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Efficacy and Resistance Potential of JPC-3210 in *Plasmodium falciparum*

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by

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Abstract

Combating drug resistant malaria has been historically challenging, and remains so today. Recent reports from Southeast Asia show that *Plasmodium falciparum* is developing resistance to even our best defenses; artemisinin-based therapies. This development threatens to become a significant challenge in controlling malaria infections worldwide, making research into developing and characterizing new antimalarial drugs increasingly important. The purpose of this study was to characterize the resistance potential of novel antimalarial compound JPC-3210 *in vitro* using *P. falciparum* clones. JPC-3210 is a new long acting drug with potential to be used in combination with fast-acting drugs like artemisinins to cure drug resistant malaria. In this study several methods were used to characterize the efficacy and resistance potential of JPC-3210. To determine the frequency of resistance generation in *P. falciparum* clones, parasites were kept under continuous drug pressure for thirty days, at which point drug pressure was removed and cultures were observed for signs of recrudescence. *P. falciparum* clones also were exposed to increasing levels of intermittent drug pressure that involved 3-4 days of drug exposure followed by a recovery period. The step-wise experiment was conducted over three months with drug pressure being increased step-wise until a maximal concentration of 700 ng/ml of JPC-3210; resistance was measured phenotypically in drug susceptibility assays at multiple time points. Additionally, the ability of JPC-3210 to induce dormant stage parasites, and its effect on dihydroartemisinin (DHA)-induced dormant stages was assessed in both a chloroquine resistant parasite (W2) and in an artemisinin resistant clone (4G). Results showed that the frequency of resistance against JPC-3210 in W2 clones was less when compared to that of atovaquone. The step-wise pulse exposure of JPC-3210 induced resistance in W2 clones, however, resistance proved unstable. Dormant stage parasites were not induced by JPC-3210, even at high concentrations in W2 or 4G clones, furthermore, the effect of JPC-3210 on dormant-induced parasites was found to be dose dependent, yet the drug did not kill DHA-induced dormant rings.

JPC-3210 appears to be a good drug to use in combination with other antimalarial compounds for treatment of *P. falciparum*, but further research is needed. Future studies to assess the field performance of new antimalarial compounds by investigating resistance and dormancy profiles *in vitro*, and thereby maximizing out understanding of such drugs and their optimal implementation, are of the utmost importance.

Introduction

Malaria is a vector borne infectious parasitic disease. In 2013, there were about 200 million cases reported worldwide, around 600,000 deaths, and it is considered a major global public health concern (Roll Back Malaria, 2013a). The word "malaria" comes from the Italian language, meaning "bad air," and the disease has had a substantial impact on human history (CDC, 2012). According to the World Health Organization's (WHO) World Malaria Report in 2014, malaria is found in 97 countries and it is estimated that 3.2 billion people are currently at risk (WHO, 2014a).

Malaria is caused by the protozoan *Plasmodium*, and is a member of the phylum Apicomplexa, as these protozoans possess an apical complex structure which facilitates entry into host cells (Bogtish et al., 2013). There are over 50 species of *Plasmodium* that infect an array of animals, of which five are known to infect humans: *P. falciparum, P. vivax, P. ovale, P. malariae*, and *P. knowlesi* (Bogtish et al., 2013). The most common cause of malaria is *P. vivax*, which is endemic to many tropical and subtropical regions worldwide. However, the most deadly form of malaria is caused by *P. falciparum*, which is found in Sub-Saharan Africa, Asia, and South America, is most often implicated in severe malaria, and is responsible for 90% of deaths attributed to malaria (WHO, 2014a). *Plasmodium* is transmitted by up to 40 species of female *Anopheles* mosquitos; however, the particular species of *Anopheles* mosquito involved in transmission depends on location and climate (CDC, 2014a).

The life cycle of *P. falciparum* involves a vector stage and a human stage, with the human stage being further subdivided into liver and blood stages. The life cycle involves a female mosquito picking up malarial parasites in the blood when feeding on an infected human, from which a portion of these parasites are in the dormant asexual stage known as gametocytes; the necessary stage for survival within the mosquito. When the blood cools within the mosquito the asexual parasites develop into a male or

female gamete; the female becomes an egg and the male becomes a sperm, both of which are known gametes. The fertilized cell, known as an ookinete, exits the mosquito's stomach lining and turns into an oocyst, which by asexual replication produces sporozoites that infest the salivary glands. Saliva is injected to stop blood from clotting when the female mosquito takes a blood meal, and malarial sporozoites within the saliva are passed to the human host, entering into the capillaries and migrating to the liver via the blood stream. Inside the liver they enter hepatocytes and develop into schizonts, which then rupture, releasing merozoites into the bloodstream. As a mechanism to evade the immune system, merozoites target and enter red blood cells and continue to develop into immature trophozoite rings. At this point development can continue on from schizonts to merozoites, or the parasites can develop into male and female gametocytes (Bogtish et al., 2013). If within the erythrocyte, the trophozoites continue to develop to schizonts, merozoites are released once the red blood cells rupture; this process may release up to 20 merozoites per erythrocyte. This lysing of the red blood cells induces a clinical presentation of periodic fever, also known as paroxysm, during which toxins are released from the rupturing erythrocytes (Karunaweera, Grau, Gamage, Carter, & Mendis, 1992). Depending on the species of *Plasmodium* that causes the malaria infection, this periodic fever may be seen every 24, 48, or 72 hours; the length of the period mirrors the length of the asexual development cycle in erythrocytes and varies in different species of *Plasmodium* that infect humans. The complexity of the lifecycle, and a lack of knowledge about how our immune system interacts with the parasite are indicative of some of the challenges associated with treatment and why an effective vaccine remains an elusive goal (CDC, 2014c).

Infection from malaria is classified as either uncomplicated or severe. Clinical manifestations of uncomplicated malaria include periods of fever, chills, sweating, and lethargy. Severe cases can be life threatening and result in organ failure, severe anemia, and cerebral malaria, potentially resulting in coma and death. *P. falciparum* is the protozoan species capable of causing cerebral malaria, in which the parasite produces a protein on the red blood cell wall that causes cytoadherence to receptors on the endothelial lining of blood vessels to the brain (Chen, Schlichtherle, & Wahlgren, 2000). This

mechanism causes infected and uninfected red blood cells to adhere in clumps, leading to vascular occlusion that ultimately blocks blood flow to the brain. Severe malaria can be life threatening and must be treated immediately, as fatal complications may result, particularly in children and pregnant women. In fact, mortality rates are 50% higher during pregnancy in severe malaria cases, as compared to severe cases in a non-pregnant adult (WHO, 2014). In addition, there is increasing evidence that *P.vivax* can cause severe disease and death, particularly in patients with other health complications such as malnutrition or HIV infection (Douglas et al., 2014).

There are about 600,000 cases of cerebral malaria annually, mostly occurring in children in sub-Saharan Africa. Of the children who survive, many suffer from long-term neurological problems (Murphy & Breman, 2001). Populations especially susceptible to complicated malarial infections include young children, infants, pregnant women, and people visiting endemic areas with no previous exposure to malaria. Children are particularly affected because they have not yet built up a resistance to the malaria parasite, and carry a greater risk of complications from cerebral malaria. In pregnant women, malaria may lead to low birth weight or infant mortality, due to cytoadherence of infected red blood cells to the placenta (Muthusamy, Achur, Bhavanandan, Fouda, Taylor, & Gowda, 2004).

Clinical diagnosis is based on the presentation of symptoms. Additionally, the Centers for Disease Control and Prevention (CDC, 2014b) recommends confirmation through microscopy, antigen detection using Rapid Diagnostic Tests, and when available, molecular diagnostics such as PCR and ELISA. Furthermore, drug resistance tests should be performed to indicate which treatment would be most effective (CDC, 2012).

Malaria is found primarily in tropical and sub-tropical regions, where its prevalence and that of the vector depends on factors such as temperature, humidity, and rainfall (CDC, 2014e). More specifically, it has been shown that rainfall must be greater than 80 mm monthly, with average temperatures between 18°C and 32°C, and humidity levels must be greater than 60% (Grover-Kopec,

Blumenthal, Ceccato, Dinku, Omumbo, & Connor, 2006). Malaria can be present in locations throughout the year or have a seasonal pattern, depending on temperatures and rainfall.

P.vivax is the most common of the malaria species to infect humans, and demonstrates widespread distribution. This species is attributed with causing the majority of malaria cases outside of Africa, although these infections are rarely life threatening. While Africa bears the greatest malaria burden, with the majority of deadly cases occurring there, malaria is also found in Latin America, Asia, some parts of the Middle East, and even the U.S., whereby about 1,500 people (mostly returning travelers) are diagnosed each year (CDC, 2014f). In the U.S., malaria first appeared alongside European settlers in the mid-18th century, and was present until the early 1950s, when elimination was achieved primarily through the use of pesticides such as DDT and efforts to eliminate mosquito-breeding sites.

Fevers and symptoms matching that of clinical malaria presentation have been documented as early as 5,000 years ago in China, 4,000 years ago in ancient Egyptian texts, 3,000 years ago in India, and was noted in Ancient Greece and Rome. It is believed that malaria migrated out of Africa and dispersed with populations where vectors and climate were suitable, with agriculture playing a role in stabilizing transmission. Evidence of strong selective pressure is exhibited in traits that appear to have come at a cost, including Thalassemia, glucose-6-phosphate dehydrogenase deficiency (G6PD), sickle cell traits, and the Duffy negative RBC phenotype (Carter & Mendis, 2002). These mutations offer protection against malaria and have been passed on, occurring in high frequency in some populations, thus indicating that malaria has been a strong acting force in our evolutionary history.

Many factors work together to contribute to a person's risk of acquiring malaria. Living in an endemic area is a prominent factor, but there are numerous other factors that play a role as well. Malaria has often been identified as a disease of the poor, and there have been many challenges in the implementation of control programs due to poverty and poor infrastructure in communities of low socioeconomic status (Enayati & Hemingway, 2009; Yadav, Dhiman, Rabha, Saikia, & Veer, 2014). A Gallup poll in 2013 reported that the top ten countries in which people are living in extreme poverty are

all located in sub-Saharan Africa, which is where 90% of deaths from malaria occur (Gallup, 2013). Socioeconomic barriers contributing to increased risk of malaria infection are poor housing, inability to afford preventive measures such as bed nets and insecticides, employment such as agricultural work which increases contact with the vector, lack of education about prevention and treatment, and the inability to adhere to treatment regimens due to financial restrictions. Additionally, limited access to healthcare facilities is problematic, as according to the WHO treatment guidelines for 2014, it is very important to get to a hospital within 24 hours of the first appearance of symptoms of a *P. falciparum* infection (WHO, 2014b). Unfortunately, reaching such facilities is often challenging in rural settings where transportation is limited.

The global economic impact of malaria was quantified at $12 billion (USD) in direct losses in 2013, with a loss of 1.3% of gross domestic product per year in Africa (Roll Back Malaria, 2013). According to a study published by the American Journal of Tropical Medicine and Hygiene in 2001, "countries with intensive malaria grew 1.3% less per person per year, and a 10% reduction in malaria was associated with 0.3% higher growth" (Gallup & Sachs, 2001). Over time, these losses result in large economic differences between counties with and without malaria. On a smaller scale, malaria leads to individual hardships due to the financial burden of morbidity and mortality, but also in terms of lost productivity due to sickness and long term neurological deficits in children that survive severe *P. falciparum* induced malaria.

Although not fully understood, immunity to malaria can be acquired naturally as the result of multiple exposures and protects people who are in malaria-endemic areas. Children who are routinely exposed to malaria parasites, and adults in endemic areas, rarely get very ill, excluding those people who are pregnant, considered immune-suppressed, or suffering from other diseases. However, immunity is lost quickly if a person goes to a region without malaria and is not continuously exposed to the bites of infectious mosquitos. Previous studies have shown that when programs aimed at reducing malaria in

endemic regions do not fully stop transmission, malaria typically rebounds, with potentially catastrophic results for people who had lost their immunity (Doolan, Dobano & Baird, 2009).

According to the CDC (2014b), the types of treatment being considered should take into account what type of malaria is implicated in the infection, the location of the acquired infection (to assess drug resistance), whether it is complicated or uncomplicated malaria, and if the patient is immunosuppressed or pregnant. The WHO recommends artemisinin combination therapy (ACT) to treat all uncomplicated malaria. This treatment consists of two medications that act on the schizont stages of the parasites, with each medication acting on different targets within the parasite. This therapy reduces the chance that resistance to both medications will develop. Using monotherapy to treat malaria is no longer recommended, especially since using chloroquine, sulfadoxine-pyrimethamine, or even artemisinin monotherapy has resulted in parasites resistant to these drugs. ACTs are combinations, wherein artemisinin, or its derivatives, is paired with another compound. Artemisinin and its derivatives are fast-acting drugs with short half-lives and are recommended to be administered for three days in combination with a slower acting drug with a longer half-life, for which resistance has not been shown in that area (WHO Treatment Guidelines, 2014). ACTs recommended by the WHO include: artemether plus lumefantrine, artesunate plus amodiaquine, artesunate plus mefloquine, artesunate plus sulfadoxine-pyrimethamine, and dihydroartemisinin plus piperaquine (WHO, 2014). For treating severe malaria infections, the WHO recommends the administration of intramuscular or intravenous artesunate; artemether, quinine, or rectal artesunate. Second line therapies for severe malaria should not be used if artesunate is available since it has been shown to be much more effective at saving lives than other therapies. In addition, other measures must typically be taken if a patient is severely anemic, in a coma, suffering from seizures, or has another serious health complication. Failures in treatment may be the result of drug resistance, re-infection, or recrudescence of the parasite, which is defined as a low-level persistence of parasites despite treatment (Bogtish et al., 2013). Moreover, failure may result from

substandard medication. Substandard and counterfeit anti-malarial drugs are a problem in many part of the world and can be a driving factor in drug resistant malaria (Karunamoorthi, 2014).

Travelers are particularly vulnerable to malaria, and the CDC (2014c) provides country-specific guidelines for choosing which drugs to use for prophylaxis. These guidelines depend on resistance in a given area, and are noted not to be 100% effective; care must still be taken to avoid contact with the vector. Potential options for prophylaxis recommended by the CDC include: Atovaquone/Proguanil (Malarone), Chloroquine, Doxycycline, Mefloquine (Lariam), and Primaquine (CDC, 2014b).

