

# Synthesis of Artemiside and Its Effects in Combination with Conventional Drugs against Severe Murine Malaria

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**This research describes the use of novel antimalarial combinations of the new artemisinin derivative artemiside, a 10-alkylamino artemisinin. It is a stable, highly crystalline compound that is economically prepared from dihydroartemisinin in a one-step process. Artemiside activity was more pronounced than that of any antimalarial drug in use, both in *Plasmodium falciparum* culture and *in vivo* in a murine malaria model depicting cerebral malaria (CM). *In vitro* high-throughput testing of artemiside combinations revealed a large number of conventional antimalarial drugs with which it was additive. Following monotherapy in mice, individual drugs reduced parasitemias to nondetectable levels. However, after a period of latency, parasites again were seen and eventually all mice became terminally ill. Treatment with individual drugs did not prevent CM in mice with recrudescent malaria, except for piperazine at high concentrations. Even when CM was prevented, the mice developed later of severe anemia. In contrast, most of the mice treated with drug combinations survived. A combination of artemiside and mefloquine or piperazine may confer an optimal result because of the longer half life of both conventional drugs. The use of artemiside combinations revealed a significant safety margin of the effective artemiside doses. Likewise, a combination of 1.3 mg/kg of body weight artemiside and 10 mg/kg piperazine administered for 3 days from the seventh day postinfection was completely curative. It appears possible to increase drug concentrations in the combination therapy without reaching toxic levels. Using the drug combinations as little as 1 day before the expected death of control animals, we could prevent further parasite development and death due to CM or anemic malaria. Earlier treatment may prevent cognitive dysfunctions which might occur after recovery from CM.**

Three hundred to 500 million people are infected annually with malaria, and about 1 million, mostly children under 5 years, die (47, 48). *Plasmodium falciparum* causes most cases of severe malaria, which sometimes is associated with coma and death; young children and pregnant women are especially vulnerable. Splenomegaly, severe headache, cerebral ischemia, hepatomegaly, hypoglycemia, and renal failure may occur (4, 23). Malaria induced by *P. falciparum* can cause irreversible neurocognitive damage, especially in children. This neurologic damage is associated with cerebral malaria (CM), which is the most severe central nervous system complication of *P. falciparum* infection (6, 24, 45). A significant number of *P. falciparum* malaria cases are expressed as CM (24, 32).

As no successful vaccine for malaria is available, the elimination of the disease is dependent on antimalarial drugs. Unfortunately, in countries where malaria is endemic, many *P. falciparum* strains now have become resistant to formerly effective antimalarial drugs, making alternative drugs a necessity. Derivatives of the natural compound artemisinin constitute a recent group of antimalarial agents in clinical use or under development (Fig. 1a). These derivatives include dihydroartemisinin (DHA), artesunate, artemether, artemisone, and artemiside, the thiomorpholine precursor of artemisone (21). A key strategy to prevent the emergence and spread of resistance is to use artemisinin derivatives in combination with partner drugs in artemisinin-based combination therapy, termed ACT (2, 33, 49). Because artemisinin monotherapy leads to a high rate of recrudescence, other drugs are required to clear all parasites and prevent recurrence. When used as a drug combination, the artemisinin derivatives are effective in a

3-day course because the partner drug, with a longer plasma half-life, fully eliminates remaining parasites. This combined therapy is more efficient and also protects both components of the ACT from causing the selection of resistant parasites (1, 18).

Among the artemisinin derivatives, artemisone and artemiside are newer semisynthetic 10-alkylaminoartemisinins that can be synthesized from DHA (Fig. 1a). Artemisone is highly effective against *P. falciparum* in monkeys and has been used in phase IIa clinical trials for nonsevere malaria in humans (34). Recent work also indicates the marked superiority of artemiside over artemisone in both *in vitro* and *in vivo* studies involving the apicomplexan parasite *Toxoplasma gondii* (12). Artemiside has an advantage over artemisone in that its preparation from DHA requires only a one-step procedure (Fig. 1b). Artemiside has been shown to be potent in treating cerebral malaria (46), which we hypothesize is due to its antiplasmodial capacity and its interactions with the immune system.

The hypothesis of this research is that artemiside is a very potent member of its class for the treatment of severe malaria, with

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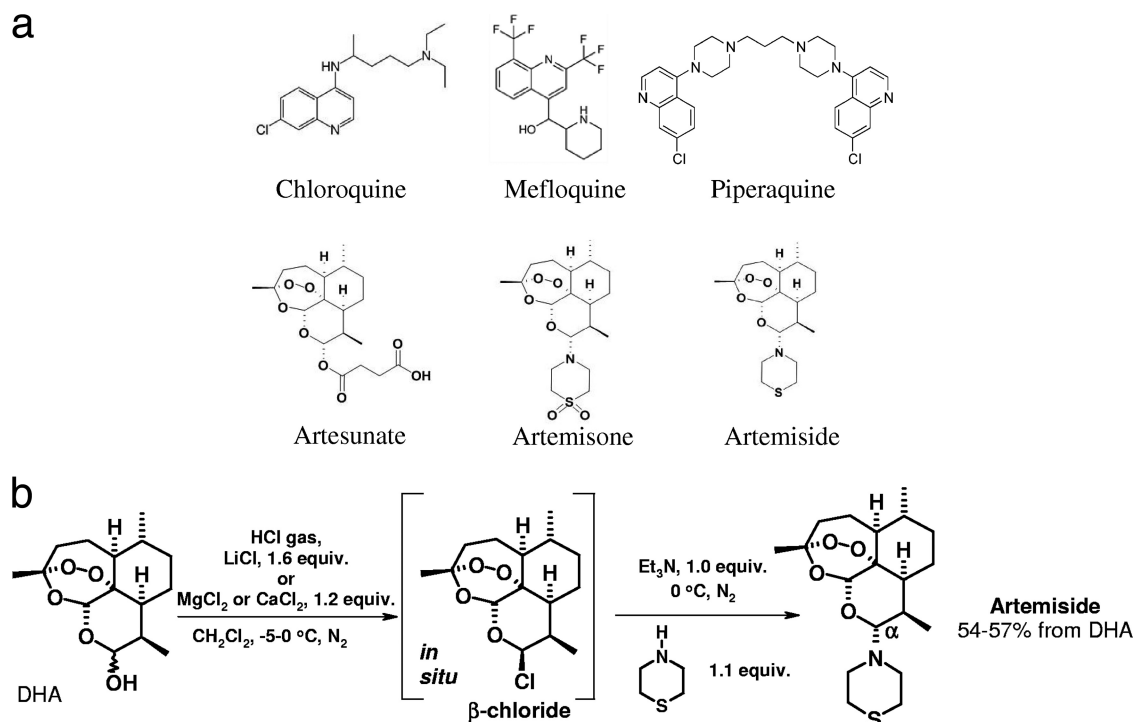
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**FIG 1** (a) Drug structures used for treatment of infected mice. (b) Preparation of artemiside in one step from dihydroartemisinin (DHA). DHA is treated with HCl gas in the presence of a group I or II metal salt, and then, after the degassing of the reaction mixture, with thiomorpholine. The formation of the  $\beta$ -chloride in this reaction is easily detected by conducting  $^1\text{H}$  nuclear magnetic resonance (NMR) spectroscopic examinations of reactions conducted in deuterated dichloromethane ( $\text{CD}_2\text{Cl}_2$ ). Other approaches also have been used (21).

an emphasis on CM prevention, especially when used in combination therapy with conventional antimalarial drugs.

