Title: Modified *Plasmodium falciparum* Ring-stage Survival Assay with ML10 kinase inhibitor

Running title: Modified RSA

Lucien Platon[1,2,3], David A. Baker[4], Didier Ménard[1,3,5,6]

[1]Institut Pasteur, Université Paris Cité, Malaria Genetics and Resistance Unit, INSERM U1201, F-75015 Paris, France.

[2]Sorbonne Université, Collège doctoral ED 515 Complexité du Vivant, F-75015 Paris, France.

[3] Institut Pasteur, Université Paris Cité, Malaria Parasite Biology and Vaccines Unit, F-75015 Paris, France.

[4]Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London WC1E 7HT, UK.

[5]Université de Strasbourg, Institute of Parasitology and Tropical Diseases, UR7292 Dynamics of Host-Pathogen Interactions, F-67000 Strasbourg, France.

[6]CHU Strasbourg, Laboratory of Parasitology and Medical Mycology, F-67000 Strasbourg, France

Corresponding author: Lucien Platon (lucien.platon@pasteur.fr)

Abstract 75 words

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 0-3 hours post-invasion 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.

Main text 1249 words

Plasmodium falciparum malaria is a vector-borne parasite disease, responsible for 627,000 deaths and 241 million cases in 2021, predominantly in Sub-Saharan Africa (1). Artemisinin-based combination therapies (ACTs), that 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 (WHO) as the front-line treatment of uncomplicated *falciparum* malaria (1, 2). Unfortunately, *P. falciparum* partial resistance to artemisinin (ART-R), that emerged in Southeast Asia in 2006-2007 (3, 4) has been detected recently in Africa (5, 6). ART-R, defined as delayed parasite clearance in patients treated with artemisinin monotherapy or 3-days 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 (0-3 h post-invasion) 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 dimethyl sulfoxide (DMSO, the vehicle used to dissolve DHA)-exposed parasites (***Figure 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-hour periodicity, *in vitro* and transcriptomic studies showed that *P. falciparum* isolates

can have different period lengths that can vary substantially from 48 hours (from 36 to 54 hours) (12, 13). This difference, 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. Two parasite lines (3D7-K13-wild-type, an African laboratory strain and a culture-adapted Cambodian strain, Cam1-K13-wild-type) were used (see table S1 for the detailed strain list). The asynchronous cultures were firstly treated with 5%-sorbitol to achieve 0-12h ring-stage synchronization. The cells were cultivated for 20 hours to reach 20-32h trophozoite stage and later exposed to 50, 100, 150 and 200 nM of ML10 or complete RPMI culture medium (RPMI, used as control) for 17h in order to obtain ML10-treated mature 37-49h parasites, when the merozoite egress is happening. This range of concentrations was used to validate the ML10 safety and ensure schizonts tight synchronization 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, 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) compared to RPMI-treated lines, regardless of the concentrations of ML10: 83% and 78.5% 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 respectively. ML10 treatment, regardless of the concentrations used, was highly effective at increasing the proportion of schizonts in both strains (***Figure 2***). ML10 was used at 200 nM in the following experiments as it provides satisfactory schizont yield in all strains tested without visible toxicity to parasites.

Next, we estimated the range of the exposure time of ML10 (from 17h to 24h) allowing 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 hours and exposed to ML10 200 nM for 17, 20, 22 or 24 hours, respectively. After ML10 exposure, parasites were isolated using 75%-percoll gradient, washed, placed in culture flasks at 37°C in 5% O2,

5% CO2, and 90% N2 for 3h to allow for re-invasion, treated with 5%-sorbitol to remove residual mature forms and cultivated for additional 24h. 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 in all tested conditions (***Figure 3A***). As expected, we noticed a decrease of the parasitaemia from 17h to 24h exposure for both strains. The observed decrease was associated with schizont death caused by prolonged egress inhibition, notably beyond 20 hours exposure to ML10. Of note, we observed in any conditions, all ML10-treated parasites were highly synchronous (at trophozoite-stage) for both strains. Our data suggest that ML10 treatment (from 17h to 20h pulse) is a practical step to produce large amounts of synchronized schizonts, especially when multiple strains are assayed simultaneously.