In the mid-1950s, the WHO launched The Global Malaria Eradication Programme, which aimed to eradicate malaria through the use of the insecticide DDT and the use of chloroquine as a treatment method. These efforts had success in parts of the Americas, Europe, and Asia, but the program did not target Africa. Ultimately, resistance developed to chloroquine and DDT, and this outcome, in addition to other factors, led to the program's end in 1972 (Greenwood et. al., 2008). Malaria research today involves a variety of components, including ongoing research in the areas of vector control, vaccine development, preventive methods development, and laboratory research to find and characterize new antimalarial drugs that may one day replace current drugs, which are developing or have already developed resistance. Campaigns—such as Roll Back Malaria, which is a partnership between WHO, UNICEF, UN, the World Bank, and many other organizations—are working towards the reduction and ultimate elimination of malaria worldwide on a country-by-country basis. Elimination efforts include improving health care systems, providing prevention and treatment to vulnerable people at risk (particularly those of low income), putting monitoring systems in place, increasing access to care, investing in research, and getting communities involved in prevention efforts (Roll Back Malaria, 2014). Additionally, many public and private organizations in the U.S. and abroad are very actively participating in efforts to research and reduce malaria worldwide. The U.S. government supports research and development through institutions such as the National Institute of Allergy and Infectious Diseases (NIAID) and the National Institutes of Health (NIH). Furthermore, organizations in the private sector support this effort, particularly the Bill

and Melinda Gates Foundation, which has donated billions of dollars toward the goal of eradication through various methods (Gates Foundation, 2014). On a positive note, according to the Gates foundation, in the past decade there has been a 25% decline in malaria incidence worldwide and a 42% drop in deaths from malaria (Gates Foundation, 2014). Despite these advances, many factors remain which contribute to the difficultly of achieving eradication, such as rapid emergence of drug and insecticide resistance, mobility of populations, political instability, natural disasters, economics, and poverty (Liu, Modrek, Gosling, & Feachem, 2013). Cultural challenges also exist, including the risk perception of malaria, and challenges associated with infrastructure, such as poor access to hospitals and a limited ability to diagnose and treat the disease (Shah, 2013).

Plasmodium parasites capable of developing drug resistance are a persistent threat to global public health. Parasites with resistance to the best antimalarial compounds can be induced in laboratories, and have been reported clinically in certain regions of the world. Drug resistance has emerged and spread in the past, and will almost certainly continue to happen in the future. To combat treatment failure from recrudescing or resistant parasites it will be necessary to find and characterize new antimalarial compounds and investigate better drug combinations.

Quinine was the first effective antimalarial compound, which was extracted from the bark of the cinchona tree in Peru in the early 17th century (CDC, 2012). Quinine was used almost exclusively until the mid-1900s, when due to supply and demand issues, chloroquine (CQ) was synthesized and became the premier drug, as it was able to be mass-produced and had fewer side effects (Achan et al., 2011). By 1960 chloroquine was widely affordable and available, and is reported to have contributed to—along with the use of DDT—a reduction in infant mortality and a population increase in Africa during this time period (Webb, 2009). However, *P. falciparum* resistance to chloroquine began emerging in the late 1950s, spreading out of South America and Southeast Asia, and by the 1980s and 1990s, resistance was widespread across the globe (CDC, 2012). Resistance to chloroquine resulted in increased mortality rates from malaria, most markedly in Sub-Saharan Africa (Packard, 2014). In the early 1970s, other drugs

were synthesized to replace chloroquine, including sulphadoxine-pyrimethamine (SP), which became a common first defense in areas with chloroquine resistance. As with CQ, SP resistance emerged rapidly out of the same areas in Southeast Asia and South America (WWARN, 2014). The next antimalarial drug, mefloquine, was introduced in Southeast Asia, and resistance occurred rapidly throughout that region during the 1990s (WWARN, 2014).

Currently, the newest and best treatments for malaria are artemisinin derivatives, which were originally isolated from a plant used in herbal Chinese medicine in the 1960s, but for political reasons and doubts about their effectiveness, they were not widely available or used outside China until the 1990s (Faurant, 2011). In 2005, the WHO called for an end to monotherapy with artemisinin derivatives to prevent resistance from developing as quickly as with other drugs in the past, and recommended that ACTs be the first treatment for cases of *P. falciparum* (WHO, 2014c). During treatment, clearance of the malaria parasite is more effective when ACT is utilized. ACTs combine artemisinins (fast-acting antimalarial drugs with a short half-life) with an additional (longer-acting) drug; these combinations are used globally against *P. falciparum*, with the drugs used in varying combinations depending on the region and local resistance patterns.

An example of the benefits of switching to ACTs is represented in a study, in which initially, mefloquine in combination with sulphadoxine-pyrimethamine had an effective cure rate of 95%, but after resistance developed, the cure rate subsequently dropped to 71%. However, when mefloquine—to which resistance had already developed—was combined with artesunate, the cure rate reached 100% (Nosten et al., 2000). This result highlights how new drug combinations can be highly effective even if resistance has already developed to the component used in combination with an artemisinin derivative.

Although ACTs are currently the best therapy, longer drug regimens are needed to treat malaria in Western Cambodia and along the Thailand-Myanmar boarder, where there are fears that resistance is once again popping up (Dondorp et al., 2009). The specific site in western Cambodia is where much of the past and current antimalarial resistance has emerged (e.g., chloroquine and SP resistance), and continues

to emerge. This development may be the result of the unregulated use of antimalarial drugs, counterfeit pills, and/or dosage issues. There is also evidence that there are multiple populations of resistance in this small area due to high genetic diversity (Miotto et al., 2013). If artemisinin resistance begins to spread out from this region, as has happened in the past with other drugs, the result could be devastating to many people all over the world, and especially in sub-Saharan Africa where *P. falciparum* is endemic (Hay et al., 2009). It should be noted that infections from *P. malariae*, *P. vivax*, and *P. ovale* are treatable with chloroquine, and while there has been some resistance based on the location of the acquired infection, it has not been nearly as problematic as has been seen with *P. falciparum* (CDC, 2014b). Locations where chloroquine resistance has been documented in *P. vivax* infections include Papua New Guinea, where resistance was first seen in the 1980s, Indonesia, Myanmar, and India (Baird, 2004). Current research through genomic analysis of artemisinin-resistant parasites indicates that the resistance mechanism is thought to be linked to mutations in the kelch protein propeller domains (Ariey et al., 2014). It is important not only to investigate this further, but also characterize resistance against other antimalarial compounds currently in use, and in those being developed through the use of stable resistant lines.

The first study for which resistance was selected *in vitro* was published by Nguyen-Dinh and Trager (1978) and the induced resistance was to chloroquine in a *P. falciparum* strain. This study was flawed however since the parasite used already was resistant to chloroquine. Regardless, at the time it suggested that drug resistance could be induced *in vitro* and this opened the door for many following studies to induce resistance and subsequently investigate the mechanism by which resistance occurs (Nzila & Mwai 2009). In a notable study published by Gassis and Rathod (1996), the researchers developed an *in vitro* method for measuring the frequency of drug resistance in *P. falciparum* clones, through which they determined at what drug concentration and initial parasite population parasites recrudesced under drug pressure. Their research focused specifically on the frequency of resistance to atovaquone and 5-fluoroorotate, both independently, and in combination. They showed that it is important to find the frequency of resistance of a parasite to a drug, because even if an antimalarial

compound looks promising, resistance can develop if the total population of parasites is not eliminated. It was noted in this study that increasing the concentration of a drug will decrease the frequency of resistance, but it is also an important way to determine the line between eliminating parasites and having drug toxicity. This study supported the use of multiple antimalarial compounds to reduce the frequency of resistance, since combination therapy results in a lesser chance of recrudescence (Gassis & Rathod, 1996). Later studies showed that through analysis of atovaquone resistance *in vivo*, cytochrome b mutations were associated with the resistance mechanism and acted by inhibiting the drug from affecting the mitochondrial electron transport system (Korsinczky, Chen, Kotecka, Saul, Rieckmann, & Cheng, 2000). Also, the kelch mutations associated with artemisinin resistance were first identified in an *in vitro* derived artemisinin-resistant parasite (Ariey et al., 2014). Although these studies were unrelated, these examples highlight how the development of resistant parasites *in vitro* can lead to a greater understanding of resistance mechanisms.

Newly discovered antimalarial drugs typically undergo characterization methods which include a component of assessing the resistance potential of drugs prior to devoting resources into development of a compound for clinical use. Often these studies include a frequency of resistance comparison to atovaquone in *P. falciparum* clones by using methods developed by Gassis & Rathod (1997). Therefore, the first aim of this study will be to determine the frequency of resistance of a new novel antimalarial compound, JPC-3210 in relation to the known frequency of resistance to atovaquone in *P. falciparum* W2 clones. From this experiment there will be two potential outcomes: either the parasites recrudesce and require phenotypic and genotypic evaluation, or no parasites will emerge.

The use of pulse exposure to antimalarial drugs on various *P. falciparum* lines in an attempt to induce resistance has been used throughout the years by many researchers. In addition to Nguyen-Dinh and Trager (1978), Oduola, Milhous, Weatherly, Bowdre, and Desjardins, (1988), Chavchich, Gerena, Peters, Chen, Cheng, and Kyle (2010), and Tucker, Mutka, Sparks, Patel, and Kyle (2012) all have used pulse exposure *in vitro* to develop resistance to various drugs. In Oduola et al. (1988), *P. falciparum*

parasites were made resistant to mefloquine hydrochloride *in vitro* by exposing W2 parasites through increasing concentrations of drug over 96 weeks, which resulted in stable mefloquine resistant W2 parasites (W2-mef). Chavchich et al. (2010), selected for parasites with resistance to artelinic acid and artemisinin, and analyzed the genotypic changes, which resulted in several *P. falciparum* lines which displayed resistance to artelinic acid and multiple artemisinin derivatives. They found that exposure to increasing amounts of artelinic acid over time resulted in reduced susceptibility, not only to artelinic acid, but to other drugs as well. This study was one of the first to demonstrate that artemisinin resistance can be produced in the lab. This also increased resistance to mefloquine, a common drug often used in combination with artemisinin derivatives clinically. Additionally, this study correlated observed changes in coding sequences in parasites that had developed resistance.

Other studies attempted to develop resistance to artemisinin *in vitro* and were successful, however, resistance often proved to be unstable. Phenotypes resulting from increased exposure to a drug may become unstable quickly or over a longer period of time in the absence of drug pressure in continuous culture (Nzila & Mwai 2009). An explanation for instability hypothesized by Preechapornkul et al. (2009), which looked at *pfmdr1* in mefloquine resistant parasites, is that through developing mechanisms to resist drug effects there is a resulting loss of fitness to the parasite, and the phenotype reverts back to the original phenotype because it is more advantageous in the absence of drug pressure. A key difference in this study by Chavchich et al. (2010) was that resistance was not achieved with continuous drug pressure with incremental increases, but through pulse exposures to the drug from which parasites were allowed to recover in between dosages, resulting in a stable phenotype. Tucker et al. (2012), expanded on work by Chavchich et al. (2010), by producing artemisinin-resistant parasites that had stability and were resistant to higher levels of artemisinin, similar to dosages that are used clinically. Resistance was measured in the shifting IC50 values (the concentration of drug which inhibits 50% of parasite growth) and through genomic analysis, specifically of *pfmdr1*. Overall, resistance was measured and interpreted using IC50 data throughout these experiments. The IC50 and IC90 values used in this

study, and many others, reflect the concentration of drug that inhibits 50% and 90% of parasite growth. An increase in these values reflects a reduced susceptibility of the parasite to the drug's effects, and when these values shift higher than that of the parental clones, it is an indication that resistance is occurring.

IC50 and IC90 values can be determined using the 3H-hypoxanthine uptake inhibition assay, which was first developed by Desjardins, Canfield, Haynes, and Chulay (1979). In this assay inhibition of the uptake of Tritium radiolabeled hypoxanthine, which is incorporated during nucleic acid synthesis as a purine, indicates the susceptibility of the parasites to the compound being evaluated as amount of hypoxanthine incorporated is proportional to the number of parasitized erythrocytes present (Chulay, Haynes, & Diggs, 1983). Desjardins et al. (1979) noted that the "Inhibition of uptake of a radiolabeled nucleic acid precursor by the parasite served as the indicator of antimalarial activity" (p. 710).

A second component of assessing for the development of drug resistance in *P. falciparum* clones includes the application of discontinuous drug pressure to see if resistance develops. These types of resistance experiments would be expected to produce one of three potential outcomes: either parasites become resistant and require evaluation, resistant parasites emerge but resistance is unstable, or no parasites emerge from drug pressure.

Previous experiments have shown that interrupting the ring stage of *P. falciparum* parasites with dihydroartemisinin (DHA), a derivative of artemisinin, will induce dormant parasite forms. This finding led to the hypothesis that some parasites enter a stage of dormancy that allows them to withstand drug pressure in response to artemisinin, and then recover when that pressure is removed (Kyle & Webster, 1996). Kyle and Webster (1996) further hypothesized that dormancy serves as an innate protection mechanism, which could explain recrudescence seen after artemisinin monotherapy, and could explain delayed treatment times seen in some Cambodian cases (Noedl, Se, Schaecher, Smith, Socheat, & Fukuda, 2008). Dormant parasites seen in cultures after exposure to artemisinin have been described as having "distinct morphological features: the vacuole is not present, the cytoplasm is condensed and tightened towards the nucleus and the nucleus is condensed" (Cheng, Kyle, & Gatton, 2012, p. 250).

Teuscher, Gatton, Chen, Peters, Kyle, and Cheng (2010) found that exposing parasites to 200 ng/ml of DHA for six hours halted parasite growth, followed by a period of dormancy where growth was arrested, after which recovery and growth of normal asexual parasites was observed several days later. Furthermore, when looking at DHA in combination with exposure to mefloquine, it was found that parasites took longer to recover than with DHA alone, which indicated that mefloquine was acting on dormant-stage parasites, or parasites that were recovering from dormancy. According to Teuscher et al. (2010), dormancy could explain why artemisinin is less effective against combating malaria than when paired with a long acting drug, which would kill off the remaining dormant parasites (Cheng, Kyle, & Gatton, (2012). In 2011, Codd, Teuscher, Kyle, Cheng, and Gatton designed a model to predict the impact dormancy would have on treatment clinically, which indicated that dormant parasite recovery could account for the high number of treatment failures seen in artemisinin monotherapy. Additionally, this paper also described the difference between recrudescence and resistance, as a slower parasite clearance over time implies resistance.

Tucker et al. (2012), performed quantitative recrudescence assays drawing on methods developed by Teuscher et al. (2010). Daily slides of recovering dormant parasites were measured to determine the ratios of dormant parasites to normal parasites, as cultures recrudesced and gave a clearer picture of what is observed during recovery from DHA. This study also showed that both artemisinin-sensitive and artemisinin-resistant parasites were capable of entering and recovering from dormancy. Tucker et al. (2012) described dormancy as a "novel resistance mechanism that can result in parasite recrudescence." Recently, researchers investigated the metabolic activity in dormant parasites induced by exposure to DHA, and found primarily that there was a down regulation of metabolic pathways, with only two metabolic pathways found to be active (Chen et al., 2014). The active metabolic pathways were the pyruvate pathway and the fatty acid metabolism pathway. Such information could be used to find more effective antimalarial drug combinations that eliminate dormant parasites, thus reducing treatment recrudescence.

Given the likely importance of artemisinin induced dormancy and its potential for being a component of novel drug resistance, new drug discovery efforts should include studies to assess the potential for candidate compounds to eliminate dormant parasites. The methods for these studies have been developed by Teuscher et al. (2010) and Tucker et al. (2012) and can easily be applied to other drugs in the development pipeline. Accordingly, the third component of this study is to assess the effect JPC-3210 has on dormant parasites, and to determine if JPC-3210 is capable of inducing dormancy in both artemisinin-resistant and non-artemisinin-resistant clones, as it has been shown that non-artemisinin antimalarial drugs can also induce dormant parasites (Nakazawa, Maoka, Uemura, Ito, & Kanbara, 2002).

