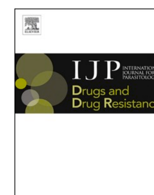
International Journal for Parasitology: Drugs and Drug Resistance 17 (2021) 150–155

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr



MEFAS, a hybrid of artesunate-mefloquine active against asexual stages of *Plasmodium vivax* in field isolates, inhibits malaria transmission

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ARTICLE INFO

Keywords:
Plasmodium vivax
Plasmodium falciparum
Transmission blocking
Drug resistant
Antimalarial
New drugs

ABSTRACT

Human malaria continues to be a public health problem and an important cause of morbidity and mortality in the world. Malaria control is achieved through both individual protection against mosquito bites and drug treatment, which is hampered by the spread of *Plasmodium falciparum* resistance to most antimalarials, including artemisinin derivatives. One of the key pharmacological strategies for controlling malaria is to block transmission of the parasites to their mosquito vectors. Following this rational, MEFAS, a synthetic hybrid salt derived from artesunate (AS) and mefloquine has been previously reported for its activity against asexual *P. falciparum* parasites *in vitro*, in addition to a pronounced reduction in the viability of mature gametocytes. Herein, MEFAS was tested against asexual forms of *Plasmodium vivax* and for its ability to block malaria transmission in *Anopheles darlingi* mosquitoes in a membrane feeding assay using *P. vivax* field isolates. MEFAS demonstrated high potency, with a IC50 of 6.5 nM against asexual forms of *P. vivax*. At 50 μM, MEFAS completely blocked oocyst formation in mosquitoes, regardless of the oocyst number in the control group. At lower doses, MEFAS reduced oocyst prevalence by greater than 20%. At equivalent doses, AS irregularly reduced oocyst formation and caused only slight inhibition of mosquito infections. These results highlight the potential of MEFAS as a novel transmission-blocking molecule, as well as its high blood schizonticidal activity against *P. vivax* and *P. falciparum* field isolates, representing a starting point for further development of a new drug with dual antimalarial activity.

1. Introduction

Among the estimated 815,543 cases of malaria in the Americas, 22% were in Brazil; of those, 76% were caused by *Plasmodium vivax* (World Malaria Report, 2020). The most important current strategies for combating malaria transmission include diagnosis and treatment of the infected patients with artemisinin-based combination therapy (Naing et al., 2013; WHO, 2015a).

Artemisinin Combined Therapy (ACTs) is active against asexual blood stages and gametocytes have also shown a beneficial impact on decreasing transmission (Stepniewska et al., 2008; Bousema et al., 2010). In previous *in vitro* drug screening assays using Mefloquine (MQ) it was shown that it affects gametocyte maturation, which would explain why after initiation of ACTs regimens using a combination of MQ, it causes consequences for gametocytemia post-treatment (Bolscher et al., 2015).

A meta-analysis carried in which the effect of a combination of artesunate (AS) and lumefantrine (AL) suggests that AL is superior to non-ACT regimens in reducing gametocyte carriage and interrupting transmission in individuals with uncomplicated falciparum malaria (Ippolito et al., 2020). Previous studies have shown that the combinations of AS and MQ and AL are superior ACT options in preventing gametocytes soon after treatment compared to non-ACT treatments (WWARN, 2016).

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https://doi.org/10.1016/j.ijpddr.2021.09.003
Received 19 December 2020; Received in revised form 9 September 2021; Accepted 15 September 2021
Available online 22 September 2021
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In this study the appearance of gametocytemia was lowest after AS-MQ and AL and significantly higher after dihydroartemisinin-piperaquine. Among individuals who had gametocytemia before treatment, gametocytemia clearance was significantly faster with AS-MQ and slower with dihydroartemisinin-piperaquine compared to AL (WWARN, 2016).

