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Modified *Plasmodium falciparum* Ring-Stage Survival Assay with ML10 Kinase Inhibitor

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ABSTRACT The ring-stage survival assay is the reference assay to measure *in vitro* *Plasmodium falciparum* artemisinin partial resistance. The main challenge of the standard protocol is to generate 0-to-3-h postinvasion ring stages (the stage least susceptible to artemisinin) from schizonts obtained by sorbitol treatment and Percoll gradient. We report here a modified protocol facilitating the production of synchronized schizonts when multiple strains are tested simultaneously, by using ML10, a protein kinase inhibitor, that reversibly blocks merozoite egress.

KEYWORDS artemisinin resistance, malaria, *Plasmodium falciparum*, ring-stage survival assay

Plasmodium falciparum malaria is a vector-borne parasite disease and was responsible for 627,000 deaths and 241 million cases in 2021, predominantly in sub-Saharan Africa (1). Artemisinin-based combination therapies (ACTs), which include a fast and potent artemisinin derivative (ART) and a long half-life companion drug to kill persistent parasites that may survive after ART is metabolized, are currently recommended by the World Health Organization as the front-line treatment of uncomplicated *falciparum* malaria (1, 2). Unfortunately, *P. falciparum* partial resistance to artemisinin (ART-R), which emerged in Southeast Asia in 2006 to 2007 (3, 4), has been detected recently in Africa (5, 6). ART-R, defined as delayed parasite clearance in patients treated with artemisinin monotherapy or a 3-day ACT course, is due to the decreased susceptibility of ring-stage parasites to ART. The decreased susceptibility of ring-stage parasites to ART can be measured *in vitro* using the ring-stage survival assay (RSA^{0-3h}), and single point mutations in the gene coding for the propeller domain of the Kelch13 protein (*Pfkelch13*, PF3D7_1343700) strongly correlate with ART-R (7-10).

The most challenging step in performing the standard RSA^{0-3h} is to obtain tightly synchronized ring-stage parasites (0 to 3 h postinvasion) by sequential use of sorbitol and Percoll solutions (9, 11). The subsequent steps, which involve pulsing ring stages with 700 nM dihydroartemisinin (DHA; the active metabolite of all artemisinin derivatives) for 6 h, washing them, and culturing them for 66 h, are straightforward. The survival rate of the assayed parasites (from *ex vivo* isolates or *in vitro* culture-adapted strains) is then calculated relative to that in parasites exposed to dimethyl sulfoxide (DMSO; the vehicle used to dissolve DHA) (Fig. 1). While effective, this protocol is laborious and time-consuming and requires multiple steps over many hours. Moreover, although *in vivo* *P. falciparum* infection suggests a 48-h periodicity, *in vitro* and transcriptomic studies have shown that *P. falciparum* isolates can have different period lengths that can vary substantially from 48 h (from 36 to 54 h) (12, 13). This difference,

