T he dwindling arsenal of drugs for treatment of malaria and the need for developing and selecting new ones is a continuing problem that has been extensively discussed. Most drugs that recently have been approved for human use are artemisinin derivatives (1). However, some artemisinin derivatives that have been introduced have already encountered various degrees of resistance and consequently have been used in drug combinations (2, 3, 4).

Artemisone is a recent artemisinin derivative, a semisynthetic 10-alkylamino artemisinin that can be synthesized from dihydroartemisinin. It is an attractive drug because, in comparison to current artemisinins, it is not neurotoxic. In in vitro screens, it elicits no cytotoxicity toward brain stem cell cultures and neurofilaments at concentrations up to 25 μM and has no effect on the respiratory chain (5, 6). Lack of neurotoxicity was also verified in filaments at concentrations up to 25 μM/100 μM (7).

Artemisone was found to be highly effective in culture against Plasmodium falciparum (8) and in vivo against murine cerebral malaria (CM) induced by P. berghei ANKA (4) and against P. falciparum in monkeys (9). It has been used in a phase Ia clinical trial for nonsevere malaria in humans (10). Artemisone can cure Toxoplasma gondii (11) and Neospora caninum (12) in animal models.

We recently have shown in a mouse model of CM that artemisone could prevent death even when administered at relatively late stages of cerebral pathogenesis. No parasite resistance to artemisone was detected and coadministration of artemisone and chloroquine was more effective than monotherapy with either drug, leading to complete cure (4). These results suggest the use of artemisone for combination therapy. However, a thorough study is needed to establish the efficacy of additional combinations of artemisone with commercially available antimalarial drugs. For this purpose, we used high-throughput in vitro screening against P. falciparum and a reliable CM model (P. berghei ANKA in C57Bl mice) for in vivo validation (2).

When choosing a multiple testing procedure for screening combinatorial drug libraries, natural products, or any compound reservoir, the results suggesting further investigation or rejection of a candidate drug often ignore a possible significant effect on the outcome of treatment following the use of these drugs: attenuation of immune responses may alleviate clinical symptoms that are caused by immunopathology. In this context, various forms of severe malaria, including CM, are the result of immunopathology (13). Therefore, immunomodulators represent an interesting new approach to CM treatment. Likewise, fasudil, a Rho kinase inhibitor, was suggested as an adjunctive therapeutic agent in the man-

The decreasing effectiveness of antimalarial therapy due to drug resistance necessitates constant efforts to develop new drugs. Artemisinin derivatives are the most recent drugs that have been introduced and are considered the first line of treatment, but there are already indications of Plasmodium falciparum resistance to artemisinins. Consequently, drug combinations are recommended for prevention of the induction of resistance. The research here demonstrates the effects of novel combinations of the new artemisinin derivative, artemisone, a recently described 10-alkylamino artemisinin derivative with improved antimalarial activity and reduced neurotoxicity. We here investigate its ability to kill P. falciparum in a high-throughput in vitro assay and to protect mice against lethal cerebral malaria caused by Plasmodium berghei ANKA when used alone or in combination with established antimalarial drugs. Artemisone effects against P. falciparum in vitro were synergistic with halofantrine and mefloquine, and additive with 25 other drugs, including chloroquine and doxycycline. The concentrations of artemisone combinations that were toxic against THP-1 cells in vitro were much higher than their effective antimalarial concentration. Artemisone, mefloquine, chloroquine, or piperaquine given individually mostly protected mice against cerebral malaria caused by P. berghei ANKA but did not prevent parasite recrudescence. Combinations of artemisone with any of the other three drugs did completely cure most mice of malaria. The combination of artemisone and chloroquine decreased the ratio of proinflammatory (gamma interferon, tumor necrosis factor) to anti-inflammatory (interleukin 10 [IL-10], IL-4) cytokines in the plasma of P. berghei-infected mice. Thus, artemisone in combinations with other antimalarial drugs might have a dual action, both killing parasites and limiting the potentially deleterious host inflammatory response.
agement of severe malaria (14, 15). IDR-1018, an adjunctive anti-inflammatory peptide, was partially protective against murine CM (16). Moreover, antiplasmodial drugs, including artemisinins, may affect immune responses, in addition to exerting direct effect on the parasites (17, 18).