In the 1960s and 1970s, the U.S. Army Research Program in Malaria screened around 250,000 (WHO, 1981) compounds for antimalarial activity as a way to combat chloroquine resistance. Of these compounds, 43 were selected for evaluation through clinical trials. Included in the trials was WR-194,965, an aminomethylphenol Mannich base (Figure 1). This compound was first synthesized in the early 1940s during World War II as part of the campaign to discover new drugs to combat malaria infection in allied troops (WHO, 1981). Analysis of this compound, and others with similar structural features was not compelling and research was abandoned in favor of other drugs with longer acting profiles (Schmidt & Crosby 1978). Soon thereafter, research into WR-194,965 was resumed and it was found to be active against malaria infection in monkeys, and comparable to chloroquine with a long half-life. In 1978, Schmidt & Crosby looked at dosage variations, and found that WR-194,965 had low toxicity, with the amount of drug required being more important than how the dosages were administered. In 1984, a small human study was performed to test the compound involving six volunteers who were infected with malaria. Of these volunteers, four were cured and the remaining two had parasite recrudescence. Ultimately, WR-194,965 was determined to have limited potency as compared with other compounds in development at the time (e.g., mefloquine) and research on WR-194,965 compound was discontinued (Cosgriff, 1984).

Recently, a study analyzed and compared the novel antimalarial compound JPC-2997 to WR-194,965, both of which are aminomethylphenols and have very similar structures (Birrell et al., 2015). JPC-2997 was found to be active against chloroquine-sensitive and chloroquine-resistant cell lines, in addition to a multidrug resistant line of *P. falciparum*, and featured a long half-life, more activity (2-4 fold), and less toxicity than WR-194,965. JPC-2997 was also found to be more active *in vivo* using mouse and monkey models than chloroquine, DHA and WR-194,965, and was determined to have the potency that WR-194,965 was originally cited as lacking. Researchers suggested that due to these factors, JPC-2997 would be a good candidate as a partner to artemisinin in a new ACT regimen.

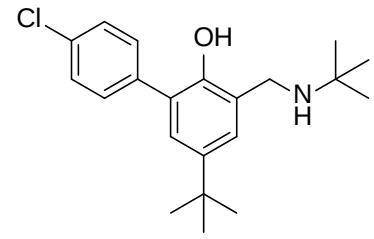
Another promising antimalarial compound, JPC-2583, was presented at the 60th annual meeting of the American Society of Tropical Medicine and Hygiene in 2011 by Chavchich et al. (unpublished results). JPC-2583 was reported to have antimalarial activity *in vitro* without inducing dormancy, was effective at eliminating parasites in combination with DHA, and did not induce dormancy with exposure to high drug concentrations. Trophozoite stages were determined to be the most susceptible, and the activity of JPC-2583 was assessed to be comparable with that of piperaquine. Since then additional studies have been conducted by Jacobus Pharmaceutical Co., Inc. to continue optimization of this series. As a result, JPC-3210 (Figure 1) has been identified as the best candidate for further development.

In 1999, the nonprofit Medicines for Malaria Venture (MMV) was formed and works today in collaboration with researchers around the globe toward the discovery and development of new drugs. MMV estimates that it takes an average of 12 to 15 years for an antimalarial drug to be approved for use due to the multiple layers involved in the process (Burrows, Huijsduijnen, Möhrle, Oeuvray, & Wells, 2013). A drug has to be characterized i*n vitro*, *in vivo*, various combination therapies have to be explored, and a drug has to work in malaria-endemic countries, where different resistance profiles may exist. According to MMV, the desirable traits of drug candidates include safety and efficacy, low resistance potential, affordability, and a combination of drugs that have fast parasite clearance, which can be combined with a longer acting drug to eliminate all blood stage parasites (Burrows et al., 2013). In the

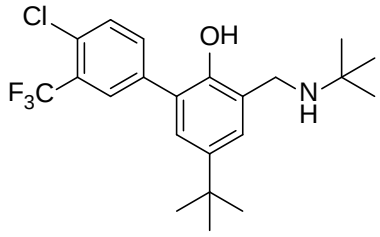
most recent report from MMV, JPC-3210 was designated as part of the Global Malaria Portfolio, with JPC-3210 being in the preclinical stage of development. Jacobus Pharmaceutical Co., Inc. are finalizing the preclinical data package for submission to the US Food and Drug Administration so that JPC-3210 can be developed as a new, long acting antimalarial drug for use in a new ACT regimen. As part of this data package, what would be needed is information on the resistance potential for the compound.

Aims for this study are 1) to assess the frequency of resistance for JPC-3210, 2) to select for resistance through pulse drug exposure, and 3) evaluating the drug effects on dormant ring-stage parasites, all within the context of characterizing the new novel antimalarial compound JPC-3210, developed by Jacobus Pharmaceutical Co., Inc.

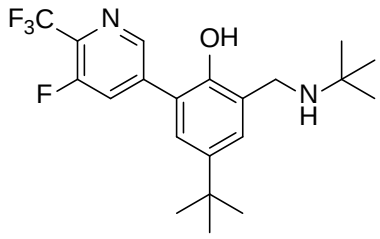
Chemical Structures



WR 194,965



JPC-2583



JPC-3210

Figure 1. Chemical structures of related compounds; WR-194,965, JPC-2583 and JPC-3210. Compounds JPC-2583 and JPC-3210 were optimized by Jacobus Pharmaceutical Co., Inc. and are considered structural analogs of WR-194,965.

Materials and Methods

Parasites

P. falciparum clones and isolates used in this study included W2, 4G, 7G8, NF10-01, PF185, F09N30, F09A28, and F09A35. W2 (Indochina) chloroquine resistant clones were used in assessing the frequency of drug resistance to JPC-3210, in pulse drug exposure to induce resistance, in dormancy assays investigating the effect of JPC-3210 on dormant parasites, and its ability to induce dormancy. 4G (Cambodia) artemisinin resistant clones were also used in parallel with W2 to explore the effect of JPC-3210 on dormant parasites and inducing dormant parasites. *P. falciparum* isolates 7G8, NF10-01, PF185, F09N30, F09A28, and F09A35 were provided to the Kyle lab by Liwang Cui, Pennsylvania State University; some of these were thought to be piperaquine resistant and were used in addition to W2 and 4G to determine the IC50 of JPC-3210 in a variety of parasites with different genetic profiles. Additionally, *P. falciparum* clones and isolates W2, 7G8, NF10-01, PF185, F09N30, F09A28, and F09A35 were used to compare the IC50 values of JPC-3210, atovaquone, and piperaquine in triplicate to look at the parasites varying susceptibility to these compounds. Cultures were maintained in T75cm^3 flasks in O+ red blood cells at 4% hematocrit in 10ml of complete RPMI-1640 media containing 25 mM HEPES, 3.2% sodium bicarbonate, and 10% AB plasma in a mixed gas environment of 5% CO^2, 5% O^2, and 90% N^2 at 37°C. Cultures were fed daily and the percentage of parasitemia was calculated by counting the number of infected red blood cells in relation to the total number of red blood cells on slides with thin and thick smears stained in Giemsa; parasites were maintained routinely at levels below 10% parasitemia. These methods have been previously described by Trager & Jensen (1976).

Estimation of the frequency of drug resistance to JPC-3210 in vitro

The frequency of drug resistance to JPC-3210, as compared with that of atovaquone, was performed in W2 clones. The starting population of W2 parasites for selection was 10^8 parasitized erythrocytes per T75cm^3 flask, and was set up in triplicate for pressure with JPC-3210 (Figure 2). Additionally, one control T75cm^3 flask with 10^8 parasitized erythrocytes was set up for pressure with atovaquone. To estimate the initial population of parasites, the erythrocyte concentration was determined using a hemocytometer to count the number of red blood cells, and the parasitemia was calculated based on Giemsa-stained slides taken from the initial culture. All cultures were synchronized prior to the start of this experiment with 5% D-Sorbitol to ensure that greater than 90% of parasites were in ring stage as described by Lambrose and Vanderberg (1979). To start there were four flasks, each with 10^8 parasitized erythrocytes total, in 10 ml of media with 2% hematocrit. On Day 1 three flasks were put under 10x (7 ng/ml) the IC50 pressure of JPC-3210 and one flask was put under 10x (1.1 ng/ml) the IC50 concentration of atovaquone. Media with fresh drug was replaced twice weekly at either 72- or 96-hour intervals, with slides containing thin and thick smears taken and stained with Giemsa to monitor for emerging parasites. Cultures were expanded 1:3 on Day 10 and Day 20, with fresh erythrocytes added to maintain a 2% hematocrit. Drug pressure was continuous for 30 days, at which point all cultures were centrifuged at 4,000 RPM for two minutes and washed three times in RPMI stock containing 25 mM HEPES. Cultures were maintained for 30 days post drug exposure to monitor for parasite recrudescence; if parasites were not observed at any point during this period, cultures were disposed of on Day 60. If parasites were observed, portions of the culture were frozen for later DNA extraction, cryopreserved in glycerolyte, and drug susceptibility (IC50/IC90 values) was determined for all emerging parasites using the 3H-hypoxanthine uptake inhibition assay. According to Rathod, McErlean, and Lee (1997), “a flask was expected to yield mutants whenever the initial parasite numbers were greater than or equal to the inverse of the frequency of resistance" (p. 915). Therefore, if parasites emerged from flasks containing 10^8 parasites, one could conclude that the frequency of resistance associated with that drug would be 10^{-8}.

The process for running the 3H-hypoxanthine uptake inhibition assay began with the synchronization of the culture and the determination of the parasitemia percentage. Calculations were performed to determine the correct amount of infected erythrocytes that would be needed per assay (0.5%); these erythrocytes were then added to uninfected red blood cells (2% hematocrit) and complete media. Drug dilutions started at predetermined values that were serial diluted 1:2. Next, 10µl of drug dilutions were added to a 96 well tissue culture-treated plate, then 90µl of infected erythrocyte suspension was added to the drug dilution wells and the positive control wells, while uninfected red blood cells constituted the negative control wells. The assay plate was incubated for 48 hours, then 10µl of Tritium radiolabeled hypoxanthine was added to each well (pulled from a 1:20 stock dilution of hypoxanthine) and incubated with mixed gas for an additional 24 hours. At 72 hours, plates were removed from the incubator and frozen at -80°C. Plates were then thawed to lyse the red blood cells, which were collected onto 96 well filter paper plates using a cell harvester. The filter plates were then dried and liquid scintillation fluid was added to each well, after which incorporated radioactivity was measured as corrected counts per minute (cpm) on the liquid scintillation counter (Top Count) machine. Data was then exported, and IC50 and IC90 values were determined using the DataAspects Plate Manager program and GraphPad Software. Thus, the procedure followed was similar to that of Desjardins et al. (1979).

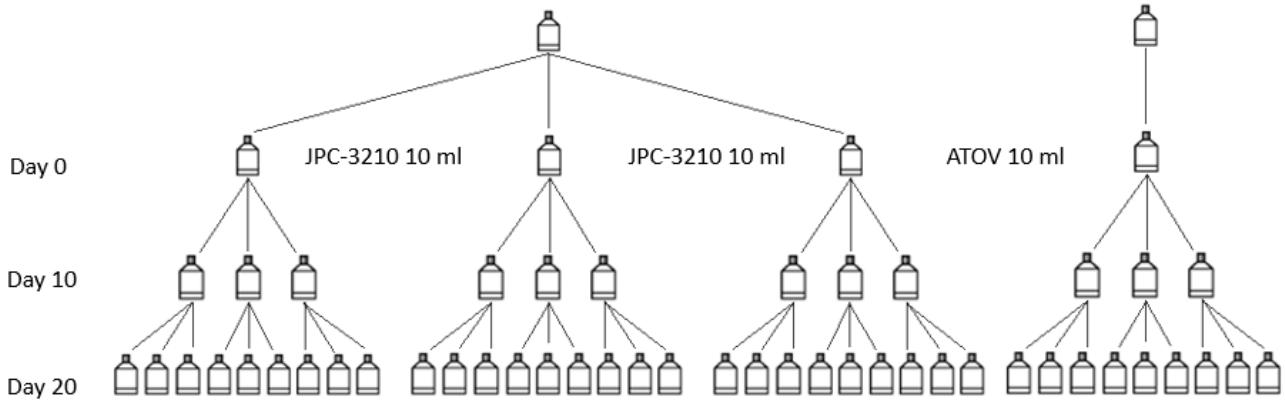


Figure 2. Schematic representation of the frequency of resistance expansion in JPC-3210 and atovaquone (ATOV) treated *P. falciparum* W2 clones in T25cm3 flasks over the course of 30 days.

Stepwise pulse drug exposure to JPC-3210 in vitro

The stepwise pulse experiments were performed on W2 clones, with increasing concentrations of JPC-3210 added in a discontinuous stepwise manner over time to induce resistance to JPC-3210. An initial asynchronous culture began at 4% parasitemia in 10 ml of complete RPMI media in a T75cm^3 flask with 2% hematocrit. Drug exposure was applied incrementally for 96 hour periods, with recovery allowed between increasing drug pressure. The first pressure was administered for 96 hours at 3.5 ng/ml of JPC-3210; which is 5x the IC50 of JPC-3210 (0.7ng/ml) in W2. At the end of the 96 hour period, the culture was centrifuged and washed three times with stock RPMI to remove the drug, with fresh red blood cells added to maintain a 2% hematocrit. Media was replaced and slides taken to monitor parasite growth every 48 hours. When parasites emerged they were grown above 4%, with a portion of the culture frozen for later DNA extraction, cryopreserved for future analysis, and then drug susceptibility (IC50) was assessed through measuring the inhibition of 3H-hypoxanthine uptake. IC50 values for emerging parasites at each recovery was determined for JPC-3210, atovaquone, and piperaquine, and compared to parent W2 control. To continue the stepwise pressure, the culture was again brought to 4% parasitemia and drug pressure was added for 96 hours at 10x the IC50 of JPC-3210 (7 ng/ml). The duration of the pulse exposures to JPC-3210 totaled 96 days, and the exposures to JPC-3210 were applied at concentrations of 5x (3.5 ng/ml), 10x (7 ng/ml), 100x (70 ng/ml), 100x (repeat-70 ng/ml), 200x (140 ng/ml), 500x (350 ng/ml), and 1,000x (700 ng/ml) the IC50 of JPC-3210. After the recovery of parasites from 500x the IC50 drug pressure from JPC-3210, the stability of resistance was assessed by continuously growing the recovered parasites for three weeks without drug pressure, then determining the IC50 of JPC-3210 using the 3H-hypoxanthine uptake inhibition assay, and comparing the IC50 values taken immediately after recrudescence to the value taken three weeks later. This procedure was based on methods described by Oduola et al. (1988) and Chavchich, Gerena, Peters, Chen, Cheng, and Kyle (2010).

