AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Transmission-Blocking Potential of MEFAS, a Hybrid Compound Derived from Artesunate and Mefloquine

Julia Penna-Coutinho,[a,b,c] Maria Jesús Almela,[c] Celia Miguel-Blanco,[c] Esperanza Herreros,[c] Paula M. Sá,[d] Núbia Boechat,[d] Antoniana Ursine Krettli[a,b]

Centro de Pesquisas René Rachou, Malária Experimental e Humana, Fiocruz-Minas, Belo Horizonte, Minas Gerais, Brazil[a]; Faculdade de Medicina, Programa de Medicina Molecular, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil[b]; GlaxoSmithKline R&D, Malaria Discovery Performance Unit, Madrid, Spain[c]; Farmanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brazil[d]

Most antimalarial drugs target asexual parasites without reducing gametocyte formation or development. Drugs with dual roles, i.e., those that can target both asexual parasites and gametocytes, would improve the control of malaria. In the current study, MEFAS, a hybrid drug derived from mefloquine and artesunate that has been shown to be an active blood schizonticidal drug, was assessed to determine its ability to block the infectivity of *Plasmodium falciparum* gametocytes. MEFAS was 280 and 15 times more effective than mefloquine alone and artesunate alone, respectively.

Despite significant recent advances in the treatment of malaria, the eradication of this disease, which is a goal of the World Health Organization, has not yet been achieved. An ideal drug should have dual effects, i.e., it should cure the disease and should reduce the infectivity of gametocytes in mosquitoes, thereby limiting transmission (1). Reports of delayed parasite clearance rates in patients treated with artemisinin derivatives have triggered the development of novel antimalarials that target multiple stages of the parasite life cycle, thus blocking infection and transmission (2). Gametocytes, the sexual forms of the parasite that are responsible for parasite development in mosquito vectors, are affected by a few drugs, such as primaquine (PQ), that prevent parasite transmission. This is especially important in *Plasmodium falciparum*, because the gametocytes of this species survive longer than the asexual forms (3). Although the initial gametocyte stages (stages I to III) are sensitive to most schizonticidal antimalarials, the mature stages (stages IV and V) are sensitive only to PQ (4–6).

Single-dose PQ is used as a *P. falciparum* transmission-blocking drug; long-term PQ treatments (14 days) are used for *Plasmodium vivax*. In this case, PQ blocks transmission and prevents late relapse (7). However, prolonged use of PQ requires medical supervision, because this drug may cause gastrointestinal problems and severe hemolytic anemia in patients who lack the glucose-6-phosphate dehydrogenase (G6PD) enzyme (8, 9).

New antimalarial drugs with different biological functions and distinct pharmacophores include hybrids that are covalently linked to single compounds (10). MEFAS, a hybrid salt derived from artesunate (AS) and mefloquine (MQ), was synthesized on a large scale and evaluated for chemical purity (N. Boechat, M. V. N. de Souza, A. L. Valverde, and A. U. Krettli, international patent application WO 2005/100370 A1). MEFAS was less toxic and more effective than AS and MQ applied separately against chloroquine-resistant (clone W2) and chloroquine-sensitive (3D7 strain) *P. falciparum in vitro*. Additionally, MEFAS was able to cure *Plasmodium berghei* malaria in experimentally infected mice (11). In this work, the ability of MEFAS to interfere with gametocyte infectivity, male gamete exflagellation, and female gamete activation was evaluated using mature *P. falciparum* gametocytes cultured *in vitro*.

Two gametocyte-producing *P. falciparum* strains, 3D7 (MR4, MRA-102) and NF54 (MR4, MRA-1000), were cultured at 37°C in an atmosphere containing 3% O_2, 5% CO_2, and 92% N_2 (12, 13); these conditions were used for all experiments. Human biological samples were ethically processed, and they were used in accordance with the terms of the informed consent. Written informed consent was obtained from the blood donors for the use of samples in this research. The human blood was supplied by the Banc de Sang i Teixits (http://www.bancsang.net/) (Barcelona, Spain). The blood provision from Banc de Sang i Teixits was approved by the Clinical Research Ethics Committee of the Vall d'Hebron Hospital in Barcelona, Spain. The malaria project research was approved by the Ethics Research Committee of the Universidad Autónoma de Madrid, Spain.

