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Pharmacokinetics and *Ex Vivo* Antimalarial Activity of Artesunate-Amodiaquine plus Methylene Blue in Healthy Volunteers

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ABSTRACT High rates of artemisinin-based combination therapy (ACT) failures in the treatment of *Plasmodium falciparum* malaria in Southeast Asia have led to triple-drug strategies to extend the useful life of ACTs. In this study, we determined whether methylene blue [MB; 3,7-bis(dimethylamino)phenothiazin-5-ium chloride hydrate] alters the pharmacokinetics of artesunate-amodiaquine (ASAQ) and enhances the *ex vivo* antimalarial activity of ASAQ. In an open-label, randomized crossover design, a single oral dose of ASAQ (200 mg AS/540 mg AQ) alone or with MB (325 mg) was administered to 15 healthy Vietnamese volunteers. Serial blood samples were collected up to 28 days after dosing. Pharmacokinetic properties of the drugs were determined by noncompartmental analysis. After drug administration, plasma samples from seven participants were assessed for *ex vivo* antimalarial activity against the artemisinin-sensitive MRA1239 and the artemisinin-resistant MRA1240 *P. falciparum* lines, *in vitro*. MB significantly increased the mean area under the curve of the active metabolite of AS, dihydroartemisinin (1,246 ± 473 versus 917 ± 405 ng·h/ml, $P = 0.009$) but did not alter the pharmacokinetics of AQ, AS, or desethylamodiaquine. Comparing the antimalarial activities of the plasma samples from the participants collected up to 48 h after ASAQ plus MB (ASAQ+MB) and ASAQ dosing against the MRA1239 and MRA1240 lines, MB significantly enhanced the blood schizontocidal activity of ASAQ by 2.0-fold and 1.9-fold, respectively. The ring-stage survival assay also confirmed that MB enhanced the *ex vivo* antimalarial activity of ASAQ against MRA1240 by 2.9-fold to 3.8-fold, suggesting that the triple-drug combination has the potential to treat artemisinin-resistant malaria and for malaria elimination. (This study has been registered in the Australian New Zealand Clinical Trials Registry [https://anzctr.org.au/] under registration number ACTRN12612001298808.)

KEYWORDS malaria, artesunate-amodiaquine, methylene blue, *ex vivo* antimalarial activity, pharmacokinetics

Artemisinin-based combination therapies (ACTs) are currently recommended worldwide for first-line treatment of uncomplicated *Plasmodium falciparum* malaria (1). However, high ACT failure rates in Southeast Asian countries (2–4) are alarming and new treatment strategies are urgently required to extend the useful life of ACTs until more effective antimalarial drugs are developed by agencies such as the Medicines for Malaria Venture (MMV). Clinical trials of the efficacy of a new partner drug added to an established ACT (i.e., artemether-lumefantrine plus

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amodiaquine [AQ] and dihydroartemisinin [DHA]-piperaquine plus mefloquine) for the treatment of uncomplicated *P. falciparum* malaria are under way in Southeast Asia (5). The concept of triple-drug combinations extending the utility of ACTs is not new, as the use of combinations of more than two drugs for the treatment of multidrug-resistant tuberculous has been reported previously (6). Various groups have advocated the use of triple-drug combinations to address the development and spread of multidrug-resistant malaria and for mass drug administration where malaria elimination is the goal (7–9).

Methylene blue [MB; 3,7-bis(dimethylamino)phenothiazin-5-ium chloride hydrate], a tricyclic phenothiazine, was the first synthetic antimalarial drug used for the treatment of malaria over 100 years ago (10), but interest in the drug declined due to the development of its derivatives, mepacrine and chloroquine. However, there has been renewed interest in MB as a partner drug to combat chloroquine resistance, particularly in sub-Saharan Africa (11). The mechanism of action of MB as an antimalarial is not fully understood. MB is a redox-cycling oxidant and a potent inhibitor of the antioxidant enzyme glutathione reductase of *P. falciparum* causing oxidative stress to the parasite (12). It readily complexes with heme and prevents its sequestration into hemozoin, with the unsequestered heme being cytotoxic to the parasite, similarly to the proposed mode of action of other quinolines such as chloroquine and mefloquine (13).

In vitro studies have shown Proveblue, a methylene blue preparation that contains levels of limited organic impurities and heavy metals, to be highly active against *P. falciparum* strains from Africa, South America and Southeast Asia, with 50% inhibitory concentrations (IC_{50}s) in the low nanomolar range (14, 15). High *ex vivo* efficacy of MB has been shown against clinical field isolates of *P. falciparum* and *P. vivax* from an area of known multidrug resistance of the *Plasmodium* species in Indonesia with the highest potency against ring-stage parasites (i.e., IC_{50}s < 7 nM) (16).

In vitro drug combination studies have also demonstrated MB or Proveblue to potentiate the activity of artemisinins against *P. falciparum* lines (15, 17). This potentiation may be due to the redox-active cycler activity of MB enhancing the damaging effects of reactive oxygen species produced through the iron-mediated endoperoxide cleavage of artemisinins (18), which in turn creates a fatal overload of oxidative stress on the fragile parasitized erythrocyte redox-dependent architecture (19).

