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Defining the Timing of Action of Antimalarial Drugs against *Plasmodium falciparum*

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Most current antimalarials for treatment of clinical *Plasmodium falciparum* malaria fall into two broad drug families and target the food vacuole of the trophozoite stage. No antimalarials have been shown to target the brief extracellular merozoite form of blood-stage malaria. We studied a panel of 12 drugs, 10 of which have been used extensively clinically, for their invasion, schizont rupture, and growth-inhibitory activity using high-throughput flow cytometry and new approaches for the study of merozoite invasion and early intraerythrocytic development. Not surprisingly, given reported mechanisms of action, none of the drugs inhibited merozoite invasion *in vitro*. Pretreatment of erythrocytes with drugs suggested that halofantrine, lumefantrine, piperaquine, amodiaquine, and mefloquine diffuse into and remain within the erythrocyte and inhibit downstream growth of parasites. Studying the inhibitory activity of the drugs on intraerythrocytic development, schizont rupture, and reinvasion enabled several different inhibitory phenotypes to be defined. All drugs inhibited parasite replication when added at ring stages, but only artesunate, artemisinin, cycloheximide, and trichostatin A appeared to have substantial activity against ring stages, whereas the other drugs acted later during intraerythrocytic development. When drugs were added to late schizonts, only artemisinin, cycloheximide, and trichostatin A were able to inhibit rupture and subsequent replication. Flow cytometry proved valuable for *in vitro* assays of antimalarial activity, with the free merozoite population acting as a clear marker for parasite growth inhibition. These studies have important implications for further understanding the mechanisms of action of antimalarials, studying and evaluating drug resistance, and developing new antimalarials.

Malaria is a mosquito-borne disease causing an estimated 800,000 deaths each year, occurring predominantly in children under 5 years in sub-Saharan Africa (1), and the majority of disease results from infection with *Plasmodium falciparum*. The multiple life cycle stages of *Plasmodium* spp. provide a number of targets for antimalarial chemotherapy. However, to date, all drugs in clinical use for the treatment of malaria act primarily against the intraerythrocytic development of *Plasmodium* parasites. The most important drugs currently in use, or previously widely used, for the treatment of clinical *P. falciparum* malaria are focused either on the food vacuole of ring-stage and trophozoites of blood-stage malaria (2–5) or on enzymes in the trophozoite folic acid biosynthesis pathway (6). Drugs that have been used clinically that have one of these two modes of action include chloroquine, amodiaquine, quinine, sulfadoxine-pyrimethamine, artemisinin derivatives (predominantly artemether and artesunate), and lumefantrine. Unfortunately, drug resistance has rendered many of these compounds ineffective, and there is increasing evidence that resistance to artemisinin derivatives, currently the most effective drugs, has begun to develop (7, 8).

The identification of compounds or drugs that act against other steps in blood-stage replication, or against other life cycle stages, may aid the development of new therapeutics for the treatment and prevention of malaria. Antimalarials with alternate blood-stage targets, such as atovaquone, which inhibits mitochondrion electron transport (9, 10), and the apicoplast ribosomal protein translation inhibitors (clindamycin, azithromycin, and doxycycline) (11, 12), are not in widespread use in countries in which malaria is endemic, due to a number of factors, including concerns about drug resistance. Only one drug in use clinically, primaquine, targets the sexual stages of the *P. falciparum* life cycle. Primaquine is thought to act against the mitochondria and is active against gametocytes and the dormant liver-stage hypnozoites of *Plasmodium vivax* (13, 14). The efficacy of many existing drugs in inhibiting different stages of red blood cell (RBC) infection remains unclear.

Recently, the development of methods for the purification of viable *P. falciparum* merozoites and improvements in methods to track and quantify development stages by flow cytometry have made it possible to precisely and efficiently examine two previously intractable life stages, merozoite invasion and late schizont development (15, 16). The likelihood that many of the parasite proteins involved in invasion and schizont rupture are unique to the parasite makes proteins involved in these stages important vaccine and drug targets. Merozoite invasion of the red blood cell (RBC) is a complex process involving the coordinated interaction between multiple parasite proteins and between parasite proteins and receptors on the RBC surface (17, 18). To date, few clearly defined drug targets have been identified in this life stage because

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of a lack of precise and efficient methods to measure invasion inhibition and a limited understanding of invasion events. Only a small number of compounds, including protease inhibitors (19–22) and heparin-like compounds (23), have been reported to have inhibitory activity against merozoite invasion. While heparin is used clinically as an anticoagulant, it is not licensed for use as an antimalarial, and studies to date suggest that it has limited effectiveness as an adjunctive treatment for complicated malaria (24). The life cycle stage immediately preceding merozoite invasion, schizont rupture, is another potential target for antimalarial chemotherapy. Several protease inhibitors target this stage *in vitro* by blocking protease-mediated cell rupture (16, 21, 25–27), but few other compounds are known to specifically target this life stage.

In this study, we sought to more precisely define the timing of action of antimalarial agents, particularly those with a history of clinical use, by determining the inhibitory effects of these drugs on merozoite invasion, schizont rupture, and intraerythrocytic development. In addition to known antimalarials, we have included the histone deacetylase (HDAC) inhibitor trichostatin A, which modifies gene expression and is part of a family of drugs under development for treatment of malaria (28), and the antibiotic cycloheximide, which is a potent inhibitor of protein translation (29), for comparison. The approaches developed in this study provide novel insights into inhibitory phenotypes of antimalarials that can be applied in studies of drug resistance and in the identification and development of novel inhibitory compounds.

MATERIALS AND METHODS

***P. falciparum* culture and synchronization.** D10 wild-type and D10-PfPHG (30) parasites were cultured in human O^+ erythrocytes as previously described (31) with modifications. Growth of D10 parental cells and that of the D10-PfPHG (pyrimethamine and blasticidin-HCl) line were compared in a 56-h assay in the presence of several of the antimalarials, and there was no difference in 50% inhibitory concentrations (IC_{50}) (see Table S1 in the supplemental material). Synchronization of parasite life stages using heparin has been described previously (16, 23). In this study, sterile injectable heparin (Pharmacia) was added to cultures to inhibit invasion and the formation of new ring-stage parasites and was removed to allow a "window" of invasion of 4 h for schizont rupture experiments and 6 to 8 h for merozoite experiments.

Intracellular drug inhibition assays. Parasite growth inhibition assays were performed as previously described (30). Briefly, trophozoite-stage (24- to 28-h-old) drug inhibition assays (50 μl) were set up at 1% parasitemia and 1% hematocrit with a 1-in-10 dilution of drug added. Assay mixtures were cultured for 56 to 60 h through to the next cycle of replication until mainly mature trophozoites appeared (32 to 36 h) and then stained with 10 μg/ml ethidium bromide (EtBr; Bio-Rad) for 1 h prior to flow cytometry assessment of parasitemia.

For schizont rupture assays, tightly synchronized ring-stage (2- to 6-h postinvasion) or schizont-stage (42- to 46-h postinvasion) parasites at 2% parasitemia were incubated with drug until flow cytometry analysis of parasite populations 4 h after the expected completion of schizont rupture.

Merozoite invasion inhibition assays. The protocol for filtration of viable merozoites is modified from the work of Boyle et al. (16). Typically, two or three 150-ml flasks at 2 to 3% hematocrit of parasites that were loosely (6 to 8 h) heparin synchronized were harvested using magnet purification (Miltenyi Biotech). Purified schizonts were eluted into a volume of 30 ml medium per 200 ml of culture with 10 μM E64 and left to develop for 5 to 6 h prior to filtration in RPMI-HEPES plus $NaHCO_3$ and human serum.

Merozoites were isolated by passage through a 1.2-μm syringe filter (Acrodisc, 32 mm; Pall), and 22.5 μl of the resulting filtrate was added to 2.5 μl of inhibitor and incubated for 10 min at 37°C (21) prior to addition of uninfected RBCs (0.5% final concentration) and agitation at 400 rpm for 10 min. Plates were gassed and left for a further 40 min to allow invasion events to finalize. Assays were assessed by flow cytometry after 1 h or washed for growth through to late trophozoite stage (40 h) and analyzed by flow cytometry. For washing, medium (165 μl) was added prior to centrifugation at 300 × *g* for 2 min, the supernatant was replaced with 180 μl of fresh medium, and the wash step was repeated before final resuspension in 100 μl of fresh medium. This process resulted in an approximately 1/80 dilution of the inhibitor. After 24 h, 80 μl of supernatant was removed and the assay mixtures were supplemented with 80 μl of fresh medium.