P. falciparum gametocyte cultures were initiated as described previously (14). Briefly, on day 0, blood cultures were synchronized at the ring stage using sorbitol and were diluted to 0.5% parasitemia and 4% hematocrit in 40 ml complete RPMI 1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 50 mg/liter hypoxanthine (Sigma-Aldrich), 2 g/liter sodium bicarbonate (Sigma-Aldrich), 5% pooled human A^+ serum, and 2.5 mg/ml AlbuMAX II solution (Sigma-Aldrich). The medium was changed daily for 15 days without the addition of fresh erythrocytes. The percentages of asexual forms and gametocytes were evaluated in Giemsa-stained smears. Cultures with predominant stage IV to V gametocytes (day 14 onward) were concentrated using NycoPrep 1.077 cushions (Axis-Shield, Norway) and loaded onto an LS column (Miltenyi Biotech, United Kingdom) for a second purification

Received 10 September 2015 **Returned for modification** 11 October 2015
Accepted 6 February 2016

Accepted manuscript posted online 22 February 2016

Citation Penna-Coutinho J, Almela MJ, Miguel-Blanco C, Herreros E, Sá PM, Boechat N, Krettli AU. 2016. Transmission-blocking potential of MEFAS, a hybrid compound derived from artesunate and mefloquine. Antimicrob Agents Chemother 60:3145–3147. doi:10.1128/AAC.02216-15.

Address correspondence to Antoniana Ursine Krettli, akrettli@cpqrr.fiocruz.br.

TABLE 1 *In vitro* activity of the hybrid molecule MEFAS, compared with standard antimalarials (artesunate and mefloquine) and epoxomicin, against mature gametocytes of *P. falciparum*, as evaluated in an ATP bioluminescence-based assay

Compound	IC_{50} (μM)			
	Expt. 1	Expt. 2	Expt. 3	Average ± SD
MEFAS	2.5	5.8	5.4	4.6 ± 1.8[a]
Artesunate	25	7.1	6.0	12.7 ± 10.7[a]
Mefloquine	13.4	18.8	17.2	16.5 ± 2.8[a]
Epoxomicin	0.0016	0.0006	0.0006	0.0009 ± 0.0006

[a] *P* values of <0.05 were considered significant.

TABLE 2 Inhibition of female and male gamete formation by MEFAS, the antimalarials artesunate and mefloquine, and methylene blue (as control)

Compound	IC_{50} (μM)		Activity ratio (MEFAS/other compound)	
	Female gamete formation	Male exflagellation	Female	Male
MEFAS	0.02 ± 0.01	0.017 ± 0.008		
Methylene blue	0.71 ± 0.08	NT[a]	35	
Artesunate	0.3 ± 0.2	0.12 ± 0.01	15	7
Mefloquine	5.6 ± 2.1	0.57 ± 0.2	280	33

[a] NT, not tested.

step using a VarioMACS magnetic separator (Miltenyi Biotech, United Kingdom).

The density of the purified mature gametocytes was determined using a hemocytometer and was adjusted to 5×10^4 gametocytes per well in a final volume of 100 μl or 1.25×10^4 gametocytes per well in a final volume of 50 μl, in 96-well plates or 384-well plates, respectively. The plates were incubated at 37°C for 48 h. The ATP level in each well was determined using BacTiter-Glo reagent (Promega), based on the presence of a "glow-type" luminescence signal produced by the luciferase reaction (15). Epoxomicin, a proteasome inhibitor, was used as a control (16).

