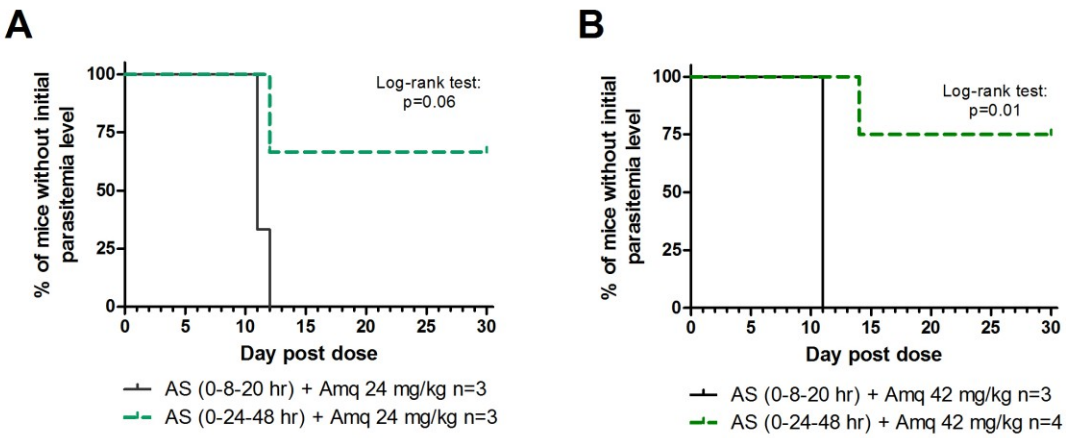

FIG 2.5 Kaplan-Meier curves without return to initial parasitemia for two artesunate durations in combination with amodiaquine. Single doses of amq (24 mg/kg (A); 42 mg/kg (B)) were administered at time 0 with the three doses of 50 mg/kg of artesunate administered at either 0, 8, and 20 hours (solid line) or 0, 24, and 48 hours (dashed line, green) to female BALB/cJ mice. Log-rank test ***p<0.001; **p<0.01; *p<0.05. Upon return to initial parasitemia (~10%), the mice were euthanized. Mice were monitored until day 30 for recrudescent parasitemia.

In order to determine if this observation was reproducible with another ACT partner compound, we tested both durations of artesunate in combination with piperaquine. Single doses of piperaquine at 9 and 18 mg/kg resulted in a return to initial parasitemia by day 4 and 6, respectively (Figure 2.6A). As 9 mg/kg piperaquine alone did not result in substantial parasite killing, we chose 18 mg/kg to test in combination with both of the artesunate durations. In combination with the extended interval of artesunate and 18 mg/kg piperaquine, 100% of the mice cured the infection and survived until day 30 (Figure 2.6B-C). By contrast, only 33% of the mice receiving the single asexual cycle duration of artesunate and 18 mg/kg piperaquine were able to cure the infection and survive until day 30. Of those mice that did not survive the infection, recrudescent parasitemia exceeded pre-treatment levels by day 12.

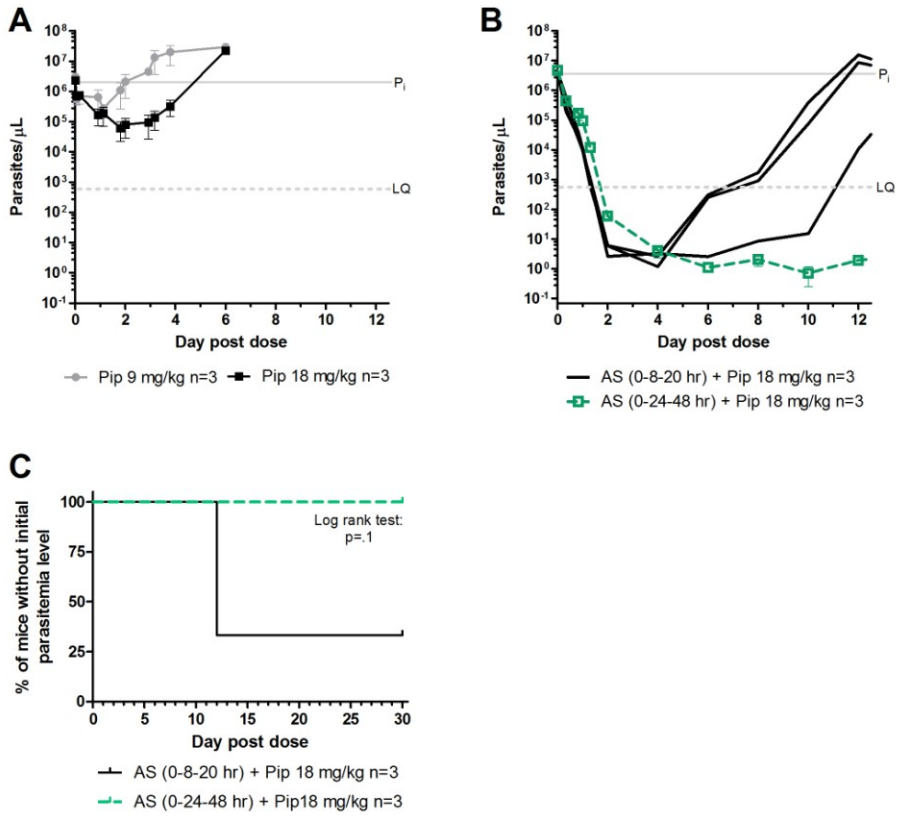

FIG 2.6 Effect of increased artesunate duration in combination with piperaquine. Single doses of piperaquine (Pip) at either 9 mg/kg or 18 mg/kg were administered at time 0 to female BALB/cJ mice (A). Time 0 corresponds to a parasitemia of ~10%, as determined by counting the percentage of infected erythrocytes in Giemsa-stained blood films. A single dose of pip (18 mg/kg) was administered at time 0 with three doses of 50 mg/kg artesunate (AS) administered at either 0, 8, and 20 hours (solid line) or 0, 24, and 48s hours (dashed line, green) to female BALB/cJ mice (B). Data is represented as mean ± SEM or individual mice. Lack of return to initial parasitemia is represented on the Kaplan-Meier curve and analyzed using the log-rank test (C). Upon return to initial parasitemia (~10%), mice were euthanized. Mice were monitored until day 30 for recrudescent parasitemia.

DISCUSSION

By the mid 1990s, chloroquine resistant and sulfadoxine-pyrimethamine resistant *P. falciparum* had emerged and spread on every malarious continent, requiring a new approach to antimalarial treatment regimens (32). Several clinical trials had compared the efficacy of different treatment durations for artemether-lumefantrine. A 3-day duration of four doses resulted in 28-day cure rates of 69%-85% in Thailand and 71% in The Gambia (33–35). A comparison of a 3-day or 5-day duration of six doses revealed 28-day cure rates of 93% and 97% in Bangkok, Thailand and 99% and 100% in MaeLa, Thailand (36). This led to the conclusion that no significant difference in efficacy existed between treatment regimens of 3 or 5 days and thus the 93%-99% cure was determined to be an acceptable standard. In 2001, the World Health Organization convened a technical consultation on antimalarial combination therapy to address the dwindling of effective treatment options. Combining the artemisinin derivatives with a short half-life with a partner drug with a longer half-life was determined to allow for a reduction in the duration of treatment. It was concluded that, if cost allowed, the following options were available for implementation: 3 days of artemether-lumefantrine, artesunate (3 days) plus amodiaquine, and artesunate (3 days) plus sulfadoxine-pyrimethamine where high efficacy remained (37). And for over a decade, the 3-day dosing regimen of ACTs was sufficiently effective. However, initial reports of decreased *in vitro* susceptibility to the artemisinin compounds arose in 2005 and reports from Southeast Asia continue to document the decreasing *in vivo* efficacy of dihydroartemisinin-piperaquine against *P. falciparum* infections (38–41). This emphasizes the need for both novel antimalarials and more effective implementation of current antimalarials.

Our study aimed to address the latter by focusing on the role that treatment duration has on the cytocidal activity of the artemisinin derivative artesunate in a mouse model of malaria with a 24-hour asexual lifecycle. The 3-day dosing regimen of ACTs and the very short plasma half-life of artesunate result in approximately a single life cycle exposure of the parasite to artesunate. In terms of antimicrobial therapy, this single life cycle exposure of *P. falciparum* is an anomaly. With a 24-hour doubling time, *Mycobacterium tuberculosis* is treated with 6 months of combination therapy (42). With a 30-minute doubling time, *Streptococcus pneumonia* infections are treated with a recommended minimum 5 day course of therapy (43). We hypothesized that the same total dose of artesunate would achieve greater reduction in the *P. berghei* biomass when administered over 3 asexual life cycles versus a single asexual life cycle.

We adapted the luciferase-reporter *P. berghei* for use in evaluating cytocidal activity of artesunate, amodiaquine, and piperaquine over a 3-log range of parasite density. In mice, dihydroartemisinin, the metabolite of artesunate, has a half-life of 25 minutes, while piperaquine has a significantly longer half-life of 18 days (44, 45). Amodiaquine has a half-life of 2.8 hours in mice, but it is rapidly metabolized to desethylamodiaquine, which has a half-life in humans of 10 days (46, 47). By using this cytocidal murine model of malaria, we determined that extending the interval of three doses of artesunate from spanning one asexual cycle to spanning three asexual cycles results in a significantly greater reduction in parasite density from the same total amount of drug. This was evident by the 2-3 day delay in time to return to initial parasitemia in both the luciferase assay and the qPCR. Comparing the strikingly different efficacy of the same total dose of artesunate administered at a single time point or spread out evenly

across 7 days further highlights the importance of artesunate treatment duration. Our results support the findings of a previous study demonstrating that increased survival is achieved when the same total dose of artesunate was administered in 3 or 4 daily doses versus a single dose in a *P. vinckei* model (48). Furthermore, a previous study on pharmacodynamic modeling in *P. berghei* found that three doses of 30 mg/kg dihydroartemisinin would produce a 12-fold lower nadir than a single dose of 100 mg/kg (25) However, to our knowledge, ours is the first to demonstrate the importance of the specific timing of 3 artesunate doses with respect to parasite life-cycle progression.

In addition to investigating the effect of monotherapy treatment duration, we determined the effect of an increased artesunate duration in combination with subcurative and sub-human-equivalent doses of two widely used 4-aminoquinolines: amodiaquine and piperaquine. With two different doses of amodiaquine at 24 mg/kg and 42 mg/kg, we have demonstrated that increasing the duration of artesunate enables cure in 66% and 75% of mice, respectively. By contrast, neither 24 mg/kg nor 42 mg/kg of amodiaquine was sufficient to cure any mice when combined with a single asexual exposure of artesunate. Furthermore, 18 mg/kg of piperaquine in combination with a single asexual exposure of artesunate cured 33% of mice, but was 100% curative in combination with the increased artesunate duration. Improving noncurative dosing regimens with amodiaquine and piperaquine to curative dosing regimens through an alteration in treatment duration, we have demonstrated the biological significance of targeting the parasite with artesunate over multiple life cycles. Although we have analyzed the effect of treatment duration in a *P. berghei* model, the concept of a single versus multiple life cycle hit of the artemisinin compounds may have strategic application to *P. falciparum*

chemotherapy. Our results support those of a recent pharmacokinetic/pharmacodynamic modeling study of dihydroartemisinin-piperaquine suggesting that administering the same total dose over an increased duration would result in increased *P. falciparum* killing and a three-fold reduction in treatment failure rates in humans (49)

Early studies on the efficacy of artemisinin monotherapy for human *P. falciparum* cases have shown, not surprisingly, that an increased duration of treatment and subsequent increase in total dose is associated with increasing 28-day cure rates. Ittarat *et al.* reported a 69% cure rate when 600 mg artesunate was administered over 3 days (50). The same total dose of artesunate was reported by Bunnag *et al.* to result in a 92.5% cure with a 7-day duration and an 85% cure with a 5-day duration. However it is worth noting that the 7-day duration involved placebo administered on days 3 and 4, with only 5 actual days of drug administration (51). In a similar study, Bunnag *et al.* investigated the schedule of artesunate monotherapy by comparing the same total dose of a 5-day duration with daily or twice daily dosing. They observed 28 day cure rates of 72% for daily dosing and 76% for split daily dosing. Although a slight increase in efficacy was observed, the authors concluded that duration, rather than dosing schedule, may be a more important factor for antimalarial efficacy (52). More recently, in Western Cambodia, Bethell *et al.* compared the efficacy of a 7-day duration of artesunate monotherapy at 2, 4, or 6 mg/kg for an approximate total dose of 700, 1400, or 2100 mg. At 28 days, the cure rates were 92%, 94%, and 84%, respectively, with the 6 mg/kg arm being halted due to several individuals developing neutropenia (53). This study indicates that dose escalation of artesunate is insufficient to combat treatment failures, as 2100 mg of artesunate is not well tolerated and doubling the total dose from 700 mg to 1400 mg did not significantly

improve outcome. Although the duration of artemisinin compounds was recognized as a key factor for successful treatment in monotherapy, it has been largely overlooked in recent discussions of improving combination therapy.

As has been shown in the ring-stage survival assays, artemisinin resistant parasites exhibit stage specific drug susceptibility (12). Similarly, other studies have demonstrated the stage-specific drug susceptibility of wild-type *P. falciparum* with respect to the artemisinins and quinolines (13, 14). Given that ACTs remain the current first line therapy against *P. falciparum* infections, both drug sensitive and drug resistant, modifying the duration of artemisinin derivatives deserves consideration. It has previously been suggested that the artemisinin derivatives, despite their rapid cytocidal activity, must be present over three *P. falciparum* asexual life cycles, in order to effect combination drug cure (18). If we are able to increase drug efficacy by extending treatment duration and achieving a greater reduction in the total parasite biomass, the opportunity remains to increase the efficacy of the ACTs and subsequently decrease selective pressures on the partner compounds. While there are several promising leads in the antimalarial pipeline, such as KAE609 and KAF156, these compounds have not yet entered phase III clinical trials (32). Thus, the lack of novel compounds on the immediate horizon requires a reevaluation of how to best implement ACTs, specifically the artemisinin derivatives. Triple ACTs involving an artemisinin component plus two partner compounds, as well as cycling the use of ACTs on a population level have been suggested as possible solutions (54, 55). Artemether-lumefantrine plus amodiaquine and dihydroartemisinin-piperaquine plus mefloquine are currently under investigation and are registered at ClinicalTrials.gov as NCT02453308. Based on our data, we suggest

doubling the dosing duration of current ACTs from 3 days to 6 days in order to expose multiple life cycles of the *P. falciparum* parasite to drug.

By adapting a luciferase-reporter *P. berghei* to study cytocidal activity of antimalarials over at least a 30 day period, we have demonstrated the importance of artesunate duration in combination therapy. Our data suggests that, keeping total dose constant, greater parasite killing can be achieved by extending the duration of artemisinin derivatives. This work may have relevance for current dosing guidelines of WHO-recommended antimalarials and future efforts geared toward the development of novel antimalarial treatment regimens.