Flow cytometry and microscopy analysis of inhibition. All flow cytometry assay mixtures were analyzed on a Becton, Dickinson FACSCalibur with a 96-well plate reader using Fl-2 (EtBr; excitation wavelength, 488 nm) and Fl-1 (green fluorescent protein [GFP]; excitation wavelength, 488 nm) where appropriate. Typically, 20,000 to 40,000 RBCs were counted for each well. Samples were analyzed using FlowJo software (Tree Star Inc.). Parasites for flow cytometry analysis of ring-stage and schizont rupture experiments were stained with 5 μg/ml EtBr for 20 min with no wash, and trophozoite-stage (40 h postinvasion) parasites were stained with 10 μg/ml EtBr for 1 h prior to washing. Ring-stage parasitemia was counted using an Fl-1-high (GFP)–Fl-2-low (EtBr) gate (Fig. 1A and B). Late stages were gated with a forward scatter (FSC)- and Fl-2-high gate, and free merozoites were gated using an FSC-low, Fl-2-high gate (Fig. 1C). Thin smears for microscopy were fixed with methanol and stained in fresh 10% Giemsa stain (Merck) for 10 min.

Estimation of IC_{50} and IC_{80}. The IC_{50} and IC_{80} data were determined for each antimalarial using GraphPad Prism (GraphPad Software) according to the recommended protocol for nonlinear regression of a log-(inhibitor)-versus-response curve. An IC_{80} was used in preference to an IC_{90} value since some merozoite inhibition curves were too variable to obtain an accurate IC_{90}. Inhibitory concentrations for assays with drug added at trophozoite stage are denoted with IC^{troph} while those where drug was added at merozoite stage are denoted with IC^{mero}.

Antimalarial drugs. Quinine-HCl, chloroquine diphosphate salt, amodiaquine dihydrochloride, lumefantrine, piperaquine tetraphosphate tetrahydrate, artemisinin, artesunate, atovaquone, cycloheximide, and trichostatin A were supplied by Sigma. Mefloquine-HCl was supplied by Hoffmann-La Roche, and halofantrine-HCl was supplied by Monash University, Victorian College of Pharmacy. Stock concentrations were made at 2 mg/ml in H_2O (chloroquine, amodiaquine, mefloquine, and cycloheximide), ethanol (halofantrine and quinine), and dimethyl sulfoxide (DMSO) (artemisinin, artesunate, lumefantrine, piperaquine, atovaquone, and trichostatin A).

RESULTS

Initial testing for potential inhibition of merozoite invasion by drugs. To identify possible merozoite invasion-inhibitory activity of antimalarials, the panel of drugs was first screened in a merozoite inhibition assay using a newly developed technique for the purification of viable *P. falciparum* merozoites (16). Inhibition of invasion was tested by incubating merozoites with a range of concentrations of each drug for 10 min, followed by addition of RBCs to allow invasion to occur. The cells were washed 50 min later, resulting in a total drug exposure time of 1 h (Fig. 2A). Parasitemia was assessed by flow cytometry after 40 h of incubation, to allow parasites to develop to mature trophozoite stages, and the IC_{80} was determined in order to obtain an inhibitory drug concentration (IC_{80}^{mero}) for direct testing of invasion inhibition in subsequent assays. Activity of drugs in merozoite inhibition assays was compared to standard drug inhibition curves in which parasites were cultured for 56 h with constant drug treatment, and the IC_{80} was calculated (IC_{80}^{troph}). Drugs were classified as having no merozoite-

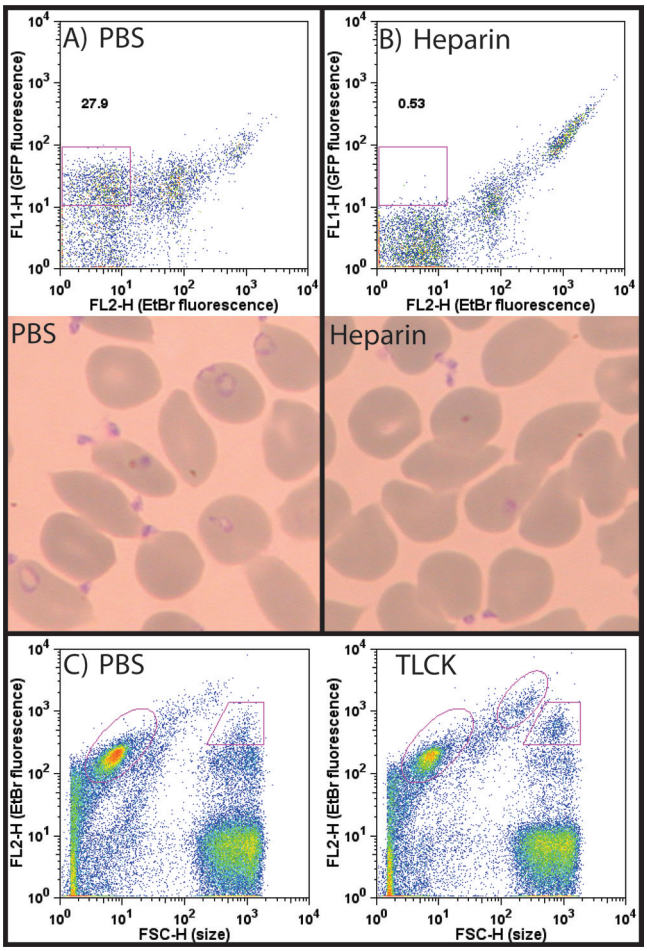

FIG 1 Flow cytometry assessment of merozoite invasion inhibition. (A and B) Flow cytometry gating of ring-stage parasites 1 h postinvasion defined by an EtBr-low/GFP-high population after treatment with noninhibitory PBS (A) or invasion-inhibitory heparin (B) treatments where the population is completely absent. Flow cytometry plots represent Fl-2 (EtBr fluorescence) versus Fl-1 (GFP fluorescence). Giemsa-stained thin smears (below) confirm the absence of early ring-stage parasites for the heparin-treated culture. (C) Representative flow cytometry plots showing basic parasite populations seen after schizont rupture in the presence of noninhibitory PBS control (left panel) and inhibition of schizont rupture by the protease inhibitor TLCK (right panel) (*N*α-*p*-tosyl-L-lysine chloromethyl ketone) (16). Flow cytometry plots show FSC (size) versus Fl-2 (EtBr fluorescence). Merozoites are gated as the oval using the total population as shown; comparison between populations was done using merozoite numbers. Late stages are gated as the polygon gate as shown. A second late-stage population (oval gate) typical of E64-treated schizonts lies above the main late-stage gated population. Bound merozoites typically lie below the main late-stage population. Assessment of late stages was always done on RBCs (infected and uninfected) gated out from the total population and not on the total population as shown here.

inhibitory activity if they failed to have an effect when tested at concentrations that were 10 times that of the IC_{80}^{troph} determined in the standard growth inhibition assay (referred to as 10× IC_{80}^{troph}).

The panel of drugs could be classified into two inhibitory profiles. Chloroquine, quinine, artemisinin, artesunate, atovaquone, and trichostatin A were found to inhibit parasite growth in standard assays but did not inhibit growth after merozoite treatment ($P < 0.0001$, extra sum-of-squares F test; Table 1; Fig. 2B), indicating that they do not act on merozoite invasion. We also tested the active metabolite of artemisinin, dihydroartemisinin, and found that it had no activity against merozoites at concentrations of up to 200 ng/ml (700 nM). Our findings are consistent with previous reports on the activity of chloroquine (32, 33). Amodiaquine, mefloquine, piperaquine, halofantrine, and lumefantrine all showed inhibition of parasite growth in merozoite inhibition assays (Table 1; Fig. 2B). The level of merozoite invasion and growth inhibition relative to inhibition measured in standard assays varied between drugs. Amodiaquine, mefloquine, and piperaquine all had lower IC_{50}^{troph} than IC_{50}^{mero} values. Conversely, halofantrine and lumefantrine both showed lower IC_{50}^{mero} than IC_{50}^{troph} values. For all five drugs with evidence of inhibition upon treatment of merozoites, the IC_{50}^{troph} was found to be significantly different from the IC_{50}^{mero} ($P < 0.0001$, extra sum-of-squares F test).

