CHARACTERIZATION OF THE NOVEL ANTIMALARIAL 2741-19: BARRIER TO RESISTANCE AND STAGE-SPECIFIC ACTIVITY

By

MICHAEL CHAN

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Thesis

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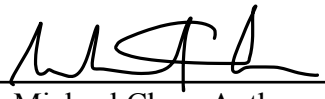
Date 01/16/2026 

Michael Chan, Author

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Date 01/16/2026 Signature: Jonathan Kurtis MD/PhD

Dr. Jonathan Kurtis, Advisor

Date 01/16/2026 Signature: Tanbir Najrana

Dr. Tanbir Najrana, Reader

Date 01/16/2026 Signature: Hannah Wu

Dr. Hannah Wu, Reader

Approved by the Graduate Council

Date _____ Signature: _____

Dr. David Lindstrom, Dean of the Graduate School

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Abstract of "Characterization of the Novel Antimalarial 2741-19: Barrier to Resistance and Stage-Specific Activity", by Michael Chan, ScM, Brown University, February 2026

Malaria imposes an immense global health burden, and remains a significant driver of child mortality in sub-Saharan Africa. The emergence and spread of parasite strains resistant to artemisinin derivatives, the global first-line therapy, presents an imminent threat to public health. New antimalarials with novel mechanisms of action are desperately needed. This thesis details two preclinical studies supporting the development of 2741-19, a small-molecule drug discovered within the Kurtis Laboratory at the Center for International Health Research at Brown University. The compound is believed to target PfGARP, a parasite antigen expressed on the exofacial surface of infected trophozoite-stage erythrocytes.

An *in vitro* selection for resistance study was performed to characterize the barrier to resistance of 2741-19 by establishing the minimum inoculum for resistance (MIR). Atovaquone was included as a positive control. Cultures of the Dd2 strain were seeded in triplicate with an inoculum of 3.85×10^9 parasites and maintained under continuous drug pressure at $3 \times IC_{50}$ until recrudescence or for a maximum of 60 days. Selection for resistance to atovaquone was achieved, with recrudescence in all three replicates on day 28. Atovaquone-selected lineages exhibited a 10.5-fold increase in IC_{50} relative to the parental strain, validating the experimental design. In contrast, selection for resistance to 2741-19 yielded no resistant parasites, with no recrudescence observed through day 60. These results establish an MIR of > 9 for 2741-19, indicating an exceptionally high genetic barrier to resistance, comparable to chloroquine and artemisinin.

Modified growth inhibition assays were also performed to characterize the compound's pharmacodynamic profile. 2741-19 exhibited trophozoite-specific killing, which is consistent with PfGARP-mediated toxicity, the hypothesized mechanism of action. Maximum growth

inhibition required slightly over 12 h of exposure. This demonstrates strong compatibility with the established 32–38 h elimination half-life, and reinforces the compound's clinical viability as a prophylactic and therapeutic agent.

Collectively, these findings position 2741-19 as a high-priority candidate for next-generation antimalarial development, driven by its robust genetic barrier to resistance and favorable pharmacodynamic profile.

Chapter 1: Introduction

Malaria continues to impose an immense global health burden. The disease is caused by an infection of protozoan parasites of the *Plasmodium* genus. There are five species that infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*, but severe manifestations of the disease are almost entirely attributed to *P. falciparum*.[1] Parasites are transmitted by infected female *Anopheles* mosquitoes.

The World Health Organization (WHO) estimates that in 2024, there were 282 million cases and 610,000 fatalities across the world's 80 malaria-endemic countries.[2] This burden is predominantly concentrated in sub-Saharan Africa, which accounts for over 95% of global cases and deaths. The latest Global Burden of Disease study (GBD 2023) indicates that malaria has the greatest global health burden of any single-agent pathogenic disease, as measured in disability-adjusted life years (DALYs).[3] At 52 million, its impact surpasses that of HIV/AIDS (45 million) and tuberculosis (44 million). This substantial burden is primarily driven by the disease's high child mortality rate; children under five years old account for 75% of fatalities in Africa.[2] Other vulnerable demographics of malaria infection are pregnant women and girls, fetuses, and newborns. Malaria parasites preferentially sequester in the placenta, inducing maternal anemia which severely worsens pregnancy outcomes. Mothers face an elevated risk of mortality, and increased incidence of adverse outcomes like stillbirth and preterm birth. Surviving offspring frequently suffer from impaired cognitive development and stunted growth.[2]

The impact of malaria on Africa's economic development cannot be understated. A highly-cited study from Harvard's Center of International Development quantified the economic impact of malaria between 1965–1990.[4] The economic growth of 75 countries with varying malaria burdens were compared, controlling for socioeconomic and geographic factors including

poverty level, quality of schooling, access to healthcare, economic policies, colonial history, climate, and coastal access. All else equal, countries with the highest possible malaria burden experienced 1.3% lower annual growth in GDP per capita compared to malaria-free countries. The maximum burden was defined as a scenario in which a country's entire population resides in an area of stable malaria transmission, and all malaria cases are attributable to *P. falciparum*. When extrapolated over a century, this 1.3% growth gap implies that after 100 years, a country facing the maximum malaria burden would have a GDP per capita just 27% of an otherwise identical malaria-free country. The control and eventual eradication of malaria in Africa is crucial for lifting its rural communities out of poverty and unlocking their economic potential.

1.1 Pathophysiology and Clinical Manifestations of Malaria

Clinical malaria is broadly classified as either uncomplicated or severe, based on the symptoms presented. Uncomplicated malaria is characterized by non-specific flu-like symptoms, such as fever, chills or rigors, headaches, fatigue, nausea, vomiting, and muscle pains.[1,5] Progression to severe malaria is defined by the emergence of any of the following severity markers: impaired consciousness, acidosis, prostration, hypoglycemia, renal impairment, jaundice, pulmonary edema, significant bleeding, repeated seizures, shock, or hyperparasitemia.[6] Severe malaria is almost exclusively attributed to *P. falciparum* infection, and if left untreated, has a 90% fatality rate.

