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NEUROTOXICITY AND EFFICACY OF ARTEETHER RELATED TO ITS EXPOSURE TIMES AND EXPOSURE LEVELS IN RODENTS

Q. G. LI, S. R. MOG, Y. Z. SI, D. E. KYLE, M. GETTAYACAMIN, AND W. K. MILHOUS

Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Division of Pathology, Naval Medical Research Center, Silver Spring, Maryland; Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

Abstract. The neurotoxicity of β-arteether (AE) is related to drug accumulation in blood due to slow and prolonged absorption from the intramuscular injection sites. In this efficacy and toxicity study of AE, the traditional sesame oil vehicle was replaced with cremophore to decrease the accumulation and toxicity of AE. Dihydroartemisinin (DQHS), a more toxic and active metabolite of AE, was also analyzed. When administered at a daily dosage of 25 mg/kg for seven days, blood accumulation of AE with sesame oil (AESO) was used had a 7.5-fold higher area under the curve (AUC) (on last versus first day dosing), while AE with cremophore (AECM) had only a 1.8-fold higher AUC. Although the accumulation of AECM was greatly reduced, its total exposure level (46.29 μg · h/ml) was 2.7-fold higher than with AESO (16.92 μg·h/ml) due to a higher bioavailability of AECM (74.5%) compared with AESO (20.3%). Total exposure time (calculated at over the minimal detected neurotoxicity level of 41.32 ng/ml) of AECM was 103 hours during the whole treatment period (192 hours), which was more than one-third (37%) less than with AESO (162 hours). Similar pharmacokinetic results were also shown with the active metabolite, DQHS. Anorexia and gastrointestinal toxicity with AESO were significantly more severe than with AECM ($P < 0.001$). Histopathologic examination of the brain demonstrated neurotoxic changes; the AESO rat group was significantly more severe than the AECM rat group. The brain injury scores with AECM were mild to moderate (2.3–3.0), and with AESO they were moderate to severe (3.0–4.7) on day 7 and day 10, respectively. In addition, the results of a 50% cure dose (CD_{50}) against *Plasmodium berghei* in mice were 34.1 mg/kg for AESO and 14.2 mg/kg for AECM, indicating a significant higher efficacy was found in the AECM animals. Toxicity and efficacy of DQHS were also dependent on its exposure time and level, which was the same as its parent drug (AE). In conclusion, following the seven-day treatment in rats, AE and DQHS exposure time and level varied based on the vehicle used. The extension of drug exposure time and the low peak level of AE and DQHS were more associated with severe neurotoxicity and lower antimalarial efficacy, whereas the high level and short exposure time of AE and DQHS resulted in higher efficacy and milder toxicity.

INTRODUCTION

In recent years there have been major advances in our knowledge of central nervous system (CNS) neurotoxicity in mice, rats, dogs, and rhesus monkeys administered multiple doses of artemether (AM) or β-arteether (AE).[1–5] It has been shown that AM and AE are toxic to the central nervous system; neuropathologic changes were noted to occur in those animals.

Pharmacokinetic data demonstrated that accumulation of AE and AM was observed in the plasma of rats,[6] beagle dogs,[7] rhesus monkeys (Q. G. Li, unpublished data), and humans[8,9] following multiple intramuscular (im) injections. The rat data demonstrated that the accumulation of AE in plasma was due to a slow and prolonged absorption from the injection sites.[6] The drug formulation, administration route, and plasma accumulation appeared to be associated with general toxicity and neurotoxicity.[5,6,10–12]

Except during im injection of AE and AM with sesame oil vehicles, no plasma accumulation is found for all artemisinin-derivative drugs by other administration routes in humans. Conversely, four artemisinin drugs (artemisinin, artesunate, AM, and dihydoartemisinin [DQHS]) were reported to decrease in plasma concentration during multiple oral treatments in malaria patients and healthy subjects. The Cmax and area under the curve (AUC) values were markedly reduced from one-third to one-seventh on the last dose day compared with the first day. The decrease in drug exposure levels during treatment was not malaria disease-related, since the artemisinin drug pharmacokinetics (PK) on the first day was similar to those reported in healthy subjects.[13–18] One possible explanation for the decrease in plasma concentration-time during treatment is an increase in metabolic capacity due to autoinduction of hepatic drug-metabolizing enzymes.[17]