Because of the promising *in vitro* potentiation between MB and the artemisinin derivatives, the clinical efficacy of MB as a potential partner drug with either artesunate (AS) or AQ was evaluated as an ACT in northwestern Burkina Faso, West Africa, in 2006. A randomized controlled phase II study of the use of the combinations AS plus MB (AS+MB), AS+AQ, and AQ+MB in children was evaluated (20). The 3-day regimens evaluated were AS at 4 mg/kg of body weight/day, AQ at 10 mg/kg/day, and MB at 20 mg/kg/day. By day 28 posttreatment, the PCR-based correction for recrudescence revealed cure rates of 62% for AS+MB, 82% for AS+AQ, and 95% for AQ+MB. The high efficacy of AQ+MB was demonstrated in an area where the cure rate with AQ alone was previously reported at 82% (21). More recently, in 2016, MB (15 mg/kg/day for 3 days) added to a 3-day course of the fixed-dose ACT, artesunate-amodiaquine (ASAQ), was superior to the same course of ASAQ and primaquine (PQ; 0.25 mg/kg) added at day 2 in clearing falciparum asexual parasites in children from Burkina Faso (22). ASAQ+MB treatment was also more effective than ASAQ+PQ in reducing the prevalence and density of *P. falciparum* gametocytes following treatment (22).

On the basis of the promising findings with respect to AQ+MB efficacy and on the potential of MB as a partner drug with ASAQ, we determined the pharmacokinetic (PK) properties of ASAQ and ASAQ+MB in healthy volunteers, with the aim of determining whether MB alters the PK of ASAQ. Applying the PK data of the ACTs, we then evaluated the *ex vivo* antimalarial activity of ASAQ with and without MB in plasma samples collected from healthy volunteers to determine whether MB could enhance the blood schizontocidal activity of ASAQ.

RESULTS AND DISCUSSION

Dose selection of MB and ASAQ. *In vivo* studies have revealed that MB *per se* is not a highly potent antimalarial drug for the treatment of uncomplicated falciparum malaria administered either alone at an oral dose of approximately 13 mg/kg of body weight/day for 3, 5, or 7 days (23) or in combination with chloroquine (total dose of 25 mg/kg chloroquine plus 12 mg/kg, 18 mg/kg, or 24 mg/kg MB daily for 3 days) (24). High oral doses of MB (i.e., ≥15 mg/kg/day for 3 days) are associated with adverse gastrointestinal effects and dysuria (20, 25). However, because of the synergy between MB and DHA (15), we hypothesized that a small dose of MB combined with ASAQ would generate enhanced antimalarial activity due to high oxidative stress on the malaria parasites. As a proof of concept that MB enhances the antimalarial activity of ASAQ, we selected a single oral dose of approximately 5.5 mg/kg of MB combined with a standard first-day treatment oral dose of ASAQ given to adults. The same oral MB formulation (i.e., Urolene Blue) administered to healthy volunteers in this study was previously used in efficacy studies of MB for the treatment of falciparum malaria patients in Burkina Faso (20, 23).

Safety and tolerability of ASAQ and ASAQ+MB. The ACTs were safe and well tolerated at the administered doses. Blood chemistries (biochemical and hematology indices) were comparable before and after administration of the combinations (data not shown). No serious adverse events were reported in the participants administered either ASAQ or ASAQ+MB. All participants given ASAQ+MB had green-blue stools and urine; those color changes disappeared 24 h and 72 h, respectively, after dosing. Four of 16 participants also experienced a mild episode of diarrhea without abdominal pain between 2.5 and 4.5 h after ASAQ+MB administration, and one volunteer had nausea and abdominal pain about 15 min after dosing. One participant vomited the ASAQ+MB dose and was subsequently withdrawn from the study. Mild diarrhea and completely reversible green-blue staining of stools and urine are common occurrence in individuals administered MB, with these adverse events not considered clinically significant (26).

There was a significant difference ($P < 0.001$) in the volunteers' ECGs with QT interval (QTc) immediately before (364 ± 13 ms) and 6 h after (383 ± 22 ms) ASAQ administration. Similarly, there was a significant difference ($P = 0.002$) in the volunteers' QTc intervals before (359 ± 14 ms) and 6 h after (383 ± 22 ms) ASAQ+MB administration. These changes in QTc intervals were not considered clinically significant.

Pharmacokinetics of ASAQ with and without MB in healthy volunteers. The mean (standard deviation [SD]) plasma concentration-time curves of AS and dihydroartemisinin (DHA) after ASAQ and ASAQ+MB administration are shown in Fig. 1A and of AQ and desethylamodiaquine (DAQ) in Fig. 1B. The PK properties of AS, DHA, AQ, and DAQ with and without MB are summarized in Table 1. Overall, the PK properties of AS, DHA, AQ, and DAQ obtained in this study are in broad agreement with those obtained in other studies in healthy volunteers given either AS or AQ alone or in combination with a longer-acting antimalarial drug (27–30).