The severity markers that define severe malaria frequently arise from two primary clinical manifestations: severe malarial anemia and cerebral malaria. Severe malarial anemia is characterized by systemic hypoxia caused by massive hemolysis.[7] Malarial infection can induce clearance of both infected and uninfected erythrocytes in the spleen, whilst suppressing the

production of new erythrocytes in the bone marrow. The depleted erythrocyte counts prevent adequate oxygen transport, triggering systemic tissue hypoxia and metabolic acidosis that can progress to multi-organ failure and death. It is diagnosed by low hemoglobin (<5 g/dL in children, <7 g/dL in adults) or low hematocrit (<15% in children, <20% in adults).[7]

Cerebral malaria is characterized by impaired consciousness caused by the sequestration of parasites in the brain microvasculature. Infected erythrocytes can cytoadhere to the vascular endothelium within the brain, creating blockages that impede blood flow and cause localized hypoxia. This triggers a neuroinflammatory response that compromises the blood-brain barrier. The subsequent vascular leakage can cause cerebral edema and increased intracranial pressure, inducing neurological collapse that leads to coma and death. It is diagnosed by a coma that persists for >1 h after a seizure (Blantyre coma score <3 in children, Glasgow coma score <11 in adults).[5]

Cerebral malaria is considered the most serious and lethal complication of severe malaria, carrying a poor prognosis with a fatality rate 2 to 5 times higher than that of severe malarial anemia.[8] While patients with severe malarial anemia are typically very responsive to blood transfusions and antimalarial drugs, patients with cerebral malaria are not, as parasite clearance does not directly reverse the presented disease, and neurological damage is less reversible than other organ impairments. Furthermore, survivors of cerebral malaria also experience a higher incidence of long-term neurological deficits and cognitive impairment.[8]

1.2 Rising Threat of Artemisinin Resistance

Artemisinin-based combination therapies (ACTs) have been the first-line therapies around the world since the early 2000s due to their exquisite potency and rapid action.[9]

Artemisinin derivatives can reduce parasite biomass by 10,000-fold in a single 48 h intraerythrocytic cycle.[10] However, the emergence and spread of artemisinin partial resistance (ART-R) is an urgent concern, with models predicting that widespread resistance could cause over 100,000 additional deaths per year.[9]

The ART-R phenotype is conferred by various mutations in the Kelch13 (K13) gene, and is characterized by delayed clearance of parasites after treatment. Genomic surveillance has identified at least six distinct K13 mutations that have independently emerged in East Africa.[10] This represents a shift from historical trends; the mutations that conferred resistance to chloroquine and sulfadoxine-pyrimethamine were imported into Africa from Southeast Asia.

The emergence of ART-R in Africa can be largely attributed to improper drug use.[10] Specifically, the unendorsed use of artemisinin as a monotherapy. Combination therapies are particularly important for artemisinin derivatives. While artemisinins kill parasites quickly, they possess a short half-life. Partner drugs with longer half-lives are required to clear any residual parasites. The standard of care for uncomplicated malaria is a full ACT course, while the standard of care for severe malaria is intravenous artesunate (monotherapy) followed by a full ACT course. In practice, however, intravenous artesunate is commonly administered for the treatment of both uncomplicated and severe malaria without the subsequent ACT course, resulting in incomplete treatment, and providing the perfect environment for the selection of artemisinin resistance.[10]

In Southeast Asia, where ART-R was first reported in 2008, the prevalence of K13 mutations has become so high that ACTs are effectively functioning as monotherapies.[10] This has accelerated the selection of resistance to partner drugs like mefloquine, piperaquine, and amodiaquine. Consequently, treatment failure has been observed for artesunate-mefloquine,

dihydroartemisinin-piperaquine, and artesunate-amodiaquine.[10] These treatment failures are relatively manageable in the low-transmission regions of Southeast Asia, where low patient volumes permit close monitoring. However, widespread treatment failures in the high-transmission areas of sub-Saharan Africa would be catastrophic. The development of new antimalarials with novel mechanisms of action is desperately needed before ART-R becomes firmly established across Africa.

1.3 Origin and Development of 2741-19

The Kurtis Laboratory at the Center for International Health Research at Brown University identified PfGARP as a novel vaccine candidate and drug target through differential screening of pooled plasma collected from their Tanzanian birth cohort.[11] PfGARP is a parasite antigen expressed on the exofacial surface of infected erythrocytes during the trophozoite stage. Differential biopanning was performed on a blood-stage T7 phage display cDNA library, probing the library with plasma from children who were the most-resistant to high parasitemia versus those who were the most-susceptible. This process identified 11 parasite genes uniquely recognized by antibodies in the resistant group, with PfGARP emerging as the most highly enriched.[11]

Subsequent experiments discovered that the binding of anti-PfGARP antibodies kills parasites by inducing programmed cell death, as established by multiple cellular signatures including the activation of caspase-like proteases, fragmentation of parasite DNA, and the loss of mitochondrial membrane potential and food vacuole integrity. The clinical significance of PfGARP was validated in a longitudinal study of the entire Tanzanian birth cohort, in which children lacking anti-PfGARP antibodies had 2.5-fold higher risk of severe malaria.[11]

The Kurtis Lab sought to develop a drug that could kill parasites through PfGARP-binding, and performed a high-throughput screen of 30 million small-molecule compounds from the Torrey Pines Combinatorial Library. Scaffolds that inhibited the binding of polyclonal anti-PfGARP antibodies to PfGARP were identified, and sub-libraries of those scaffolds were screened for killing activity. The scaffold with the highest-potency killers was identified. The group contained 36 active compounds, and the compound with the lowest IC_{50}, 2741-19, was selected as the lead candidate (Figure 1).

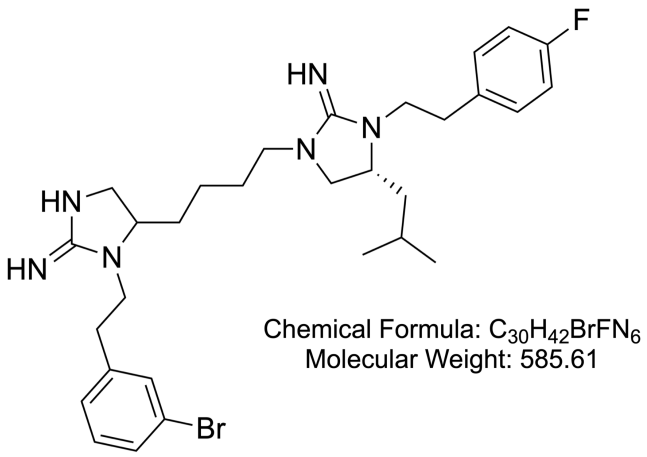

Figure 1. Structure of 2741-19.

1.4 Study Rationale and Experimental Objectives

Current antimalarial development guidelines emphasize the importance of assessing resistance risks early in preclinical development to deprioritize any high-risk compounds.[12,13] This involves both cross-resistance studies and *in vitro* selection for resistance studies. Cross-resistance studies determine whether the candidate drug has reduced potency against strains resistant to current antimalarials. The Kurtis Lab has challenged Dd2 (chloroquine, sulfadoxine, pyrimethamine, cycloguanil, and mefloquine-resistant) and MRA-1251

(artemisinin-resistant) with 2741-19, and found no difference in potency compared to drug-sensitive strains. This lack of cross-resistance suggests 2741-19 operates through a novel mechanism of action.