ACKNOWLEDGMENTS

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CHAPTER 3

Enhanced Efficacy of Fosmidomycin and Clindamycin Against *Plasmodium berghei* by Implementing a Modified Dosing Regimen and Demonstrated Efficacy Against Delayed Clearance *Plasmodium falciparum* Isolates

Adapted from the following manuscript to be submitted to Antimicrobial Agents and Chemotherapy:

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ABSTRACT

With over 200 million cases of malaria annually, the development and spread of drug resistant *P. falciparum* is a consistent challenge for malaria control efforts. Fosmidomycin and clindamycin target the *Plasmodium* apicoplast and are generally well tolerated. Clinical trials of the combination of the two antibiotics have produced mixed results with the primary problem being the frequency of recrudescent infections by day 28. Given that antibiotic efficacy against bacterial infections often depends on the constant presence of drug over several days, we hypothesized that the antimalarial efficacy of fosmidomycin and clindamycin could be improved by optimizing the dosing regimen. Using a *P. berghei* GFP-luciferase inhibition and cytocidal model, our data indicates that the same total dose of fosmidomycin and clindamycin, alone and in combination, are more efficacious when administered in smaller, more frequent doses. Further, our data indicates that a noncurative combination regimen can be improved to a curative dosing regimen with a 44% reduction in overall total dose. This was achieved by increasing the frequency of dosing from 2x to 4x per asexual life cycle and extending the treatment duration from two asexual life cycles to four. We have also demonstrated *in vitro* efficacy of fosmidomycin and clindamycin against *P. falciparum* C580Y with IC_{50}s similar to those for drug sensitive *P. falciparum*. Fosmidomycin and clindamycin have the potential to be safe and efficacious against drug sensitive and drug resistant *P. falciparum* when the dosing regimen is optimized to maximize time above minimum inhibitory concentration rather than maximize peak concentration.

INTRODUCTION

In 2016, the *Plasmodium* parasite was responsible for approximately 216 million cases of malaria and 445,000 deaths across 91 countries (1). The majority of morbidity and mortality is caused by *P. falciparum* and occurs in the WHO African region. Despite continued efforts to increase distribution and use of insecticide treated mosquito nets, rapid diagnostic tests, and artemisinin combination therapies (ACTs), from 2014-2016, the malaria case incidence rate has remained unchanged globally and has increased in all WHO regions except Europe. Similarly, between 2015 and 2016, a slight increase in the malaria mortality rate was noted in all WHO regions except the African region, in which it remained relatively unchanged (1). Antimalarial drugs are essential in decreasing the malaria incidence and mortality rates in the future. However, the development and spread of drug resistant parasites has posed a continuous challenge since the early 20th century.

Across the African region, artemether-lumefantrine, artesunate-amodiaquine, and dihydroartemisinin-piperaquine are the recommended first lines of therapy and remain highly efficacious. In the Greater Mekong subregion of Southeast Asia, which has historically been the birthplace of many forms of malaria drug resistance, there is widespread partial resistance (or delayed clearance) to the artemisinins and some ACT partner compounds (2). Beyond ACTs, *Plasmodium* parasites are susceptible to antibiotics such as fosmidomycin (FOS) and clindamycin (CLIN) due to the presence of the apicoplast, which harbors prokaryote-like metabolic pathways (3). FOS was initially intended for the treatment of urinary tract infections (4). CLIN is clinically used against anaerobic bacterial infections, gram positive infections caused by *Staphylococcus* or *Streptococcus,* and is a second line therapy for pneumocystis and toxoplasmosis, the

latter of which is caused by the apicomplexan *Toxoplasma gondii* (5–7). FOS has a pharmacokinetic terminal half-life of ~3 hours and is known to inhibit a key enzyme in the nonmevalonate pathway for the synthesis of isoprenoid precursors, 1-deoxy-D-xylulose 5-phosphate (DOXP) reductoisomerase (8–10). Importantly, FOS has a poor oral bioavailability of approximately 33% (11). CLIN also has a terminal half-life of ~3 hours, but targets the 23s rRNA and ribosomal translocation (12, 13). Parasites treated with CLIN progress through the asexual cycle in which they are treated. The inability of the apicoplast to successfully replicate results in parasite death in the second generation of parasites and leads to the phenomenon of delayed generational death. By contrast, FOS has been reported to have an immediate death phenotype (14).

It is important to note that treatment regimens involving antibacterials commonly include 3-4 doses per day for up to 7 days, but antimalarial drug regimens tend to be confined to once or twice daily for 3 days. Seven clinical trials of FOS and CLIN for the treatment of malaria have produced differing results. For each of the clinical trials, FOS and CLIN were dosed at approximately 30 mg/kg and 10 mg/kg, respectively. The treatment regimen involved 2 or 4 doses daily for 3-5 days. Two studies in Thai adult populations resulted in 28-day cure rates exceeding 90%; however, 28-day cure rates among pediatric populations ranged from 46%-100% (9, 15–20). The geographic location of the study and the age and immune status of the children within the pediatric population may explain some of the variance in efficacy.

Our study was based on three primary considerations: novel antimalarials are urgently needed to combat the spread of drug resistance and diversify the treatment landscape, FOS and CLIN exhibit antimalarial effects with low-toxicity, and we believe

the utility of FOS and CLIN is undervalued due to sub-optimal dosing regimens. To this end, we investigated the optimization of FOS and CLIN dosing regimens *in vivo* in an inhibition model and a cytocidal model using *P. berghei* GFP-luciferase, which has previously been utilized to analyze quinine, chloroquine, and artesunate efficacy (21). We also assessed the *in vitro* efficacy of FOS and CLIN against *P. falciparum* bearing the Kelch-13 C580Y mutation which is most commonly associated with delayed clearance to the artemisinins (22). Broadly, our study aimed to couple the *in vivo* pharmacodynamics of several different dosing regimens of FOS and CLIN against *P. berghei* with *in vitro* activity against a clinically relevant *P. falciparum* strain in order to better understand how to most effectively implement FOS and CLIN for the treatment of malaria.

MATERIALS AND METHODS

Ethics statement. All experimentation was carried out under an approved protocol by the Animal Care and Use Committee of the Johns Hopkins University (MO15H319)

Maintenance of *P. berghei* culture and infection of naïve mice. All *in vivo* experimentation was carried out using a *P. berghei* ANKA strain expressing GFP-luciferase (MRA-868) that was obtained through BEI Resources Repository, NIAID, NIH and previously contributed by Chris J. Janse and Andrew P. Waters. Maintenance of *P. berghei* included passaging through naïve female BALB/cJ mice (The Jackson Laboratory) and periodically through *Anopheles stephensi* mosquitoes. A frozen stock was used to generate a donor mouse for each set of experiments and the number of passages in mice did not exceed 5. Giemsa-stained blood films were used to determine parasitemia of the donor mouse. For infection of naïve mice, tail blood from the donor mouse was diluted in phosphate buffered saline (PBS) to 500,000 – 1,000,000 infected erythrocytes per 200 µL and administered via intraperitoneal injection.

Drug preparation for *in vivo* dosing. Fosmidomycin, sodium salt (FOS) (FR-31564, Thermo Fisher Scientific) and clindamycin phosphate (CLIN) (1138008, USP) were dissolved in nuclease-free water and administered via intraperitoneal injection in a volume of 200 µL. For FOS, a 100 mg/kg intraperitoneal injection is equivalent to a 300 mg/kg oral dose as measured by achievable blood levels, as only 33% of FOS is absorbed. In contrast, CLIN has good bioavailability of greater than 85% (23). The intraperitoneal injection avoids first pass metabolism and the subsequent difference in oral bioavailability. Thus, for our experiments we dosed FOS and CLIN at the same dose

rather than at the 3:1 ratio of FOS:CLIN that is commonly chosen for human clinical trials. Depending on the experiment, FOS and CLIN were dosed at 25 mg/kg, 50 mg/kg, or 100 mg/kg. The dose of 100 mg/kg was chosen as a benchmark for the first experiments (Figures 3.1-3.2) as a rough approximation of the effective human equivalent doses of FOS and CLIN (10 mg/kg) using interspecies allometric scaling and a K_m factor of 12 (24). For combination dosing, each mouse received a single dose of fosmidomycin followed by a single dose of clindamycin.

Determination of *in vivo* drug activity. Two different *in vivo* assays were carried out to assess the efficacy of fosmidomycin and clindamycin on *P. berghei*: a suppression assay and a cytocidal assay. The suppression assay was used to determine the significance of increased dosing frequency during a single asexual life cycle of *P. berghei.* For the suppression assay, mice were infected with 1,000,000 infected *P. berghei* ANKA erythrocytes and drug treatment with FOS, CLIN, or combination was initiated 24 hours after infection. Two drug regimens were tested in the suppression assay: a single 100 mg/kg dose and four doses of 25 mg/kg administered 6 hours apart (q6). Untreated mice were also included in the suppression assay. Recurrent parasitemia was monitored for 10 days using the luciferase assay and statistical analysis was carried out using two-way ANOVA between the two treatment regimens. The cytocidal assay was used for subsequent determination of *in vivo* drug efficacy. Specifically, the cytocidal assay was used to determine the significance of increased dosing frequency, dose escalation, and an extended duration of FOS, CLIN, and the combination. Mice were infected with 500,000 infected *P. berghei* ANKA erythrocytes and drug treatment with FOS, CLIN, and combination was initiated 5-8 days following infection once a high

parasitemia (~5-10%) was reached. Mice were monitored for recrudescent parasitemia for up to 30 days and were euthanized when parasitemia levels exceeded that of the parasitemia on the day of drug treatment initiation or when a 20% decrease in body weight was detected. The approximate drug concentration-time profile of the combination dosing regimens was determined using the conversion factor (1000 kg/1 m^3) and assuming a 3 hour half-life of FOS and CLIN.

Luciferase assay and analysis. Generation of a standard curve translating the luciferase signal (photons per second) into number of parasites per well was previously described in chapter 2 (25). Briefly, blood samples from 24 *P. berghei* infected mice were collected for the luciferase assay and a Giemsa-stained blood film was made to determine parasitemia. A dilution series and nonlinear regression of a log-log line was performed, giving an equation that allowed for the quantitative estimation of the number of parasites in a well within a range of 300 to 600,000 parasites/well. During experimentation, blood was regularly sampled to monitor the progression of parasitemia and efficacy of drug treatment. At each time point, 5 µL of tail blood was drawn from each mouse and deposited into a 96 well plate containing 45 µL of lysis buffer (21). A total of 5 µL of blood/lysis buffer was transferred to a black, opaque 96-well plate (3792, Costar) containing 95 µL of luciferase buffer (20 mM Tricine, 100 µM EDTA, 1.07 mM K_2CO_3, 2.67 mM $MgSO_4$, 17 mM DTT, 250 µM ATP, 250 µM D-Luciferin). Luciferase activity was measured in the IVIS Spectrum *In Vivo* Imaging System and analyzed using Living Image v. 4.4 software. Data analysis was carried out using GraphPad Prism 5 software.

Maintenance of *P. falciparum* culture. *In vitro* experimentation involved either *P. falciparum* CamWT (MRA-1250) or *P. falciparum* CamWT_C580Y (MRA-1251), which were obtained through BEI Resources, NIAID, NIH and were previously contributed by David A. Fidock. Parasite cultures were maintained under a modification of the Trager and Jensen method (26). Specifically, parasites were cultured at 2% hematocrit in RPMI 1640 media supplemented with L-glutamine, 25 mM HEPES, 0.25% $NaHCO_3$, 0.37 mM hypoxanthine, 50 µL of 50 mg/ml gentamycin, and 10% human serum. Cultures were incubated at 37°C in 5%CO_2/5%O_2/Balanced N_2.

***In vitro P. falciparum* inhibitory drug assay.** *P. falciparum* cultures were synchronized to ring stage by incubation in 5% sorbitol. Parasitemia was assessed by light microscopy of a Giemsa-stained blood film. IC_{50} values were determined using a modified version of the [^{3}H]-hypoxanthine incorporation assay (27). Fosmidomycin, sodium salt, clindamycin phosphate, and chloroquine diphosphate salt (C6628, Sigma-Aldrich) were dissolved in nuclease-free water. Dihydroartemisinin (D7439, Sigma-Aldrich) was dissolved in acetone. Each drug was dissolved to 10 mM, sterile syringe filtered (SLHV013SL, Millex-HV) and subsequently diluted in RPMI 1640 media. Each drug concentration was performed in three technical replicates. Negative growth control wells (n=8) contained 10 µM of chloroquine. Positive growth control wells contained either hypoxanthine-free culture media (n=8) or 0.3% dimethyl sulfoxide (n=8). Parasite cultures were plated in tissue culture treated 96-well plates (353072, Falcon) at 2% hematocrit and 0.25% parasitemia in a final volume of 200 µL of hypoxanthine-free complete media. Parasite cultures were incubated with drug for either 72 hours or 144 hours. Twenty four hours before harvesting the parasite cultures, 0.5 µCi of [^{3}H]-

hypoxanthine was added to each well in 5 μL of hypoxanthine-free culture media. Upon completion of the incubation period, 96-well plates were frozen at -80°C until ready for sample harvesting. The 96-well plates were thawed and samples were harvested onto glass fiber filters (GF/C, Brandel) by a cell harvester (MB48, Brandel). Incorporation of [^{3}H]-hypoxanthine was measured on a liquid scintillation counter. Parasite growth was determined by comparing the disintegrations per minute of control wells to test wells. IC_{50} curves and IC_{90} values were generated using nonlinear regression analysis (log (inhibitor) vs. response) in GraphPad Prism software.

RESULTS

***In vivo* dosing frequency.** We first assessed the significance of dosing frequency for fosmidomycin (FOS) and clindamycin (CLIN) in an *in vivo P. berghei* GFP-luciferase suppression model. Twenty four hours following infection, two dosing regimens were initiated: a single dose of 100 mg/kg and four doses of 25 mg/kg given 6 hours apart. As evidenced by the *P. berghei* suppression on days 2 and 3, the same total dose of FOS was more efficacious when administered in smaller, more frequent doses (Figure 3.1A). Similarly, CLIN was more efficacious when administered in 25 mg/kg q6 rather than a single large dose of 100 mg/kg; however, due to the delayed activity of CLIN, the enhanced activity is noted on days 5 and 6 (Figure 3.1B). In combination, the enhanced suppressive activity of the 25 mg/kg, q6 regimen is significantly more effective than the single dose of 100 mg/kg. The early activity of FOS on days 2 and 3 is combined with the delayed activity of CLIN on days 4 and 5 and is sustained until day 8 (Figure 3.1C). Two-way ANOVA indicates that the combination regimen dosed every 6 hours provides significantly greater parasite inhibition than the single dose on days 2-8.