Assessment of invasion-inhibitory activity. Amodiaquine, mefloquine, piperaquine, halofantrine, and lumefantrine were tested further to determine whether they inhibited merozoite invasion directly. This was achieved by examining the inhibitory activity of the drugs when they were added at different time periods around merozoite invasion and assessing subsequent parasite growth over 32 to 46 h (Fig. 3A). For the drugs amodiaquine, mefloquine, piperaquine, halofantrine, and lumefantrine, we assessed the inhibitory effect of treatment of purified merozoites and ring-stage parasites at 20 min after invasion and pretreatment of RBCs for 1 h at an IC_{80}^{mero} drug concentration (Fig. 3B). High levels of inhibition were seen for all three treatments, including pretreatment of RBCs. This suggests that the drugs do not inhibit merozoite invasion directly. Rather, these data suggest that the drugs persist in the RBCs after being removed from the medium and act on the intraerythrocytic parasite at some point after invasion. Supporting this conclusion was the detection of parasite populations with reduced fluorescence compared to that of control populations for parasite cultures treated with each of these five drugs at merozoite and early ring stages (Fig. 3C). The low-fluorescence population evident for these drug-treated cultures is not present for the invasion inhibitor heparin and is consistent with dying intracellular parasites. In these experiments, heparin was also included because of its known specific invasion-inhibitory activity (23). As expected, heparin inhibited invasion of merozoites but had little activity when added 20 min postinvasion or when RBCs were preincubated with heparin.

Microscopy and flow cytometry examination of ring-stage parasites assessed 1 h postinvasion were used to further assess any invasion-inhibitory activity of the drugs directly (Fig. 4A). After halofantrine, lumefantrine, mefloquine, amodiaquine, and piperaquine treatment of merozoites during invasion, invasion rates measured by microscopy and flow cytometry were indistinguishable from those of phosphate-buffered saline (PBS)-treated control preparations (Fig. 4B and C), confirming that these drugs do not inhibit merozoite invasion but inhibit parasite development postinvasion.

Inhibition of intraerythrocytic development. The inhibitory activity of the panel of antimalarial drugs was assessed during intraerythrocytic development by addition of drugs at either the early ring stage (0 to 6 h postinvasion) or the late schizont stage (44 to 48 h postinvasion) (Fig. 5A). The number of ring-stage parasites, free merozoites, and late-stage parasites relative to a noninhibitory control was assessed by flow cytometry 4 h after the ex-

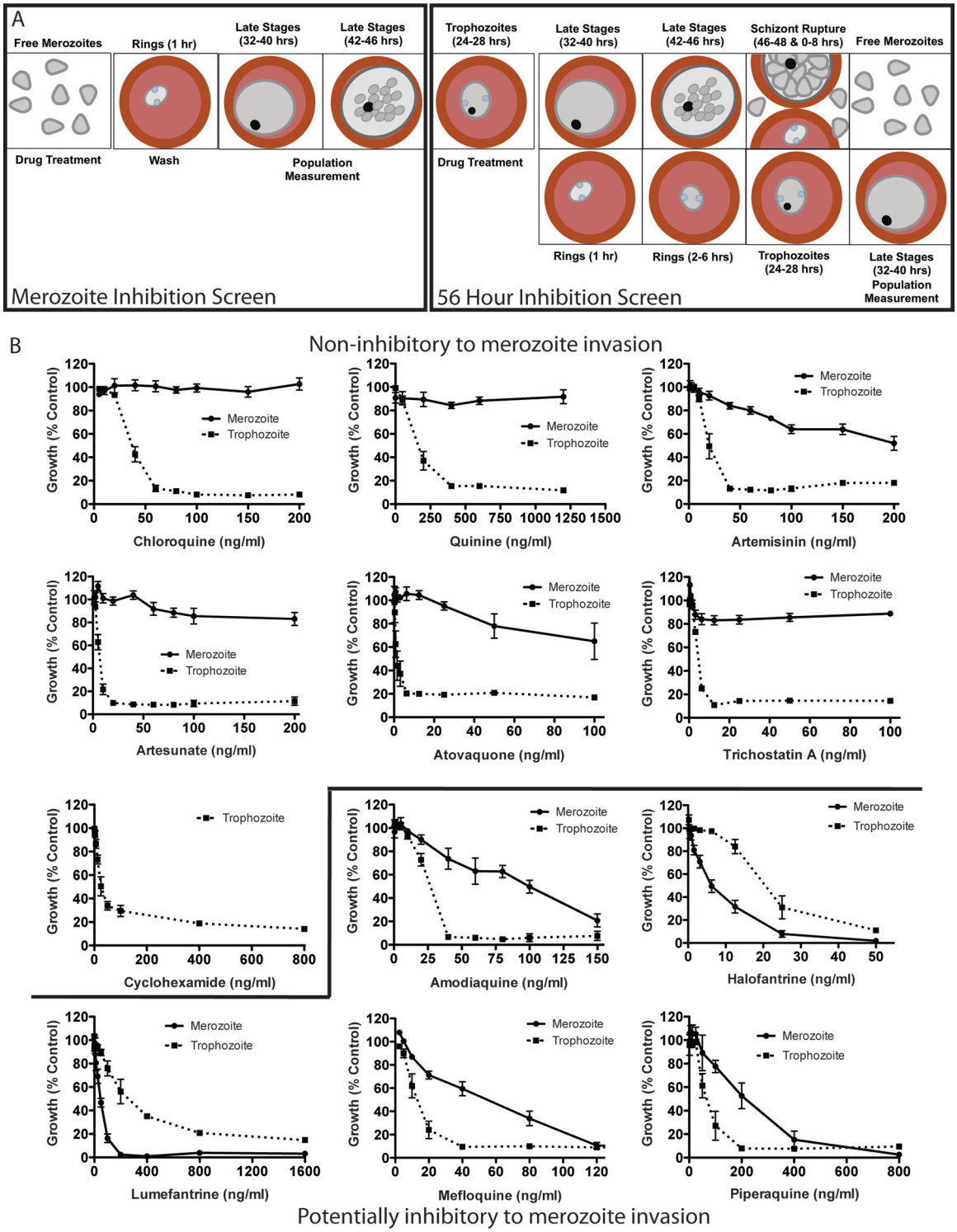

FIG 2 Comparison between merozoite invasion inhibition screens and 56-h parasite growth inhibition. (A) Schematic of treatment regimens for merozoite (left) and trophozoite (right) drug screens outlining the time of treatment and analysis of parasitemia. (B) Results from initial assays for growth-inhibitory activity of drugs when incubated with merozoites during and immediately postinvasion (merozoite), with a total incubation time of 1 h before drugs were washed out and parasitemia was counted at late trophozoite stage. The merozoite invasion inhibition screens were compared to 56-h growth inhibition assays (trophozoite) where the parasites were incubated with drug at the early trophozoite stage (24 to 28 h postinvasion), and the parasitemia was counted by flow cytometry at the mature trophozoite stage (32 to 36 h postinvasion). The first 7 drugs showed no inhibition of merozoite invasion at up to a 10-fold-higher concentration than the IC_{80}^{troph}. Cycloheximide was tested for invasion-inhibitory activity at a concentration above the 10-fold threshold, and the data are not shown. The final 5 drugs had evidence of merozoite invasion inhibition during the initial screen. Drug names are written under each graph. Data represent the means of three or more experiments; error bars represent the standard errors of the means.