However, the widespread parasite resistance characterized by delays in parasite clearance in patients after administration of artemisinin derivatives (Noedl et al., 2008; Dondorp et al., 2009; Phyo et al., 2012; Cheeseman et al., 2012), threatens the efficacy of current drug treatments (Dondorp et al., 2017). The development of new antimalarials with activity comparable to artemisinin that block mosquito infections and transmission will aid in global efforts to eliminate malaria (Bousema et al., 2010; Noedl et al., 2008; Dondorp et al., 2009; Kuhen et al., 2014; Delves et al., 2018).

Tafenoquine is a long-acting 8-aminoquinoline (Llanos-Cuentas et al., 2014) recently registered by the Food and Drug Administration as a single-dose treatment for the radical cure of *P. vivax* (Lacerda et al., 2012). Although tafenoquine blood schizonticidal activity has been demonstrated in nonhuman primate models (Obaldia et al., 1997), there is still no strong evidence proving its schizonticidal or gametocytocidal activity against *Plasmodium falciparum* or *P. vivax* in humans (Fukuda et al., 2017). However, there are ongoing clinical studies to analyze this hypothesis (https://clinicaltrials.gov/ct2/show/NCT04609098).

MEFAS is a single organic substance that contains MQ base associated with AS as an organic salt ($MQ^{+}AS^{-}$). MEFAS is considered a promising new compound for the following reasons: (i) it has a strong schizonticidal effect against *P. falciparum* chloroquine-resistant (clone W2) and chloroquine-sensitive strain (3D7) and cures malaria in mice with blood-induced infection by *Plasmodium berghei* (de Pilla Varotti et al., 2008); and (ii) it shows transmission blocking potential against *P. falciparum* mature gametocytes *in vitro* (Penna-Coutinho et al., 2016). To date, MEFAS activity against asexual forms of *P. vivax* and the development of this parasite in the mosquito vector has not been studied, which was one of the objectives of the present work.

Using *P. vivax* isolates from patients in the Brazilian Amazon and an *Anopheles darlingi* colony maintained in Porto Velho, Rondônia, MEFAS activity was evaluated against *P. vivax* using asexual forms in a schizont maturation assay (SMA), as well as the sexual stages in a membrane feeding assay (MFA) with *An. darlingi* mosquitoes, the primary vector of malaria in the Amazon Region (Tadei et al., 2000; Pimenta et al., 2015).

2. Materials and methods

2.1. Ethical approval

This study was approved by the Ethics Committee from *Centro de Pesquisa em Medicina Tropical de Rondônia* (CEPEM) (CAAE 61442416.7.0000.0011) for tests of experimental MEFAS activity against asexual forms of *P. vivax* and *P. falciparum*. The protocol to investigate MEFAS activity against sexual forms was approved by the Ethics Committee from the *Instituto René Rachou* - FIOCRUZ (CAAE – 67011617.8.0000.5091). All participants signed a written informed consent before blood collection.

2.2. Blood donors for experiments with asexual and sexual forms of P. vivax

Adults volunteers (≥18 years old), microscopically diagnosed with *Plasmodium* at the CEPEM, in the city of Porto Velho/Brazil, were invited to participate in the study. Patients who had used antimalarial treatment in the previous month and/or those who presented severe symptoms of malaria were excluded from this study. Mono-infected patients, with either *P. falciparum* or *P. vivax*, having parasitaemia between 2,000 and 80,000 parasites/μL with at least 70% ring stage parasites were recruited specifically for the experiments with asexual forms of *P. vivax*. Patients with *P. vivax* circulating gametocytes were recruited for the MFA experiments.

Approximately 10 mL of blood were collected from each volunteer by venous puncture in heparinized tubes and placed in a constant 37 °C water bath to avoid gametogenesis until the time experiments were performed. Immediately after blood collection, all patients were treated for *P. vivax* or *P. falciparum* infection following the guidelines of the Brazilian Health Ministry (Brasil, 2019).

2.3. Malaria diagnosis and parasite densities

Malaria diagnosis was performed by microscopy in thick smears prepared using finger-prick blood collected immediately before the test, stained with 10% Giemsa and examined for the presence of sexual and asexual parasites. Results were independently confirmed by two well trained microscopists, and inconsistencies were solved by a senior microscopist. The number of asexual parasites and mature gametocytes were counted under light microscopy using a 100X oil immersion lens and was estimated relative to 200 white blood cells. Gametocytemia was expressed as the number of gametocytes per microliter (μL) of blood, assuming leukocyte counts of 6000/μL of blood.