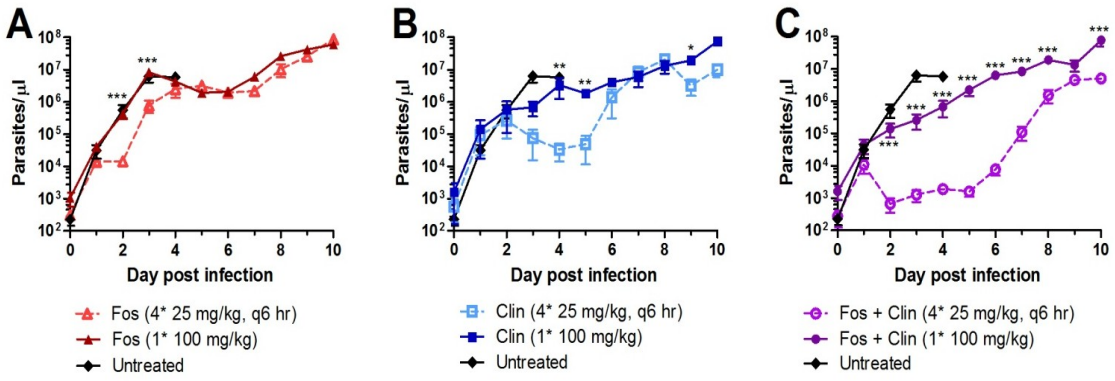

FIG 3.1 Increased dosing frequency of fosmidomycin and clindamycin in the suppression model. Inhibitory effects of fosmidomycin (Fos, red) (A), clindamycin (Clin, blue) (B), and both drugs in combination (purple) (C) were assessed. Each drug (alone or in combination) was administered as a single dose of 100 mg/kg (solid line) or four doses of 25 mg/kg given in 6 hour intervals (dashed line). Each drug treatment group contains 3 mice. Data is represented as mean ± SEM. Statistical analysis compared the single 100 mg/kg dose versus the four 25 mg/kg doses using two-way ANOVA. ***$p<0.001$; **$p<0.01$

Next we sought to verify the importance of dosing frequency in the cytocidal model. Five days following infection, the same drug regimens that were applied in the suppression model were initiated. For FOS, CLIN, and the combination, the 25 mg/kg, q6 regimen provided more cytocidal activity than did the 100 mg/kg single dose as evidenced by the lower nadir parasitemia for the former treatment regimens (Figure 3.2). At 25 mg/kg q6, the nadir parasitemia occurred on day 1.5 for FOS, day 4 for CLIN, and days 3-4 for the combination. This difference in cytocidal activity was statistically significant for CLIN on day 5 and the combination on days 5 and 6. The cytocidal assay confirms the results of the suppression assay and provides a second set of data indicating that FOS and CLIN are more efficacious at inhibiting and killing *P. berghei* when a low

concentration of drug is present over a continuous period of time.

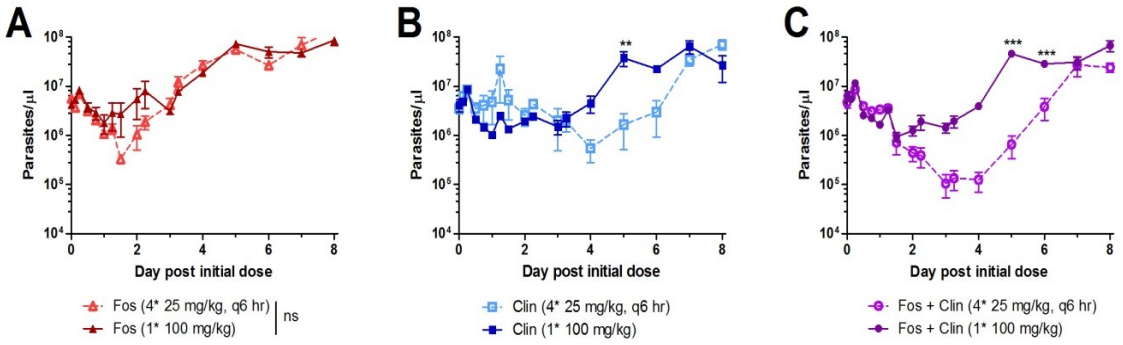

FIG 3.2 Increased dosing frequency of fosmidomycin and clindamycin in the cytocidal model. Cytocidal effects of fosmidomycin (Fos, red) (A), clindamycin (Clin, blue) (B), and both drugs in combination (purple) (C) were assessed. Each drug (alone or in combination) was administered as a single dose of 100 mg/kg (solid line) or four doses of 25 mg/kg given in 6 hour intervals (dashed line). Each drug treatment group contains 3 mice. Data is represented as mean ± SEM. Two-way ANOVA. ***p<0.001; **p<0.01

In vivo treatment duration. To investigate the significance of the duration of treatment with FOS and CLIN, we administered 25 mg/kg q6 of each drug over one or two days for a total dose of 100 mg/kg or 200 mg/kg, respectively. The two dosing regimens compare dose escalation as well as the impact of drug treatment over an additional asexual cycle of *P. berghei*. Both FOS regimens resulted in early cytocidal activity; the two day regimen resulted in significantly more parasite killing on days 3, 4, and 5 (Figure 3.3A). By contrast, both CLIN regimens demonstrated a delay in parasiticidal activity with the two day regimen demonstrating significantly more parasite killing on days 5-9 (Figure 3.3B). As expected, the combination regimen mirrors the early FOS killing and the delayed CLIN killing with the two day regimen demonstrating significantly more cytocidal activity on days 2.25-9 (Figure 3.3C, 3.3D). As an indicator of overall number of parasites killed, we compared the length of time it took for each

treatment group to return to the pre-treatment parasitemia levels. The mice receiving the two day treatment regimens received twice as much drug and approximately 24 additional hours of drug exposure; however, the parasite recrudescence for each drug group was delayed beyond 24 hours. For FOS, mice that received a single day of drug experienced recrudescent infections to pre-treatment parasitemia levels by day three, but those that received two days of drug did not experience pre-treatment parasitemia levels until day five (Figure 3.3A). For CLIN, mice that received a single day of drug recrudesced to pre-treatment parasitemia levels by day six, but those that received two days of drug did not experience pre-treatment parasitemia levels during the nine day follow-up (Figure 3.3B). Similarly, mice that received the combination regimen for a single day recrudesced to pre-treatment parasitemia levels by day six, but those that received two days of drug did not experience pre-treatment parasitemia levels by day nine (Figure 3.3C).

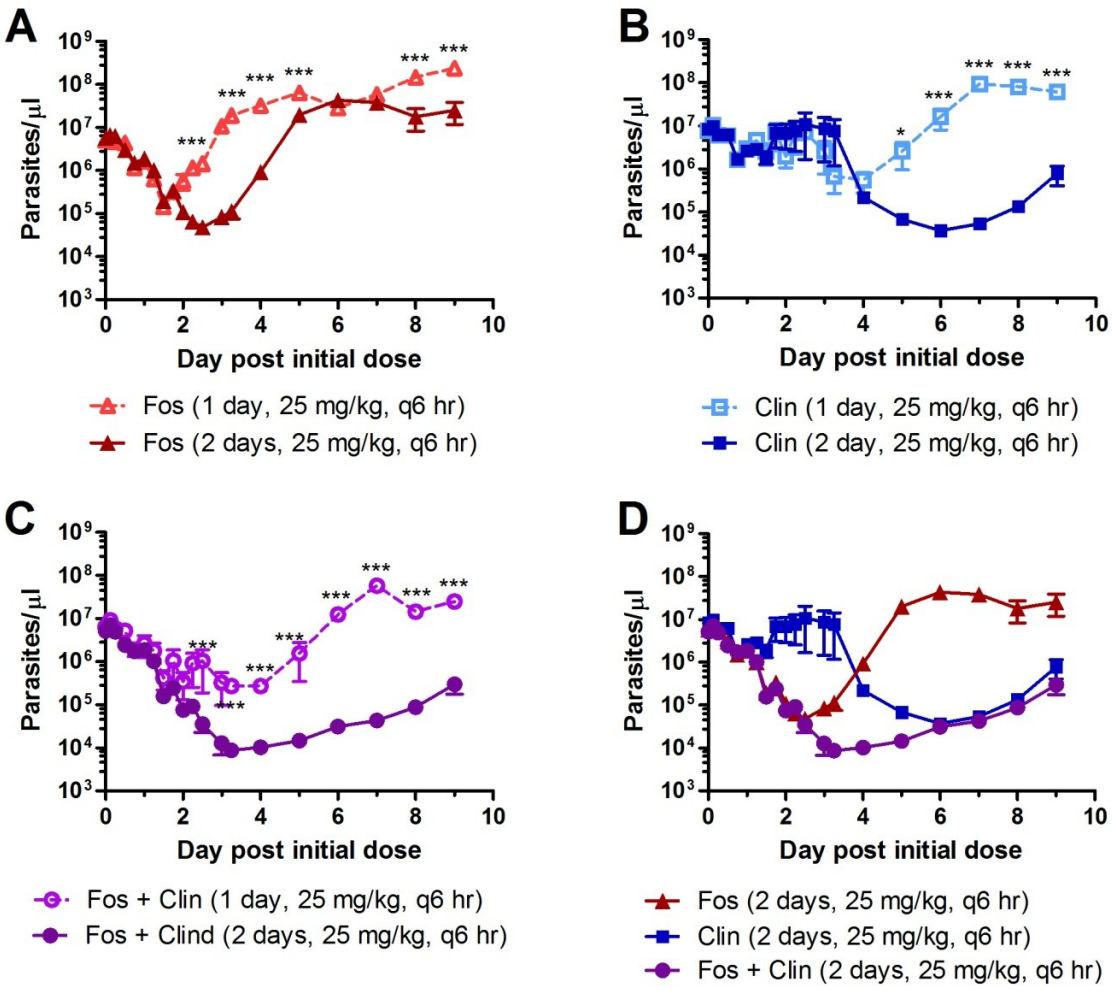

FIG 3.3 Fosmidomycin and clindamycin dose escalation and extended dosing duration. Five days following infection with 500,000 infected erythrocytes, drug treatment was initiated with fosmidomycin (Fos, red) (A), clindamycin (Clin, blue) (B), or both drugs in combination (purple) (C). Each drug was administered at 25 mg/kg given every 6 hours for 24 hours (1 day, dashed line) or 48 hours (2 days, solid line). Each drug treatment group contains 3 mice, except for Fos + Clin (1 day, 25 mg/kg, q6) which contains 2 mice. Data is represented as mean ± SEM and analyzed using two-way ANOVA. ***p<0.001; **p<0.01; *p<0.05. The 2 day durations for each drug individually and in combination are overlaid in (D).

***In vivo* FOS dosing frequency.** Building on the previous data, we sought to determine the importance of varying dosing frequencies of FOS. Three drug regimens were compared over three days: 25 mg/kg q6, 50 mg/kg q12, and 100 mg/kg q24. The same total dose of 300 mg/kg was administered to each treatment group. Relative to the 100 mg/kg q24 treatment group, mice that received 25 mg/kg q6 experienced approximately two log greater parasite killing as of day 3.5 (Figure 3.4). This difference in parasiticidal activity was also evident by the extra two day delay in return to starting parasitemia in the 25 mg/kg group. Similarly, relative to the 100 mg/kg q24 treatment group, mice that received 50 mg/kg q12 experienced approximately one log greater parasite killing as of day 3.5. On days five and six, there was significantly higher parasitemia in the 100 mg/kg group relative to both the 50 mg/kg and 25 mg/kg group. Comparing the mice that received 25 mg/kg q6 and 50 mg/kg q12, there was approximately a single log greater reduction in parasitemia as noted on day 4. No statistically significant difference was observed between the treatment regimens of q6 and q12; however, this does not indicate a lack of biological relevance.

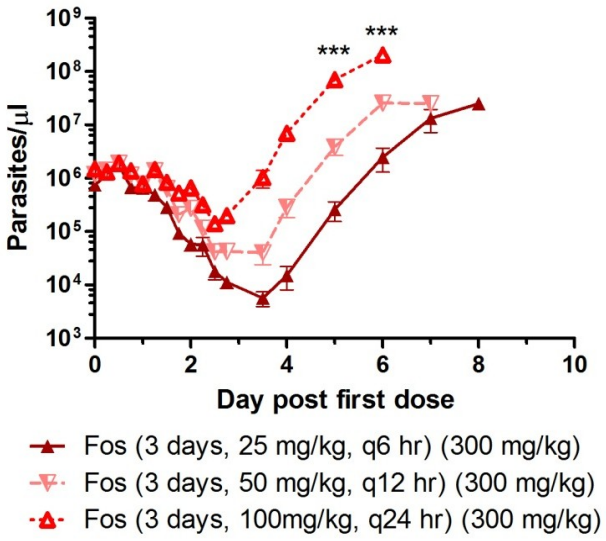

FIG 3.4 Increased fosmidomycin dosing frequency. Cytocidal activity of fosmidomycin (Fos) was assessed. Three drug regimens were administered over 3 days: 25 mg/kg every 6 hours, 50 mg/kg every 12 hours, or 100 mg/kg every 24 hours. Each drug treatment group contains 3 mice. Data is represented as mean ± SEM and analyzed using two-way ANOVA. Statistical significance was detected on days 5 and 6 between the q24 and q12 regimen and the q24 and q6 regimen ***$p<0.001$

Increasing CLIN dose and dosing duration *in vivo*. As previous data indicated the importance of dosing frequency and dosing duration, we sought to investigate the relationship between dose and dosing duration. Three drug regimens were initiated: 100 mg/kg q12 for two days, 100 mg/kg q6 for two days, and 25 mg/kg q6 for 4.5 days. A comparison of 100 mg/kg q12 for two days and 25 mg/kg q6 for 4.5 days demonstrates that a similar total dose (400 mg/kg is 89% of 450 mg/kg), is significantly less effective when administered over a shorter duration. Administered over two asexual cycles, 100 mg/kg q12 results in recrudescence by day eight (Figure 3.5). Administered over 4.5 asexual cycles, 25 mg/kg q6 delays recrudescence until day 12. Increasing the total dose to 800 mg/kg, as in the 100 mg/kg q6 regimen, does not compensate for the shorter treatment duration. Recrudescence occurs on day 11, but increases much more rapidly than the parasitemia in the mice dose with 25 mg/kg for 4.5 days. On day 16 following treatment initiation, 450 mg/kg administered over 4.5 days every 6 hours demonstrates significantly more parasite killing than the two day regimens of 800 mg/kg ($p<0.05$) and 400 mg/kg ($p<0.001$).

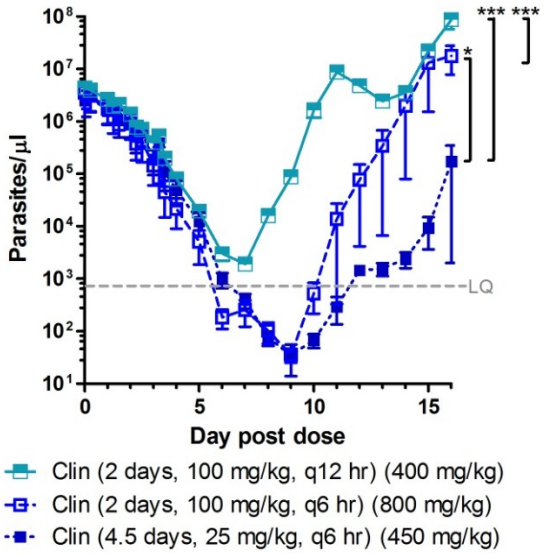

FIG 3.5 Increased dose and extended duration of clindamycin. Cytocidal activity of clindamycin (Clin) was assessed. Three drug regimens were administered: 100 mg/kg every 12 hours for 2 days ; 100 mg/kg every 6 hours for 2 days; or 25 mg/kg every 6 hours for 4.5 days. Each drug treatment group contains 3 mice. Data is represented as mean ± SEM. Two-way ANOVA was performed on day 16 between each drug regimen. ***$p<0.001$; **$p<0.01$; *$p<0.05$

***In vivo* curative combination regimen.** Finalizing the *in vivo* experimentation, we sought to identify a curative dosing regimen of FOS and CLIN in our cytocidal model. The same dosing regimens that were administered for CLIN alone were administered for the combination: two days of 100 mg/kg, q12; two days of 100 mg/kg, q6; 4.5 days of 25 mg/kg, q6. Recrudescence occurred on day nine in the 100 mg/kg, q12 regimen (Figure 3.6A). Doubling the total dose to 800 mg/kg and administering 100 mg/kg more frequently at q6 prolonged the recrudescence to day 11. A 44% reduction from the total dose of 800 mg/kg was able to cure 100% of the mice by extending the dosing duration to 4.5 days. Mice in the two day treatment groups were euthanized on day 16, as their parasitemia exceeded that of their pre-treatment parasitemia level, whereas mice in the 4.5 day treatment group were cured and survived until day 30 (Figure 3.6B). A visualization of the relationship between approximate drug concentration and time for each dosing regimen is shown in Figure 3.6C. The least effective regimen (two days, 100 mg/kg, q12) has four peaks of drug concentration and drops to just > 10µg/ml within two asexual cycles of *P. berghei*. The next regimen (two days, 100 mg/kg, q6) similarly has four peaks of drug concentration and a constant drug concentration >50 µg/ml over two asexual cycles of *P. berghei*. The curative regimen (4.5 days, 25 mg/kg, q6) does not achieve the same high drug concentrations of ~100µg/ml; however, the drug concentration exceeds 10µg/ml for over four asexual cycles.