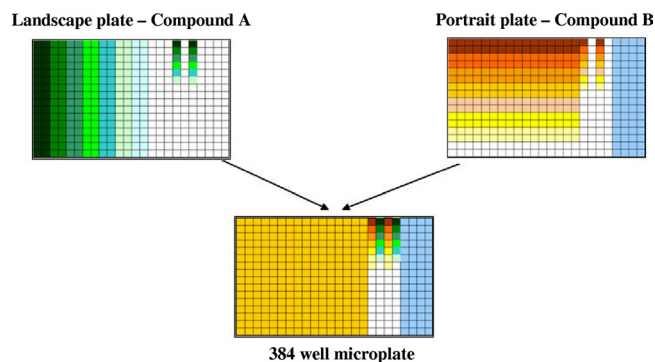
## MATERIALS AND METHODS

**Parasites.** *Plasmodium berghei* ANKA was maintained *in vivo* by the serial transfer of parasitized erythrocytes (PE) from infected to naïve mice. Experimental mice were infected by the intraperitoneal (i.p.) injection of  $5 \times 10^4$  (Jerusalem, Israel) to  $5 \times 10^5$  (Sydney, Australia) PE from peripheral blood of infected donor mice, an inoculum that caused fatal experimental cerebral malaria (ECM) in at least 80% of infected C57BL/6 mice. The link between early death and ECM in mouse models has been discussed previously (46): mice that died at a parasitemia of 20% or below with accompanying neurological symptoms and drastic reductions in body weight and temperature were considered to have died of ECM, which usually was confirmed by the presence in the central nervous system of hemorrhages, edema, and intravascular leukocyte accumulation upon histopathological analysis. Untreated mice which did not die from ECM went on to succumb to severe anemia and hyperparasitemia, as has been reported in all other cases where mice are resistant to *P. berghei* ANKA-induced ECM (14, 35).

The 3D7 strain of *P. falciparum* (purchased from the American Type Culture Collection [ATCC]) was grown in culture as specified later.

**Animals.** C57BL/6 mice (C57BL/6 from Harlan, Israel, or from the Animal Resource Centre, Perth, Australia) aged 7 to 8 weeks were used in all experiments, with 6 to 10 mice per group. The mice were housed under standard light and temperature conditions and provided with unlimited access to water and food. All experiments were carried out in accordance with institutional guidelines for animal care by following protocols approved by the Animal Ethical Care Committee of The Hebrew University of Jerusalem or the University of Sydney. Parasitemia was monitored every other day, from the time of inoculation until death or sacrifice, by

thin-blood Giemsa-stained smears prepared from tail blood. The slides were examined under a light microscope, and parasitemia was determined as the number of infected red blood cells per 10,000 erythrocytes. Clinical score was evaluated and used for scoring disease severity (using a scoring



**FIG 2** Combination experiment strategy. Compound A was dispensed in all wells of column 1 and then 2.5-fold serial diluted down the row (landscape plate) from left to right until column 16, each in duplicate. Compound B was dispensed in wells A1 to A16 and then 2.5-fold serial diluted from top to bottom from row A through P, each in duplicate. Compounds A and B also were 2.5-fold serial diluted in columns 17 and 19 (landscape plate) and in columns 18 and 20 (portrait plate) to determine their individual  $\text{IC}_{50}$ s. Controls included the following columns: 22, 100% DMSO as a negative control (0% growth inhibition); 23, 10 mM mefloquine solution as a positive control (100% growth inhibition); 21 and 24, 3-fold serial dilutions of 10 mM mefloquine and 10 mM chloroquine, respectively. Binary combinations were performed by combining a portrait plate (compound A) and a landscape plate (compound B) in a 384-well microplate. Additional controls included combining a landscape plate with its portrait counterpart.

TABLE 1 IC<sub>50</sub>s of individual drugs in *P. falciparum* cultures

Compound	IC <sub>50</sub> of <i>P. falciparum</i> strain 3D7 in:	
	nM	ng/ml
Artemiside	5.70	2.10
Artemisone	2.30	0.92
Artesunate	9.70	3.70
Chloroquine	42.9	13.7
Mefloquine	31.2	11.8
Piperaquine <sup>a</sup>	52.4	28.1

<sup>a</sup> Piperaquine was examined manually (i.e., not by the high-throughput system).

chart [46]). Mice were euthanized when they reached a degree of disease severity that inevitably would have led to their death.

**Histology.** Mice were deeply anesthetized with isoflurane and sacrificed by terminal intracardial perfusion with 10 ml ice-cold phosphate-buffered saline (PBS). Organs were removed and fixed overnight in 10% (vol/vol) neutral buffered formalin (Fronine, Australia). Paraffin-embedded tissues were cut into 5- to 7- $\mu$ m slices, deparaffinated, and stained with hematoxylin and eosin before coverslipping.

**Drugs.** DHA and artesunate were purchased from the Kunming Pharmaceutical Corporation.

Artemisone was synthesized from DHA and purified by flash column chromatography, followed by recrystallization according to the procedure previously reported (21).

Artemiside was prepared according to a new procedure (Fig. 1b) as fol-

lows. HCl gas was passed over a stirred mixture of DHA (5.0 g; 17.6 mmol) and LiCl (1.2 g; 28.3 mmol) in dichloromethane (80 ml) at 0°C. The reaction mixture changed from colorless to pale purple. The system was gently flushed with nitrogen gas for 5 min. After degassing further under reduced pressure with alternate nitrogen flushing, CH<sub>2</sub>Cl<sub>2</sub> (20 ml) was added to rinse the reaction flask and to dilute the reaction mixture. The reaction mixture was treated with the slow dropwise addition of a mixture of thiomorpholine (2.0 ml, 2.1 g, and 19.9 mmol) and triethylamine (2.5 ml, 1.8 g, and 18.0 mmol). The color of the reaction mixture changed to pale yellow. After 45 min, the reaction mixture was quenched with brine (50 ml). The organic layer was separated, and the aqueous layer was extracted with dichloromethane (twice with 20 ml). The combined organic extracts were dried (MgSO<sub>4</sub>). The filtration and evaporation of the filtrate gave a pale yellow solid, which was recrystallized by the addition of methanol (80 ml) to give 10 $\alpha$ -(thiomorpholino) dihydroartemisinin as a fine white microcrystalline solid (3.58 g; 55%). Further material was able to be recovered from the mother liquor by concentration and crystallization. An analytical sample of artemiside was obtained by recrystallization from methanol to give the product as white needles. m.p. 147.0 to 147.6°C; [ $\alpha$ ]<sub>D</sub><sup>20</sup> = +17° (c 0.021, CHCl<sub>3</sub>); IR (film)  $\nu_{\max}$  2,924, 2,872, 1,454, 1,418, 1,376, 1,326, 1,278, 1,226, 1,198, 1,184, 1,154, 1,130, 1,100, 1,056, 1,038, 1,018, 988, 940, 926, 880, 850, 828, 756;  $\delta_{\text{H}}$  5.26 (1H, s, H-12), 3.93 (1H, d, *J* = 10.21 Hz, H-10), 3.23 to 3.31 (2H, m), 2.92 to 2.96 (2H, m), 2.60 to 2.67 (5H, m), 2.25 to 2.45 (1H, m), 1.95 to 2.05 (1H, m), 1.80 to 1.92 (1H, m), 1.67 to 1.70 (2H, m), 1.14 to 1.52 (5H, m), 1.36 (3H, s, H-14), 0.90 to 1.04 (1H, m), 0.91 (3H, d, *J* = 6.14 Hz, H-16), 0.76 (3H, d, *J* = 7.18 Hz, H-15);  $\delta_{\text{C}}$  104.20, 92.28, 91.42, 80.11, 52.20, 51.30, 46.60, 37.90, 36.80, 34.90, 28.45, 26.35, 25.1, 22.10, 20.85, 13.90; MS (CI, NH<sub>3</sub>) *m/z* 370 (M<sup>+</sup>+1, 100), 324