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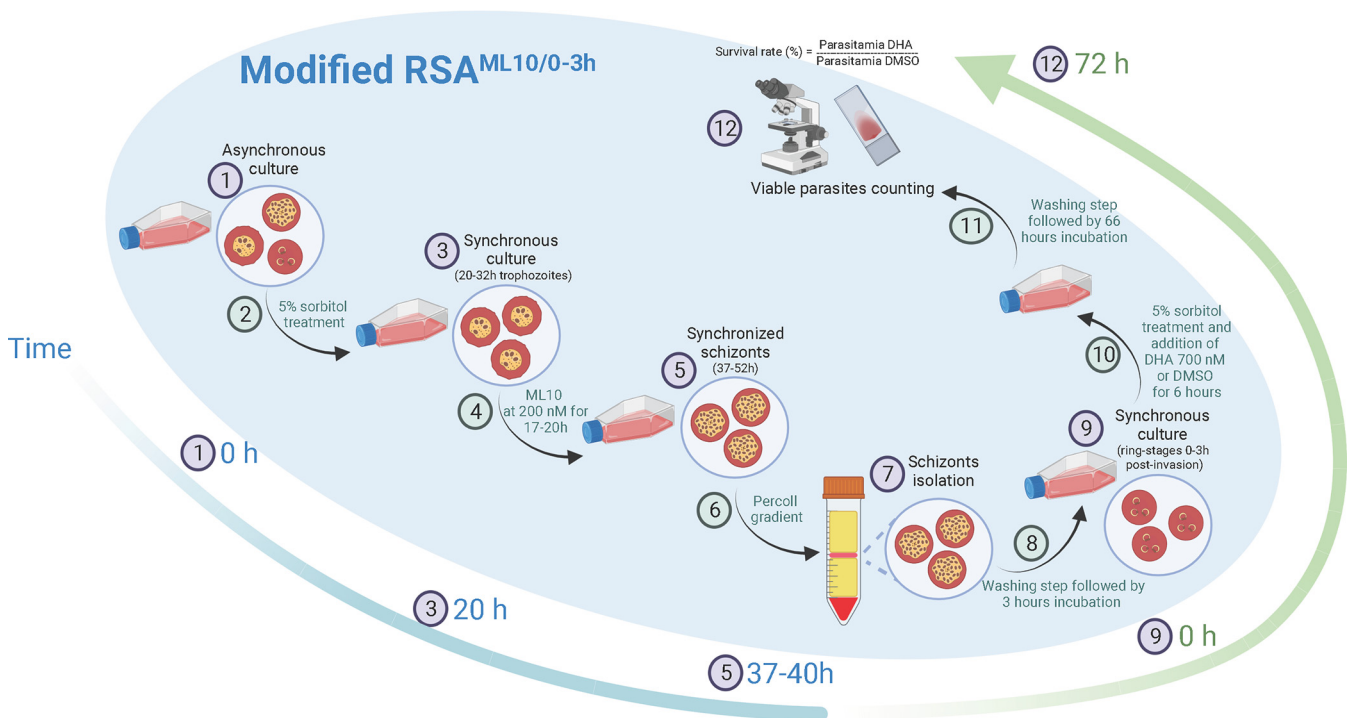


FIG 1 Overview of the ring-stage survival assay. (1) Parasites were cultivated to reach 1% parasitemia (all blood stages). (2) Parasites were treated with 5% sorbitol to eliminate mature parasites and preserve ring stages (~0 to 12 h postinvasion). (3) Parasites were cultivated for 20 h to obtain 20-to-32-h trophozoites and exposed to ML10 at 200 nM for 17 to 20 h. (4) Schizonts (~37 to 52 h) were isolated using a 75% Percoll gradient. Red blood cells were then washed to remove ML10 and cultivated with fresh blood cell for 3 h for reinvasion. (5) Synchronous 0-to-3-h ring stages were recovered and pulsed with 5% sorbitol to eliminate any remaining schizonts. (6) Parasites were treated for 6 h with 700 nM DHA or 0.1% DMSO (control), then washed and cultivated for an additional 66 h (72 h total). (7) Red blood cells were collected and used to prepare Giemsa-stained blood smears. The mean survival was then calculated as follows: (parasitemia with DHA)/(parasitemia with DMSO) × 100.

genetically controlled, between strains represents a major challenge when multiple strains are tested simultaneously. ML10 is a specific inhibitor of the cGMP-dependent protein kinase that arrests *P. falciparum* growth immediately prior to merozoite egress (14). This compound allows parasite cultures to be synchronized so that all parasites are within a window of development of several minutes, with a simple washing step. As this compound is of unquestionable interest for enrichment of tightly synchronized schizonts required for the RSA^{0-3h}, we developed a modified RSA protocol facilitating simultaneous synchronization of different *P. falciparum* strains.