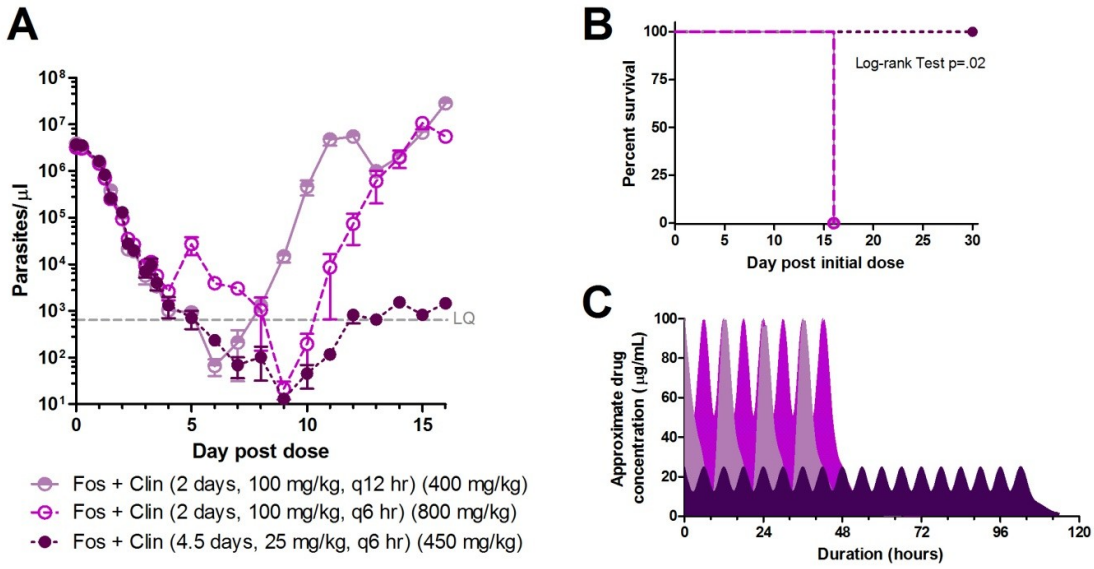

FIG 3.6 Extended duration of fosmidomycin and clindamycin. Cytocidal activity of the combination of fosmidomycin (Fos) and clindamycin (Clin) was assessed. Three drug regimens were administered: 100 mg/kg every 12 hours for 2 days at a total dose of 400 mg/kg; 100 mg/kg every 6 hours for 2 days at a total dose of 800 mg/kg, or 25 mg/kg every 6 hours for 4.5 days at a total dose of 450 mg/kg. Each drug treatment group contains 3 mice. Data is represented as mean ± SEM. LQ = Limit of Quantification (A). Survival is represented on the Kaplan-Meier curve and analyzed using the log-rank test. Mice were monitored until day 30 for recrudescent parasitemia and euthanized upon return to initial parasitemia (B). For a single drug (FOS or CLIN) the relationship between approximate drug concentration and time is shown (C).

***In vitro P. falciparum* inhibitory drug assay.** Following the *in vivo* studies, we assessed the efficacy of FOS and CLIN on two clinically relevant *P. falciparum* isolates: CamWT, an artemisinin sensitive strain, and CamWT_C580Y, a strain displaying delayed clearance to the artemisinins. Each strain was incubated continuously with dihydroartemisinin (DHA), CLIN, and FOS for 72 and 144 hours. In both the 72 hour and 144 hour DHA assay, CamWT and CamWT_C580Y resulted in IC_{50} values less than 10 nM (Figure 3.7A, 3.7D). Additionally, the IC_{90} of DHA was determined for CamWT and CamWT_C580Y. In the 72 hour assay, the IC_{90} for CamWT and CamWT_C580Y was

12.9 nM (11.5-14.4) and 24.4 nM (6.9-86.0). In the 144 hour assay, the IC_{90} for CamWT and CamWT_C580Y was 8.0 nM (2.8-23.1) and 20.6 nM (3.4-125.0). For CLIN, the 72 hour assay did not generate an IC_{50} for either strain as the highest concentration tested (10 μM) barely inhibited parasite growth 25% (Figure 3.7B). This was not unexpected given the mechanism of CLIN activity and its delayed generational effect on the apicoplast. By contrast, the CLIN 144 hour assay resulted in a CamWT IC_{50} of 19.5 nM (7.1-53.8 nM) and a CamWT_C580Y IC_{50} of 25.6 nM (12.8-51.2 nM) (Figure 3.7E). For FOS, the 72 hour and 144 hour assays displayed similar results with CamWT IC_{50} values of 1.5 μM (400 nM – 6 μM) and 1.27 μM (230 nM – 7.1 μM) and CamWT_C580Y IC_{50} values of 2.7 μM (1.1-7 μM) and 1.3 μM (500 nM – 3.4 μM) (Figure 3.7 C, 3.7F). Overall, there were no statistically significant differences in the IC_{50} or the IC_{90} values between the two strains.

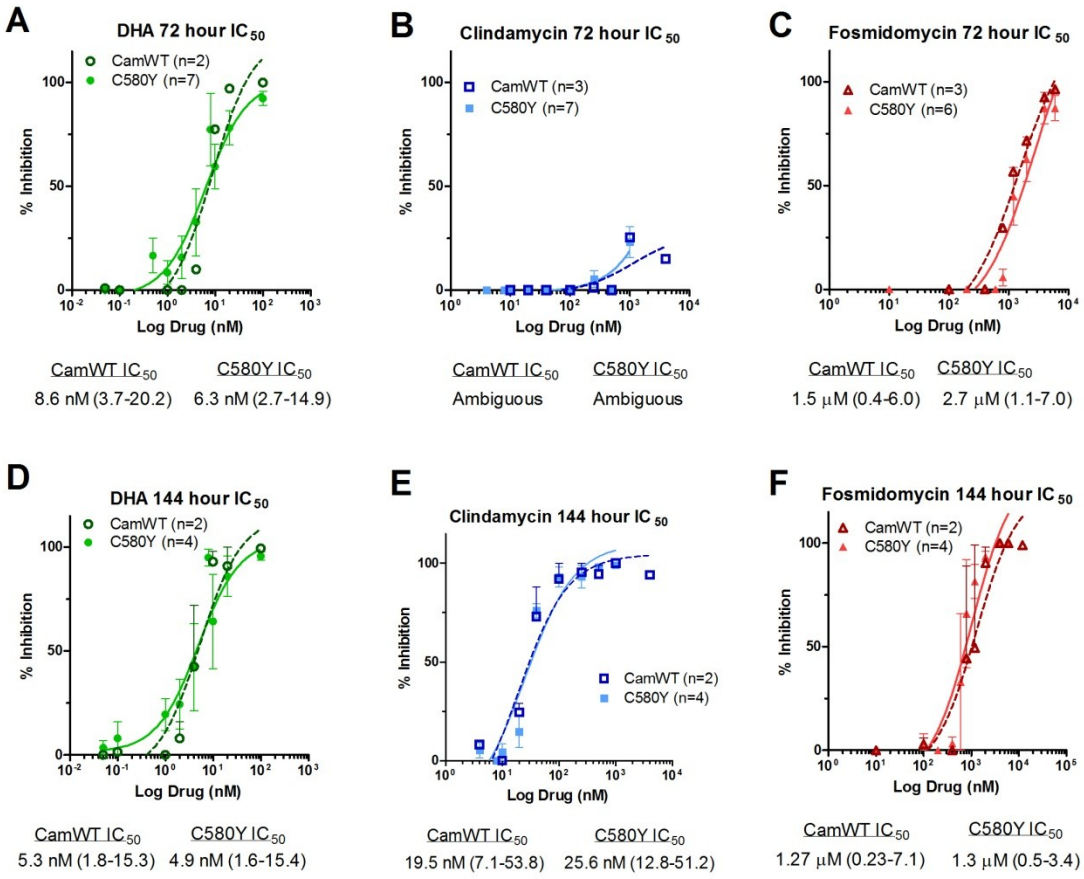

FIG 3.7 *In vitro* drug inhibitory assay. Cytostatic activity of dihydroartemisinin (DHA, green), clindamycin (blue), and fosmidomycin (red) was assessed against *P. falciparum* CamWT and *P. falciparum* CamWT_C580Y for 72 hours (A-C) and 144 hours (D-F).

DISCUSSION

Antibacterials are recognized to possess different pharmacokinetic and pharmacodynamic parameters that affect their clinical efficacy. Bactericidal activity can be divided into two groups: concentration-dependent killing, in which the higher the drug concentration, the greater amount of bacteria are killed, and minimal concentration-dependent killing, in which the longer the bacteria are exposed to the drug, the more bacteria that are killed (28). This concept has also been applied to the antimalarial drug artemisinin as *in vitro* studies have demonstrated the efficacy of artemisinin is driven by exposure to peak concentrations (29). Taken together, our *in vivo* data suggests that the anti-plasmodial activity of FOS and CLIN is driven, at least in part, by minimal concentration-dependent killing.

The significance of dosing frequency for the antibiotics fosmidomycin (FOS) and clindamycin (CLIN) was assessed in the suppression and cytocidal models. In the suppression model, the single drug data indicated that a single dose of 100 mg/kg was less effective than four doses of 25 mg/kg administered 6 hours apart. The cytocidal data for FOS and CLIN alone showed the same trend of smaller, more frequent doses being more efficacious; however, the statistical significance was not as strong as it was in the inhibition assay. We believe this is due to a larger dose of drug being required to kill parasites in a higher density infection. It has previously been noted that total parasite burden is a main determinant of therapeutic response (30) Taken together, these data also underscore the importance of evaluating both cytostatic and cytocidal drug efficacy, as has been previously discussed for the quinoline compounds' interaction with heme crystals (31). The single day versus two day duration of FOS, CLIN, or combination

dosing suggests that treatment duration is also a critical component of successful antimalarial drug regimens. Previously, it has been demonstrated that extending the duration of artesunate treatment from spanning a single *P. berghei* asexual life cycle to spanning three asexual life cycles results in a biologically relevant increase in overall cytocidal activity (25).

Further demonstrating the importance of dosing frequency, our FOS data indicates that the same total dose of drug administered over three days results in a 1-2 log greater reduction in parasitemia if dosed more frequently during a single asexual cycle. Due to the short terminal half life of FOS (< 3 hours), when dosed once a day, less than 5% of the drug remains when the second dose is administered on the second day. However, when dosed four times a day, 25% of the drug remains when the next dose is administered. Of course, the concentration of the drug is less during the q6 dosing. However, our data shows that the consistent presence of the drug results in a greater overall reduction in parasites.

The importance of dose and dosing frequency was also demonstrated with CLIN monotherapy. A doubling of the total dose of CLIN from 400 mg/kg to 800 mg/kg did result in enhanced parasite killing, as evidenced by the three day delay in recrudescence. However, implementing a dosing strategy in which CLIN is administered four times in a single asexual cycle for 4.5 asexual cycles further enhances the efficacy and utilizes only 56% of the 800 mg/kg. We wanted to understand if this difference in cytocidal activity was biologically relevant in the combination regimen. With 450 mg/kg of each FOS and CLIN, an extended treatment regimen with q6 dosing was a curative dosing regimen. This is in contrast to 800 mg/kg of FOS and CLIN q6 for two days as well as 400 mg/kg

of FOS and CLIN q12 dosing for two days, which resulted in recrudescence and death by day 16.

Clinical trials of FOS and CLIN also support the notion that increased dosing frequency may result in slightly enhanced efficacy against cases of uncomplicated *P. falciparum*. A clinical trial in Thailand comparing two dosing regimens of either FOS 900 and CLIN 300 mg q6 versus FOS 1,800 and CLIN 600 mg q12 for three days found cure rates of 91.3% (n=23) and 89.7% (n=78) (8). The authors noted that the q12 regimen resulted in large fluctuations of the peak and trough plasma concentrations of FOS. The authors concluded that FOS (900 mg) and CLIN (300-600 mg) in a q6 hour dosing regimen for 5 days would be necessary for achieving greater than a 95% cure rate with these two apicoplast inhibitors (8). However, most clinical trials have focused on twice daily dosing. In a clinical trial of asymptomatic schoolchildren in Gabon, FOS at 30 mg/kg and CLIN at 4 mg/kg dosed twice daily for five days resulted in a 100% 28-day cure for the 11 patients (18). Similarly, in Thailand, combination therapy of 900 mg FOS and 600 mg CLIN given q12 for seven days was 100% curative at day 28 for 12 patients (9). However, a shorter treatment duration in Mozambican children, 30 mg/kg of FOS and 10 mg/kg of CLIN dosed twice daily for three days resulted in a poor 28-day cure in 17 of 37 patients (16). There have also been some studies demonstrating efficacy of twice daily dosing in shorter duration treatments. In Gabonese children aged 3-14 years, 30 mg/kg of FOS and 10 mg/kg of CLIN q12 for three days resulted in 46 of 49 patients cured on day 28. This regimen was simultaneously compared to sulfadoxine-pyrimethamine at doses of 25 mg/kg and 1.25 mg/kg, which also resulted in 46 of 49 patients cured on day 28 (17). Given the high endemicity of malaria in Gabon, the

immune status of the children may have impacted the cure rates, as immune status is known to impact the therapeutic response (30, 32). Taken together, these studies suggest that the limitations of a twice daily dosing frequency may be compensated for by an extended treatment duration. Our data expands upon this and suggests that the efficacy of the desired three day regimen of FOS and CLIN may be improved with q6 dosing rather than q12 dosing.

With drug resistance a primary concern for malaria control efforts, it is worth mentioning that resistance to fosmidomycin has been generated *in vitro* in *E. coli*; however, these mutations have not been identified *in vivo* in *P. falciparum* (33). Analysis of the previously mentioned clinical trial in Mozambique determined that the 17 recrudescent infections were not due to the acquisition of fosmidomycin resistance by the parasites (16, 34). This suggests that the failure of the twice daily and three day duration of FOS and CLIN combination therapy was due to inadequate dosing and not drug resistance.