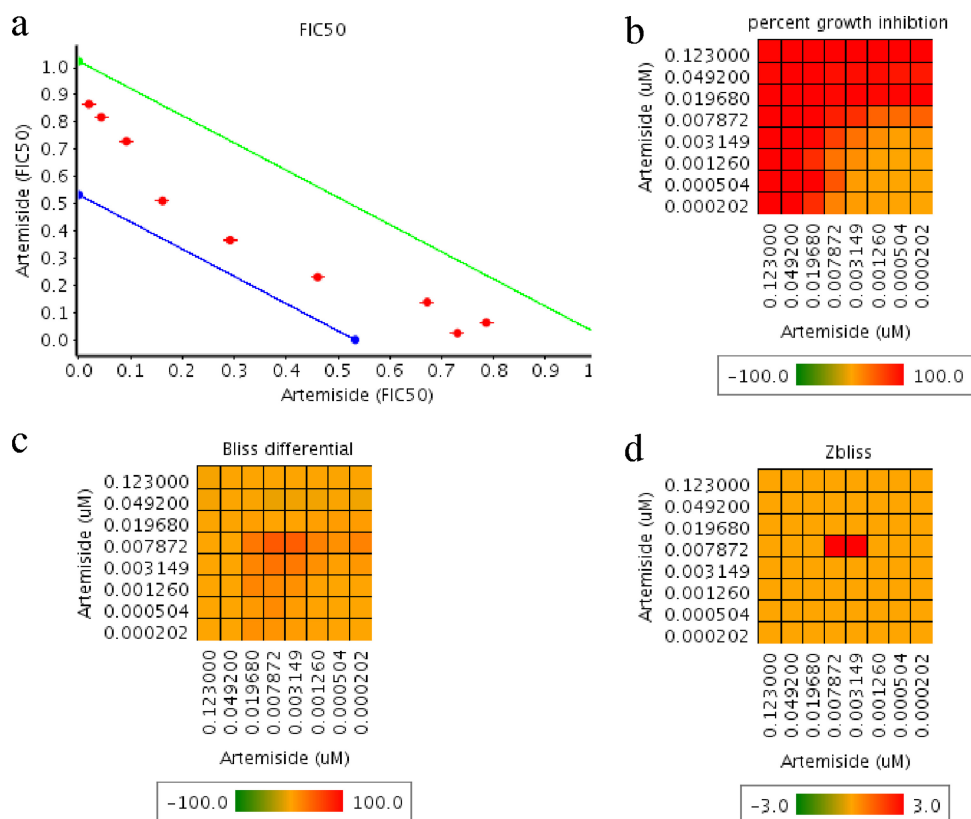


FIG 3 Artemiside pairwise combinations examined *in vitro*. Artemiside combined with itself was used as a control for additivity. (a) Fractional IC<sub>50</sub> of artemiside in combination with itself. The surface between the green and the blue lines defines the additivity area. The distance between these two lines equals 95% of the FIC<sub>50</sub> confidence interval. (b) Heat map representing the percentage of the growth inhibition of the tested drug combinations. (c) Heat map representing the Bliss differential of the drug combinations, the difference in growth inhibition between observed and predicted values. (d) Zbliss is derived from data shown in panel c using Z scores, considering all Bliss differential values as additive values. Value in panels b, c, and d are averages from four dependent replicates.

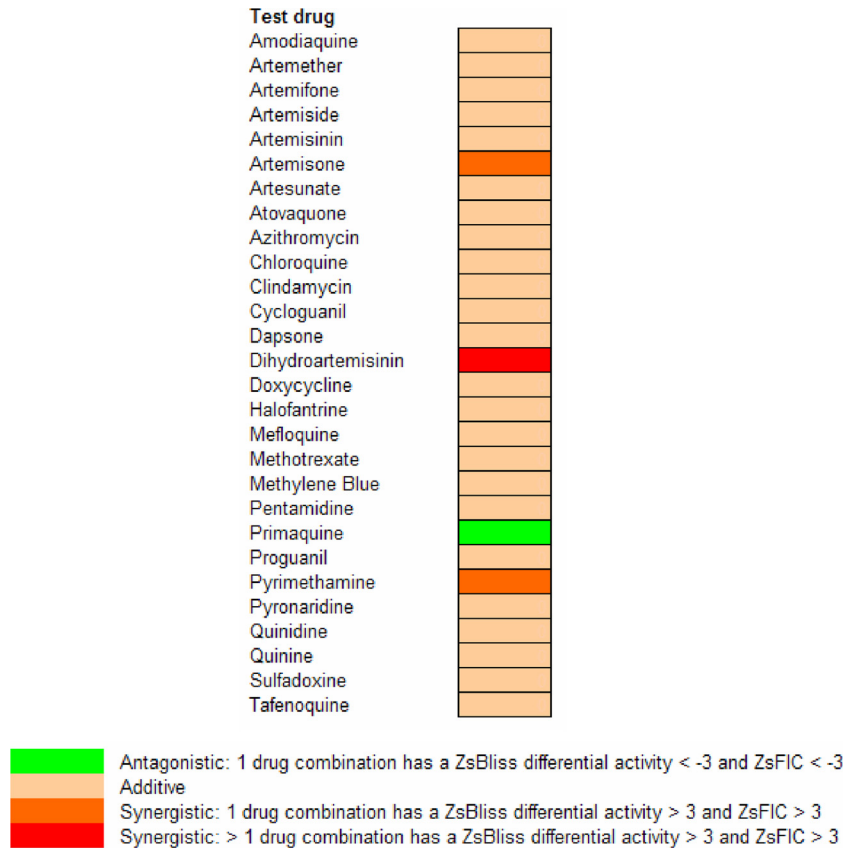


FIG 4 Artemiside pairwise combinations examined *in vitro*. A color-coded summary of the pairwise combinations examined by the high-throughput system is shown. For each pair, FIC<sub>50</sub>s and Bliss values were calculated (as shown in Fig. 3), and the degree of synergy was estimated according to defined criteria based on Z scores (Zs).