In view of growing information on parasites resistant to artemisinin derivatives, malaria treatments now recommended by the World Health Organization are artemisinin-based combination treatments (ACT). These are combinations of an artemisinin derivative and another structurally unrelated and more slowly eliminated antimalarial (19). Such pairings might include drugs that are not effective as a monotherapy but are useful in combination; for example, combined atovaquone and proguanil (Malarone) are considered a useful malaria therapeutic agent. However, there are indications of resistance to atovaquone-proguanil (20), stressing the need for a constant search for both new individual antimalarial compounds and drug combinations. Consequently, we decided to examine the effects of artemisone, a recently discovered 10-alkylaminooxidized antimalarials. In this study, we focused on murine cerebral malaria, including an examination of the effect of a representative drug combination on cytokine responses that are relevant to CM induction.

MATERIALS AND METHODS

Parasites. *P. berghei* ANKA was maintained in vivo by serial transfer of parasitized erythrocytes (PE) from infected to naive mice. Experimental mice were infected by intraperitoneal (i.p.) injection of 5 × 10⁸ 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 (2, 4): 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 where possible was confirmed by the presence in the central nervous system (CNS) of hemorrhages, edema, and intravascular leukocyte accumulation upon histopathological analysis. Untreated mice that 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 ECM induced by *P. berghei* ANKA (21, 22).

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 (Harlan, Jerusalem, Israel; Animal Resources Centre, Perth, Australia) 7 to 8 weeks old were used in all experiments, 8 to 10 mice per group (as described). 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 protocols approved by the Animal Ethical Care Committee of The Hebrew University of Jerusalem, and in accordance with the guidelines under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the University of Sydney Animal Ethics Committee. Parasitemia was monitored microscopically in thin blood Giemsa-stained smears prepared from tail blood. Clinical score was evaluated and used for scoring disease severity (see the scoring chart in reference 4). Mice were euthanized when they reached a degree of disease severity that would inevitably 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. Paraffin-embedded tissues were cut into 5- to 7-μm slices, deparaffinized, and stained with hematoxylin and eosin before being coverslipped.

Drugs. Dihydroartemisinin (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 (8). Piperaquine was donated by Cipla Ltd., Mumbai, India. It was dissolved in double-distilled water, adjusted to pH 3.5 with HCl, and injected in 100 μl. Dimethyl sulfoxide (DMSO) and chloroquine diprophosphate 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 μl by intraperitoneal injection. Chloroquine diprophosphate (Sigma) was dissolved in PBS and administered in a 50-μl volume by intraperitoneal injection. Mefloquine (Sigma) was dissolved in DMSO and used in the same way as the artemisinin derivatives. Drug structures have been shown elsewhere (2). Artemisone and chloroquine were injected 6 times, twice on days 6, 7, and 8. Piperaquine was injected once a day due to its longer half-life in mice (23).

Drug toxicity. Drug toxicity was determined in THP-1 cells (human monocytic, ATCC, USA) as previously described (2) using alamarBlue viability assay in 96-well flat-bottom plates (Nunc). The alamarBlue method has been questioned concerning the use of redox-active drugs and/or adherent or fast-growing cells. However, in our system, where the cells were exposed to the drugs before the addition of the indicator and the cells were not adherent or fast growing, it is unlikely that there is a significant aberration of the results. Percent growth inhibition of the cells was calculated according to the equation % inhibition = (Fluorescence control – Fluorescence test)/Fluorescence control × 100.

Automated screening of *in vitro* antimalarial activity. Automated screening was described elsewhere (2). Briefly, parasites were incubated for 72 h and parasitemia was estimated by using a DNA dye solution (SYBR green). Three-fold serial dilutions resulting in 10 different concentrations were examined. Tests were run in triplicates in two independent runs to determine 50% inhibitory concentrations (IC₅₀) against the 3D7 *P. falciparum* strain for each drug. Synergy or antagonism was determined using Bliss independence (24) and fractional inhibition concentration (FIC₅₀) (25). Drug combination were defined as nonadditive when more than one binary combination effect lay outside the predicted effect (Bliss) and when more than one FIC₅₀ was outside the 95% confidence interval of a control FIC₅₀ (using a compound against itself as a control).