We tested first the effectiveness of different concentrations of ML10 for blocking merozoite egress (ML10 is available at MR4, BEI Resources). Two parasite lines (3D7-K13-wild-type, an African laboratory strain, and Cam1-K13-wild-type, a culture-adapted Cambodian strain) were used (see Table S1 in the supplemental material for a detailed strain list). The asynchronous cultures were first treated with 5% sorbitol to achieve 0-to-12-h ring-stage synchronization. The cells were cultivated for 20 h to reach the 20-to-32-h trophozoite stage and later exposed to 50, 100, 150, or 200 nM ML10 or complete RPMI culture medium (used as a control) for 17 h in order to obtain ML10-treated mature 37-to-49-h parasites, the time when the merozoite egress is happening. This range of concentrations was used to validate the ML10 safety and ensure tight synchronization of schizonts necessary for the RSA. Red blood cells were then collected to prepare Giemsa-stained blood smears. Microscopic examination showed that the proportions of RPMI-treated 3D7-K13-wild-type and Cam1-K13-wild-type schizonts were 7.5% and 20.5%, respectively, with most of the other blood stages being ring stages. For ML10-treated 3D7-K13-wild-type and Cam1-K13-wild-type parasites, the proportions of schizonts were significantly higher (~3- to 16-fold) than those for the RPMI-treated lines, regardless of the concentration of ML10: 83% and 78.5% schizonts at 50 nM, 92% and 84% at 100 nM, 89.5% and 78.5% at 150 nM, and 95% and 79.5% at 200 nM for the 3D7-K13-wild-type and the Cam1-K13-wild-type parasites,