(70), 310 (10); Anal. Calc. for C<sub>19</sub>H<sub>31</sub>NO<sub>4</sub>S: C, 61.76; H, 8.46; N, 3.79; found C, 62.04; H, 8.39; N, 3.65.

Piperaquine was donated by Cipla Ltd., Mumbai, India. It was dissolved in distilled deionized water at pH 3.5 (reached with HCl) and injected in 100  $\mu$ l of solution.

Dimethyl sulfoxide (DMSO) and chloroquine diphosphate were purchased from Sigma-Aldrich, Ltd. All artemisinin derivatives were prepared in DMSO according to the required dosage and administered in a volume of 20  $\mu$ l by intraperitoneal injection.

Chloroquine diphosphate (Sigma) was dissolved in PBS and administered in a 50- $\mu$ l volume by intraperitoneal injection.

Mefloquine (Sigma) was dissolved in DMSO and used as the artemisinin derivative.

Drug structures that were used in the current research are presented in Fig. 1a. Drugs were injected six times, either (i) starting in the evening of day 5 postinfection (after inoculation with parasites), twice on days 6 and 7, and once on day 8 (morning), or (ii) twice a day on days 7 to 9 postinfection. All drugs were injected twice a day except for piperaquine, which was injected once a day due to its longer retention time in mice.

**Cytotoxicity assay in THP-1 cell line.** A monocyte THP-1 cell line (ATCC) was cultured in suspension in flat-bottom flasks (Nunc) at 37°C in a humidified 5% CO<sub>2</sub> incubator. The growth medium (Biological Industries, Beit-Haemek, Israel) was RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100  $\mu$ g/ml), and streptomycin (100  $\mu$ g/ml). Before each experiment the cells were washed, and an appropriate volume was centrifuged, resuspended, and diluted in growth medium to the desired cell concentration. Drug toxicity was determined in THP-1 cells using an alamarBlue viability assay. THP-1 cells

were plated into 96-well flat-bottom plates (Nunc) at a density of  $4 \times 10^5$  to 8 cells/ml, which corresponds to  $0.5 \times 10^5$  to  $1 \times 10^5$  cells/well. Using an automated dispenser, 125  $\mu$ l cell suspension was added to the wells. Compounds to be tested were diluted in complete medium, and 125  $\mu$ l was aliquoted in triplicate to the wells. After 48 h of incubation at 37°C, the cell viability indicator alamarBlue (AbD Serotec, United Kingdom) was added (25  $\mu$ l/well), the plates were incubated for an additional 3 h at 37°C, and the fluorescence (excitation wavelength, 544 nm; emission wavelength, 590 nm) was read using a microplate reader (Fluoroskan Ascent FL, Finland). The percent growth inhibition of the cells was calculated according to the equation % inhibition = [(fluorescence control – fluorescence test)/(fluorescence control)]  $\times$  100.

**High-throughput analysis of *in vitro* antiplasmodial activity.** (i) *P. falciparum* drug susceptibility assay. Parasites were grown as previously described (44). For drug susceptibility assays, 20  $\mu$ l of RPMI 1640 (L-glutamine and HEPES; Invitrogen) with 5  $\mu$ g/ml gentamicin was dispensed in each well with a liquid dispenser (Matrix Wellmate; Thermo Scientific) in an assay plate (384-well black polystyrene microplate, clear bottom, tissue culture treated; Corning). DMSO inhibitor stock solutions were pin transferred (V&P Scientific) into the assay plate. Twenty  $\mu$ l of a synchronized culture suspension (1% rings, 4% hematocrit) was added to each well, thus making a final hematocrit and parasitemia of 2 and 1%, respectively. Assay plates were incubated for 72 h, and parasite development was determined based on a modified protocol (42). Briefly, 10  $\mu$ l of mixed DNA dye solution in RPMI (10 $\times$  SYBR green I, 1% [vol/vol] Triton X-100, 1 mg/ml saponin) was added to each well. Assay plates were shaken for 30 s at 2,000 rpm and incubated in the dark for 4 h, and then



fluorescence was measured (excitation at 485 nm, emission at 535 nm) in an Envision plate reader (Perkin Elmer).

(ii) **IC<sub>50</sub> determination and combination experiments in synergy experiments.** To determine the 50% inhibitory concentrations (IC<sub>50</sub>s) of drugs on cultures of *P. falciparum*, 3-fold serial dilutions resulting in 10 different concentrations in DMSO were prepared from stock solutions. Tests were run in triplicate in two independent runs to determine IC<sub>50</sub>s against the 3D7 strain for each drug. Based on IC<sub>50</sub>s, the top concentration for each compound was calculated using the following formula to center the compound's IC<sub>50</sub> around the median dilution point of the plate: top concentration = IC<sub>50</sub> × 1.5 × 2.5<sup>3</sup>. If IC<sub>50</sub>s were greater than 0.5 μM, then a top concentration of 10 μM was used. Landscape and portrait compound plates for drug combination experiments (Fig. 2) were made with a liquid handler (Tecan robot Freedom EVO; Tecan Group Ltd.).

(iii) **Data analysis.** Data were parsed with an in-house algorithm, RISE (for robust investigation of screening experiments). IC<sub>50</sub>s were calculated using Pipeline Pilot v7.5 and GraphPad software. Synergy or antagonism was determined using the following models.

(a) **Bliss independence (3).** The Bliss noninteraction for two drugs in combination is expressed by the following equation:  $E_{ab} = E_a + E_b - (E_a \times E_b)$ , where  $E$  is the fractional effect and  $a$  and  $b$  are the doses of two compounds. The expected effect ( $E_{ab}$ ) is compared to the experimental data and plotted as a heat map for visual analysis. A modified version of the Bliss analysis was done using a Z score. The average Z score was derived from controls in which a compound was tested against itself.

(b) **Fifty percent fractional inhibition concentration (5) (FIC<sub>50</sub>).** A derivative of the Loewe additivity model, FIC was determined using the following equation:  $FIC_{50} = IC_{50a}/IC_{50A} + IC_{50b}/IC_{50B}$ , where IC<sub>50a</sub> and IC<sub>50b</sub> are the respective IC<sub>50</sub>s of the individual drug in the combination and IC<sub>50A</sub> and IC<sub>50B</sub> are the respective IC<sub>50</sub> of each drug alone.