Lastly, 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) were assayed in triplicate and processed simultaneously using the standard (RPMI) or the modified protocol (ML10 at 200 nM for 17h). 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 (±SEM) were 0.20%±0.10% (standard) vs. 0.26% ±0.10% (modified) (p=0.65, Mann-Whitney test) and for the Cam2-K13-C580Y strain, 8.50%±1.10% (standard) vs. 7.80%±0.80% (modified) (p=1.0, Mann-Whitney test) (***Figure 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 mutations known to confer moderate and high levels of resistance to DHA, including C580Y, R622I and R539T. As shown in Figure S1, 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 is maintained not only for 72 hours after DHA but also through successive cycles, we also followed the parasitaemia of two ART-resistant strains (3D7-K13-C580Y and Cam2-K13-C580Y). Both strains remain viable and reach >2% parasitemia in 8 and 12 days for 3D7-K13-C580Y and Cam2-K13-C580Y respectively (see Figure S2). All of these results confirm that ML10 does not alter the survival rate obtained with RSA0-3h and can be successfully used to test a variety of strains simultaneously compared to the standard protocol.

We show here that the use of ML10 can improve the standard RSA protocol for assessing ART-resistance, by facilitating tight 0-3 h ring stage synchronization when multiple strains that might have different period length 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 (15-17) (see supplementary table S2 for detailed comparisons). However, additional research is required for assessing its potential impacts on cell signaling, gene transcriptions, metabolomics, and epigenetic regulation.

Acknowledgment.

We would like to thank Simon Osborne from LifeArc for supplying ML10. ML10 is available at MR4 (BEI Resources) https://www.beiresources.org/Collection/54/MR4-Malaria-Resources.aspx?f_displaysearchname=Proteins%23%7E%23Monoclonal%2BAntibodies&page=1

References

1. World Health Organization. 2021. World Malaria Report 2021.

2. Nosten F, White NJ. 2007. Artemisinin-based combination treatment of falciparum malaria. Am J Trop Med Hyg 77:181-92.

3. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 361:455-67.

4. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, Artemisinin Resistance in Cambodia 1 Study C. 2008. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med 359:2619-20.

5. Balikagala B, Fukuda N, Ikeda M, Katuro OT, Tachibana SI, Yamauchi M, Opio W, Emoto S, Anywar DA, Kimura E, Palacpac NMQ, Odongo-Aginya EI, Ogwang M, Horii T, Mita T. 2021. Evidence of Artemisinin-Resistant Malaria in Africa. N Engl J Med 385:1163-1171.

6. Uwimana A, Legrand E, Stokes BH, Ndikumana JM, Warsame M, Umulisa N, Ngamije D, Munyaneza T, Mazarati JB, Munguti K, Campagne P, Criscuolo A, Ariey F, Murindahabi M, Ringwald P, Fidock DA, Mbituyumuremyi A, Menard D. 2020. Emergence and clonal expansion of in vitro artemisinin-resistant *Plasmodium falciparum* kelch13 R561H mutant parasites in Rwanda. Nat Med 26:1602-1608.

7. Kite WA, Melendez-Muniz VA, Moraes Barros RR, Wellems TE, Sa JM. 2016. Alternative methods for the *Plasmodium falciparum* artemisinin ring-stage survival assay with increased simplicity and parasite stage-specificity. Malar J 15:94.

8. Straimer J, Gnadig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, Dacheux M, Khim N, Zhang L, Lam S, Gregory PD, Urnov FD, Mercereau-Puijalon O, Benoit-Vical F,

Fairhurst RM, Menard D, Fidock DA. 2015. Drug resistance. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. Science 347:428-31.

9. Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, Lim P, Mao S, Sopha C, Sam B, Anderson JM, Duong S, Chuor CM, Taylor WR, Suon S, Mercereau-Puijalon O, Fairhurst RM, Menard D. 2013. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. Lancet Infect Dis 13:1043-9.