After identifying superior dosing regimens of FOS and CLIN *in vivo*, we wanted to assess their efficacy against *P. falciparum* delayed clearance isolates. In both the 72 hour and 144 hour assays, the FOS IC_{50} values for CamWT and CamWT_C580Y were in agreement with a recently published IC_{50} for drug sensitive *P. falciparum* D10 of 600 nM-1.2 μM (14). For CLIN, as expected, the 72 hour assay did not reveal an IC_{50} for CLIN at the tested concentrations, which did not exceed 10 μM. However, IC_{50} values for CamWT and CamWT _C580Y in the 144 hour assay were determined to be 19.5 nM and 25.6 nM which were also in agreement with recently published values of 30-50 nM (14). A limitation of the *in vitro* data is that the 72 and 144 hour constant drug assays are not

used in determining the sensitivity of *P. falciparum* delayed clearance isolates to the artemisinin compounds, as ring-stage survival assays involving short pulses of drugs are now the standard approach (35). However, our *in vivo* data supports a dosing regimen for FOS and CLIN that involves frequent dosing and extended treatment duration to ensure constant drug presence over several asexual life cycles. Thus, we believe there is value in the *in vitro* demonstration that *P. falciparum* CamWT_C580Y exhibits similar susceptibility to FOS and CLIN as *P. falciparum* isolates lacking delayed clearance to the artemisinin compounds.

In order to further characterize the efficacy of FOS and CLIN against delayed *P. falciparum* isolates, two sets of experimentation are planned. First, a 144 hour isobologram should be performed in order to identify possible synergy between FOS and CLIN. A previous study found that FOS was synergistic with the lincosamide antibiotics CLIN and lincomycin *in vitro* (36) Importantly, this isobologram analysis was performed using a 72 hour assay, which, as demonstrated, does not capture the delayed generational effect of CLIN. A related lincosamide antibiotic, mirincamycin, demonstrated *ex vivo* additivity with DHA on *P. falciparum* field samples from Bangladesh (37). Similarly, an *in vivo* study of a drug-susceptible *P. berghei* indicated mild synergism or additivity between CLIN and artemisone, an artemisinin derivative (38). Second, pulse drug assays are planned in order to mimic the *in vivo* studies and determine the importance of constant drug pressure versus intermittent drug-free recovery time for the parasites.

The goal of chemotherapy is to maximize the therapeutic benefit while minimizing toxicity to the patient. Aside from occasional, mild gastrointestinal upset, FOS and CLIN are well tolerated in both children and adults (18, 39, 40). We recognize

the push to constrain antimalarial drug regimens to once or twice daily dosing for three days; however, these data indicate that FOS and CLIN have potential to be safe and efficacious antimalarials if dosed more frequently than q12 and for longer than three days. Of course, patient adherence is a primary concern for antimalarial regimens. A better understanding of the prevalence and impact of patient adherence is necessary. It was recently shown that it is possible to model the relationship between treatment adherence and treatment failure with the common ACT, artemether-lumefantrine (41). Additionally, our data suggesting that a more complex treatment regimen may be necessary in order to achieve sustained antimalarial efficacy is not alone. In an effort to preserve the future efficacy of ACTs throughout Africa, sequential use of two different ACTs is currently under investigation for superiority over a single three day regimen (42). Broadly, our data reinforces the notion that dose, frequency of dosing, and treatment duration are essential components of establishing successful chemotherapy regimens. In conclusion, our data indicate that FOS and CLIN deserve further consideration as antimalarial agents and that dosing four times daily may lead to enhanced efficacy.

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CHAPTER 4

Analyzing the Phenotype and Genotype of *Plasmodium berghei* SANA for Utility as a Model of Artemisinin Tolerance

ABSTRACT

The spread of *Plasmodium falciparum* isolates exhibiting delayed clearance to the artemisinins in Southeast Asia represents a major concern for malaria control efforts worldwide. Currently, there are no well-established *in vivo* models of this delayed clearance phenotype. In 2004, *P. berghei* SANA was exposed to over 100 rounds of sub-lethal artesunate and was reported to be artesunate resistant. Our initial hypothesis was that this resistance was epigenetically controlled and the epigenetic regulation of drug susceptibility would be removed by passaging through the definitive host of the parasite, *Anopheles* mosquitoes. However, further experimentation led to the conclusion that *P. berghei* SANA does not produce infectious gametocytes. Thus, we then set out to compare the phenotype and genotype of *P. berghei* SANA to the artemisinin-sensitive *P. berghei* ANKA GFP-luciferase. Our data indicates that *P. berghei* SANA displays reduced susceptibility to the artemisinin compound artesunate, but is fully susceptible to the quinoline chloroquine. No mutations were detected in the *P. berghei* SANA kelch13 gene that have been identified and linked to delayed clearance to the artemisinins in *P. falciparum*. A comparison of the lipidomics and metabolomics between the two strains was also performed. Several future experiments are outlined to further determine the utility of *P. berghei* SANA as a model for artemisinin tolerant *P. falciparum*.

INTRODUCTION

Mouse models have been used extensively to analyze the efficacy of antimalarial drugs (1). However, there is currently no widely used *in vivo* model of delayed clearance to the artemisinins. This led us to investigate a murine malaria parasite that was developed to analyze resistance to the artemisinin compounds before *P. falciparum* isolates exhibiting reduced susceptibility were widespread on the Greater Mekong sub region (2).

P. berghei SANA was developed in the early 2000s using the 2% relapse technique. This involves infection of a naïve mouse immediately followed by a single, high dose of 300 mg/kg of artesunate. Passage into a new mouse occurs when the initial mouse reaches a 2% parasitemia. A decrease in the time taken to reach 2% parasitemia is indicative of decreasing susceptibility to the drug. Interestingly, the creation of this artesunate "resistant" parasite was motivated by the spread of chloroquine, mefloquine, and sufadoxine-pyrimethamine resistance (3). The endoperoxides, the drug class of the artemisinins, had recently been introduced for clinical use against *P. falciparum* infections and the creators of *P. berghei* SANA sought to understand the rate at which artemisinin parasites would acquire resistance to the endoperoxides. After 600 passages through artesunate-treated mice, drug pressure was removed for 21 passages and partial sensitivity to artesunate returned (4).

There have been two relatively recent studies that utilized the *P. berghei* SANA parasite to evaluate drug efficacy. Vivas *et al.* utilized an *in vivo* suppression model to demonstrate the efficacy of pyronaridine against both artesunate sensitive and artesunate resistant parasites (5). The authors also demonstrated that *P. berghei* SANA had a similar

artesunate effective dose 50 (ED_{50}) as an artesunate sensitive strain of *P. berghei*; however, the ED_{90} was approximately 10x that of the sensitive strain. Henrich *et al.* compared the drug susceptibility of *P. berghei* SANA to that of the parental strain, *P. berghei* N (6). The data indicated that the *P. berghei* SANA strain had differing susceptibilities to artesunate and dihydroartemisinin, with the latter being more potent. Similar to Vivas *et al*., the authors determined the combination and, now approved ACT, pyronaridine-artesunate was effective against *P. berghei* SANA.

We hypothesized that the reduced susceptibility of *P. berghei* SANA to artesunate was due to epigenetic modifications rather than genetic mutations. Additionally, we hypothesized that the epigenetic modifications would be eliminated by passaging the parasites through the definitive host, the mosquito. To answer this question, we first eliminated the lactate dehydrogenase virus (LDV) from the *P. berghei* SANA clone and then ensured we had a clonal population. Then, we assessed the susceptibility of *P. berghei* SANA to artesunate and chloroquine relative to *P. berghei* ANKA, which is sensitive to both drugs. Next, we attempted to passage the *P. berghei* SANA clones through *An. stephensi.* We then sequenced the *P. berghei* kelch 13 gene and performed a metabolomics and lipidomics analysis.

MATERIALS AND METHODS

Ethics statement. All experimentation was carried out under an approved protocol by the Animal Care and Use Committee of the Johns Hopkins University (MO15H319)

***Plasmodium berghei* strains.** Female BALB/cJ mice (Jackson Labs) were used for all experimentation. Three strains of *P. berghei* were used for experimentation: *P. berghei* ANKA GFP-luciferase (*P. berghei* ANKA), *P. berghei* SANA, and *P. berghei* RC. We obtained the original stocks of *P. berghei* ANKA and *P. berghei* SANA from BEI Resources American Type Culture Collection (ATCC). The depositor supplied stock of *P. berghei* SANA was found to carry the adventitious pathogen, lactate dehydrogenase elevating virus (LDV). A single vial was released to us on the condition that we would attempt to purify the *P. berghei* SANA stock from LDV. *P. berghei* RC was obtained from Dr. Jian Wu at the National Institutes of Health.

Lactate dehydrogenase elevating virus detection. Removal of LDV from *P. berghei* SANA was performed as previously described (7). The initial stock of *P. berghei* SANA was injected into a naïve mouse in a BSL-3 facility. Blood was harvested and erythrocytes were separated from the mononuclear leukocytes on a Percoll gradient. Erythrocytes were then washed and injected into three naïve mice. *P. berghei* RC was also cloned prior to any drug testing. *P. berghei* SANA and *P. berghei* RC samples were sent for testing of the presence of lactate dehydrogenase elevating virus. Fecal pellets or 200 µL of undiluted plasma from infected mice were sent to Charles River Research Animal Diagnostic Services (Wilmington, MA) and tested by PCR. Following the LDV PCR, *P. berghei* SANA was cloned.

Monitoring *P. berghei* parasitemia. Parasitemia was monitored by blood film analysis and counted under light microscopy (100x). Thin blood smears were made from tail blood. Microscope slides were fixed in methanol and stained using a 10% Giemsa solution (32884, Sigma-Aldrich).

Drug preparation and dosing Artesunate (A3731, Sigma-Aldrich) was dissolved in 5% $NaHCO_3$. Chloroquine diphosphate salt (C6628, Sigma-Aldrich) was dissolved in nuclease-free water and dosed on salt weight. Artesunate and chloroquine were administered via intraperitoneal injection in 200 μL.

***Plasmodium berghei* infection and drug susceptibility testing.** For each strain, a frozen stock was injected into a donor mouse, which was then passaged through no more than two mice before starting from a new fresh frozen stock for a new set of experiments. To establish a new infection, parasitemia of a stock mouse was counted by Giemsa-stained blood film. Infected blood was diluted to 500,000 infected erythrocytes per 200 μL of phosphate buffered saline (PBS). Several days following infection, when the parasitemia had reached approximately 5-10%, drug treatment was initiated with either artesunate or chloroquine. Drug susceptibility was evaluated by monitoring parasitemia and recrudescence over a 30 day period.

***Anopheles stephensi* infection.** Naïve mice were infected with frozen stocks of *P. berghei* ANKA and *P. berghei* SANA clones 3, 4, and 9. Four days later, a second naïve mouse was infected with 1% parasitemic blood diluted into 200 μL PBS for each clone. Five days later, mice were anesthetized with ketamine and starved *An. stephensi* female mosquitoes were fed on a mouse for 30 minutes. Blood fed mosquitoes were separated

and re-fed on naïve mice on days 19 and 20. Mice were monitored for patency until day 14 by Giemsa-stained blood film.

***P. berghei* exflagellation assay.** 7.5 µL of complete ookinete medium was mixed with 1 µL of 1 mg/ml heparin in PBS solution. 1.5 µL of *P. berghei* infected tail blood was added and incubated at room temperature for 15 minutes. Samples were loaded onto microscope slides with glass coverslips and observed under light microscopy at 40x. Incomplete ookinete medium is composed of the following reagents and filter sterilized: 1L RPMI 1640, 50 mg hypoxanthine, 2g sodium bicarbonate, 50,000 U penicillin, 50 mg streptomycin, xanthurenic acid to a final concentration of 100 µM, pH=8.3. For complete ookinete medium, 20% heat inactivated fetal bovine serum was added.

PCR and sequencing. *P. berghei* kelch13 gene was amplified using the Platinum SuperFi PCR Master Mix (12358250, ThermoFisher Scientific) according to the manufacturer's protocol. PCR primers are listed in Table 4.1. The PCR protocol was as follows: 98ºC for 30 seconds; 35 cycles of 98ºC for 7 seconds, 63ºC for 10 seconds, and 72ºC for 1 minute; 72ºC for 5 minutes.

Target		Sequence (5′ – 3′)
Plasmodium berghei kelch13	Fwd	ATGGAGGATGATAAAATAAAGTCAAACAG
	Rev	TTAAATATTTGCAACCAAAACGGAATG

Table 4.1 PCR primers for *P. berghei* kelch13

Purified PCR product was obtained through ethanol extraction. At a concentration of 35-100 ng/µL, 2 µL of PCR product was mixed with 4 µL of either the forward or reverse primer at a concentration of 10 µM. Sequencing primers are listed in table 4.2.

Samples were sent to the Synthesis and Sequencing Facility at the Johns Hopkins School of Medicine. Sequences were analyzed using Sequence Scanner Software v1.0 (Applied Biosystems).

Target		Sequence (5′ – 3′)
Plasmodium berghei kelch13 A	Fwd	ATGGAGGATGATAAAATAAAGTCAAACAG
	Rev	CATCATCAAATCGTTTTCGATGTTCTTC
Plasmodium berghei kelch13 B	Fwd	GCTATAGAAGAACAAAAATTACAAGATG
	Rev	CCATGTATCTCTTAATCTATCATATACTTC
Plasmodium berghei kelch13 C	Fwd	GGAACTTTTAGATATAAGCCAACAATGC
	Rev	TTAAATATTTGCAACCAAAACGGAATG

Table 4.2 Sequencing primers for *P. berghei* kelch13

Amino Acid and Lipid Composition Analysis. *P. berghei* infected mice were anesthetized with avertin and cardiac punctures were performed. A saponin lysis was performed and the hemozoin quantity was estimated for normalization of sample quantity. Parasite pellets were sent to Dr. Vladimir Shulaev at the University of North Texas. Liquid chromatography-mass spectrometry (LC-MS) and flow injection analysis-mass spectrometry (FIA-MS) were performed to identify any differences in metabolites or lipids, respectively, between *P. berghei* SANA or *P. berghei* ANKA.

RESULTS

Lactate dehydrogenase elevating virus detection. The initial stock of *P. berghei* SANA and three post-Percoll samples of *P. berghei* SANA were tested for lactate dehydrogenase LDV. The initial stock received from ATCC was positive, the three post-Percoll samples tested negative. Limited dilution cloning was performed on one of the *P. berghei* SANA LDV negative samples resulting in nine clones. Three clones of *P. berghei* RC were tested in duplicate and found to be negative for LDV.

***Plasmodium berghei* infection and drug susceptibility testing.** Prior to performing drug susceptibility assays, an analysis compared the relative percentages of asexual stages in *P. berghei* ANKA vs. *P. berghei* SANA clones 3, 4, 5, 6, 7. The number of erythrocytes that were infected with ring stages versus trophozoite or schizont stages were counted as a percentage. *P. berghei* ANKA averaged 33.6% rings, while the SANA clones trended higher (Table 4.3).

Clone	Mouse	% Rings	Average %
SANA 3	1	35.4	39.7
	2	37.0	
	3	46.6	
SANA 4	1	46.3	46.8
	2	44.2	
	3	50.0	
SANA 5	1	62.3	52.1
	2	43.5	
	3	50.6	
SANA 6	1	57.2	53.6
	2	46.7	
	3	57.0	
SANA 7	1	40.0	38.8
	2	35.0	
	3	41.4	
P. berghei ANKA	1	39.8	33.6
	2	35.9	
	3	25.2	

Table 4.3 Asexual stage percentage of *P. berghei* SANA. Female mice were infected with *P. berghei* SANA clones 3-7, or *P. berghei* ANKA. Prior to drug treatment, Giemsa-stained blood films were made. The percentage of ring stages was counted out of all infected erythrocytes.