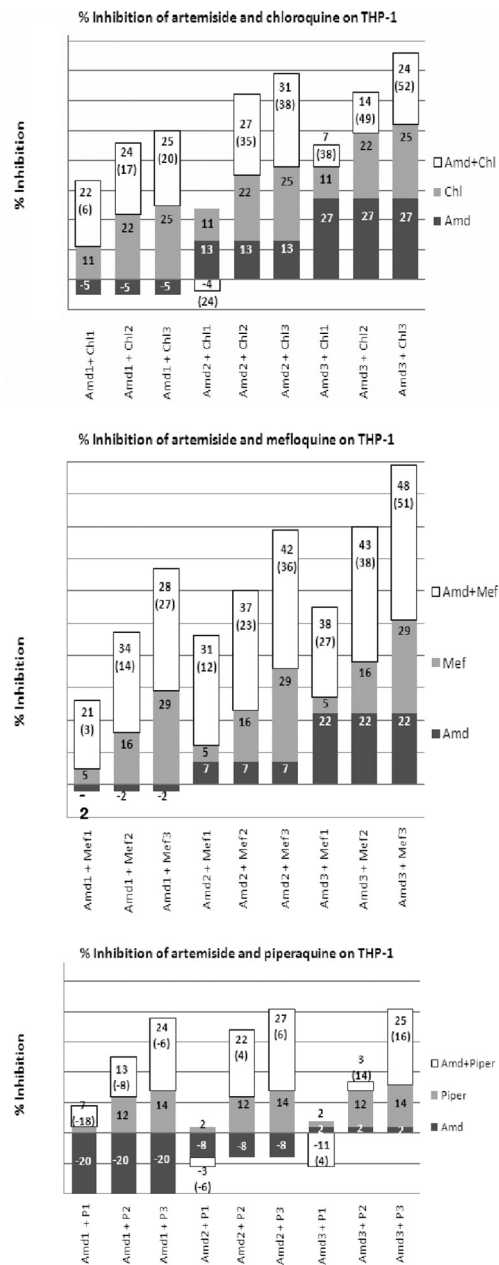
Drug combinations were defined as nonadditive when more than one binary combination effect lay outside the predicted effect (Bliss) and when more than one FIC value was outside the 95% confidence interval of the control FIC<sub>50</sub> (using a compound against itself as a control).

**Manual analysis of *in vitro* antiplasmodial activity.** The effect of piperazine and its combinations with artemiside against *P. falciparum* were manually evaluated *in vitro* by a luciferin-luciferase bioluminescence assay. For this experiment, we use erythrocytic stages of *P. falciparum* stably expressing the luciferase gene by the *hrp2* promoter from a chromosomal locus (*PfLUC*). Cultures containing predominantly ring forms were used. Upon reaching 3% parasitemia, cultures were transferred to a 96-well flat-bottom sterile plate (200 μl/well). Diluted drugs were added to the appropriate wells and tested in triplicate. The plate was incubated in a sealed culture chamber for 24 h in an atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub>. After 48 h the medium was removed and the erythrocytes lysed by lysis buffer of the Bright-Glo luciferase assay system (100 μl/well). Fifty μl of the Bright-Glo substrate was added to each well, and the luminescence was measured by a luminometer (Fluoroskan Ascent FL; Thermo).

**Statistics.** When comparing parasitemia,  $P$  values were calculated using Student's  $t$  test; for the analysis of survival curves, the Kaplan-Meier test was employed. In both cases, values below 0.05 were considered significant.

## RESULTS

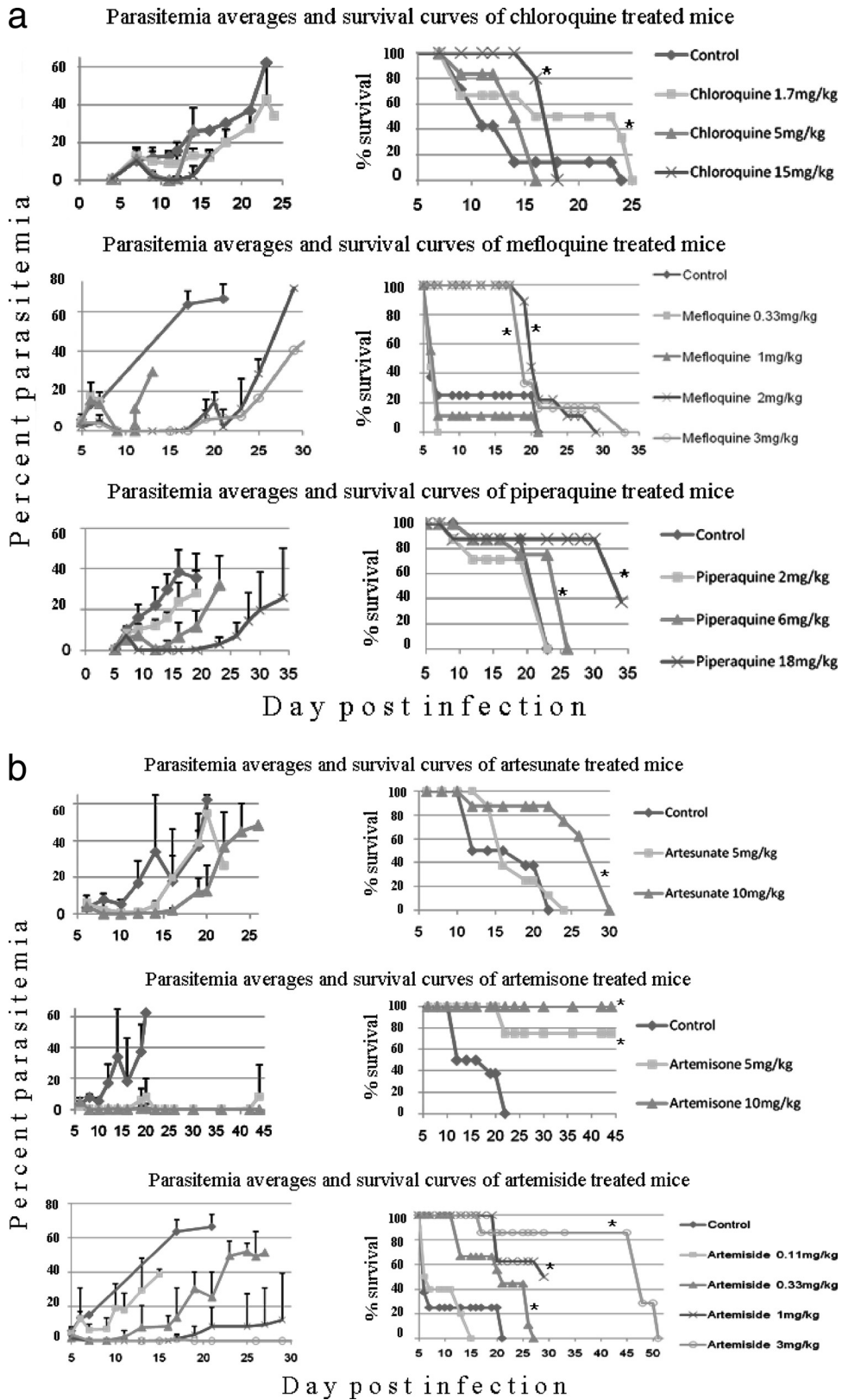
Drug susceptibility assays were performed in *P. falciparum* cultures using high-throughput techniques. Piperazine and its combinations with artemiside were manually examined in microplates. The IC<sub>50</sub>s for chloroquine, mefloquine, piperazine, artesunate, artemisone, and artemiside are shown in Table 1. All IC<sub>50</sub>s were within an average of 2.3 to 52.4 nM. The counts for individual drugs were within 10% of the average result. To evaluate synergistic combinations, we used two orthogonal methods (FIC<sub>50</sub> and Bliss independence), and the combination of artemiside with itself was used to define the additive background (Fig. 3). A sum-