Analysis of *in vitro* antimalarial activity. The effects of piperazine and its combinations with artemisone against *P. falciparum* were evaluated manually *in vitro* by a luciferin-luciferase bioluminescence assay (2). Briefly, we used erythrocytic stages of *P. falciparum* stably expressing the luciferase gene by the hsp2 promoter from a chromosomal locus (PfLUC) in 96-well flat-bottom sterile plates. After 48 h, medium was removed and the erythrocytes were lysed by the lysis buffer of the BrightGlo lucerase assay system (100 μl/well). A total of 50 μl of the Bright-Glo substrate was added to each well, and the luminescence was measured by a luminometer (Fluoroskan Ascent FL; Thermo). ELISA. Enzyme-linked immunosorbent assays (ELISAs) for murine plasma cytokine analysis of interleukin 10 (IL-10), gamma interferon (IFN-γ), IL-4, and tumor necrosis factor (TNF) were purchased from BioLegend, Israel. C57BL/6 mice were injected with *P. berghei* ANKA and treated with different drugs at days 6, 7, and 8 postinfection (p.i.). On days 0, 5, 8, and 12 p.i., mice were sacrificed and blood samples were collected in heparin for plasma cytokine analysis of IL-10, IFN-γ, IL-4, and TNF by ELISA according to the manufacturer’s instructions.

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

RESULTS Evaluation of drug combinations in *P. falciparum*. Drug susceptibility assays were performed in *P. falciparum* cultures, using high-throughput sequencing (HTS) techniques. To evaluate syn-
ergistic combinations, we used two orthogonal methods (FIC50 and Bliss independence), and the combination of artemisone with itself was used to define the additive background (Fig. 1). A summary of the overall results of examining combinations of artemisone with conventional antimalarial drugs is shown in Fig. 2. Artemisone in combination with most antimalarials currently used in the clinic was additive, except with halofantrine and mefloquine, where synergy was identified. No antagonism was identified.

Manual analysis of in vitro antiplasmodial activity revealed identical results (data not shown). Artemisone-piperaquine combinations that were examined only manually depicted a synergistic effect.

Cytotoxicity assays. Drug toxicity was determined against THP-1 cells using the alamarBlue viability assay. For positive control (maximal growth inhibition), we used KuRei, a cell inhibitor (26). The assay was performed in triplicates, and the standard deviation of the activities was within 10% of the mean for each drug. The IC50s of artemisone and chloroquine were >282 and 313 nM, respectively; the IC50s for mefloquine and piperaquine were >12.1 and 1.5 μM, respectively. These data, and the results of drug combinations (Fig. 3), should be compared to the effects of the drugs on P. falciparum cultures (2). The antiplasmodial effects were evident at much lower concentrations (about 80-fold lower). It is obvious that the drug combinations had no synergistic cytotoxicity.

The effects of artemisone combination therapy on P. berghei-infected mice. Drug concentrations suitable for in vivo combination experiments were selected based on the HTS screening and previous results (2, 4): those that induced a temporary reduction in parasitemia and clinical score. Combination therapy of infected mice with artemisone and either mefloquine, chloroquine, or piperaquine was applied to try to prevent the cerebral symptoms and achieve complete cure. Figure 4 and 5a and b depict resulting parasitemias, survival curves, body temperatures, weights, and clinical scores. An abrupt decline in temperature and loss of weight were reflecting initial symptoms of the disease that were alleviated by the drugs. However, all mice treated with the individual drugs, despite an initial delay in parasitemia increase, eventually succumbed to the disease. In contrast, the combinations prevented CM, delayed recrudescence, and prevented death in most mice. Artemisone-mefloquine combinations were superior to the individual drugs but not as efficient as artemisone combinations with chloroquine or piperaquine.
Histology. Brains were assessed at different intervals postinfection (at least four matching animals from each group). The results paralleled the clinical score: in control untreated mice, hemorrhages and intravascular leukocyte accumulation were abundant, but in drug-treated animals depicting low clinical score, there were no such manifestations (data not shown).

Analysis of cytokines involved in development of CM. The purpose of this part of the study was to examine a possible correlation between the outcome of the treatment and the immunological status of the treated animals. We examined the effects of different therapies on the plasma levels of representative cytokines in the malaria-infected mice treated at days 6, 7, and 8 p.i. with artemisone (10 mg/kg of body weight twice per day), chloroquine (15 mg/kg twice per day), or their combination. These concentrations were chosen to allow maximal antimalarial activity (without approaching toxic levels). TNF and IFN-γ represent proinflammatory cytokines (Th1 type). IL-4 and IL-10 represent anti-inflammatory cytokines (Th2 type). All injected animals had shown early CM symptoms (coat ruffled, hunched, slight decrease in body weight and temperature) before treatment was started (e.g., ruffled coat, wobbly gait; scoring chart, see reference 4). Cytokine plasma levels were estimated using ELISA on days 0, 5, 8, and 12 p.i. Control groups included uninfected untreated, uninfected treated, and infected untreated mice.

On day 0 (Fig. 6A), in uninfected untreated mice, the IL-10 level in the plasma was lower than the levels of the other cytokines.
On day 5 (Fig. 6B), in infected untreated mice, the most striking events were a significant increase in TNF and a decrease in IL-4 levels, in comparison both with the other cytokines and with their level in the control group on day 0.