The female gametocyte activation assay was performed as described previously (17). Briefly, gametocytes (8,000 gametocytes per well) from 16-day-old cultures were incubated for 48 h in predispensed plates containing the test and control drugs. Activation was achieved by decreasing the temperature from 37°C to 26°C and adding ookinete medium (RPMI 1640 medium with 25 mM HEPES, 50 mg/liter hypoxanthine, 2 g/liter sodium bicarbonate, 100 μM xanthurenic acid, and 20% human serum) containing the antibody anti-Pfs25-Cy3 (final concentration, 0.5 μg/ml) (18). Female gametocytes become morphologically round when activated and express the Pfs25 protein on the gamete membrane, as revealed with a specific monoclonal antibody (18). Methylene blue (MB) and thiostrepton were used as control drugs (16, 19, 20).

For the exflagellation assay, sterile 1.5-ml tubes containing 100 μl culture medium and the compounds to be tested, at 2 times the final concentrations, were prewarmed to 37°C in a heat block. Aliquots (100 μl) of the cell suspensions were quickly dispensed into each assay tube. The tubes were maintained for 48 h at 37°C in a humidified incubator. Exflagellation events were counted 14 min after addition of the ookinete medium, in one chamber of a FastRead disposable hemocytometer slide. To detect and to quantify male gametocyte exflagellation, a computer-aided assay was used. Erythrocyte movement was visualized in a microscopic field at the "exflagellation center" (16).

The concentration at which the sexual development of *P. falciparum* was inhibited by 50% (i.e., 50% inhibitory concentration [IC_{50}]) was calculated from dose-response curves using GraphPad Prism 5 software. The differences between the IC_{50}s of experimental and control drugs were evaluated with nonparametric *t* tests using GraphPad Prism 5.

MEFAS was tested in parallel with the standard antimalarials AS and MQ and with epoxomicin. The latter antimalarial is gametocytocidal against stage V gametocytes at nanomolar concentrations (21). MEFAS was significantly more active against gametocytes (IC_{50} = 4.6 ± 1.8 μM) than were the standard antimalarial drugs AS (IC_{50} = 12.7 ± 10.7 μM) and MQ (IC_{50} = 16.5 ± 2.8 μM) (Table 1).

MEFAS inhibited female gametocyte activation (IC_{50} = 0.02 ± 0.01 μM) and was 15 times more effective than AS (IC_{50} = 0.3 ± 0.2 μM), 280 times more effective than MQ (IC_{50} = 5.6 ± 2.1 μM), and 35 times more effective than MB (IC_{50} = 0.71 ± 0.08 μM). The latter antimalarial is known for its potent activity against gametocytes (6).

The male gametocyte exflagellation assay was performed with 14-day-old cultures; 0.5% dimethyl sulfoxide (DMSO) was used as a control. MEFAS (IC_{50} = 0.017 ± 0.008 μM) was 7 times more active than AS (IC_{50} = 0.12 ± 0.01 μM). Dihydroartemisinin (DHA) was tested only at 10 μM as an internal control, to ensure 100% inhibition of exflagellation. MEFAS inhibited 99% of exflagellation at 500 nM; this dose is 20-fold lower than the DHA dose used in this experiment. MQ also showed inhibitory activity against exflagellation (IC_{50} = 0.57 ± 0.2 μM) (Table 2). In another experiment, MEFAS (IC_{50} = 0.023 ± 0.005 μM) showed 5 times greater inhibitory activity than did AS (IC_{50} = 0.11 ± 0.01 μM) (data not shown).

The combination of AS and MQ is currently being used in a few areas in which malaria is endemic and drug-resistant *P. falciparum* parasites are present (22). Knowledge about this combination provides a starting point from which to evaluate the possible dual effects of MEFAS, a potent schizonticidal drug (11). In the current study, the ability of MEFAS to interfere with the final critical steps of *P. falciparum* gametocyte maturation and gamete formation was demonstrated *in vitro*. MEFAS was compared with AS and MQ, which were tested in parallel.