Concentration *ng/ml			
Amd1	10*	Ch1	11.1
Amd2	30	Ch2	33.3
Amd3	90	Ch3	100
		Mef1	507
		Mef2	1520
		Mef3	4560
		P1	200
		P2	400
		P3	800

FIG 5 Cytotoxicity assays of artemiside (Amd) combinations with the standard antimalarial drugs chloroquine (Ch), mefloquine (Mef), and piperazine (P). The numbers represent the experimental results. The numbers in parentheses are the theoretical additive values, e.g., Amd2 (30.0 ng/ml), Ch2 (33.3 ng/ml), and their combination inhibit 13, 22, and 27% of untreated THP-1 growth, respectively. The theoretical additive value is 35% (13% ± 22%).

mary of the overall results of examining combinations of artemiside with conventional antimalarial drugs is shown in Fig. 4. Primaquine was the only antagonistic drug, while most drugs were additive. The artemiside-artemisinin combination was additive, as



**FIG 6** (a) Treatment of *P. berghei* ANKA-infected mice with individual conventional antimalarials. An asterisk indicates significance ( $P < 0.01$ ). (b) Treatment of *P. berghei* ANKA-infected mice with individual artemisinin derivatives. An asterisk indicates significance ( $P < 0.01$ ) in each panel.

expected, but some other artemiside-artemisinin derivative combinations were synergistic in one run (and additive in two runs). Synergism among various artemisinins could be explained by the fact that they may affect the parasites by multiple mechanisms (see Discussion) that are effective at different concentrations. In view of these results and the availability of reference results of other ACTs (18, 36, 37, 43), we conducted *in vitro* cytotoxicity experiments with THP1 cells and *in vivo* combination experiments to monitor artemiside combinations with mefloquine and piper-quinine. Chloroquine also was examined due to its proven *in vivo* synergistic effect with artemisone, another 10-alkyl amino artemisinin (46). The cytotoxicity results show that all compounds tested, including their combinations with artemiside, are nontoxic at doses much higher than needed to kill *P. falciparum* in culture (Fig. 5).

The individual effects of artemiside, chloroquine, mefloquine, and piper-quinine on *P. berghei* ANKA infections in C57BL/6 mice were determined. Artesunate also was used for comparison with artemiside. Figures 6a and b depict the resulting parasitemias and survival curves. Following treatment, parasitemias were reduced and survival prolonged, in parallel with an (temporary) increase in body weight and temperature (data not shown). All parameters were affected in a dose-dependent manner. However, the most pronounced effects were recorded for artemiside: 0.66 mg/kg treatment generally was as beneficial as 10-fold greater concentrations of the other compounds. In general, individual drugs at the concentrations that were examined failed to prevent malarial death or CM (Table 2). However, most of the mice were rescued by using high concentrations of artemisone and artemiside (>5 and >3 mg/kg for artemisone and artemiside, respectively). The results obtained with the individual drugs enabled us to select concentrations that induced a temporary reduction in parasitemia (Fig. 6a and b) and clinical score (data not shown) for further *in vivo* combination experiments.

Figure 7 depicts a representative experiment in which artemiside, mefloquine, and their combination were used. Vehicle control (DMSO) and mefloquine alone failed to prevent the death of the infected mice. Mefloquine and artemiside prevented the early death of the mice by the initial reduction of the parasitemias to a nondetectable level. Both drugs delayed recrudescence by about a week. However, artemiside reduced the death to 4 out of 9 mice, while all mefloquine-treated mice eventually developed fatal illness and had to be euthanized. All control mice died of CM at parasitemias lower than 20%. Most animals that died in the mefloquine and artemiside groups also suffered from CM following recrudescence. In contrast, most mice (8/9) that were treated by the drug combination were free of parasites even after three injections and survived. One animal in this group died of CM at day 23 postinfection.

Figure 8 depicts a representative experiment that is similar in its design to the previous one, and it demonstrates the effect of artemiside combined with chloroquine. The results generally are similar to those obtained with the artemiside-mefloquine combination: the drugs delayed recrudescence after the initial reduction of parasitemia, and the combination gave a significant increase in survival.

Figure 9 shows the results of treatment using an artemiside-piper-quinine combination. The figure depicts the results of individual mice to demonstrate the reproducibility of the results. Drugs alone (artemiside at 0.66 mg/kg and piper-quinine at 10 mg/

TABLE 2 Summary of *in vivo* treatments<sup>a</sup>

Compound(s) (in mg/kg)	No. of mice		
	dCM	dSM	Survived
Control saline	17	8	0
Control DMSO	13	4	0
Chloroquine (1.7)	3	3	0
Chloroquine (5)	4	2	0
Chloroquine (15)	7	5	0
Mefloquine (0.33)	8	1	0
Mefloquine (1)	8	1	0
Mefloquine (2)	7	2	0
Mefloquine (3)	5	1	0
Piper-quinine (2)	4	3	0
Piper-quinine (6)	3	5	0
Piper-quinine (10)	0	7	1
Piper-quinine (18)	2	4	2
Artesunate (5)	6	2	0
Artesunate (10)	3	5	0
Artemisone (5)	8	3	5
Artemisone (10)	4	3	8
Artemiside (0.11)	9	1	0
Artemiside (0.33)	3	6	0
Artemiside (0.66)	8	0	0
Artemiside (1)	3	1	4
Artemiside (3)	5	0	2
Chloroquine (5) and artemisone (10)	1	0	6
Chloroquine (15) and artemisone (10)	0	0	7
Piper-quinine (10) and artemisone (5)	0	0	8
Chloroquine (10) and artemiside (0.66)	1	3	2
Mefloquine (2) and artemiside (0.33)	2	3	4
Mefloquine (1) and artemiside (1)	1	0	5
Mefloquine (2) and artemiside (1)	1	0	7
Piper-quinine (10) and artemiside (0.66)	0	0	9

<sup>a</sup> The table summarizes *in vivo* experiments in which mice were injected with *P. berghei* ANKA and treated with drugs alone or in combination with artemiside. Mice that died at a parasitemia below 20% with accompanying neurological symptoms, matching clinical score, and brain histology were considered to have died of CM (dCM). Mice which did not die of CM went on to die at higher parasitemias, with severe anemia (dSM).

kg) reduced the initial peak parasitemia and delayed the recrudescence but did not prevent the mortality (except for that of one mouse in the piper-quinine group). In contrast, the combination reduced the initial peak, there was no recrudescence, and all mice survived. Parasitemia monitoring in this experiment was accompanied by measuring weight and temperature and the estimation of the clinical score. Weight, temperature, and clinical score were in accordance with the parasitemias.