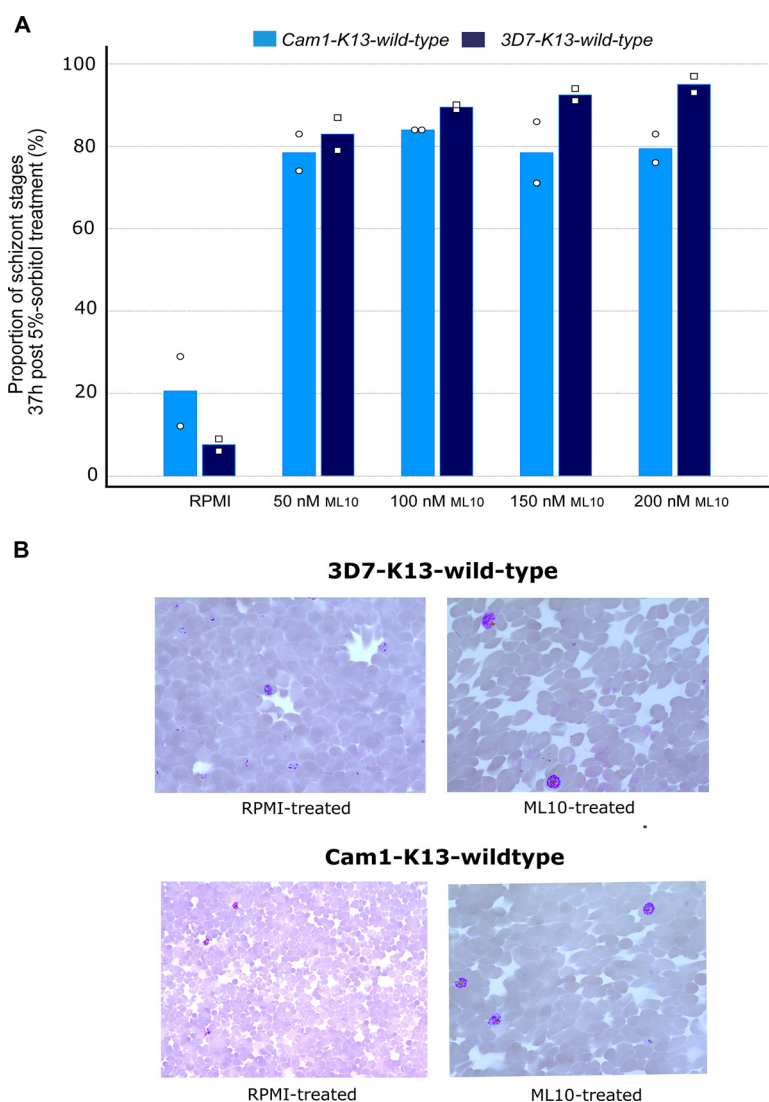


FIG 2 Effectiveness of different concentrations of ML10 for blocking merozoite egress. (A) Proportion of parasite stages (schizonts versus other blood stages) detected at 37 h post-5% sorbitol treatment for the 3D7-K13-wild-type and Cam1-K13-wild-type strains after a 17-h pulse in complete RPMI culture medium (control) or in ML10 at 50 nM, 100 nM, 150 nM, or 200 nM (data are from biological duplicates). (Detailed data are available in Table S3 in the supplemental material.) (B, upper) Giemsa-stained blood smears of 3D7-K13-wild-type parasites in complete RPMI culture medium and treated with ML10. (Lower) Giemsa-stained blood smears of Cam1-K13-wild-type in complete RPMI culture medium and treated with ML10. Each image is representative of the culture; the ML10 concentration used for ML10-treated parasites was 150 nM.

respectively. ML10 treatment, regardless of the concentration used, was highly effective at increasing the proportion of schizonts in both strains (Fig. 2). ML10 was used at 200 nM in the following experiments, as it provided a satisfactory schizont yield in all strains tested without visible toxicity to parasites.

Next, we estimated the range of exposure times to ML10 (from 17 h to 24 h) that allowed the schizonts to remain viable when treated at 200 nM. Two parasite lines (3D7-K13-wild-type and Cam2-K13-C580Y), treated with 5% sorbitol, were cultivated for 20 h and exposed to ML10 at 200 nM for 17, 20, 22, or 24 h. After ML10 exposure, parasites were isolated using 75% Percoll gradient, washed, placed in culture flasks at 37°C in 5% O₂, 5% CO₂, 90% N₂ for 3 h to allow for reinvasion, treated with 5% sorbitol to remove residual mature forms, and cultivated for an additional 24 h. Red blood cells were then collected to prepare Giemsa-stained blood smears to estimate the parasite density. All experiments were carried out in duplicate. Viable parasites were detected

