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Synthesis, Antimalarial Activity, and Intracellular Targets of MEFAS, a New Hybrid Compound Derived from Mefloquine and Artesunate▽

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A new synthetic antimalarial drug, a salt derived from two antimalarial molecules, mefloquine (MQ) and artesunate (AS), here named MEFAS, has been tested for its pharmacological activity. Combinations of AS plus MQ hydrochloride are currently being used in areas with drug-resistant *Plasmodium falciparum* parasites; although AS clears parasitemia in shorter time periods than any other antimalarial drug, it does not cure infected patients; in addition, MQ causes side effects and is rather expensive, important problems considering that malaria affects mostly populations in poor countries. Here, we show that MEFAS is more effective than the combination of AS and MQ, tested in parallel at different mass proportions, against *P. falciparum* (chloroquine-resistant clone W2 and chloroquine-sensitive clone 3D7) in vitro and in mice infected with *Plasmodium berghei*, promoting cure of this infection. MEFAS tested against HepG2 hepatoma cells exhibited lower toxicity than the antimalarials AS and MQ alone or combined. Possible targets of MEFAS have been studied by confocal microscopy using fluorescent probes (Fluo-4 AM and BCECF-AM) in *P. falciparum* synchronous culture of W2-infected red blood cells. Dynamic images show that MEFAS exhibited intracellular action increasing cytoplasmic Ca^{2+} at 1.0 ng/ml. This effect was also observed in the presence of tapsigargin, an inhibitor of SERCA, suggesting an intracellular target distinct from the endoplasmic reticulum. Trophozoites loaded with BCECF-AM, when treated with MEFAS, were still able to mobilize protons from the digestive vacuole (DV), altering the pH gradient. However, in the presence of bafilomycin A1, an inhibitor of the H^+ pump from acidic compartments of eukaryotic cells, MEFAS had no action on the DV. In conclusion, the endoplasmic reticulum and DV are intracellular targets for MEFAS in *Plasmodium* sp., suggesting two modes of action of this new salt. Our data support MEFAS as a candidate for treating human malaria.

Malaria is a human disease caused by infections with protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum* causes severe cases of malaria, leading to death if not promptly treated. At least 1 million people die each year, mostly in sub-Saharan Africa (36). The worldwide spreading of chloroquine-resistant *P. falciparum* parasites led to the use of other drugs to treat malaria, including amodiaquine, sulfadoxine-pyrimethamine, mefloquine (MQ), and artemisinin derivatives like artesunate (AS) (41).

During its intraerythrocytic life cycle, *P. falciparum* digests the host erythrocyte hemoglobin, using it as a source of amino acid in the digestive vacuole (DV). Heme is released in the process and presumably detoxified through crystallization into inert hemozoin (11). The antimalarial activity of 4-aminoquinolines is linked to the quinolinic ring modifying the parasite heme metabolism (14). In the case of AS, a sesquiterpene lactone that contains a 1,2,4-trioxane ring system (20), the endoperoxide bond is essential for reactions with iron and thereby damages the parasite (31).

The mechanisms of most antimalarial action and resistance are still unclear. Chloroquine resistance in *P. falciparum* has been found to be associated with mutations in a parasite DV membrane protein, *P. falciparum* CRT (PfCRT) (4). MQ resistance appears to be linked to the function of PfMDR1, a P glycoprotein expressed on DV (30, 32). AS has been shown to inhibit PfATPase6, a SERCA-type ATPase, in a heterologous system (10). Specific mutations on PfATPase6 have been found in parasites from field isolates, and the selection of these mutations could be attributed to the noncontrolled use of artemisinin derivatives to treat chloroquine-resistant *P. falciparum* (18).

At present, the guidelines for malaria treatment recommend artemisinin in combination therapy (ACT) with other antimalarials or one antimalarial plus antibiotics provided that there is adequate evidence of their efficacy and safety in areas where malaria is endemic (39, 42). The principle behind the choice of drug combinations is that they act synergistically (25) or present different modes of action in order to prevent the evo-

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lution of drug resistance (40). The ACTs containing AS and MQ, adopted for malaria treatment in areas of intense drug resistance (12, 27), combine the immediate effect of AS, which rapidly clears asexual blood-stage parasites and gametocytes (40), with the prolonged schizontocidal effect of MQ, which has a long half-life of about 2 weeks (23). However, MQ causes side effects such as vomiting, headaches, gastrointestinal problems, fatigue, and sleeping disorders (6), whereas AS is not a curative drug by itself (42).