To build on this work, I performed an *in vitro* selection for resistance study to characterize the barrier to resistance of 2741-19. The primary objective of this experiment was to determine the minimum inoculum for resistance (MIR): the number of parasites required to reliably select for resistance *in vitro*, reported as a log10.[12] This value serves as a critical proxy for how quickly resistance will emerge in the clinic. This experiment would enable us to determine whether to continue development of this specific compound or pivot to other candidates, as a high barrier to resistance is a critical attribute for next-generation antimalarials. Successful selection of 2741-19 resistant strains would enable the characterization of the molecular basis of resistance through whole genome sequencing, fitness cost assays, and cross-resistance assays, which could inform modifications that improve the drug's efficacy.[12]

In addition to characterizing the resistance profile, I performed growth inhibition assays (GIAs) that characterized the pharmacodynamic profile of 2741-19. Prior GIAs in the Kurtis Lab have demonstrated that the binding of anti-PfGARP antibodies induces definitive early markers of programmed cell death within 12-24 h of treatment.[11] The objective of this experiment was to determine whether parasite killing induced by 2741-19 is consistent with PfGARP binding, and to define the minimum duration of exposure required for the maximum killing effect, a critical intrinsic pharmacodynamic property of the drug. This duration directly informs the pharmacokinetic parameters, notably half-life and dosing frequency, that are required for clinical viability. The results could indicate whether the drug's intrinsic profile aligns with the intended target product profile, or whether new delivery systems should be considered.

Chapter 2: Methods

2.1 *In vitro* Selection for Resistance

The protocol for this experiment was derived from the standard *in vitro* selection for resistance methods published by Ng and Fidock.[14]

Parasites were cultured in RPMI 1640 medium containing GlutaMAX and 25 mM HEPES (Gibco Cat# 72400-047), supplemented with 0.5% (w/v) AlbuMAX II (Gibco Cat# 11021-037), 100 µM hypoxanthine and 16 µM thymidine (Gibco Cat# 11067-030), 25 µg/mL gentamicin (Gibco Cat# 15710-064), and human O+ erythrocytes (Rhode Island Blood Center). Cultures were gassed with a mixture of 5% CO_2, 5% O_2, and 90% N_2, and incubated at 37 ºC.

3D7 and Dd2 *P. falciparum* strains were obtained from BEI Resources, NIAID, NIH. The Dd2 clone is the recommended strain for this assay due to its accelerated resistance to multiple drugs (ARMD) phenotype. The strain has defective DNA mismatch repair mechanisms that increase the frequency of mutations and its ability to select for resistance.[15] It is resistant to chloroquine, sulfadoxine, pyrimethamine, cycloguanil, and mefloquine.[13]

Stock solutions of 100 mM 2741-19 and 136.5 µM atovaquone (Sigma Cat# A7986) were prepared in DMSO and stored at –80 ºC and –20 ºC, respectively. Atovaquone was selected as the positive control for this assay due to its known low genetic barrier to resistance. It has an MIR of 5–8, depending on factors such as strain and drug pressure.

GIAs were performed to determine the IC_{50} of 2741-19 and atovaquone against Dd2. Additionally, 2741-19 was tested against the drug-sensitive 3D7 strain to evaluate potential cross-resistance or collateral sensitivity. The protocol for GIAs is described in Section 2.2.

Over a one-week period, the Dd2 culture was expanded to a population of 2.31×10^{10} parasites (5.05% parasitemia, 1.25% hematocrit). The culture was confirmed to be majority

ring-stage via blood film. Complete culture media supplemented with 3 × IC_{50} of either 2741-19 or atovaquone was prepared in bulk. Final DMSO concentrations were negligible (< 0.001%). Six T225 flasks were prepared, each with 3.85×10^9 parasites (2.50% parasitemia, 4.00% hematocrit, 295 mL of drug-supplemented media), yielding three replicates per drug. This study prioritized screening an inoculum of 10^9 parasites, the maximum feasible inoculum in a laboratory setting. If a resistant mutant was selected, the experiment would be expanded to include inocula of 10^7 and 10^8 parasites to accurately characterize the MIR.

Drug pressure was maintained throughout the assay with thrice-weekly media changes. Cultures were centrifuged (4000 × g for 8 mins) in 50 mL conical tubes, supernatants were aspirated to a residual volume of 2 mL, and fresh drug-supplemented media was added. As described by Ng and Fidock, beginning on day 7, fresh erythrocytes were added weekly to ensure sufficient supply of viable erythrocytes, and beginning on day 14, the culture volume was reduced weekly.[14] A detailed schedule of specific erythrocyte and media volume adjustments is provided in Table 1.

Giemsa-stained blood films were prepared at every media change to estimate parasitemia and parasite viability. Blood films were scanned and analyzed with the CellaVision automated microscopy system, which classifies infected and uninfected erythrocytes. All infected erythrocytes were manually verified. Parasitemia values were only quantified beginning on day 14, as drug-induced crisis forms precluded reliable classification during the initial clearance phase.

Table 1. Erythrocyte and media volume adjustments as described by Ng and Fidock.[14]
On day 7, fresh RBCs equivalent to an additional 0.5% hematocrit were added. On day 14 and weekly thereafter, total culture volume was reduced by 25%, and fresh RBCs equivalent to 1% of the new total culture volume were added. T225 flasks were used until day 21. T175 flasks were used until day 49. T75 flasks were used until day 60. **RBCs**: Red Blood Cells in culture; **uRBCs**: fresh uninfected Red Blood Cells; **CM**: Complete Media; **V**: Total Culture Volume; **HC**: Hematocrit.

