Efficacy of a novel anti-malarial against multi-drug resistant *P. falciparum*

By

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Thesis

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Abstract

Abstract of Efficacy of a novel anti-malarial against multi-drug resistant P. falciparum, by Nazrin Rustamzade, ScM, Brown University, May 2024

Malaria is a deadly, infectious, but also preventable disease. Despite ongoing efforts in the development of intervention strategies against malaria, drug resistance mandates innovation that would not only bring efficacious anti-malarials but also therapeutics that can capture and eradicate malaria across continents, overcoming the burden of multi-drug resistant strains.

This study focuses on exploring a potential antimalarial compound through the use of growth inhibition assays to assess its efficacy at attenuating growth of both drug-sensitive and drug-resistant parasite strains, establish an understanding of the compound's potency through calculating half maximal inhibitory concentration (IC_{50}) for each of the respective strains, and evaluate the toxicity of the compound across various mammalian cell lines. Ultimately, the study lays the groundwork for establishing robust, clinically relevant in-vivo safety and toxicity studies; and contributes to the overarching research efforts in identifying and validating an anti-malarial agent that will be efficacious in overcoming the current antimalarial drug-resistance-driven limitations.

Chapter 1: Introduction

Unveiling the Global Impact: Understanding the Burden of Malaria

In 2024, malaria remains one of the most devastating infectious diseases in the world and continues to remain an afterthought of the global public health agenda. According to the World Health Organization (WHO) World Malaria Report, 249 million malaria cases and 608,000 malaria deaths in 85 countries have been recorded in 2022. With Sub-Saharan Africa carrying the brunt of morbidity and mortality by being home to 94% of malaria cases and 95% of malaria deaths, this tropical disease also affects regions of Asia, Oceania, and Latin America. High-risk groups for malaria include pregnant women and children, with children under 5 accounting for about 80% of all malaria deaths in the African region.[1] Vaccination is undoubtedly the most effective method of preventing infectious diseases known to mankind. It is the epitome of one of the greatest contributions of immunology to human health and is further underscored by the triumph of global health against polio, smallpox, measles, tetanus, diphtheria, etc. Therefore, it is clear that developing an efficacious vaccine against malaria is a direct path towards eradication. As the global health community unites in its efforts to advance the research in favor of materializing a vaccine and developing new anti-malarials, it is important to reflect on and evaluate the control strategies at our disposal today.[2]

Effective intervention strategies against malaria remain one of the most significant challenges. The main focus of such efforts has been turned to vector control strategies and protective mechanisms targeting high-risk groups such as pregnant women and children. Most

national malaria control programs have adopted the use of insecticide-treated mosquito nets (ITNs), committing to universal coverage. While ITNs have contributed to reductions in disease prevalence, ownership of these nets does not often translate to actual usage, particularly in areas farther from distribution sites. Only partial subsidization also adds to the cost-related inaccessibility of the ITNs. Next, indoor residual spraying (IRS), a measure often referred to as the main strategy of the Global Malaria Eradication Campaign, has been successful at greatly reducing the disease burden and contributing to malaria elimination. IRS often targets areas with low and/or seasonal transmission. One of the biggest challenges with transitioning to high transmission areas is the cost of IRS on a larger scale. Other issues that stem from the use of insecticides more amply are the risks to human health and the environment as well as insecticide resistance, which is something that ITNs are subject to as well. Last, but not least is the Larval Source Management (LSM) strategy which focuses on targeted management of mosquito breeding sites to prevent the completion of development of the immature stages of larvae through habitat modification and manipulation, larviciding, and biological control. It can be used to supplement the aforementioned vector control measures but not replace them because due to the extremely large number of larval habitats in the African region, it becomes very difficult to locate and treat them promptly. Undoubtedly, socio-economic inequities tend to dictate people's health-seeking behaviors, therefore, the success of vector control methods is directly dependent on community mobilization and combined efforts from all stakeholders to ensure equitable access to healthcare.[3] Reshaping malaria intervention strategies through the development of vaccines and novel antimalarial therapeutics should remain one of the utmost priorities of international health research. Current

malaria control is plagued with impediments: the diverging transmission dynamics within endemic areas to insufficient understanding of the best parasitic stage to target and thus the timing of intervention; environmental factors; and last but not least the emergence of antimalarial drug-resistant parasites.

Trials and Triumphs: A Journey Through the Development of Antimalarial Drugs

Malaria derives from words for bad air- mala aria- coined by an Italian physician, Francesco Torti. It aligned closely with the then-held miasma theory which attributed many infectious diseases to the noxious vapors of foul air. This informed a lot of the public health measures resulting in the removal of waste and the draining of stagnant waters as a way to fight infections. This was not too far removed from reality since stagnant waters cultivated the spread of malaria, as this is where mosquitoes lay their eggs and where larvae develop. The spread of malaria throughout history has been shaped by changes to the environment through the development and expansion of agricultural practices and population movements.[4]

The New World was an impactful period in the history of malaria as the first effective treatment for the disease was discovered. The powdered bark of the Cinchona tree, native to the tropical Andes, was deemed as a miracle cure by Jesuit missionaries. With time, it became known that the quality largely depended on the species of the Cinchona genus that introduced variability in bark's therapeutic effect. The treatment was also not readily accessible and considered a largely scarce commodity. As scientists and physicians searched for alternatives, the active principle responsible for the therapeutic properties of the Cinchona bark, called cinchonin, was discovered.

The procedure was then modified to isolate the alkaloid quinine, shifting the scientific community's interest toward clinically testing purified salts of quinine. This critical innovation in drug discovery allowed for the emergence of a potent and effective pure substance, which was unrivaled for many more years to come. Administering quinine did not come without its problems: as its low therapeutic index and adverse effects of varying severity became more pronounced, the term cinchonism was coined to describe the common side effects of nausea, headache, tinnitus, and hearing loss. Resistance to quinine was first reported in the early 1900s. While the WHO no longer recommends quinine as a first-line treatment, almost 400 years since its efficacy was first reported, it remains an important anti-malarial today.[4]

After World War I, the need for safer and more effective alternatives to quinine became more apparent, a synthetic analog of quinine with the same mechanism of action, chloroquine (CQ), emerged, quickly becoming one of the most successful single drugs for treatment and prophylaxis. As the scientific and medical community sought to develop better anti-malarials, the WHO Malaria Eradication Program was enacted in 1955 to achieve vector control using insecticides, focusing on the use of dichlorodiphenyltrichloroethane (DDT) for indoor residual spraying. Around the same time, pyrimethamine was discovered and became widely used together with chloroquine as programs aimed at identifying and treating infected individuals expanded. The eradication program proved to be a successful tool in Europe and North America largely due to the region's ability to implement robust infrastructure for disease monitoring. The success was not well-rounded and Less Economically Developed Countries (LEDCs) remained at a disadvantage- one that is ever so pronounced today.[4]

In the 1960s, CQ became one of the top-selling medications due to being generally well-tolerated with a long half-life and its ability to prevent reinfection. Chloroquine, unfortunately, proved to not be immune to the inevitable part of an anti-malarial's life cycle and chloroquine-resistant *P. falciparum* infections began to spread in Southeast Asia, South America, and Papua New Guinea. In 1978, chloroquine resistance made its way to Africa and since then remained unwaveringly endemic to the region. This promoted governments, academic, and commercial organizations to collaborate, and as a result over a quarter million compounds were screened for antimalarial activity, leading to the discovery of mefloquine, structurally related to quinine. It became the drug of choice for treating uncomplicated malaria in parts of Southeast Asia, but soon enough resistance caught up to mefloquine and was reported in Indochina.[4]

Once again as the global public health community faced yet another drug-resistance-driven hurdle, Professor Tu Youyou at the Chinese Academy of Chinese Medical Sciences, turned to traditional Chinese medicine and led the team in searching through the ancient literature. A total of 640 recipes that had potential antimalarial activity were located, but the most frequently mentioned species of plants for intermittent fevers caught her attention: Artemisia annua. As the extract was tested for the inhibition of parasite growth, it became clear that poor extraction of the active ingredient led to low levels of inhibition. The methodology was modified to a low-temperature extraction protocol with the additional step of removing the toxic acidic component. This was all taking place during the Cultural Revolution in China, when it was virtually impossible to carry out clinical toxicity trials, so Youyou together with her team of investigators carried out toxicity studies on themselves. These were followed by efficacy trials on patients with chloroquine-

resistant *P. falciparum* infections. The isolated crystals of the active principle were named artemisinin (ART), which eventually was reduced to a derivative, dihydroartemisinin with greater potency and higher bioavailability in both uncomplicated and cerebral malaria. WHO followed up by issuing guidelines, recommending the use of artemisinin-based combination therapies (ACTs) as the first-line treatment for *P. falciparum*. This was important because WHO strongly advocated for a ban on artemisinin monotherapies, acknowledging the high risk of drug resistance development due to its unregulated use. However, by that time, artemisinin resistance had already begun to emerge, spreading throughout Southeast Asia and Africa, leading to resistance to ACT partner drugs as well. Today, five artemisinin combination therapies are recommended by the WHO for uncomplicated *P. falciparum* infections. Unfortunately, as no alternative to artemisinin therapy is expected to become available for the next few years, the prescription and use of ACTs must be closely monitored.[4]