In this study, MEFAS was shown to be more active against mature *P. falciparum* gametocytes (male and female) *in vitro* than was AS or MQ. At nanomolar concentrations, MEFAS inhibited exflagellation and female gamete activation. Additionally, these results indicate that, due to its intense *in vitro* gametocytocidal activity, MEFAS could be a potential candidate for use in clinical trials in areas in which malaria is endemic. However, other studies should be conducted to investigate the ability of MEFAS to block the sporogonic cycle of mosquitoes.

ACKNOWLEDGMENTS

We appreciate the financial support provided by the Brazilian funding agencies MCT/CNPq/CT-Saúde/MS/SCTIE/DECIT (Edital PRONEX 55675/2009-2 and Edital Doenças Negligenciadas 404455/2012-3), PDTIS/FIOCRUZ/MS, FAPEMIG (PRONEX project 16712), and

FAPERJ (grant E-26/110.610/2012). We thank CNPq for providing fellowships to N.B. and A.U.K. and the CNPq program Ciência sem Fronteiras for providing a fellowship to J.P.-C. as part of her Ph.D. support.

We thank GlaxoSmithKline for providing the facilities to perform the assays and José Luis Llergo for help with the exflagellation assays.

FUNDING INFORMATION

This work, including the efforts of Julia Penna-Coutinho, Núbia Boechat, and Antoniana Ursine Krettli, was funded by MCTI | Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (55675/2009-2 and 404455/2012-3). This work, including the efforts of Paula Miranda Sá and Núbia Boechat, was funded by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (E-26/110.610/2012). This work, including the efforts of Julia Penna-Coutinho and Antoniana Ursine Krettli, was funded by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (16712).