In the neurotoxic experiments with AM and AE, an anorectic toxicity was always concomitant in the animals.[19,20] Anorectic toxicity of AE resulted in the reduction of food consumption and body weight after multiple im treatments in various animal species. Swiss scientisits[21] found anorexia due to AE in rats and dogs following im treatments of 10–36 mg/kg/day for 14 or 28 days. Brewer and others[19] observed reduced body weights in rats when treated with AE at a concentration of 25–50 mg/kg/day for 28 days. Petras and others[4] reported anorexia in all monkeys after multiple im injections of AE of 8–24 mg/kg/day for 14 days. Li and others[20] demonstrated the anorectic toxicity of AE was due to the inhibition of gastrointestinal (GI) motility (delayed gastric emptying, decreased gastric transit, and decreased food consumption) resulting in significant body weight loss, and even death following daily multiple im injections > 12.5 mg/kg of AE in rats.

Although we do not fully understand the anorectic mechanism that occurs during feeding control and treatment with artemisinin drugs, it may be similar to other anorectic agents such as amphetamines.[22] The toxicity of concomitant anorexia when compared with CNS neurotoxicity was found to be closely related in this study. Therefore, using both anorexia and neuropathology data as the toxic index, we compared the toxic effects of artemisinin drugs with two different formulations, sesame oil and cremophore.

Cremophore EL is a polyethylene oxide modified castor oil used as a solubilizing agent for oral and parenteral administration, and has been used in humans and animals.[23–25] Cremophore EL emulsion has fewer lipophilic characteristics and

easily crosses membranes and distributes among serum lipoproteins. Drug given with cremophore is also related to a slower clearance from the plasma and the parallel slower uptake in the liver. The slow clearance from the circulation of the cremophore-delivered drugs can be due to the high stability of the lipidic droplets of this particular emulsion in the presence of serum lipoproteins.[26–28] Thus, rapid absorption and longer residence were observed when using the cremophore formulation with various drugs.[24–28] The formulations of AM or AE with sesame oil (slow absorption) and cremophore (fast absorption) were also compared with their efficacy potency against *Plasmodium berghei* in mice. In addition, DQHS, an active metabolite of both AE and AM, is known to be extremely toxic in rats;[20] we also included it for comparison with AE and AM in this study. To compare the two formulations in efficacy and toxicity, the relationship study with pharmacokinetics and drug accumulation of AE was also designed.

MATERIALS AND METHODS

The drugs (AM, AE, and DQHS) used in the present study were obtained from the repository of the Walter Reed Army Institute of Research (Washington, DC). These compounds were > 99% pure. Dimethylacetamide (DMAC), cremophore EL, sesame oil, and other chemicals used were of analytical grade and obtained from the Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). The high-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, n-butyl chloride, and ethyl acetate were obtained from Burdick and Jackson (Muskegee, MI). High-performance liquid chromatography with reductive electrochemical detection was performed using liquid chromatography (model BAS 200B; Bioanalytical Systems, West Lafayette, IN).

Anorectic toxicity. Male Sprague-Dawley rats weighing 242–268 g at the time of dosing were housed individually in a controlled environment (22 ± 1°C, 60% relative humidity) with a 12-hour light:12-hour dark reverse cycle room for at least one week before use. Rats were trained more than one week to consume their daily food allotment (chow pellets) during an 8-hour (8:00 am to 4:00 pm) period. At this time, the baseline for food consumption was established. Rat consummatory behavior was measured using a modification of the Nichols method.[29] Groups of 6–8 rats received multiple im doses of 25, 50, or 100 mg/kg/day of either AE or AM, and 25 mg/kg/day of DQHS for seven days. A 14-day clinical observation was kept for monitoring food consumption, body weight, behavioral disorders, and physical or neurologic deficits. The AM and AE dose solutions were prepared in sesame oil or 1:2 cremophore/saline, while 10% DMAC/sesame oil or cremophore vehicles were used for DQHS.

Rats were individually housed and food-deprived except for the 8-hour free access to food from 8:00 am to 4:00 pm. Rats were dosed im with the appropriate drug 30 min before the feeding period. At the end of the feeding period (4:00 pm), cumulative food consumption was determined by measuring the weight difference between the beginning and end of the period. Food intake was recorded to the nearest 0.1 g after correction for spillage, if any. Rats had continued access to tap water, and water intake was recorded to the nearest 0.5 ml until every morning at 7:00 AM. At day 14 or when moribund, rats were killed by overexposure to carbon dioxide gas and a necropsy was performed.