An ideal antimalarial candidate should have a high efficacy, a short half-life, and low toxicity, reducing the risk of resistant parasites emerging (21). A series of hybrid molecules recently synthesized from trioxane and quinoline fragments tested against *Plasmodium* sp. provided rather promising results (5, 34). They exhibited potent antiplasmodial activity in vitro against *P. falciparum* chloroquine-sensitive and chloroquine-resistant strains (50% inhibitory concentration [IC_{50}] ranging from 4 to 32 nM), including the sexual stages (gametocytes); they were active in rodent malaria against *Plasmodium vinckei petteri* and *Plasmodium yoelii nigeriensis* in suppressive and curative tests (5). Here, we have evaluated the antimalarial activity of another hybrid drug, MEFAS, synthesized from MQ and AS at low cost. It is also important that an ideal antimalarial should have a low price to be accessible to the poor populations exposed to drug-resistant *P. falciparum* malaria in Africa, Southeast Asia, and Latin America.

MATERIALS AND METHODS

Chemicals. [^{3}H]hypoxanthine was obtained from Amersham Life Science. Thiazolyl blue salt (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma. The antimalarials chloroquine, MQ, and AS were provided by Knoll/Abbott (Germany). MEFAS was synthesized at the Instituto de Tecnologia e Fármacos (Farmanguinhos/Fiocruz) and is covered by a patent (N. Boechat et al., international patent application WO 2005/100370 A1).

Synthesis. MEFAS was prepared using a solution of MQ hydrochloride (2 g) in 50 ml of H_2O-MeOH (8:3). The same volume of ethyl ether was added to this solution at room temperature with stirring. Sodium bicarbonate was added until the effervescence had ceased. The two phases were separated, the organic layer was dried over anhydrous sodium sulfate, and the solvent was evaporated. The free base obtained (1.48 g) was dissolved in ethyl ether (40 ml), and a solution of AS (1.5 g) in ethyl ether (40 ml) was added. The mixture was stirred at room temperature for 12 h. The solid form was collected, washed with ethyl ether, and dried under a vacuum. MEFAS was obtained in 80% yield (2.38 g) and characterized by ^{1}H nuclear magnetic resonance (NMR) (400 MHz; $CDCl_3$) and ^{13}C NMR spectra, mass spectra, and elementary analysis. Thermogravimetric analysis, differential scanning calorimetry, and high-performance liquid chromatography analyses of MEFAS were also performed. All melting points were determined using a Mettler FP90 instrument attached to an FP81HF oven. Infrared spectra were recorded in KBr disks using a Nicolet 205 infrared spectrometer. Mass spectra were recorded using a Waters micromass ZQ. NMR spectra were recorded on a Bruker HC400 instrument: internal standards used were tetramethylsilane for ^{1}H and ^{13}C. MQ AS melting point, 122°C to 125°C. CHN anal. calc. for $C_{36}H_{44}F_6N_2O_9$: C, 56.69; H, 5.81; N, 3.67, found C, 56.54; H, 6.04; N, 3.18; IR (KBr). $\nu_{max/cm}{}^{-1}$: 2,934, 1,752, 1,310, 1,143, and 1,109; ^{1}H NMR (400 MHz; DMSO-d_6; δ 8.73 [d, J 8.8 Hz, ^{1}H]); 8.40 (d, J 8.8 Hz, ^{1}H); 8.08 (s, ^{1}H); 7.93 (t, J 7.9 Hz, ^{1}H); 5.66 (d, J 5.7 Hz, ^{1}H); 5.55 (s, ^{1}H); 5.43 (d, J 4.5 Hz, ^{1}H); 2.48 (m, ^{4}H, —$COCH_2CH_2CO$—); 1.28 (s, ^{3}H); 0.89 (d, J 6.2 Hz, ^{3}H) and 0.76 (d, J 7.1 Hz, ^{3}H); ^{13}C NMR (100 MHz; $CDCl_3$, δ 11.68 [CH_3]), 20.00 (CH_3), 20.97 (CH_2), 24.14 (CH_2), 25.47 (CH_3), 29.20 (CH_2), 29.25 (CH_2), 29.29 (CH_2), 31.62 (CH_2), 33.66 (CH_2), 33.82 (CH), 35.87 (CH_2), 35.93 (CH_2), 36.28 (CH), 44.55 (CH), 45.12 (CH), 51.29 (CH_2), 60.07 (CH), 79.83 (C<u>H</u>-OH), 90.55 (O-C<u>H</u>-O), 91.61 (O-C<u>H</u>-O), 80.57 (C<u>-</u>O-O), 103.53 (O-C<u>-</u>O-O), 115.49 (CH-Ar), 119.87 (q, J 274 Hz, C<u>F</u>$_3$,), 122.34 (C-Ar), 122.61 (q, J 274 Hz, CF_3), 126.81 (CHAr), 127.16 (q, J 36 Hz, C<u>-</u>CF_3), 127.91 (C<u>H</u>-Ar), 129.04 (C<u>H</u>-Ar), 142.73 (C-Ar), 146.73 (q, J 34 Hz, C<u>-</u>CF_3), 152.72 (C-Ar), 171.24 (CO), and 174.02 (CO). m/z: 763 (40%); 379 (100%). Ongoing studies are being developed in order to establish the solubility property, log P value, and stability of MEFAS by comparisons with parent MQ and AS using precise quantification methods.