Effect of JPC-3210 on dormant parasites

The effect of varying concentrations of JPC-3210 on DHA induced dormant parasites was observed in both W2 and 4G *P. falciparum* clones. Bulk cultures of synchronized W2 and 4G clones were brought to 2% parasitemia with 4% hematocrit in 55 ml of complete media. To induce dormancy, DHA was added at a concentration of 200 ng/ml (700 nM) and incubated with cultures for 6 hours. After 6 hours, cultures were centrifuged and washed with stock RPMI to remove DHA, and both W2 and 4G bulk cultures were divided out into 10 flasks, each containing 5 ml, with 4% hematocrit and 2% parasitemia. JPC-3210 was added to the flasks in duplicate at concentrations of 100 ng/ml, 500 ng/ml, 1,000 ng/ml and 2,000 ng/ml, with dimethyl sulfoxide (DMSO) controls at <0.05%. Dilutions for JPC-3210 were made in stock RPMI to keep DMSO concentrations <0.05%. Drug pressure was applied for 24 hours, after which cultures were washed of the drug and slides were taken, and parasitemia was calculated daily until viable parasites exceeding 5% parasitemia were observed. W2 and 4G clones were chosen as they have different resistance profiles, which could potentially give clues as to the mechanistic action of the experimental drug JPC-3210. Similar methods were described by Tucker, Mutka, Sparks, Patel, and Kyle (2012).

JPC-3210 induction of dormancy

To determine if JPC-3210 could induce dormant parasites as shown with DHA, W2 and 4G clones were exposed to varying concentrations of JPC-3210. Bulk cultures of synchronized W2 and 4G clones were each divided into 10 flasks, with a volume of 5 ml in duplicate at 2% parasitemia and 4% hematocrit. JPC-3210 was added to flasks in duplicate at concentrations of 100 ng/ml, 500 ng/ml, 1,000 ng/ml and 2,000 ng/ml, with controls at <.05% DMSO. Drug pressure was applied for 24 hours, then washed out, with slides and parasitemia calculated daily until the level of parasitemia exceeded 5%. Each day, in addition to calculating the parasitemia, the stages of the parasites' development were recorded, while monitoring for and quantifying parasites morphologically similar to dormant parasites, as described by Tucker, Mutka, Sparks, Patel, and Kyle (2012).

Results

Frequency of resistance induction studies with JPC-3210 in vitro

In determining the frequency of drug resistance to experimental compound JPC-3210, while using atovaquone as a control in W2 clones, it was found that the frequency of resistance was less than 10^8 parasites for atovaquone and a number greater than 10^8 parasites for JPC-3210. Parasites treated with atovaquone recrudesced over the course of the study, while in cultures exposed continuously to JPC-3210, no recrudescence was observed either during the thirty days of continuous drug pressure, or during the subsequent thirty days without drug pressure (Figure 3). Drug pressure from both the experimental compound and the control were applied at a constant 10x the IC50 for JPC-3210 (7 ng/ml, 70 ng total for 10 ml culture), and 10x the IC50 for atovaquone (1.1 ng/ml, 11 ng total for 10 ml culture) for thirty days. The initial inoculum of 10^8 parasites per flask was determined to be at 4.7% parasitemia. Slides taken on Day 7 revealed that in the atovaquone flask there were no remaining visible parasites, while it took until Day 17 for cultures under pressure from JPC-3210 to be cleared of visible parasites in the thin smear. On Day 27 under pressure with atovaquone, several flasks had small numbers of what appeared to be visible parasites in the thick smears; the number of parasites was not quantified. On Day 30, drug pressure was removed from all cultures, and by Day 34 parasitemia within the nine flasks of atovaquone averaged 0.61%, while by Day 41, the average parasitemia was 2.9% (Figure 4). Analysis of parasites that recrudesced under atovaquone pressure revealed a ten-fold shift in the average IC50 from 0.11 ng/ml to 1.1 ng/ml (Figure 5). Although this is less than the resistance normally selected in this type of study with atovaquone, the presence of viable parasites in every flask (n=9) and the decreased susceptibility is consistent with selection of atovaquone resistance.

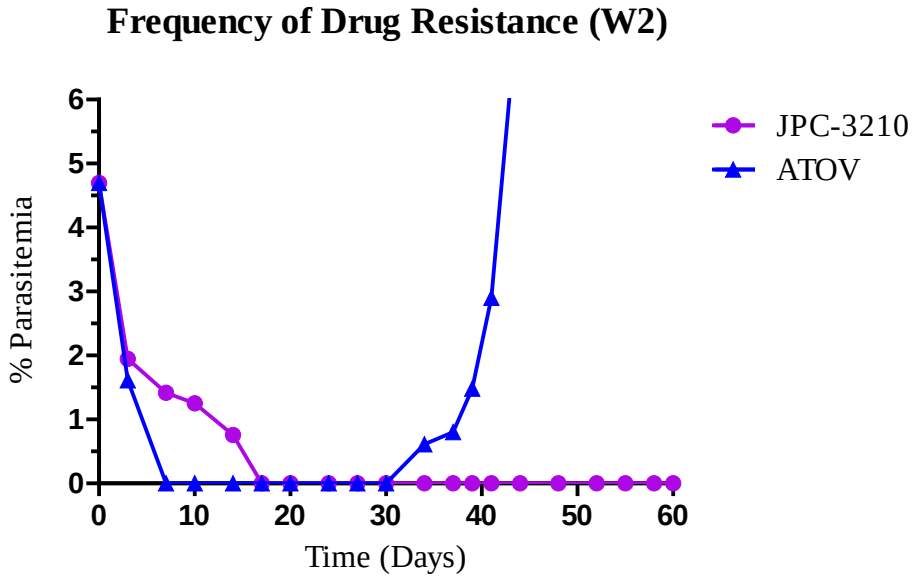


Figure 3. W2 parasites kept under constant drug pressure at 10x the IC50 for JPC-3210 (7 ng/ml) and atovaquone (ATOV) (1.1 ng/ml) for 30 days. Selection was performed from a starting concentration of 10^8 parasites with cultures expanded every 10 days.

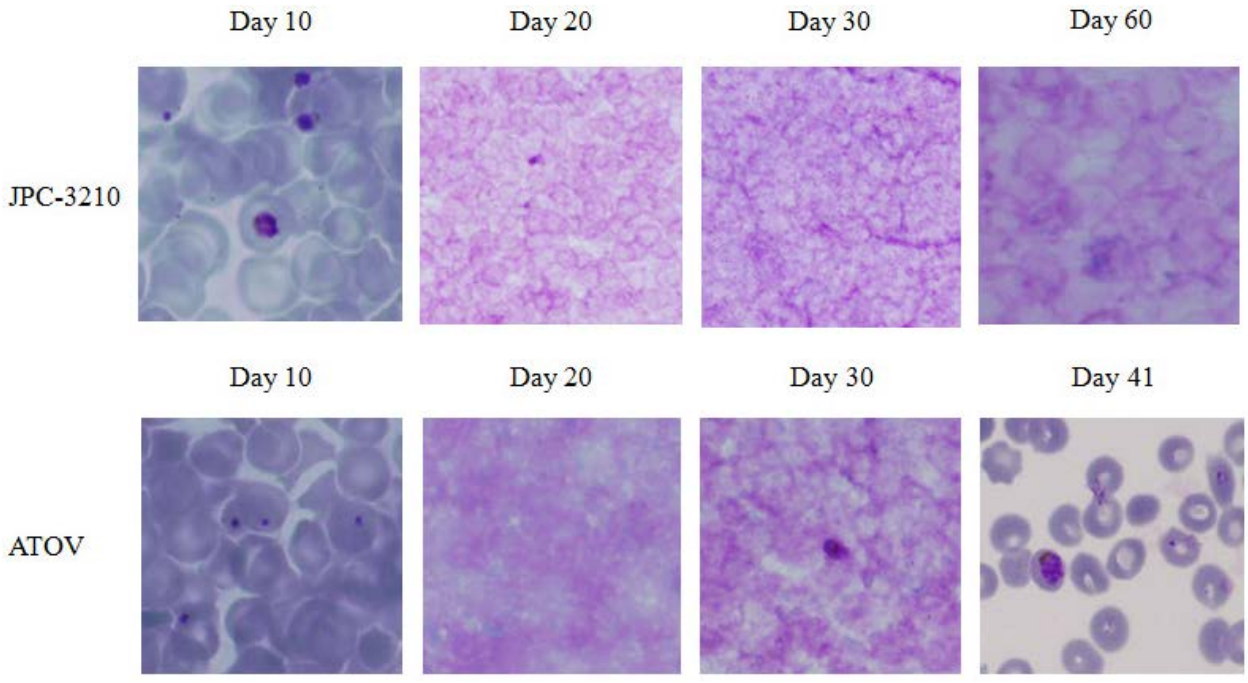


Figure 4. Microscopic images taken during and post drug exposure for JPC-3210 and atovaquone (ATOV), for Day 10, Day 20, Day 30, Day 41 (ATOV), and Day 60 (JPC-3210).

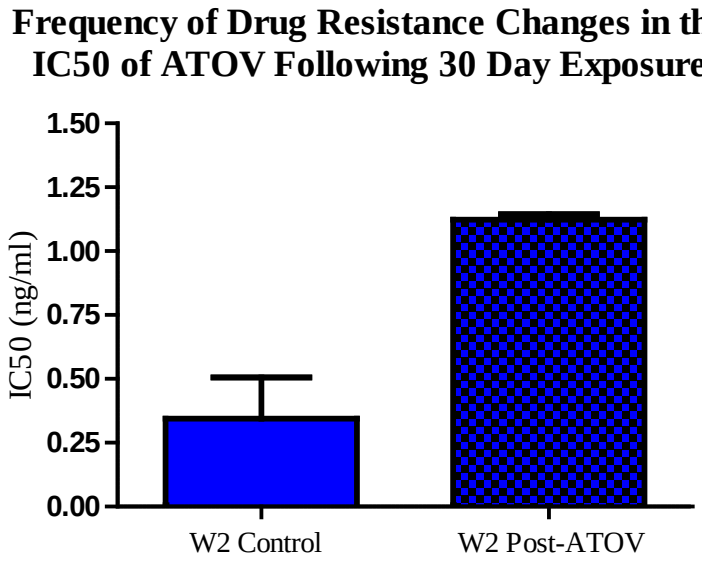


Figure 5. IC50 of atovaquone (ATOV) pre and post drug exposure to 1.1 ng/ml atovaquone (10x the IC50) for 30 days.

Effect of discontinuous pulse drug exposure to JPC-3210 in vitro

Drug exposure was applied incrementally for 96-hour periods, with recovery monitored between intermittent pulses of increasing drug pressure (Figure 6). The first pulse drug exposure of JPC-3210 on W2 parasites was at 3.5 ng/ml in 10 ml of an asynchronous culture containing 4% parasitemia with a 2% hematocrit level. Drug pressure was removed after 4 days, cultures were monitored, and 14 days post drug removal the parasites recovered to 4% parasitemia. The initial starting IC50 for JPC-3210 in W2 parasites before pulse exposure to drug was found to be 0.7 ng/ml, and the changes in IC50 values over the course of pulse exposure recovery were monitored at each recovery using the 3H-hypoxanthine uptake inhibition assay (Figure 7). Resulting from pressure with 3.5 ng/ml (5x the IC50 of JPC-3210), the IC50 shifted to 7.6 ng/ml. The second pulse with JPC-3210 was applied at 7 ng/ml (10x the IC50) with recrudescence above 4% observed 7 days post drug exposure, and the IC50 of these parasites was found to be 2.12 ng/ml. The third pulse JPC-3210 was applied at 70 ng/ml (100x the IC50) with greater than 4% parasitemia observed 17 days post drug exposure, resulting in an IC50 value of 5.21 ng/ml. For the

fourth pulse, the conditions of the third pulse were repeated at 100x the IC50 pressure, and recrudescence was observed 19 days after drug pressure removal with an IC50 of 8.54 ng/ml. The fifth pulse with JPC-3210 was applied at 140 ng/ml (200x the IC50) and 4% parasitemia was reached in 14 days, with an IC50 value of 9.32 ng/ml. For the sixth pulse with JPC-3210, pressure was applied at 500x the initial IC50 with 350 ng/ml, with recrudescence occurring in 7 days; recovered parasites were determined to have an IC50 value of 10.53 ng/ml. To assess stability of the resistance induced to JPC-3210, a portion of the parasites recovering from 500x the IC50 pressure with JPC-3210 were kept off drug pressure and grown continuously for three weeks. After the three-week period it was determined that the IC50 for JPC-3210 dropped from 10.53 ng/ml immediately after parasite recrudescence to 3.55 ng/ml. The final pulse of drug pressure was applied at 700 ng/ml of JPC-3210 (1,000x the initial IC50 value); parasites recovered in 18 days and the IC50 was found to be 5.28 ng/ml

At each point of recovery from JPC-3210, the IC50 values were determined in addition to JPC-3210 for piperaquine and atovaquone (Figure 7). The starting IC50 of piperaquine was 28.65 ng/ml in parent W2 clones and reached its highest shift in the IC50 at 101.74 ng/ml, resulting from 350 ng/ml of JPC-3210 (500x pressure), although after a three-week stability assessment the IC50 of piperaquine had dropped to 13.79 ng/ml. For atovaquone the starting IC50 in the W2 strain was 0.11 ng/ml. The highest IC50 value for atovaquone in response to JPC-3210 was 0.863 ng/ml, which occurred after 5x the IC50 value of JPC-3210 was applied. Following recovery from 500x the IC50 drug pressure of JPC-3210, atovaquone had an IC50 of 0.224 ng/ml that dropped to 0.15 ng/ml after the three-week stability assessment.

The highest IC50 values recorded for JPC-3210 and piperaquine in response to JPC-3210 pressure was found in parasite recovering from 350 ng/ml (500x the IC50 of JPC-3210). Reduced susceptibility to JPC-3210 and piperaquine in W2 parasites recovering from 500x the IC50 pressure from JPC-3210 as compared with W2 parent control parasites was compared using the 3H-hypoxanthine uptake inhibition assay (Figure 8).

Due to the instability of resistance after parasite recovery from JPC-3210 at 350 ng/ml (500x the IC50), DNA extractions were not performed on parasites recovering from each pulse drug exposure however samples were preserved for extraction at a later date if necessary. Some general observations were that on days immediately following drug pressure cultures appeared to have dying and dead parasites with lots of extracellular parasite material. At the lower drug pressure, defined by pulses at 3.5 ng/ml and 7 ng/ml (5x and 10x the IC50 of JPC-3210), dying parasites and extracellular debris were observed in the thin and thick smears for up to a week. However, once pressure was increased to 70 ng/ml (100x) and above, the cultures cleared out faster following drug pressure removal.