On day 8 (Fig. 7), in chloroquine- and combination-treated mice, the plasma level of the anti-inflammatory cytokine IL-4 was higher than in infected untreated mice. The IL-10 level had increased in all treated groups compared to that in untreated mice, where it was below detection. Plasma IFN-γ levels were comparable in all groups, although there was a slightly lower level in the combination-treated mice. It is interesting that infected untreated mice displayed much higher TNF in plasma than all the treated mice. These results are consistent with the involvement of proinflammatory cytokines, such as IFN-γ and TNF in CM.

**DISCUSSION**

Drug combinations are the current strategy in malaria treatment to contain resistance to individual drugs or postpone its induction. Vivas et al. (27) found, by using isobolograms obtained with susceptible 3D7 and drug-resistant K1 *P. falciparum* strains at the IC₅₀ level, slight antagonistic trends between artemisone and chloroquine, amodiaquine, tafenoquine, atovaquone, or pyrimethamine. Additive to slight synergistic interactions were seen with artemisone and mefloquine, lumefantrine, or quinine. *In vitro* automated screening allows for the examination of multiple drug combinations in order to identify potential partner drugs to be used in the clinic or to avoid combinations that would be antagonistic (Fig. 1 and 2). We tested artemisone in combination with most antimalarials currently used in the clinic and found that it was additive with all drugs tested except halofantrine and mefloquine, where synergy was identified (Fig. 2). Most importantly, no antagonism was identified, implying that artemisone could be paired with current antimalarials for further *in vivo* combination studies.

We selected combinations of artemisone with mefloquine, piperaquine, or chloroquine for *in vivo* evaluations. This decision was based partly on the *in vitro* synergistic effect of artemisone with...
FIG 5 Effect of artemisone, chloroquine, and piperaquine on *P. berghei* ANKA infection. Infected mice were treated on days 6 to 8 postinfection with artemisone (art; twice each day) and chloroquine (chl; twice each day) or artemisone (twice each day) and piperaquine (piper; once per day). (a) Data are parasitemias and survival curves of infected mice; (b) data are clinical scores, weights, and body temperatures of infected mice. Values are means ± standard deviations (SD); *n* = 8 to 10/group.
mefloquine and additive effect with piperaquine or chloroquine (Fig. 2) and also our experience where piperaquine or chloroquine, in combination with artemiside (another alkyl-aminoartemisinin), were found additive in vitro and synergistic in vivo (2). Vivas et al. (27) found slight antagonism between the in vitro effects of artemisone and chloroquine, while we found additivity. The difference in these in vitro results is not great and may have its origin in the different experimental conditions. However, both groups report synergistic in vivo effects. This is important because often in vitro results are not translated into the in vivo domain (28). It is especially interesting to examine artemisone (which is already in clinical trials) in combination with chloroquine. The latter drug played an important role in malaria eradication, but it remains attractive because of its low cost and the possibility that when combined with other drugs, treatment success will be significantly increased. Also, it is desirable to elucidate the use of combination of drugs that are less effective alone because of parasite resistance but are efficient together. A clear example is provided by the combination of atovaquone and proguanil (resistance to the individual drugs in this combination has been unequivocally demonstrated).

Schmuck et al. (29) suggest that artemisone is embryotoxic. However, in that paper there are no comparative experimental results; it focuses on artemisone alone, ignoring other artemisinin derivatives. In a direct in vivo comparison of artemisone and artesunate, artemisone was not found neurotoxic, in contrast with artesunate (7). Moreover, artesunate in low doses was embryotoxic in rats (30). In addition, a fatal artesunate toxicity was reported in a child (31). Yet, artesunate alone or in drug combinations is considered a first-line antimalarial treatment. Indeed, in a limited study involving pregnant women, there was no evidence of artesunate embryotoxicity (32). Artemisone is more effective than

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**FIG 6** Plasma cytokine levels in control (A) and infected untreated (B) mice on day 5. Columns and vertical bars represent means ± SD; n = 8 to 10/group.

**FIG 7** Effect of artemisone and chloroquine on cytokines in infected mice on day 8 postinfection. Plasma cytokine levels in mice treated on days 6 to 8 (twice each day) postinfection by artemisone (Art), chloroquine (Chl), or their combination. Columns and vertical bars represent means ± standard errors of the means (SEM); n = 8 to 10/group.
artesunate in vitro and against murine models of malaria (4, 27).
In our experiments, artemisone combinations with the tested antimalarial drugs were not toxic, both in vitro (Fig. 3) and in vivo. The highest concentrations of artemisone (10 mg/kg), in drug combinations, when injected twice a day for 3 days, had no visible effects on the mice. Overall, artemisone might be considered for treatment of malaria, especially in drug combinations, where toxicity of individual drugs can be reduced.