The mean (SD) plasma concentration-time curves of MB and azure B (AZB) after ASAQ+MB administration are shown in Fig. 1C, and the PK properties of both analytes are summarized in Table 1. Following the single oral dose of 325 mg of MB (~5.5 mg/kg), the mean plasma maximum concentrations (C_{max}) of MB and its monodemethylated active metabolite AZB were 965 ± 247 ng/ml and 168 ± 44 ng/ml, respectively, with a time to achieve the C_{max} (T_{max}) of ~1.9 h. The parallel plasma concentration profiles of MB and AZB revealed mean elimination half-life ($t_{1/2}$) values of 14.9 h and 14.4 h, respectively. The PK values of T_{max} and $t_{1/2}$ for MB in this study were comparable to those determined with healthy volunteers who received a single oral dose of 500 mg MB (7.5 mg/kg) taken as an aqueous solution (mean T_{max} and $t_{1/2}$ of MB of 2.2 h and 18.3 h, respectively) (26).

MB administration significantly increased the mean area under the curve (AUC) of DHA (1,246 ± 473 ng.h/ml versus 917 ± 405 ng.h/ml, $P = 0.009$). This increase in DHA concentrations as well as the presence of MB would have contributed to the enhanced

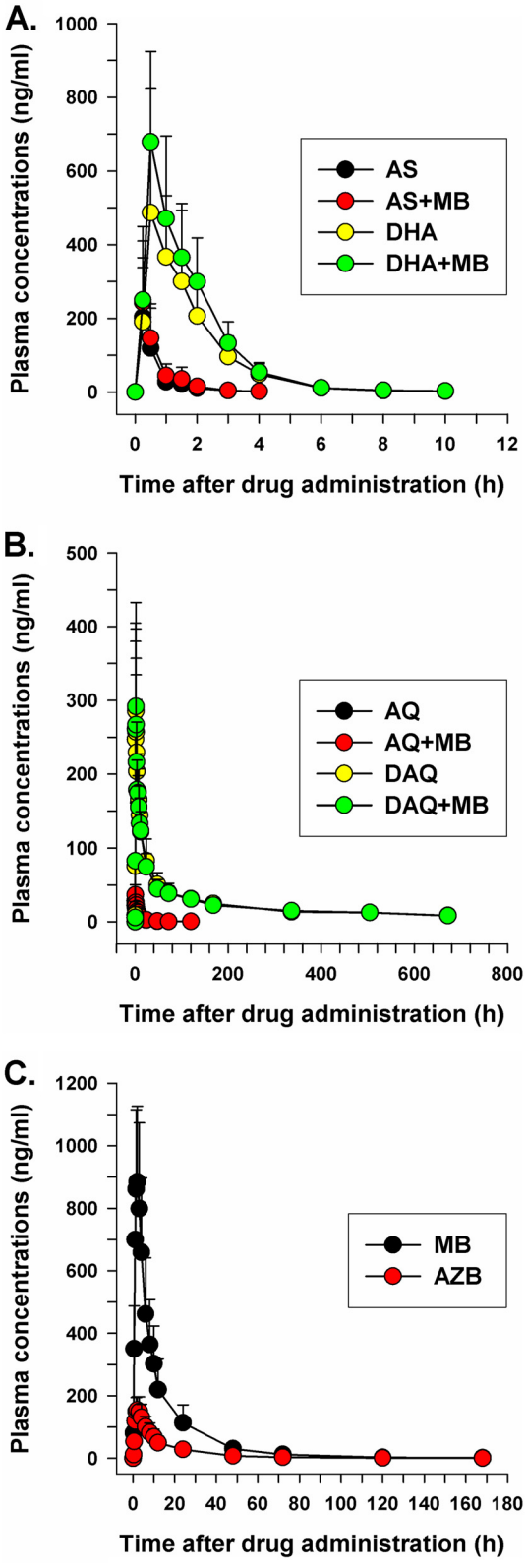

FIG 1 Mean (SD) plasma concentration-time profiles of artesunate (AS) and dihydroartemisinin (DHA) (A) and of amodiaquine (AQ) and desethylamodiaquine (DAQ) (B) after either artesunate-amodiaquine (ASAQ) or artesunate-amodiaquine plus methylene blue (ASAQ+MB) administration in 15 healthy volunteers or of methylene blue (MB) and azure B (AZB) (C) after ASAQ+MB administration. The single oral dose of ASAQ (200 mg AS/540 mg AQ) either alone or with MB (325 mg MB). (Note the different time and concentration scales for the analytes).