***Plasmodium falciparum* continuous culture.** Two *P. falciparum* clones were used, one chloroquine resistant (W2) and one chloroquine sensitive (3D7). The infected red blood cells were maintained in continuous culture on human erythrocytes (blood group O^+) in RPMI medium supplemented with 10% human plasma (complete medium), as previously described (37). The synchronization of the parasites was achieved by sorbitol treatment (22) and parasitemias were determined microscopically in Giemsa-stained smears.

Antimalarial tests in vitro. The antimalarial effects of MEFAS and of the controls compounds were measured with the [^{3}H]hypoxanthine incorporation assay performed as described previously (8), with minor modifications (1). Briefly, ring-stage parasites in sorbitol-synchronized blood cultures were added to 96-well culture plates at 2% parasitemia and 2% hematocrit and then incubated with the test drugs, alone or in combination. MEFAS, the antimalarial controls (chloroquine, AS, and MQ), and the combination of AS and MQ (1:1, 1:2, and 2:1 mass proportions) were diluted in complete medium without hypoxanthine, from 10-mg/ml stock solutions in DMSO, at a final concentration of 0.002% (vol/vol) and stored at −20°C. After a 24-h incubation period, 25 μl of medium containing [^{3}H]hypoxanthine (0.5 μCi/well) was added per well, followed by another 18-h incubation at 37°C. The plates were frozen (−20°C for 24 h) and thawed, and the cells were harvested (Tomtec 96-Harvester) on glass fiber filters (Wallac, Turku, Finland) and then placed onto sample bags (Wallac) and immersed in scintillation fluid (Optiphase super mix; Wallac). Radioactive emission was counted in a 1450 Microbeta reader (Wallac). The inhibition of parasite growth was evaluated from the levels of [^{3}H]hypoxanthine incorporation; i.e., IC_{50} and IC_{90} values were evaluated by comparing the incorporation in drug-free control cultures and estimated by linear interpolation (17) using curve-fitting software (Microcal Origin software 5.0). All experiments were performed three times, and each sample was tested in triplicate.

Antimalarial tests in vivo. *P. berghei* strain NK65, used for the tests, was maintained in liquid nitrogen and by serial weekly passages of infected blood in mice (2). The method used for the antimalarial screening in the infected animals was performed as described previously (28), with minor modifications (3). Briefly, adult outbred Swiss mice, 20 g ± 2 g body weight, were inoculated by the intraperitoneal route with 1×10^5 infected red blood cells and then divided randomly into groups of three to five mice per cage. The mice were treated orally each day for 3 days with the test and control drugs or with only the drug vehicle. All solutions were prepared on the day of the experiment. MEFAS, the antimalarial controls AS and MQ, and the combination of AS plus MQ (1:1 mass proportion) were dissolved in an aqueous solution of 0.2% (vol/vol) DMSO and diluted to the desired final volume with saline so that each mouse received 200 μl. One control group of five nontreated mice was used in each test. On different days after parasite inoculation up to day 30, blood smears were prepared from the mouse tail, methanol fixed, stained with Giemsa stain, and microscopically examined by counting parasitemia in up to 6,000 erythrocytes. Inhibition of parasite growth in the drug-treated groups was calculated in relation to the nontreated control mice. The cumulative mortality of the animals was monitored daily. All compounds and combinations were tested in parallel in three independent experiments. Blood from the presumably cured mice was tested by PCR for *P. berghei* using specific primers for the alfa-tubulin gene (A. de Menezes-Neto, unpublished data) and were also subinoculated in normal mice to ensure that they were negative. The blood recipient mice were monitored daily during 15 consecutive days through blood smears examined by microscopy.

Cytotoxicity test. Cultures of Hep G2 A16 hepatoma cells were kept at 37°C in RPMI medium supplemented with 5% fetal calf serum (complete medium) in a 5% CO_2 environment. Cells from confluent monolayers were trypsinized, washed, counted, diluted in complete medium, distributed in 96-well microtiter plates (4×10^4 cells/well), and then incubated for another 18 h at 37°C. The compounds to be tested (with or without the test drugs added to the cultures) were diluted in ethanol (final concentration of 0.02%). After 24 h incubation at 37°C, 20 μl of MTT solution (5 mg/ml in RPMI 1640 medium) without phenol red was added to each well (7). After 4 h of incubation at 37°C, the supernatant was removed, and 200 μl of acidified isopropanol was added to each well. The culture plates were read by a spectrophotometer with a 570-nm filter and a background of 630 nm. The minimum lethal dose that killed 30% of the cells (MLD 30%) was determined (24); each assay was performed two or three times.