Resistance is at the center of the threats to the eradication of malaria. The spread of resistance is sustained by long-term low-dose antimalarial prophylaxis and selective survival of resistant parasites in treated hosts. Interestingly, antimalarial resistance in non-*falciparum* species has been slower to develop, perhaps due to lower parasite numbers in the human host and therefore fewer mutation events.[5] It is extremely difficult to strike a balance between increasing the number of partner drugs in a given combination therapy to slow down the development of drug resistance and facing the increased risk of adverse side effects and implementation costs. Infected human hosts who have a degree of resistance and are asymptomatic, can act as a reservoir for malaria parasites to be then passed on to the biting mosquitoes. Therefore, it is of utmost importance for

malaria prevention strategies to focus on sensitive diagnostic field technologies that would be able to rapidly screen for malaria infection in asymptomatic patients in endemic regions. Undoubtedly, treatment guidelines should be informed by advancing genetic research to not only better understand the *P. falciparum* genome but also to facilitate the discovery of new anti-malarials and insecticides to account for drug resistance-associated genes. Ultimately, the success of effective treatments and control measures in endemic regions will depend on the extent of how multifaceted and preemptive they are.

Resistance on the Rise: Delving into the Battle Against Malaria's Defenses

Resistance has historically coursed through the development of anti-malarials and today it controls the effectiveness of malaria control efforts. However, to wage a battle against parasites' defense mechanisms, it is important to understand molecular underpinnings and intricacies that need to be considered as we embark on antimalarial and vaccine development. While it will be difficult to pinpoint all the diverse intricacies of molecular mechanisms of resistance in *P. falciparum*, it is significant to touch upon some of them to underscore the unique nature of *P. falciparum* resistance-conferring tactics, which are ultimately responsible for producing major setbacks in the development of new class anti-malarials.

According to the WHO, antimalarial drug resistance is defined by the parasite's ability to survive despite the administration of medicine given in doses that are equal to or higher than recommended but within tolerance of the subject.[1] Time and time again, it was highlighted that *P. falciparum* has a complicated biology where as parasites progress through the various stages of their life cycle, they express a plethora of stage-specific antigens, each responsible for inducing a

specific response. *P. falciparum* is an extremely advanced and smart parasite that can respond to specific interventions such as rapid diagnostic tests, host immune pressure, and mosquito vector environments. As the parasite responds to administered anti-malarials and other aforementioned malaria control strategies, it simultaneously continues to evolve through genetic mutations and sexual recombinations, constantly moving the target for these interventions. Therefore, all antimalarial drug development should be centered around the identification and validation of drug-resistance molecular markers. The molecular markers of drug resistance are helpful tools in detecting resistance in both clinical and field isolates. Thus, focusing on creating the right infrastructure that will enable earlier detection of drug-resistant parasites and result in appropriate treatments, reducing mortality and the spread of resistance, is of the essence.[6]

Detection of molecular markers of antimalarial drug resistance can be a feasible way to gain insight into resistance-associated parasite genetics. Hemoglobin catabolism in *P. falciparum* is a source of amino acids for protein synthesis, making it the target of multiple first-line antimalarial drugs available today. The digestive vacuole membrane proteins of *P. falciparum* Chloroquine Resistance Transporter (PfCRT) and *P. falciparum* Multidrug Drug Resistance (PfMDR) genes are the primary molecular determinants of resistance. The once widespread use of CQ selected for mutant PfCRT alleles that independently emerged in Southeast Asia and South America, eventually making their way into Africa. What is unique about these PfCRT variants is that they can revert parasites to CQ-sensitive status, but at the same time they can mediate resistance to the active metabolite of amodiaquine (AQ), a structural analog of CQ that was once considered to be active against CQ-resistant *Plasmodium* species, but its usage has been restricted

due to hepatic and hematological toxicities. All mutant CQ-resistant PfCRT alleles increase *P. falciparum* susceptibility to ART derivatives, providing yet another reason for the transition towards ACTs. Mechanistically, mutant PfCRT operates through the H+-dependent drug efflux out of the digestive vacuole by preventing CQ from binding its heme receptor. Moreover, it possesses pleiotropic drug-transport properties that also allow it to impact other anti-malarials by altering their concentration at the site of action. Most mutant PfCRT alleles come with a fitness cost due to less efficient hemoglobin catabolism; however, the Cambodian parasites have been found to harbor a polymorphic PfCRT allele that confers CQ resistance without exuding fitness costs. New mutations in both PfMDR1 and PfCRT are constantly being documented across Asia and Africa, suggesting that a shift from treating with CQ towards ACTs is selecting novel variants of multi-drug resistant transporters.[7]

Resistance is conferred through structural, functional, or quantitative changes in proteins which is usually mediated by genetic changes such as single nucleotide polymorphisms (SNP) or gene amplification in target catalytic enzymes or efflux pumps. *P. falciparum* Kelch 13 (PfKelch13), for example, located on chromosome 13, is a molecular determinant of artemisinin resistance and is acquired by SNPs in the parasite's kelch propeller domain. Interestingly enough, mammalian orthologs of PfKelch13 confer resistance to cancer drugs which kill tumors through the induction of proteopathy. According to Haldar et al., PfKelch13-driven artemisinin resistance supports the idea that this may play a role in parasitical proteostasis which promotes the parasite's survival even after artemisinin-induced proteopathy.[8] When the PfKelch13 mutation was first acquired, there was a more substantial fitness cost to the parasite; however, the latter C580Y

mutation conferred essentially no fitness cost, suggesting that these K13 fitness defects were mitigated. When resistance to ART was first reported in Cambodia, in a clinical setting, infected patients displayed prolonged parasite clearance following treatment with both artemisinin derivatives and ACTs. Slow parasite clearance is also associated with the higher number of gametocytes in patients pre and post-treatment suggesting the transmission advantage of ART-resistant *P. falciparum*. There is also no substantial obstacle to mutant K13's ability to be transmitted by various Anopheles species.[7]

P. falciparum isolates from various geographic regions showcase differences in infectivity to the same mosquito vector, pointing to the fact that natural selection was at play to allow parasites to adapt to different mosquito vector species. In Canepa et al. analysis of *Plasmodium* evasion of mosquito immunity, a complement-like system is proposed as a determinant of vector-parasite compatibility. In Anopheles gambiae, a thioester containing protein-1 (TEP1) gene binds to the surface of the *Plasmodium* ookinetes when they come into contact with the mosquito hemolymph, activating the formation of a complex that would kill that strain of parasites. However, any disruption of the TEP1 preferentially eliminates certain strains of parasites while allowing others to evade the mosquito immune system and survive. Since TEP1 is a polymorphic gene, which can suggest its susceptibility to mutations, if mutations do occur, parasites, with conferred mechanisms of resistance, which would otherwise be eliminated, persist and eventually find their way into the human host.[9] This is similar to the storyline of artemisinin-resistant parasites, where mosquito-vector differences have the possibility of eventually not posing a hurdle for the successful dissemination of mutated alleles of Asian origin across Africa.

A commonly described anti-malarial for prophylaxis, atovaquone, effectively clears gametocytes by inhibiting the electron transport chain; however, its solo use can result in a single point mutation in the parasite's cytochrome b leading to the conferral of drug resistance- a fact that was unknown during the therapy's rollout. This prompted the global health community to focus on testing new drug combinations with atovaquone in clinical trials, but, unfortunately, only post-factum.[10] Another unique adaptation of malaria parasites is the loss of classical nonhomologous end joining (cNHEJ), a mechanism that is responsible for the repair of DNA double-stranded breaks. This has been hypothesized to give parasites a smaller and more streamlined genome to allow them to multiply faster at a lower metabolic cost. Last, but not least, evolutionary history also plays an important role as surface antigens exhibit extensive genetic diversity and are subject to prolonged selective pressure by the human immune response. Parasites are extremely successful at evading the human host antibody-mediated clearance through their ability to vary the antigens they express on the surface of erythrocytes, extending the length of infections and thus the spread of resistance.[11]

Nowadays, treatment relies heavily on the continued efficacy of ACTs. They have been successful so far because they effectively combine both artemisinin-derivatives and other long-acting anti-malarials: when these therapies are combined, each one employs a different mechanism of action to maximize the chances of eliminating parasites. Cambodia is a valuable case study for studying multi-drug resistance and understanding how these patterns can be partially mirrored in other endemic regions of the world. In Cambodia, most *P. falciparum* infections are monoclonal, which decreases the fitness costs associated with resistance, allowing parasites to optimize their

mechanisms and disseminate. In Africa, on the other hand, most infections are polyclonal and parasite strains need to exhibit robust growth rates to remain competitive. Host immunity also plays an important role in the clearance of *P. falciparum* infections, and in areas where host immunity is lower, meaning there is a reduced frequency of infections as compared to higher transmission rates in Africa, there is a weaker ability to clear drug-resistant parasites naturally. Additionally, unlike other infectious diseases, parasites are capable of inducing resistance in the exact cellular target of the drug.[12] However, if we have learned our lesson through time, we know that ACTs are bound to reach a point of conferring resistance. According to Lubell et al., if we were to model the scenario of widespread ACT resistance in malaria-endemic countries, there would be an approximate impact of 100,000 additional deaths per year.[13] Indeed, resistance to artemisinin derivatives is no longer in the distant future and is the reality today.