Day	RBCs (mL)	uRBCs (mL)	CM (mL)	V (mL)	HC (%)
1–6	12.308		295.39	307.70	4.00
7		+1.61			
7–13	13.92		295.39	309.31	4.50
14	–6.96	+2.32	–72.69		
14–20	9.28		222.70	231.98	4.00
21	–4.64	+1.74	–55.10		
21–27	6.38		167.61	173.99	3.67
28	–3.19	+1.30	–41.61		
28–34	4.49		126.00	130.49	3.44
35	–2.25	+0.98	–31.35		
35–41	3.23		94.64	97.87	3.30
42	–1.61	+0.73	–23.59		
42–48	2.35		71.05	73.40	3.20
49	–1.17	+0.55	–17.73		
49–55	1.72		53.33	55.05	3.13
56	–0.86	+0.50	–4.69		
56–60	1.36		48.64	50.00	2.72

Selection was considered successful if recrudescent parasites emerged. Cultures were removed from the study protocol upon reaching 1–2% parasitemia, and prepared for GIAs whilst under continuous drug pressure. GIAs quantified the shift in IC_{50} values between the resistant strains and the parent strain in parallel. The resistant strains were also cryopreserved at –80 ºC. Cultures were maintained for a maximum of 60 days. If recrudescence was not observed through day 60, the selection was deemed unsuccessful. To confirm the absence of viable parasites, a minimum of 80,000 cells per replicate were examined before the cultures were discarded.

2.2 Standard Growth Inhibition Assays

The Dd2 (or 3D7) culture was synchronized twice with 5% D-sorbitol, with parasitemia maintained < 5% throughout the two replication cycles. The assay was performed in a sterile 96-well round-bottom culture-treated polystyrene plate. Media of various drug concentrations were prepared via 1:1 serial dilution directly in the plate. As the drugs were solubilized in DMSO, media with the corresponding DMSO concentrations were prepared in separate wells as vehicle controls. The synchronized ring-stage parasites were diluted and added to each well, such that each well contained 0.4% parasitemia, 1% hematocrit, and a final volume of 150 µL. Each condition was performed in triplicate. The plates were placed in an incubator chamber, gassed with a mixture of 5% CO_2, 5% O_2, and 90% N_2, and incubated at 37 ºC for 66-72 h.

Following the incubation period, parasitemia was quantified via flow cytometry. Aliquots were transferred to a new plate, stained with 4 µM Hoechst solution in PBS, incubated for 30 minutes in the dark, and then washed with PBS. Fluorescence at 450 nm was measured with a CytoFLEX (Beckman Coulter) flow cytometer; since mature erythrocytes lack DNA, only infected erythrocytes fluoresce. Parasitemia values were exported to GraphPad Prism, and the IC_{50} was calculated with a non-linear regression model. Blood films were prepared at select drug concentrations to confirm the flow cytometry results.

2.3 Stage-Specific Exposure Growth Inhibition Assays

Modified GIAs were performed using synchronized early-ring stage and early-trophozoite stage 3D7 parasites at a predetermined drug concentration: the lowest concentration that yields the maximum killing effect in a standard 66–72 h GIA. Initial parasitemia, hematocrit, total volume, and gas mixture were as described for the standard GIA.

Parasites were exposed to 2741-19 for durations of 1, 4, 8, or 12 h, or for the full incubation period. For the shorter exposure intervals (≤ 12 h), drug pressure was removed, and parasitemia was quantified after the full incubation period. This provided sufficient time for all parasites that had committed to programmed cell death to complete the apoptotic process. For samples treated at the early-ring stage, the incubation period was the standard 66–72 h. For samples treated at the early-trophozoite stage, the incubation period was shortened to 50–56 h. This aligned the life stages of both groups at the endpoint and enabled a direct comparison of the parasitemia values.

To remove drug pressure, the 150 µL samples were transferred to Eppendorf tubes, washed with 1 mL of complete media three times, and transferred to new wells. As described for the standard GIA, parasitemia was quantified via flow cytometry, the IC_{50} was calculated with a non-linear regression model, and blood films were prepared to confirm the flow cytometry results. Each condition was performed in triplicate.

Chapter 3: Results

3.1 *In Vitro* Selection for Resistance Did Not Yield 2741-19 Resistant Parasites

GIAs were performed to assess the baseline sensitivity of the parent *P. falciparum* Dd2 strain to 2741-19, and the positive control drug atovaquone. 2741-19 exhibited potent antimalarial activity in the nanomolar range, with an IC_{50} of 166 nM (Figure 2A). Atovaquone also exhibited potent antimalarial activity, with an IC_{50} of 0.215 nM (Figure 2B). In addition, 2741-19 exhibited similar potency in the drug-sensitive 3D7 strain, with an IC_{50} of 189 nM (Figure 6A). There were thus no concerns with potential cross-resistance or collateral sensitivity.

An *in vitro* selection for resistance assay was performed to evaluate the genetic barrier to resistance to 2741-19. Cultures were seeded in triplicate with an inoculum of 3.85×10^9 parasites and maintained under continuous drug pressure at $3 \times IC_{50}$ (500 nM for 2741-19; 0.534 nM for atovaquone) until recrudescence or for a maximum of 60 days.

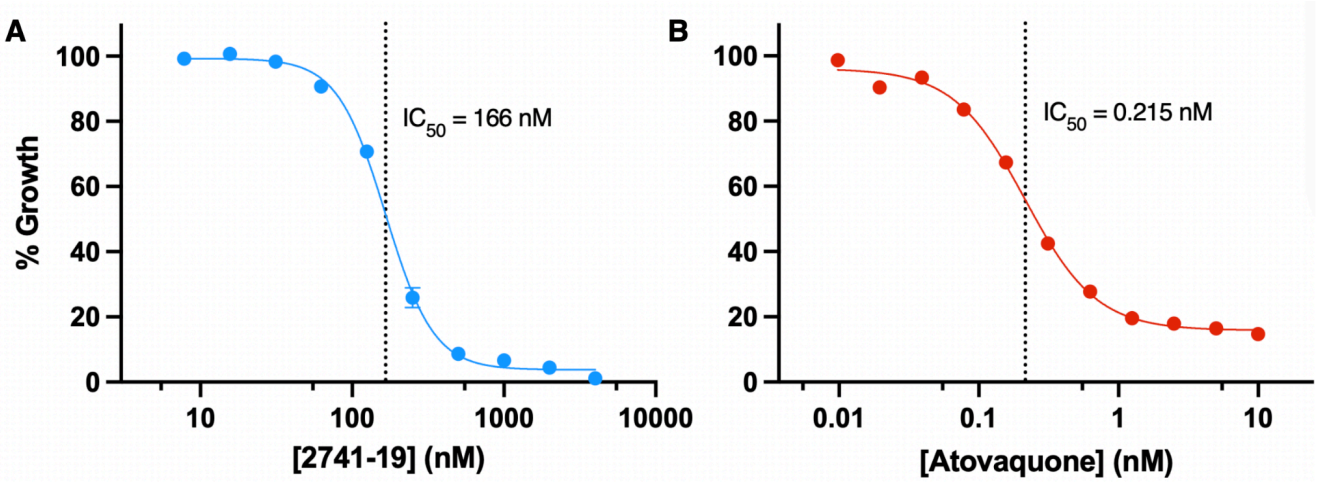

Figure 2. *In vitro* susceptibility of parental Dd2 strain to 2741-19 and atovaquone. Dose-response curves were generated from a 72 h growth inhibition assay. Ring-stage Dd2 parasites were exposed to serial dilutions of 2741-19 **(A)** or atovaquone **(B)**. Parasitemia was quantified by Hoechst fluorescence via flow cytometry and normalized to DMSO-treated controls. Data represent mean ± SEM (n = 3). Curves were fitted using a non-linear regression (four-parameter logistic) model to calculate the half-maximal inhibitory concentration (IC_{50}).