2.4. Pre-dosed plates containing test and control drugs for SMA

MEFAS, covered by an international patent application (Boechat et al., 2005) and antimalarial AS were both synthesized at the *Instituto de Tecnologia e Fármacos* (Farmanguinhos/FIOCRUZ) on a large scale, where they were also evaluated for chemical purity. Antimalarial MQ was purchased from Sigma (USA).

MEFAS, MQ and AS were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and diluted in RPMI in 96-well plates (20 μL per well) with variable maximum drug concentration, according to each previously determined activity, i.e., chloroquine (CQ) (5000 nM), MQ (1000 nM) and AS (1000 nM). A serial dilution factor of two was then performed to obtain 11 different test concentrations. Next, plates were stored at 4 °C until further use. Each lot of plates was assayed for quality control based on the profile response of antimalarials controls to the *P. falciparum* 3D7 laboratory strain to the *P. falciparum* 3D7 laboratory strain.

2.5. Schizont maturation assay (SMA)

The drug susceptibility of *P. vivax* malaria parasites was measured *ex vivo* as previously described (Renapurkar et al., 1989) with modifications. Briefly, white blood cells were removed by filtration in a CF 11 cellulose column as previously described (Sriprawat et al., 2009). Immediately before the assays, the packed red blood cells containing parasites (iRBC) were diluted to 2% hematocrit using McCoy's 5A medium plus 20% AB human serum. The infected red blood cells (iRBC) (200 μL per well) were distributed in predosed drug plates with the compounds in a final concentration, i.e., MEFAS (500–0.048 nM), MQ (100–0.097 nM) and AS (100–0.097 nM). For maturation of the ring stages to schizonts, plates with parasites were maintained in a gas mixture (90% N_2, 5% O_2 e 5% CO_2), at 37 °C as previously described (Trager and Jensen, 1976). The control wells were iRBCs cultured with drug-free complete medium. A total of 6 extra control wells were prepared for evaluation of the schizont maturation, in order to conclude the experiment. The parasite-drug incubation was stopped only when 40% of the ring stages had reached the schizont stage (at least four distinct nuclei per parasite) in the drug-free control wells. Thick blood films were then made from each well, dried, stained with 5% Giemsa solution for 30 min, and examined microscopically.

The number of schizonts per 100 asexual stage parasites was determined for each drug concentration and normalized by comparing with the schizont number in the drug-free control wells (considered 100%).

2.6. Drug dilution for the membrane feeding assay (MFA)

For experiments with sexual stages of *P. vivax,* MEFAS and AS drugs were first diluted in DMSO in 10 mM stock solution followed by dilution with the vehicle (RPMI 1640 medium supplemented with 10% human serum) to reach the final test concentrations. Diluted drugs and the vehicle were added to 15 mL Falcon tubes and mixed with 1 mL of *P. vivax*-infected blood (final DMSO concentration of 0.5%) known to be non-toxic (Ziegler *et al.*, 2017), which was then offered as a blood meal to the mosquitoes in the MFA.

Concentrations of MEFAS (10, 25 and 50 μM) and AS (5, 12.5 and 25 μM) were stoichiometrically equivalent, considering that the MEFAS hybrid salt contains two antimalarial functionalities, i.e., one quinolinic ring from MQ and one endoperoxide ring from AS (de Pilla Varotti et al., 2008; Prado et al., 2020).

Drug concentrations were determined considering the limitation of *P. vivax* field isolates and the absence of incubation time of drug with the parasite. The pilot experiment was with An. *darlingi* colony at Iquitos Peru using MEFAS at 5 μM (Moreno et al., 2014). *P. vivax* donors in this case were enrolled at the outpatient department of the Hospital Apoyo Iquitos, Peru (Amazonia-ICEMR: International Centers of Excellence for Malaria Research, approval number 2000024610). The other drug concentrations tested were performed using An. *darlingi* colony established at Porto Velho, Brazil (Araujo et al., 2019), as described below and higher concentrations of all test and control drugs.