TABLE 1 Inhibitory concentration of drugs tested in merozoite invasion inhibition and growth inhibition assays for the D10-PfPHG line

Drug	Abbreviation	Drug class	Trophozoite inhibition[a]		Merozoite inhibition[b]	
			IC_{50}, ng/ml (nM)	IC_{80}, ng/ml (nM)	IC_{50}, ng/ml (nM)	IC_{80}, ng/ml (nM)
Chloroquine	CHL	4-Aminoquinoline	31 (60)	125 (242)		
Amodiaquine	AMO	4-Aminoquinoline	21 (49)	85 (198)	114 (265)	456 (1,063)
Piperaquine	PPQ	4-Aminoquinoline	70 (70)	279 (279)	206 (206)	824 (824)
Quinine	QUN	Amino alcohol	138 (382)	551 (1,526)		
Halofantrine	HAL	Amino alcohol	23 (43)	94 (175)	6 (11)	23 (43)
Lumefantrine	LUM	Amino alcohol	265 (501)	1,059 (2,002)	41 (78)	162 (306)
Mefloquine	MFQ	Amino alcohol	124 (299)	496 (1,196)	413 (996)	1,652 (3,982)
Artemisinin	ART	Endoperoxide	21 (74)	83 (294)		
Artesunate	ATS	Endoperoxide	6 (16)	26 (68)		
Atovoquone	ATO	Naphthoquinone	2 (6)	7 (19)		
Trichostatin A	TSA	HDAC inhibitor	5 (17)	20 (66)		
Cycloheximide	CYX	NPT inhibitor[c]	33 (117)	132 (469)		

[a] Drug treatment of trophozoite-stage parasites (24 to 28 h postinvasion) until late-trophozoite-stage parasites (32 to 36 h postinvasion) in the next cycle (approximately 56 to 60 h of treatment). Parasitemia was measured by flow cytometry.
[b] Drug treatment of merozoites for 10 min prior to addition of RBCs and a further 50 min after RBC addition before the drug was washed out. Parasitemia was measured 40 h later by flow cytometry.
[c] Inhibitor of eukaryotic protein translation (nuclear).

pected finish of schizont rupture. To simplify comparisons, the drugs have been broadly grouped based on their inhibitory phenotype at an IC_{80}^{troph} value (Table 2; Fig. 5B, left panels). Data similar to those described were obtained at a 2× IC_{80}^{troph} concentration for all drugs, suggesting that the inhibitory phenotypes are broadly representative (data not shown). The results from testing drugs added at early ring stage are presented first, with the results from testing drugs added at late schizont stage following.

The invasion inhibitor heparin showed little impact on parasite growth when added at early ring stages of development. Only minor differences in free merozoites (34% increase) and unruptured late-stage (27% reduction) parasite populations relative to the PBS control were seen, suggesting that there is no substantial inhibition of intraerythrocytic development of parasites. As expected, heparin treatment blocked invasion, resulting in a loss of ring-stage (92% reduction) parasite populations.

When chloroquine, halofantrine, lumefantrine, and quinine were added to early-ring-stage cultures, there was a modest and variable effect on the proportion of late-stage parasites in the population (ranging from a 58% increase to a 38% reduction). However, treatment of ring-stage parasites (representing newly invaded erythrocytes) with these drugs resulted in almost complete inhibition of free merozoites (>96% reduction) and subsequent ring stages (>86% reduction), suggesting that parasites had failed to grow and release merozoites. From microscopic examination of cultures, the late-stage parasites appeared to be pyknotic remnant parasites that maintained enough nuclear material to be detected by flow cytometry using the nucleic acid stain ethidium bromide. In contrast, mefloquine, amodiaquine, piperaquine, artesunate, and artemisinin treatment of ring stages led to a substantial loss of free merozoites (>97%) and ring-stage (range, 59 to 89% reduction) and late-stage (range, 69 to 93% reduction) parasite populations, suggesting that early exposure to these drugs was severely inhibitory to parasite development and growth. Pyknotic remnant parasites could be detected by microscopy with low and variable nuclear staining detected by flow cytometry.

Treatment of ring stages with trichostatin A resulted in parasites of a fluorescence similar to that of late-ring-/early-trophozoite-stage parasites but with few late-stage parasites (57% reduction) or free merozoites detected compared to noninhibitory control. The majority of parasites detected in RBCs had reduced EtBr fluorescence compared to typical late-stage parasites. Comparison of the EtBr low-staining RBC population with microscopy of thin smears suggests that parasite growth arrested several hours after the addition of trichostatin A. In contrast, cycloheximide-treated parasites examined more than 48 h after drug treatment appeared to have stalled not long after drug addition since the remaining parasites appear to be early ring stages, with few late-stage parasites observed (71% reduction). The cycloheximide-treated, stalled ring-stage parasites had no EtBr fluorescence but remained GFP fluorescent when analyzed by flow cytometry, a pattern typical of newly invaded ring stages. Given that there were no free merozoites (98% reduction for both trichostatin A and cycloheximide) evident for these two treatments, the ring-/early-trophozoite-stage parasites are likely to be from the original ring-stage population rather than being newly formed rings arising from recent invasion.

Atovaquone treatment of ring stages resulted in a loss of merozoite (71% reduction) and ring-stage (79% reduction) populations. However, unique among the drugs tested, there was a corresponding pronounced increase in the late parasite population (2.5-fold increase. The flow cytometry and microscopy data suggest that the intracellular parasite can develop to a late-stage parasite after atovaquone treatment of rings but fails to form daughter merozoites and to propagate the next generation.

Schizont rupture inhibition. Adding the antimalarial drugs around the schizont stage of development (44 to 48 h postinvasion) highlighted the potency of some of the drugs tested on parasite development and schizont rupture, despite the relative short periods of treatment. As with the ring-stage treatments, the drugs have been broadly grouped based on their inhibitory phenotype at a drug concentration based on the IC_{80}^{troph} (Table 2; Fig. 5B, right panels).

Free merozoite and unruptured late-stage parasite populations in the presence of the invasion inhibitor heparin were little different from PBS controls. However, as expected there was a complete