Susceptibility of *P. berghei* SANA to artesunate was first compared to *P. berghei* ANKA. Artesunate was administered at 50 mg/kg over 5 days to *P. berghei* SANA clones 3 and 9 and *P. berghei* ANKA. There was some variability in initial parasitemia ranging from 2-8% (Figure 4.1) Parasitemia was not detected on day 8 following initiation of artesunate treatment in *P. berghei* ANKA. However, all six mice infected with *P. berghei* SANA had visible parasitemia by blood film.

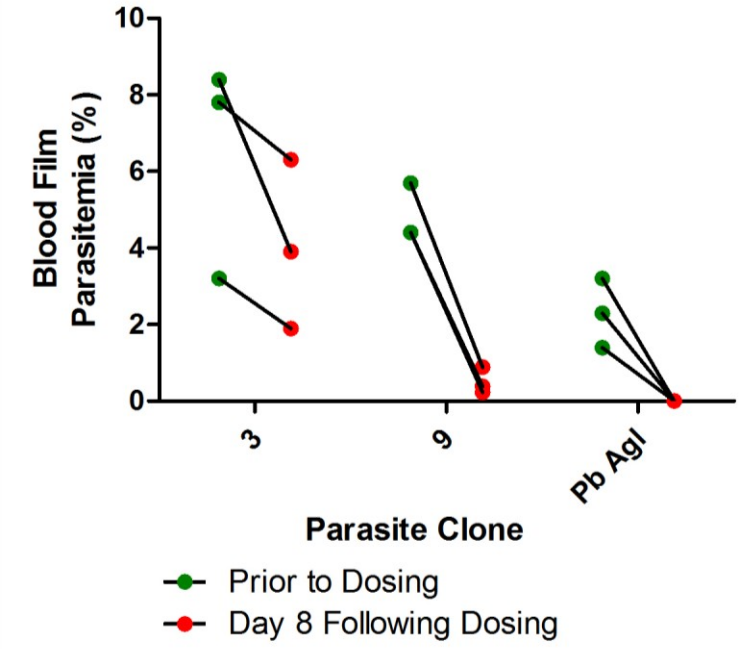


FIG 4.1 Trial 1 of preliminary artesunate susceptibility of *P. berghei* SANA. Female mice were infected with *P. berghei* SANA clone 3, *P. berghei* SANA clone 9, or *P. berghei* ANKA. Five doses of 50 mg/kg artesunate were administered over five days. Parasitemia was monitored with Giemsa-stained blood films. Individual mice are shown (n=3).

Due to the variability in starting parasitemia, this assay was repeated with five clones of *P. berghei* SANA (Figure 4.2). For the second trial, all mice had initial parasitemia ranging from 3-9%. Eight days following initiation of artesunate treatment,

each mouse infected with *P. berghei* SANA clone 3 or 4 had parasitemia exceeding 1%. Of the 3 mice infected with clone 5, 6, or 7, 1 mouse that was blood film negative.

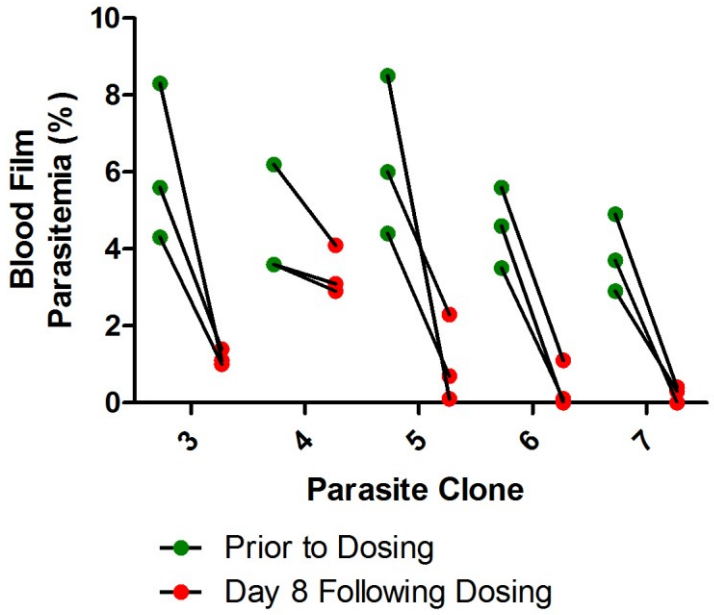


FIG 4.2 Trial 2 of preliminary artesunate susceptibility of *P. berghei* SANA. Female mice were infected with *P. berghei* SANA clones 3, 4, 5, 6, or 7. Five doses of 50 mg/kg artesunate were administered over five days. Parasitemia was monitored with Giemsa-stained blood films. Individual mice are shown (n=3).

To further interrogate the artesunate susceptibility of SANA clones 3, 4, and 9, a more rigorous test of drug resistance was performed. Instead of 5 days of artesunate treatment (250 mg/kg), mice were administered 8 days of artesunate treatment (400 mg/kg). The 8 day duration of artesunate treatment was chosen as previous studies in chapter 2 demonstrated that 7 days of 50 mg/kg of artesunate were curative in the drug sensitive *P. berghei* ANKA model (8). Additionally, chloroquine was chosen for a second drug comparison. The parental strain of *P. berghei* SANA is *P. berghei* N and was reported to be chloroquine sensitive. Thus, a single dose of 100 mg/kg of chloroquine was administered. The results of this assay are presented in Table 4.4. This

more rigorous phenotypic assay confirmed that *P. berghei* SANA clones 3 and 4 exhibit reduced susceptibility to artesunate. While the mice infected with *P. berghei* ANKA strain were cured on day 11, except for one mouse which displayed a 0.8% parasitemia, the mice infected with *P. berghei* SANA 3 or 4 were highly parasitemic on day 11. For chloroquine, all mice in each group were parasitemic on day 14.

Drug	Duration (days)	Dose (mg/kg)	Parasite	Outcome (% Parasitemia Range)	
Artesunate n=5	8	50	PbAgl GFP-luc	Day 11	(0-0.8)
			SANA 3		(5.7-9.8)
			SANA 4		(4.9-9.1)
Chloroquine n=3	1	100	PbAgl GFP-luc	Day 14	(4.8-8.3)
			SANA 3		(10-17.6)
			SANA 4		(8.8-34.1)

Table 4.4 Artesunate and chloroquine susceptibility of *P. berghei* SANA. Female mice were infected with *P. berghei* ANKA or *P. berghei* SANA clones 3 or 4. Eight doses of 50 mg/kg artesunate were administered over 8 days. A single dose of 100 mg/kg of chloroquine was administered. Parasitemia was monitored with Giemsa-stained blood films.

No drug susceptibility assays have been performed with any of the *P. berghei* RC clones. Relative to infection with *P. berghei* ANKA or *P. berghei* SANA, the infection with *P. berghei* RC has 3 differences. The parasites seem to have a stronger reticulocyte preference, the infection proceeds slower, and infection in the absence of drug treatment does not necessarily result in anemia and death.

***An. stephensi* infection.** The presence of both male and female gametocytes of *P. berghei* SANA were confirmed by an experienced microscopist (GM). Three independent experiments were performed to infect *An. stephensi* mosquitoes with *P. berghei* ANKA and *P. berghei* SANA clones 3, 4, and 9. Following the blood feeding of infected

mosquitoes onto naïve mice, mice were monitored for patency. Each experiment resulted in a patent mouse for *P. berghei* ANKA, but no patent mice for *P. berghei* SANA were detected.

***P. berghei* exflagellation assay.** Two exflagellation assays were performed comparing *P. berghei* ANKA to *P. berghei* SANA clones 3, 4, and 9. Over 10 fields of view with exflagellation of *P. berghei* ANKA microgametocytes were observed. No visible centers of exflagellation were observed for any of the *P. berghei* SANA clones.

PCR and sequencing. The kelch13 gene was amplified for *P. berghei* ANKA and *P. berghei* SANA clones 3, 4 and 9 from genomic DNA (Figure 4.3). The mutations in the *P. falciparum* kelch13 gene which have most commonly been associated with the delayed clearance phenotype are C580Y and R539T (2). A sequence alignment of several different human and mouse *Plasmodium* species indicates that cysteine (580) is conserved within the genus. Following a sequence alignment between the *P. falciparum* (Pf3D7_1343700) and *P. berghei* (PbANKA_135670) kelch13 loci, the corresponding mutatios in *P. berghei* are C592Y and R551T. Following sequencing, a cysteine was present at position 592 in *P. berghei* SANA clones 3, 9, and *P. berghei* ANKA GFP-luciferase. Similarly, an arginine was present at position 551 in *P. berghei* SANA clones 3, 9, and *P. berghei* ANKA GFP-luciferase. The sequencing for *P. berghei* SANA returned poor quality sequence and needs to be repeated.

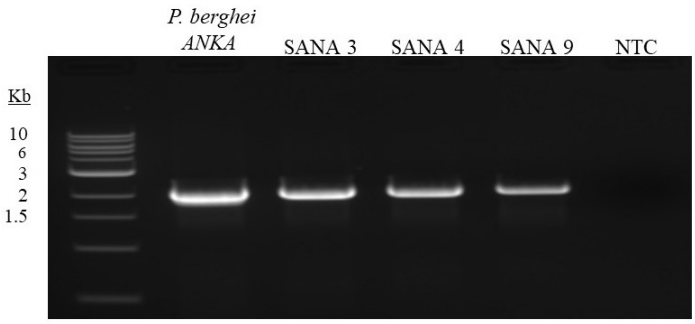
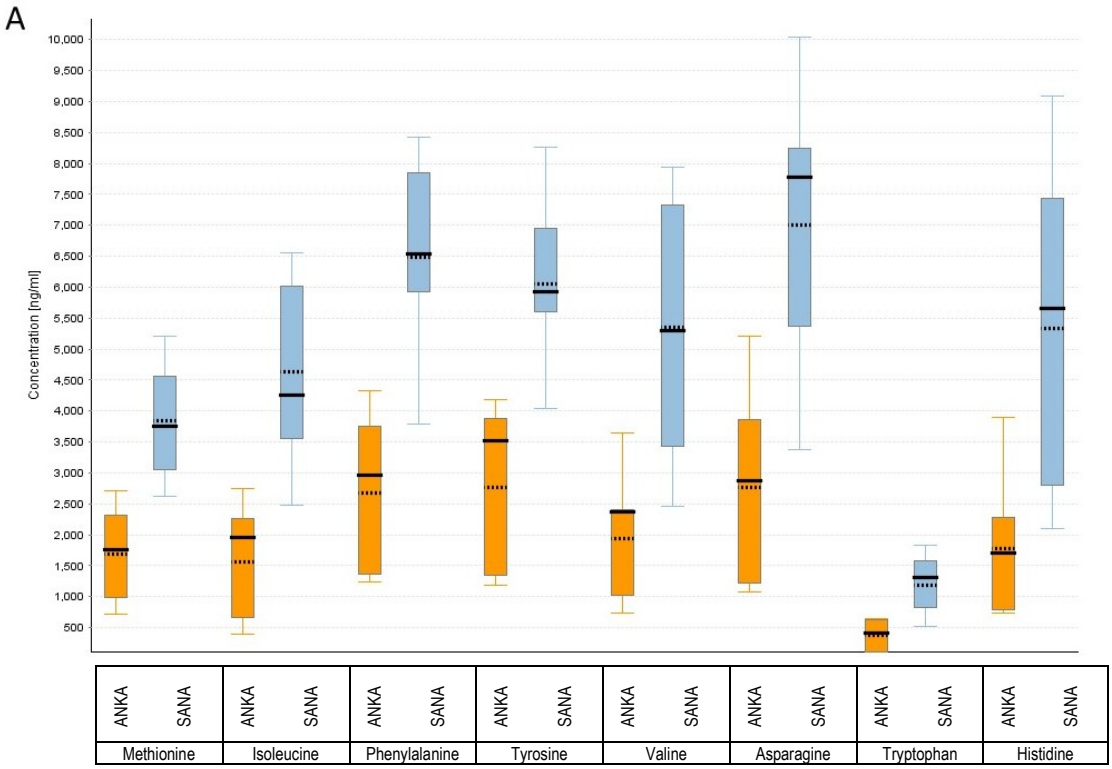


FIG 4.3 PCR amplification of P. *berghei* kelch13. PCR products of *P. berghei ANKA* and clones 3, 4, and 9 of *P. berghei* SANA were amplified and visualized on a 1% agarose gel.

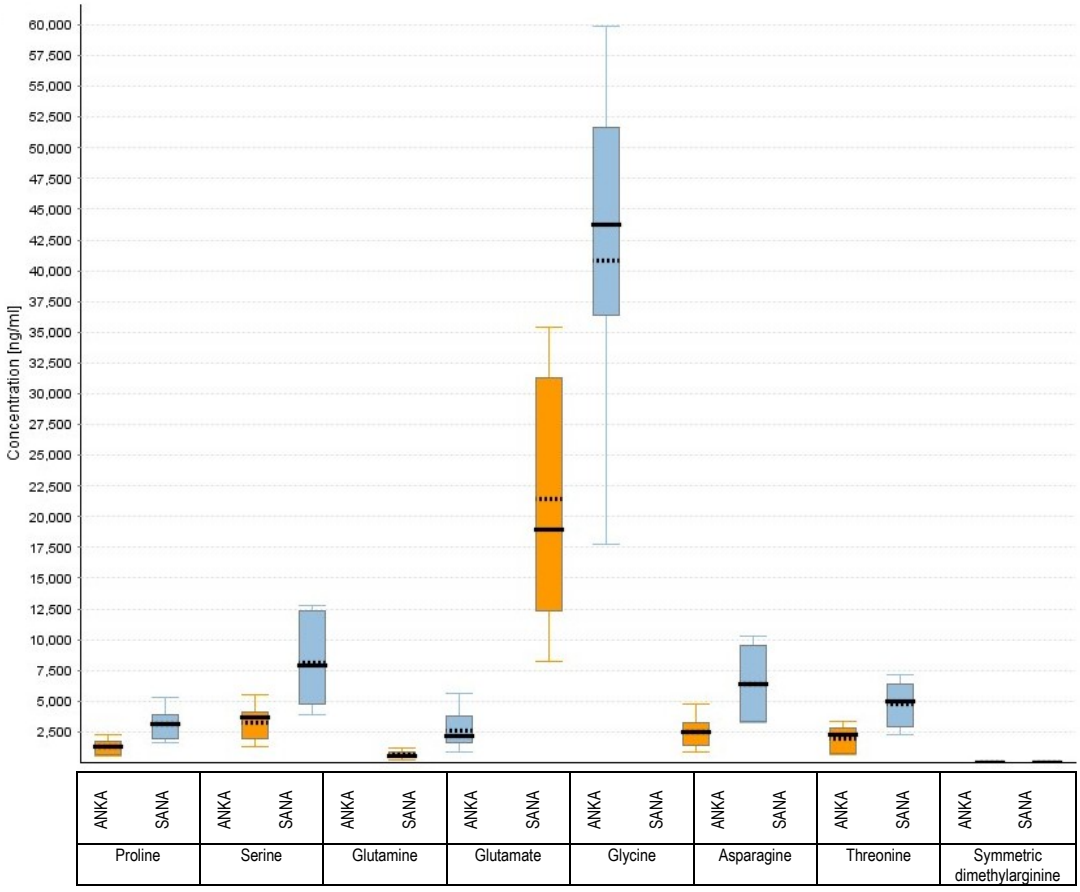
Amino Acid and Lipid Composition Analysis. The metabolomic and lipidomic profiles were compared for *P. berghei* ANKA and *P. berghei* SANA. In general, the SANA parasites had greater concentrations of amino acids than the *P. berghei* ANKA parasites; however, no statistically significant fold changes were observed (Figure 4.4). This trend was also observed for the lipids with, in general, more lipids present in *P. berghei* SANA (Figure 4.5).