Yet, when tackling the distinct molecular mechanisms of resistance at play, social and economic conditions cannot be overlooked. *P. falciparum* parasites are undoubtedly smart and have weaved intricate mechanisms of resistance through time, but resistance would be conferred at a much slower rate if the socio-economic context in endemic regions did not accommodate for the dissemination of resistance. The socio-economic conditions singlehandedly shape the norms and behaviors and therefore people's choices. Factors ranging from the level of education, occupation, and income to housing will all affect individuals' decision to seek malaria treatment and the mode in which they choose or not to do so. In LEDCs, the lack of regulations regarding the use and sale of medication is very common, allowing pharmacies to distribute counterfeit products.[14] This is coupled with the fact that people experience the lack of means to pay for anti-malarials from

pharmacies, so they inevitably resort to self-medication through the use of traditional medicine and/or self-prescription with anti-malarials that lack the active gradient or mixing different anti-malarials that were unintended for combined therapy, diminishing the efficacy of the administered drugs by exposing parasites to sub-therapeutic doses and spreading resistance. When financial constraints are at play, it is tough to expect people to also make use of malaria diagnostic tests before proceeding with self-medication. Presumptive treatment can alter the within-host ecology of drug-resistant parasites, desensitizing the patient to antimalarial treatments in the long run. Self-medication persists due to health and financial inequities, particularly those pertaining to minimal or nonexistent infrastructure to support people seeking professional medical advice and lack of information surrounding malaria and the appropriate course of action to be undertaken in the case of. When people try to treat themselves, the temporary short-lived relief of symptoms such as fever masks the fact that the infections persist. Moreover, there is a risk of adverse reactions and antimalarial drug overuse, which eventually result in hospitalization that only prolongs the infection. Furthermore, the qualitative study by Anyanwu et al. revealed that drug resistance is encouraged by the fact that people often share malaria treatments or stop treatments prematurely to save the anti-malarial for use at a later time.[15]

Control of antimalarial drug resistance will rely on the continuous identification of molecular resistance markers. Tools such as genome-wide association studies (GWAS) and next-generation sequencing (NGS) must be utilized to make sure that emerging polymorphisms that confer decreased susceptibility to anti-malarials are captured in real-time to effectively inform and prompt malaria control programs to evolve as more characterization of the molecular

epidemiology of antimalarial resistance becomes available. However, addressing socio-economic factors is equally vital. Integrating healthcare access, education, and regulatory measures is imperative for effective malaria control efforts.

Unraveling Nature's Intricacy: Deciphering the Malaria Parasite's Life Cycle

Malaria in humans is caused by five protozoan parasite species of the *Plasmodium* genus: *P. falciparum*, which causes virtually all malaria-attributed deaths, *P. vivax, P. ovale, P. malariae*, and *P. knowlesi*. For the purposes of the study, the focus will solely be on the *P. falciparum* species. Malaria spreads through the bites of infected female Anopheles mosquitoes which kickstarts the cascade of the *P. falciparum* life cycle in humans. The pre-erythrocytic stage initiates the infection, the asexual erythrocytic stage causes the disease, and the gametocyte stage infects mosquitoes that ultimately transmit the parasite. The cycle begins with the female Anopheles mosquito inoculating several *P. falciparum* parasites by piercing the skin and taking up a blood meal. Sporozoites then travel to the liver and infect a number of hepatocytes. It takes only up to 30 minutes for the sporozoites to reach liver cells. A single sporozoite then can give rise to tens of thousands of asexual parasites, called merozoites. After approximately a week from the initial liver infection, the infected hepatocytes burst, releasing merozoites into the bloodstream. As of October 2023, there are two vaccines, RTS,S/AS01 and R21/Matrix-M, recommended by the WHO. While both vaccines show some efficacy and promise of reducing symptomatic cases of malaria, in theory, their main shortcoming arises from the fact that they both aim to target the pre-erythrocytic stage, as described above. They aim to prevent the invasion of hepatocytes and also

elicit a cellular response enabling the destruction of already infected hepatocytes by targeting the circumsporozoite (CS) protein on the surface of the parasite. This is an extremely small window of opportunity. If at least one sporozoite infects a hepatocyte, disease pathogenesis progresses, making it an unattractive vaccine target in practice. Once merozoites are released into the bloodstream, they invade an erythrocyte and can multiply up to 20-fold during the course of the 48-hour life cycle which encompasses invasion, replication, rupture, and release of infectious merozoites from an erythrocyte. During this two-day life cycle, parasites undergo asexual multiplication in the erythrocyte, changing their morphology within the red blood cells from an infecting merozoite, ring-like to trophozoites, to schizonts, which mature, and finally lead to the pinnacle of rupture which releases merozoites to infect more erythrocytes and the cycle repeats itself. These blood-stage parasites are responsible for the clinical manifestation of the disease. The timing of the paroxysmal fevers, chills, myalgia, vomiting, and severe anemia, which are the most common clinical manifestations of malaria, is closely associated with the timing of the rupture of erythrocytic stage schizonts.[16] Therefore, identifying antigens that are expressed during the asexual blood stage of the *P. falciparum* infection and that play a significant role in erythrocyte invasion is of utmost importance as they could be attractive targets for vaccine and anti-malarial development because the morbidity is closely associated with the repeated cycles of asexual replication in the host's red blood cells.[17] It is crucial to understand that the eukaryotic parasite is equipped for survival in humans, as its intermediate host, and in mosquitoes, as its definitive host. Further complications and manifestations of severe malaria occur when parasitized or infected erythrocytes cyto adhere to the endothelium, platelets, and uninfected erythrocytes. This leads to

the sequestration of infected erythrocytes that can block microvasculature and result in cerebral malaria, stroke, or death. As the parasite population increases every 48 hours, so does the parasite burden. The goal of the parasites is not to lead to the intermediate host's demise, it is to continue proliferation until they can commit to sexual development and transmit the parasites to the next mosquito vector. A developmental switch will then transpire within a fraction of the asexually replicating parasites, causing them to exit and commit to sexual development.[10]

A small percentage of blood-stage asexual parasites spontaneously convert to gametocytes, initiating the sexual cycle. Only this form of parasite enables the transmission of parasites from the host to the mosquito. Once gametocytes reach maturity, they are picked up by the mosquito during a blood meal. They become activated in the mosquito midgut, differentiating into male and female gametes, which fertilize to form a zygote. The zygote develops into an ookinete, which is motile and is thus able to glide into and penetrate the gut epithelium, forming an oocyst. Upon maturation oocyst release sporozoites that migrate to the salivary glands of the mosquito, waiting to be transmitted to yet another host through the infected bite.[18] *P. falciparum* gametocytes and therefore, the sexual stage development can be considered as significant targets of malaria intervention, such as a transmission-blocking approach, which could inhibit the maturation of gametocytes. However, the molecular mechanisms and genes governing sexual reproduction and sexual stage commitment and development; as well as the strategies that the parasites have adapted to maximize the success of transmission remain largely unknown.[19]

It is important to understand that as the parasite proceeds through different stages of the life cycle, it undergoes morphological changes and consequently displays antigenic variations. The life

cycle itself is dependent on a variety of specialized protein expressions each with respect to survival, invasion, and evasion of host immune response. The successful development of novel anti-malarials and vaccines is dependent on the comprehensive understanding of the *P. falciparum* biology because the ability to interrupt the life cycle of a parasite of this complexity requires targeting proteins pertaining to different life stages. Therefore genome sequencing and proteomics will have to be at the center of any antimalarial development pursuits.[20]

In the description of the intricacies of the *P. falciparum* life cycle, it was mentioned that the recently approved and recommended vaccines fall short due to the fact that they focus on the pre-erythrocytic stage, characterized by its extremely small window of opportunity to intervene. This is precisely why the Jonathan D. Kurtis Lab focused its research efforts on identifying an antimalarial and vaccine candidate that would target the asexual blood cycle or the erythrocytic stage. Targeting this cycle, instead, extends the intervention window to at least 24 hours, offering a promising alternative to current vaccine strategies.