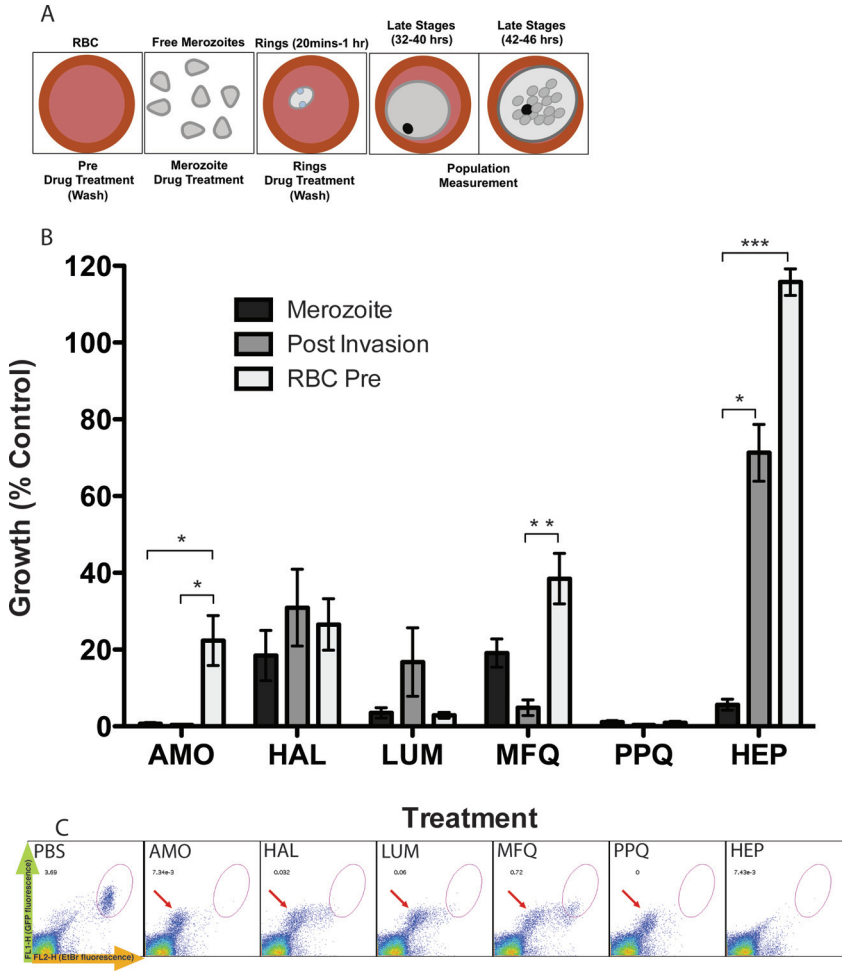

FIG 3 Merozoite treatment time and parasite growth inhibition. (A) Schematic of treatment regimen for merozoite invasion inhibition assay outlining the time of treatment and analysis of parasitemia at 40 h postinvasion. (B) Merozoite invasion-inhibitory potential was initially assessed by treatment of merozoites (10 min), treatment of early ring stages 10 min postinvasion (for 1 h), and pretreatment of uninfected RBCs (for 1 h) prior to invasion. The resulting parasitemia was assessed 40 h postinvasion and suggested that the 5 antimalarials tested are not inhibitory to merozoite invasion at 2× IC_{80}^{mero}. However, the high levels of growth-inhibitory activity for all treatments indicate that exposure of RBCs, both infected and noninfected, to the drugs was ultimately inhibitory to parasite growth. Data represents the means of 3 or more experiments, and error bars represent standard errors of the means. Significant results are indicated as follows: ***, <0.001; **, 0.001 to 0.01; *, 0.01 to 0.05. All other comparisons show no significant differences (Kruskal-Wallis test with Dunn's multiple comparison test). (C) Examination of flow cytometry plots 40 h after treatment of invading merozoites shows a population of cells with low fluorescence relative to noninhibitory controls (likely dead parasites) that is absent from merozoites treated with the invasion-inhibitory compound heparin. The green arrow indicates increasing GFP fluorescence; the orange arrow indicates increasing EtBr fluorescence. AMO, amodiaquine; HAL, halofantrine; LUM, lumefantrine; MFQ, mefloquine; PPQ, piperaquine; HEP, heparin.

loss of the ring-stage parasite population (88% reduction) for late stages treated with the invasion inhibitor heparin. This result confirms the precision of synchronization, with the majority of parasites being unruptured late schizonts at the time of antimalarial addition.

The inhibitory activity of artemisinin when added to schizont-stage parasites was striking; there was a clear loss of free merozoites (67% reduction) and ring stages (68% reduction) after treatment of schizonts. This was associated with a >600% increase in the number of unruptured schizonts, indicating that artemisinin acts rapidly against late-stage parasites and inhibits schizont rupture. Similarly, both trichostatin A and cycloheximide showed strong schizont rupture inhibition, reflected in a late-stage parasite population of around 600% compared to control; there was a corresponding loss of free merozoites (>60% reduction) and ring-stage parasites (>80% reduction). Microscopy of thin smears from these assays suggested that the daughter merozoites had failed to develop upon treatment of late stages with artemisinin, trichostatin A, and cycloheximide, despite the overall size and staining of the parasite resembling those of a mature schizont by flow cytometry and Giemsa-stained thin smears. These three drugs can therefore be considered to be extremely fast-acting and potent inhibitors of intracellular parasite development.

Treatment of schizont stages with chloroquine, halofantrine, lumefantrine, and quinine led to a small reduction in free merozoites (range, 0 to 32% reduction) and ring-stage parasites (range, 5% increase to 54% reduction) but up to a 2-fold increase in late-stage parasites. Similarly, mefloquine, amodiaquine, and piperaquine treatment showed a larger reduction in free merozoites

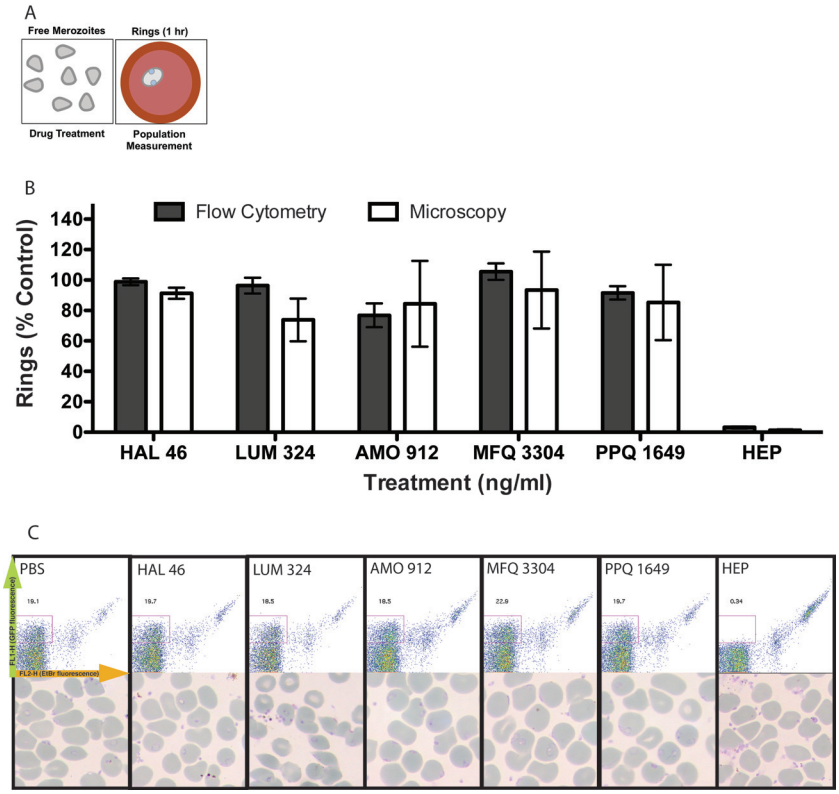

FIG 4 Confirmation that the antimalarials tested are not inhibitory to merozoite invasion. (A) Schematic of treatment regimen for merozoite invasion inhibition assay outlining the time of treatment and analysis of parasitemia at 1 h postinvasion. (B) Flow cytometry and microscopy assessment of merozoite invasion inhibition confirm that the antimalarials halofantrine (HAL), lumefantrine (LUM), amodiaquine (AMO), mefloquine (MFQ), and piperaquine (PPQ) are not inhibitory to merozoite invasion at 2× IC_{80}^{mero}. Invasion-inhibitory activity was demonstrated using heparin (HEP). Data represent the means of 2 (in duplicate) or more experiments, and error bars represent standard errors of the means. Differences between flow cytometry and ring-stage counts for each inhibitor were not significant (Mann-Whitney test, two-tailed). (C) Representative flow cytometry plots and microscopy thin smears highlight the lack of invasion-inhibitory activity for the 5 antimalarials compared to heparin. The green arrow indicates increasing GFP fluorescence; the orange arrow indicates increasing EtBr fluorescence.

(range, 28 to 38% reduction) and ring stages (range, 33 to 66% reduction) after treatment of schizonts, associated with an up to 4-fold increase in late stages. These results suggest that these drugs are only partially effective at preventing schizont rupture and reinvasion when added to very mature blood-stage parasites.

Both artesunate and atovaquone showed little difference in the merozoite and ring- and late-stage populations relative to control upon treatment of schizonts. These results suggest that neither atovaquone nor artesunate should be considered a fast-acting inhibitor of schizont maturation and rupture *in vitro*.

Flow cytometry-based *in vitro* microtest. We examined whether the observations of schizont rupture during drug treatment could be developed as a flow cytometry-based *in vitro* microtest assay for *P. falciparum* drug susceptibility using a method modeled on that approved by WHO (34). The WHO microtest of drug susceptibility is reliant upon microscopy-based assessment of thick films. The assay determines the number of late-stage parasites with fewer-than-expected daughter merozoites relative to untreated control after drug treatment of late-ring- to early-trophozoite-stage parasites through to late schizont stage (treatment time, 24 to 30 h). In this study, with a similar assay setup and utilizing flow cytometry, we examined drug susceptibility using the markers of unruptured late stages, free merozoites, and newly invaded rings.