One limitation of the amino acid and lipid data is that *P. berghei* ANKA does not have the same genetic background as *P. berghei* SANA. We were unable to obtain the *P. berghei* SANA parental strain, N. However, we did identify a parasite that shared the parental strain *P. berghei* N. This strain, *P. berghei* RC, had similarly undergone cycles of drug treatment for induction of chloroquine resistance. *P. berghei* RC was obtained and three clones were obtained in January 2018. However, as previously mentioned, the infection kinetics between *P. berghei* RC and *P. berghei* SANA vary significantly and thus, its utility as a relevant comparator may be limited.

FIG 4.4 Amino acid concentrations for *P. berghei* SANA and ANKA. Amino acid concentrations are compared for *P. berghei* ANKA (orange) and *P. berghei* SANA (blue) (A-C).



B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57,500
55,000
52,500
50,000
47,500
45,000
42,500
40,000
37,500
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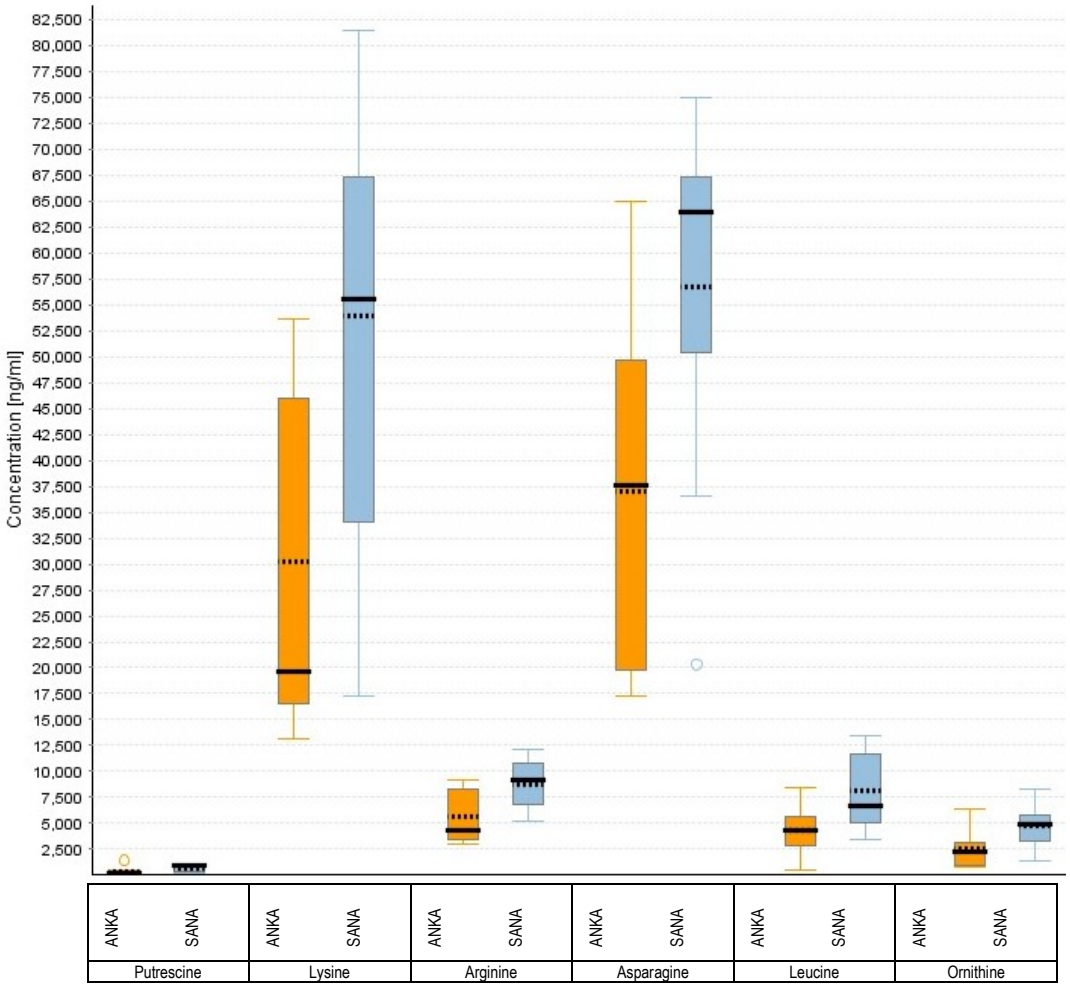
32,500
30,000
27,500
25,000
22,500
20,000
17,500
15,000
12,500
10,000
7,500
5,000
2,500
ANKA
SANA
Proline
Serine
Glutamine
Glutamate
Glycine
Asparagine
Threonine
Symmetric dimethylarginine

C


Concentration [ng/ml]
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75,000
72,500
70,000
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65,000
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60,000
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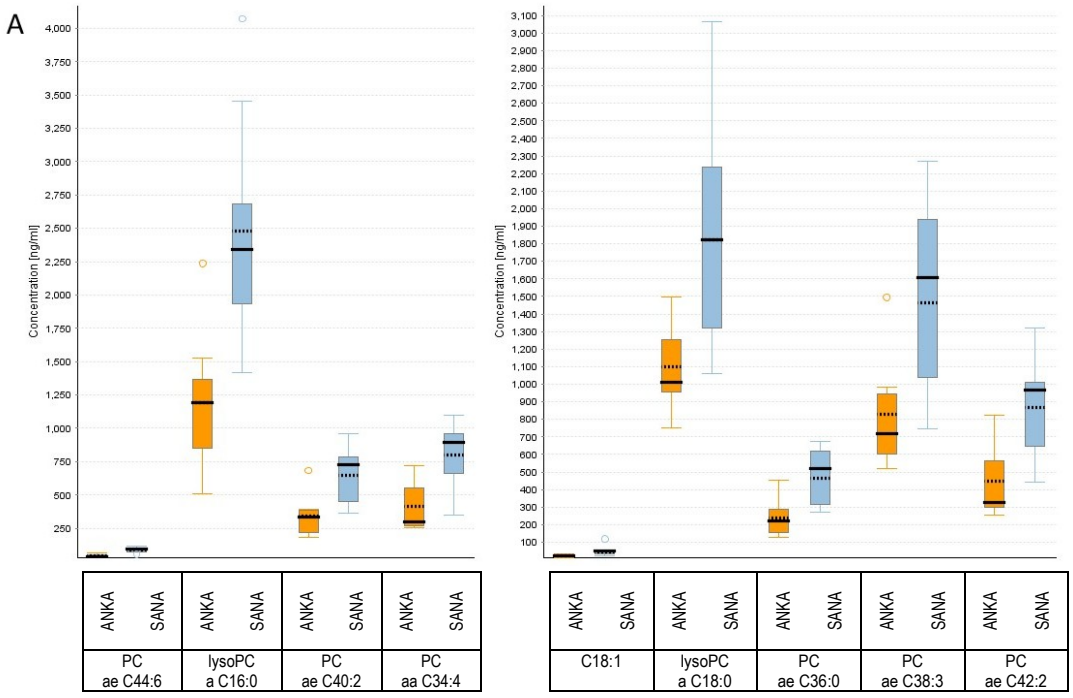
50,000
47,500
45,000
42,500
40,000
37,500
35,000
32,500
30,000
27,500
25,000
22,500
20,000
17,500
15,000
12,500
10,000
7,500
5,000
2,500
ANKA
SANA
Putrescine
Lysine
Arginine
Asparagine
Leucine
Ornithine

FIG 4.5 Lipid concentrations for *P. berghei* SANA and ANKA. Lipid concentrations are compared for *P. berghei* ANKA (orange) and *P. berghei* SANA (blue) (A-D). PC=phosphatidylcholine; lysoPC=lysophosphatidylcholine; SM=sphingomyelin



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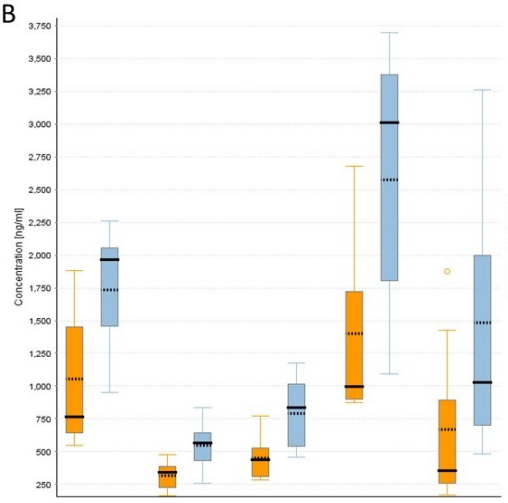
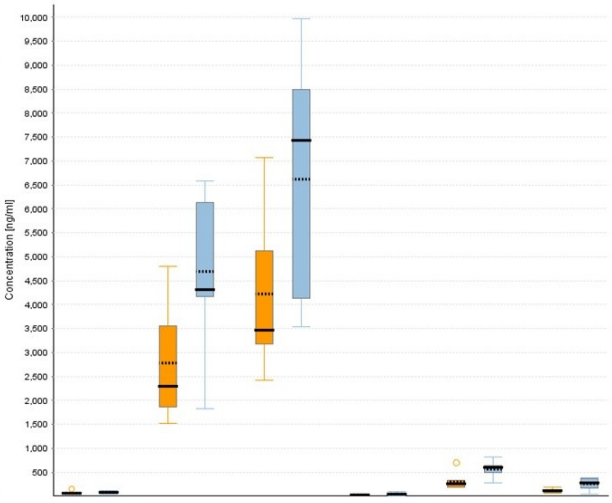
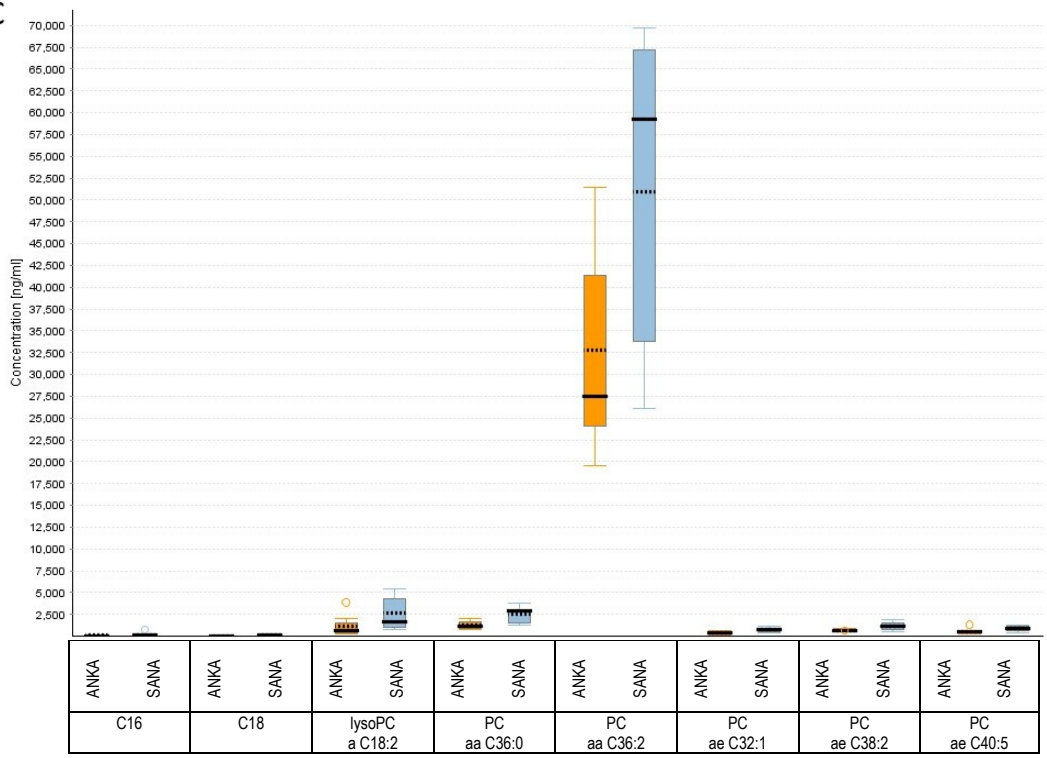


ANKA	SANA	ANKA	SANA	ANKA	SANA	ANKA	SANA	ANKA	SANA
PC aaC32:2		PC ae C36:5		PC ae C42:1		PC ae C42:3		lysoPC a C18:1	



ANKA	SANA	ANKA	SANA	ANKA	SANA	ANKA	SANA	ANKA	SANA	ANKA	SANA
PC aa C24:0		PC aa C30:0		PC aa C38.3		PC ae C30:2		PC ae C44:5		SM C26:0	

C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65,000
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55,000
52,500
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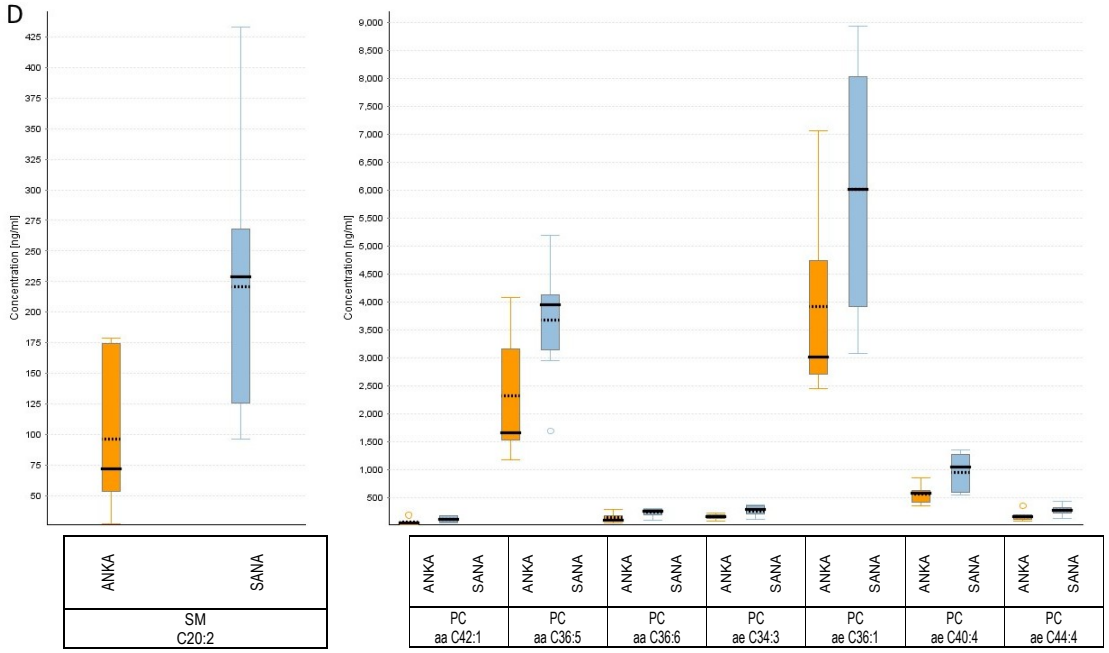
42,500
40,000
37,500
35,000
32,500
30,000
27,500
25,000
22,500
20,000
17,500
15,000
12,500
10,000
7,500
5,000
2,500
ANKA
SANA
C16
C18
lysoPC a C18:2
PC aa C36:0
PC aa C36:2
PC ae C32:1
PC ae C38:2
PC ae C40:5

D


Concentration [ng/ml]
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SANA
SM C20:2
PC aa C42:1
PC aa C36:5
PC aa C36:6
PC ae C34:3
PC ae C36:1
PC ae C40:4
PC ae C44:4

DISCUSSION

Lactate dehydrogenase elevating virus detection. As LDV has been noted to lead to altered immune function in mice, it was essential that the virus be removed from the parasite stocks prior to any drug efficacy assays. Mononuclear leukocytes are the hosts for LDV; therefore, separation of whole blood on a density gradient and limited dilution cloning of *P. berghei* SANA was able to separate parasitized erythrocytes from the source of LDV. As a precaution to remove any LDV, and to ensure a clonal population for drug assays, *P. berghei* RC was also cloned. Following our experimentation, stocks of *P. berghei* SANA will be resubmitted to ATCC so that this parasite may be available to other investigators.