2.7. *Anopheles darlingi* mosquito colony

The *An. darlingi* laboratory-established colony was maintained at FIOCRUZ-RO in an acclimatized insectary at 26 ± 1 °C and 70 ± 10% relative humidity with a photoperiod of 12:12 h light/dark cycle. Larvae were reared in pans (30.3 × 22.1 × 7.5 cm) containing approximately 200 larvae each and fed daily with finely powdered fish food (Tetra Marine Granules®). Pupae were transferred daily using Pasteur pipettes (3 mL) into small plastic containers containing distilled water placed in a screened cage (46 x 46 × 46 cm) for adult emergence. Adult mosquitoes were maintained in 15% honey solution *ad libitum.* Premated three to five-day old adult females were blood-fed in a chicken (Moreno et al., 2014; Araujo et al., 2019). F09 and F10 colony generations were used for the present studies.

2.8. Blood membrane feeding assay (MFA) and oocyst quantification

Four- and five-day old *An. darlingi* female mosquitoes deprived of sucrose overnight were placed in small plastic cups with approximately 50 specimens each and were subsequently used for the feeding assays. Working as quickly as possible, heparinized blood from each human volunteer with malaria was mixed with the vehicle (control), MEFAS or AS at various concentrations and immediately transferred to 1.5 cm diameter discs (Hemotek Systems) fitted with a parafilm membrane and maintained at 37 °C to avoid gametocyte exflagellation. Mosquitoes were allowed to feed for 30 min in the dark, and then, fully engorged females were transferred to different mosquito cages and maintained in the insectary with 15% honey solution *ad libitum* until the day of dissection.

On day seven after the infectious blood meal, midguts from the surviving females were dissected in phosphate buffered saline (PBS 1 X) and stained with 0.2% mercurochrome, then microscopically examined (40X objectives) under a glass coverslip. The presence of oocysts was registered in each dissected midgut and the number of oocysts was counted per midgut.

2.9. Data analysis

The half-maximal drug inhibitory response (IC_{50}) in the SMT was estimated in a curve fitting software (OriginLab Corporation, Northampton, MA, USA). The statistical analysis was conducted in Prism 9 (GraphPad Software, San Diego, USA) using the *t*-test to evaluate possible correlations between continuous skewed data with a significance level of ≤0.05.

The transmission-reduction activity - TRA (% intensity inhibition) and transmission-blocking activity - TBA (% prevalence inhibition) of MEFAS and AS on parasite sporogony were calculated as: 100 x [100 - (mean number of oocysts in the test group)/(mean number of oocysts in control group)] and 100 x [100 - (proportion of mosquitoes with oocysts in the test group)/(proportion of mosquitoes with oocysts in the control group)], respectively. After calculating the mean of TRA and TBA of each repeat, the Shapiro-Wilk test was used to assess data normality. Once normality assumptions were satisfied, the parametric unpaired T-test was used to verify the difference between AS and MEFAS groups of each concentration. Prism 9 (GraphPad Software, San Diego, USA) was used to draw the graphs plotting the % TRA and TBA. Data presented are the mean of three independent experiments for each drug concentration.

3. Results

3.1. MEFAS is a potent inhibitor of *P. vivax* asexual forms in the blood

The potential of MEFAS to inhibit asexual forms of *P. vivax* was evaluated using blood collected from *P. vivax* mono-infected patients. MEFAS and standard antimalarials (AS and MQ) were assayed on 10 *P. vivax* fresh isolates, all of which had achieved schizont maturation. The initial features of these patients are summarized in Table 1. The processing time of all blood samples was approximately 55 min, and parasitemia ranged from 2,090–13,246 parasites/μL. Suitable parasite growth for harvest of the isolates was achieved in 100% of samples. All 10 *P. vivax* isolates included in the study were incubated with the tested drugs ≥40 h.