For both chloroquine and artemisinin, treatment of ring-stage cultures led to a gradual loss of ring-stage and free merozoite populations with increasing concentrations of drug (Fig. 6A and B). Interestingly, there was a peak in the late-stage parasite population corresponding to the initial drop in ring-stage parasites, indicating that parasites had grown to late stages but had failed to rupture and release merozoites at that drug concentration. Increasing artemisinin concentrations during treatment of late-stage parasites resulted in a reverse correlation between increasing late-stage parasites and decreasing free merozoite and ring-stage parasites (Fig. 6C). Importantly, there is a clear correlation between readily distinguished free merozoites and ring stages for these drugs that are not inhibitory to invasion. These results suggest that a flow cytometry-based approach for the assessment of schizont rupture inhibition is a viable alternative to microscopy-based schizont development assays. The use of the free merozoite population, which is clearly distinguishable from background and other cell populations when stained with EtBr, is an advantage of this approach over microscopy-based *in vitro* microtests.

DISCUSSION

In this study, we took advantage of the recent development of a robust method for the purification of viable *P. falciparum* merozoites (15, 16) to develop a high-throughput flow cytometry ap-

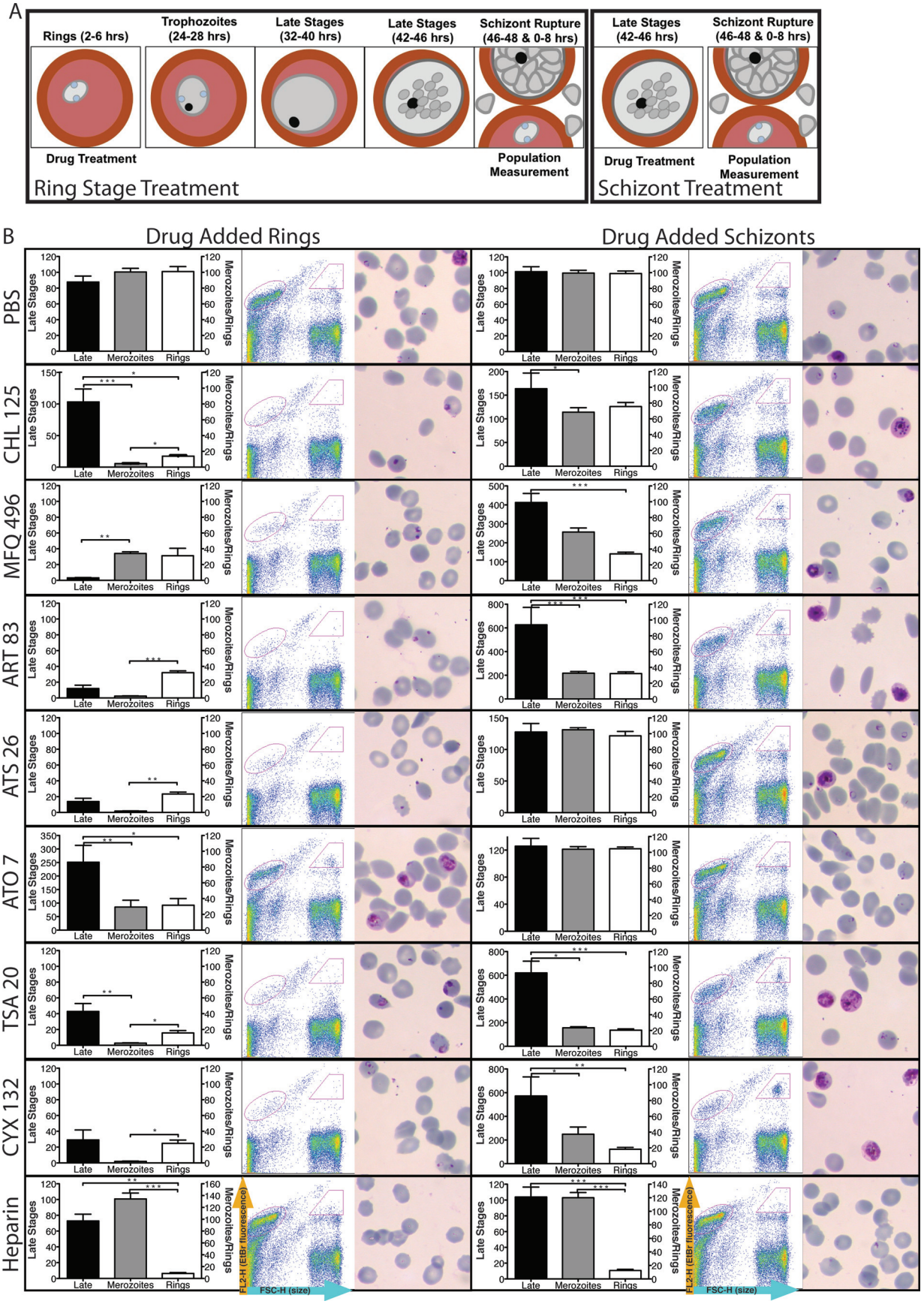

TABLE 2 Populations of drug-treated *P. falciparum* blood stages following schizont rupture as assessed by flow cytometry[c]

Drug (ng/ml)	Ring-stage addition[a]			Schizont-stage addition[b]		
	M	R	LS	M	R	LS
CHL (125)	4	14	96	68	76	164
HAL (94)	2	3	158	74	56	181
LUM (1,059)	3	6	62	100	105	136
QUN (551)	3	13	81	86	46	204
MFQ (496)	3	11	31	62	34	412
AMO (85)	2	41	10	66	78	251
PPQ (279)	3	15	7	72	77	328
ART (83)	2	32	12	33	32	627
ATS (26)	2	23	14	105	97	128
ATO (7)	29	21	251	104	105	126
TSA (20)	2	16	43	23	20	620
CYX (132)	2	25	29	38	18	572
HEP	134	8	73	120	12	104

[a] Antimalarials were added at early ring stages (0 to 6 h postinvasion) and were left in the culture until approximately 4 h after the expected rupture of schizonts (48 to 54 h of treatment). Free merozoites, newly invaded ring stages, and unruptured late stages were assessed by flow cytometry and expressed as a percentage of the same population observed for the noninhibitory PBS control.

[b] Antimalarials were added at late schizont stage (42 to 46 h postinvasion) and were left in the culture until approximately 4 h after the expected rupture of schizonts (10 h of treatment). Parasite populations measured are the same as those for ring-stage treated cultures.

[c] Abbreviations: CHL, chloroquine; HAL, halofantrine; LUM, lumefantrine; QUN, quinine; MFQ, mefloquine; AMO, amodiaquine; PPQ, piperaquine; ART, artemisinin; ATS, artesunate; ATO, atovaquone; TSA, trichostatin A; CYX, cycloheximide; HEP, heparin; M, free merozoites; R, ring stages; LS, late stages.

proach to screen for inhibitors of invasion and schizont rupture. Compounds targeting these stages, such as protease inhibitors (19–22) and heparin (23), have been proposed as candidates for the development of antimalarials (15), but to date, no drug approved for clinical use against malaria has been shown to directly inhibit these life stages, in part due to the lack of appropriate assays. In total, 12 drugs, 10 of which have been used extensively clinically, were screened for their invasion and growth-inhibitory activity using optimized high-throughput flow cytometry (Table 3).

The complementary methods of flow cytometry and microscopy-based assessment of ring-stage parasitemia within 1 h postinvasion clearly distinguished between invasion-inhibiting drugs, such as heparin, and compounds that rapidly kill the developing ring-stage parasites after invasion has occurred. Initial experiments assessing parasite growth 40 h after exposure of merozoites and very early ring (less than 1 h postinvasion) stages identified five drugs (amodiaquine, mefloquine, piperaquine, halofantrine, and lumefantrine) with a possible invasion-inhibiting effect; however, our assays testing these compounds for 1 h during invasion and immediately postinvasion showed that none of these drugs inhibit merozoite invasion of the RBC. Rather, pretreatment of RBCs with the five invasion-inhibiting candidates suggests that these drugs are absorbed and retained by the red blood cell, where they inhibit postinvasion development of parasites. The increased potency of halofantrine and lumefantrine when applied in early ring stages rather than at the mature trophozoite stage, as used in standard assays, suggests that there are stage-specific effects of these drugs and that they lose efficacy over the 56- to 72-h time course of the standard *in vitro* drug assay.