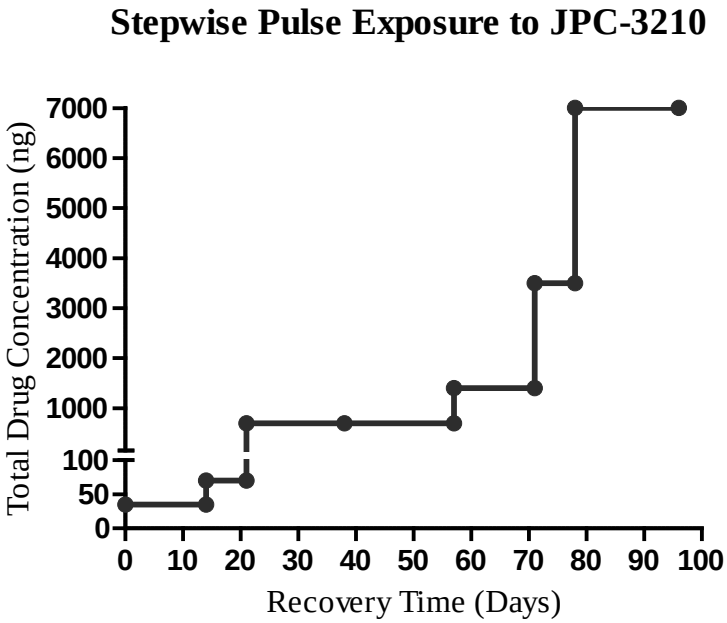
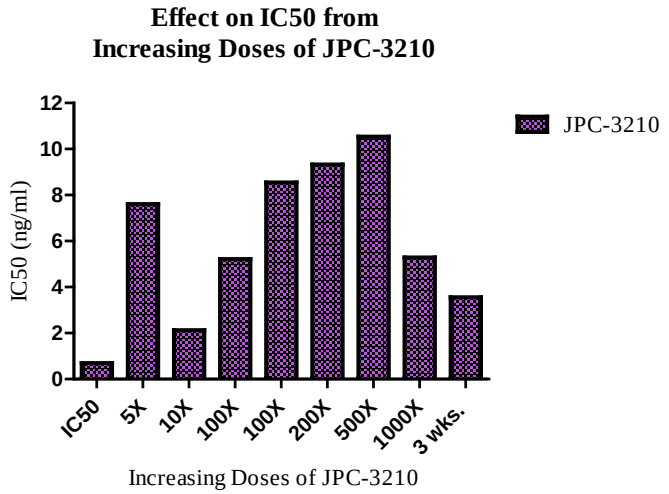
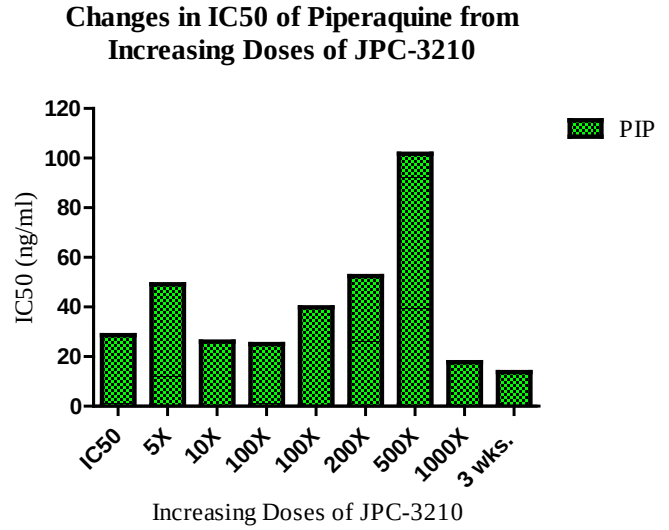


Figure 6. Representation of the increasing discontinuous stepwise pulse exposure of JPC-3210 to W2 parasites over the course of 100 days.





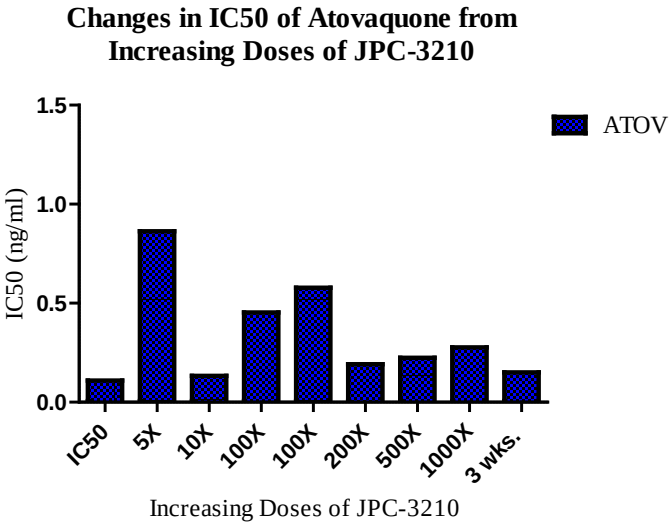


Figure 7. IC50 changes in JPC-3210, piperaquine (PIP), and atovaquone (ATOV) over time resulting from increasing pulse drug exposure to JPC-3210.

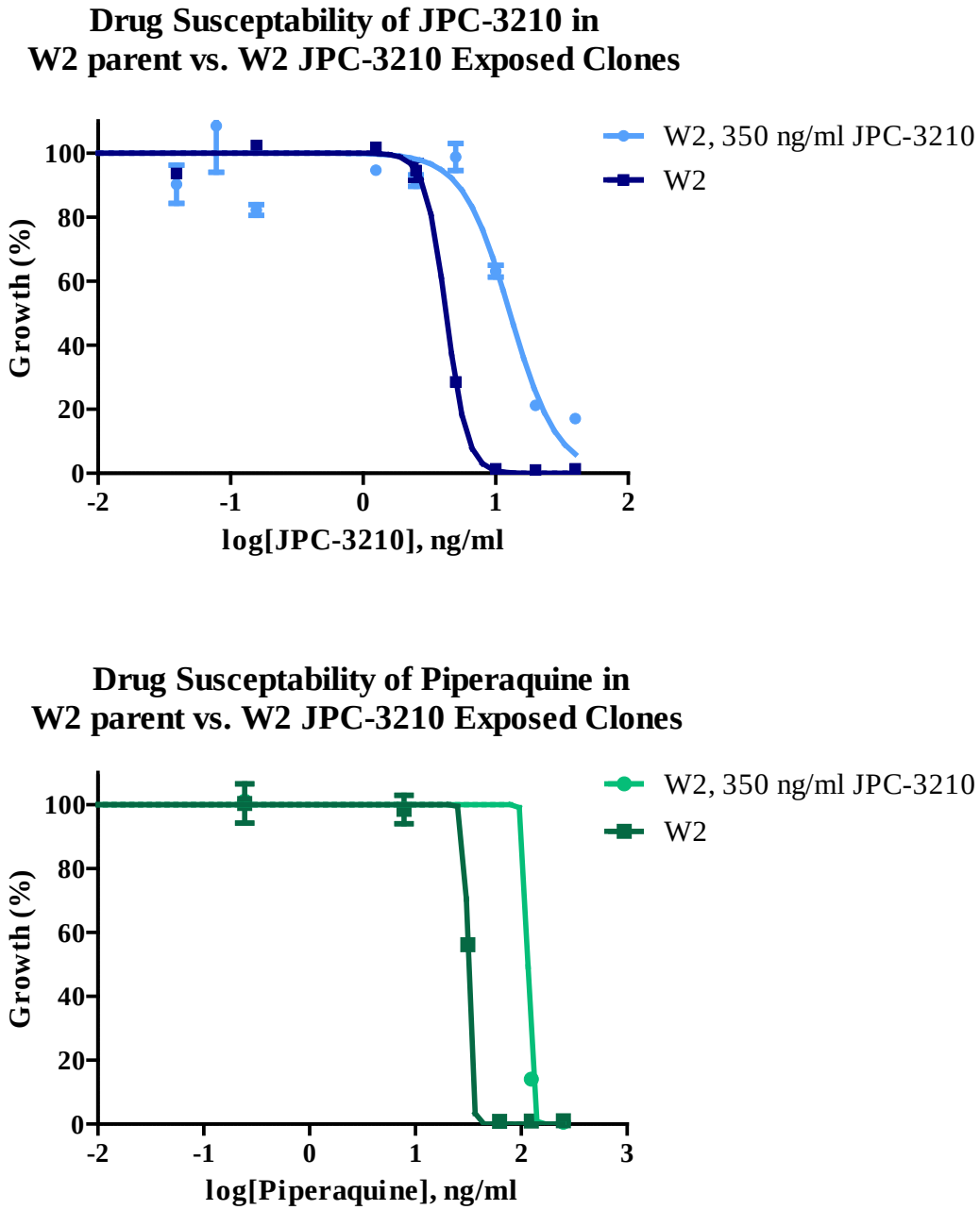


Figure 8. Drug susceptibility determined by the 3H-hypoxanthine uptake inhibition assay for piperaquine and JPC-3210 in W2 parent clones and W2 clones post recovery from 350 ng/ml JPC-3210 (500x the IC50 of JPC-3210) pulse drug exposure.

Effect of JPC-3210 on DHA-induced dormant parasites

To measure the effect of varying concentrations of JPC-3210 on dormant parasites, synchronized W2 clones were exposed to DHA at 200 ng/ml (700 nM) for 6 hours. At the end of the 6 hour period, a blood smear was prepared that showed parasites entering ring stage dormancy as previously described (Figure 9). Cultures were then exposed to varying concentrations of JPC-3210 in 5 ml cultures for a 24-hour period. The monitoring of parasite growth and recrudescence was measured based on calculating the daily parasitemia in each culture (Figure 10). As a SHAM control, parasites were induced into dormancy using DHA, then treated with DMSO (<0.05%), and in 4 days the cultures reached the initial starting parasitemia of 2%. For the W2 experimental treatment conditions of exposure to 100 ng/ml and 500 ng/ml of JPC-3210 for 24 hours following DHA, exposure parasitemia reached above the initial starting parasitemia by Day 6. In parasites treated with 1,000 ng/ml of JPC-3210, the initial parasitemia level was reached by Day 7, and in cultures with 2,000 ng/ml of JPC-3210, by Day 8. 4G parasites were used in a parallel experiment, with the same concentrations and incubation times of both DHA and JPC-3210 as performed with W2, and parasitemia and recrudescence were measured daily (Figure 11). 4G control parasites that were exposed to DHA for 6 hours, then DMSO (<.05%) for 24 hours, all grew to above the initial starting concentration of 2% parasitemia in 5 days. For the treatment conditions of JPC-3210 exposure at 100 ng/ml and 500 ng/ml of JPC-3210 for 24 hours following DHA, the initial starting parasitemia was reached by Day 6. In parasites treated with 1,000 ng/ml, the initial starting parasitemia was reached by Day 7, and in the 2,000 ng/ml condition parasites were back at 2% by Day 9. The results of these experiments demonstrated that JPC-3210 failed to eliminate dormant parasites induced by DHA in both W2 and 4G and that the recrudescence of JPC-3210-treated parasites was dose dependent.

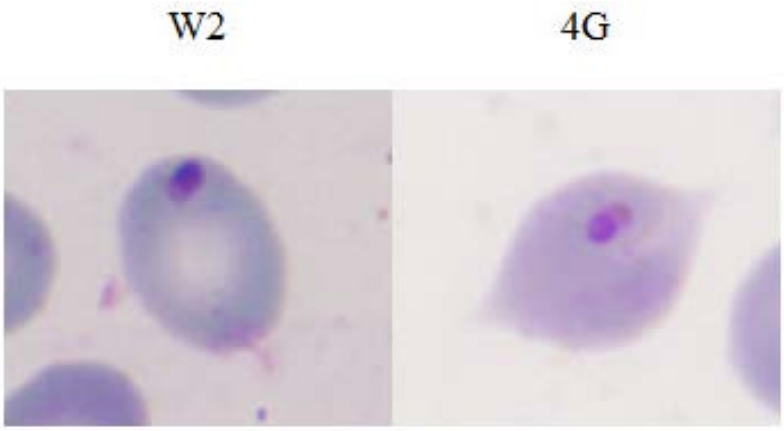


Figure 9. Dormant parasites induced from 6 hour exposure to dihydroartemisinin (DHA).

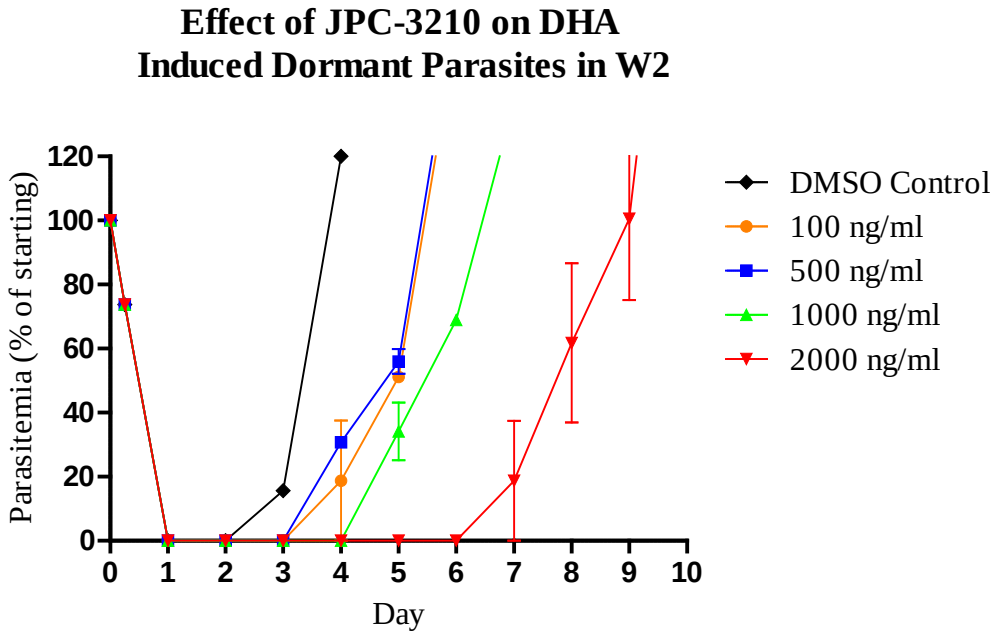


Figure 10. Effect of varying concentrations of JPC-3210 on dormant *P. falciparum* W2 clones *in vitro*. Dormant parasites were induced using 200 ng/ml (700 nM) dihydroartemisinin (DHA) for 6 hours followed by 24 hour exposure to JPC-3210.