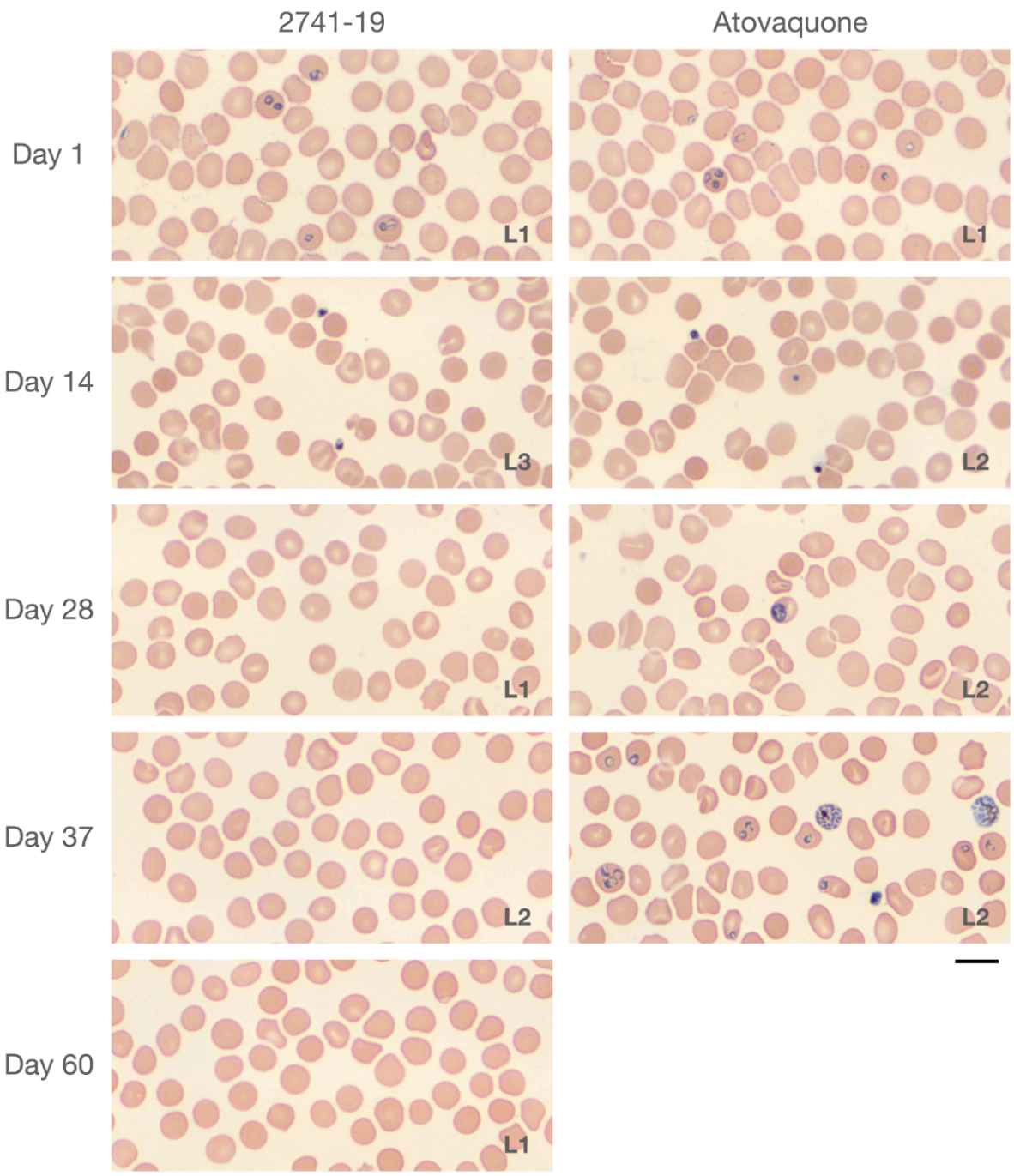

Figure 3. Blood films demonstrate recrudescence solely in atovaquone treatments. Representative Giemsa-stained blood films show the progression of Dd2 cultures maintained under continuous pressure of 3 × IC_{50} of 2741-19 (left panels) or atovaquone (right panels). **Day 1:** Healthy ring-stage parasites before introduction of drug pressure. **Day 14:** Absence of viable parasites, only pyknotic forms visible. **Day 28:** Recrudescence observed in atovaquone-treated cultures. 2741-19 treated cultures remain devoid of parasites. **Day 37:** High parasitemia in atovaquone-treated cultures, with healthy parasites of all intraerythrocytic stages. 2741-19 treated cultures remain devoid of parasites. **Day 60:** No late-stage recrudescence in 2741-19 treated cultures. Labels L1–L3 indicate the specific biological replicate from which the image was captured. Scale bar represents 10 µm.

Upon the introduction of drug pressure, rapid parasite death occurred in all cultures, as evidenced by an abundance of crisis or pyknotic forms on blood films (Figures 3 and 4). Since these dying parasites confounded reliable classification of viable and non-viable forms, the quantification of parasitemia was deferred to day 14, by which point the parasitemia in all cultures had fallen below the limit of detection (1 parasite per 30,000 erythrocytes = 0.0033%; Figures 3 and 4).

Recrudescence was observed in all three atovaquone replicates on day 28, and the cultures expanded to 1–2% parasitemia by days 36–37 (Figures 3 and 4). Selection for atovaquone-resistant parasites was deemed successful, and the cultures were removed from the study protocol and prepared for GIAs. In contrast, recrudescence was not observed in any of the three 2741-19 replicates through day 60, and the selection for 2741-19 resistant parasites was deemed unsuccessful (Figures 3 and 4).