The aim of this study was to demonstrate the activity of MEFAS against *P. vivax*. IC_{50} median values for the *P. vivax* isolates are shown in Table 2 and Fig. 1. MEFAS presented a median IC_{50} of 6.5 nM against *P. vivax* isolates. The isolates were sensitive to AS, with an IC_{50} of 4.5 nM, and to MQ, with a median value of 15.1 nM. Values against blood stages of *P. vivax* isolates were similar to those found for the *P. falciparum* 3D7 lab strain, indicating that MEFAS has equivalent power in both species. In addition, MEFAS was active against seven *P. falciparum* fresh isolates, with an IC_{50} of 5.2 nM (see Table S1 for data).

3.2. MEFAS is a potential inhibitor of *P. vivax* sporogonic development in *An. darlingi* mosquitoes

MEFAS was initially tested at 5 μM using An. *darling* colony from Iquitos, Peru. The results show less than 5% of transmission blocking activity against *P. vivax* sporogonic development (Fig. S1 and Table S2). Further experiments were performed using 10, 25 and 50 μM of MEFAS in An. *darling* colony in Porto Velho, Rondônia, Brazil. MEFAS at 50 μM completely inhibited *P. vivax* sporogony development in *An. darlingi* ($p < 0.05$), while AS, at the equivalent concentration (25 μM) resulted in TBA of only 26.95%. Moreover, even without statistical difference of TRA between MEFAS (50 μM) and AS (25 μM), the hybrid of artesunate-

Table 1
Characteristics of all isolates of *Plasmodium vivax* for *ex vivo* assays.

Baseline characteristics	*Plasmodium vivax*
Isolates reaching harvest (number/%)	10 (100%)
Initial parasites/μL mean (range)	6,756 (2,090–13,246)
Delay from venipuncture to start of the culture, mean (range)	55 (40–70 min)
Duration of the assay, mean (range)	45 h (40–52 h)
Parasite at ring stage, mean (range)	90% (82–100%)
Schizont count at harvest, mean (range)	58 (49–70)

Table 2
Plasmodium vivax ex vivo susceptibility to MEFAS and to control antimalarial drugs Artesunate (AS) and Mefloquine (MQ).

Compound	*Plasmodium falciparum* 3D7 IC_{50} nM*	*Plasmodium vivax* clinical field isolates
		Median (range) IC_{50} nM
MEFAS	2.1 ± 0.9	6.5 (2.3–12.9; 3.78 and 8.22)
AS	4 ± 1	4.5 (1.2–11; 2.21 and 5.79)
MQ	13 ± 3	15.1 (11.7–22; 13.07 and 16.93)

There were no significant differences between MEFAS and AS activities. IC_{50} levels against *P. falciparum* were not significantly different from those for *P. vivax*. *A total of 3 experiments in triplicate were performed to determine the IC_{50}.

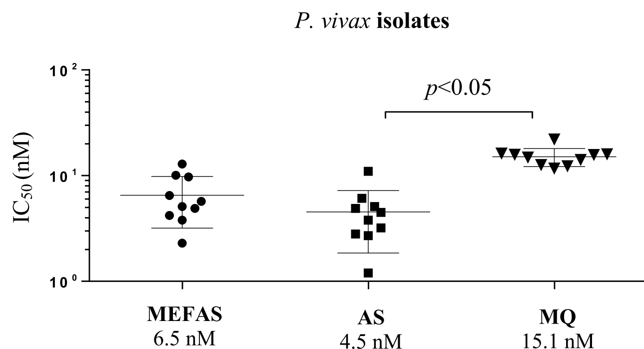

Fig. 1. *P. vivax ex vivo* susceptibility (IC_{50} values) to the control drugs (AS and MQ) and the MEFAS test compound. Median values per group are shown in each graph below the compound name. MQ was significantly less active than MEFAS and AS. No significant differences were found between the IC_{50} values of MEFAS and AS.

mefloquine also showed 100% of TRA in all three repeats (Fig. 2A, Table S3).

