PHARMACODYNAMICS OF PYRONARIDINE IN COMBINATION WITH CURRENT ANTIMALARIALS IN A CYTOCIDAL MURINE MALARIA MODEL

by

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Abstract

While there has been a progress in understanding *P. falciparum* genomics, and exciting discoveries of promising leads in the antimalarial pipeline, the efficacies of existing artemisinin combination therapies are threatened by incorrect dosing and non-compliance with duration of dosing regimen, which might hasten the emergence of resistant parasites. Understanding of the effects of drug interactions on combination therapy with existing antimalarial agents may provide useful information such as their mechanism of action, and influence the development of new drug combination. A traditional way of evaluating antimalarial drug efficacy and combined drug interactions has been relying on *in vitro* drug sensitivity assays using cultured cells. This method measures the rate of suppression or inhibition of parasite growth during 48 – 72 hours in comparison to untreated controls. However, extrapolating *in vitro* results from cultured parasites to humans remains challenging as cytocidal activity of antimalarials (reduction in absolute parasite numbers)

in patients is measured at a high asynchronous parasitemia at close to 1 trillion (per adult).

In this thesis, we adapted a transgenic luciferase reporter, *P. berghei* ANKA to study cytocidal activity of tafenoquine and pyronaridine in mice for combinations of three current antimalarials, artesunate, amodiaquine, and azithromycin at clinically relevant dosage. We initiated each drug treatment at a high parasitemia (~10%) and followed for 30 days. Three metrics of measures we used in characterizing pharmacodynamic properties are 30-day survival, parasite log reduction over 48 hours, and recrudescent parasitemia (i.e., return to initial parasitemia). We adapted the parasite reduction ratio (PRR), or fractional log reduction in parasitemia as the PRR can be analogous to the killing rate, thus serving as a measure to compare the different intrinsic pharmacodynamic between the drugs in question. Pyronaridine when compared to tafenoquine at the same dose exhibited more potent cytocidal activity with approximately 1,000-fold more reduction in the number of parasite over the single 24-hour parasitic life cycle. Tafenoquine synergized with artesunate. In contrast, additive interactions were evident for pyronaridine in combination with artesunate. In addition, while pyronaridine synergized amodiaquine, differential interactions were shown with azithromycin, suggesting that synergy may only be achieved at certain dose ratios.

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An introduction to *Plasmodium* spp. and antimalarial chemotherapy

1.1 Malaria

Malaria, a mosquito-borne, acute febrile illness caused by a protozoan parasites of the genus *Plasmodium,* remains one of the life-threatening infectious diseases with 216 million cases and 445,000 deaths(1). In humans, five different *Plasmodium* species, namely *P. falciparum, P. vivax, P. ovale, P. malariae*, and *P. knowlesi* can cause malaria through the bites of infected female mosquitoes of the genus *Anopheles.* While other *Plasmodium* species remain important causes of significant disease, including severe illness, *P. falciparum* and *P. vivax* pose the greatest public health challenge globally. Infection with *P. falciparum* is most

prevalent on the African continent where more than two thirds (70%) of all fatal malaria cases occur in children under age 5. *P. vivax* has a wider geographical distribution, covering most countries outside of sub-Saharan Africa owing to its ability to develop in the *Anopheles* mosquito at lower temperatures (below 20°C) and survive at higher altitudes. In case of *P. vivax* and *P. ovale* malaria, parasites have an advantage of having a dormant liver-resident hypnozoite stage, producing no clinical manifestation, but causing recrudescence or relapse months or years after initial antimalarial treatment (2, 3)

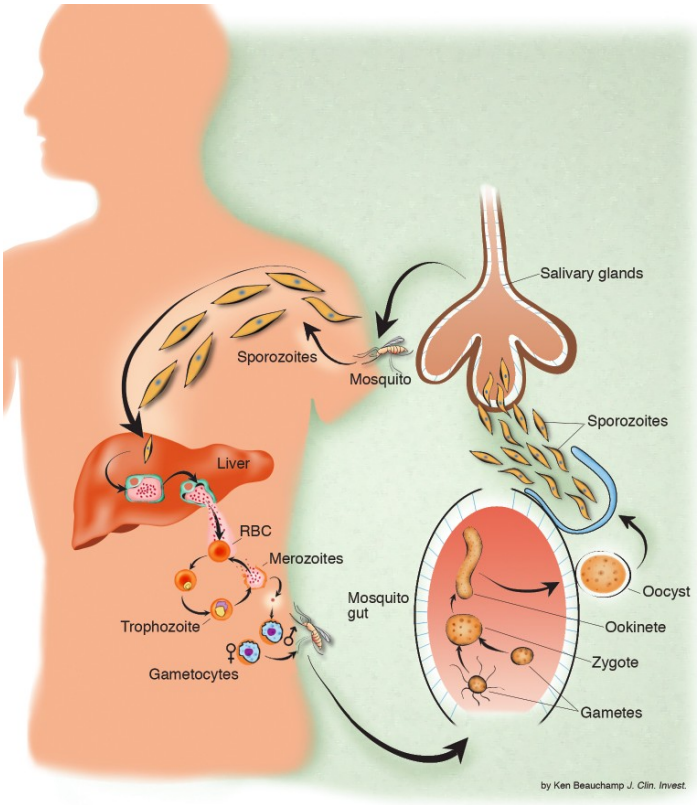


Figure 1.1 *Plasmodium* Life Cycle. Stages in both human host and mosquito vector. Reprinted by permission from American Society for Clinical Investigation: the Journal of Clinical Investigation. "Antimalarial drug resistance" by Nicholas J. White, copyright (2004).

Plasmodium spp. have a complex life cycle. A complete cycle involves both humans and female *Anopheles* mosquitoes and distinct forms at specific life stages to invade different cell types (Fig. 1.1). Human-to-human transmission of malaria occurs through an infected female *Anopheles,* serving as a vector. In vertebrate hosts, the parasites are injected as highly motile "sporozoites" into the host dermis during a blood feed. Only a proportion exits the dermis, entering the bloodstream and quickly transported to the liver, where the sporozoites invade hepatocytes. The remaining parasites in the skin are cleared by a host immune response. Upon successful infection of hepatocytes, the sporozoites develop to an exo-erythrocytic form in the liver, releasing up to 40,000 merozoites per hepatocyte into the bloodstream in packets of merosomes. Free merozoites can infect erythrocytes at any age within a very short time, and the parasites develop to asexual, erythrocytic (ring) stage. While little is understood about the molecular details of interactions with erythrocytes, merozoites surface protein 1 (MSP1), the major glycophosphatidylinositol (GPI)-associated protein on the merozoites surface, is largely involved in merozoites invasion of erythrocytes (4). The initial interactions of merozoites are irreversible, resulting in deformation of the red cells. Once erythrocytic infection is established, the parasites take 48-hour life cycle to asexually multiply approximately tenfold in the red cells (erythrocytic schizogony) and mature into schizonts which then eventually rupture, releasing a brood of merozoites to invade new host cells. The blood stage parasites are responsible for the clinical symptoms of malaria. In fact, malaria pathology in human hosts is caused by *Plasmodium*-infected erythrocytes binding to vascular endothelial cells in various organs, such as the heart, brain, kidney,

lungs and liver(5). The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, extremely diversified proteins expressed on the surface of the *P. falciparum*-infected red cells, plays the central role in host immunity and pathogenesis as their display exposes them to host antibodies. PfEMP1 proteins, however, are able to evade immune destruction, thus escaping splenic clearance, and maintain a chronic infection via antigenic variation. In addition to PfEPMP1 family, a wide array of extrinsic proteins is exported to the infected erythrocytes. This process of renovation of erythrocyte structure allows parasites to scavenge nutrients and hide from host immune responses(6). The main source of food for *Plasmodium spp.* is the host cell hemoglobin. The parasites digest hemoglobin and crystalize heme, a potentially toxic byproduct of the process, producing hemozoin (or malaria pigment). At some points in a schizogony cycle, merozoites in an infected red cell develop into either asexual schizonts or sexual male or female gametocytes. While molecular details of this transition remain inconclusive, it is evident that parasites can regulate gametocyte production by sensing their environmental stimuli e.g., high parasitemia and drug exposure (7)).

The sexual stage of *Plasmodium* begins when gametocytes are ingested by a female *Anopheles* mosquito during a blood feed. A zygote is formed after the fusion of gametes in the mosquito's stomach. The zygote develops into motile and elongated ookinetes which can penetrate into the mosquito's midgut and develop into the next stage, oocysts. The oocysts undergo multiplication, result in explosive release of sporozoites. The free sporozoites travel to the mosquito's salivary glands, and are injected with the mosquito's saliva during a blood meal.

Clinical symptoms of human malaria infection in a non-immune individual involve fever, chills and non-specific headache and muscle pains approximately 14 days after the infective mosquito bite. When *P. falciparum* malaria is untreated or partially treated, initial infection progresses to severe malaria, which may lead to anemia, multi-organ failure and death(8). Asymptomatic infections may occur in older children and adults with naturally acquired immunity in stably endemic areas in which exposure to infected mosquitoes is high, and thus transmission level is more consistent year-to-year. In the case of *P. vivax* and *P. ovale* malaria, a prolonged treatment is required as reactivation of the dormant liver-resident hypnozoite may occur at intervals. Historically, due to a very low incidence of deaths reported from *P. vivax* malaria, it has been regarded as a relatively benign parasite. However, this perception is increasingly challenged by recent studies reporting a substantial amount of morbidity, severe disease, and mortality due to *P. vivax,* particularly among infants(9). Generally, malaria case fatality is much higher in individuals exposed to multiple and concurrent health risks such as HIV infection. Malaria infection is also associated with an increased prevalence of invasive bacterial disease and inflammatory bowel disease (IBD)(10, 11).

Accurate and prompt diagnosis of malaria remains a critical factor for effective disease management and surveillance. Due to variable and non-specific clinical symptoms of malaria, parasite-based diagnostic testing is recommended prior to initiating antimalarial treatment on patients with suspected malaria. Such practice helps prevent the emergence and spread of antimalarial drug resistance by limiting antimalarials use to test-positive cases. Microscopy of blood films with Giemsa stain and malaria rapid diagnostic

test (RDT) are the two primary options for diagnosing malaria. Three most vital pieces information that microscopy diagnosis can provide are the presence of parasites in patient's blood, identification of *Plasmodium* species and the parasitemia. RDT dipstick is another rapid way of detecting specific malaria antigens in a patient's blood specimen. The test is able to differentiate between antigen specific for *P. falciparum* and one found in all 4 human malaria species. RDTs, however, may fail to detect infections with low parasitemia, and *P. ovale* and *P. malariae* malaria. Therefore, microscopy diagnosis of malaria is required to speciate or quantify the parasite load. In addition to microscopy and malaria RDT, serology and molecular diagnosis are available. While these techniques may be more sensitive than microscopy and RDT, serology measures previous exposure, thus not representing the current infection, and molecular diagnosis using polymerase chain reaction (PCR) is not readily available in many resource-limited, malaria endemic countries.