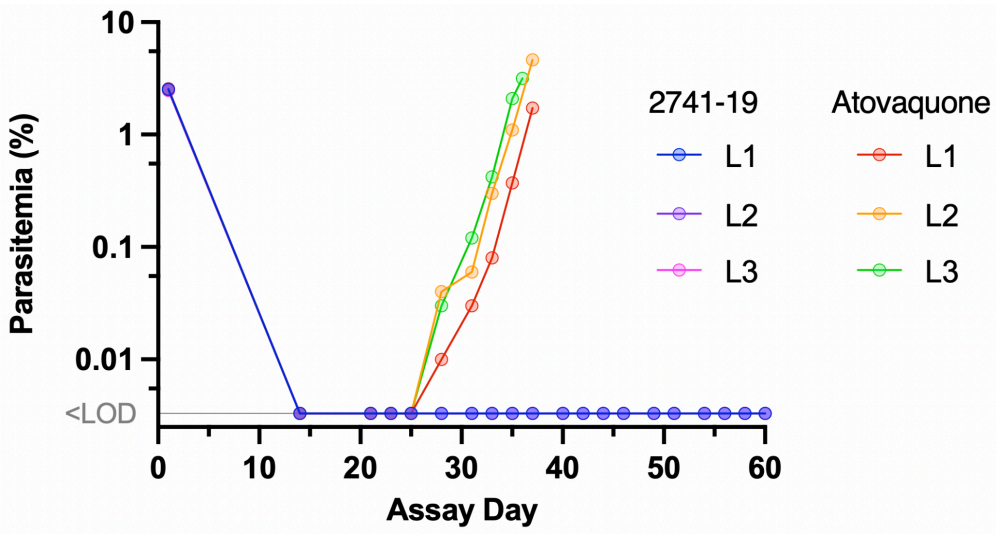

Figure 4. Parasitemia quantified from blood films demonstrates recrudescence solely in atovaquone treatments. Dd2 cultures were maintained in triplicate under continuous pressure of 3 × IC_{50} of 2741-19 (blue/purple/pink) or atovaquone (red/orange/green) for up to 60 days. Labels L1–L3 indicate the specific biological replicate. Parasitemia was monitored via microscopy. A minimum of 30,000 cells were scanned per replicate; the gray horizontal line indicates the limit of detection (LOD, 0.0033%). All three atovaquone-treated replicates exhibited recrudescence on day 28, while none of the three 2741-19 treated replicates exhibited recrudescence by day 60.

To confirm the atovaquone-resistant phenotype, GIAs were performed in parallel on the parent Dd2 strain and the recrudescent lineages. The mean IC_{50} of the atovaquone-selected strains was 10.5-fold higher than the parent strain (Figure 5). This shift confirms the successful selection of resistant strains and validates the effectiveness of the protocol. The absence of recrudescence in the 2741-19 replicates can thus be confidently attributed to a high barrier to resistance rather than experimental failure. Based on these results, the MIR for 2741-19 is > 9, and the MIR for atovaquone is ≤ 9.

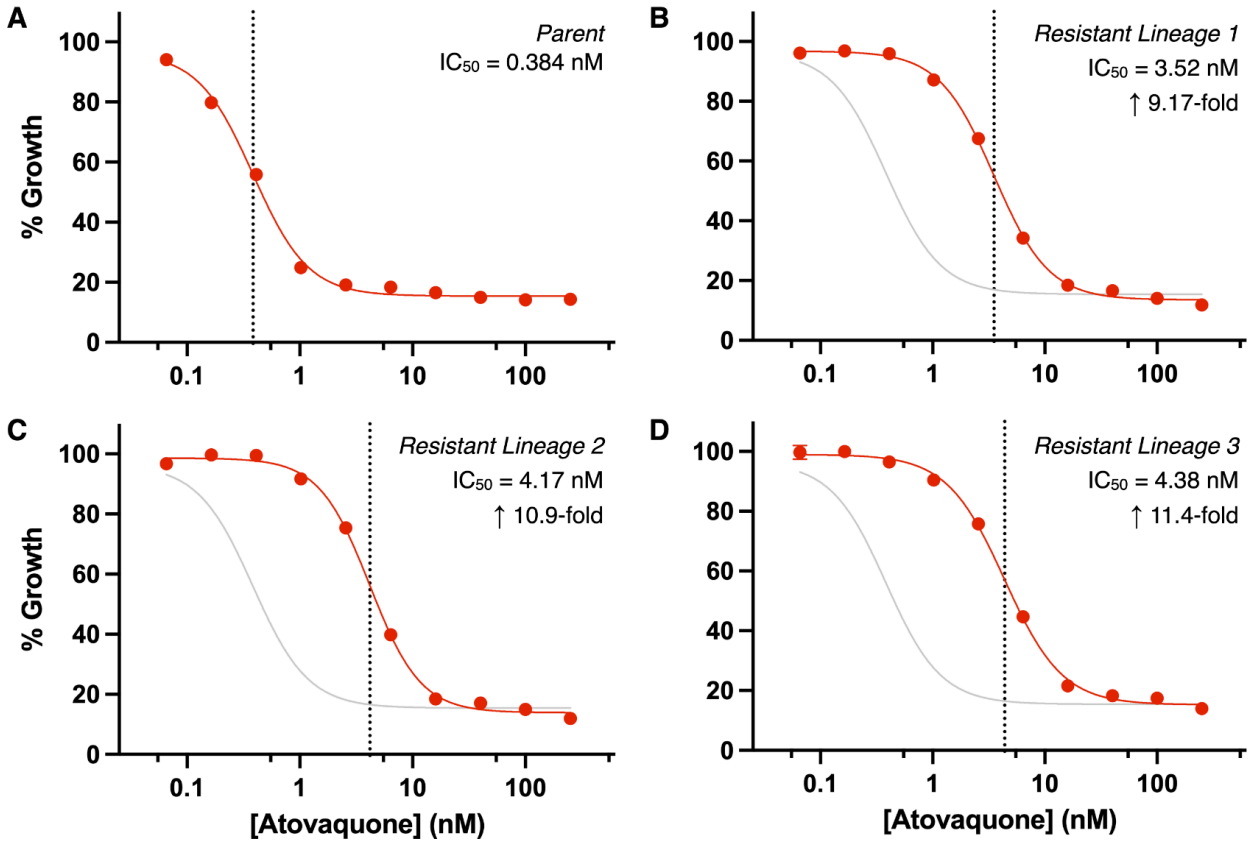

Figure 5. Atovaquone-selected Dd2 lineages exhibit a 10.5-fold increase in IC_{50} relative to the parent strain. Dose-response curves were generated from a 72 h growth inhibition assay. Ring-stage parasites of the parent strain **(A)** and resistant lineages **(B–D)** were exposed to serial dilutions of atovaquone. For reference, the parental dose-response curve is overlaid in gray in panels B–D. Parasitemia was quantified by Hoechst fluorescence via flow cytometry and normalized to DMSO-treated controls. Data represent mean ± SEM (*n* = 3). Curves were fitted using a non-linear regression (four-parameter logistic) model to calculate the half-maximal inhibitory concentration (IC_{50}).