The inability of artemisinin to inhibit merozoite invasion was somewhat surprising given reports that this drug binds to SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase), a calcium ATPase with a role in microneme release during invasion in *Toxoplasma gondii* (35). *In vitro* binding of artemisinin to ATPase 6, the SERCA homologue in *P. falciparum*, has also been reported previously (36). We found no invasion-inhibitory effects of artemisinin at concentrations up to 2 M (data not shown), suggesting that the binding of artemisinin to SERCA proteins does not inhibit merozoite invasion of the red blood cell. Further, the active metabolite of artemisinin (dihydroartemisinin) has no invasion-inhibitory activity up to 200 ng/ml (700 nM), indicating that the artemisinin-derivative drugs have little activity against merozoite invasion.

Application of the flow cytometry-based approach for the assessment of rings, free merozoite, and late-stage parasites allowed us to compare the effects of antimalarial drugs during the blood stage and on schizont rupture in particular. All of the aminoquinolone-related compounds (chloroquine, amodiaquine, mefloquine, halofantrine, quinine, lumefantrine, and piperaquine) showed an expected drug response: parasite death during the trophozoite stage and no effect on schizont rupture. An earlier study reported that chloroquine can inhibit the development of ring stages to pigmented trophozoites (33). However, the presence of pyknotic cells at half the reported drug concentration in this study suggests that parasite death occurred later in the life cycle. There was a complete loss of all developmental stages when parasites were treated with artesunate or artemisinin at ring stages, indicating that both drugs were extremely effective when applied early in the intracellular parasite life cycle. This appears consistent with their ability to cause rapid clearance of parasitemia when used to treat malaria. Interestingly, the effects of artemisinin and artesunate differed markedly in the schizont rupture assay. Artemisinin treatment led to a dramatic reduction in schizont rupture and the formation of new ring-stage parasites, while artesunate treatment had no effect. If these differences in activity also occur *in vivo* when the drugs are used clinically to treat malaria, this may have impor-

FIG 5 Characterization of growth and schizont rupture phenotype by flow cytometry. (A) Schematic of treatment regimen for schizont rupture assays outlining the time of treatment and analysis of parasitemia at >4 h postinvasion of the second cycle of growth. (B) Tightly synchronized (4-h window of invasion) parasites were treated with an IC_{80}^{troph} concentration of antimalarials at late schizont stage (42 to 48 h postinvasion; right panels) or early ring stage (0 to 6 h postinvasion; left panels). The inhibitory phenotype was then assessed by flow cytometry and microscopy 4 to 8 h after the expected following cycle of invasion. Bar graphs represent the mean late-stage (black bars), free merozoite (gray bars), and ring-stage (white bars) populations measured by flow cytometry from three or more experiments expressed as a percentage of noninhibitory control populations. Error bars represent the standard errors of the means. Significant results are indicated as follows: ***, <0.001; **, 0.001 to 0.01; *, 0.01 to 0.05. All other comparisons show no significant differences (Kruskal-Wallis test with Dunn's multiple comparison test). Representative flow cytometry dot plots and microscopy thin smears are presented for each drug to highlight the inhibitory effects. Late-stage and free merozoite populations are denoted by the polygon and oval gates, respectively. The orange arrow indicates increasing EtBr fluorescence; the blue arrow indicates increasing forward scatter (size). CHL, chloroquine; MFQ, mefloquine; ART, artemisinin; ATS, artesunate; ATO, atovaquone; TSA, trichostatin A; CYX, cycloheximide.

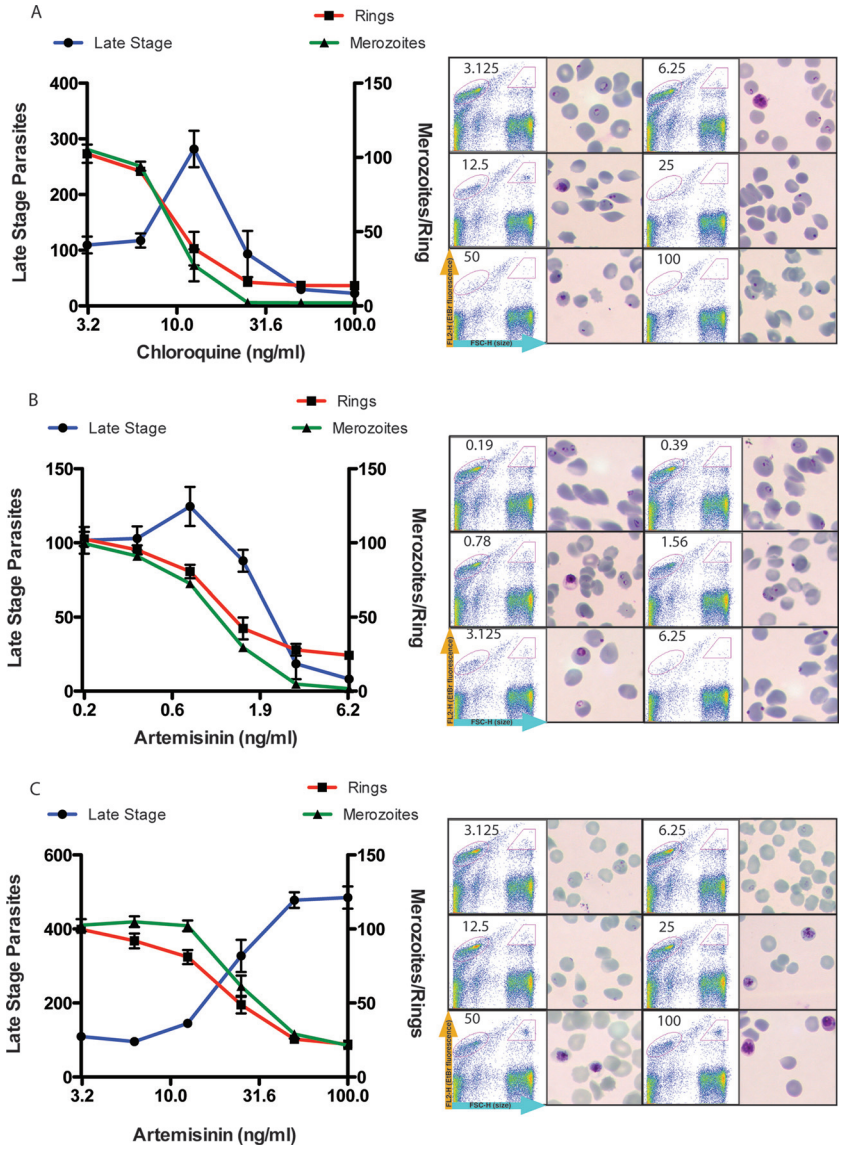

FIG 6 Flow cytometry characterization of schizont rupture for drug screens. Proof-of-principle experiments highlight the inhibitory effects of doubling dilutions of chloroquine added at early ring stages (A), artemisinin added at early rings (B), and artemisinin added at late schizont stage (C) on the rupture of schizonts as measured by flow cytometry 4 to 8 h after the expected second round of invasion (left panels). Data represents the means of 3 or more experiments expressed as a percentage of noninhibitory control populations. Error bars represent the standard errors of the means. Representative flow cytometry dot plots and microscopy thin smears (right panels) highlight the different inhibitory parasite populations corresponding to the points on the associated graph. Late-stage and free merozoite populations are denoted by the polygon and oval gates, respectively. The orange arrow indicates increasing EtBr fluorescence; the blue arrow indicates increasing forward scatter (size).

tant implications for treatment of severe malaria where there is thought to be a large biomass of sequestered late-stage parasites (37).

Atovaquone had a unique phenotype where treatment of ring stages led to an accumulation of late-stage parasites with a corresponding loss of rings and free merozoites, whereas treatment of schizonts gave no inhibitory effect. This drug response is consistent with the primary activity of atovaquone, which inhibits electron transport in the mitochondria but ultimately kills *Plasmodium* through downstream effects on pyrimidine synthesis (38). We would not expect effects on late schizont stages as DNA replication has been completed at this stage. The slow onset of atovaquone inhibitory activity relative to aminoquinolones and artemisinin was also reflected by the continued development of ring-stage treated parasites. At the time of assaying, atovaquone-treated parasites were approximately equal in size to late-stage parasites but lacked merozoites. This is similar to the response seen after treatment with the isoprenoid synthesis inhibitor fosmidomycin (39) and may represent a common pattern of response to drugs inhibiting bulk synthetic pathways.