In recent work by Raj et al., *P. falciparum* glutamic-acid-rich-protein (PfGARP) was identified as a parasite antigen that is recognized by antibodies in the plasma of children who are relatively resistant, but not in those who are susceptible to *P. falciparum* malaria. PfGARP is a parasite antigen that is expressed exofacially on the surface of erythrocytes infected by early-to-late-trophozoite-stage parasites. Raj et al. were able to demonstrate that antibodies against PfGARP kill trophozoite-infected erythrocytes in culture by inducing “crisis” forms (Fig. 1) and consequently programmed cell death- that, in addition to other data led the group to believe that PfGARP could be a not only a potential vaccine but also an antimalarial candidate that would

allow to target the asexual blood cycle. One of the results that was particularly important and informative to this study was that human anti-recombinant-PfGARP IgG purified from pooled serum collected from individuals who had been exposed to malaria inhibited the growth of three different strains of *P. falciparum,* which include both drug-sensitive and drug-resistant strains- 3D7, DD2 and D10- by 94–99% (Fig. 2).[21]

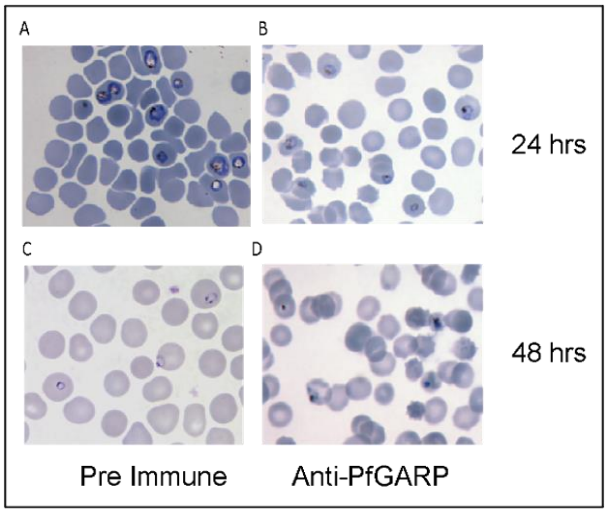


Fig. 1. Raj et al., Nature 2020. 3D7 *P. falciparum* parasites were synchronized to the ring stage and plated at 5% parasitemia in the presence of pre-immune and anti-recombinant-PfGARP-A mouse serum at a 1:10 dilution. Parasites were cultured for 24 h or 48 h, stained with Giemsa, and photographed by light microscopy. Anti-PfGARP-A-treated parasites displayed a dysmorphic, pyknotic appearance on Giemsa-stained blood smears, consistent with "crisis" forms that are associated with dying or dead parasites. Parasites treated with pre-immune sera, containing no PfGARP progressed into another stage of the life cycle.

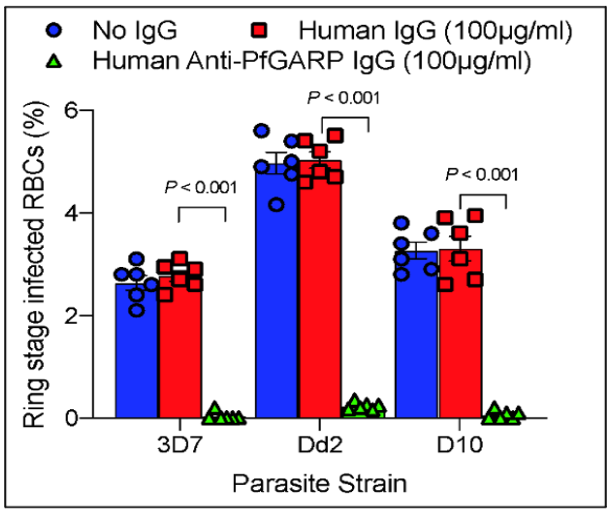


Fig. 2. Raj et al., Nature 2020. Human anti-recombinant-PfGARP IgG purified from pooled serum collected from individuals who had been exposed to malaria inhibits the growth of three different strains of *P. falciparum* (3D7, DD2, and D10) by 94–99%. Negative controls were no serum (blue) and pre-immune mouse serum (red). Data are mean ± SEM of five biologically independent replicates.

The scientific premise of PfGARP as a druggable target is based on its expression on the surface of infected erythrocytes; limited sequence variation in over 3,000 sequenced field isolates; absence of amino acid homology with host proteins; and its ability to initiate parasite programmed cell death. Moreover, since PfGARP is expressed on the surface of infected erythrocytes during the early-to-late-trophozoite-stage of the parasite life cycle, it allows us to effectively target the asexual blood cycle.

To develop a drug based on PfGARP binding, the Jonathan D. Kurtis lab screened a total of 10,000 compounds from the ChemBridge DIVERSe small molecule compound library and in collaboration with the Center for Translational Science at Florida Atlantic University, the 30 million compound library to identify compounds that inhibit the binding of anti-PfGARP antibody to the recombinant PfGARP protein. Two compounds (#579 and #424) had enhanced parasite

killing with an IC_{50} of 2-20 μM in 3D7 parasites and no activity in PfGARP-KO parasites, indicating specificity. Confirmation of specific binding to PfGARP was achieved through surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) spectroscopy. With the hopes of discovering a compound that could be effective at a lower molar range, the lab proceeded to identify two sub-libraries (#2253 and #1664) containing compounds that bind to PfGARP. Using a positional scanning method, the group deconvoluted sub-library #2353 to 36 individual compounds of the 2741 series, 7 of which killed 3D7 parasites at a nanomolar range. Surprisingly, even though these 7 compounds were effective at attenuating parasite growth in vitro and intended for PfGARP targeting, the compound exhibited parasite-killing effects in PfGARP-KO culture. This changed the scientific premise of this study but left us with potent compounds that are successful at inducing "crisis" forms and consequently programmed cell death, but the mechanisms of which remain unknown.

Chapter 2: Methods

2.1 Parasite Strains

P. falciparum strains (3D7 and D10) were obtained from the MR4 *P. falciparum* strain. DD2 (MRA-156) was obtained through BEI Resources, NIAID, NIH, contributed by Thomas E. Wellems. *P. falciparum strains* MRA-1250 (CamWT) and MRA-1251 (CamWT_C580Y) were obtained through BEI Resources, NIAID, NIH, contributed by David A. Fidock and directly through David. A. Fidock, Department of Microbiology and Immunology at Columbia University Irving Medical Center, New York, NY, USA.

2.2. *P. falciparum* Culture and Maintenance

P. falciparum strains were grown and maintained using RPMI 1640 GlutaMAX™, supplemented with HEPES (Gibco Cat# 72400-047), 10% Albumax (Gibco Cat# 11021-045), hypoxanthine (Gibco Cat # 11067-030), and gentamycin (Gibco Cat# 15710-064). Cultures consisted of infected erythrocytes and O+ human erythrocytes. Cultures were treated with a gas mixture containing 5% CO_2, 5% O_2, 95% N_2 and incubated at 37°C. The media in the flask was changed every 48 hours. Parasitemia (ratio of infected RBCs to uninfected RBCs) as well as parasite visualization was done by preparing blood films and staining with Giemsa to be looked at under the microscope as well as CellaVision, a digital hematology microscopy analyzer. Strains were maintained in continuous culture with backup stored in liquid nitrogen.

2. 3 Growth Inhibition Assays (GIAs)

The experimental set-up for all 5 strains, was consistent throughout all GIAs performed. 3D7, D10, DD2, MRA-1250, and MRA-1251 parasite strains (cultured in RPMI 1640 GlutaMAX™, supplemented with HEPES, 10% Albumax, hypoxanthine, and gentamycin were synchronized to the ring stage by 5% sorbitol for at least two successive replication cycles while keeping the parasitemia below 5%. On the day of the GIA, parasites were diluted to 0.2% parasitemia and plated in wells at 2% hematocrit in complete media. From the 32 μM stock concentration of compound 2741-19 800 nM was prepared. 150 μL of the 800 nM concentration were then diluted 1:2 and 75 μL of parasites were added, resulting in the following experimental conditions: 0.2% parasitemia, 1% hematocrit, and 400 nM, as the highest dose and 0.78125 nM as

the lowest dose in 96-well flat-bottom microtiter plates. Vehicle control wells received DMSO treatments, corresponding to the compound 2741-19 dilutions. Each treatment condition was performed in triplicate. After incubation at 37°C for 72 hours, blood films, flow cytometry analysis using Hoechst staining, and *Plasmodium* lactate dehydrogenase (pLDH) assays were performed to assess parasitemia levels. Apart from performing the GIAs in triplicate doses, all of them were repeated independently three times for each of the strains. IC_{50} post-treatment for each respective strain was determined using a four-parameter logistic regression model on GraphPad Prism through either plotting the optical density values (OD) after a pLDH assay or the parasitemia levels (%) after flow cytometry analysis on the y-axis against the effector concentrations.