3.2 Killing Activity of 2741-19 is Specific to the Trophozoite Stage

A GIA was performed to assess the sensitivity of the 3D7 strain to 2741-19, to determine the appropriate concentration for the stage-specific exposure studies. 2741-19 exhibited potent antimalarial activity, with an IC_{50} of 189 nM (Figure 6A). The dose-response curve indicated that 500 nM was the lowest drug concentration that yielded near-maximal growth inhibition, and was thus selected as the working concentration for these assays.

GIAs were performed to evaluate the stage-specificity of the 2741-19 killing effect, and determine the minimum duration of 2741-19 binding required to induce irreversible parasite death. Parasites were exposed to the drug for durations of 1, 4, 8, or 12 h, or for the full incubation period (69 h for early rings, and 54 h for early trophozoites).

At 500 nM, the killing effect of 2741-19 was specific to the trophozoite stage (Figure 6B). Early trophozoites showed significant growth inhibition after 8 h of exposure, though durations exceeding 12 h were required to achieve maximal growth inhibition. In contrast, ring-stage parasites demonstrated no sensitivity to the drug, tolerating exposures of up to 12 h without meaningful growth inhibition. The incomplete clearance of early-trophozoite parasites after 54 h of exposure indicates that parasites may be particularly susceptible during the ring-to-trophozoite transition.

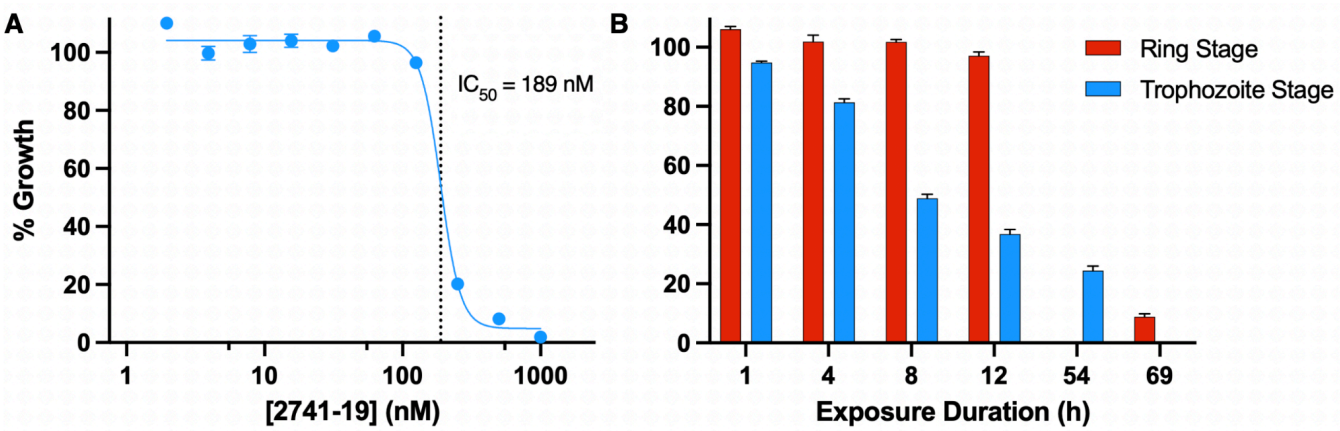

Figure 6. Killing activity of 2741-19 is specific to the trophozoite stage. (A) A dose-response curve was generated from a 72 h growth inhibition assay. Ring-stage 3D7 parasites were exposed to serial dilutions of 2741-19. Data represent mean ± SEM (*n* = 3 replicates), and are representative of three independent experiments. The curve was fitted using a non-linear regression (four-parameter logistic) model to calculate the half-maximal inhibitory concentration (IC_{50}). **(B)** Percent growth data were generated from a timed-exposure growth inhibition assay. Early rings and early trophozoites were exposed to 500 nM of 2741-19 for various durations. Early rings were insensitive to treatment. Early trophozoites showed significant growth inhibition after 8 h of exposure, though over 12 h of exposure was required to achieve maximal growth inhibition. Data represent mean ± SEM (*n* = 3 replicates), and are representative of three independent experiments.

Chapter 4: Discussion and Conclusions

This study demonstrates that 2741-19 has a high barrier to resistance, a critical attribute for next-generation antimalarials. Selection for 2741-19 resistance was unsuccessful. Recrudescence did not occur despite a total challenge of 1.16×10^{10} parasites (3.85×10^{9} parasites across three replicates). The MIR for 2741-19 is > 9, surpassing most antimalarials in clinical use or development.[12] The successful selection of resistance in the atovaquone positive control treatments validates the experimental design, yielding resistant strains with a 10.5-fold increase in IC_{50} relative to the parent strain. The data are consistent with prior research; *in vitro* selection for resistance studies of DSM265 reported an MIR of 7 for atovaquone, and a comparable 9.6-fold shift in the IC_{50}.[16] In addition, this study indicates that 2741-19 exhibits trophozoite-specific killing activity at 500 nM. Trophozoites required upwards of 12 h of exposure for maximal growth inhibition, and incomplete clearance after 54 h of exposure suggests parasites may be particularly susceptible during the ring-to-trophozoite transition. Rings demonstrated no sensitivity even after 12 h of exposure. These pharmacodynamic properties provide insights into the compound's mechanism of action and enable the evaluation of pharmacokinetic compatibility, which confirms its clinical viability and outlines potential applications. Collectively, these findings position 2741-19 as a high-priority candidate for prophylactic and therapeutic antimalarial applications.

2741-19 satisfies the latest antimalarial development guidelines, which emphasize deprioritizing any candidates with an MIR < 7.[12,13] While its high MIR confirms a high barrier to resistance, an understanding of parasite mutational dynamics provides more insights into the nature of any potential mechanisms of resistance.

Malaria parasites have an average mutation rate of 1×10^{-9} per base pair per replication.[17]

Unlike humans, malaria parasites have relatively similar mutation rates across the genome. Therefore, a population of 10^9 parasites is statistically likely to harbor at least one individual with a point mutation at any specific base pair, given that the mutation is non-lethal. Since this experiment treated a total of 1.16×10^{10} parasites across the three replicates, we can be confident that if a single point mutation in the entire parasite genome could confer resistance, recrudescence would have been observed in at least one of the replicates.