10. Zhang J, Feng GH, Zou CY, Su PC, Liu HE, Yang ZQ. 2017. Overview of the improvement of the ring-stage survival assay-a novel phenotypic assay for the detection of artemisinin-resistant *Plasmodium falciparum*. Zool Res 38:317-320.

11. Lambros C, Vanderberg JP. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 65:418-20.

12. Smith LM, Motta FC, Chopra G, Moch JK, Nerem RR, Cummins B, Roche KE, Kelliher CM, Leman AR, Harer J, Gedeon T, Waters NC, Haase SB. 2020. An intrinsic oscillator drives the blood stage cycle of the malaria parasite *Plasmodium falciparum*. Science 368:754-759.

13. Wockner LF, Hoffmann I, Webb L, Mordmuller B, Murphy SC, Kublin JG, O'Rourke P, McCarthy JS, Marquart L. 2020. Growth Rate of *Plasmodium falciparum*: Analysis of Parasite Growth Data From Malaria Volunteer Infection Studies. J Infect Dis 221:963-972.

14. Ressurreicao M, Thomas JA, Nofal SD, Flueck C, Moon RW, Baker DA, van Ooij C. 2020. Use of a highly specific kinase inhibitor for rapid, simple and precise synchronization of *Plasmodium falciparum* and *Plasmodium knowlesi* asexual blood-stage parasites. PLoS One 15:e0235798.

15. Amaratunga, C., Neal, A. T. & Fairhurst, R. M. 2014. Flow Cytometry-Based Analysis of Artemisinin-Resistant *Plasmodium falciparum* in the Ring-Stage Survival Assay. Antimicrob Agents Chemother 58, 4938–4940.

16. Kite, W. A., Melendez-Muniz, V. A., Moraes Barros, R. R., Wellems, T. E. & Sá, J. M. 2016. Alternative methods for the *Plasmodium falciparum* artemisinin ring-stage survival assay with increased simplicity and parasite stage-specificity. Malar J 15, 94.

17. Davis, S. Z., Singh, P. P., Vendrely, K. M., Shoue, D. A., Checkley, L. A., McDew-White, M., Button-Simons, K. A., Cassady, Z., Sievert, M. A. C., Foster, G. J., Nosten, F. H., Anderson, T. J. C., Ferdig, M. T. 2020. The Extended Recovery Ring-Stage Survival Assay Provides a Superior Association with Patient Clearance Half-Life and Increases Throughput. Malar J, 19, 54.