2. 4 *Plasmodium* lactate dehydrogenase (pLDH) assays

pLDH assays are a way of measuring the level of lactate dehydrogenase (LDH). LDH is an enzyme that is present in all cell types and turns sugar into energy. LDH is released into the fluid when the cells are damaged or injured. Therefore, LDH assay can be used to assess the extent of tissue damage or cell death. After 72 hours of incubation had elapsed, a sample from each of the treated and untreated control wells was aliquoted to be frozen and thawed twice to ensure RBC lysis, for at least an hour at -80°C for each freeze-thaw. After the second freeze-thaw cycle, nitro blue tetrazolium (NBT), phenazine ethosulfate (PES), and Malstat reagent were added and the plate was incubated on a shaker for 1 hour. After one hour had elapsed, the plate was read at 560 nm. The LDH assay generated optical density (OD) values at various concentrations of the compound as raw data. The parasite growth value at each compound concentration was obtained

by adjusting OD values from compound-treated wells by subtracting parasite-free red blood cells to account for background.

2.5 Hep3B and THP-1 Culture and Maintenance

Both Hep3B and THP-1 cells were grown and maintained using RPMI 1640 Gibco Cat# 11875093 and 10% FBS (Corning Cat # 35-011-CV). Hep3B cells were trypsinized and counted using a hemocytometer twice a week. THP-1 cells were counted using a hemocytometer and the media was changed twice a week.

2.6 Hep3B Cytotoxicity Assay

Hep3B cells were trypsinized, washed, counted, and resuspended so that there were 20,000 cells plated per 200 µl in each of the wells. Following a 24-hour incubation, by visualizing the cells under the microscope, it was determined whether the cells had reached 60-80% confluency to be able to proceed with the treatment. 200 µM stock of compound was prepared 2741-19 and serially diluted 1:2 so that the final concentration in the well was 100 µM. Negative control wells received DMSO treatments, corresponding to the compound 2741-19 dilutions. Positive control wells received Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Invitrogen™ Cat #M34152). CCCP is considered a chemical inhibition of oxidative phosphorylation, which destroys living cells. It reduces the ability of ATP synthase to function optimally. At the relevant time points, the samples were analyzed using an MTT assay.

2.7 MTT Assay

MTT assay is a colorimetric assay for assessing cell metabolic activity. For the purposes of our study, it was used for cytotoxicity assessment of adherent Hep3B cells, a human liver cancer cell line that was originally derived from a patient with a well-differentiated hepatocellular carcinoma. NAD(P)H-dependent cellular oxidoreductase enzymes can reflect the number of viable cells present in a sample by reducing the yellow tetrazolium dye MTT into purple insoluble formazan crystals in living cells. A 5mg/ml solution of MTT was prepared. Once added to the plate, samples were incubated for 4 hours. After 4 hours, DMSO was added to dissolve the insoluble purple formazan crystals. The absorbance of the colored solution was then quantified by reading the plate at 560 nm. Similarly to the pLDH assay, the MTT assay also generated OD values at various concentrations of the compound and a four-parameter logistic regression model on GraphPad Prism was used to plot the OD values on the y-axis against the effector concentrations.

2.8 THP-1 Cytotoxicity Assay

THP-1 cells were counted and resuspended so that there were 100,000 cells per 100 μL in each well. 200 μM stock of compound was prepared 2741-19 and serially diluted 1:2 so that the final concentration in the well was 100 μM. Negative control wells received DMSO treatments, corresponding to the compound 2741-19 dilutions. Positive control wells received 20% DMSO (10% after serial dilution), a concentration known to be toxic to mammalian cells.[22] Two 96-well,

flat-bottom plates were set up, one for the 24-hour time point and another for the 48-hour time point. Cytotoxicity was then assessed using 7-AAD Staining.

2.9 Flow cytometry staining for P. *falciparum* growth assessment and THP-1 cells cytotoxicity assessment

Flow cytometry staining was used to assess parasite growth post-treatment with compound 2741-19 in GIAs and cytotoxicity of compound 2741-19 on THP-1 cells. For P. *falciparum* growth assessment post-treatment, samples were aliquoted and stained with Hoechst. Usually, this type of staining can be used to distinguish between live and dead cells. In live cells, Hoechst stains the nucleus uniformly, whereas in dead cells, the dye may penetrate the compromised cell membrane and result in diffuse or absent nuclear staining. For the purposes of our experiment, since assessment of parasitemia levels was prioritized, Hoechst was used to preferentially stain parasites and not enucleated red blood cells. A 20mM stock of Hoechst (Thermo Fisher Cat #62249) was used to prepare a 4 μM solution for staining. The stained samples were then washed in PBS twice and analyzed using flow cytometry.

For cytotoxicity assessment in THP-1 cells, 7-AAD powder (Invitrogen™ Cat # A1310) was resuspended in DMSO and then prepared using 1:500 dilution in Complete Media. The samples were stained and then analyzed using flow cytometry. 7-AAD is a useful cell viability stain. Cells with compromised membranes will stain with 7-AAD, helping assess cytotoxicity.

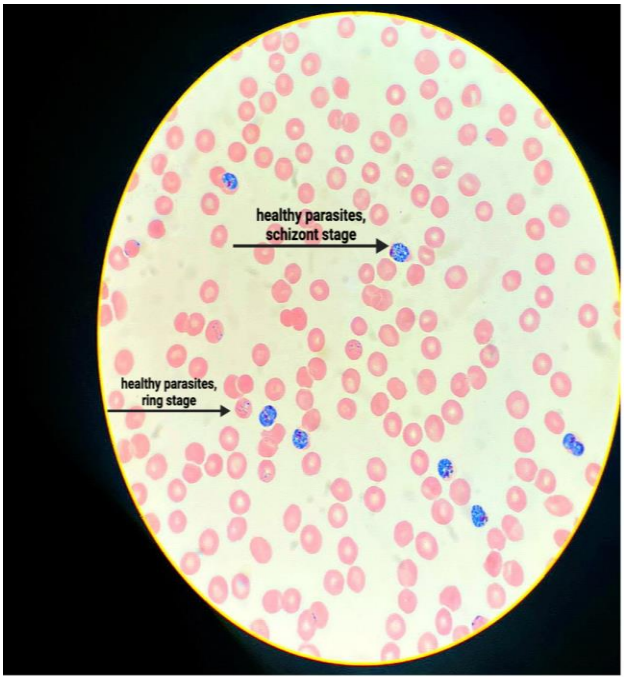
Chapter 3: Results

It was previously mentioned that although screening a 30 million compound library to identify PfGARP-targeting compounds, did not yield the intended specificity, it resulted in the identification of 7 potent compounds. This initiated a series of in vitro research using five *P. falciparum* strains (3D7, D10, DD2, MRA-1250, MRA-1251) and 3 lead compounds out of the previously mentioned 7 that reproducibly demonstrated killing parasite from both drug-sensitive and drug-resistant strains, laying the foundation for advancing a potential anti-malarial. This study is focused solely on lead compound 2741-19 and therefore the results presented here will be reflective of that. The data presented in this study will showcase the efficacy of compound 2741-19 and additionally tap into preliminary toxicity data using two mammalian cell lines such as THP-1 and Hep3B.

In vitro studies using a lead compound 2741-19 comprised of two main approaches: growth inhibition assays to determine whether a 72-hour treatment using 2741-19 could not only initiate parasite death in vitro in drug-sensitive and drug-resistant strains but also show enhanced killing with low Molar range IC_{50} and cytotoxicity assays to assess the compound's safety profile. Parasitemia post-treatment was determined using three readouts: blood films for microscopic analysis to determine parasitemia and observe any morphological differences between treated and untreated parasites; flow cytometry analysis using a nucleic acid stain; and lastly, *Plasmodium* lactate dehydrogenase (LDH) assays to assess parasite growth inhibition using optical density. Cytotoxicity in mammalian cell lines was assessed using either an MTT assay or flow cytometry analysis using fluorescent markers for DNA post-treatment. The results presented are

representational of 3 independent sets of microscopy, flow cytometry, and LDH data confirming parasite killing post GIAs and 2 independent cytotoxicity experiments for each respective mammalian cell line.