Historically, malaria prevention tools, including indoor residual spraying (IRS), insecticide-treated bed nets (ITN), and environmental strategies such as reduction or permanent destruction of mosquito larval habitats have contributed greatly to malaria control and elimination efforts. IRS prevents human-to-human transmission by targeting endophilic mosquito's resting behaviors. A few examples of key malaria vectors in sub-Saharan Africa that IRS is most successful with killing are *Anopheles gambiae s.s.* and *Anopheles funestus*. ITN provides a protective barrier around people sleeping under them by killing and repelling mosquitoes, as well as other insects. Pyrethroids are the only chemicals approved for use on ITNs. In addition to IRS and ITN, the development of

vaccines against malaria has been a major focus of the malaria research since the early 20th century. Many of these studies can be broadly categorized into three approaches: pre-erythrocytic stages which include sporozoites and hepatocyte invasion to stop establishment of blood stage infection, asexual stages to control clinical symptoms, and sexual stages to interrupt the transmission cycle between human and mosquito vectors. The majority of vaccine attempts have been focused on the most significant strain, *P. falciparum* using circumsporozoite protein (CSP), a highly repetitive region on the sporozoites. However, the complexity of *Plasmodium* complicates the development of preventive malaria vaccine. Highly polymorphic nature of *Plasmodium* antigens and poor immunogenicity are few examples. Ideally, a safe and effective malaria vaccine induce sterile protection with as few doses as possible, and an ideal strategy may be targeting multiple lifecycle stages. With the current inconclusive understanding of the human immune system and its interactions with malaria parasites, malaria control today heavily relies on safe and effective antimalarial drugs for both treatment of established infection and prevention of malaria.

1.2 Antimalarial chemotherapy

The primary goal of antimalarial drug treatment in malaria is to kill asexual, intraerythrocytic-stage parasites (the stage that causes disease), thereby reducing parasite number and leading to their immediate removal from the peripheral circulation(12). Most antimalarial drugs target these stages, and are called blood schizonticides. Today, the use of single drugs is not recommended in the clinic for various reasons, including the

emergence of resistant parasites. Current international guidelines recommend the use of artemisinin-based combination therapy (ACT) as a first-line treatment on a three-day course schedule, as artemisinin derivatives, particularly, artesunate, can rapidly reduce the parasitic load by at least 10,000-fold within 48 hours of the parasitic life cycle, resulting in greater than 95% clearance of initial infection. Based on the mode of action, common partner drugs for ACTs are classified into three groups: antifolates, quinolines (4-aminoquinolines, quinoline alcohols and 8-aminoquinolines), and antibiotics.

Artemisinin derivatives

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 (DHA)(13). Artemisinin derivatives and DHA are activated by iron and or heme to cause radical damage to surrounding proteins, resulting in reduction of the total parasite burden more rapidly than other antimalarials(14). While fast-acting schizonticidal activity remains the greatest advantage, both artemisinin derivatives and DHA have a short half-life of less than a few hours, requiring a partner drug with a longer half-life to clear residual parasites(15, 16).

Antifolates

Antifolate antimalarial drugs (e.g., pyrimethamine, sulfonamide) interfere with folate metabolism, a pathway essential to malaria parasite survival, thereby

interrupting the synthesis of DNA(17). Similar to other antimalarial drug classes, the antifolates appear to affect only the actively dividing schizonts rather than the young parasites in their early stages during the first 24 hours(18). This class of drugs include effective causal prophylactic and therapeutic agents against *P. falciparum*, some of which act synergistically when used in combination. Unfortunately, due to mutations in the genes in two key target enzymes i.e., dihydrofolate reductase (DHFR), dihydropteroate synthase (DHPS), resistance to the antifolates has arisen relatively rapidly and is now common worldwide(19).

Quinolines

The quinoline-containing drug class includes some of the most commonly used antimalarials, such as aryl-amino alcohols quinine and mefloquine. Quinine, a component of the bark of the cinchona tree offered the most effective treatment of malaria as early as the 1600s with cure rates of greater than 98%(20). Quinine continues to play a major role today in the management of malaria in pregnancy, although its use for the treatment of uncomplicated malaria has been replaced by newer and better tolerated therapies such as ACT. The primary mode of action of quinine involves interfering with heme detoxification process of intra-erythrocytic-stage parasites. Quinine and structurally related compound, mefloquine share similar modes of antimalarial action against *falciparum* and *vivax* malaria as noted by Schmidt in 1978(21). One example is quinine- and mefloquine-induced morphological changes in the early ring stages. While

resistance to quinine and mefloquine, and cross resistance between these two compounds limited the clinical usefulness of these compounds, particularly in Southeast Asia, mefloquine remains a priority antimalarial chemoprophylaxis for travelers to high risk malarial endemic areas(22, 23).

The rest of quinoline-related compounds can be broadly categorized into three classes: 4-aminoquinoline, quinoline alcohols and 8-aminoquinoline. The primary mode of action for the non 8-aminoquinoines is much like that of quinine, involving interference with intraerythrocytic parasite, particularly its heme detoxification by inhibiting the dimerization or crystallization process that produces hemozoin. A number of key 4-aminoquinoline compounds include chloroquine, pyronaridine, and amodiaquine. For the purpose of the present study, the following discussion is focused on pyronaridine, amodiaquine, and tafenoquine.

4-aminoquinolines

- **Pyronaridine** was first synthesized in 1970 in China, and developed for the treatment of acute uncomplicated *P. falciparum* and blood stage *P. vivax* malaria. In previous *in vitro* and *ex vivo* studies, the drug demonstrated high efficacy against both chloroquine and amodiaquine-resistant strains of *P. falciparum*(24, 25). Pyronaridine is a promising therapeutic partner to use in artemisinin combination therapy, as it appears to delay the development of resistance and show synergistic interactions with

artemisinin derivatives, such as dihydroartemisinin (DHA) and artesunate, against strains resistant to the individual components or both in *in vivo* animal studies(26, 27). The drug is administered as pyronaridine tetraphosphate (56.89% free base), and as with other 4-aminoquinolines, pyronaridine works by interfering with parasite's food vacuole, and inhibits the heme crystallization by blood stage parasite(28, 29).

- **Amodiaquine** is structurally related to chloroquine and pyronaridine, and has a potent blood schizonticidal activity against chloroquine-resistant strains of falciparum malaria in humans (30). While it was widely used in the past for both treatment and prevention, the clinical use of amodiaquine, specifically for prophylactic use has been severely limited today because of associations with hepatotoxicity and agranulocytosis (31). When used as treatment, however, amodiaquine has a number of advantages, including affordability, efficacy against many (not all) chloroquine-resistant strains of *P. falciparum*, and relatively fair tolerability when used as treatment(32). In a randomized trial led by Muller et al in 1996, amodiaquine demonstrated its superior efficacy over chloroquine in the treatment of uncomplicated malaria in Gambian children (33). While the drug shares a similar mode of action with other 4-aminoquinolines, and cross-resistance

is possible, its unique ability to remain at high concentration in parasitized erythrocytes in falciparum malaria explains its effectiveness in previous *in vitro,* and *in vivo* studies in areas with a high level of chloroquine resistance (34-36).

8-aminoquinolines

While mechanisms of action have not been clearly defined, the 8-aminoquinolines e.g., pamaquine, primaquine and tafenoquine show a number of attractive antimalarial properties, including causal prophylactic activity as well as acting against the transmission-stage gametocytes. Particularly, primaquine has a marked gametocytocidal activity against *P. falciparum,* and remains the drug of choice for treatment of vivax (and ovale) malaria, as radical cure (eradication of the dormant liver hypnozoites) can only be achieved by the drug. However, a short biological half-life (4-6 hours), lengthy treatment course of 14 days, and severe side effect i.e., hemolysis to people who are deficient in glucose-6-phosphate dehydrogenase (G6PD) limit its use. Tafenoquine, a new synthetic primaquine analog with a longer elimination half-life (14-28 days) and fewer adverse effects, has demonstrated greater activity than primaquine against blood- and liver-stage parasites *in vitro,* including multi-drug resistant *P. falciparum* strains, and *in vivo* animal studies(37-39). However, like primaquine, tafenoquine can cause G6PD deficient patients to experience hemolysis. Tafenoquine is formulated as salt, and

dosed as tafenoquine succinate salt whose free base is 80% of the total salt (40). Its mode of action against erythrocytic parasites is similar to that of 4-aminoquinolines, with an additional mechanism involving interference with mitochondrial function of parasites in the liver- and gametocyte stage(41, 42). While currently not approved for use, tafenoquine is in stage 3 of clinical trials, and presents a potential alternative to primaquine for both prevention and treatment of malaria. *In vivo* studies of drug interactions of tafenoquine with the quinolones or artemisnins are understudied.

Naphthoquinones atovaquone

Atovaquone is a hydroxynaphthoquinone, used for both the treatment and prevention of malaria in fixed combination with proguanil (Malarone®). The combination is highly synergistic *in vitro* and effective against strains that are resistant to a variety of other antimalarial drugs(43). Clinical studies demonstrated enhanced efficacy of atovaquone in combination with proguanil with a significant reduction in the rate at which parasites resistant to both drugs develop(44). The mode of action and synergy with proguanil are not fully understood while the combination is believed to act on the parasite in the early liver stages of *P. falciparum*, and the erythrocytic stages of *P. vivax, P. ovale, and P. malariae*, and atovaquone acts primarily on mitochondrial functions, inhibiting the electron transport system at the level of cytochrome bc_1 complex(45, 46). Malaria

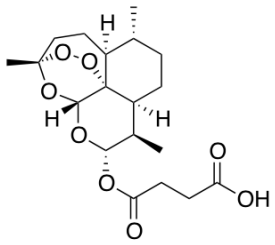
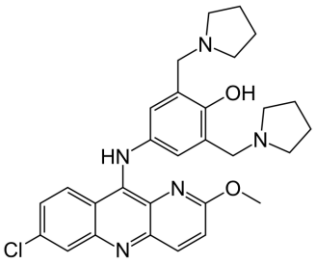
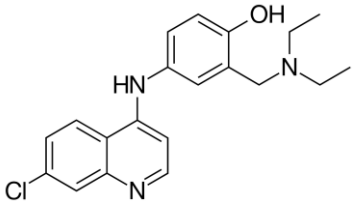
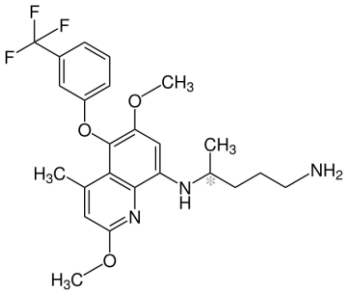
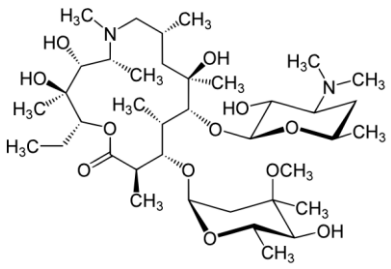
parasites require a coupling of pyrimidine biosynthesis and electron transport, and the novel mechanism of action of atovaquone, in fact, takes an advantage of the fact that *Plasmodium spp.* are dependent on *de novo* pyrimidine biosynthesis while mammalian cells can simply salvage and recycle pyrimidines(47). The inability of parasites to retrieve premade pyrimidines results in atovaquone blocking nucleic acid synthesis and thus replication.