Figure legends

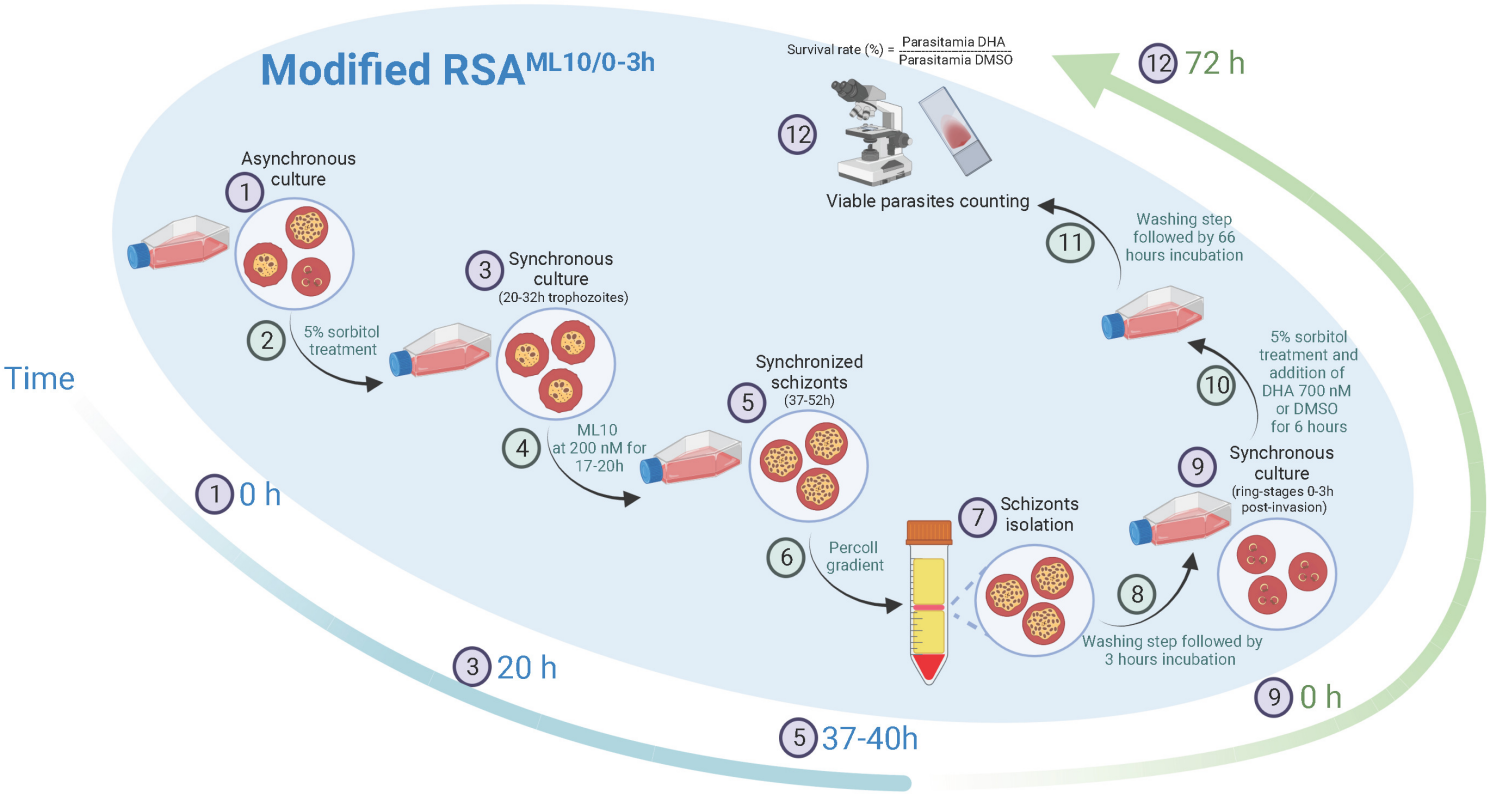
Figure 1: Overview of the Ring-stage Survival Assay. 1) Parasites were cultivated to reach 1% parasitaemia (all blood stages); 2) Parasites were treated with 5%-sorbitol to eliminate mature parasites and preserving ring-stages (~0 to 12 hours post invasion); 3) Parasites were cultivated for 20 hours to reach 20-32h trophozoites and exposed to ML10 at 200 nM for 17 to 20 hours; 4) Schizonts (~37-49h) were isolated using 75%-percoll gradient. Red blood cells were then washed to remove ML10 and cultivated with fresh blood cell for 3 hours for reinvasion; 5) Synchronous 0-3 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 dihydroartemisinin (DHA) or 0,1% Dimethyl sulfoxide (DMSO) (control), then washed and cultivated for additional 66 h (72 h total); 7) Red blood cells were collected and used to prepared Giemsa-stained blood smears. The mean survival was then calculated as following: $(Parasitemia\ DHA)\ /\ (Parasitemia\ DMSO)\ *100$

Figure 2. Effectiveness of different concentrations of ML10 for blocking merozoite egress.