Microscopy, is one of the first readouts post 72-hour GIA. When analyzing blood films, it was revealed that ring-stage parasites treated with compound 2741-19 (Fig. 3b.) for 72 hours are shrunken and pyknotic- a morphology referred to as “crisis” forms compared to those that were left untreated in media (Fig. 3a.) which have continued into another life cycle and are now either healthy rings or schizonts which upon full maturation will rupture into rings. Even though the mechanism of action of compound 2741-19 is unrelated to the PfGARP pathway, morphological changes, similar to the above-stated ones that initially inspired the pursuit to screen and identify compounds that are PfGARP specific, can be induced when treating using compound 2741-19 as well.



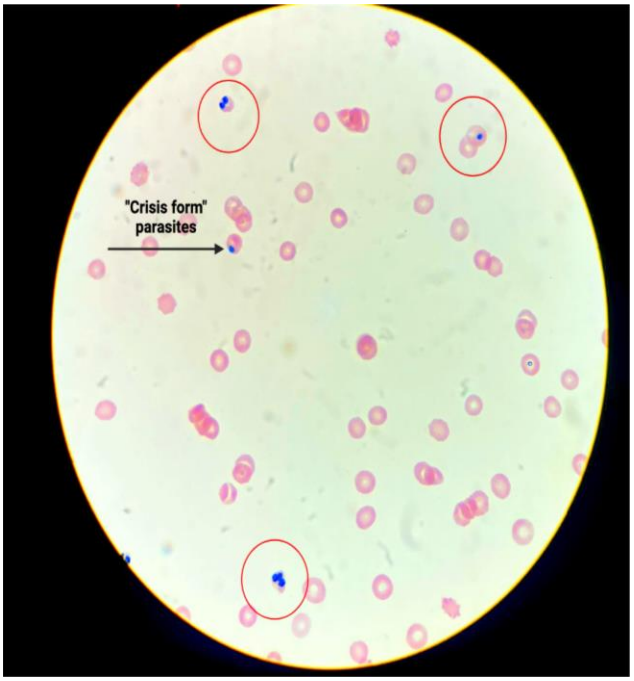
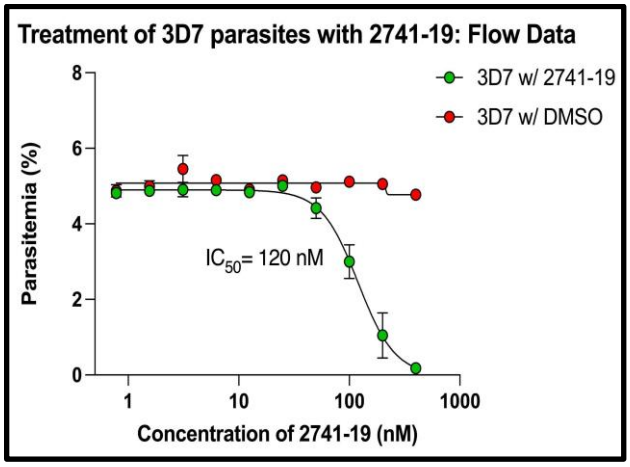


Fig. 3a **Fig. 3b**

Fig. 3a and 3b. *P. falciparum* parasites were synchronized to the ring stage and plated at 0.2% parasitemia in the presence of compound 2741-19. After a 72 h incubation samples were stained with Giemsa and photographed by light microscopy. Compound 2741-19-treated parasites (Fig. 3b) displayed a dysmorphic, pyknotic appearance on Giemsa-stained blood smears, consistent with “crisis” forms that are associated with dying or dead parasites. Untreated parasites (Fig. 3a) progressed into another stage of the life cycle. Treated parasites in the images received the highest dose of 400nM within the serial dilution range. Images are representative of three independent experiments.

3D7 strain was clinically isolated from a Dutch resident who had not left the country and is considered a case of airport malaria. It has been cultured for over 30 years and has been the standard in antimalarial and vaccine development, and therefore it is the strain that initiated our in vitro studies. When treated with 2741-19, ~90% of 3D7 parasites are killed in culture in the nM range with an IC_{50} of 67-120 nM (Fig. 4a and 4b).



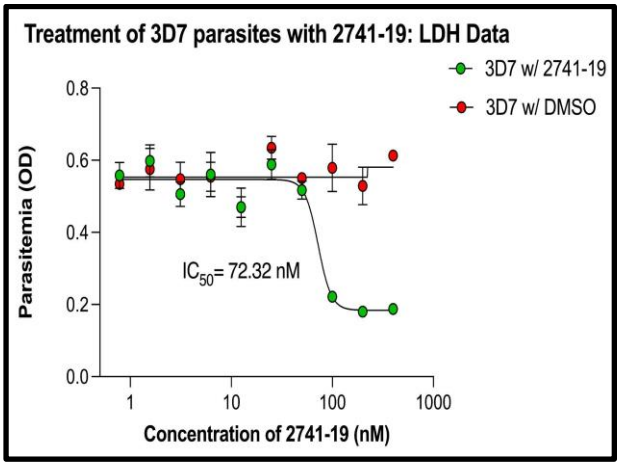
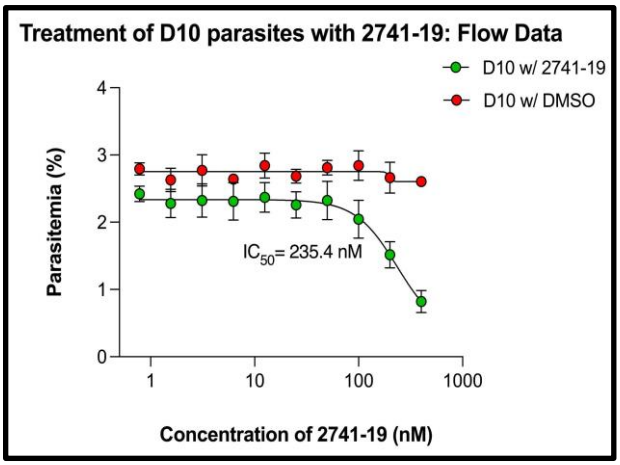


Fig. 4a **Fig.4b**

Fig. 4a and 4b. 2741-19 kills 3D7 *P. falciparum* parasites. 3D7 parasites were synchronized to the ring stage and incubated with a 1:2 dilution series of 2741-19 or vehicle controls for 72 h followed by quantification of parasitemia by flow cytometry using Hoechst staining (4a) and pLDH assay (4b). Experiments were performed in triplicate doses and error bars present represent SD. Results are representative of 3 independent experiments.

Next, the D10 strain was isolated in Papua New Guinea and was deposited as drug-sensitive. Treatment with compound 2741-19 resulted in ~70% killing and this strain also happens to have the highest IC_{50} across the five strains (Fig. 5a and 5b).



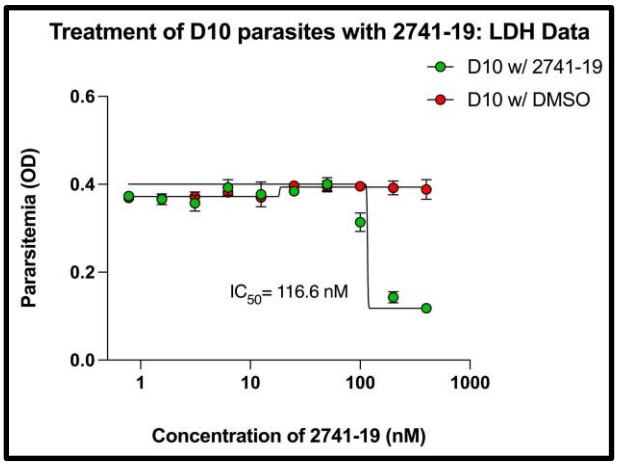
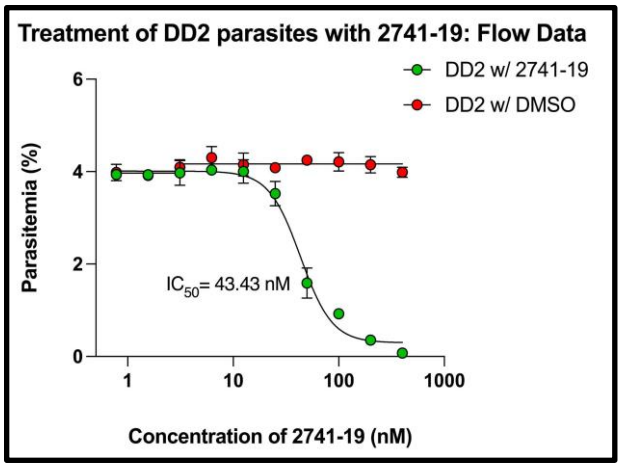


Fig. 5a **Fig. 5b**

Fig. 5a and 5b. 2741-19 kills D10 *P. falciparum* parasites. D10 parasites were synchronized to the ring stage and incubated with a 1:2 dilution series of 2741-19 or vehicle control for 72 h followed by quantification of parasitemia by flow cytometry using Hoechst staining (5a) and pLDH assay (5b). Experiments were performed in triplicate doses and error bars present represent SD. Results are representative of 3 independent experiments.