Confocal microscopy. Synchronized cultures with *P. falciparum* trophozoites in red blood cells (10^7 parasites/ml) were loaded with FLUO-4 AM (10 μM) for 40 min at 37°C or BCECF-AM (8 μM) for 10 min at room temperature in load buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, 50 mM MOPS [morpholinepropanesulfonic acid], 2 mM $CaCl_2$ [pH 7.4]) and washed twice with the same buffer (9,000 × g for 10 min) at room temperature. The

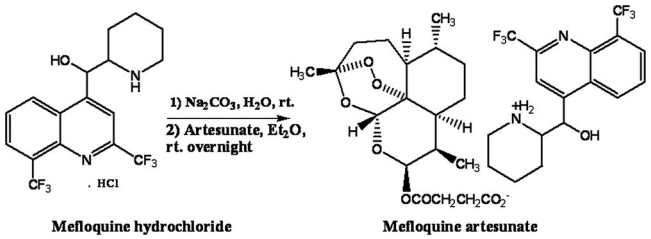

FIG. 1. Synthesis and structure of MEFAS. rt., room temperature.

labeled parasites were deposited onto a coverslip coated with L-polylysine (1 mg/ml) and kept at room temperature for approximately 10 min before use. The coverslip was then introduced into a stainless steel chamber (Attofluor cell chamber; Molecular Probes) containing 100 μl of load buffer, which was placed onto the stage of the microscope (Zeiss Axiovert 100 M). Tapsigargin (TAP) and other chemicals were added directly to the chamber containing the loaded cells. Dynamic imaging was performed with the LSM 510 laser scanning microscope (Carl Zeiss), using LSM 510 software (version 2.5), in the Axiovert 100 M microscope equipped with a 63× oil immersion objective. The samples were illuminated at an excitation wavelength of 488 nm by Argon laser, and emission wavelength was collected by bandpass filter at 505 to 530 nm. Transmitted light observations were performed at the beginning and end of each experiment in order to monitor the integrity of the cells.

The data were analyzed by media and standard error of fluorescent probe ratio intensity (maximal fluorescence after drug addition/fluorescence before drug addition). Software-based analysis allowed fluorescence imaging of the whole field of view or in a selected cell as a function of time. This was accomplished by defining areas of interest on a given image frame and using the software to graphically represent intensity against time (38).

Statistical analysis. The average of IC_{50} and MLD 30% were compared using Tukey's test. Differences between IC_{50}s were evaluated with Biostat 1.0 MCT-CNPq. A P value of <0.01 was considered to be statistically significant.

RESULTS

MEFAS is a new hybrid salt that contains two antimalarial functionalities: one quinolinic ring from MQ and one endoperoxide ring from AS (Fig. 1). The melting point of MEFAS is 122°C to 125°C, which is significantly different from those of MQ hydrochloride (170°C to 172°C) and AS (139°C to 140°C). The mass spectrum of MEFAS exhibits m/z peaks at 763 $[M^+]$ and 379 $[M^- \text{ AS} = \text{MQ}]^+$. The ^{1}H NMR spectrum of the new salt does not show the signal at δ 12.26 observed in the spectrum of the original AS. Furthermore, the IR spectrum of MEFAS does not show the absorption at 3,276 cm^{-1} (νO-H) observed in the spectrum of AS (N. Boechat et al., unpublished data). We need to perform a more precise quantification method to determine the solubility of MEFAS, and possible stabilizing interactions between the MQ and AS moieties using solid-phase experiments need to be performed.

Different concentrations of MEFAS were tested for antimalarial activity in parallel with AS and MQ alone or in combinations at different mass proportions as shown in Table 1. A fresh solution was prepared each time for the tests in which the 1:1 mass proportion was used, which was very close to an equimolar ratio of these two drugs since their respective molecular weights are 384 and 415, resulting in a 1:1.05 molar ratio. MEFAS was active against chloroquine-resistant (W2) and chloroquine-sensitive (3D7) *P. falciparum* parasites. The average inhibitory concentrations for W2 and 3D7 parasites at the IC_{50} were 1.0 and 1.1 ng/ml, respectively, and at the IC_{90}, they were 4.2 and 4.0 ng/ml, respectively (Table 1), in three experiments. The IC_{50} and IC_{90} values show that MEFAS was at least five times more potent than MQ alone, more potent than AS against 3D7, as effective as AS against W2, and more potent than the mixtures of AS with MQ tested at different mass proportions. Statistical analysis ($P < 0.01$ by Tukey's test) indicated that the differences observed between MEFAS and the combination of AS plus MQ were significant.