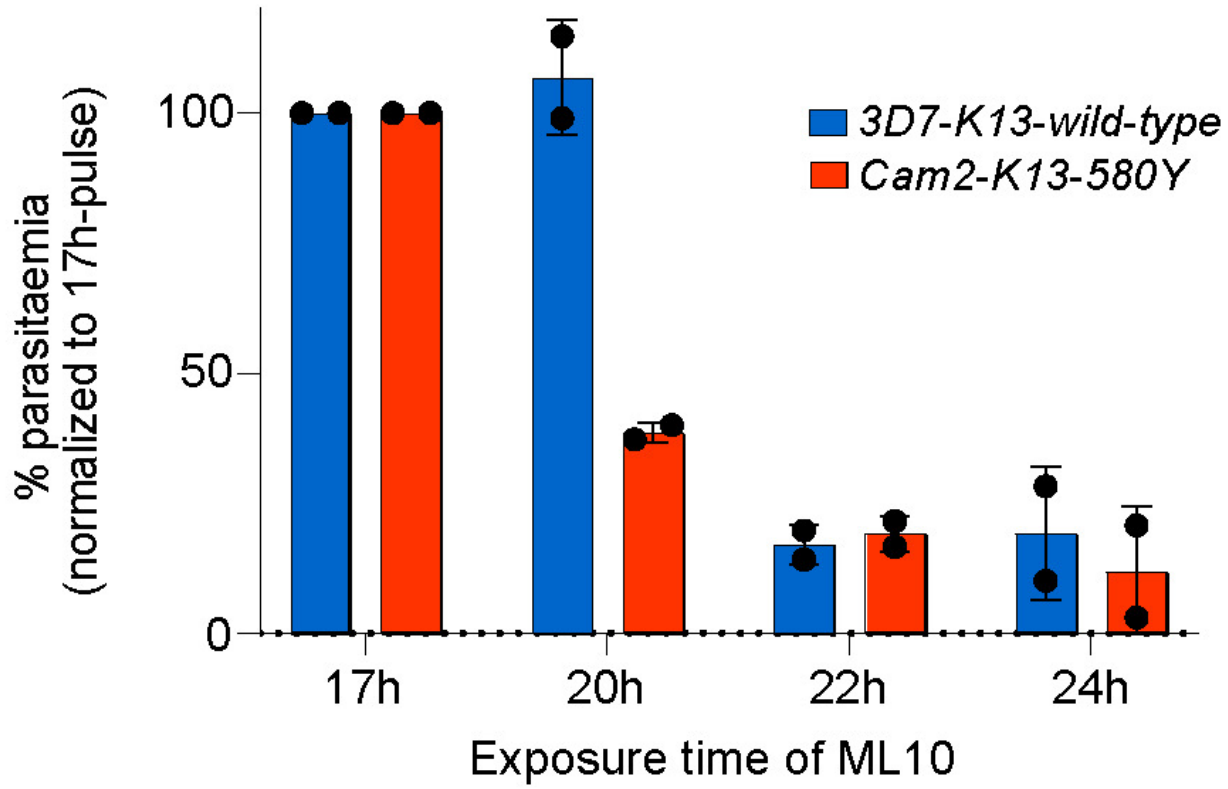
Panel A. Proportion of the parasite stages (schizonts vs. other blood stages) detected at 37 h post 5%-sorbitol treatment for the 3D7-K13-wild-type and Cam1-K13-wild-type strains after 17 h pulse complete RPMI culture medium (control) or ML10 at 50 nM, 100 nM, 150 nM and 200 nM (data from biological duplicate, data are available in Table S3).

Panel B. Upper. Giemsa-stained blood smears of 3D7-K13-wild-type complete RPMI culture medium-treated and ML10-treated. Lower. Giemsa-stained blood smears of Cam1-K13-wild-type complete RPMI culture medium-treated and ML10-treated. Each image is representative of the culture, the concentration used for ML10 treated parasites is 150 nM.