TABLE 1 Pharmacokinetic properties of artesunate, dihydroartemisinin, amodiaquine, desethylamodiaquine, methylene blue, and azure B after a single oral dose of either artesunate-amodiaquine or artesunate-amodiaquine plus methylene blue administered to healthy volunteers based on a randomized crossover design[a]

Drug	Drug combination	C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0-\infty}$ (ng·h/ml)
AS ($n = 15$)	ASAQ	224 ± 132	0.4 ± 0.2	0.5 ± 0.2	174 ± 208
	ASAQ + MB	274 ± 113	0.4 ± 0.3	0.4 ± 0.1	170 ± 60
	P	0.178	0.879	0.279	0.935
DHA ($n = 15$)	ASAQ	571 ± 325	0.7 ± 0.4	1.0 ± 0.2	917 ± 405
	ASAQ + MB	690 ± 257	0.6 ± 0.2	0.9 ± 0.2	1,246 ± 473
	P[b]	0.245	0.189	0.288	0.009
AQ (n=15)	ASAQ	28.7 ± 14.0	1.0 ± 0.8	9.2 ± 2.3	203.2 ± 94.5
	ASAQ+MB	38.2 ± 13.1	0.9 ± 0.7	8.6 ± 1.2	258.6 ± 89.2
	P[b]	0.08	0.746	0.279	0.068
DAQ (n=15)	ASAQ	342 ± 121	2.2 ± 1.8	254 ± 46	18,555 ± 4,346
	ASAQ+MB	315 ± 96	1.8 ± 1.3	268 ± 53	18,611 ± 6,590
	P[b]	0.232	0.415	0.536	0.97
MB (n=15)	ASAQ+MB	965 ± 247	1.9 ± 0.7	14.9 ± 4.2	10,547 ± 3,588
AZB (n=15)	ASAQ+MB	168 ± 44	1.8 ± 0.7	14.4 ± 6.9	2,268 ± 694

[a]AS, artesunate (200 mg); DHA, dihydroartemisinin; AQ, amodiaquine (540 mg); DAQ, desethylamodiaquine; MB, methylene blue (325 mg); AZB, azure B; ASAQ, artesunate-amodiaquine; ASAQ+MB, artesunate-amodiaquine plus methylene blue; C_{max}, maximum observed plasma concentration after oral administration; T_{max}, observed time to reach C_{max}; $t_{1/2}$, terminal elimination half-life; $AUC_{0-\infty}$, area under the plasma concentration-time curve from zero time to infinity. $n = 15$ healthy volunteers. Values represent means ± SD. *P* values were calculated using paired *t* tests.

ex vivo antimalarial activity of ASAQ with MB compared to that of ASAQ alone. None of the other PK properties of ASAQ were significantly altered by MB. The geometric mean percentages (90% confidence interval [CI]) of the ASAQ+MB/ASAQ ratios for the logarithmically transformed values of DAQ C_{max} and AUC from 0 to infinity ($AUC_{0-\infty}$) were 99.0% (96.6% to 101.3%) and 99.8% (98.6% to 101.0%), respectively. The corresponding values for DHA C_{max} and $AUC_{0-\infty}$ were 105.3% (100.2% to 109.4%) and 105.3% (102.4% to 107.8%), respectively. These values meet the criterion of falling between 80% and 125% for assuming bioequivalence of DAQ and DHA exposure after administration of ASAQ alone and with MB. Because of limited funding, the effect of ASAQ altering the PK of MB was not determined, but such an evaluation would be warranted for future studies to fully characterize potential drug-drug interactions of ASAQ with MB.

***Ex vivo* antimalarial activity of ASAQ versus ASAQ+MB.** Information concerning the *ex vivo* activity and PK of antimalarials, including their active metabolites in blood collected from healthy volunteers or malaria patients following drug treatment, has aided the selection and development of drugs, including ACTs (31–34). Distinct advantages of the *ex vivo* assay are that it measures the overall activity of drugs and their metabolites at physiological concentrations achieved in the participant's blood after treatment and that it is independent of the immune status of individuals.

In order to gain an insight into the *ex vivo* antimalarial activity of ACTs, it is necessary to acquire information on the intrinsic activity of the drugs and their active metabolites using *in vitro* assays and culture-adapted field isolates. In the present study, the prodrugs AS and AQ were rapidly metabolized and eliminated (31), with most of the antimalarial activity coming from their respective metabolites, DHA and DAQ. Using conditions approximating the characteristics of blood (i.e., 50% plasma) in the *in vitro* drug susceptibility assay, the mean (± SD) IC_{50}s against the artemisinin-sensitive MRA1239 line were 1.2 ± 0.7 nM for DHA, 16.2 ± 4.3 nM for DAQ, and 7.8 ± 2.5 nM for MB. In contrast, the artemisinin-resistant MRA1240 line was less susceptible to DHA

TABLE 2 Comparison of the inhibitory dilutions of plasma samples collected from seven healthy volunteers at 0.5, 2, 6, 24, and 48 h after administration of artesunate-amodiaquine or artesunate-amodiaquine plus methylene blue against the artemisinin-sensitive MRA1239 and artemisinin-resistant MRA1240 *Plasmodium falciparum* lines[a]