MEFAS was also active in vivo in mice infected with *P. berghei* parasites and cured all animals treated with 10 and 20 mg/kg of body weight/day of MEFAS; no recrudescence of parasitemia was observed during the 30 days of blood examination (Table 2). Mice treated with the combination of AS and MQ at 5 and 10 mg/kg/day showed an initial reduction of parasitemia; after 9 days of inoculation, however, a relapsing parasitemia started to occur. Similar results were achieved with AS tested alone. A clearance of parasitemia with no recrudescence effect was also achieved with MQ alone or combined with AS at the highest dose tested (Table 2).

The overall mortality in all infected groups is shown in Fig. 2. Treatment with 10 mg/kg/day of MEFAS increased the survival of infected mice in relation to nontreated control mice, indicating a protective effect of this new compound, like MQ and the combination of AS plus MQ.

The malaria curative effect of MEFAS at doses of 10 and 20 mg/kg/day was further confirmed by the subinoculation of blood from the treated animals into normal mice, which did not show circulating *P. berghei* blood parasites in the next 15 days of examination. A similar curative action was observed with MQ (5 and 10 mg/kg/day) and with the combination of AS plus MQ (dose of 20 mg/kg/day). The PCR tests confirmed that these infected and treated groups did not harbor subpatent malaria infections (data not shown).

The in vitro cytotoxicity of MEFAS against Hep G2 cells tested in parallel with the original antimalarials shows that it has toxicity that is five times lower than that of MQ. The combination of AS and MQ (1:1 mass proportion), tested in duplicate, was three times more toxic than MEFAS (Table 3). The toxicity of MEFAS was thus considered low since its MLD 30% was 30.5 μg/ml, at least 30,000 times higher than the mean IC_{50} in vitro.

TABLE 1. Inhibitory concentrations of MEFAS, of the antimalarial controls AS and MQ, alone or combined, and of chloroquine used in parallel tests against *P. falciparum* clones W2 and 3D7[a]

Compound[b]	W2		3D7	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
MEFAS	1.0 ± 0.05[c]	4.2 ± 0.10[c]	1.1 ± 0.10[c]	4.0 ± 0.20[c]
MQ	7.2 ± 0.12[d]	25.2 ± 0.30[d]	11.1 ± 0.76[d]	30.5 ± 1.50[f]
AS	1.4 ± 0.08[c]	4.1 ± 0.10[c]	2.5 ± 0.10[e]	9.3 ± 0.15[e]
AS + MQ (1:1)	2.3 ± 0.12[e]	8.8 ± 0.20[e]	2.2 ± 0.15[e]	9.5 ± 0.52[e]
AS + MQ (1:2)	2.5 ± 0.20[e]	8.9 ± 0.30[e]	3.0 ± 0.10[e]	11.0 ± 0.50[e]
AS + MQ (2:1)	1.9 ± 0.05[e]	8.2 ± 0.10[e]	2.2 ± 0.15[e]	9.5 ± 0.30[e]
CQ	152.3 ± 2.50[f]	490.5 ± 3.20[f]	28.6 ± 1.50[f]	100.2 ± 2.30[f]

[a] Values are averages ± standard deviations of data from three different experiments. The different superscript letters (*c*, *d*, *e*, and *f*) indicate significant statistical differences in each column ($P < 0.01$ by Tukey's test).

[b] Numbers in parentheses represent mass proportions. CQ, chloroquine.

TABLE 2. Parasitemia and inhibition of parasitemia by *P. berghei* in mice treated by the oral route with different doses of MEFAS or the antimalarials AS and MQ, alone or combined, compared to not-treated control mice

Compound	Dose (mg/kg/day)	Parasitemia (% reduction) at day after inoculation[a]:				
		5	7	9	14	18
MEFAS	5	0.2 (96.3)	0.8 (95.0)	4.9 (90.3)	21.7 (53.4)	66.5 (0.0)
	10	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
	20	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
AS	5	3.1 (42.6)	11.4 (40.8)	29.3 (37.9)	39 (16.3)	+
	10	0.2 (96.3)	1.6 (94.2)	23.6 (53.2)	34.5 (26)	+
MQ	5	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
	10	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)
AS + MQ (1:1)[b]	5[c]	0 (100)	0.4 (98.6)	8.9 (82.3)	24.5 (47.4)	42 (30)
	10	0 (100)	0 (100)	0 (100)	1.8 (96.1)	7.8 (87)
	20	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)

[a] Results are from one representative experiment. +, dead animals (further details including nontreated controls are described in the legend of Fig. 2).
[b] Mass proportion.
[c] Treatment with 5 mg/kg/day of AS plus MQ (1:1) means that mice received together the equivalent of 2.5 mg/kg/day of AS and 2.5 mg/kg/day of MQ administered in fresh solution.