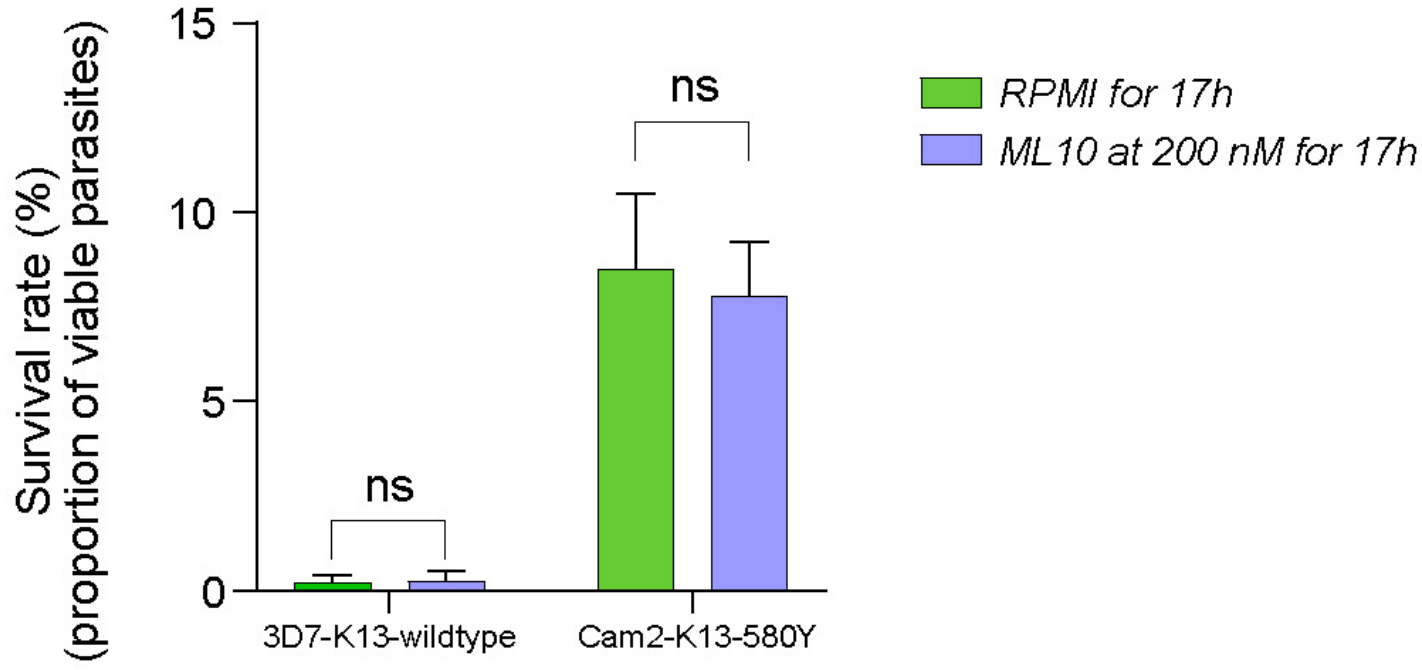
Figure 3: Impact of ML10 treatment on schizont availability. *Panel A*. Impact of the exposure time of ML10 (from 17 h to 24 h) on schizonts viability. Parasitaemia of 3D7-K13-wild-type and Cam2-K13-C580Y are expressed as percentage compared to 17 hours pulse exposure time (biological duplicates). *Panel 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).


Modified RSA ML10/0-3h
Survival rate (%) = Parasitamia DHA / Parasitamia DMSO
12 72 h
12
Viable parasites counting
11
Washing step followed by 66 hours incubation
10
5% sorbitol treatment and addition of DHA 700 nM or DMSO for 6 hours
9
Synchronous culture
(ring-stages 0-3h post-invasion)
8
Washing step followed by 3 hours incubation
7
Schizonts isolation
6
Percoll gradient
5
Synchronized schizonts
(37-52h)
4
ML10 at 200 nM for 17-20h
3
Synchronous culture
(20-32h trophozoites)
2
5% sorbitol treatment
1
Asynchronous culture
Time
1 0 h
3 20 h
5 37-40h
9 0 h