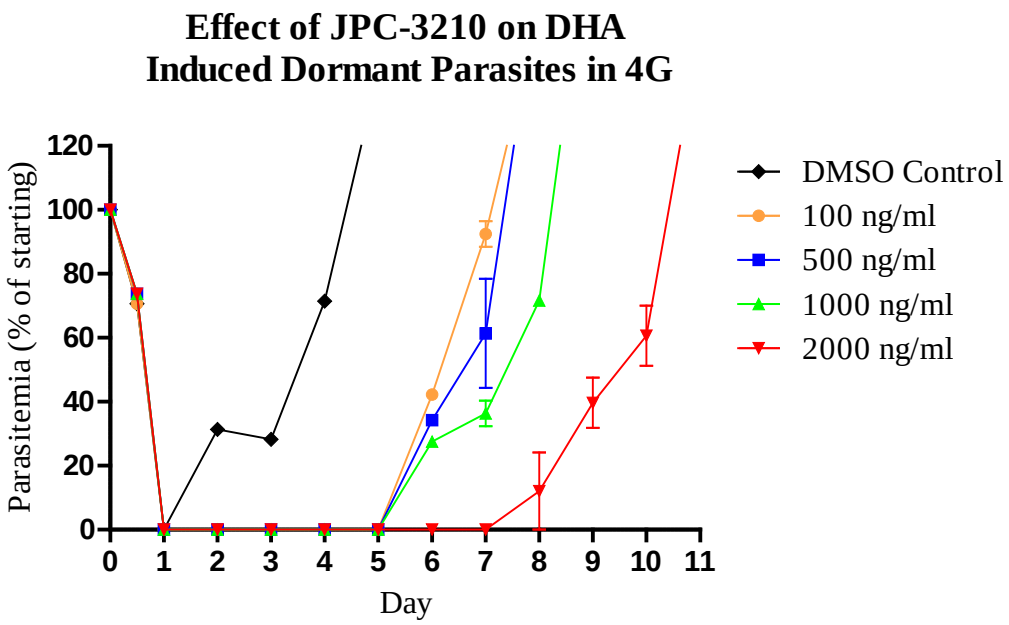


Figure 11. Effect of varying concentrations of JPC-3210 on dormant *P. falciparum* 4G clones *in vitro*. Dormant parasites were induced using 200 ng/ml (700 nM) dihydroartemisinin (DHA) for 6 hours followed by 24 hour exposure to JPC-3210.

Induction of dormancy with JPC-3210

To determine if JPC-3210 could induce dormancy in W2 and 4G clones similarly to that of DHA, synchronized parasites at 2% parasitemia were exposed to varying concentrations of JPC-3210 in 5 ml cultures for 24 hours, then monitored daily for parasite growth and staging. In DMSO-treated W2 controls, the parasitemia level reached above 5% within 4 days, and was followed by 100 ng/ml, 500 ng/ml, and 1,000 ng/ml of JPC-3210, all by Day 5. The treatment of 2,000 ng/ml with JPC-3210 reached 5% by Day 7. Using these concentrations of JPC-3210, no dormant parasites were observed, and only the 2,000 ng/ml concentration had parasitemia levels drop below 0.5% (Figure 12). For 4G clones, the DMSO control parasites grew above 5% parasitemia within three days. The JPC-3210-treated parasites at 100 ng/ml, 500 ng/ml and 1,000 ng/ml were all above 5% parasitemia by Day 5. For JPC-3210-treated parasites under 2,000 ng/ml, they were above 5% by Day 7. No dormant parasites were observed in any of the 4G cultures, and in all cultures parasitemia never dropped below 0.5% (Figure 13).

In both W2 and 4G clones, during the induction of dormancy experiments the stages of recovering parasites were recorded each day. In W2 the control parasites were primarily in late trophozoite and early schizont stages at 24 hours, while in parasites exposed to 100 ng/ml, 500 ng/ml, and 1,000 ng/ml of JPC-3210, at 24 hours the majority of parasites were in ring stages. At exposure to 2,000 ng/ml of JPC-3210, recrudescing parasites were found to be around 75% in ring stage for the final four days of the experiment (Figure 14). In 4G the DMSO control parasites were in late trophozoite and early schizont stages at 24 hours, similar to what was observed in the W2 control. In 4G clones exposed to 24 hours of JPC-3210 at 100 ng/ml and 500 ng/ml, the majority of parasites were in ring stages at 24 hours, and for the concentrations of 1,000 ng/ml and 2,000 ng/ml of JPC-3210. The parasites were also primarily in late trophozoite and early schizont stages at both 24 and 48 hour time points (Figure 15). Only viable parasites, assessed as possessing normal ring, trophozoite, or schizont morphology, were reported for staging; drug affected schizonts were observed at the higher concentrations of 1,000 ng/ml and 2,000 ng/ml in JPC-3210 treated cultures in both W2 and 4G, and were excluded from staging and parasitemia counts (Figure 16).

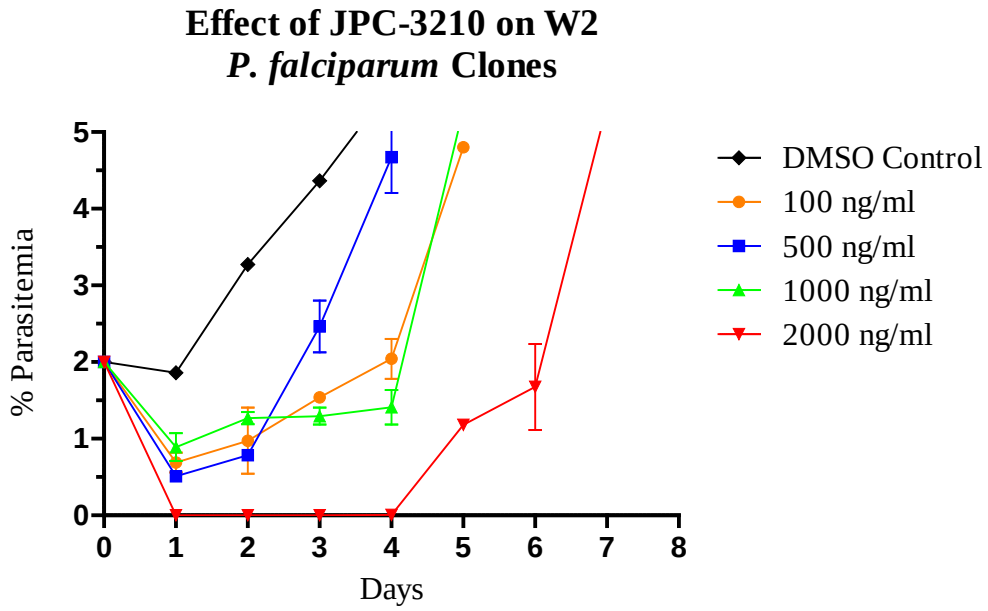


Figure 12. Effect of varying concentrations of JPC-3210 on W2, *P. falciparum* clones *in vitro*.

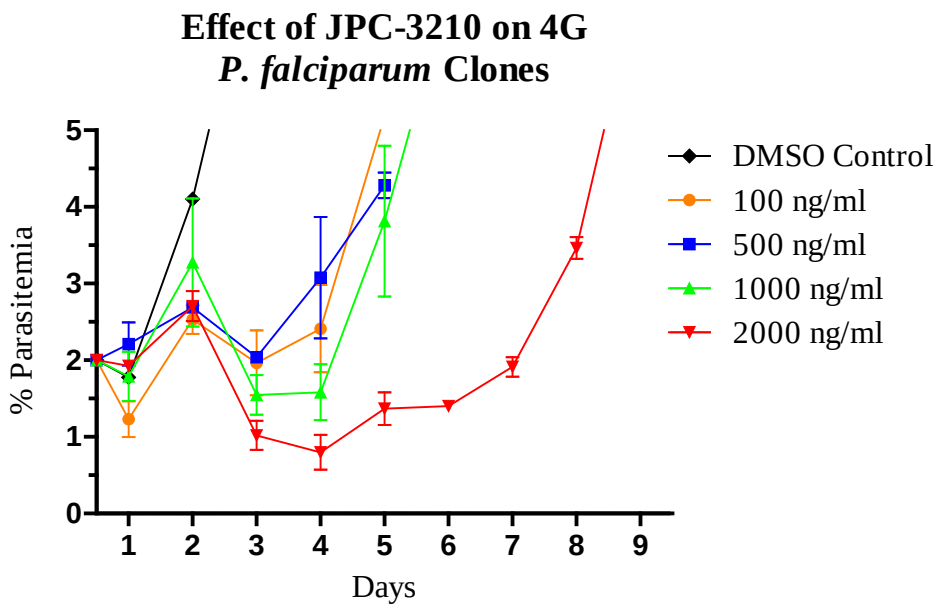


Figure 13. Effect of varying concentrations of JPC-3210 on 4G, *P. falciparum* clones *in vitro*.

Cross-resistance to JPC-3210 across various P. falciparum isolates

For each measure of the concentration of JPC-3210 needed to inhibit 50% and 90% of parasite growth, the 3H-hypoxanthine uptake inhibition assay was run in triplicate for each strain of *P. falciparum* used throughout the experiments in this study (Figure 17). Inhibitory concentrations of the IC50 for JPC-3210 in W2 were determined to be 0.7 ng/ml; in 4G it was 12.5 ng/ml; in F09A28, 2.9 ng/ml; in F09A35, 4.9 ng/ml; in F09N30, 2.7 ng/ml; in PF185, 2.3 ng/ml; in NF10-01, 5.83 ng/ml; and in 7G8, 1.0ng/ml. Additionally, the concentrations of JPC-3210, atovaquone, and piperaquine needed to inhibit 50% of parasite growth were determined and compared in the *P. falciparum* clones and isolates W2, 7G8, NF10-01, PF185, F09N30, F09A28, and F09A35 (Figure 18). It was found that W2 and 7G8 parasites were the most susceptible to JPC-3210, with F09A35 and NF10-01 determined to be the least susceptible. Limited susceptibility to piperaquine within this group of clones and isolates was found when looking at the IC50 values which had an average of 19.51 ng/ml, (*SD*= 4.29 ng/ml) and parasites were found to be sensitive to atovaquone.

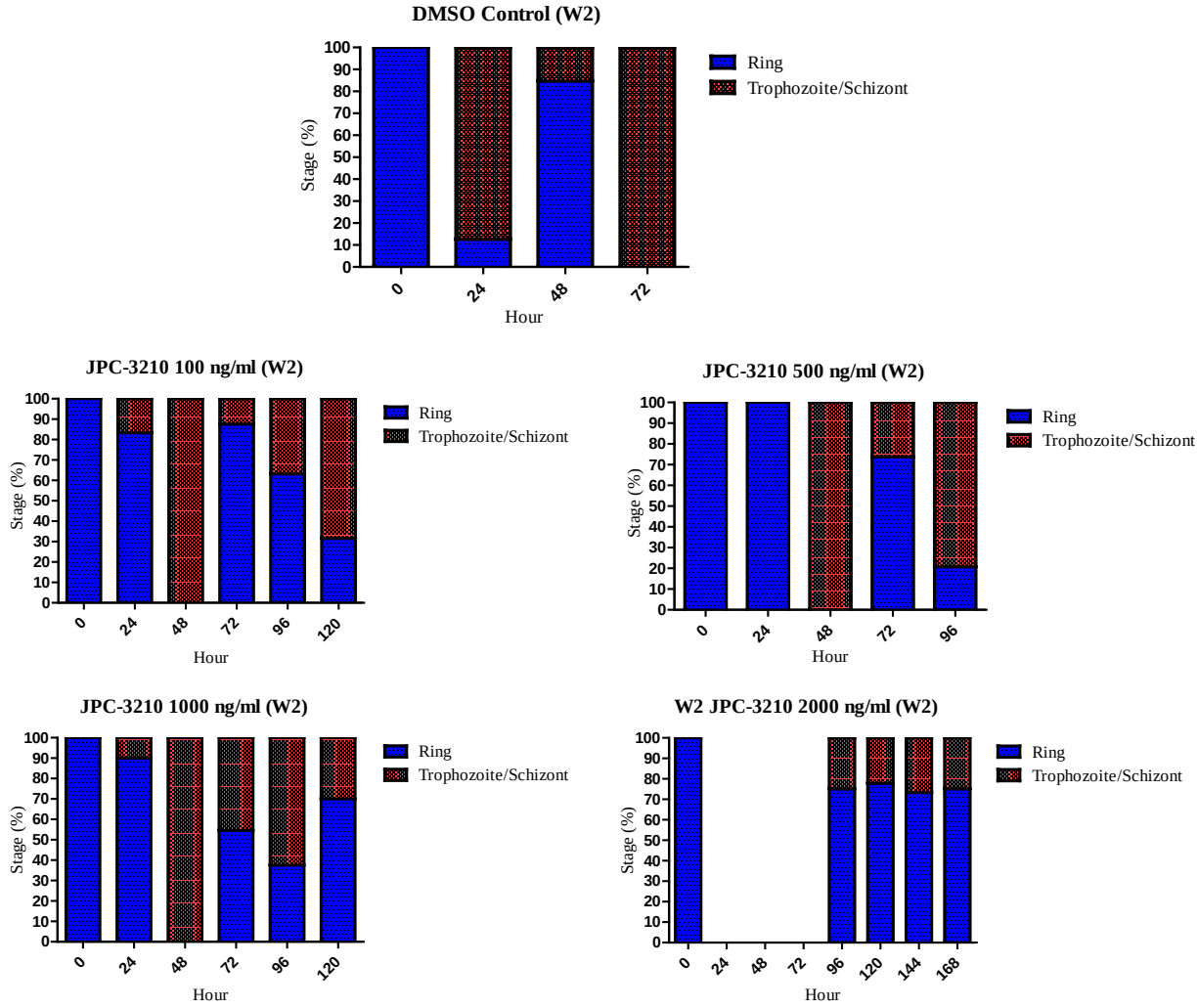


Figure 14. Parasite stages measured in response to multiple concentrations of JPC-3210 in W2 clones.

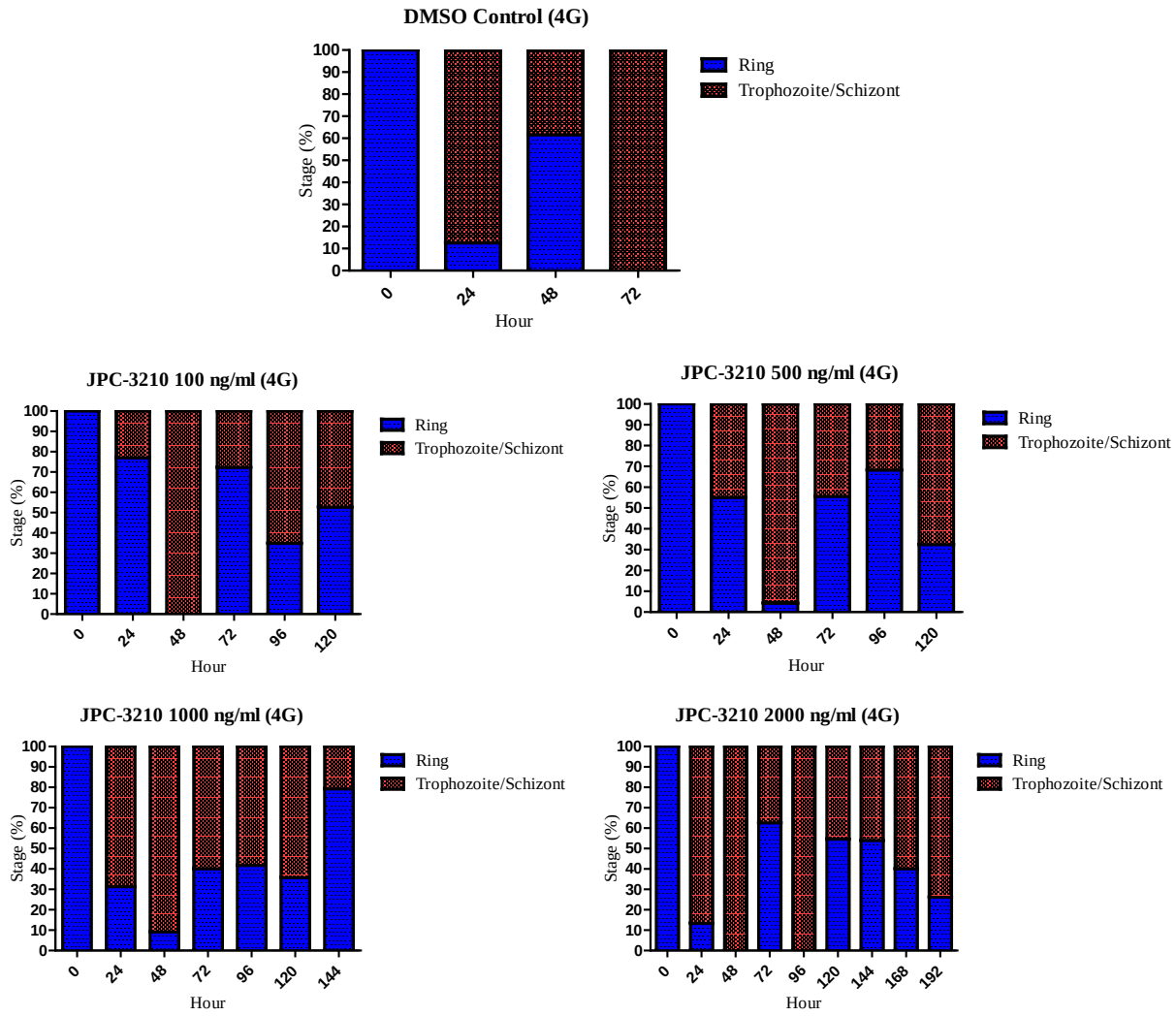


Figure 15. Parasite stages measured in response to multiple concentrations of JPC-3210 in 4G clones.