To investigate the intracellular targets of MEFAS in *P. falciparum* clone W2, the fluorescence changes in live trophozoites were measured in infected red blood cells using synchronous cultures loaded with the fluorescent probes FLUO-4 AM (10 μM) and BCECF-AM (8 μM). MEFAS at 1.0 ng/ml promoted an increase in levels of cytoplasmic Ca^{2+} (Fig. 3). In the presence of TAP (10 μM), an inhibitor of the Ca^{2+}-ATPase pump (from the endoplasmic reticulum) capable of mobilizing

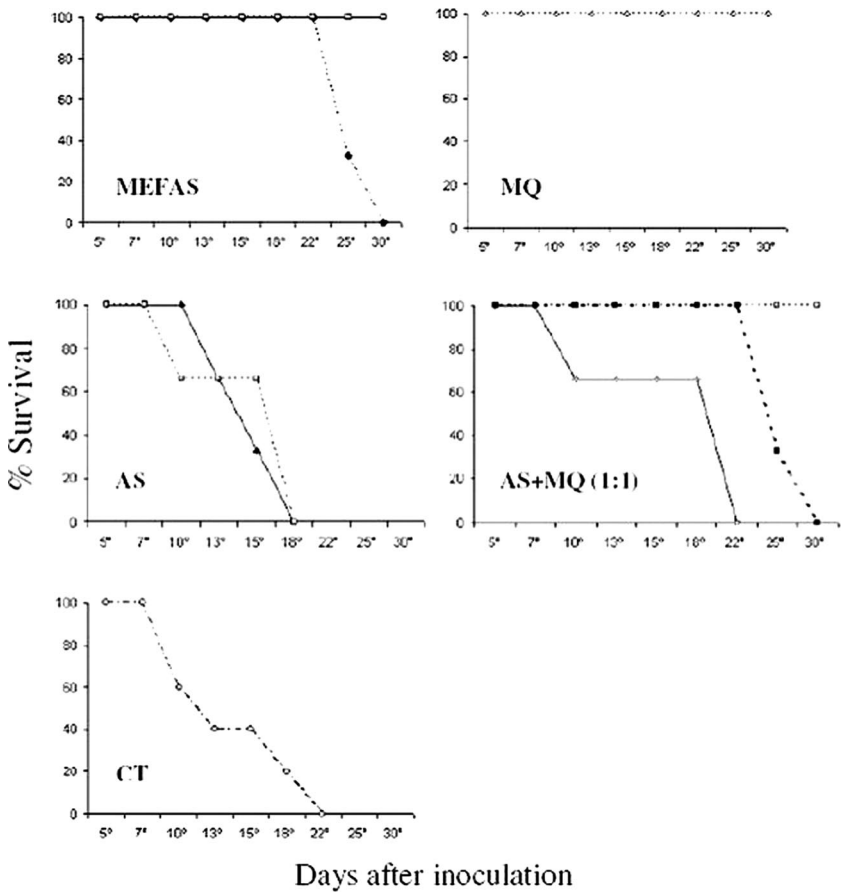

FIG. 2. Survival of mice infected with *P. berghei* after oral treatment with the antimalarials MEFAS (◆, 10 mg/kg/day; □, 5 mg/kg/day), MQ (◇, 5 and 10 mg/kg/day), AS (◆, 5 mg/kg/day; □, 10 mg/kg/day), AS plus MQ (◇, 5 mg/kg/day; ■, 10 mg/kg/day; □, 20 mg/kg/day), and nontreated controls (CT) (○). Results from one representative experiment are shown. Data in parentheses represent mass proportions.

TABLE 3. Mean lethal dose of MEFAS and of the antimalarials AS, MQ, and CQ, alone or combined, that killed 30% of Hep G2 cells in vitro

Compound (mass proportion)[a]	MLD 30% (μg/ml) ± SD[b]	
	Expt A[c]	Expt B[d]
MEFAS	30.5 ± 6.5[e]	30.0 ± 5.0[e]
AS	62.5 ± 12.5[e]	55.3 ± 10.1[e]
MQ	5.7 ± 1.3[f]	4.9 ± 2.0[f]
AS + MQ (1:1)	ND	10.5 ± 1.5[f]
CQ	>100.0[e]	>100.0[e]

[a] CQ, chloroquine.
[b] Values are averages ± standard deviations. Different superscript letters (*e* and *f*) indicate significant statistical differences ($P < 0.01$ by Tukey's test). ND, not done.
[c] Three independent experiments.
[d] Two independent experiments.

calcium in *P. falciparum* parasites (38), MEFAS still increased levels of cytoplasmic Ca^{2+}, suggesting another intracellular drug target (Fig. 3G). However, no fluorescence increase was recorded by TAP after a previous addition of MEFAS (Fig. 3H). AS, whose target on the parasite is predicted to be PfATPase6 (10), was tested in parallel and under the same conditions as those used for MEFAS; both showed similar responses to TAP (Fig. 3D to G).