Antibiotics

The greatest advantage of using antibiotics in the treatment of malaria comes from the fact that many antibiotics have mechanisms of action likely to be different from those of most traditional antimalarials. This could contribute to combination therapies as the probability of developing drug resistance is greatly reduced. Antibiotics inhibit the translation machinery of the apicoplast which is essential for the synthesis of fatty acids, isoprenoids, and heme, thereby arresting the parasites in the schizont stage and preventing further formation of merozoites in the following replication cycle. Interestingly, this unique activity of antibiotics leads to a slow killing of the parasite, affecting the next replication cycle rather than the time point when the antibiotic is first introduced(48-50). This is termed "delayed death effect," and is observed in most antibiotics active against malaria, including clindamycin and azithromycin(51, 52). Antibiotics are used in combination with traditional antimalarial drugs, particularly against multidrug-resistant *P. falciparum.* A number of successful cases in the past include

combination of tetracyclines with quinine and mefloquine to treat multidrug-resistant *P. falciparum* in Thailand, and the artesunate-tetracycline combination in Brazil(53, 54). However, the use of tetracyclines is limited by its poor safety profiles in young children and pregnant women. Clindamycin is commonly combined with either chloroquine or quinine to treat children with uncomplicated falciparum malaria(55). Erythromycin has demonstrated its potent antimalarial activity against *P. falciparum in vitro* and rodent malaria, *P. berghei in vivo* in mice (56, 57). Azithromycin, a structurally similar derivative of erythromycin is a macrolide antibiotic with a superior toxicological profile, and offers a number of advantages, including its safety in both children and pregnant women (58). The drug has demonstrated its efficacy for the treatment of uncomplicated falciparum malaria when used in combination with artesunate(59). In another *in vitro* combination study of azithromycin with artesunate demonstrated additive to synergistic interaction against clinical isolates (60). In the earlier study of azithromycin in combination with artesunate against culture-adapted parasite strains, however, Ohrt et al. suggested an additive to antagonistic interaction, explaining the limited success of this combination in early clinical trials(61, 62). While predicting clinical outcomes based on the results from *in vitro* study is largely limited and there remains a loose correlation between *in vitro* data and clinical outcomes, previous studies of azithromycin both *in vitro* and *in vivo* studies suggest that pharmacodynamics properties of azithromycin in combination with traditional antimalarial drugs should be further investigated.

TABLE 1.2 Structural classification of antimalarial drugs used in study

Antimalarial	Chemical structure	Classification
Artesunate		Artemisinin
Pyronaridine		Aza-acridine mannich base
Amodiaquine		4-aminoquinoline
Tafenoquine		8-aminoquinoline
Azithromycin		Macrolide antibiotics

1.3 Rationale

While there has been a dramatic progress in understanding *P. falciparum* genomics, and exciting discoveries of promising leads in the antimalarial pipeline, such as KAE609 and KAF156, both the safety and efficacy of these novel compounds remain to be assessed before testing in humans(63). Moreover, the efficacies of existing ACTs are threatened by incorrect dosing and non-compliance with duration of dosing regimen, which might hasten the emergence of resistant parasites. The increasing spread of *P. falciparum* isolates with a delayed clearance phenotype in the Mekong subregion of Southeast Asia has been a growing concern, and antimalarials in combinations, rather than as single drugs, are now recommended in order to prevent the emergence and spread of resistance (64-66). Understanding of the effects of drug interactions on combination therapy with existing antimalarial agents may provide useful information such as their mechanisms of action, and influence the development of new drug combinations in the future.

In the treatment of malaria, combinations of two or more drugs interact in either higher (synergism), or lower (antagonism) than would be expected from the individual activities simply added together while additive interactions simply indicate no interactions between drugs in combination. If antimalarial agents in combination is synergistic, it is favored as it suggests that the doses of each drug used may be reduced while maintaining its antimalarial effect and minimizing side effects. Conversely, combinations showing antagonistic interactions are generally avoided. Evaluation of drug interactions can be presented in an isobolograms, employing fractional inhibitory concentrations (FIC). The FIC is defined as the concentration of a drug present in the

combination divided by the concentration of the drug alone that gives the same effect(67). In an additive combination, the sum of FICs of two drugs at the mid-point of a curve is 1, lying a straight line. With synergistic compounds, the sum of FICs is < 1, forming a concave line while > 1 with convex line is seen in an antagonistic one. An advantage of employing FICs in an isobolograms, which dates back over 100 years, lies in its ability to show not only the difference between synergism or antagonism, but also the degree of interactions. In this type of illustration, median inhibitory concentration (IC_{50}) (or any given effect such as killing or cure) values for one drug in the presence of various concentrations of another drug are plotted on the x-axis and vice versa on the y-axis. While a wide range of concentrations/ratios can be tested to generate isobolograms, interactions may dramatically differ at different ratios of two drugs, resulting in a skewed isobole. Two compounds in combination and their interactions are illustrated in Figure 1.2 (68).

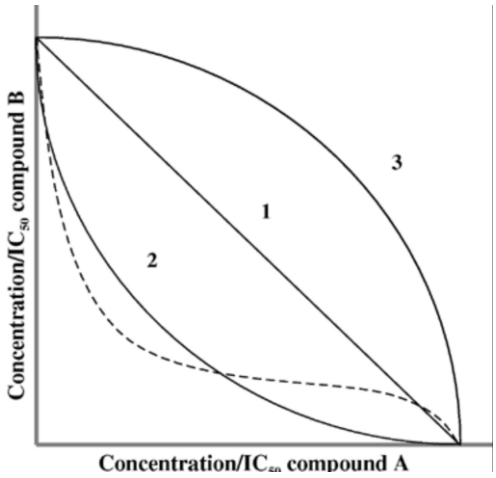


Fig 1.3 Isobologram showing possible interactions between two compounds, A and B. Curve 1: additivity (no interaction); Curve 2: synergism; Curve 3: antagonism. A skewed isobole represented in dashed line. Reprinted by permission from Federation of European Microbiological Societies: Elsevier B.V. "Antimalarial drug synergism and antagonism: Mechanistic and clinical significance" by Angus Bell, copyright (2005).

Traditionally, *in vitro* drug sensitivity assays have been commonly used to evaluate antimalarial drug efficacy and combined drug interactions in cultured cells at a very low parasitemia in a small volume of dilute blood by measuring the rate of suppression or inhibition of parasite growth during a 48-72 hour period. Such *in vitro* study captures a time dependent snapshot inhibition at different intervals of the intraerythrocytic *P. falciparum*, and measures antimalarial activity of drugs in question against the ring-stage parasites in comparison to untreated controls. In *in vivo* patient studies, however, cytocidal activity (or inhibition of parasite multiplication) is measured at a high asynchronous parasitemia (~ 1 trillion per adult). In severe *falciparum* malaria, the primary goal of antimalarial treatment is to save the life, and thus, therapeutic response in this case is determined by the speed of parasite killing, particularly the killing of ring-stage parasites in the circulation to prevent the progression to severe disease. Fast acting artemisinin and its derivatives such as artesunate show a superior effect on these young, developing-stage parasites over other antimalarial drugs(69-71). This argues in favor of an artemisinin-derivative in combination with slowly eliminated antimalarial drug in current guidelines of WHO-recommended antimalarials. Due to different measures (e.g., parasite lactate dehydrogenase (pLDH) enzyme, histidine-rich protein 2 (HRP2), radio-labeled hypoxanthine) being assessed in *in vitro* drug suppression studies, however, results obtained from different assays may obscure their interpretations and become challenging to arrive at a sound conclusion(72-74). Moreover, the relationship between inhibition of parasite growth *in vitro* and parasite killing *in vivo* in respond to antimalarial drugs is not established, and it is unclear how well the standard *in vitro*

susceptibility measures (e.g., 50% inhibitory concentrations [IC_{50}], IC_{90}) of static antimalarial drug activity correspond with drug interactions in humans. Extrapolating the results from cultured parasites to humans, therefore, requires *in vivo* studies in the relevant host. In recent years, rodent models have been developed to mimic human infections and predict therapeutic responses in the field(75). Examples of interactions between two antimalarial compounds both in animal and *in vitro* models are summarized in Table 1.3. The *Plasmodium berghei* model has been previously used to characterize pharmacokinetics and pharmacodynamics (PK/PD) profiles of antimalarials(76-78). In our study, we adapted a transgenic luciferase reporter, *P. berghei* ANKA to study cytocidal activity of antimalarials (i.e., tafenoquine, pyronaridine) in combination with artesunate, amodiaquine, and azithromycin at clinically relevant dosage. We initiated each drug treatment at a high parasitemia (~10%), and followed for 30 days. Three measures used in characterizing pharmacodynamics properties are 30-day survival, measure of parasite reduction over the first 24 to 48 hours, and recrudescent parasitemia (returned to initial parasitemia; 10^6 parasites/μL). The parasite clearance time, which heavily depends on initial parasite biomass prior to drug treatment, is a measure that has been frequently used in previous studies. In the present study, we adapted the parasite reduction ratio (PRR) - the fractional reduction in total parasite numbers per asexual cycle – with pre-treatment parasitemia normalized to 10^6 parasites/μL(79). The PRR or fractional log reduction in parasitemia is analogous to the killing rate, and the differences in PRR between antimalarial compounds reflect the different intrinsic pharmacodynamics

between the drugs. In assessing interactions between two antimalarial compounds, 100% cure at day-30 was taken as a stringent measure to define synergy.

TABLE 1.3 Drug interactions between antimalarial agents on *Plasmodium spp.*

Plasmodium spp.	**Drug A**	**Drug B**	**Interaction**	**Model**	**Reference**
P. falciparum K1	PYR	AZI	Add.	*in vitro*	(80)
P. falciparum K1, 3D7	TQ	AS	Ant.	*in vitro*	(81)
P. falciparum K1,	PYR	AS	Ant.	*in vitro*	(82)
P. falciparum 3D7	PYR	AS	Ant.	*in vitro*	(82)
P. falciparum VS1 FCB	PYR	AS	Ant.	*in vitro*	(82)
P. falciparum TM9072a	PYR	AS	Ant.	*in vitro*	(82)
P. falciparum FCR3	PYR	AS	Ant.	*in vitro*	(82)
P. yoelii	PYR	ART	Syn.	*in vivo* in mice	(83)
P. falciparum W2/Indochina	PYR	Mono-desethylAMQ	Add.	*in vitro*	(84)
P. falciparum C2A,B	AZI	Q	Syn.	*in vitro*	(61)
P. berghei	ERT	CQ	Syn	*in vivo*	(85)

PYR, pyronaridine; *TQ*, tafenoquine; *AZI*, azithromycin; *ERT*, erythromycin; *AS*, artesunate; *ART*, artemisinins; *AMQ*, amodiaquine; *Q*, quinine; *CQ*, chloroquine
Ant, antagonism; *Syn*, synergism; *Add*, additivity.

1.4 Research Aims

We hypothesize that two quinoline-related compounds i.e., 8-aminoquinoline tafenoquine, and 4-aminoquinoline pyronaridine at various sub-curative doses synergize with three current antimalarial drugs, including artesunate, amodiaquine, and azithromycin, extending days return to initial parasitemia with greater log reduction in *Plasmodium berghei* parasites *in vivo* in mouse over a single parasitic life cycle (24 hours), and making difference between cure and no cure.

Aim 1. Determine the single, curative dose of pyronaridine or tafenoquine alone *in vivo.*

Aim 2. Compare the pharmacodynamic interactions of tafenoquine versus pyronaridine *in vivo.*

Aim 3. Characterize the interactions between tafenoquine or pyronaridine with artesunate *in vivo.*

Aim 4. Evaluate the interactions between pyronaridine and another 4-aminoquinoline, amodiaquine, or antibiotics, azithromycin *in vivo.*

Chapter 2

Methodology

Antimalarial Drug Preparation and Dosing:

Artesunate which constitutes 100% parent or acid, was dissolved in 5% sodium bicarbonate ($NaHCO_3$). Stock solutions of the 4-aminoquinolines, amodiaquine and pyronaridine at 20 mg/mL and 5 mg/mL, respectively were prepared in deionized water and then diluted to the desired concentrations. The 8-aminoquinoline, tafenoquine at 5 mg/mL was initially prepared in 1x phosphate-buffer saline (PBS 1x), sterile and then diluted with 12M HCl and 2M NaOH in 1:5 ratio in PBS 1x, sterile to the desired concentrations. Azithromycin was dissolved in 1M HCl, ethyl alcohol, 10 mM potassium-phosphate buffer (PPB) in 1:4:35 ratio. All drug stock solutions were prepared on the same day each dosing regimen was initiated and stored at -4^{o}C until ready for use. Artesunate, pyronaridine, and azithromycin were administrated via intraperitoneal

injection in 200µL; amodiaquine and tafenoquine were administrated via oral gavage in 200µL. The quinoline-related compounds – tafenoquine succinate (MW = 581.58, 80% free base), pyronaridine tetraphosphate (MW = 910.03, 56% free base), amodiaquine dihydrochloride (MW = 464.81, 76% free base) were dosed on salt weight. The conversion of the base/salt weight percentage is shown in the Table 2 alongside the human equivalent doses of each drug. All drugs were obtained from the Sigma-Aldrich.