The failure to select for 2741-19 resistant mutants *in vitro* is resemblant of two antimalarials with incredible resistance profiles: chloroquine and artemisinin.[13] Resistance to both of these drugs requires simultaneous point mutations, as single mutations alone are detrimental to survival. For example, chloroquine resistance requires the K76T and A220S mutations in PfCRT.[18] The K76T mutation allows chloroquine to exit the digestive vacuole, while the A220S mutation induces a compensatory conformational shift that helps retain native PfCRT protein function. The probability of a parasite acquiring both of these specific mutations in a single generation is $\sim 10^{-18}$, which explains why a chloroquine-resistant parasite has never been selected for *in vitro*. Artemisinin resistance also requires two simultaneous mutations, although there is less specificity. Resistance requires a mutation in K13, and a compensatory mutation in a different protein that is contingent on the specific K13 mutation.[19] These drugs are in stark contrast with atovaquone, in which high-level resistance is conferred by a single mutation in Cytb.[9] This explains atovaquone's low MIR of 5–8, and why the drug is preferred as a positive control for *in vitro* selection experiments.

In addition to point mutations, resistance to antimalarials can evolve through mechanisms like gene amplification. The malaria parasite genome is particularly subject to unequal sister chromatid exchange. The genome has high frequencies of A/T-rich regions, which causes

frequent misalignment between sister chromatids during DNA repair, and can result in the transfer of large genomic segments from one sister chromatid to the other.[20] This drives gene amplification and gene deletion in tandem, as some daughter parasites may possess two copies of a gene (amplification), while others possess none (deletion). While gene amplification is a proven mechanism for antimalarial resistance, gene deletion is not, likely because such deletions often yield non-viable daughter parasites. Unlike point mutations, the frequency of these gene amplifications varies across the genome. The central core regions that house essential metabolic genes have a lower mutation rate ($\sim 10^{-8}$), while subtelomeric regions that contain the var genes can have a much higher rate ($\sim 10^{-4}$).[21] Since the 2741-19 selection experiment treated a total of 1.16×10^{10} parasites across the three replicates, we can be confident that if a single gene amplification or deletion event could confer resistance, recrudescence would have been observed in at least one of the replicates.

When traditional *in vitro* selection for resistance studies fail to yield resistant mutants, stepwise selection studies can be implemented as a secondary approach. These studies can select for resistant mutants in cases where multiple gene amplification events are required for high-level resistance, a mechanism best illustrated by mefloquine. High-level resistance to mefloquine is conferred by the accumulation of ≥ 3 copies of the *pfmdr1* gene, which sufficiently increases the expression of Pgh1 transporters that pump the drug out of the parasite cytosol.[22] The experiment begins at a lower drug pressure ($0.5 \times IC_{50}$), and is gradually increased in a stepwise fashion when the culture recovers. This process allows parasites initially possessing two copies of *pfmdr1* to accumulate sufficient copies to resist previously lethal drug pressure.

Stepwise selection studies are not performed to assess the relative risk of resistance, as the exposure to incremental drug pressure is not a fair representation of real clinical dosing.

Rather, as with standard *in vitro* selection studies, the value of these experiments lies in providing direct molecular evidence for the drug's mechanism of action. These findings could inform potential modifications that improve the drug's efficacy, identify optimal partner drugs for use in a combination therapy, and enable the monitoring of the emergence of resistance in the field from the onset of clinical deployment. However, stepwise selection studies are highly resource-intensive, as successful selection can require over a year of continuous culture.[22] Accordingly, these studies are generally prioritized only when preliminary evidence implicates gene amplification as a potential mechanism of resistance.

Current evidence indicates PfGARP is the putative target of 2741-19. PfGARP is expressed on the exofacial surface of infected erythrocytes during the trophozoite stage.[11] While the precise function of PfGARP is unproven, it is hypothesized that like PfEMP1, it is involved in the cytoadherence of erythrocytes to the vascular endothelium, enabling parasites to sequester and evade splenic clearance. The Kurtis Lab has demonstrated that anti-PfGARP antibody binding induces apoptosis, and 2741-19 binding is hypothesized to induce the same effect. Given this mechanism, gene amplification is unlikely to mediate resistance to 2741-19. Amplification of *PfGARP* would increase drug sensitivity, whilst amplification of membrane transport proteins would have no effect, as 2741-19 binds PfGARP without crossing a membrane. The most plausible mechanisms of resistance instead involve point mutations in *PfGARP* and/or downstream signaling proteins. Consequently, performing stepwise selection assays with 2741-19 is inadvisable and is considered a low priority for further investigation.

Furthermore, emerging evidence from the Kurtis Lab indicates that 2741-19 may have a second mechanism of action. In addition to triggering apoptosis mediated by PfGARP binding, 2741-19 appears to inhibit proteasomes, which prevents heme consumption and causes toxic

accumulation of protein aggregates. If true, parasites may need to simultaneously evolve resistance to both PfGARP-mediated apoptosis and proteasome inhibition to survive, an extremely high barrier to resistance.

The stage-specific exposure assays also provided valuable insights into the pharmacodynamic properties of 2741-19. As stated, the killing activity of 2741-19 is hypothesized to be mediated by PfGARP binding. The susceptibility of trophozoites contrasted with the complete insensitivity of rings substantiates this mechanism of action, as PfGARP is expressed exclusively in trophozoite-infected erythrocytes. This validation is significant because PfGARP was originally identified as a high-value target through differential screenings; young children that produced anti-PfGARP antibodies exhibited significantly stronger protection against high parasitemia and severe malaria. 2741-19 effectively provides a therapeutic means to mimic this naturally acquired immunity, especially in individuals who lack anti-PfGARP antibodies.

This study also defined the previously uncharacterized exposure thresholds of 2741-19. Prior murine pharmacokinetic studies have demonstrated an elimination half-life of 32–38 h. The findings that trophozoites required slightly over 12 h of exposure for maximal growth inhibition demonstrate strong compatibility between the pharmacodynamic and pharmacokinetic profiles, and impart no related concerns for clinical viability. In addition, it reinforces the compound's potential as both a prophylactic and therapeutic agent. Further studies could involve conducting stage-specific exposure assays at higher, more clinically-relevant drug concentrations, which could reveal more meaningful insights about the drug's properties and potential applications, such as for the treatment of severe malaria.

Collectively, these findings position 2741-19 as a high-priority candidate for next-generation antimalarial development, driven by its robust genetic barrier to resistance and favorable pharmacodynamic profile.

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