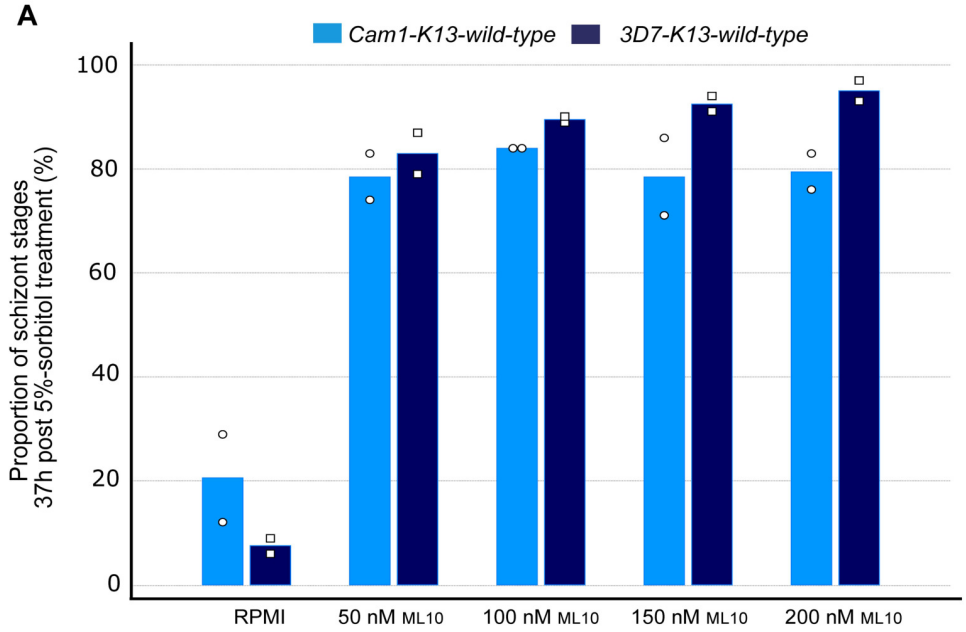
A


% parasitaemia
(normalized to 17h-pulse)
100
50
0
3D7-K13-wild-type
Cam2-K13-580Y
17h
20h
22h
24h
Exposure time of ML10

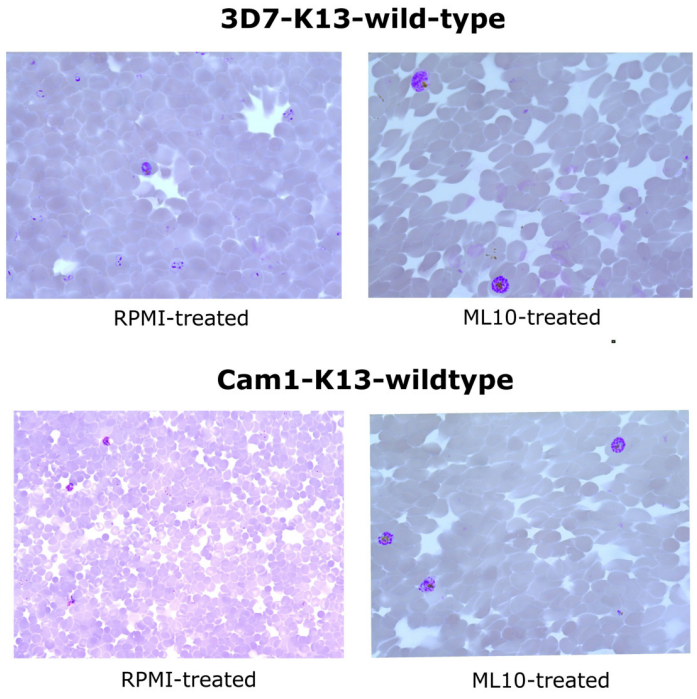
B


Survival rate (%)
(proportion of viable parasites)
15
10
5
0
ns
ns
RPMI for 17h
ML10 at 200 nM for 17h
3D7-K13-wildtype
Cam2-K13-580Y

A


Cam1-K13-wild-type
3D7-K13-wild-type
Proportion of schizont stages
37h post 5%-sorbitol treatment (%)
100
80
60
40
20
0
RPMI
50 nM ML10
100 nM ML10
150 nM ML10
200 nM ML10

B


3D7-K13-wild-type
RPMI-treated
ML10-treated
Cam1-K13-wildtype
RPMI-treated
ML10-treated