***Plasmodium berghei* drug susceptibility testing.** It has been reported that in *P. falciparum* delayed clearance isolates, the ring stage is prolonged (9). As the ring stage is less susceptible to killing by drugs such as chloroquine and artesunate, it has been hypothesized that this altered asexual stage progress confers a fitness advantage against the artemisinins, which have *in vivo* half-lives of less than 1 hour (10, 11). Thus, we were interested to see if the *P. berghei* SANA parasites exhibited altered asexual development that resulted in a greater percentage of rings over trophozoites present in the peripheral circulation. The results in Table 4.3 indicate that there may be slightly more rings present in SANA clones relative to *P. berghei* ANKA. As this analysis was completed prior to any drug susceptibility assays, future experimentation should include a comparison of SANA clones 3, 4, and 9 relative to *P. berghei* ANKA. This line of inquiry can be further investigated by using a flow cytometric assay to distinguish between the asexual stages, which may provide more quantitative data.

The variability of artesunate susceptibility of the *P. berghei* SANA clones 3, 4, 5, 6, 7, and 9 suggests that the initial stock did not contain a clonal population. Choosing to proceed with clones 3, 4, and 9 ensured that there were 3 different biological replicates. Taken together, the results presented in Figure 4.1, Figure 4.2, and Table 4.4 indicate that *P. berghei* SANA clones do exhibit reduced susceptibility to artesunate. Interestingly, the 8 doses of 50 mg/kg of artesunate was found to be curative in experiments by Henrich *et al.* (6). This further confirms the importance of testing drug efficacy on a clonal population of parasites. The demonstration that the SANA clones are as sensitive to chloroquine treatment as they are to artesunate treatment indicates that this reduced drug susceptibility is likely artesunate specific. Of course, further drug susceptibility assays would be required to demonstrate that the SANA clones do not exhibit altered sensitivity to other drugs. It would be interesting to test the clones' sensitivity to other artemisinin compounds such as dihydroartemisinin, artemisinin, or artemether. The hypothesis is that the SANA clones would display a similar reduced susceptibility pattern and these assays would strengthen the argument that SANA exhibits reduced susceptibility to the artemisinins.

The variability in the infection dynamics of *P. berghei* RC may prohibit its use as a comparator for *P. berghei* SANA. Further experimentation will be required to determine the cellular preferences and differences in infection kinetics between *P. berghei* ANKA, *P. berghei* SANA, and *P. berghei* RC.

An. stephensi* infection and *P. berghei exflagellation. The inability to passage the *P. berghei* SANA clones through *An. stephensi* and the lack of microgametocyte exflagellation leads to the conclusion that *P. berghei* SANA does not produce infectious

gametocytes. In hindsight, the discovery that *P. berghei* SANA did not produce infectious gametocytes could have been expedited by two alterations to the experimental design. The exflagellation assay should have been performed prior to any attempts to passage *P. berghei* SANA through *An. stephensi*. Additionally, the presence of oocysts in the fed mosquitoes could have confirmed the presence or absence of an infection on day 14 following blood feeding.

PCR and sequencing. The sequencing data indicates that the analogous mutation to the most prevalent *P. falciparum* delayed clearance mutation, C580Y, is not present in the *P. berghei* SANA clones. This is one data point in support of our initial hypothesis that the decreased susceptibility to artesunate was not due to genetic changes. The literature has greatly expanded with publications identifying mutations in the kelch13 gene that are or are not associated with delayed clearance to the artemisinins. Future experimentation should include a re-analysis of the sequencing data to evaluate the presence of any of these mutations. Whole genome sequencing may provide important insights for genes outside of kelch13 that are contributing to the reduced artesunate susceptibility.

Amino Acid and Lipid Composition Analysis. There was no statistically significant difference between *P. berghei* ANKA (n=3) or the aggregate of *P. berghei* SANA (n=9) for the amino acid or lipid composition analysis. Future experimentation should evaluate the metabolomic and lipidomic profiles of *P. berghei* SANA and *P. berghei* ANKA in response to artesunate drug treatment. Then, the steady-state profiles could be compared to the drug response profiles. Additionally, it may be worthwhile to isolate a *P. berghei* SANA clone that is highly resistant to artesunate (ex. *P. berghei*

SANA clone 9) and focus further analyses on that clone. Future analyses should focus on identifying any alterations in lipids, metabolites, or transcripts that may be related to delayed clearance *P. falciparum*. For example, delayed clearance to the artemisinins is thought to arise, in part, from an increased ability of ring stage parasites to cope with cellular stress as well as an increase in the activity of *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K) (12). Thus, up regulation of transcripts related to the unfolded protein response, ubiquitination, and the proteasome may indicate a shared method of decreased susceptibility to the artemisinins between *P. berghei* SANA and *P. falciparum* delayed clearance parasites (13, 14). Similarly, it would be interesting to identify alterations in the level of the lipid phosphatidylinositol-3-phosphate (PI3P) in order to understand the role of PfPI3K in artesunate susceptibility. Furthermore, as it has been observed that *P. falciparum* delayed clearance isolates exhibit an altered progression through the asexual erythrocytic cycle, future studies should investigate the expression of stage specific transcripts between *P. berghei* SANA and an artesunate sensitive *P. berghei* (9, 13).

Conclusion. Many future experiments, such as transcriptomics, are needed to fully characterize the source of decreased artesunate susceptibility of *P. berghei* SANA. However, the data presented here is encouraging that SANA may be useful as a model of delayed clearance to the artemisinins in the future.

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CHAPTER 5

Conclusions & General Discussion

CONCLUSIONS

This thesis lays the groundwork for many more experimental opportunities in the laboratory. The transgenic luciferase-reporter *P. berghei* in a cytocidal assay is currently being used in three lines of inquiry: to compare the parasite clearance rates of the quinoline compounds, to identify *in vivo* synergy between currently approved antimalarials, and to compare the effect of parasite density on the rate and efficacy of parasiticidal activity of different antimalarials. Development of this *in vivo* model, which evaluates drug efficacy in high density infections, allows a more accurate understanding of the necessary activity of antimalarial drugs for the treatment of human infections. This assay could be further expanded upon by studying the pre-erythrocytic and sexual stages of the parasite.

Beyond the continued use of the cytocidal luciferase assay in the laboratory, this work has led to two key conclusions on how to most effectively implement antimalarial treatment regimens. First, the data has indicated that the treatment duration of the artemisinin component of artemisinin combination therapies is a significant contributing factor to therapeutic efficacy. Specifically, expanding the treatment duration from targeting the parasite over a single asexual life cycle to targeting the parasite over three asexual life cycles may enhance the efficacy of our first line antimalarials. Second, data has indicated that the efficacy of fosmidomycin and clindamycin can be enhanced by increasing the daily dosing regimen from twice to four times. This work has also shown that fosmidomycin and clindamycin are effective against artemisinin delayed clearance *P. falciparum* isolates. The well-established safety profiles of fosmidomycin and

clindamycin, coupled with this research, suggests that fosmidomycin and clindamycin are important to consider in the malaria treatment landscape with a proper dosing schedule.

Additionally, the work performed in the *P. berghei* SANA model indicates that we may be on track to identifying an *in vivo* model of artemisinin-tolerant parasites. Evaluating the pharmacodynamics of novel antimalarials on artemisinin-tolerant parasites may prove to be an important pre-clinical tool. This model is limited by its requirement of generating parasitemia counts with Giemsa-stage blood films; however, in the future, it may be possible to insert the GFP-luciferase transgene into this strain.

GENERAL DISCUSSION

Several questions remain that may be addressed in the *P. berghei* ANKA-GFP luciferase model or the *P. berghei* SANA model. One particular line of inquiry may be to investigate the phenomenon known as parasite dormancy, which has been observed in *P. falciparum* isolates that have been treated with sorbitol or antimalarial drugs *in vitro* and artemisinin compounds *in vivo* (1, 2). Dormant parasites are ring stage parasites, which enter a state of arrested development characterized by reduced metabolic rate, condensed nuclei, condensed cytoplasm, and reduced drug susceptibility. Dormant parasites are not considered to have acquired drug resistance as, once they emerge from dormancy, they retain the drug sensitivity of the initial clonal population. Rather, it has been suggested that dormancy is a response to general stressors. It has been suggested that the *P. berghei* transcriptional response to the artemisinins is similar to that observed in *P. falciparum* (3). Thus, it would be interesting to investigate the concept of dormancy in *P. berghei*. Is dormancy in response to the artemisinins observed in either of our mouse models? Is

dormancy triggered *in vivo* by drugs other than the artemisinin compounds? What can be done to cause the parasites to exit from dormancy and resume normal metabolic activity? Is dormancy a stochastic event, or are certain merozoites programmed to be dormant, similar to a merozoite's commitment to become a gametocyte? Beyond the question of dormancy, either *P. berghei* model may be used to evaluate the efficacy, identify potential synergies, and optimize the dosing regimens of novel antimalarial candidates as they are identified. Specifically, it would be interesting to further validate this model's utility in having predictive accuracy of necessary treatment durations for cure in humans. While the current treatment landscape is focused on 3 day treatments, there is much historical data on various monotherapies and the corresponding clinical outcome. A comparison of existing human data with monotherapy data obtained in our model for drugs such as fosmidomycin, chloroquine, amodiaquine, and the artemisinins may provide insight into how well our model predicts successful human treatment.

Looking forward, there are several promising antimalarial drugs in the pipeline. Specifically, the spiroindolone KAE609, the imidazolopiperazine KAF156, and the triazolopyrimidine DSM265, all which have novel chemical scaffolds relative to current antimalarials (4). KAE609, an inhibitor of *PfATP4*, has activity against both the asexual stages and sexual stages of *P. falciparum*, and resulted in parasite clearance times of 12 hours against *P. falciparum* (n=11) and 8 hours against *P. vivax* (n=10) in a small Phase II trial with a three day treatment regimen (5). KAF156 has an unknown mechanism of action, but has demonstrated *in vitro* activity against *P. falciparum* and *P. vivax, in vivo* activity against *P. berghei* sporozoites, and *in vitro* activity against *P. falciparum* gametocytes (6). In a small population of Thai and Vietnamese patients, a three day

regimen of KAF156 demonstrated median parasite clearance times of 44 hours against *P. falciparum* (n=10) and 24 hours against *P. vivax* (n=10). DSM265, an inhibitor of dihydroorotate dehydrogenase, was shown to sterilely protect 33% of individuals following a controlled human infection model with *P. falciparum* sporozoites (7). While these results are encouraging, it is unlikely that any of these compounds will be ready for widespread clinical use before 2026 (8). Thus, optimizing our currently approved antimalarials based on pharmacokinetic and pharmacodynamic principles is crucial. There has certainly been an increased focus on the pharmacokinetics of the pipeline compounds; however, the interaction of pharmacokinetics with the parasite's stage-specific drug susceptibility and the effect of treatment duration have largely been ignored. Research outlined in this thesis suggests these are two critical factors for the success of antimalarial drugs and we believe the field of antimalarial chemotherapy would benefit from an increased emphasis on treatment duration and the relationship between parasite stage-specific drug susceptibilities and pharmacokinetics.

Continuing to progress towards malaria elimination for more countries will undoubtedly require a multifaceted approach involving novel vector control methods, vaccine development and strategic deployment, bolstering of the healthcare infrastructure, and maximizing the efficacy of our antimalarial drugs. History has shown us that the evolutionary drive of the parasite will likely overcome the challenges that we put in place to try to decrease the incidence of new infections. In fact, increases in a key gametocyte transcription factor, AP2-G, have recently been reported in areas of low transmission, suggesting that the parasite is adapting to changing transmission dynamics as malaria control efforts intensify (9). Changing transmission dynamics from hyper-

endemicity to hypo-endemicity will require public health officials and scientists to adapt new control strategies. Emphasis will be increasingly placed on detection and treatment of asymptomatic malaria cases in order to eliminate the infectious reservoir of parasites to the mosquito. This raises questions about how antimalarial drugs will be deployed in the future. Will mass drug administration or seasonal chemoprevention become more widespread control strategies? Novel drugs, surveillance and diagnostic tools, and increased infrastructure will certainly aid in the future fight against malaria. In the near term, public health officials and malariologists must manage to do better with currently available drugs. This body of work informs those efforts by demonstrating, in a cytocidal *in vivo* model, how the efficacy of currently available antimalarials can be improved.

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EDUCATION

Johns Hopkins Bloomberg School of Public Health – *Baltimore, MD* expected May 2018
PhD, Molecular Microbiology and Immunology
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University College London – *London, United Kingdom* Sept 2012
MSc, Infection & Immunity *(with Distinction)*
Nominee, UCL Faculty of Medical Sciences Dean's Research Prize

University of Notre Dame – *Notre Dame, IN* May 2011
BS, Biological Sciences, Minor in Africana Studies

RESEARCH EXPERIENCE

Johns Hopkins University PhD Researcher Sept 2013 - present
- Identified more efficacious malaria treatment regimens by investigating drug pharmacodynamics in newly developed assay
- Presented research at 5 national and international conferences with 250+ attendees and 6 departmental and school-wide seminars with 50+ attendees
- Trained and advised 3 students in technical expertise and oral presentation skills

University of Pittsburgh Laboratory Manager Jan 2013-Sept 2013
- Produced and analyzed datasets of >30k miRNA leading to candidate biomarker identification for HIV-associated dementia

BUSINESS DEVELOPMENT EXPERIENCE

Johns Hopkins Technology Ventures Business Development Fellow Oct 2016 - present
- Identify and summarize JHU research specialties for prospective sponsored research agreements with 5 industry partners
- Collaborate with 3 academic and 1 industry partner on research consortium launch aiming to develop novel cancer therapies
- Evaluate commercialization potential of JHU invention disclosures

LEADERSHIP EXPERIENCE

Co-Organizer of Inaugural Young Investigator Malaria Symposium Oct 2015
- Selected by Nobel Laureate Peter Agre to chair conference of young investigator-driven research as part of 3 member team
- Conceived of the scientific program and organized logistics for 13 oral presentations and >55 poster presentations
- Recruited >160 attendees from 18 countries, achieving 100% capacity a month in advance of registration deadline

Johns Hopkins Biomedical Careers Initiative Student Advisory Group Member Jan – Dec 2017
- Coordinate with 8 member team to develop programming and internship opportunities for non-academic career tracks

PUBLICATIONS

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