Time (h)	ID_{50} (mean ± SD)		*P* value
	ASAQ	ASAQ+MB	
MRA1239			
0	0.7 ± 0.9	0.9 ± 0.7	
0.5	1,504 ± 967	2,877 ± 1,109	0.030
2	551 ± 239	1,236 ± 521	0.008
6	26.1 ± 12.1	60.7 ± 32.5	0.022
24	3.8 ± 1.2	5.6 ± 2.1	0.072
48	2.7 ± 0.8	2.7 ± 1.1	0.985
MRA1240			
0	0.7 ± 1.1	0.7 ± 1.1	
0.5	469 ± 260	824 ± 300	0.035
2	149 ± 74	369 ± 96	0.0001
6	11.3 ± 3.8	26.2 ± 12.0	0.009
24	2.9 ± 0.7	4.7 ± 1.5	0.014
48	2.1 ± 0.7	1.5 ± 1.0	0.258

[a]ID_{50}, number of dilutions of plasma sample that produces a 50% inhibition of [^{3}H]hypoxanthine uptake into malaria parasites compared to drug-free plasma control samples; ASAQ, artesunate-amodiaquine; ASAQ+MB, artesunate-amodiaquine plus methylene blue.

(IC_{50}, 4.2 ± 2.1 nM) and DAQ (IC_{50}, 33.7 ± 10.7 nM) but equally susceptible to MB (IC_{50}, 8.7 ± 1.8 nM). The activity of AZB is about 2-fold to 3-fold lower than that of MB *in vitro* (35).

A subset of seven participants was randomly selected from the 15 participants for the *ex vivo* activity assessment of their plasma samples after ASAQ and ASAQ+MB administration. In this assay, parasite growth in culture media containing plasma (at a 50% concentration) collected from the participants is evaluated and the activity of the drug(s) can be appraised by comparing the inhibitory dilutions (ID) that are required to produce the same effect on parasite growth (i.e., 50% inhibition). No significant differences was seen in the mean C_{max} and AUC values of DHA and DAQ for the seven participants whose plasma samples were used for the *ex vivo* activity assessment compared to those determined for the other eight participants (data not shown). The mean values representing the 50% inhibitory dilutions (ID_{50}) of the plasma samples collected from the seven volunteers at 0.5, 2, 6, 24, and 48 h postdose against the MRA1239 and MRA1240 *P. falciparum* lines are shown in Table 2. Over the 48-h asexual cycle after drug administration, the ID_{50} AUC_{0-48h} values obtained with ASAQ+MB were significantly (2.0-fold and 1.9-fold) higher against the MRA1239 and MRA1240 lines, respectively, than those obtained with ASAQ alone. This demonstrates that the addition of MB to ASAQ markedly enhanced the blood schizontocidal activity of the ACT.

Since the ring-stage survival assay (RSA) is the preferred *in vitro* assay for the quantification of artemisinin resistance (36), we used a modified RSA to appraise the *ex vivo* activity of the plasma samples collected from the participants at time points that corresponded with the C_{max} of the active metabolites (i.e., 0.5 h for DHA and 2 h for both DAQ and MB after the administration of the ACTs). When the ring stages of MRA1239 were treated with DHA for 6 h as the reference drug, the majority of parasites were killed, with a small fraction of live parasites (0.18% ± 0.01%) detected at 72 h after commencement of the assay (i.e., the endpoint of the assay) (Fig. 2, left panel). Unlike the MRA1239 parasites, when MRA1240 parasites were treated with DHA, about 7.38% ± 1.27% of parasites were alive at 72 h.

When rings were exposed for 6 h to the participants' plasma samples collected at 0.5 h and 2 h after ACT administration, growth of the MRA1239 parasites was similar at 72 h to that in wells treated with DHA alone (i.e., 0.16% ± 0.02% and 0.16 ± 0.02% at

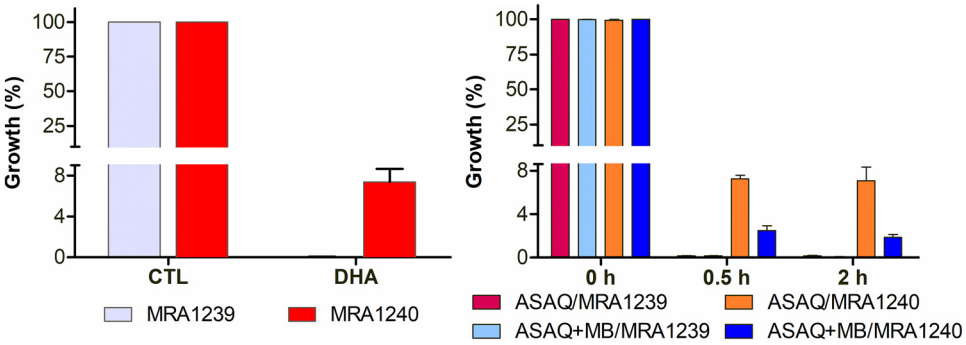

FIG 2 (Left panel) Parasite growth profile of the artemisinin-sensitive MRA1239 and artemisinin-resistant MRA1240 *Plasmodium falciparum* lines after exposure to DHA (reference drug) for 6 h in media containing 50% plasma, followed by 66 h of incubation in drug-free media under standard growth conditions. (Right panel) Parasite growth of MRA1239 and MRA1240 parasites in media containing 50% plasma collected from seven healthy volunteers at 0 h (before dosing) and 0.5 or 2 h after the administration of either artesunate-amodiaquine (ASAQ) or artesunate-amodiaquine plus methylene blue (ASAQ+MB) for 6 h, followed by 66 h of incubation in drug-free media under standard growth conditions.