Neuropathologic study. Twelve male Sprague-Dawley rats received multiple intramuscular doses of 25 mg/kg of AE with sesame oil (AESO) or 1:2 cremophore/0.9% saline vehicles (AECM) daily for seven days. Eight vehicle control rats, four for each vehicle, received multiple doses of only the respective vehicle daily for seven days. Six AESO-treated rats, six AECM-treated rats, and eight vehicle control rats were killed on day 7 or day 10 for neurohistopathologic evaluation. Typical pharmacodynamic measures included food consumption, body weight, and clinical assessment at the time of killing, and subsequent histopathology.

A necropsy was performed and the head and cranium were carefully removed, avoiding pressure on the underlying brain. The head and exposed brain were immersion-fixed in Bouin's fixative solution. The brain remained *in situ* for several days before its removal from the skull to avoid the development of neuronal hyperchromatosis. Whole brains were then removed and immersed in fresh Bouin's fixative. Brains were blocked transversely at the caudal aspect of the pons, immersed in daily changes of ethanol (70%) to remove excess picric acid, dehydrated in ascending grades of ethanol, vacuum-paraffin-infiltrated, and embedded. A rotary microtome was used to cut and collect 6 μm transverse serial sections, three sections per slide. Serial sections sets of the brain stem were stained with standard hematoxylin and eosin. Slides were examined and evaluated microscopically for neuronal chromatolysis and other evidence of cellular pathology in the target nucleus, the nucleus of the trapezoid body (*nucleus trapezoideus).*

Cell injury (chromatolysis) and death (necrosis) were assessed using a severity rating or a scaled scoring system (normal = 0, minimal = 1, mild = 2, moderate = 3, marked = 4, and severe = 5). Briefly, the number of chromatolytic neurons is higher with the more severe or higher grades. A score of 0 (normal or no neurons affected), 1 (1–2 neurons affected/target nucleus), 2 (3–4), 3 (5–10), 4 (11–20), and 5 (≥ 21) was assigned to each rat case based on the slide set examined. Each brain stem section evaluated had two bilateral target nuclei; scoring was averaged from all nuclei counted. The pathologist evaluating the slides was masked, and did not know the drug, vehicle, or dosage for individual cases.

Efficacy study in mice. The *P. berghei*-mouse test system provides a measure of the antimalarial efficacy of candidate drugs administered intramuscularly in graded doses by the response of *P. berghei* KBG 173 malaria parasites in young ICR mice at 5–8 weeks of age. An efficacy study of AE and AM against *P. berghei* was performed in infected mice. AE dissolved in either sesame oil or cremophore was administered intramuscularly once a day for three consecutive days commencing on day 3. The dose levels of compounds given were 0, 5, 10, 20, 40, and 80 mg/kg/day. Blood smears were taken on days 3, 6, 13, 20, 27, 34, 41, 48, 55, and 60. Blood schizonticidal activity was determined by monitoring blood films for the appearance of parasites. When the mice survived and showed negative smears at 60 days, they were considered cured. Compounds were considered active when the survival time of the treated mice was greater than twice the control mice. There were eight mice in each dose group.

Pharmacokinetic studies. Male Sprague-Dawley rats weighing 220–280 g were used. AE in sesame oil or 1:2 cremophore/0.9% saline was administered to three groups of rats

intramuscularly at a dosage of 25 mg/kg/day for seven days. This included four samples collected during the absorption, distribution, and elimination phases as a full PK analysis on the first and last dosing days. Additionally, trough levels (predose) samples are collected in between the first and last doses to follow drug accumulation and one time point sample was taken from one rat. Thus, each group was dosed and plasma samples were obtained for up to eight days. A total of 3 × 29 (0, 5, 15, 30, 45, and 60 minutes, and 1.5, 2, 3, 5, 8, 12, 24, 25, 48, 72, 96, 120, 144, 144.08, 144.25, 144.5, 145, 146, 149, 152, 156, 168, and 176 hours) samples were collected from 87 rats per each formulation into heparinized tubes using ethyl ether anesthesia. A single intravenous (iv) injection of AE in 1:2 cremophore/saline was administered to three groups of rats at the same dose (25 mg/kg). Extraction and HPLC analysis of the samples were performed as previously described.[30]