MEFAS concentrations of 25 μM and 10 μM did not show a statistical difference between AS, at the equivalent concentration (12.5 μM and 5 μM, respectively). However, the mean of each repeat showed higher activity when MEFAS was used (Fig. 2B and C). MEFAS at 25 μM showed a TBA of 22.83%, while the AS groups' TBA did not show any block activity (Fig. 2B). The TRA of sporogony in the mosquitoes fed with MEFAS (25 μM) treated-blood was high in all groups; only in one experiment using mosquitoes fed with the AS (12.5 μM) treated-blood the TRA was higher than 50% (Table S3).

Surprisingly, MEFAS used in a concentration of 10 μM showed a higher mean of TRA and TBA than the concentration of 25 μM (Fig. 2B and 2C and Table S3). Probably due to the mean of gametocytemia of the patients tested for each concentration (data not shown).

Blood treated with either MEFAS or AS did not modify the mosquito survival: there was less than 41% mortality for MEFAS group; whereas less than 37% mortality was observed in the AS group and control group fed with blood treated only with vehicle (Fig. 2).

4. Discussion

Malaria is the deadliest and most prevalent parasitic disease in the world, and although fatalities are primarily due to *P. falciparum* infections, *P. vivax* is responsible for higher morbidity related to severe malaria (Anstey et al., 2009; Lacerda et al., 2012). *P. vivax* is a human malaria parasite with a dormant hypnozoite stage in the liver which may cause multiple relapses after a primary infection. A main challenge for malaria elimination is the lack of safe anti-relapse drugs (Olliaro et al., 2016) and drugs which inhibit transmission of this parasite. Identification of new compounds that exhibit a dual-stage activity, i.e., is able to cure the disease and block malaria transmission, is an important strategy for the development of new antimalarials (Raphemot et al., 2016; Burrows et al., 2017).

In spite of the difficulties to work with the blood feeding membrane assay, we performed tests with MEFAS in two different conditions, in malaria endemic areas in South America. The initial preliminary test was performed in Iquitos, Peru, using a small dose of MEFAS; most of the assays were performed in Porto Velho, RO, Brazil. All patients came to the malaria laboratories directly to search for the malaria diagnosis and treatment; this avoid the blood transport from the field to the local of mosquitoes blood meal; making the tests feasible and reliable.

MEFAS has been previously tested against *P. falciparum* W2, a multidrug resistant strain maintained in continuous blood cultures, as well as against malaria in mice infected with *P. berghei* blood parasites. In both tests, MEFAS was more effective than a combination of AS and MQ (de Pilla Varotti et al., 2008), which is used in endemic areas of malaria in Brazil. In recent work, the acute toxicity of MEFAS and its precursors has been investigated in mice acutely administered with these compounds, demonstrating that MEFAS is safer than the combination of AS and MQ (de Lima et al., 2019). In addition, a new synthetic method employing a mechanochemical reaction, considered an environmentally friendly method of synthesis, has been successfully used to synthesize MEFAS, and this method has shown an additional advantage of cost reduction with respect to drug synthesis (Prado et al., 2020).

In the present work, MEFAS activity was tested against the asexual blood forms of *P. vivax* and *P. falciparum* in *ex-vivo* tests using fresh parasite isolates. Drugs eliminating the asexual forms of the parasites that also inhibit transmission are important since circulating strains of *Plasmodium* currently suffer selective driving forces, such as drug pressure and various patterns of genetic recombination, which may affect the phenotypic pool of parasites in a given region at any time point. Therefore, it is essential to confirm the antiplasmodial activity of new drug candidates presently used in tests against the current strains of parasite infecting at-risk populations to ensure the widest potential applicability of the final antimalarial drug to be developed.