0.5 h for ASAQ and ASAQ+MB, respectively, and 0.16 ± 0.04% and 0.09% ± 0.016% at 2 h for ASAQ and ASAQ+MB, respectively) (Fig. 2, right panel). For MRA1239, the levels of parasite growth were not significantly different between the two time points ($P = 0.750$) and the two ACTs ($P = 0.100$) compared to the results seen with DHA-treated cultures of the same line. In contrast, there was a significant difference between the two ACTs against the artemisinin-resistant MRA1240 parasites. After ASAQ administration, the participants' plasma samples did not completely inhibit the growth of the MRA1240 parasites (i.e., 7.26% ± 0.32% for the 0.5-h samples and 7.08% ± 1.26% for the 2-h samples at 72 h) (Fig. 2, right panel) and the results were similar to those seen with the wells that were treated with DHA alone. However, plasma samples from the same participants administered ASAQ+MB revealed 2.9-fold to 3.8-fold greater inhibition of the MRA1240 parasites than was seen with ASAQ alone (i.e., 2.49% ± 0.44% for the 0.5-h samples and 1.86% ± 0.28% for the 2-h samples). The level of growth of the cultures exposed to the participants' 0.5-h and 2-h plasma samples after ASAQ+MB dosing was significantly lower ($P < 0.001$ and $P = 0.003$, respectively) than that seen with the cultures exposed to plasma samples collected at the same time after ASAQ administration. Additionally, the 2-h plasma samples were significantly more potent against the MRA1240 line than the 0.5-h samples ($P = 0.030$) after ASAQ+MB administration, presumably due to the higher concentrations of MB in the participants' plasma samples at 2 h than at 0.5 h. Of note, because the participants' plasma samples were diluted 2-fold compared to the actual concentrations in blood in the *ex vivo* assays, it is quite likely that the maximum physiological drug concentrations would have been more inhibitory against both MRA lines.

Clinical importance of MB for future studies. A recent systematic review by Lu et al. (9) of the efficacy and safety of MB reported from 21 studies involving 1,504 malaria patients (2/3 were children) revealed that MB was consistently shown to be a highly effective antimalarial drug in various areas of endemicity and that it was largely well tolerated with mild gastrointestinal disturbances and green-blue discoloration of urine. The present report provides additional *ex vivo* antimalarial activity findings of the potential of MB to enhance the blood schizontocidal activity of ASAQ for the treatment of malaria infections and to potentially reduce the development of ACT resistance.

Caution, however, needs to be exercised given that the addition of MB to an ACT in an area where the *P. falciparum* parasites are already highly resistant to a long-acting partner drug such as AQ is unlikely to be successful in extending the life of the ACT. This was demonstrated for ASAQ+MB in Burkina Faso in 2011, where impaired AQ efficacy was suspected to have contributed to the suboptimal efficacy of 80% to 85% of ASAQ with and without MB (25), compared with the cure rate of 95% for AQ+MB that was achieved 5 years earlier in the same area (20).

In addition to high MB *ex vivo* activity against *Plasmodium* species asexual stages, MB possesses potent gametocytocidal action (37), particularly against mature gametocytes, which suggests that MB is a suitable partner drug for mass drug administration with an ACT with the goal of malaria elimination. The gametocytocidal benefits of MB were clearly demonstrated in the posttreatment period, with gametocyte prevalence being significantly lower in patients treated with ASAQ+MB than in those treated with either ASAQ alone (25) or ASAQ+PQ (22) in Burkina Faso.

Conclusions. Head-to-head comparison of the *ex vivo* antimalarial activities of physiological plasma samples from the same volunteers following ASAQ and ASAQ+MB administration against the MRA1240 line clearly demonstrated enhanced activity of MB in inhibiting artemisinin-resistant parasites. The utility of MB in combination with an ACT for the treatment of multidrug-resistant malaria and for prevention of resistant parasites and as a partner gametocytocidal drug for malaria elimination warrants further investigation.

MATERIALS AND METHODS

Drugs. AS, DAQ, and DHA were obtained from the Worldwide Antimalarial Resistance Network (Bangkok, Thailand), and AQ hydrochloride, chloroquine disphosphate, MB hydrate (lot no. BCBH5707V) (99.5% pure; water content, 15.1%), and AZB (batch no. MKBP0145V) (89% pure) were obtained from Sigma-Aldrich (St. Louis, MO).