***In vivo* Cytocidal Model of Murine Malaria**:

All experimentation was conducted under a protocol approved by the Animal Care and Use Committee of the Johns Hopkins University (MO16H91). Female BALB/c mice aged at least 6 weeks and weighing 20g $\pm$ 20% were used for all experimentation. Each mouse was infected via intraperitoneal injection with 500,000 erythrocytes infected with *P. berghei* ANKA-green florescent protein (GFP) – luciferase from a donor mouse. The original isolate was obtained from BEI Resources/American Type Culture Collection (ATCC) and transmitted through *Anopheles stephensi* mosquitoes to generate fresh stocks in 5 mice, from which blood was collected and aliquoted for frozen stocks. A donor mouse was prepared using a frozen stock, 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 in experimental mice was monitored by Giemsa-stained thin blood film in addition to luciferase analysis until approximately 10% parasitemia – equivalent to 10^6 parasites/µL- was reached prior to initiating drug dosing regimens. Each drug treatment tested was replicated with three mice.

Parasite Monitoring:

Drug treatment was initiated at a high parasitemia (~10^6 parasites/µL). Blood samples were collected at the time of the first drug dose, indicated as Time 0, 6h, 12h, and 24 h time-points post initial drug dose. Mice were followed up for 30 days with tail blood sampling to monitor daily parasitemia and survival, and euthanized when recrudescent parasitemia exceeded the initial parasitemia.

Luciferase Assay Analysis:

Our murine parasiticidal model utilizes a transgenic luciferase reporter, *P. berghei*-ANKA to study cytocidal drug activity *in vivo*. The transgenic parasites expressing firefly luciferase enzyme, when combined with ATP-Mg^{+2} co-substrate, generate a flash of light, allowing us to detect and quantify parasite density *in vivo* at multiple end-points. Luciferase assay is comparable to blood films, and allows accurate analyses of intracellular infection mechanisms of the parasite and drug treatment outcomes. A few advantages of the assay include its ability to generate more time points than the standard method of Giemsa-stained blood films in a linear fashion over at least 4 orders of magnitude, demonstrating the reliability of this method to obtain data on relative parasite densities.

Blood Sample Collection:

Five microliters of blood samples from the tail of mice that had been infected with *P. berghei*-ANKA was collected and plated into 45µL of lysis buffer (20 mM Tris [pH 7.5],

5 mM EDTA, 0.008% [wt./vol] saponin, and 0.08% [vol/vol] Triton X-100) in a 96-well plate. Blood samples were stored in - 80^{o} until processed for luciferase analysis(86). A total of 5µL of blood-lysis buffer (20 mM Tris, 5 mM EDTA, 0.008% saponin, 0.08% Triton x-100; pH=7.5) (whole-blood equivalent, 0.5µL) was transferred to a black, opaque 96-well plate, and 95µL of the luciferase buffer (200mM Tricine, 10mM EDTA, 10mM K_2CO_3, 50mM $MgSO_4$, 250µM dithiothreitol [DTT], 250µM ATP, 20µM D-luciferin) was added. Samples were then processed for the luciferase assay, and luciferase activity was measured in the IVIS Spectrum *in vivo* imagining system and analyzed using Living Image v. 4.4 software.

Data Analysis:

The raw luciferase activity reported as radiant flux in photons per second was measured in the IVIS Spectrum *in vivo* imagining system and analyzed using Living Image v. 4.4 software. The raw luciferase data log10 total flux (photons/second) were then transformed to parasites per microliter using the standard curve equation Y = (10^(log(y)-0.55/0.05))*2 in GraphPad Prism 5 software(87). All data were normalized to 10^6 parasites per microliter. Parasite reduction ratio was achieved by taking the difference of logarithmic exponents between initial parasitemia and that at 24-hour or 48-hour post dosing. Two-way analysis of variance (ANOVA) was performed to determine any statistically significant differences between comparing groups. For the evaluation of the effectiveness of drug combinations fractional inhibitory concentration (FIC) was employed using the 30-day survival profiles of various combinations tested.

TABLE 2.1 Mouse doses and human equivalents of antimalarial drugs

Antimalarial Drugs	**Mouse Dose Reference body weight: 0.02kg (Free base)**	**Human Equivalent Dose: mouse dose x 0.081 Reference body weight: 60kg (Free base)**
Artesunate	50 mg/kg (-)	4.1 mg/kg (-)
Azithromycin	50 mg/kg (-)	4.1 mg/kg (-)
Tafenoquine succinate	5 mg/kg (4)	0.41 mg/kg (0.33)
	10 mg/kg (8)	0.81 mg/kg (0.65)
	15 mg/kg (12)	1.2 mg/kg (0.96)
	20 mg/kg (16)	1.6 mg/kg (1.3)
	25 mg/kg (20)	2.0 mg/kg (1.6)
	30 mg/kg (24)	2.4 mg/kg (1.9)
	40 mg/kg (32)	3.2 mg/kg (2.6)
Pyronaridine tetraphosphate	2.5 mg/kg (1.4)	0.20 mg/kg (0.11)
	5 mg/kg (2.8)	0.41 mg/kg (0.23)
	7.5 mg/kg (4.2)	0.61 mg/kg (0.34)
	10 mg/kg (5.6)	0.81 mg/kg (0.45)
	40 mg/kg (22.4)	3.2 mg/kg (1.8)
Amodiaquine dihydrochloride	50 mg/kg (38)	4.1 mg/kg (3.1)
	100 mg/kg (76)	8.1 mg/kg (6.2)
	150 mg/kg (114)	12 mg/kg (9.1)
	200 mg/kg (152)	16 mg/kg (12)

Reference: [USFDA 2005]. Human equivalent dose calculation factor obtained from FDA draft guidelines. FDA: Food and Drug Administration, HED: Human Equivalent Dose. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharma 2016;7:27-31.

Chapter 3

Results

3.1 The minimum curative single oral administration of tafenoquine is 30 mg/kg.

The curative efficacy of a single dose of tafenoquine was evaluated in a cytocidal *in vivo* model with dose escalation at 10, 15, 20, 30, and 40 mg/kg. Tafenoquine succinate salt has free base at 80% of the total salt, and was administered to *P. berghei*-ANKA infected mice at a high initial parasitemia (~10%). Noncurative regimens included 10 mg/kg, and 15 mg/kg with a return to initial parasitemia 5 days and 14 days post dosing, respectively. The 20 mg/kg regimen was partially curative, resulted in 66% (n=2/3) recrudescence with return to initial parasitemia 14 days and 18 days post dosing (Fig 3.1.1A). 100% (n=3/3) cure was only achievable in the mice treated with either 30 mg/kg or 40 mg/kg (Fig 3.1.1B). Parasite reduction ratio of each drug regimen for the single 24-hour life cycle,

and the first 48 hours are expressed as the parasite log reduction in Table 3.1. Observed a log and 2-log-unit kill were seen within 48 hours for 30 and 40 mg/kg regimens, respectively with cure at day 30. It is interesting to note that an observed initial downward-sloping plateau phase followed by a log-linear fall was seen for 10, 20, 30 and 40 mg/kg regimens in the single 24-hour life cycle, resulting in no statistically significant difference in the amount of parasite killing among the four regimens (data not shown).

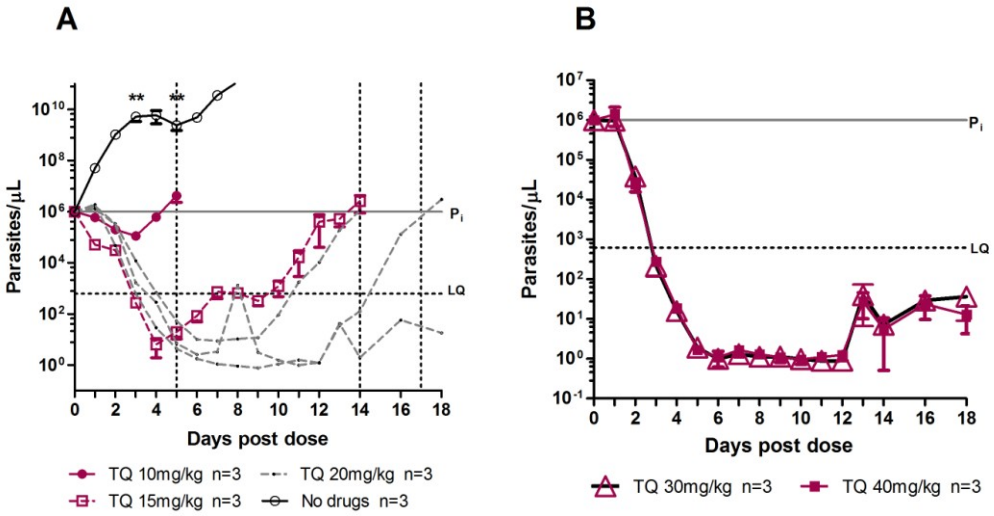


Fig 3.1.1 Determination of single minimum curative dose of tafenoquine (TQ) on *P. berghei* ANKA infection. Doses of 10, 15, and 20 (A) along with 30, and 40 mg/kg (B) of tafenoquine were administered at 0h to female BALB/c mice. Time zero corresponds to a parasitemia of ~10% as determined by the percentage of infected erythrocytes in Giemsa stained blood films as well as luciferase assay. Parasitemia were monitored only by luciferase assay thereafter. The data are represented as means ± SEM, or individual mice when group survival varied, and were analyzed using two-way ANOVA. Each graph is annotated with the initial parasitemia (P_i) and the luciferase assay limit of quantitation (LQ). **,p<0.01.

TABLE 3.1.1 Identifying the single minimum curative dose of tafenoquine, and resulting parasite log reduction on *P. berghei* ANKA infection[a]

Doses of tafenoquine (mg/kg)	24h log reduction	48h log reduction	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
10	0.2	1	3/3	5,5,5
15	1	2	3/3	14,14,14
20	-0.2	1	2/3	14,17
30	0.02	1	0/3	NA
40	-0.2	2	0/3	NA

[a]Tafenoquine (10,15,20,30,40 mg/kg) was administered at 0h to female BALB/c mice. The starting parasitemia was ~10%, as determined by the percentage of infected erythrocytes in Giemsa-stained blood films and luciferase assay. *NA* is not applicable

3.2 The minimum curative single intraperitoneal administration of pyronaridine is 10 mg/kg.

In order to determine the minimum therapeutic dose of pyronaridine *in vivo*, we evaluated the cytocidal activities of three different regimens of 5, 10, and 40 mg/kg. Pyronaridine was administrated intraperitoneally in the form of pyronaridine tetraphosphate salt, containing 56% free base. Curative regimens included 10 and 40 mg/kg (Fig 3.2.1). The 5 mg/kg regimen was partially curative, resulted in 66% (n=2/3) recrudescence with return to initial parasitemia 10, and 14 days post dosing. A 3-log-unit kill, followed by an additional log kill was observed over the single 24-hour life cycle for all dosing regimens (Table 3.2.1).

TABLE 3.2.1 Identifying the single minimum curative dose of pyronaridine, and resulting parasite log reduction on *P. berghei* ANKA infection[a]

Doses of pyronaridine (mg/kg)	24h log reduction	48h log reduction	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
5	3	4	2/3	10,14
10	3	4	0/3	NA
40	3	4	0/3	NA

[a]Pyronaridine (5,10,40 mg/kg) was administered at 0h to female BALB/c mice. The starting parasitemia was ~10%, as determined by the percentage of infected erythrocytes in Giemsa-stained blood films and luciferase assay. *NA* is not applicable.