REFERENCES

1. **World Health Organization.** 2013. World malaria report 2013. World Health Organization, Geneva, Switzerland. http://www.who.int/malaria/publications/world_malaria_report_2013/en.
2. **Bousema T, Okell L, Shekalaghe S, Griffin JT, Omar S, Sawa P, Sutherland C, Sauerwein R, Ghani AC, Drakeley C.** 2010. Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. Malar J **9:**136. http://dx.doi.org/10.1186/1475-2875-9-136.
3. **Butcher GA.** 1997. Antimalarial drugs and the mosquito transmission of *Plasmodium*. Int J Parasitol **27:**975–987. http://dx.doi.org/10.1016/S0020-7519(97)00079-9.
4. **Burgess RW, Bray RS.** 1961. The effect of a single dose of primaquine on the gametocytes, gametogony and sporogony of *Laverania falciparum*. Bull World Health Organ **24:**451–456.
5. **Smalley ME.** 1977. *Plasmodium falciparum* gametocytes: the effect of chloroquine on their development. Trans R Soc Trop Med Hyg **71:**526–529. http://dx.doi.org/10.1016/0035-9203(77)90149-3.
6. **Adjalley SH, Johnston GL, Li T, Eastman RT, Ekland EH, Eappen AG, Richman A, Sim BKL, Lee MCS, Hoffman SL, Fidock DA.** 2011. Quantitative assessment of *Plasmodium falciparum* sexual development reveals potent transmission-blocking activity by methylene blue. Proc Natl Acad Sci U S A **108:**E1214–E1223. http://dx.doi.org/10.1073/pnas.1112037108.
7. **World Health Organization.** 2012. Updated WHO policy recommendation: single dose primaquine as a gametocytocide in *Plasmodium falciparum* malaria. World Health Organization, Geneva, Switzerland. http://www.who.int/malaria/publications/atoz/who_pq_policy_recommendation/en.
8. **Carmona-Fonseca J, Alvarez G, Maestre A.** 2009. Methemoglobinemia and adverse events in *Plasmodium vivax* malaria patients associated with high doses of primaquine treatment. Am J Trop Med Hyg **80:**188–193.
9. **World Health Organization.** 2010. Global report on antimalarial drug efficacy and drug resistance: 2000–2010. World Health Organization, Geneva, Switzerland. http://whqlibdoc.who.int/publications/2010/9789241500470_eng.pdf.
10. **Walsh JJ, Bell A.** 2009. Hybrid drugs for malaria. Curr Pharm Des **15:**2970–2985. http://dx.doi.org/10.2174/138161209789058183.
11. **de Pilla Varotti F, Botelho ACC, Andrade AA, de Paula RC, Fagundes EMS, Valverde A, Mayer LMU, Mendonça JS, de Souza MVN, Boechat N, Krettli AU.** 2008. Synthesis, antimalarial activity, and intracellular targets of MEFAS, a new hybrid compound derived from mefloquine and artesunate. Antimicrob Agents Chemother **52:**3868–3874. http://dx.doi.org/10.1128/AAC.00510-08.
12. **Trager W, Jensen JB.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675. http://dx.doi.org/10.1126/science.781840.
13. **Van Huyssen W, Rieckmann KH.** 1993. Disposable environmental chamber for assessing the drug susceptibility of malaria parasites. Trop Med Parasitol **44:**329–330.
14. **Ifediba T, Vanderberg JP.** 1981. Complete in vitro maturation of *Plasmodium falciparum* gametocytes. Nature **294:**364–366. http://dx.doi.org/10.1038/294364a0.
15. **Lelièvre J, Almela MJ, Lozano S, Miguel C, Franco V, Leroy D, Herreros E.** 2012. Activity of clinically relevant antimalarial drugs on *Plasmodium falciparum* mature gametocytes in an ATP bioluminescence "transmission blocking" assay. PLoS One **7:**e35019. http://dx.doi.org/10.1371/journal.pone.0035019.
16. **Delves M, Ruecker A, Straschil U, Lelièvre J, Marques S, López-Barragán MJ, Herreros E, Sindena RE.** 2013. Male and female *Plasmodium falciparum* mature gametocytes show different responses to antimalarial drugs. Antimicrob Agents Chemother **57:**3268–3274. http://dx.doi.org/10.1128/AAC.00325-13.
17. **Miguel-Blanco C, Lelièvre J, Delves MJ, Bardera AI, Presa JL, López-Barragán MJ, Ruecker A, Marques S, Sinden RE, Herreros E.** 2015. Imaging-based high-throughput screening assay to identify new molecules with transmission-blocking potential against *Plasmodium falciparum* female gamete formation. Antimicrob Agents Chemother **59:**3298–3305. http://dx.doi.org/10.1128/AAC.04684-14.
18. **Barr PJ, Green KM, Gibson HL, Bathurst IC, Quakyi IA, Kaslow DC.** 1991. Recombinant Pfs25 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals. J Exp Med **174:**1203–1208. http://dx.doi.org/10.1084/jem.174.5.1203.
19. **Ruecker A, Mathias DK, Straschil U, Churcher TS, Dinglasan RR, Leroy D, Sinden RE, Delves MJ.** 2014. A male and female gametocyte functional viability assay to identify biologically relevant malaria transmission-blocking drugs. Antimicrob Agents Chemother **58:**7292–7302. http://dx.doi.org/10.1128/AAC.03666-14.
20. **Aminake MN, Schoof S, Sologub L, Leubner M, Kirschner M, Arndt HD, Pradel G.** 2011. Thiostrepton and derivatives exhibit antimalarial and gametocytocidal activity by dually targeting parasite proteasome and apicoplast. Antimicrob Agents Chemother **55:**1338–1348. http://dx.doi.org/10.1128/AAC.01096-10.
21. **Kiszewski AE.** 2010. Blocking *Plasmodium falciparum* malaria transmission with drugs: the gametocytocidal and sporontocidal properties of current and prospective antimalarials. Pharmaceuticals **4:**44–68. http://dx.doi.org/10.3390/ph4010044.
22. **World Health Organization.** 2006. Guidelines for the treatment of malaria. World Health Organization, Geneva, Switzerland. http://helid.digicollection.org/pdf/s13418e/s13418e.pdf.