To investigate the action of MEFAS on the DV of *P. falciparum*, trophozoites were loaded with BCECF-AM (8 μM), which was used to study acidic compartments in the parasites (15). MEFAS was able to mobilize protons altering the pH gradient in the DV, like MQ, a weak-base quinolinic antimalarial at 7 ng/ml. However, after the addition of bafilomycin A1 (4 μM), an inhibitor of the H^+ pump from acidic compartments of eukaryotic cells, MEFAS had no action on the DV (Fig. 4).

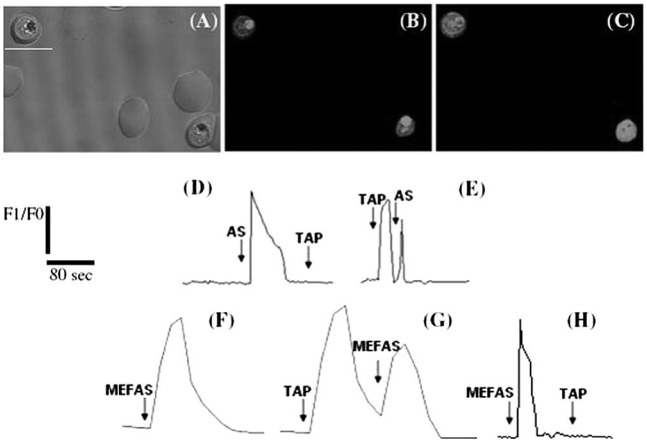

FIG. 3. Ca^{2+} mobilization by AS and MEFAS on infected red blood cells loaded with Fluo-4 AM. (A) Differential interference contrast (DIC) image. (B) Basal fluorescence image. (C) Fluorescence image after AS (1 ng/ml) addition. (D) AS (increase of 1.3 ± 0.1 fluorescence arbitrary units; $n = 8$) and TAP action. (E) TAP (increase of 1.2 ± 0.1; $n = 8$) and AS (increase of 0.7 ± 0.2; $n = 8$) action. (F) MEFAS (increase of 1.2 ± 0.2; $n = 10$) action. (G) TAP (increase of 1.2 ± 0.1; $n = 7$) and MEFAS (increase of 0.7 ± 0.1; $n = 7$) action. (H) MEFAS (increase of 1.3 ± 0.2; $n = 5$) and TAP action. Bar, 8 μm.

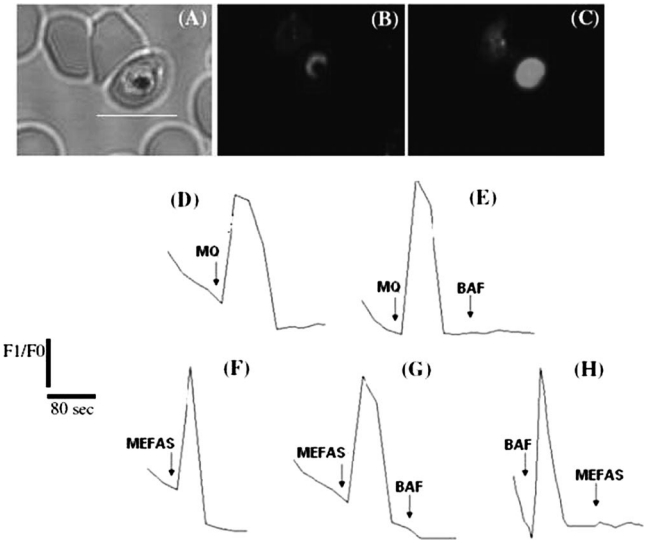

FIG. 4. H^+ mobilization by MQ and MEFAS on infected red blood cells loaded with BCECF-AM. (A) DIC image. (B) Basal fluorescence image. (C) Fluorescence image after MQ addition. (D) MQ action (increase of 1.4 ± 0.2 fluorescence arbitrary units; $n = 7$). (E) MQ (increase of 1.1 ± 0.2; $n = 8$) and bafilomycin A1 (BAF) action. (F) MEFAS action (increase of 1.4 ± 0.2; $n = 5$). (G) MEFAS (increase of 1.2 ± 0.1; $n = 7$) and BAF action. (H) BAF (increase of 1.6 ± 0.1; $n = 5$) and MEFAS action. Bar, 8 μm.