Herein, it was clearly demonstrated for the first time that MEFAS exhibits important *ex-vivo* activity against *P. vivax* sexual forms in mosquitoes *An. darlingi* laboratory reared, previously fed on blood isolates from malaria patients immediately mixed with the drug. Due to lack of reproducible *in vitro* and *in vivo* models to *P. vivax-An. darlingi* infections it has been difficult to identify new compounds that block parasite development; the attention to drugs that block *Plasmodium* transmission has resulted in attempts to identify new antimalarial drugs (Ponsa et al., 2003; Wells et al., 2009) or vaccines (Carter, 2001; Saul, 2008) able to disrupt the parasite life cycle in the mosquito vector.

Despite the considerable variation of gametocyte density, consistent results were obtained between replicates, substantiating the specificity of MEFAS activity and providing support for the robustness of the *P. vivax* field isolate model in the MFA. The results demonstrating a transmission blocking activity of MEFAS against the human parasite *P. vivax* are somewhat similar to our previous studies with *P. falciparum* in which MEFAS inhibited the development of mature gametocyte *in vitro* (Penna-Coutinho et al., 2016). The present data suggest that MEFAS has a profound effect on the final and critical steps of gametocyte maturation and exflagellation of *P. vivax* in *An. darlingi* sporogonic cycle in mosquitoes, as herein demonstrated.

In previous work, it was demonstrated that MEFAS increased the levels of cytoplasmic Ca^{2+} in *P. falciparum* (de Pilla Varotti et al., 2008), which is involved in several biological processes in *Plasmodium* development, including regulation of its sexual differentiation in the mosquito vector (Billker et al., 2004). The increase in Ca^{2+} is responsible for the following: (i) the response of *P. berghei* gametocytes to stimulation with xanthurenic acid (Billker et al., 2004); (ii) the parasite in the cell cycle and release of flagellated microgametes (Alano and Billker, 2005); and (iii) the emergence of male and female gametocytes from the host cell (Billker et al., 2004). The differentiation of later stage gametocytes