Trichostatin A and cycloheximide also had novel inhibitory phenotypes with immediate effects on ring-stage parasites and a pronounced accumulation of late stages for schizont-treated cultures. Neither drug allowed production of free merozoites or ring

TABLE 3 Inhibitory activity of antimalarial drugs at different life cycle stages

Drug	Stage of activity			
	Merozoite invasion[a]	Ring development[b]	Growth to late stages[c]	Schizont rupture[d]
Chloroquine	No	Unlikely[h]	Yes[g]	Poor
Amodiaquine	No	Unlikely	Yes[g]	Poor
Piperaquine	No	Unlikely	Yes[g]	Poor
Quinine	No	Unlikely	Yes[g]	Poor
Halofantrine	No	Unlikely	Yes[g]	Poor
Lumefantrine	No	Unlikely	Yes[g]	Poor
Mefloquine	No	Unlikely	Yes[g]	Poor
Artemisinin	No	Likely	Yes[g]	Yes
Artesunate	No	Likely	Yes[g]	No
Atovoquone	No	No	Yes[f]	No
Trichostatin A	No	Yes	NA[e]	Yes
Cycloheximide	No	Yes	NA[e]	Yes

[a] Inhibition of merozoite invasion for purified merozoites using the protocol outlined in this study.
[b] Inhibition of blood-stage development from early rings to early trophozoite stages (around 20 h postinvasion). Inferred from presence or absence of early-stage, late-stage, and pyknotic parasites as determined by flow cytometry and microscopy.
[c] Inhibition of blood-stage development from early rings to late-trophozoite/early-schizont stages (around 36 to 40 h postinvasion). Inferred from comparison of ring-treated and schizont-treated cultures.
[d] Inhibition of late-stage parasite rupture and release of free merozoites as measured by flow cytometry.
[e] NA, not assessable with the data at hand since ring and schizont treatments lead to immediate cessation of growth and development at the respective stages. It is likely, however, that such fast-acting inhibition would also occur during trophozoite-stage growth.
[f] Inhibition of parasite growth with ring-stage treatment of atovaquone is characterized by the development of parasites of size and staining similar to those of schizonts, without formation of daughter merozoites.
[g] Inferred from the body of work attributing the inhibitory activity of these drugs as targeting the food vacuole from ring stages through to late trophozoite stage.
[h] It is noted here that some studies have described potent inhibition of the development of ring stages upon treatment with chloroquine (33).

stages. It was noticeable that the remnant ring-stage parasites after trichostatin A treatment looked to have matured past early ring stages before growth arrested, whereas the cycloheximide-treated rings looked to have progressed little since drug treatment and resembled healthy rings. Cycloheximide affects protein expression by blocking cytosolic protein translation (29, 40), and so immediate effects on parasite development are expected. The histone deacetylase inhibitor trichostatin A causes changes in gene expression within 2 h of late-stage parasite treatment (41). The rapid onset of dysregulated gene expression fits with the early-stage growth arrest seen with trichostatin A-treated rings. The apparent earlier cessation of growth for the protein translation inhibitor (cycloheximide) than for the transcriptional regulator (trichostatin A) may indicate that the majority of transcript for early-ring-stage development is already present soon after invasion and that the inhibitory effect of trichostatin A is delayed. An alternative explanation is that protein translation inhibition by cycloheximide has relatively global and rapid inhibitory effects, while trichostatin A causes changes in transcript levels for only a subset of genes (41) that exert their effect later in parasite development. Trichostatin A treatment has a disproportionally large effect on the regulation of merozoite invasion proteins, suggesting that its effect on schizont rupture results from disruption of protein expression and merozoite development in the maturing parasite. The lack of merozoite invasion inhibition evident for trichostatin A and cycloheximide suggests that transcriptional regulation and translation of proteins are of limited importance for the invading merozoite. The rapid growth-inhibitory activity of these transcription and translation inhibitors for multiple stages of intracellular parasite growth could be a major benefit if *P. falciparum*-specific derivatives were to be developed. These results also suggest that trichostatin A and cycloheximide may be useful tools for studying biological processes in parasite development.

Understanding the stage specificity of drug treatment is important from a number of standpoints. Detailed stage specificity studies enhance the understanding of how drug treatments may affect parasite replication and clearance clinically. For example, in this study both artesunate and atovaquone were found to be poor inhibitors once the schizont stage is reached. If similar results are observed clinically, there may be important implications for drug treatment regimens and understanding how different drugs will affect disease progression. A drug that allows the late-stage parasite population to develop normally may not be ideal for the treatment of acute disease where rapid clearance is required and there is a large population of sequestered late-stage parasites. Merozoite release and reinvasion may exacerbate disease symptoms and the subsequent immune response, contributing to poor clinical outcomes. In such cases, drugs with strong schizont inhibition activity such as artemisinin may be an advantage. Development of an artemisinin combination therapy with drugs that act against early ring stages, as cycloheximide and trichostatin A were observed to do in this study, may be additionally important in light of recent reports that dormant ring-stage parasites can mediate resistance to dihydroartesunate *in vitro* (42, 43). A further important outcome from understanding stage specificity is that it can point to the mechanism of action. Such information is fundamental for rational drug design and assessment of clinical utility. Understanding the mechanisms of action will also improve opportunities for identification of resistance mutations.

It is also important to consider stage specificity in assessing inhibitory concentrations, particularly when assessing potential drug resistance. For example, the lower inhibitory concentrations for halofantrine and lumefantrine when applied immediately after invasion, rather than at the mature trophozoite stage, may suggest that the early ring stage is more susceptible than other stages and that these compounds lose efficacy over the course of the assay, making them less effective inhibitors of the early ring stage after 24 h under culture conditions. In this study, the inhibitory concentrations were determined by treating trophozoite stages and assessing parasitemia by flow cytometry 56 h later. This time scale allows treatment of all life cycle stages and effective assessment of late-stage parasites by flow cytometry using EtBr. However, drug treatments starting at different life cycle stages may result in very different IC_{50}, especially for drugs that are not stable after prolonged incubation *in vitro*.

The observations of schizont rupture inhibition detailed in this study were applied to an *in vitro* microtest drug susceptibility assay based on the WHO-approved *in vitro* microtest (34). Our assay demonstrated three potential markers of drug inhibition: free merozoites, ring stages, and unruptured schizonts, which are readily distinguishable by flow cytometry. In practice, detection of ring stages with non-GFP-fluorescent lines may require the application of an alternative staining method such as the combination of thiazole orange and hydroethidine (44) or Hoechst 33342 and

thiazole orange (45). Previous studies have reported an encouraging correlation between flow cytometry and microscopy counts of drug-treated cultures *in vitro* (45, 46) as well as application of flow cytometry to assess drug inhibition of late-stage parasite development (47). In this study, the correlation between numbers of free merozoites and parasite growth inhibition (newly invaded rings) has been described in detail. The clear separation of free merozoites from other cell populations, combined with the greater number of noninvaded merozoites than of ring-stage parasites, has potential applications for *in vitro* microtests of drug susceptibility where flow cytometry facilities are available.

In this study, a panel of 10 drugs with a history of extensive clinical use, as well as two broad inhibitors of transcription and translation currently not in clinical use for malaria treatment, had markedly different stage-specific activities. Although none inhibited merozoite invasion at the concentrations tested, halofantrine, lumefantrine, piperaquine, amodiaquine, and mefloquine appear to be taken up by RBCs and subsequently to inhibit intraerythrocytic development of parasites. Of the drugs in use clinically, only artemisinin showed potent activity when used to treat schizont-stage parasites. These data have important implications for understanding or predicting the clinical efficacy of drug treatment, design of drugs, or combinations with broad activity across different developmental stages and for measuring the development of and mechanisms of drug resistance. The flow cytometry approach applied in this study demonstrated that the correlation between numbers of free merozoites and growth inhibition (defined by ring-stage inhibition) could be used for the detection of developing drug resistance in low-parasitemia field samples *in vitro*. The search for potent new drugs for the treatment of malaria needs to include compounds that affect life stages of the parasite that are not targeted by currently licensed antimalarials, and studies presented here provide methods and approaches to facilitate the identification and evaluation of these compounds.

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