DD2 was an extremely crucial strain for us to incorporate into our in vitro studies as it is resistant to chloroquine, pyrimethamine, and mefloquine and is isolated from Indochina. Fig. 6a and 6b demonstrate that 2741-19 delivered the most consistent killing activity of ~95% and IC_{50} of 40-95 nM from experiment to experiment in the DD2 strain.



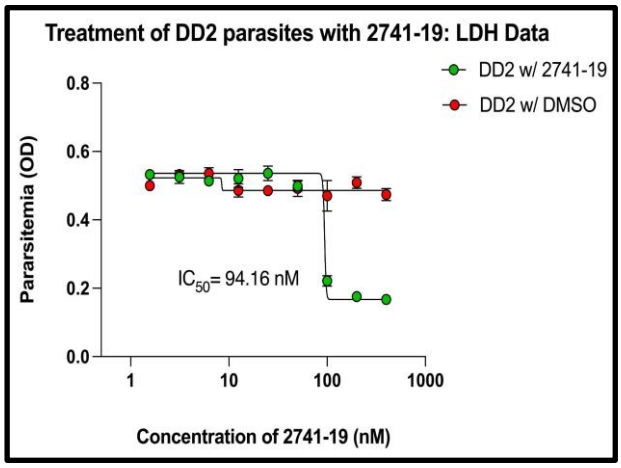


Fig. 6a **Fig.6b**

Fig. 6a and 6b. 2741-19 kills DD2 *P. falciparum* parasites. DD2 parasites were synchronized to the ring stage and incubated with a 1:2 dilution series of 2741-19 or vehicle control for 72 h followed by quantification of parasitemia by flow cytometry using Hoechst staining (6a) and pLDH assay (6b). Experiments were performed in triplicate doses and error bars present represent SD. Results are representative of 3 independent experiments.

The MRA-1250 (CamWT) strain was isolated in 2010 from a malaria patient in Western Cambodia and was deposited as artemisinin sensitive. MRA-1251, or CamWildType_C580Y, on the other hand, is a mutant of the MRA-1250 strain featuring only a single nucleotide substitution leading it to be resistant to artemisinin. 2741-19 kills ~90% of MRA-1250 parasites and ~85% of MRA-1251 parasites in culture.

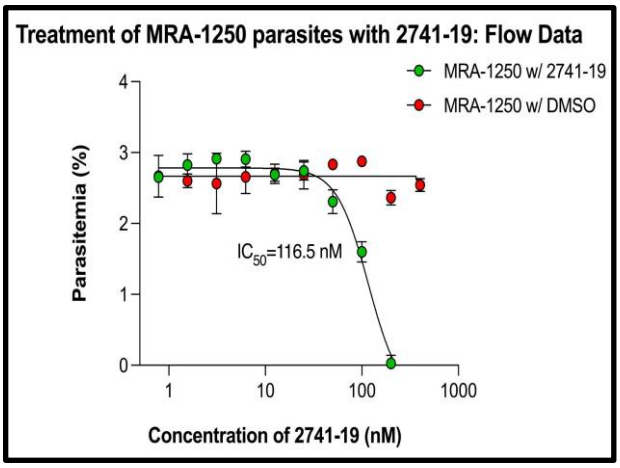


Fig. 7a

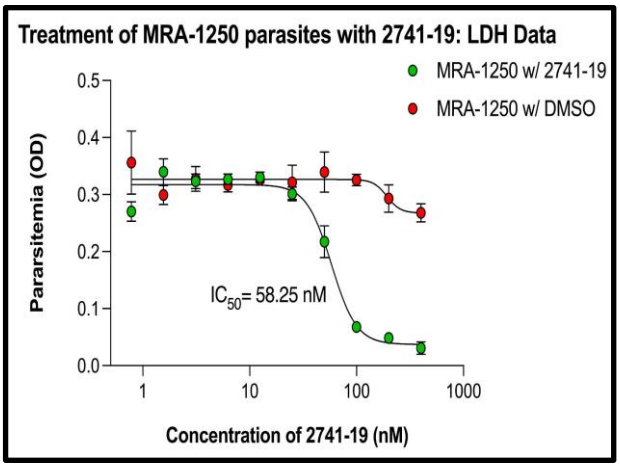


Fig.7b

Fig. 7a and 7b. 2741-19 kills MRA-1250 *P. falciparum* parasites. MRA-1250 parasites were synchronized to the ring stage and incubated with a 1:2 dilution series of 2741-19 or vehicle control for 72 h followed by quantification of parasitemia by flow cytometry using Hoechst staining (7a) and pLDH assay (7b). Experiments were performed in triplicate doses and error bars present represent SD. Results are representative of 3 independent experiments.

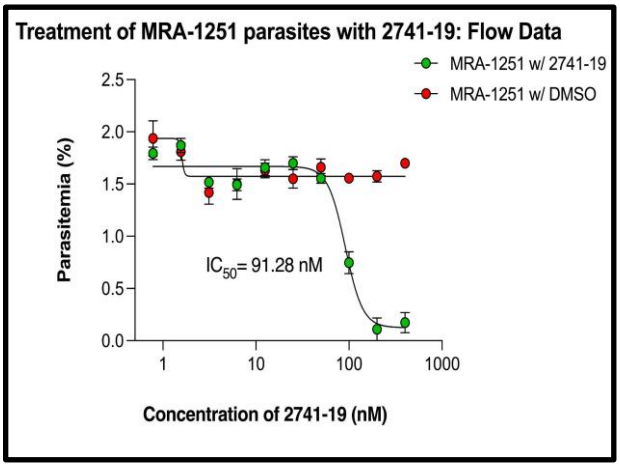


Fig. 8a

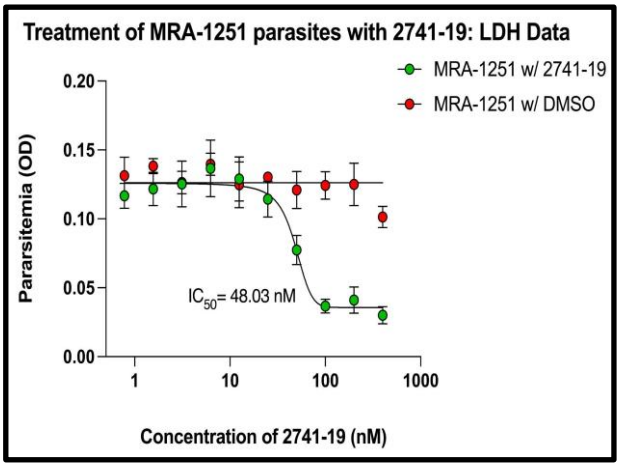
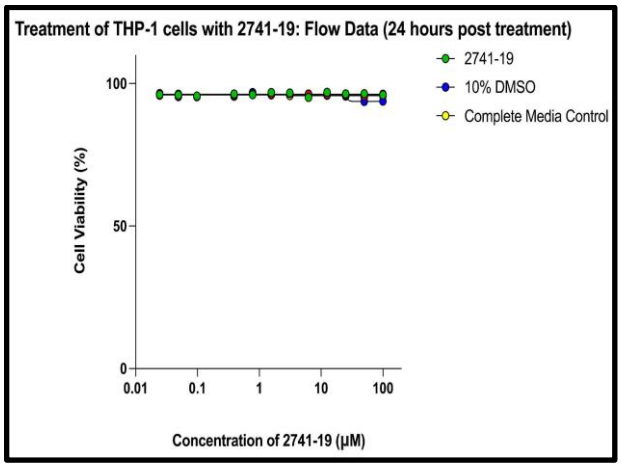


Fig. 8b

Fig 8a and 8b. 2741-19 kills MRA-1251 *P. falciparum* parasites. MRA-1251 parasites were synchronized to the ring stage and incubated with a 1:2 dilution series of compounds or vehicle control for 72 h followed by quantification of parasitemia by flow cytometry using Hoechst staining (8a) and pLDH assay (8b). Experiments were performed in triplicate doses and error bars present represent SD. Results are representative of 3 independent experiments.

The latter half of the results section will focus on cytotoxicity experiments that were performed using two mammalian cell lines- THP1 and Hep3B- to assess the toxicity of 2741-19 at a 100 µM concentration, which is approximately 1000x the IC_{50} that were observed across parasite strains in growth inhibition assays. THP-1 is a human leukemia monocytic cell line, which has been extensively used to study monocyte/macrophage functions, mechanisms, signaling pathways, and nutrient and drug transport. Cytotoxicity was assessed at 2 time points- 24 and 48 hours. The results revealed that compound 2741-19 was not toxic to THP-1 cells at both 24 and 48 hours post-treatment.