Parasite cultures of *P. falciparum*. Two laboratory-adapted *P. falciparum* lines, namely, MRA1239 (artemisinin sensitive) and MRA1240 (artemisinin resistant), were originally collected in Cambodia. Both MRA lines were obtained from BEI Resources (Manassas, VA). Parasites were cultured as previously described (31).

Volunteers and study site. Sixteen healthy male Vietnamese volunteers (mean [±SD] age, 21.01 [1.8] years; weight, 59.6 [2.2] kg) participated in the study, which was carried out in Central Military Hospital 108 in Hanoi (Vietnam). The volunteers were judged healthy based on medical history, normal vital signs, electrocardiogram (ECG) results, and laboratory testing (biochemistry and hematology) within normal range. A physical examination and laboratory blood tests were performed during screening, and the blood tests were repeated on day 7 after commencement of administration of the drug combinations. All participants were shown to be glucose-6-phosphate dehydrogenase (G6PD) normal using the CareStart G6PD deficiency screening test (Biospecific, Melbourne, Australia). Data from a 12-lead ECG examination were recorded for each volunteer during screening and at approximately 6 h after the last ACT dose. The ECGs with QT interval (QTc) (38) recorded were reviewed by the hospital's cardiologist, and no abnormalities were detected in any of the participants.

Sample size calculation. The sample size for determining whether MB altered the PK of DHA and DAQ were based on the AUC. Absence of a significant interaction was considered to have been demonstrated if the 90% confidence interval (CI) values for the geometric mean ratios of the DHA and DAQ AUC values for ASAQ versus ASAQ+MB were within the range of 0.80 to 1.25 (39). On the basis of an earlier PK study of a single oral dose of ASAQ in healthy volunteers and assuming a within-subject coefficient of variation for DAQ and DHA of about 25% (40), a sample size of 13 would provide a statistical power of approximately 80% to detect with 95% confidence a 25% increase or decrease in the DHA and DAQ AUCs. To account for the expected participant dropout rate of up to 20%, 16 participants were recruited into the study.

Study design and drug administration. The study was a randomized crossover design using a computer-generated random list. The participants received a single oral dose of either ASAQ (Winthorp; Sanofi Aventis, Morocco) (2 tablets, with each tablet containing 100 mg AS and 270 mg AQ) or ASAQ (2 tablets) plus MB [3,7-bis(dimethylamino)phenothiazin-5-ium chloride hydrate; Star Pharmaceuticals, FL] (5 tablets of MB, with each tablet containing 65 mg MB). Drug administration took place 10 to 15 min after the volunteers had ingested a low-fat breakfast of one packet of instant noodles. The drugs were administered with 200 ml of water. There was an 8-week washout period between the two drug administrations.

The administration of medication was observed. All participants were asked daily for 4 days the following nonleading question: "How do you feel since you took your antimalarial tablets?" If a volunteer reported symptoms, the timing and intensity of the adverse event were recorded by the study doctor.

Blood sampling. Immediately before the dose, an indwelling cannula was inserted into a forearm vein of the participant and kept patent with heparinized saline solution. Blood samples (7 ml) were then collected at 0 h (before dosing) and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h after drug administration and equal volumes transferred to either a fluoride oxalate tube for drug analysis or a lithium heparin tube for *ex vivo* assay. Subsequent blood samples were collected by venipuncture at days 3, 4, 7, 14, 21, and 28 for both drug combinations. The fluoride oxalate minimizes hydrolysis of AS to DHA due to plasma esterases but, unlike heparin, is toxic to and inhibits growth of malaria parasites. All blood samples were centrifuged at 1,400 × *g* for 15 min, and the separated plasma samples were stored at −80°C prior to transport to Australia on dry ice for drug analysis at the Australian Defence Force Malaria and Infectious Disease Institute (ADFMIDI). The ADFMIDI pharmacology laboratory participates in the Worldwide

Antimalarial Resistance Network (WWARN) quality control and assurance proficiency testing program with satisfactory performance (41).

***In vitro* antimalarial activity of reference drugs.** Evaluation of the *in vitro* antimalarial activity of DAQ, DHA, and MB was carried out using the [^{3}H]hypoxanthine incorporation assay in parasites, which were cultured in RPMI 1640 media with 50% human plasma as previously described (31). Drug IC_{50}s (i.e., concentrations that cause 50% inhibition of parasite growth or [^{3}H]hypoxanthine uptake compared with drug-free samples) were determined using nonlinear regression analysis (GraphPad Prism V5.0; GraphPad Software, Inc., CA). Mean IC_{50}s were based on at least two independent experiments.