DISCUSSION

AS, a fast-eliminating antimalarial drug, and MQ, a slow-eliminating drug, have been adopted over the past decade as an ideal ACT (41). However, the high cost of these drug combinations is still a problem for many countries where malaria is endemic, and sources of artemisinin are erratic (26). Aiming to develop new drugs against parasite-resistant strains and considering that hemoglobin digestion within the parasite DV is an important target, we synthesized and tested the antimalarial activity of MEFAS, a new salt derived from MQ and AS. Hybrids are defined as being chemical entities with two (or more than two) structural sites with different biological functions (26). Recently, a new promising hybrid group of compounds containing a trioxane motif and an aminoquinoline entity was developed against malaria parasites (5).

The industrial use of MEFAS could offer advantages compared to MQ plus AS used in fixed-dose AS combination therapy, as recommended by the Drugs for Neglected Diseases Initiative (9). The antimalarials adopted in treatment guidelines are considered active pharmaceutical ingredients (API). The cost of production of an API is enhanced by the number of purification processes necessary to reach a pharmaceutical standard. The cost of production of MEFAS is reduced first because its preparation requires only one set of necessary analysis, as a single API. Secondly, MEFAS would not require MQ and AS with high pharmaceutical purity as APIs adopted in fixed-dose AS combination therapy treatment guidelines (9). It is possible to purchase MQ as a base (rather than the salt MQ hydrochloride [99% pure]). The base is synthesized using a procedure that is one synthetic step less than that of the salt and without the additional purification processes required for the pharmaceutical standard of MQ hydrochloride production.

AS can also be purchased prior to the steps required for obtaining its API standard.

It was recently suggested that the uncontrolled use of artemisinin compounds may result in the emergence of resistant *P. falciparum* parasites based on a substantial PfATPase6 polymorphism detected in human blood samples collected in French Guiana and Senegal (18). Such resistance, if confirmed, may severely limit the utility of ACTs (29), although recently, authors reported no correlation between PfATPase6 mutations and artemisinin resistance in *P. falciparum* field isolates from the Brazilian Amazon (13) and China (43).

MEFAS was proven to be a potent antimalarial in vitro against *P. falciparum* and equally active against chloroquine-sensitive (3D7) and chloroquine-resistant (W2) parasites; it was more active than the different mixtures of AS and MQ at various mass proportions. Its toxicity in vitro, about 30,000 times higher than its IC_{50}, which was five times lower than that of MQ and three times lower than that of the combination of AS plus MQ, indicates a selective antimalarial action. MEFAS toxicity was investigated only in hepatoma cells in vitro, as shown in Table 3. There is no consistent information about the mechanisms of antimalarial action on the cell lineages like Hep G2 cells, and further studies will be necessary to investigate the action of MEFAS as well as its possible neurotoxicity, as described previously for AS (35).

The in vivo antimalarial tests of MEFAS in *P. berghei*-infected mice indicate its curative action at an initial dose of 10 mg/kg/day. The MEFAS-treated mice had no recrudescence until day 30, whereas all mice treated with 5 and 10 mg/kg/day of AS and AS plus MQ were dead by this time. In addition, the subinoculation of blood from the presumably cured mice confirmed the results. MEFAS was able to cure the malaria infection like MQ, an antimalarial standard, and the combination of AS plus MQ at the higher dose tested.

With the antimalarial activity described above, and knowing that MEFAS is a hybrid species containing a quinolinic ring and an endoperoxide bridge, we analyzed its role in different intracellular targets of living *P. falciparum* trophozoites in red blood cells in real time. The ability of MEFAS to increase the cytoplasmic Ca^{2+} from internal stores like the endoplasmic reticulum was similar to that shown for the AS sample tested in parallel. The absence of an observable fluorescence increase when TAP was posteriorly added suggests PfATPase6 as a possible target for MEFAS. The inhibition of a SERCA pump, resulting in a prolonged increase in levels of cytoplasmic Ca^{2+}, could change a number of important events in malarial parasites including trigger cell signaling events and developmental processes (16).

The effect of MEFAS on H^+ homeostasis monitored on the DV of the parasite in parallel with MQ, which is known to concentrate on the DV (32), showed that both drugs disrupted the pH gradient in this organelle, increasing the fluorescence intensity. The acidic pH of the DV is thought to play a key role in the hemoglobin digestion within this organelle (19). Maintaining an acidic pH involves a V-type H^+-ATPase inhibited by concanamycin A and bafilomycin A1 (33). No fluorescence increase was observed when bafilomycin A1 was added posteriorly, suggesting that the DV is a target for MEFAS action.

This work shows that MEFAS is able to act on the DV, probably due to its endoperoxide bridge and aminoquinoline entities. As expected, this hybrid salt may have a dual mode of action. Whether there is a participation of PfCRT, PfMDR1, and PfATPase6 in MEFAS action is still an open question. Its action on calcium homeostasis could limit the emergence of resistance in the malaria parasite, favoring its use as a potential alternative and less toxic antimalarial treatment.

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