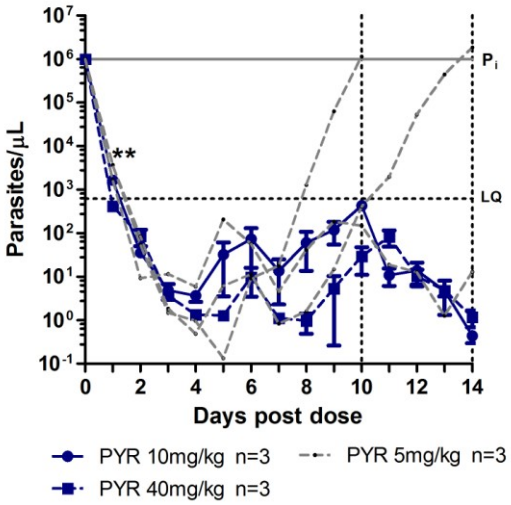


Fig 3.2.1 Determination of single minimum curative dose of pyronaridine (PYR) on *P. berghei* ANKA infection. Doses of 5,10 and 40 mg/kg of pyronaridine were administered at 0h to female BALB/c mice. Time zero corresponds to a parasitemia of ~10% as determined by the percentage of infected erythrocytes in Giemsa stained blood films as well as luciferase assay. Parasitemia were monitored only by luciferase assay thereafter. The data are represented as means ± SEM, or individual mice when group survival varied, and were analyzed using two-way ANOVA. Each graph is annotated with the initial parasitemia (P_i) and the luciferase assay limit of quantitation (LQ). **, $p < 0.01$.

3.3 Pyronaridine exhibits more rapid killing than tafenoquine.

To compare the pharmacodynamics effects of the 4-aminoquinoline pyronaridine to those of the 8-aminoquinoline tafenoquine, single dose of each drug was administered at 10 and 40 mg/kg. Tafenoquine at 10 mg/kg was not curative, resulted in a return to initial parasitemia 5 days post dosing. In contrast, 100% (n=3/3) cure was achieved with10 mg/kg pyronaridine with a 3-log-unit kill over 24-hour life cycle (Table 3.3.1). While the 40 mg/kg regimen of both tafenoquine and pyronaridine were curative, pyronaridine achieved 1,000-fold more reduction in the number of parasites over the single 24-hour parasitic life cycle, reaching the limit of quantification (~1,000 parasites/μL) nearly 48 hours faster than tafenoquine as illustrated in Figure 3.3.1.

TABLE 3.3.1 Measure of parasite log reduction of tafenoquine versus pyronaridine on *P. berghei* ANKA infection[a]

Dose (mg/kg)	24h log reduction	48h log reduction	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
TQ 10	0.2	1	3/3	5,5,5
PYR 10	3	4	0/3	NA
TQ 40	-0.2	2	0/3	NA
PYR 40	3	4	0/3	NA

[a] TQ, tafenoquine; PYR, pyronaridine. *NA* not applicable.

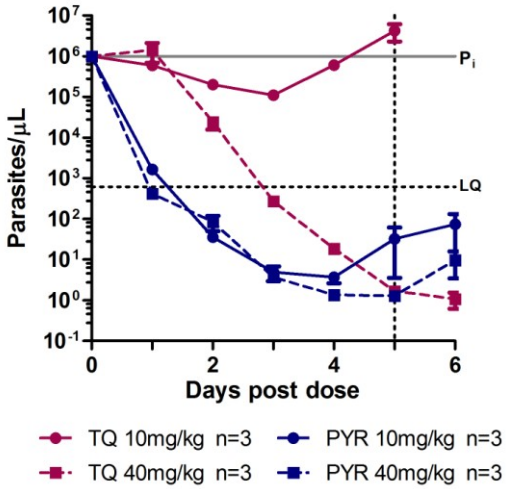


Fig 3.3.1 Curative efficacy of pyronaridine (PYR) versus tafenoquine (TQ) on *P. berghei* ANKA infection. Doses 10 and 40 mg/kg of pyronaridine or tafenoquine were administered at 0h to female BALB/c mice. Time zero corresponds to a parasitemia of ~10% as determined by the percentage of infected erythrocytes in Giemsa stained blood films as well as luciferase assay. Parasitemia were monitored only by luciferase assay thereafter. The data are represented as means ± SEM and were analyzed using two-way ANOVA. The graph is annotated with the initial parasitemia (P_i) and the luciferase assay limit of quantitation (LQ).

3.4 Checkerboard experiments to examine *in vivo* drug interactions between tafenoquine or pyronaridine with ACT partner compounds.

In order to further investigate the *in vivo* efficacy of tafenoquine or pyronaridine, we tested both curative and noncurative doses of tafenoquine (5,10,15,20, and 25 mg/kg) or pyronaridine (2.5,5, and 7.5 mg/kg) in combination with three current antimalarials, including artesunate, amodiaquine, and azithromycin. We then determined the impact of combination therapy versus each drug alone, juxtaposing the results with sets of published and unpublished data from the laboratory for comparison.

3.4.1 Tafenoquine synergizes with artesunate..

Figure 3.4.1.1 illustrates the isobolograms obtained with tafenoquine (5,10,15,20, and 25 mg/kg) and 50 mg/kg-artesunate (1,2,4, or 6 days). Overall, a synergistic trend was observed between the two compounds. The parasite log reduction over 24 to 48 hours of each combination was compared to those of tafenoquine or artesunate alone (Table 3.4.1.1). While there was no statistically significant difference observed in parasite reduction over the first 24 hours post dosing among tafenoquine (5 mg/kg)-artesunate (1,2,4-days) regimens, increasing the duration of artesunate responded with incremental 30-day survival, ultimately leading to 100% cure: 1-day (0%, n=0/3), 2-days (33%, n=1/3). 4-days (100%, n=3/3) (Fig 3.4.1.2A). When a noncurative, single dose of tafenoquine at 10 mg/kg was administered with fast acting artesunate (2-days), a reduction of parasite numbers by a factor of approximately 100,000 was observed over the first 24-hour life cycle (Fig 3.4.1.2B). This is 1,000-fold more killing in comparison to

2-days duration of artesunate alone or a single day artesunate in combination with tafenoquine at 10 mg/kg over the first 24 hours. Overall, lower doses of tafenoquine in combination with a short duration of artesunate were not curative. The results of the combination therapy are summarized in Table 3.4.1.1

A

Dose of tafenoquine (mg/kg)	Durations of artesunate treatment (days)				
	0	**1**	**2**	**4**	**7**
25	**C**	C	NT	NT	NT
20	15.5	C	C	C	NT
15	14	**C**	C	C	NT
10	5	4.3	**C**	C	NT
5	NT	11	12	**C**	NT
0		NT	NT	NT	**C**

B

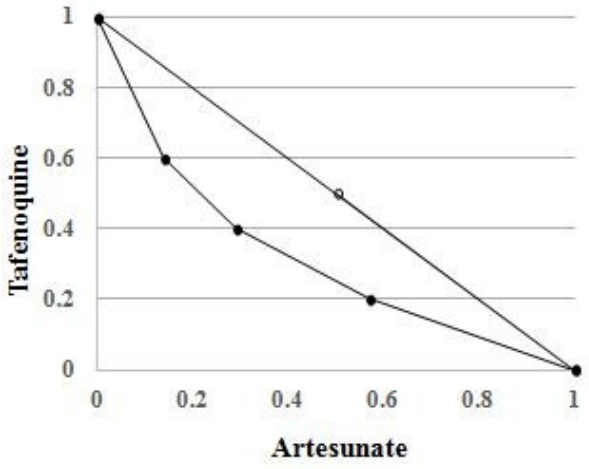


Fig 3.4.1.1 Synergistic interaction between tafenoquine (TQ) and artesunate (AS) against *P. berghei* in mice. (A) Checkerboard showing single dose of tafenoquine on day 1 (vertical axis) and number of daily doses of 50 mg/kg of artesunate (horizontal axis), with average number of days to return to initial parasitemia indicated in each checkerboard box. *C*, 100% of mice (n=3/3) cured (no detectable parasitemia for 30 days); *NT* not tested; bolded ***C***, data points used to construct isobolograms. Artesunate monotherapy results as previously reported (87). (B) Isobologram of cure at various tafenoquine dose ratios and artesunate treatment duration ratios.

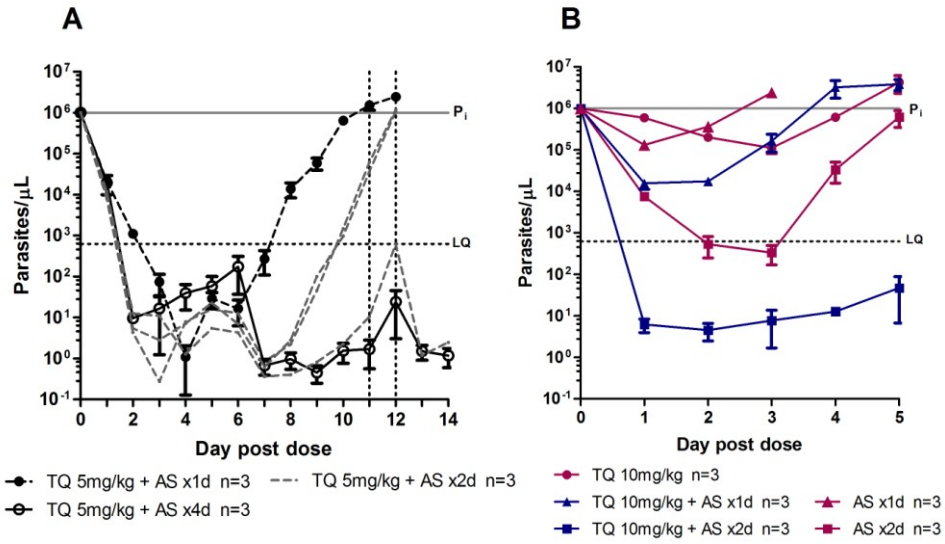


Fig 3.4.1.2 Determination of curative combination regimens of tafenoquine (TQ) with artesunate (AS) on *P. berghei* ANKA infection. Dose of 5 mg/kg of tafenoquine was administered at 0h to female BALB/c mice with artesunate (50mg/kg) for durations of 1, 2, or 4 days (A) along with 10 mg/kg of tafenoquine with 1 or 2 days of artesunate (B). Time zero corresponds to a parasitemia of ~10% as determined by the percentage of infected erythrocytes in Giemsa stained blood films as well as luciferase assay. Parasitemia were monitored only by luciferase assay thereafter. The data are represented as means ± SEM, or individual mice when group survival varied (A), and were analyzed using two-way ANOVA. Each graph is annotated with the initial parasitemia (P_i) and the luciferase assay limit of quantitation (LQ).

TABLE 3.4.1.1 Identifying curative combination regimens of tafenoquine with artesunate on *P. berghei* ANKA infection[a] and resulting parasite log reduction

Duration of artesunate treatment (days)	Dose of tafenoquine (mg/kg)	24h log reduction	48 log reduction	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
1 [b]	-	1	0.4	-	-
	5	2	3	3/3	11,11,11
	10	2	2	3/3	5,4,4
	15	2	3	0/3	NA
	20	2	4	0/3	NA
	25	2	4	0/3	NA
2 [b]	-	2	3	-	-
	5	2	5	2/3	12,12
	10	5	5	0/3	NA
	15	6	4	0/3	NA
	20	6	4	0/3	NA
4	5	2	5	0/3	NA
	10	6	6	0/3	NA
	15	6	6	0/3	NA
	20	6	5	0/3	NA
6	10	6	6	0/3	NA
	15	6	6	0/3	NA
	20	6	6	0/3	NA

[a]Single dose of tafenoquine (5,10,15,20,25 mg/kg) was administered at 0h in combination with artesunate (50 mg/kg) for durations of 1,2,4, or 6 consecutive days to female BALB/c mice. The starting parasitemia was ~10%, as determined by the percentage of infected erythrocytes in Giemsa-stained blood films and luciferase assay. *NA* is not applicable

[b]Artesunate monotherapy results as previously reported.