## DISCUSSION

The decreasing effectiveness of the antimalarial arsenal due to drug resistance necessitates constant efforts to develop new drugs. Artemisinin derivatives are considered the front line of treatment, but there already are indications of resistance to the currently used artemisinins by *P. falciparum*, especially to artemether and artesunate (11, 18). Consequently, it is critical to evaluate the antimalarial effect of newer artemisinins that could be developed as ACT formulations (31). While one important consideration is that the drug combination should assist in countering the development of resistance, it must be recognized that artemisinins have short half-lives, and to avoid recrudescence the combination partner should

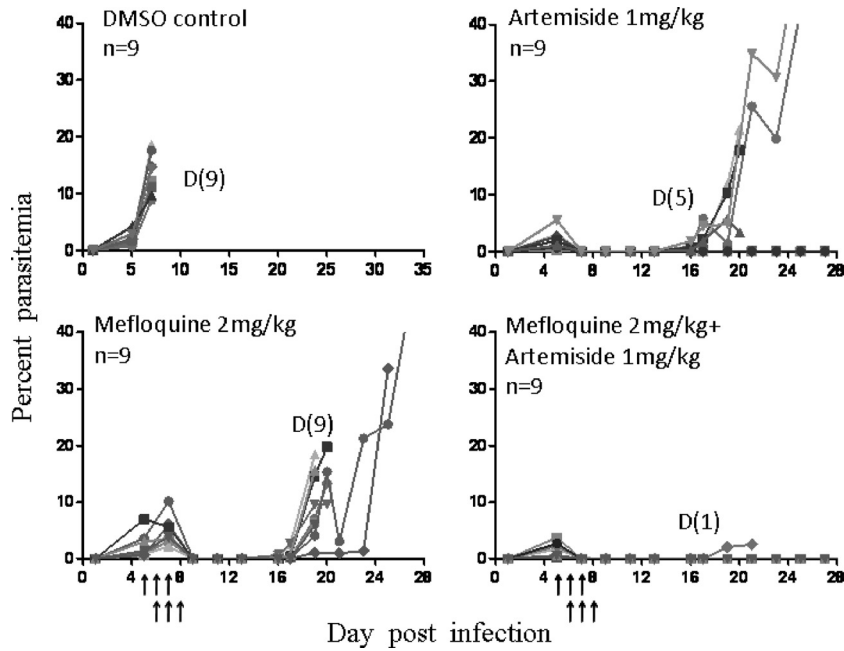


FIG 7 Artemiside-mefloquine effects in *P. berghei* ANKA-infected mice. Each line represents parasitemias of a single mouse. Arrows denote treatment times. n, number of mice in the group; D, number of dead mice in the group.

have a long half-life (13). For this reason, mefloquine was first used in combination with artemisinin by Li and coworkers, where it was most effective in curing chloroquine-resistant malaria on Hainan Island (27).

The research here describes the use of novel combinations of the new artemisinin compound artemiside, a 10-alkylamino artemisinin derivative. Artemiside was prepared in a one-step process

from DHA that requires no chromatographic purification, and because of the relative accessibility of one of the other starting materials, thiomorpholine (Fig. 1b), artemiside is more economically prepared than is artemisone, the 10-alkylamino artemisinin derivative that is currently under development for clinical use. Artemiside is a stable, highly crystalline compound that appears to have a stability profile similar to that of artemisone (results not

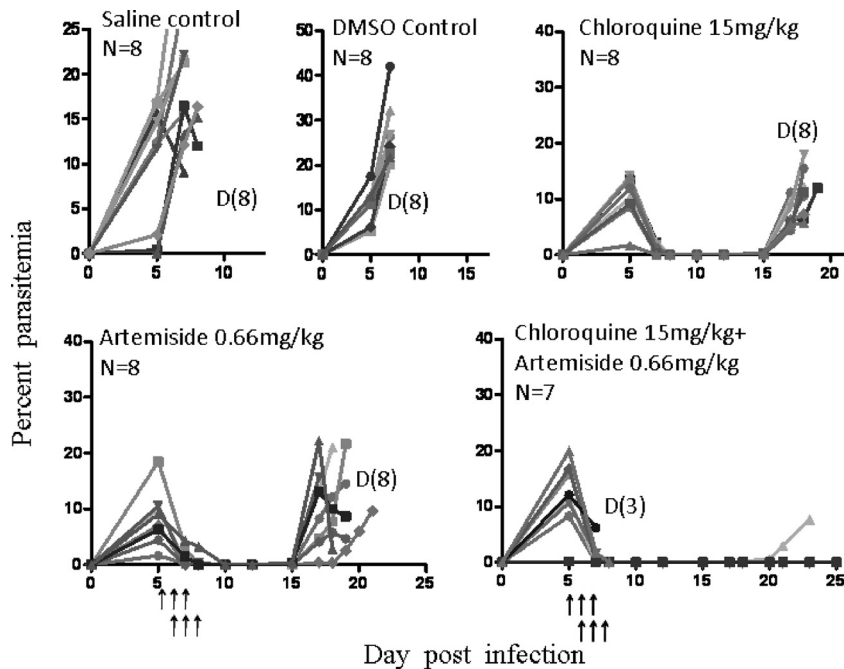


FIG 8 Artemiside-chloroquine effects in *P. berghei* ANKA-infected mice. Each line represents parasitemias of a single mouse. Arrows denote treatment times. n, number of mice in the group; D, number of dead mice in the group.