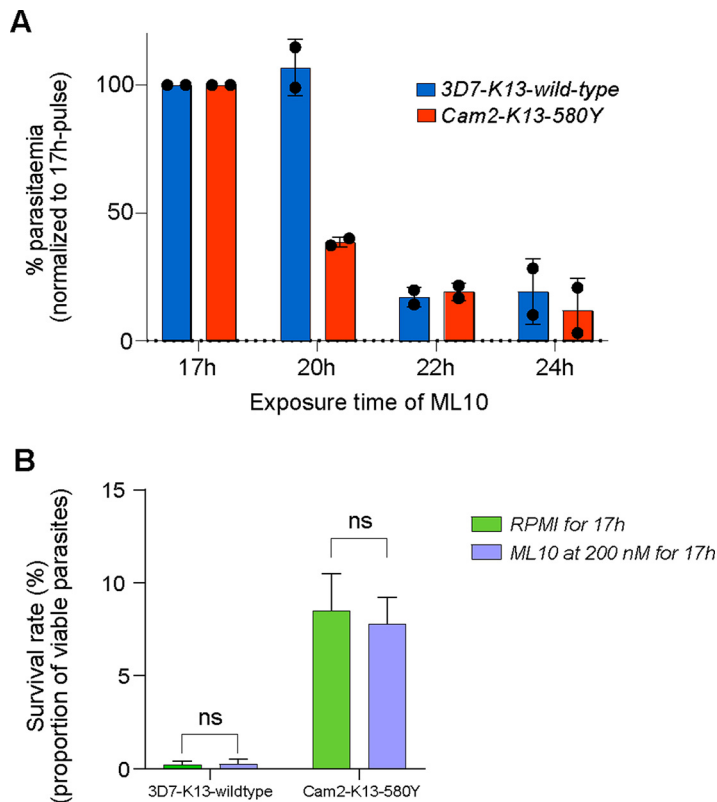


FIG 3 Impact of ML10 treatment on schizont availability. (A) Impact of the exposure time of ML10 (from 17 h to 24 h) on schizont viability. Parasitemia of 3D7-K13-wild-type and Cam2-K13-C580Y strains are expressed as a percentage relative to results of 17-h pulse exposure time (biological duplicates). (B) Impact on the survival rates expressed in the RSA^{0-3h}. Data present the survival rates (proportion of viable parasites) of 3D7-K13-wild-type (ART-S) and Cam2-K13-C580Y (ART-R) strains exposed for 17 h to complete RPMI culture medium (standard protocol) and 200 nM ML10 (modified protocol) (biological triplicates).

under all tested conditions (Fig. 3A). As expected, we noticed a decrease of the parasitemia from 17- to 24-h exposure for both strains. The observed decrease was associated with schizont death caused by prolonged egress inhibition, notably beyond 20 h of exposure to ML10. Of note, we observed under any condition that all ML10-treated parasites were highly synchronous (at trophozoite stage) for both strains. Our data suggest that ML10 treatment (from a 17- to 20-h pulse) is a practical step to produce large amounts of synchronized schizonts, especially when multiple strains are assayed simultaneously.

Finally, we estimated whether the ML10 pulse had an impact on the survival rates expressed in the RSA^{0-3h}. To this end, both 3D7-K13-wild-type (ART-sensitive) and Cam2-K13-C580Y (ART-resistant) parasites were assayed in triplicate and processed simultaneously using the standard (RPMI) or the modified protocol (ML10 at 200 nM for 17 h). We found similar survival rates between both protocols, consistent with previous published data for these *Pfkelch13* genotypes (7). For the 3D7-K13-wild-type strain, the mean survival rates (\pm standard errors of the means) were $0.20\% \pm 0.10\%$ (standard) versus $0.26\% \pm 0.10\%$ (modified; $P = 0.65$, Mann-Whitney test) and for the Cam2-K13-C580Y strain, $8.50\% \pm 1.10\%$ (standard) versus $7.80\% \pm 0.80\%$ (modified; $P = 1.0$, Mann-Whitney test) (Fig. 3B). The laboratory strains Dd2 and NF54 and three additional field isolates from Cambodia were used to validate our modified RSA protocol. The *Pfkelch13* genotypes tested were the wild type as well as three variants with mutations known to confer moderate and high levels of resistance to DHA, including a Cys-to-Tyr change at position 580 (C580Y), R622I, and R539T. As shown in Fig. S1 in the supplemental material, the survival rates of the different parasitic lines assayed were consistent with those expected from previous observations based on the *Pfkelch13* genotype. To confirm that

the viability was maintained not only for 72 h after DHA but also through successive cycles, we also followed the parasitemia of two ART-resistant strains (3D7-K13-C580Y and Cam2-K13-C580Y). Both strains remained viable and reached >2% parasitemia in 8 and 12 days for 3D7-K13-C580Y and Cam2-K13-C580Y, respectively (see Fig. S2). All of these results confirmed that ML10 does not alter the survival rate obtained with the RSA^{0-3h} and can be successfully used to test a variety of strains simultaneously, compared to the standard protocol.

We showed here that the use of ML10 can improve the standard RSA protocol for assessing ART resistance, by facilitating tight 0-to-3-h ring-stage synchronization when multiple strains that might have different period lengths are tested simultaneously (12, 13). ML10 treatment is simple to handle and does not add complex steps to the procedure, making it a convenient tool (14). This protocol constitutes a new addition to the other improvements already published regarding the RSA procedure (7, 15, 16) (see Table S2 for detailed comparisons). However, additional research is required for assessing its potential impacts on cell signaling, gene transcription, metabolomics, and epigenetic regulation.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, DOCX file, 0.1 MB.

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