3.4.2 Pyronaridine exhibits additive interactions with artesunate.

In vivo efficacy of pyronaridine was further explored in combination with artesunate in a curative model of *P. berghei*-ANKA infected mice. In combination with a low, noncurative dose of 2.5 mg/kg pyronaridine, extending the duration of artesunate from 1 day to 2 days prolonged recrudescence by a single day (Fig 3.4.2.1A). In combination with a single day artesunate, increasing the dose of pyronaridine from 2.5 mg/kg to 5 mg/kg prolonged recrudescence to initial parasitemia from day 7 until day 11. While a single, noncurative dose of pyronaridine at 2.5 mg/kg and a single day artesunate alone share similar parasite

log reduction profiles, the two compounds in combination demonstrated a 2-log-unit kill over a single 24-hour parasitic life cycle, extending recrudescence to initial parasitemia by three days. Interestingly, all of combination regimens exhibited a 2-log-unit kill over the first 24 hours (Table 3.4.2.1). The greatest log reduction of the parasite biomass was achieved by 2.5 mg/kg of pyronaridine with artesunate (2 days) over the second parasitic life cycle (48 hours post dosing) with an additional 3-log-unit kill. 100% (n=3/3) cure was only achievable in the mice treated with 5 mg/kg and 7.5 mg/kg pyronaridine in combination with longer durations of artesunate treatment (2- and 4-days). While there was no statistically significant difference observed in parasite reduction over the first 24 hours post dosing among pyronaridine (2.5,5, or 7.5 mg/kg)-artesunate (2-days) regimens, increasing the dose of pyronaridine responded with prolonged recrudescence to initial parasitemia by 5 days and incremental 30-day survival, ultimately leading to 100% cure: 2.5mg/kg (0%, n=0/3), 5mg/kg (33%, n=1/3). 7.5mg/kg (100%, n=3/3) (Fig 3.4.2.1B). The overall interactions between the two antimalarial compounds were additive, and are illustrated in isobolograms in Figure 3.4.2.2.

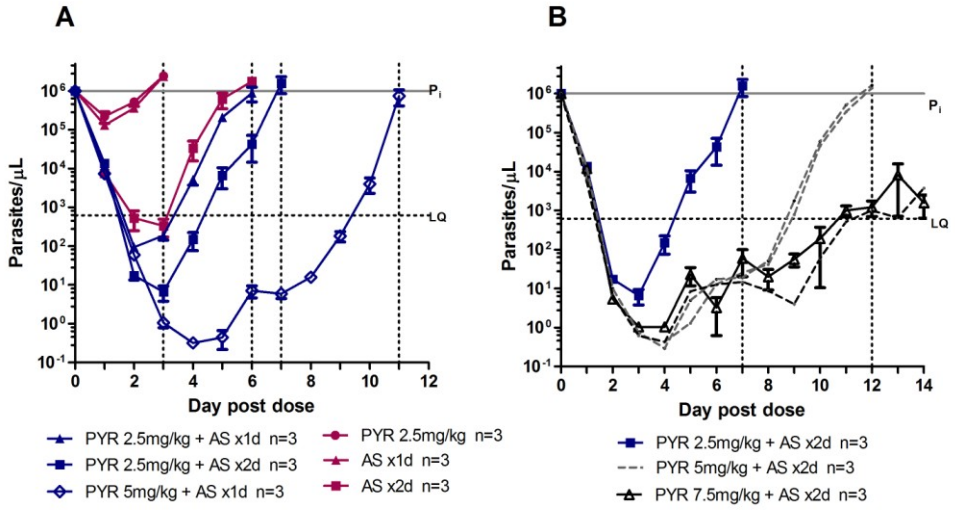


Fig 3.4.2.1 Determination of curative combination regimens of pyronaridine (PYR) with artesunate (AS) on *P. berghei* ANKA infection. Doses of 2.5, 5 and 7 mg/kg of pyronaridine were administered at 0h to female BALB/c mice with artesunate (50 mg/kg) for durations of 1, or 2 days. Time zero corresponds to a parasitemia of ~10% as determined by the percentage of infected erythrocytes in Giemsa stained blood films as well as luciferase assay. Parasitemia were monitored only by luciferase assay thereafter. The data are represented as means ± SEM, or individual mice when group survival varied (B), and were analyzed using two-way ANOVA. 1- and 2-day durations of artesunate monotherapy data obtained from separate experiments and juxtaposed for comparison. Each graph is annotated with the initial parasitemia (P_i) and the luciferase assay limit of quantitation (LQ).

TABLE 3.4.2.1 Identifying curative combination regimens of pyronaridine with artesunate on *P. berghei* ANKA infection[a], and resulting parasite log reduction

Duration of artesunate treatment (days)	Dose of pyronaridine (mg/kg)	24h log reduction	48h log reduction	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
1 [b]	-	1	0.4	-	-
	2.5	2	3	3/3	7,7,5
	5	2	4	3/3	12,14,12
	7.5	2	4	0/3	-
2 [b]	-	2	3	-	-
	2.5	2	5	3/3	7,7,7
	5	2	5	2/3	12,12
	7.5	2	5	0/3	NA
4	2.5	2	5	3/3	9, 12, 13
	5	2	5	0/3	NA

[a]Single dose of pyronaridine (2.5,5, and 7.5 mg/kg) was administered at 0h in combination with artesunate (50 mg/kg) for durations of 1,2,or 4 consecutive days to female BALB/c mice. The starting parasitemia was ~10%, as determined by the percentage of infected erythrocytes in Giemsa-stained blood films and luciferase assay. *NA* is not applicable

[b]Aretesunate monotherapy results as previously reported.

A

Dose of pyronaridine (mg/kg)						
	10	**C**	NT	NT	NT	NT
	7.5	NT	**C**	**C**	NT	NT
	5	12	12.7	12	**C**	NT
	2.5	NT	6.3	7	11.3	NT
	0		NT	NT	NT	**C**
		0	**1**	**2**	**4**	**7**
		Durations of artesunate treatment (days)				

B

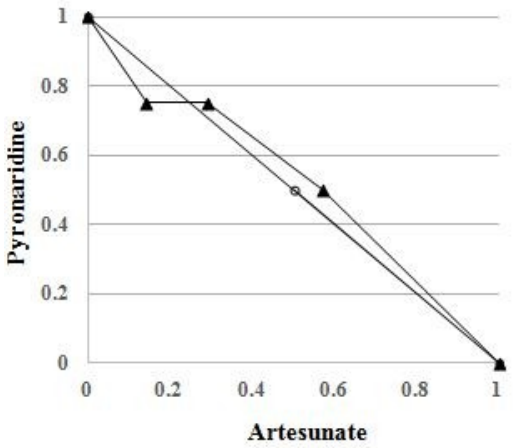


Fig 3.4.2.2 Additive interaction between pyronaridine and artesunate against *P. berghei* in mice. (A) Checkerboard showing single dose of pyronaridine on day 1 (vertical axis) and number of daily doses of 50 mg/kg artesunate (horizontal axis), with average number of days to return to initial parasitemia indicated in each checkerboard box. *C.* 100% of mice (n=3/3) cured (no detectable parasitemia for 30 days after first dose); *NT,* not tested; *bolded* ***C***, data points used to construct isobolograms. Artesunate monotherapy results as previously reported (87). (B) Isobologram of cure at various pyronaridine dose ratios and artesunate treatment duration ratios.

3.4.3 Pyronaridine synergizes with amodiaquine.

Drug interactions of pyronaridine with another 4-aminoquinoline, amodiaquine were evaluated *in vivo* against *P. berghei*-ANKA. Four different doses of amodiaquine at 50,100,150, and 200 mg/kg were orally administrated in the form of amodiaquine dihydrochloride salt, containing 76% free base. An observed 3-log-unit kill was seen over 24 hours in all treatment regimens (Fig 3.4.3.1 and Table 3.4.3.1). While 100% of the mice (n=3/3) cured the infection and survived until 30 days, when combination included 7.5 mg/kg of pyronaridine with 100 mg/kg amodiaquine only a single mouse survived, resulting in 66% (n=2/3) recrudescence, exceeding initial parasitemia levels by day 11, and 12 (data not shown). Regardless of the duration of artesunate or doses of amodiaquine, two

compounds in combination demonstrated 100-fold more reduction than amodiaquine alone over a single 24-hour life cycle. The isobolograms obtained with pyronaridine and amodiaquine are illustrated in Figure 3.4.3.2, and the results of the combination therapies are summarized in Table 3.4.3.1. Overall, synergistic effect was observed between the two compounds.

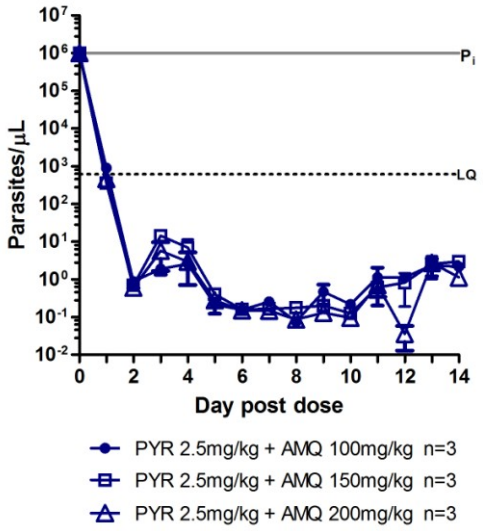


Fig 3.4.3.1 Determination of curative combination regimens of pyronaridine (PYR) with amodiaquine (AMQ) on *P. berghei* ANKA infection. Dose of 2.5 mg/kg of pyronaridine was administered at 0h to female BALB/c mice with amodiaquine (100,150, or 200 mg/kg). Time zero corresponds to a parasitemia of ~10% as determined by the percentage of infected erythrocytes in Giemsa stained blood films as well as luciferase assay. Parasitemia were monitored only by luciferase assay thereafter. The data are represented as means ± SEM and were analyzed using two-way ANOVA. The graph is annotated with the initial parasitemia (P_i) and the luciferase assay limit of quantitation (LQ).

TABLE 3.4.3.1 Identifying a curative combination regimens of pyronaridine with amodiaquine on *P. berghei* ANKA infection[a], and resulting parasite log reduction

Dose of amodiaquine (mg/kg)	Dose of pyronaridine (mg/kg)	24h log reduction	48h log reduction	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
50 [b]	-	1	3	-	-
	5	3	6	0/3	NA
	7,5	3	6	0/3	NA
100 [b]	-	1	3	-	-
	2.5	3	6	0/3	NA
	5	3	6	0/3	NA
	7.5	3	6	2/3	11.12
150 [b]	-	1	2	-	-
	2.5	3	6	0/3	NA
	5	3	6	0/3	NA
	7.5	3	6	0/3	NA
200 [b]	-	1	2	-	-
	2.5	3	6	0/3	NA
	5	3	6	0/3	NA
	7.5	3	6	0/3	NA

[a]Single dose of pyronaridine (2.5,5,and 7.5 mg/kg) was administered at 0h in combination with amodiaquine (50,100,150, or 200 mg/kg) to female BALB/c mice. The starting parasitemia was ~10%, as determined by the percentage of infected erythrocytes in Giemsa-stained blood films and luciferase assay. *NA* is not applicable.
[b]Amodiaquine monotherapy results as previously reported.