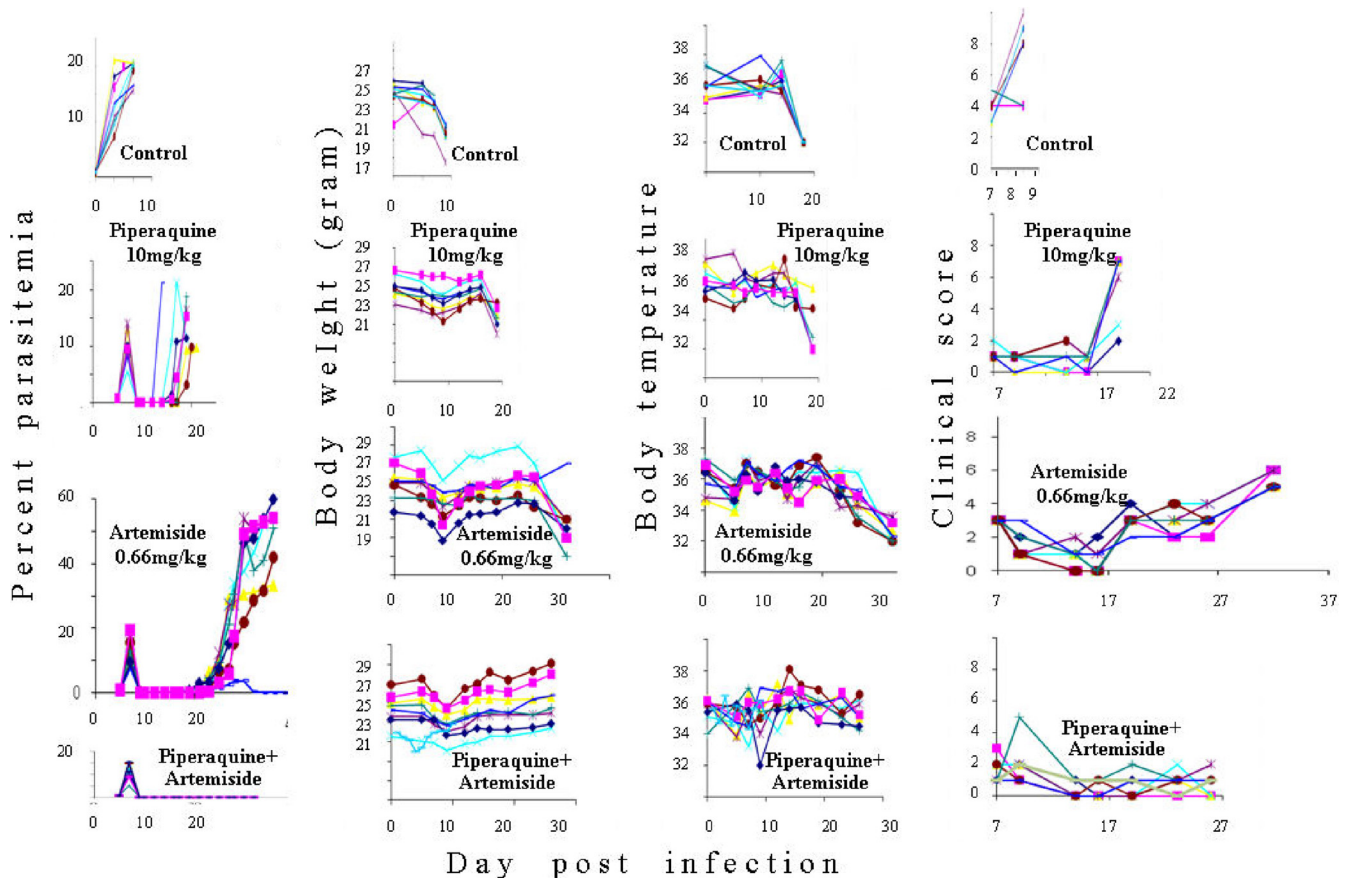


FIG 9 Artemiside-piperazine effects in *P. berghei* ANKA-infected mice. Each line represents a single mouse. Artemiside treatment was twice a day and piperazine once a day on days 7 to 9 postinfection. Each group included eight mice.

shown). We found in *P. falciparum* cultures that its activity is more pronounced than that of artesunate and artemisone. The differences in the activity of the individual drugs also were expressed in our *in vivo* model of C57BL/6 mice that were infected with *P. berghei* ANKA.

The testing of the antiplasmodial activity of various artemiside combinations *in vitro* revealed a relatively large number of conventional antimalarials that were additive with artemiside. These results and the *in vivo* testing of candidate drugs enabled the selection of drug concentrations for monotherapies that had a significant effect on the course of experimental malaria but did not prevent death. Subsequent drug combination studies used these concentrations. Following monotherapy, individual drugs reduced parasitemias to nondetectable levels. However, after a period of latency, parasites again appeared in the blood and eventually all mice died. The treatments did not prevent CM in the mice with malaria recrudescence, except for piperazine, which decreased CM frequency at its higher concentrations. Even when CM was prevented, the mice died later of severe anemia. In contrast, most of the mice treated by drug combinations survived.

Compared to artemisinins in current use, artemisone is non-neurotoxic both *in vitro* and *in vivo* (9). Artemiside, the thiomorpholine precursor of artemisone, is more lipophilic (log *P*, 4.97; calculated log *P*, 3.98) than artemisone (log *P*, 2.49; calculated log *P*, 2.08); however, in line with the facile metabolism of drugs containing thioether linkages, it is expected to be irreversibly metab-

olized *in vivo* into artemisone (15, 39). In *in vitro* neurotoxicity screens, artemiside has  $IC_{50}$ s that are approximately two orders of magnitude higher than those of artemisinin (21). *In vivo* pilot tolerability screens involving the treatment of male rats with artemiside (10 mg/kg for 14 days by gavage) in sesame oil (a vehicle that exacerbates neurotoxic effects) elicited no clinical effects. In contrast to artemisone, which has no effect, artemiside administered at 50 mg/kg for 14 days induced body weight loss, reduced motility, uncoordinated gait, the discoloration of feces, and piloerection. However, according to our current results, artemiside is a more efficient antiplasmodial drug than artemisone *in vivo*. Moreover, the use of artemiside combinations with other drugs revealed a significant safety margin of the effective artemiside doses. For example, a combination of 1.3 mg/kg artemiside and 10 mg/kg piperazine administered for 3 days from the seventh day postinfection was completely curative. Overall, in view of the outcome of the *in vitro* antiplasmodial and cytotoxicity tests and our *in vivo* results, it is most probable that the concentrations of the drugs used in the combination therapy can be increased without reaching toxic levels. Consequently, the parasites will be completely eliminated.

There are a few mechanisms of action proposed for artemisinins (10, 17, 38). It is commonly accepted that artemisinins are accumulated in parasitized erythrocytes, and that they increase oxidant stress within the parasitized cell. Most recently, it has been shown that artemisinins potently interfere with flavin cofactors

that are essential for the functioning of flavin disulfide reductases that maintain redox homeostasis in the intraerythrocytic parasite (19, 20). Other mechanisms have been suggested. Therefore, it is possible that there is more than one mechanism of action for a single artemisinin compound, and that different mechanisms are active at different drug concentrations. This also should be considered in selecting a drug combination (where the result could be a potentiation of different mechanisms). It may be possible to elucidate the use of individual drugs that are less effective alone because of parasite resistance but are efficient and synergistic in a combination (e.g., Malarone contains atovaquone and proguanil, and *P. falciparum* resistance to both of these has been shown). It has been suggested that artemisinin drugs induce *P. falciparum* dormancy, and this is the reason for recrudescence, but a recent paper by Codd et al. (8) suggests that the dormancy recovery hypothesis is not valid. In any case, a drug combination should prevent (in high enough doses) the reappearance of plasmodia. We achieved such an effect by using the artemiside-piperaquine combination. Using the drug combinations as little as 1 day before the anticipated death of control animals, we could prevent further parasite development and death due to CM or anemic malaria. However, if treatment was started at a later stage (12 h before the death of control mice), even higher doses of the drugs could not prevent terminal illness (data not shown). Obviously, the earlier the antimalarial treatment is given to a patient, the better the chance that the treatment will be successful. This is especially vital if the pathogenic process leading to CM has been initiated already, which in ECM occurs at least several days before terminal illness (7). In this case, a delayed treatment may eliminate the parasites and prevent death but not neurological damage (25, 28). Cognitive dysfunction might be sustained after rescue therapy in ECM, but it is reduced by additive antioxidant therapy (40). This might be a hint to consider drug combinations that also include components that reduce oxidant stress. In addition, immunomodulators that could alleviate CM should be considered for adjunctive therapy due to the inflammatory etiological nature of CM (and severe malaria in general) in both human malaria and experimental models (16, 22, 26, 29, 30, 41). Obviously, an adjunctive drug also should be examined in combination with the antiplasmodial drugs to exclude any deleterious antagonistic effects.

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