In long-term experiments, we found that mice treated with the individual drugs, despite an initial delay in parasitemia increase, eventually succumbed to the disease. However, most mice treated with artemisone combination therapy were completely cured (Fig. 4 and 5). Vivas et al. (27) described some in vivo artemisone-drug interactions in a rodent model by using Peter’s four-day test. This method is inadequate for estimation of the effect of drug treatment on severe malaria (e.g., CM), where pathogenesis is most pronounced a week or more after infection, and pathology (or lack of pathology) is the result of a prolonged innate immune response and early acquired immunity (4, 33).

The in vivo experiments were performed in a reliable mouse model of CM (22, 34). The underlying mechanism of CM pathogenesis remains incompletely understood, but there is widespread agreement that cytokines (and other components of the immune system) have a crucial role in CM and severe malaria in general, in mice and in humans. An imbalance between the release of proinflammatory and anti-inflammatory cytokines has been associated with the central nervous system dysfunction found in human and experimental CM (35). The current study in C57BL/6 mice infected with P. berghei ANKA aimed to investigate whether the antiplasmodial effect and prevention of CM correlate with an immunological shift, expressed in plasma cytokine levels. The inflammatory cytokines TNF and IFN-γ (Th1 type) and anti-inflammatory IL-4 and IL-10 (Th2 type) were determined in plasma by ELISA.

In clinical studies of human CM, elevated serum IFN-γ is seen in acute malaria infection in Southeast Asian (36) and African (37) patients. Murine and human studies strongly support a role for IFN-γ and downstream immune system processes in the pathogenesis of CM (38, 39, 40). Evidence suggesting that TNF is another key element in the pathogenesis of experimental CM has been reviewed extensively (41, 42). This includes the observation of high serum levels of TNF at the onset of CM (43) and the prevention of the neurological syndrome when TNF levels are low (44, 45). Anti-inflammatory cytokines such as IL-10 seem to have a host-protective role in murine malaria. For example, the clinical scores of IL-10-deficient, infected mice were significantly higher than those of WT mice. In addition, in a susceptible mouse strain, administration of IL-10 gave some degree of protection against CM induced by P. berghei ANKA (46, 47). IL-4 is an anti-inflammatory cytokine. Changes in plasma IL-4 have been reported to correlate with severe malaria (48) but, conversely, increased levels of IL-4 have been linked with reduced immunopathological symptoms (49, 50).

Antimalarial drugs may induce immunological alterations in treated patients and animals. Artemisinins can produce immunosuppression by downregulating various cytokines of both the innate and acquired immune systems. They induce an anti-inflammatory effect, neutropenia, and reduction in macrophage number and functions, which may produce immunosuppression. Artemisinins also have the ability to induce immunosuppression by inhibiting delayed-type hypersensitivity, lymphocyte proliferation, and a rise in antibody level (51). Immune deviation caused by chloroquine and other antimalarial drugs has been reported (52, 53).
We found that the plasma levels of the proinflammatory cytokines TNF and IFN-γ increased in P. berghei ANKA-infected mice (Fig. 6 to 8). In parallel with CM reduction, there was an attenuated increase in TNF plasma levels in P. berghei ANKA-infected mice, after treatment with chloroquine, artemisone, or the combination of the two. Reduction of IFN-γ was achieved with the drug combination. These results agree with the hypothesis that the CM syndrome is a result of a shift in the balance of Th1/Th2 responses toward Th1. While many immune components (cytokines, chemokines, effector cells) and metabolic pathways are involved in processes leading to the expression of CM, one drug may affect only some of these components, while another one may affect others. Thus, judicious selection of combination therapy may reduce parasitemia by direct actions on the parasite and also inhibit the severe symptoms of malaria through immunomodulatory actions. In general, all drug treatments reduced inflammatory cytokines and increased anti-inflammatory cytokines (positive effects). However, when examining carefully the data of days 8 and 12 postinfection, the positive effects induced by the drug combination was more pronounced (Fig. 6 to 8; Table 1).

Overall, experiments are needed to determine how drugs that are used in combination influence each other in terms of immunomodulation, toxicity, pharmacokinetic, pharmacodynamic, and even pharmacogenetic aspects.

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REFERENCES


32. Camposa