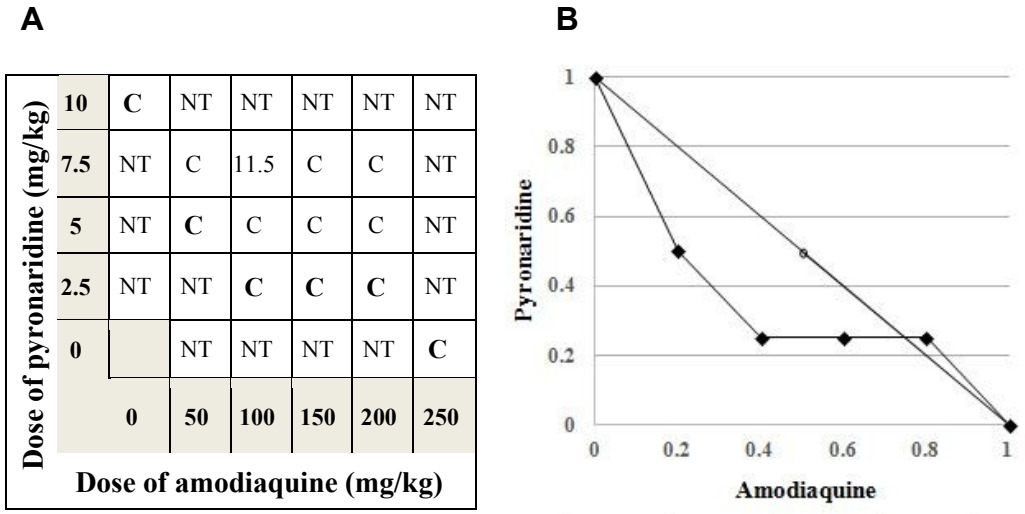


Fig 3.4.3.2 Synergistic interaction between pyronaridine and amodiaquine against murine malaria, *P. berghei* in mice. (A) Checkerboard showing single dose of pyronaridine on day 1 (vertical axis) and single dose of amodiaquine (horizontal axis), with average number of days to return to initial parasitemia indicated in each checkerboard box. *C,* 100% of mice (n=3/3) cured (no detectable parasitemia for 30 days after first dose); *NT*; not texted, *bolded* ***C***; data points used to construct isobolograms. Amodiaquine monotherapy results as previously reported. (B) Isobologram of cure at various pyronaridine dose ratios and amodiaquine dose ratios.

3.4.4 Pyronaridine exhibits differential interactions with azithromycin.

Using the same *P. berghei*-ANKA model, cytocidal activities of pyronaridine in combination with azithromycin were examined. Azithromycin at 50 mg/kg was intraperitoneally administered for 1,2,3,4,5, or 6 days along with a single dose of pyronaridine at 2.5,5, or 7.5 mg/kg. In combination with a low, noncurative dose of 2.5 mg/kg pyronaridine, extending the duration of azithromycin from 3 days to 4 days did not result in a statistically significant difference in clearing the parasite biomass over the first 24 or 48 hours, and both regimens achieved 100% survival (n=3/3) at day 30 (Fig 3.4.4.1). Similarly, in combination with 3- or 5-days durations of azithromycin treatment, there was no evidence of enhanced cytocidal effect seen by increasing the dose of pyronaridine from 5 to 7.5 mg/kg, resulting in 3-log-unit kill over the single 24-hour parasitic life cycle followed by an additional log kill over the next cycle (data not shown). This was also evident from the maximal duration of azithromycin (6 days) with pyronaridine (5 mg/kg) with no additional killing achieved (data not shown). Overall, it was concluded that interactions are varying dependent on durations of azithromycin treatment; at least 3-days or longer durations of azithromycin must be co-administered with pyronaridine in order to achieve 100% cure in mice, and the two compounds exhibited both synergy and additive interactions at varying drug ratios (Fig 3.4.4.2). The results of the combination therapies are summarized in Table 3.4.4.1.

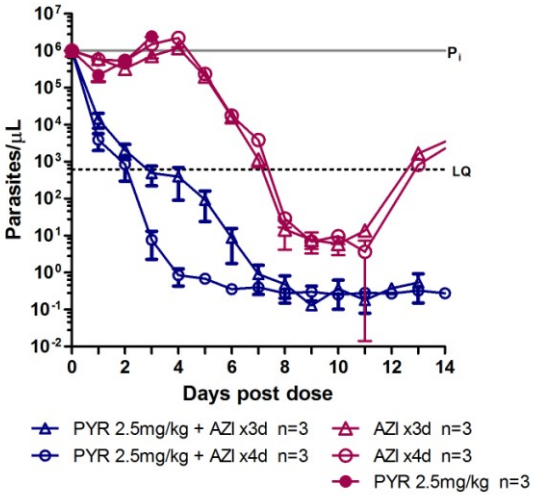


Fig 3.4.4.1 Determination of curative combination regimens of pyronaridine (PYR) with azithromycin (AZI) on *P. berghei* ANKA infection. Dose of 2.5 mg/kg of pyronaridine was administered at 0h to female BALB/c mice with azithromycin (50 mg/kg) for durations of 3 or 4 days. Time zero corresponds to a parasitemia of ~10% as determined by the percentage of infected erythrocytes in Giemsa stained blood films as well as luciferase assay. Parasitemia were monitored only by luciferase assay thereafter. The data are represented as means ± SEM and were analyzed using two-way ANOVA. Pyronaridine (2.5 mg/kg) and 3- and 4-days durations of azithromycin monotherapy data obtained from separate experiments and juxtaposed for comparison. The graph is annotated with the initial parasitemia (P_i) and the luciferase assay limit of quantitation (LQ).

TABLE 3.4.4.1 Identifying curative combination regimens of pyronaridine with azithromycin on *P. berghei* ANKA infection[a], and resulting parasite log reduction

Duration of azithromycin treatment (days)	Dose of pyronaridine (mg/kg)	24h log reduction	48h log reduction	Proportion of mice with recrudescent parasitemia	Day of return to initial parasitemia
1	2.5	-4	-5	3/3	7,8,7
	5	1	1	3/3	12,1,12
	7.5	2	1	3/3	11,11,11
2[b]	-	0.3	0.5	-	-
	2.5	-4	-5	3/3	14,14,5
	5	1	0.1	3/3	14,16,16
	7.5	1	1	3/3	14,14,14
3[b]	-	0.2	0.5	-	-
	2.5	2	3	0/3	NA
	5	3	4	0/3	NA
	7.5	3	4	0/3	NA
4[b]	-	0.2	0.3	-	-
	2.5	2	3	0/3	NA
	5	3	3	0/3	NA
	7.5	3	5	0/3	NA
5[b]	-	0.3	0.3	-	-
	5	3	4	0/3	NA
	7.5	3	4	0/3	NA
6	5	3	4	0/3	NA

[a]Single dose of pyronaridine (2.5,5,and 7.5 mg/kg) was administered at 0h in combination with azithromycin (50 mg/kg) for durations of 1,2,3,4,5,or 6consecutive days to female BALB/c mice. The starting parasitemia was ~10%, as determined by the percentage of infected erythrocytes in Giemsa-stained blood films and luciferase assay. *NA* is not applicable.

[b]Azithromycin monotherapy results as previously reported .

A

Dose of pyronaridine (mg/kg)									
	10	**C**	NT	NT	NT	NT	NT	NT	NT
	7.5	NT	11	14	C	C	C	NT	NT
	5	NT	8.3	15.3	C	C	**C**	**C**	NT
	2.5	NT	7.3	11	**C**	**C**	NT	NT	NT
	0		NT	NT	NT	NT	NT	NT	**C**
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**

Durations of azithromycin treatment (days)

B

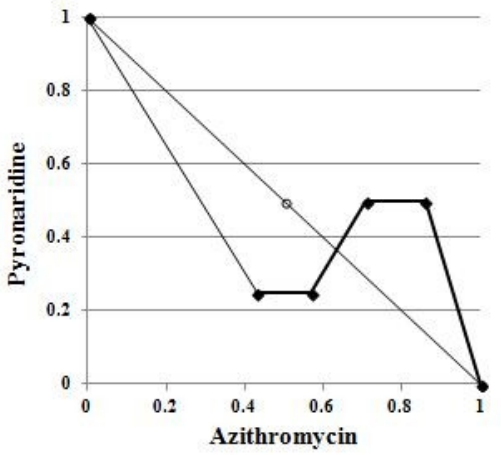


Fig 3.4.4.2 Differential interaction between pyronaridine and azithromycin against *P. berghei* in mice. (A) Checkerboard showing single dose of pyronaridine on day 1 (vertical axis) and number of doses of 50 mg/kg azithromycin (horizontal axis), with average number of days to return to initial parasitemia indicated in each checkerboard box. *C,* 100% of mice (n=3/3) cured (no detectable parasitemia for 30 days after first dose); *NT*; not tested; *bolded* ***C***; data points used to construct isobolograms. Azithromycin monotherapy results as previously reported. (B) Isobologram of cure at various pyronaridine dose ratios and azithromycin duration ratios.

Chapter 4

Discussion

International guidelines have recommended the use of ACTs as a first-line treatment on a three-day course schedule for the treatment of uncomplicated falciparum malaria. Fast acting artemisinin derivatives with a short half-life such as artesunate can reduce the parasitic load by at least 10,000-fold within 48 hours of the parasitic life cycle, resulting in more than 95% clearance of initial infection. Artemisinin-based combination therapy remains important in many parts of malaria endemic countries, however, delay in parasite clearance has been detected as evident in decreased *in vivo* efficacy of dihydroartemisinin-piperaquine against *P. falciparum* infection in Southeast Asia(88). This necessitates discovery of novel antimalarials as well as search for better dosing regimens for the existing drugs. Our study aimed to address the latter by focusing on the cytocidal activities of two quinoline-related compounds, 4-aminoquinolines pyronaridine

and 8-aminoquinoline tafenoquine in a mouse model of malaria with a 24-hour parasitic asexual life cycle. We evaluated efficacies of pyronaridine and tafenoquine alone and in combination with three established antimalarial drugs, including artesunate, amodiaquine, and azithromycin. We hypothesized that both tafenoquine and pyronaridine would achieve greater reduction in the *P. berghei* biomass when administered with another antimalarials than monotherapy at their sub-curative doses.

We adapted the luciferase reporter *P. berghei* for use in comparing the differential cytocidal activities of tafenoquine versus pyronaridine at the same dose of 10 and 40 mg/kg. Considering the active base of tafenoquine succinate (80%), and pyronaridine tetraphosphate (76%), pyronaridine showed more potent cytocidal activity against *P. berghei* at the same total dose. This was evident from its rapid reduction of parasite density with 10,000-fold reduction over the first 48 hours post dosing, and cure at day 30 at both 10 and 40 mg/kg. The observed reduction in parasite density were in agreement with previous *in vivo* experimental data(12). Tafenoquine at its curative doses (30, 40 mg/kg), on the other hand, showed a strikingly different pattern of killing with a brief plateau followed by a log-linear fall. The plateau phase resembles the documented pharmacodynamics profile of quinine(89). As described in previous studies of chloroquine, for example, rapid parasite clearance presumably reflects a broader stage-specificity of action, while initial plateau phase followed by a sustained fall as seen in tafenoquine indicates the killing of sequestered parasites rather than circulating ring stages(90, 91). In the study of morphology of falciparum parasites following pyronaridine treatment, Kawai et al. has demonstrated that the earliest and most distinct

effect of pyronaridine was on the parasite food vacuole of late trophozoites and schizonts(92). Similarly, falciparum parasites exhibit stage-specific drug response against commonly used antimalarials, quinine, 4-aminoquinoline chloroquine and artemisinin with the trophozoite and schizont stages considerably more sensitive to the drugs than the young ring stages(93).