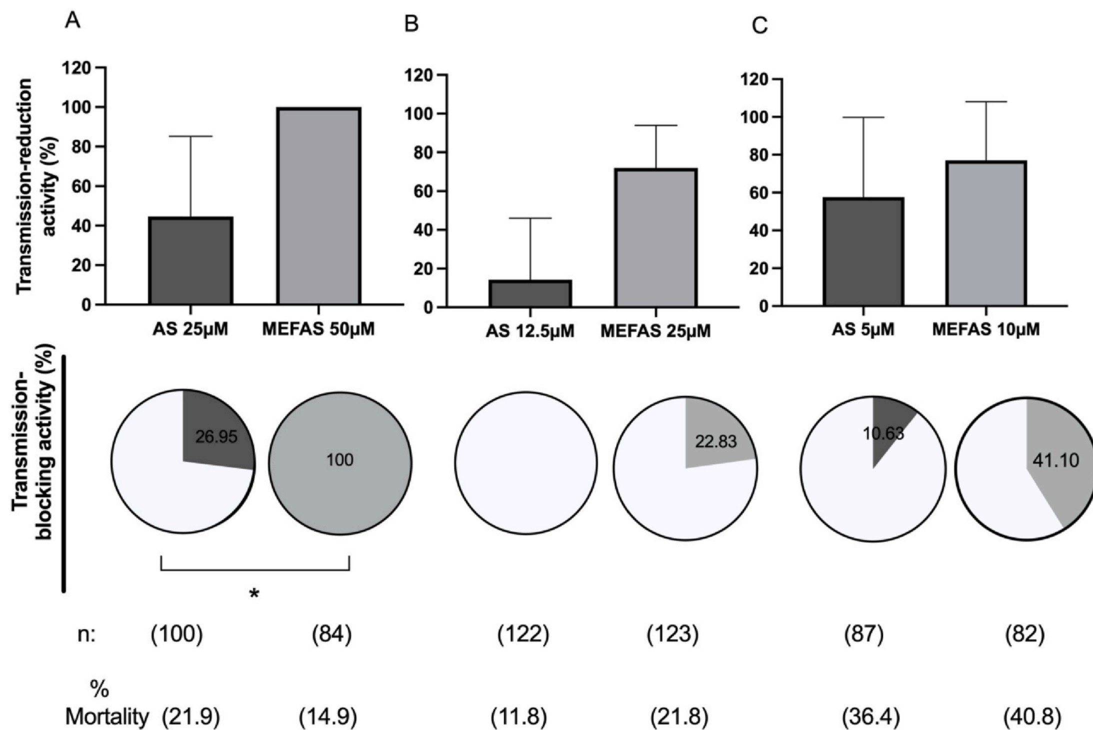

Fig. 2. Transmission-reduction and -blocking activity of MEFAS and Artesunate (AS) at different concentrations in *ex vivo* tests with *Anopheles darlingi* mosquitoes blood in the presence of the drugs. The transmission-reduction activity (top) and transmission-blocking activity (bottom) at different concentrations in the given mosquito sample sizes (n). MEFAS 50 μM and AS 25 μM (A); MEFAS 25 μM and AS 12.5 μM (B); and MEFAS 10 μM and AS 5 μM (C) resulted in the TRA and TBA mean. % mortality is also showed. Bars represent the mean of three independent experiments (patients' blood), error bars indicate the standard error of the means (SEM).*$p < 0.05$, analysis of parametric unpaired T-test.

depends on phosphorylation of *Pf*Map2, an atypical mitogen activated protein kinase, by *Pf*CDPK4, i.e., the *P. falciparum* calcium-dependent protein kinase, just before male gametes become motile and are released (Tewari et al., 2005).

MEFAS completely blocked malaria transmission at a concentration of 50 μM, illustrating potentiation of the AS effect, which at an equivalent concentration (25 μM), was unable to reach 100% transmission blocking effect. AS showed a variable dose response profile, likely due to the proximity of the tested concentrations and the high Hill coefficient of this molecule. Noteworthy, there was no time of incubation between the sexual parasites with the test and control drugs, and this probably explains why a 50 μM of MEFAS was needed to block completely the sporogony in the mosquito vectors.

Considering the limitation of the *ex vivo* tests using the membrane feeding assay, further studies are required to determinate an ideal concentration of MEFAS to block the parasite-transmission activity, and to determine a better time of incubation between *P. vivax* infected blood and drug, without decreasing parasite viability.

5. Conclusion

In conclusion, the present study demonstrates the *in vitro* activity of MEFAS against asexual stages of *P. vivax* and *P. falciparum* isolates, as well as its activity on the sporogonic development of nine different isolates of *P. vivax* in *An. darlingi* mosquitoes. Therefore, MEFAS is a potential drug with important transmission blocking activity. Considering the antiplasmodial activity of MEFAS on different life cycle stages of the parasite, this drug holds promise for the design of new, effective, multistage combination medicines to be used in malaria elimination campaigns to treat the acute disease, as well as to reduce parasite transmission, allowing a malaria eradication complain in the future.

Acknowledgments

We thank Dr. Dionicia Gamboa and Dr. Manuela Herrera (Universidad Peruana Cayetano Heredia, Lima, Peru) and Dr. Joseph Vinetz (Yale School of Medicine, USA) for collaboration with the implementation of the *An. darlingi* colony in Porto Velho/RO. We thank Dr. Carolina Bioni for allowing the laboratory and other facilities in Porto Velho to be utilized, and Dr. Ricardo Gazzinelli (Fiocruz, Belo Horizonte, MG, Brazil) for financial help in the field work. We also thank Dr. Roberto S. Rocha (Director of the Fiocruz, Belo Horizonte, MG, Brazil) for the payment for the publication of the article. We thank the Foundation for teh Research Support of the State of São Paulo (FAPESP - 2019/19708-0).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.09.003.

Funding

This work, including the efforts of JPC, PMS, NB, and AUK, was funded by MCTI/Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)/MS-SCTIE-Decit N° 40/2012 (grant number 404455/2012-3). JPC expresses her appreciation for fellowships granted by CNPq (grant number 420153/2017-9); the efforts of MSA and JFM, was in part funded by Fundação de Amparo à Pesquisa do Estado de Rondônia (FAPERO), grant number N02/2014, and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Finance Code 001.

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