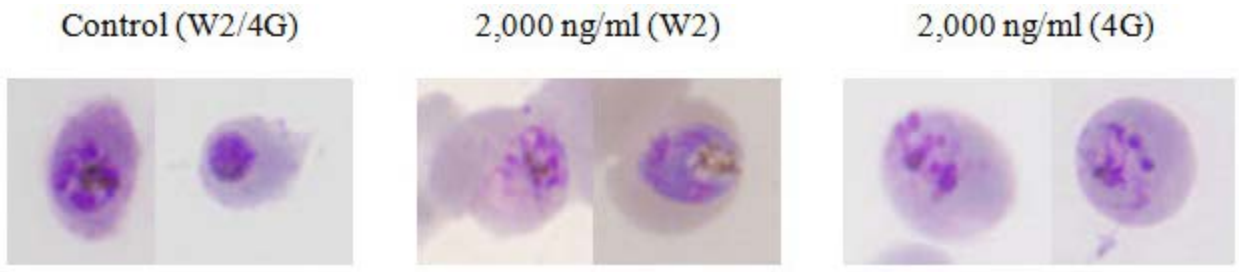


Figure 16. Control parasites (W2/4G) compared with drug effected late stage parasites from W2 and 4G cultures treated with 2,000 ng/ml JPC-3210.

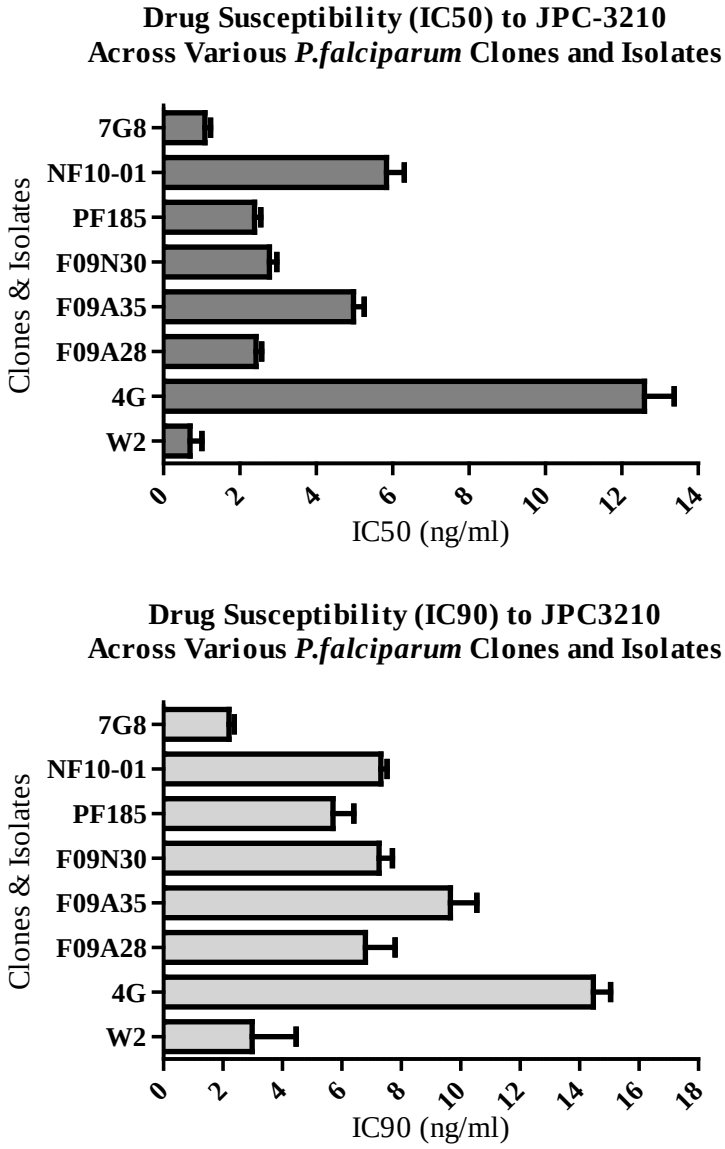


Figure 17. Drug inhibitory concentrations at 50% (IC50) and 90% (IC90) across a variety of *P. falciparum* clones and isolates. Three biological replicates were performed and the bars represent $\pm$ 1SD.

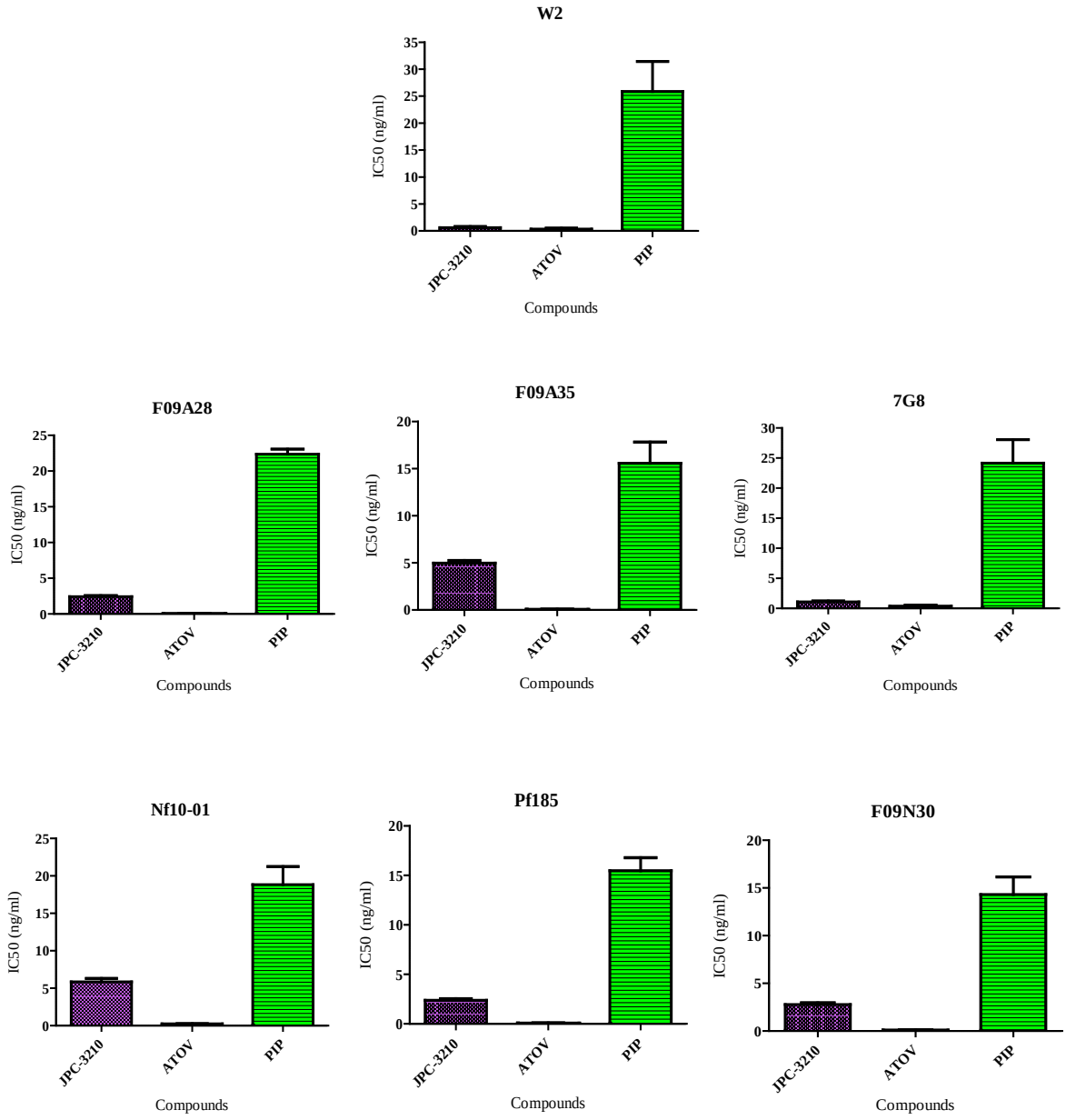


Figure 18. Drug inhibitory concentrations at 50% (IC50) across a variety of *P. falciparum* clones and isolates determined using the 3H-hypoxanthine uptake inhibition assay. IC50s were calculated for atovaquone (ATOV), piperaquine (PIP), and JPC-3210. Three biological replicates were performed and the bars represent $\pm$ 1SD.

Discussion

Novel antimalarial compounds are needed to improve treatment, and ultimately, the eradication of malaria worldwide. Drug resistant malaria is of particular concern, especially with reports of *P. falciparum* parasites emerging from areas in South East Asia with reduced susceptibility even to frontline artemisinin-based combination therapies (Dondorp et al., 2009). While such reports are not widespread, there is still fear that patterns of resistance could be spread, as was previously seen with chloroquine-resistant emergence that began moving out of South America and South East Asia in the 1950s (CDC, 2012). Today, it is important to continually monitor current treatment options and their efficacy on parasite clearance, and also to find new effective antimalarial compounds that act on novel targets within the parasite. New antimalarial compounds should be analyzed *in vitro* through various methods to determine efficacy, resistance potential, and cross-resistance with existing drugs, as was undertaken in this study of JPC-3210.

The frequency of resistance method was used to determine if at 10^8 parasites would recrudesce under continuous drug pressure for 30 days. Previous studies have shown that W2 clones with a starting population of 10^8 parasites recrudesced under 10^{-8}M pressure from atovaquone, with parasites first visible on Day 15 (Gassis & Rathod, 1996). This method, developed by Gassis and Rathod, was replicated in our experiments comparing the frequency of resistance of atovaquone to that of JPC-3210. The frequency of resistance of atovaquone was used as a control against which to measure the frequency of resistance of JPC-3210. It was hypothesized that if JPC-3210 recrudesced at a faster rate than atovaquone, then JPC-3210 would be considered subpar to atovaquone, which was previously shown to be a poor antimalarial compound when used as monotherapy (Looareesuwan, Viravan, Webster, Kyle, Hutchinson, & Canfield, 1996). The results of our findings were that JPC-3210 at 10^8 starting parasites with 10x the IC50 (7 ng/ml) did not recrudesce under the 30 days of drug pressure, or within 30 days post drug pressure

removal. This data suggests that the frequency of resistance to JPC-3210 is less than 10^{-8}, a level which has been determined to be the minimal acceptable rate of acquisition of drug resistance for a new candidate antimalarial drug.

Based on previous findings it was expected that in the atovaquone control flasks, parasites would be visible by around Day 15. However, in this experiment, it was found that the atovaquone controls did not recrudesce to a measurable level of parasitemia in thin smears until after drug pressure was removed. Although in several flasks gametocytes were seen in thick smears around Day 23, they appeared to be drug effected, and were not visible in thick smears taken on Day 27. This result could have been an indication that the parasites were stressed and unable to survive. Viable parasites in the atovaquone flasks became visible only shortly after drug removal on Day 34, at which point the parasitemia averaged 0.61%. Researchers within the Kyle Lab at the University of South Florida had previously performed this experiment outlined by Gassis and Rathod (1996), with results that aligned more closely with the published data, and it is unclear why the results from these atovaquone controls failed to do as well. Additionally, when analyzing the IC50 values of recrudesced atovaquone parasites, it was found that the IC50s had shifted from 0.11 ng/ml, which was determined to be the starting IC50 from repeat 3H-hypoxanthine uptake inhibition assays before initiating this experiment, to 1.12 ng/ml. In contrast, previous studies by Gassis and Rathod had shown that recovering parasites were thirty times more resistant. The starting IC50 was determined to be 0.11 ng/ml for atovaquone in W2, however, over the course of the study, additional W2 IC50 data was available, which raised the average IC50 value in W2 clones to 0.44 ng/ml, although this difference is considered negligible. Possible explanations for the discrepancies between our findings and the published data could be that the initial parasitemia was overestimated in the starting cultures, and that 1.1 ng/ml of drug pressure was used, whereas Gassis & Rathod used 10^{-8}M. Although it should be noted that 10^{-8}M is equal to 3.66 ng/ml, indicating that we should have seen parasites sooner than was reported in the published data. This experiment indicated that JPC-3210 had a frequency of resistance less than that of atovaquone.

Future studies could be conducted to determine if there is a higher frequency of resistance, such as at 10^9, in order to find the starting concentration at which parasites recrudesce. Surviving parasites could then be analyzed to see how drug susceptibility had changed, and genotypic analysis could be performed, which would potentially give insight into the parasites' mechanism of action. Other studies looking at parasites with atovaquone resistance have found mutations in cytochrome b, which resists inhibition of the mitochondrial transport chain by which atovaquone is thought to act (Korsinczky, Chen, Kotecka, Saul, Rieckman, & Cheng, 2000). The same concept of isolating and characterizing resistance mechanisms could have been applied to JPC-3210, had parasites emerged from the frequency of resistance assay.