In addition to tafenoquine monotherapy, we determined the cytocidal effect of tafenoquine at its sub-curative doses in combination with artesunate. With five different doses of tafenoquine at 5, 10, 15, 20, 25 mg/kg, we have demonstrated that the lowest noncurative dose of 5 mg/kg tafenoquine achieves 100% cure with increased duration of artesunate: 1-day (0%), 2-day (33%), 4-days (100%). From available data, it is apparent that artemisinin derivatives must be present at least three parasitic life cycles (i.e., > 3 days in *P. berghei*) to remove all of the parasites from the blood(12). While this seemed to hold true with the lower dose of tafenoquine (5 mg/kg), as we increased the dose of tafenoquine 2-day duration of artesunate was shown efficacious with 1,000-fold more killing with tafenoquine in comparison to artesunate alone. The observed plateau phase was not reproduced in the combination therapy. An early clinical study of combination treatment of 8-aminoquinoline primaquine with artesunate against asexual stage falciparum parasites has shown no evidence of synergy between the two compounds(94). While sharing a similar structure with primaquine, tafenoquine comes with a number of advantages over primaquine, including longer half-life, better schizontocidal action, and thus, the possibility of a shorter course of treatment. Our findings suggest that noncurative dosing regimens of tafenoquine gains the therapeutic potential when

combined with fast acting artesunate as evident from a marked enhanced cytocidal activity against *P. berghei* and 30-day survival with tafenoquine at 15, 20, or 25 mg/kg with artesunate (1, 2, 4 days). As observed in the present study, tafenoquine seems to further reduce the residual parasites, preventing recrudescence, following the rapid killing and elimination of artesunate over the single life cycle. Similarly, the pyronaridine-amodiaquine combination showed synergistic, resulting in 100% cure across most regimens. An observed 6-log-unit kill was seen in all combinations tested (3-log-unit kill in the single 24-hour life cycle), indicating no evidence of dose-response or concentration effect. This may suggest that with only one exposure at as low as 2.5 mg/kg of pyronaridine with 100 mg/kg of amodiaquine is sufficient for a maximal antimalarial effect, and the biological effects of pyronaridine-amodiaquine combination extend beyond their sub-curative concentrations in plasma.

Pyronaridine-artesunate (Pyramax) is a newly introduced ACT at fixed-dose (3:1), and the only ACT registered for the treatment of both *P. falciparum* and *P. vivax* malaria. In the present study, we observed additive effects between with 2-log-unit kill over a single 24-hour life cycle across all regimens tested, resulting in approximately 10-fold more killing compared to artesunate (2 days) alone. Despite of enhanced cytocidal activity over the first 24 hours post dosing and superiority over monotherapy, our findings indicate that the effect was not sufficient to obtain a curative effect unless a longer durations of artesunate is administered. This is in contrast to synergistic interactions demonstrated in previous animal studies (26,27). In contrast, the pyronaridine-azithromycin combination showed mild antagonistic effects. A strong

antagonism, by definition, refers to the loss of schizontocidal effect when the drugs are used in combination, requiring higher concentrations of the drugs to produce the same effect as the drugs alone(68). In the present study, azithromycin of fewer than 3-day duration failed to achieve 100% cure, suggesting that more than a total dose of 150 mg/kg must be present to obtain a curative effect with pyronaridine. Furthermore, increasing the durations of azithromycin or doses of pyronaridine did not seem to achieve greater killing than pyronaridine alone at 5mg/kg, suggesting that pyronaridine-azithromycin combinations do not interact in strict dose-response fashion. Our finding, in part agrees with a previous *in vitro* observation of pyronaridine-azithromycin, in which additive effects were determined(95). In the treatment of uncomplicated falciparum malaria in another study, higher doses of 1,000 or 1,500 mg of azithromycin for 5 and 3 days, respectively, were required in combination with quinine in order to achieve the same effects as with quinine-doxycycline combination(96). In 2003, White et al. in another study of quinine-rifampicin in a clinical trial of uncomplicated malaria observed clear antagonism. The study indicates that recrudescence rates were five times higher for the combination despite of the observed shorter parasite clearance times for the combination than for quinine alone. White et al. suggest that this was attributed to the increased conversion of quinine to its less potent 3-hydroxy derivative in the presence of rifampicin in patients. However, a clinical trial of azithromycin and chloroquine proved ineffective as single agents against uncomplicated falciparum malaria, while effective with more than 97% cure rate when combined, re-stating the therapeutic potential of the combination(97). As an antimalarial, azithromycin is relatively slow-killing, thus,

requires faster acting partner compounds such as artemisinin-derivatives or 4-aminoquinolines that will rapidly reduce the initial parasite burden. In fact, most antibiotics share a common characteristic of the late onset of antimalarial activity, called a delayed phenotype, which justifies its requirement for high initial drug concentrations to achieve moderate killing after 48 hours post dosing(98). Although not evident in the present study, long-lasting killing activity of azithromycin even after the drug is administered for 48 hours only is well documented(98). In the case of azithromycin in combination with pyronaridine, therefore, increasing the dose of azithromycin may ensure that concentrations remain at inhibitory level and longer in the plasma, allowing a greater chance of eradicating all of the parasites.

Synergistic effect is certainly favorable in combination study and the ideal drug partner of pyronaridine should exert at least additive schizontocidal action. However, synergy and additive effects are not essential for successful treatment as some nonsynergistic combinations can be beneficial for chemotherapy such as quinine or mefloquine in combination with tetracycline, which showed additive effects in *in vitro*(99). Taking the results presented in our study in context with literature reports, it is reasonable to conclude that observed additive to mild antagonism between pyronaridine and artesunate or azithromycin do not justify the rejection of the combinations, particularly where one of the compounds is rapidly eliminated.

By adapting a luciferase reporter, *P. berghei,* to study the cytocidal activities of antimalarials over at least a 30-day period, we have demonstrated the therapeutic potential of 4-aminoquinoline pyronaridine, and 8-aminoquinoline tafenoquine in

combination with current antimalarials. Our data suggest that pyronaridine and tafenoquine at noncurative doses can achieve greater parasite killing over a single parasitic life cycle and 30-day survival by combining with artesunate, amodiaquine, or azithromycin.

The above experimental data obtained from the present study, although consistent with clinical observations in part, were tested in murine malarial models in which the parasites were relatively drug sensitive, thus, parasite log reductions were maintained and reflect true intrinsic antimalarial activities of each drug. However, evidence of resistance to pyronaridine and the spread of *P. falciparum* isolates with a delayed clearance phenotype have been observed in certain areas of endemicity, suggesting the need for further investigation against drug-resistant strains of *P. falciparum* to design clinically relevant combination regimens (dose, frequency, and duration) in the field.

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Curriculum Vitae

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Bachelor of Science in Public Health Degree
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Research Experience

December 2016 – Current (40 hours per week)
Johns Hopkins University, Baltimore, MD
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Department of Molecular Microbiology & Immunology
Principal Investigator: David Sullivan, M.D.

- Characterizing pharmacodynamics of tafenoquine and pyronaridine in combination with current antimalarials in murine malaria model

August 2015 – June 2016 (10 hours per week)
UC San Diego, Department of Family Medicine and Public Health
Principal Investigator: Florin Vaida, Ph.D., Sara H Browne, M.D.

- Designing an analysis plan and testing correlations statistically using predictive analytic software, SPSS.
- Participating in clinical research trial, AHF TB001 Project: A pilot clinical trial characterizing use of ingestion sensor enabled Rifamate in comparison to directly observed therapy for the treatment of tuberculosis (TB).

- Developing a statistical analysis plan for AHF TB001 using R (programming language).

May 2015 – August 2016 (20 hours per week)
Stein Institute for Research on Aging
UC San Diego, School of Medicine, La Jolla, CA
Principal Investigator: David Smith, M.D.

- Participating in Pre-exposure Prophylaxis (PrEP) for HIV Prevention Project: Investigating attitudes/perceptions of HIV-positive and HIV-negative persons towards PrEP use. Analyze these attitudes to determine changes in sexual practices/sexual risk behavior prior to and after use of PrEP.
- Participating in End of Life Project; *Last Gift*: Investigating how many HIV-infected individuals in the CNICS study population would consider participating in clinical research near the end of their life, and assessing what risks of this research they would be willing to endure.
- Developing online survey questions for potential participants.
- Designing an analysis plan and testing correlations statistically using predictive analytic software, SPSS.
- Attending weekly lab meetings for the purpose of acquiring au courant information.

December 2014 - April 2015
Samford University, McWhorter School of Pharmacy
Department of Pharmaceutical, Social & Administrative Sciences
Principal Investigator: Jongwha Chang, Ph.D.

- Pre-exposure Prophylaxis (PrEP) as HIV prevention strategy; evaluate findings from published research studies about physician perceptions, populations at increased risk PrEP awareness, and current PrEP roll-out.

Peer-reviewed Publications

Kim SH, **Lee G**, Chang J (2015) Pre-exposure Prophylaxis (PrEP) as HIV Prevention Strategy and Public Health Implications. Int. Res. J. Pharm. 2015;6(4):227-230
Kim SH, **Lee G**, Kim E, Jung H, Chang J. (2017) Quetiapine misuse and abuse: Is it an atypical paradigm of drug seeking behavior? J Res Pharm Pract 2017;6:12-5

Publication under review

Prakash K., **Lee G**, Smith D. (2017) Willingness to Participate in Research at the End of Life.

Honors and Awards

Alpha Chapter of the Delta Omega Public Health Honor Society April 2018
Recipient of FundScholar scholarship in science/medicine January 2018
Masters Tuition Fellowship for Outstanding Academic Achievement - Johns Hopkins Bloomberg School of Public Health 2017-2018
UC San Diego Department of Public Health – Outstanding Scholar 2014-2016
Sixth College Provost Honors (Every Quarter from Fall 13') 2013-2016
National Honor Society 2005-2007
AP National Scholar 2006-2007
Academic Decathlon, Orange County- Overall Division: 2nd place / Individual: Math-1st place, Science-1st place 2006

Reader/Grader/Teaching Assistance Experience

Graduate Teaching Assistant: Biology of Parasitism Graduate course Winter 2017
Department of Molecular Microbiology & Immunology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD
Course Instructor: David Sullivan, M.D., Clive Shiff, Ph.D.

Reader: FPMU 50. Family Preventive Medicine Undergraduate Fall 2014, Fall 2015
Department of Family Preventive Medicine and Public Health, UC San Diego, La Jolla, CA
Course Instructor: Michelle Johnson, M.D.

- Assisted with grading, exam proctoring
- Assisted with development of assignments and exams

Teaching Assistant: CHEM 140C. Organic Chemistry Spring 2015
Department of Chemistry/Biochemistry, UC San Diego, La Jolla, CA
Course Instructor: Kim Albizati, PhD.

- Led weekly sections for students who need help in organic chemistry course materials.
- Held weekly office hours for students who need extra help in organic chemistry concepts, and preparing supplementary class materials.
- Graded students' exams and held review sessions before midterm and final exam.

Grader: CHEM 140A, CHEM 140B, CHEM 140C; Three Sequential Organic Chemistry Courses Spring 2014, Summer Session I,II, 2014
Department of Chemistry/Biochemistry, UC San Diego, La Jolla, CA

Course Instructor: Kim Albizati, PhD.

- Assisted with development of answer keys for midterm and final exam
- Graded and proctored students' midterm and final exams

Extracurricular Activities

UCSD World's AIDS Day Event coordinator 2014-2015
American Foundation for Suicide Prevention (AFSP)- Walk event planning committee member 2014-2015
HandsOn San Diego Team Leader of a Project, "Feeding America" 2013-2014
San Diego Symphony Orchestra –Bassoon 2012
Orange County Youth Symphony Orchestra - First Chair Bassoon 2006-2007
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