***Ex vivo* bioassay of participants' plasma samples.** Antimalarial activity of ASAQ and ASAQ+MB in plasma samples collected from seven volunteers at 0 h (before dosing) and 0.5, 2, 6, 24 and 48 h postdose was evaluated against the MRA1239 and MRA1240 *P. falciparum* lines. Briefly, the volunteers' plasma samples (50 μl) were serially diluted 2-fold in microtiter plates with drug-free human plasma. The *P. falciparum* inoculum was made up in hypoxanthine-free plain RPMI 1640, with a final hematocrit of 2% and a parasitemia (>95% rings) of 1%. A 50-μl volume of inoculum was then added to each well containing 50 μl of the participants' plasma sample such that the total volume of parasite culture in the well was 100 μl and contained 50% plasma. The next day, 20 μl of [^{3}H]hypoxanthine was added to each well and the cultures were grown for a further 24 h to determine the extent to which parasite growth was inhibited by antimalarial drugs in the volunteers' plasma samples after 48 h of incubation.

The 50% inhibitory dilution (ID_{50}) of the volunteer plasma sample was defined as the severalfold value of the volunteer's plasma samples that resulted in a 50% inhibition of uptake of [^{3}H]hypoxanthine (surrogate for parasite growth) compared to drug-free plasma samples (controls) as previously described (30–32, 34). The ID_{50} values were determined by nonlinear regression analysis of the [^{3}H]hypoxanthine incorporation versus plasma sample dilution (log-transformed value) curves using GraphPad software (Prism V5.0; GraphPad Software, Inc., CA).

***Ex vivo* ring-stage survival assay of participants' plasma samples.** The method was modified from the ring-stage survival assay reported previously by Witkowski et al. (36). However, instead of profiling the parasite lines for their susceptibility to artemisinins, it was used to compare the antimalarial activities of the participants' plasma samples against the artemisinin-sensitive MRA1239 and artemisinin-resistant MRA1240 lines. MRA1239 and MRA1240 cultures were synchronized by several rounds of D-sorbitol selection (42) and then by heparin treatment as previously described (43). The parasite growth in MRA1239 and MRA1240 cultures containing young rings (~3 h postinvasion) was evaluated after 6 h of exposure to plasma samples collected from the same volunteers at 0.5 h and 2 h after ASAQ or ASAQ+MB administrations and compared to those exposed to their plasma collected before drug administrations (0 h). The cultures were set as described above for the *ex-vivo* bioassay (i.e., 100 μl total volume, with 50% plasma, 1% parasitemia, and 2% hematocrit). DHA (200 ng/ml) was used as a reference drug and was added to drug-free plasma provided by the Australian Red Cross Blood Service, and the cultures were set as described above. Following 6 h of exposure, the drugs were removed from the wells by 3 washes, following by addition of 100 μl of complete hypoxanthine-free medium and 20 μl of [^{3}H]hypoxanthine. Cultures were grown for a further 66 h. The parasite growth (measured by [^{3}H]hypoxanthine uptake) in cultures exposed to participants' plasma samples (0.5 h and 2 h) was assessed against that in cultures exposed to plasma samples collected at 0 h (before drug administration) (Fig. 2, right panel), whereas the viability of parasites exposed to DHA was compared to that of cultures grown in media supplemented with drug-free plasma provided by the Australian Red Cross Blood Service (Fig. 2, left panel). More details of the *ex vivo* RSA are provided in the supplemental material.

Drug analysis of participants' plasma concentrations. Plasma concentrations of AS, DHA, AQ, and DAQ were measured by liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis as previously described (31). The LC/MS/MS assay for the measurement of plasma concentrations of MB and AZB is outlined in the supplemental material. All assays used 50 μl of plasma sample. The lower limit of quantification (LLOQ) and percent coefficient of variation (interassay precision), respectively, were as follows: for AS, 1.19 ng/ml and 3.7% ($n = 17$), for DHA, 1.96 ng/ml and 4.3% ($n = 24$); for AQ, 0.39 ng/ml and 3.2% ($n = 16$); for DAQ, 3.91 ng/ml and 2.1% ($n = 34$), for MB and AZB, 1 ng/ml and ≤ 3.8% ($n = 19$) for both compounds.

Pharmacokinetic analysis. PK parameters were the maximum drug concentration (C_{max}), time to reach maximum drug concentration (T_{max}), area under the concentration-time curve from 0 h to the last data point (AUC_{0-last}) and from the last data point to infinity ($AUC_{last-\infty}$), and terminal half-life ($t_{1/2}$). These parameters were determined from the plasma concentration-time data by noncompartmental analysis (PK Solutions 2.0; Summit Research Services, OH).

Statistical analysis. Data have been summarized as means ± SD. Statistical comparisons of participant's plasma sample ID_{50} values, QT intervals, and PK parameters, including log-transformed PK exposure parameters (C_{max} and $AUC_{0-\infty}$) of the drugs, were made using paired *t* tests (SigmaStat version 3.0; Jandel Scientific, CA). Results were classified as statistically significant using the 5% significance level.

Ethical approval. The Review and Scientific Board of Central Military Hospital 108 (1373QD-BV108) and the Australian Defence Human Research Ethics Committee (ADHREC 649-11) gave ethical approval for the study, and all volunteers provided written informed consent to participate. The study was registered on anzctr.org.au (registration number ACTRN12612001298808).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We have no conflicts of interest to declare.

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