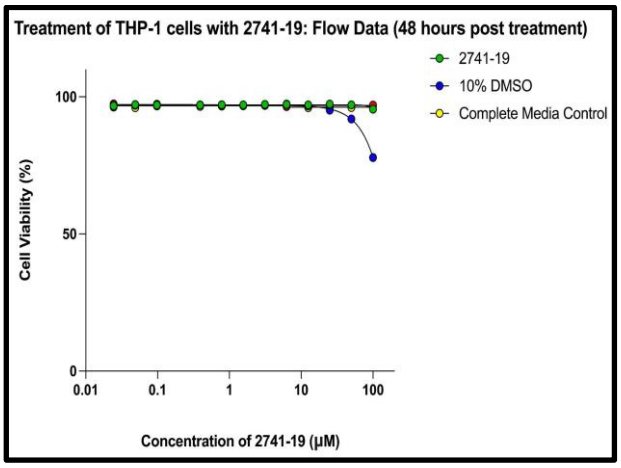


Fig. 9a **Fig. 9b**

Fig. 9a and 9b. THP-1 cells were plated at a density of 100,000 cells per well and treated with a 1:2 dilution series of compound 2741-19, vehicle control and 10% DMSO as a positive control for 24 h (Fig. 9a) and 48 h (Fig. 9b). The highest dose of 2741-19 that the cells received was 100 µM. Cytotoxicity was assessed using 7-AAD Staining. Data represent the mean ± SD of triplicate doses. Compound 2741-19 does not exhibit toxicity of THP-1 cells at 1000x the IC50 after 48 hours. Result representative of 2 independent experiments.

The Hep3B cell line was isolated from the liver tissues, namely human hepatocellular carcinoma. The liver plays a crucial role in drug metabolism, so using this cell can provide potential insights into the hepatotoxic effects of the compound, contributing to the safety assessment and overall drug development process. When Hep3B cells were treated with 2741-19 at a concentration that was 1000x the various IC_{50} that were observed across parasite strains in growth inhibition assays, the compound was revealed to be slightly toxic after 24 hours. Toxicity was more pronounced and substantial after 48 hours.

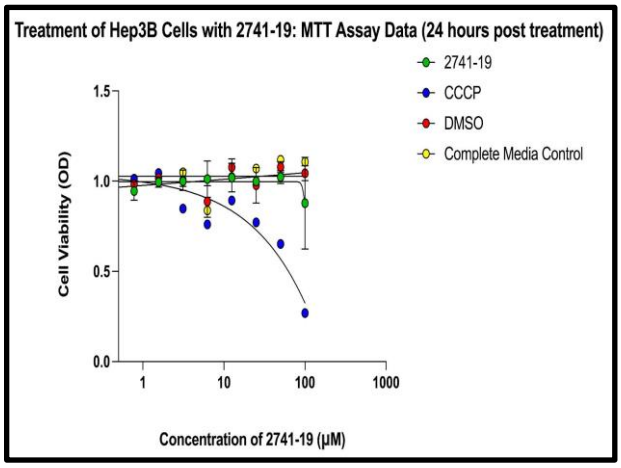


Fig. 10a

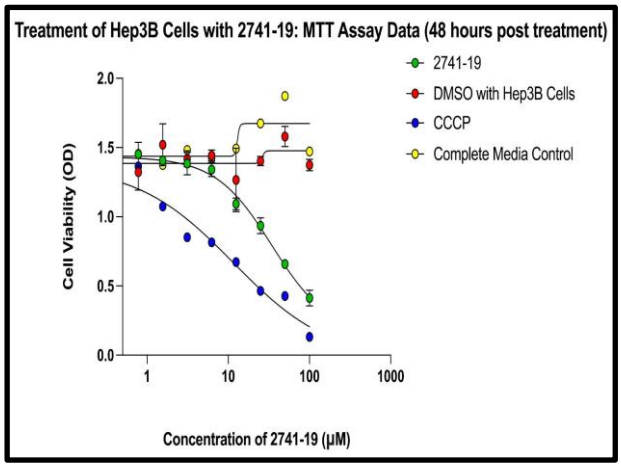


Fig. 10b

Fig. 10a and 10b. Hep3B cells were plated at a density of 20,000 cells per well and treated with 1:2 dilution series of compound 2741-19, vehicle control and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as a positive control. Cytotoxicity was assessed using an MTT assay at 24 h (Fig. 10a) and 48 h (Fig. 10b). Data represent the mean ± SD of triplicate measurements. 2741-19 exhibits toxicity in Hep3B cells at 1000x the IC50 and after 48 hours. Result representative of 2 independent experiments.

Chapter 4: Discussion

The findings presented in this study offer hope for broadening therapeutic options amidst escalating drug resistance. Since compound 2741-19 was efficacious in inhibiting parasite growth across different strains, it suggests that the compound is successful in targeting critical pathways that are essential to parasites' survival and replication within a host. As the studies further progress, it will be paramount to establish drug-resistance studies of 2741-19, to have a better understanding of the compound's potential limitations posed by parasites' resistance mechanisms. This can be done by continuously exposing *P. falciparum* cultures to increasing concentrations of 2741-19 and observing for genetic mutations and alterations in gene expression. Moreover, since the mechanism of action remains unclear, target identification should be prioritized through the use of biochemical and molecular biology techniques such as but not limited to affinity chromatography or activity-based proteomics to identify the molecular target(s) of 2741-19, but also compare it to the targets of known anti-malarials.

Combination therapies are at the forefront of available effective treatments against malaria and with monotherapies being advocated against, it will be interesting to pioneer drug combinations studies to investigate the potential synergistic or antagonistic interactions between 2741-19 and existing anti-malarials to determine if shared mechanisms of action and/or resistance are or will be at play.

This data bridges the gap between discovery and early stages of preclinical development and to ensure that progress ensues, the focus should be shifted towards establishing and validating a robust NSG/*P. falciparum* murine model. Validation will be in part dependent on gaining further

insight on absorption, distribution, metabolism, and excretion (ADME) and compound formulation optimization, so that not only appropriate dosing regimens are employed during in vivo studies. Next, toxicity data from THP-1 cells and Hep3B cell lines lays the foundation for future toxicological evaluations both in vitro and in vivo. However, as with all studies, it is important to mention limitations, such as but not limited to: Hep3B cell line is derived from hepatocellular carcinoma. Tumor-derived cell lines are inherently heterogeneous, meaning that they may not fully represent the complexity of normal liver tissue. This heterogeneity can complicate data interpretation and limit our ability to generalize findings. Additionally, these kinds of cells by the virtue of being cancer cell lines can present as more resilient. Therefore, it will be critical to continue the assessment of potential adverse effects and expand cytotoxicity studies to include more clinically relevant positive and negative controls. As for extrapolating in vitro toxicity data onto in vivo studies in NSG/*P. falciparum* murine model, compound 2741-19 should be compared to drugs that are known to be toxic and non-toxic for both humans and mice as well as those that are only toxic in either humans or mice. This will ensure that the animal model is as close as possible to being informative and predictive of the clinical settings.

The research and the efforts described in this study are a significant step toward the pursuit of novel antimalarial therapies, but they also undoubtedly emphasize the complexity of the disease pathophysiology and epidemiology. Continued research efforts, cross-sector partnerships, and continuous investment of the global health community into malaria research are essential to overcome the immense burden of malaria and defeat antimalarial drug resistance.

Conclusion

The burden of malaria remains a significant global health challenge as the current control and intervention strategies are rendered more and more helpless and less efficacious with the ever-growing and broadening drug resistance to include yet another anti-malarial. This study aims to contribute to the ongoing pursuit of the development of new anti-malarials.

While the mechanism of the action of compound 2741-19 is yet to be fully uncovered and understood, the results presented in this study show promising efficacy of a novel anti-malarial against multi-drug resistant *P. falciparum*. Compelling evidence stems from our in vitro studies extensively quantifying and validating the parasite-killing activity of our newly discovered compound in both drug-sensitive and drug-resistant parasite strains isolated from various endemic regions and demonstrated efficacy through reproducible IC_{50} in the nanomolar range. Furthermore, the attenuation of parasites' growth is complemented by data from our preliminary cytotoxicity studies using mammalian cell lines, suggestive of the compound's potentially favorable safety profile. These results ultimately lay the foundation and inform downstream drug development processes aimed at harnessing the compound's full therapeutic potential, including future in vivo studies using the NSG/*P. falciparum* murine model. With many more anti-malarials surrendering their efficacies to antimalarial drug resistance, global health progress against malaria has plateaued. We remain hopeful that the discovery of this novel compound is undoubtedly a great step towards the development of a new class of anti-malarials that is ever so needed.

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