The discontinuous stepwise pulse experiment performed in this study was done over the course of three months, and designed to determine if resistance in W2 could be induced, following increasing drug pressure from JPC-3210. Methods used in this study were drawn from previously published methods that looked at the induction of resistance though pulse drug exposures, as first described by Oduola et al. (1988), and then expanded upon by Chavchich et al. (2010) and Tucker et al. (2012). Incrementally increasing doses of JPC-3210 were added to W2 parasites over time, with three potential outcomes: either parasites would become resistant and require evaluation, resistance would emerge but would be unstable, or no resistant parasites would emerge. The experiment ran for three months, with concentrations spanning 5x the IC50 pressure (3.5 ng/ml) to 1,000x the IC50 (700 ng/ml) with JPC-3210, with an average recovery time of 14 days post drug removal. The initial IC50 of JPC-3210 was determined by using the 3H-hypoxanthine uptake inhibition assay to be 0.7 ng/ml. The highest IC50 value recorded throughout pulse exposure was in response to 500x the IC50 (350 ng/ml), although a three-week stability test post 500x recovery revealed that the inhibitory concentration dropped from 10.53 ng/ml to 3.55 ng/ml. This drop in the IC50 value indicates that the induced drug resistance was unstable, although it would have been interesting to see if in a few weeks' time the IC50 remained stable at 3.55 ng/ml or dropped back down to initial drug sensitivity levels.

The results of this experiment show that parasites can adapt to survive very high drug levels quickly, as parasites were surviving 4 days of exposure to 700 ng/ml after only three months of intermittent drug pressure. The observed instability was most likely the result of the cost of fitness to the surviving parasites, in which parasites are able to resist the drug effects to some degree; but this resistance comes at a cost of a reduction in the overall fitness of the surviving parasites. Similar outcomes have been observed in studies looking at chloroquine, mefloquine, and artemisinin resistance, where within days or months resistance developed quickly and corresponded to genotypic changes. However, the resistance proved unstable with the development of parasite line, with stable resistance taking a much longer period of time to develop (Nzila & Mwail, 2010). In regards to the instability of resistance, one hypothesis is that the resistance mechanism was acting on a membrane transport system, which is a common mechanism of resistance and often unstable (Mwai et al., 2012). Potential future studies could involve continued pulse drug exposure over a longer period of time, in an effort to induce greater levels of resistance and stability, to determine the mechanism of action through genotypic analysis of JPC-3210. In addition, we could clone from the resistant population immediately after parasites recrudesced so the highly drug resistant clones would not be outcompeted by growth of less susceptible parasites.

As illustrated by Tucker et al. (2012), stable resistance was induced in W2 to artelinic acid and artemisinin. However, their experiment went on for upwards of 800 days, in comparison to this three-month study. Additionally, Tucker et al. performed multiple repeat drug exposures at the same drug concentrations for their pulses, which was only done once in this experiment, at 100x the IC50. A longer-term study could be performed with JPC-3210 to potentially achieve a stable resistant line, however, given time constraints, this course of action was not possible. If this experiment were to be repeated, it would be interesting to analyze the percentage of early to late stage parasites in the asynchronous cultures before drug pressure was administered, to observe if recovery times are correlated to parasite stages. Tucker et al. also analyzed genotypic changes such as the amplification of *pfmdr 1* in relation to drug susceptibility. However, this study was unable to find or analyze targets of this nature, as due to the instability of the recovering parasites, genotypic analysis was not preformed. If stable resistance had been

observed, comparisons between parental W2 and JPC-3210 resistant W2 clones would have been made to characterize the differences in both lines through sequence analysis.

In response to pulse exposures of artemisinin, Chavchich et al. (2010) found that parasites became more susceptible to chloroquine, and observed cross-resistance to other artemisinin derivatives. In this study, the susceptibility to atovaquone and piperaquine in surviving parasites from exposures to JPC-3210 was analyzed. For atovaquone (IC50: 0.11 ng/ml), there were minimal changes to the IC50 data in response to increasing JPC-3210 drug pressure, with the highest recorded IC50 value throughout the experiment being 0.8 ng/ml, which was recorded after the first recovery from 3.5 ng/ml of JPC-3210. This finding indicates that JPC-3210 is not acting on the same pathway as atovaquone; if it had been, the IC50 of atovaquone would have increased over time indicating cross-resistance.

In looking at the IC50 changes to piperaquine (IC50: 28.65 ng/ml) over time, it was observed that parasites recovering from the first pressure of JPC-3210 (3.5 ng/ml) had an increased IC50, which leveled off to the initial IC50 level after exposure to 10x (7 ng/ml) and 100x (70 ng/ml) the IC50 pressure of JPC-3210. This step was followed by a jump in the IC50 of piperaquine to 101.74 ng/ml in response to 500x (350 ng/ml) the IC50 pressure from JPC-3210, which was the highest recorded IC50 value of piperaquine throughout this study. However, the IC50 of piperaquine in response to 1,000x (700 ng/ml) the IC50 of JPC-3210, dropped below the initial IC50 of piperaquine to 17.77 ng/ml, and further to 13.79 ng/ml after three weeks of continuous culture. These changing IC50 values of piperaquine in response to JPC-3210 reflected instability with no clear pattern of cross-resistance. Perhaps the 700 ng/ml concentration of JPC-3210 was too great an increase and overwhelmed the parasites ability to overcome the drug's effects, although it is unclear why after three weeks without exposure to JPC-3210, parasites were found to be more sensitive than in the parent W2 strain. Perhaps JPC-3210 is acting on a similar pathway or has a similar resistance mechanism as piperaquine, however, further investigation would be needed to make any definitive conclusions. In looking at drug susceptibility comparing parasites recovering from 500x drug pressure from JPC-3210 to parental W2 clones for JPC-3210 and piperaquine it was clear that resistance was being seen. This resistance was highlighted by the shift in the drug dose response between

the two cultures with and without discontinuous exposure to JPC-3210 which is indicative that resistance is occurring, even if this ultimately proved unstable. Overall, this experiment highlighted how feasible the development of resistant parasites is when exposed to drug dosages that are insufficient to kill all parasites. This outcome can happen not only in laboratory settings, but also clinically if patients are improperly treated with errors in dosing or though counterfeit drugs.

It has been hypothesized that treatment failures resulting in the recrudescence of parasites may be the result of parasites temporarily entering a state of dormancy, where growth is arrested to avoid drug effects, with parasites recrudescing at a later time (Cheng, Kyle, & Gatton, 2012). It is important when evaluating a new drug to assess its effects on artemisinin-induced dormant parasites, as drugs are typically administered in conjunction with artemisinin derivatives. Drugs which are partnered with artemisinin in ACTs typically have longer half-lives, and thus are able to eliminate recovery of dormant parasites resulting from artemisinin, which has been shown to be fast-acting but exhibits a short half-life (Cheng, Kyle, & Gatton, 2012). Additionally, it is important to look at whether new compounds can induce dormant parasites, since it has been hypothesized by Cheng, Kyle, & Gatton, that dormancy is an innate function that allows parasites to survive drug pressure and which may not be specific to artemisinin or its derivatives. Entering a dormant state is thought to be an innate mechanism of the parasite, as a result of studies which have shown that the *in vitro* IC50 values for parasites exposed to high levels of drug which recovered do not show changes in their IC50s and do not have any genotypic changes associated with resistance (Witkowski et al., 2010). This outcome suggests that dormancy is an explanation for recrudescing parasites, although not resistance. However, dormancy could contribute to the development of resistance if parasites are subjected to sub-optimal levels of drug.

To observe the effect of JPC-3210 on dormant parasites, dormancy was induced with 200 ng/ml (700nM) DHA, using methods developed by Teuscher et al. (2010), as this was shown to successfully induce dormant parasites. Following exposure to DHA, dormant parasites were observed in a manner consistent with the description of dormant parasites as defined by Tucker et al. (2012). In following methods developed by Teuscher et al. (2010), the experimental compound JPC-3210 was only applied for

a 24-hour interval following DHA exposure; this procedure was to ensure that only dormant parasites—and not recovering parasites—were exposed to JPC-3210. For W2, control parasites in which dormancy was induced by DHA, and then treated with DMSO for 24 hours, dormant parasites recrudesced to 100% the starting concentration of parasites by Day 4. In 4G, controls recrudesced to 100% the starting concentration of parasites by Day 5, as was expected. Parasites recrudesced faster in the W2 clones than 4G, and the highest concentrations of JPC-3210 (2,000 ng/ml) had the lengthiest recovery time. At all drug concentrations in either W2 or 4G, all parasites recrudesced to 100% the starting concentration of parasites by Day 11, and the response appeared to be dose dependent. These results indicated that JPC-3210 was not effective at eliminating dormant parasites.

To observe if treatment with JPC-3210 induced dormant parasites, drug was applied for 24 hours, and parasitemia was calculated daily, with the stages of the parasites recorded to detect the presence of dormant parasites in the cultures. In W2 and 4G, dormant parasites were not observed in either the controls or in any of the drug concentrations. The growth of the cultures appeared to be dose dependent, with only the 2,000 ng/ml concentration in W2 dropping below 0.5% parasitemia throughout the experiment. 4G clones appeared to be less effected by JPC-3210 than W2 clones, however, all cultures reached 5% overall parasitemia by Day 9. This dormancy experiment indicated that dormant parasites are not induced by JPC-3210. However, the fast recovery time from 24 hour treatments with concentrations up to 2,000 ng/ml, indicates that either the duration of drug exposure needs to be longer or other drug combinations that complement JPC-3210 should be explored.

In the analysis of parasite staging across all drug concentrations, it was observed that there were differences between lifecycle stages in controls in comparison to drug-treated parasites. These dissimilarities were most pronounced at the 24 and 48 hour time points. To eliminate confusion between early and late stage parasites, designations were made only between ring stages, whereas trophozoite and schizont stages were grouped together. The 24 hour time point occurred when drug pressure from JPC-3210 and DMSO in the controls was removed, and slides were taken immediately after drug removal. In W2 control flasks, parasites were observed to be 87.5% in trophozoite and schizont stages immediately

following drug removal (Hour 24), while all cultures recovering from JPC-3210 exposure (excluding 2,000 ng/ml) were primarily in ring stage (> 83%). The highest concentration of JPC-3210 at 2,000 ng/ml was excluded from staging analysis until Day 4 when cultures recrudesced above 0.5% parasitemia. Parasites recovering from JPC-3210 reached later stages of development by hour 48, at which point they were exclusively in trophozoite and schizont stages. In 4G controls, parasites were primarily found to be in trophozoite and schizont stages (87.5%) at Hour 24. Parasites recovering from exposure to JPC-3210 at 100 ng/ml and 500 ng/ml at Hour 24 had a greater percentage of ring stage parasites (>55%) and were primarily trophozoites and schizonts by Hour 48. Parasites recovering from 1,000-2,000 ng/ml were observed to have a higher percentage of trophozoites and schizonts at Hour 24 and 48, however, the staging of these cultures was challenging due to the presence of many drug affected schizonts. In documenting of parasite stages, patterns of both the W2 and 4G cultures revealed that differences were observed between controls and parasites recovering from exposure to JPC-3210. It appears that JPC-3210 induced an alteration in the life cycle, slowing down the progression of parasites though the ring stage. This cell cycle regulation could potentially delay parasites from entering into later stages of development where they would be more vulnerable to the effects of the drug, as most antimalarial compounds are schizonticidal.

It has been reported that an analog of JPC-3210, JPC-2583, acts on late stage parasites in the *P. falciparum* D6 line (Chavchich et al., 2011), and therefore, it is believed that the same holds true for JPC-3210. Chavchich et al. also showed that JPC-2583 did not induce dormant parasites when cultures were exposed for 6 hours with 1,884 ng/ml, and that parasites exposed to concentrations greater than 471 ng/ml of JPC-2583 did not recover within four weeks. These concentrations of JPC-2583 kept parasites from recrudescing for up to four weeks with only a 6-hour exposure, while JPC-3210 had recrudescence within days. This difference between the two was unexpected due to the relative similarity between the chemical structures for JPC-3210 and JPC-2583. A recent paper published in January 2015 on another Jacobus Pharmaceutical Co., Inc. compound, JPC-2997, showed that this compound was very active *in vivo* with low toxicity and a long half-life. However, data was not reported as to the effect of JPC-2997 on dormant

parasites, inducing dormant parasites, or the frequency of resistance. Nevertheless, JPC-3210 most likely also has low toxicity, due to the high amount of drug concentrations that were withstood throughout these experiments.

All IC50 and IC90 values were determined using 3H-hyypoxanthine methods, as previously described. The IC50 and IC90 values for JPC-3210 were found to be highest in 4G, which is an artemisinin resistant strain of *P. falciparum* with an average IC50 of 12.5 ng/ml and an IC90 of 14.46 ng/ml, as compared to W2, which is chloroquine resistant, and had an IC50 at 0.7 ng/ml, with an IC90 of 2.98 ng/ml. Meanwhile, the Penn State piperaquine resistant isolates had IC50 levels for JPC-3210 ranging from 1.08 ng/ml in 7G8 to 5.83 ng/ml in NF10-01, and IC90 levels ranging from 2.2 ng/ml in 7G8 to 9.65 ng/ml in F0935. This result indicates that some characteristic of the artemisinin resistant phenotype found in 4G makes these parasites less susceptible to JPC-3210. It is unlikely JPC-3210 has a similar mechanism of action as artemisinin, although ruling out cross-resistance will require testing on other artemisinin resistant *P. falciparum* strains. The difference in IC50 and IC90 values between W2 and 4G may also account for the differences seen in parasite growth rates in the inducing dormancy experiment, and indicates that the 4G clones probably could have withstood a higher drug concentration than 2,000 ng/ml. In looking further at the variation in IC50 values in W2 clones and in the Penn State isolates it was found that all of these had a relatively high tolerance of piperaquine (M=19.51 ng/ml, SD= 4.29 ng/ml), they were sensitive to atovaquone, and the greatest variability in susceptibility was found in response to JPC-3210, which was previously reported.

Ultimately, questions regarding the efficacy of JPC-3210 still remain. Experiments conducted here have shed some light on JPC-3210, however, more work needs to be done to elucidate its mechanism of action and to determine if conditions such as a longer exposure in combination with artemisinin derivatives, or other classes of compounds, would enhance the effect of JPC-3210. It was encouraging that JPC-3210 did not induce dormant parasites, and that in the frequency of resistance experiments, the JPC-3210 parasites did not recrudesce under drug pressure, indicating that JPC-3210 is more effective in comparison to atovaquone. However, parasites recovered quickly from high doses of JPC-3210 not only

in dormancy experiments, but in all the pulse experiments. Moreover, JPC-3210 had no effect on dormant parasites, and resistance (or tolerance), even if unstable, could be generated relatively quickly *in vitro*. While JPC-3210 might not be the most effective antimalarial compound, it is still worth the effort to characterize new compounds like JPC-3210, as there are no antimalarial drugs in existence that have not encountered resistance clinically or in the lab. Worldwide, many lives depend on anti-malarial drug development and characterization, and it is important to evaluate new drugs using some of these methods to ensure that the best drugs are put forward in the fight against malaria. The use of JPC-3210 in combination with an artemisinin derivative still could be quite effective if implemented before artemisinin resistance spreads further thus eroding the efficacy of the fast-acting component. In addition JPC-3210 should be assessed in combination with new fast-acting antimalarial drugs unrelated to artemisinin. These could include drugs targeting ATP4 which is involved in regulating sodium levels in the malaria parasite since the onset of action of these drugs rivals that of artemisinin (Jiménez-Díaz et al., 2014). Despite the potential for JPC-3210 resistance was observed, and the use of these two new drug classes in combination could be much more effective than an ACT where resistance has already emerged to one of both components. Therefore, additional studies are required to follow up on the clinical utility of JPC-3210.

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