Muscle absorption studies. A single dose of AE with sesame oil and 1:2 cremophore/saline vehicles were administered intramuscularly to two groups of rats (15 rats/group) at a dose of 25 mg/kg. The blood samples were collected at 0, 5, 15, 30, 45, and 60 minutes, and 1.5, 2, 3, 5, 8, 12, 24, and 168 hours following dosing. After collecting the blood sample, the muscle of the im injection site was ablated, cut into small pieces, and placed in a vial. The AE was extracted from the minced muscles by liquid-solid extraction for HPLC analysis.[6]

Statistical analysis. The concentration-time data of AE collected from plasma and muscle during the first day for single dose and through day 7 for multiple doses were fit to a two-compartment open model using a non-linear, least-square fitting procedure (WinNonlin, version 3.1; Pharsight Corp., Mountain View, CA). The parameters of the volume of distribution at steady state (Vss) and mean clearance (Cl) were estimated by WinNonlin program. The PK parameters were estimated by adjustment of im multiple doses remaining in the injection site(s) as well as daily bioavailability by computing. All other parameters were calculated according to standard methods.[31] The AUC was determined by the linear trapezoidal rule with extrapolation to infinity based on the concentration of the last time point divided by the terminal rate constant. The im bioavailability (F) was calculated by dividing the AUC_{IM} by AUC_{IV}.

The 50% cure dose (CD_{50}) in mice was estimated by the extended least-square fitting procedure with a corresponding 95% confidence interval (TableCurve 1.1; Jandel Scientific, San Rafael, CA). The present experiment used a within-group factorial design using the factors of drug (DQHS, AE, and AM) and dose (25, 50, and 100 mg/kg). At the end of the experiments, a separate analysis of variance was performed on the latency data and the 8-hour food consumption and body weight data. The anorectic index was expressed as that the percentage of maximal change of body weight divided by the day the change happened.[32] *Post hoc* comparisons between drug with different vehicles were computed by using a homoscedastic Student's *t*-test (TTEST program, Excel, Microsoft Office 2000; Microsoft Corp., Redmond, WA) to assess the toxicity of each treatment.

RESULTS

AE, AM, and DQHS were investigated in a toxicologic study with different doses and vehicle formulations in rats,

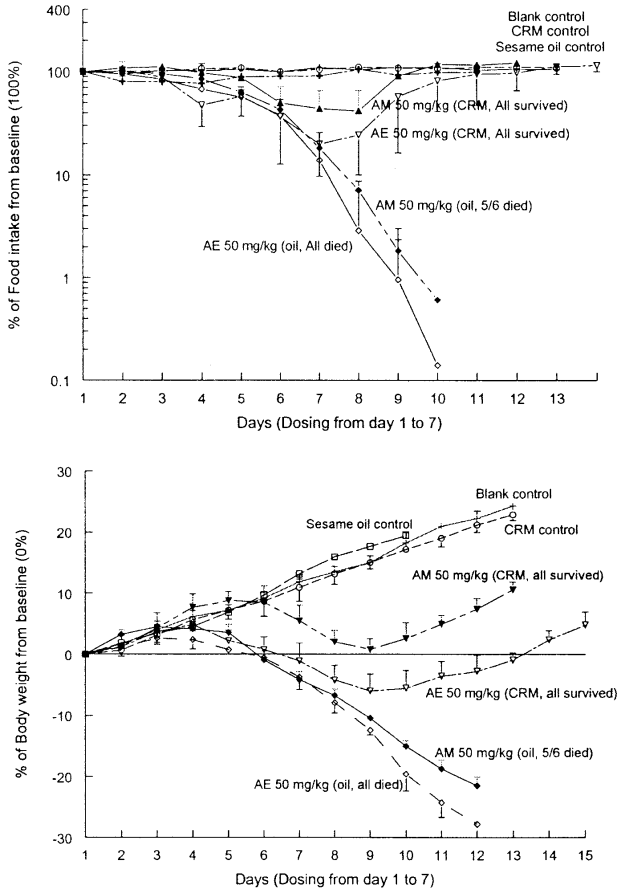

FIGURE 1. Effects of arteether (AE) and artemether (AM) with sesame oil and 1:2 cremophore/0.9% saline (CRM) vehicles on food consumption **(top)** and body weight **(bottom)** following a 50 mg/kg/day intramuscular injection for seven days in male rats during 14 days clinical observation (n = 6). Bars show the mean and SD.

and have been compared in their anorectic toxicity, neurotoxicity, and antimalarial efficacy under the same experimental conditions. The toxicities of AE are related to the drug accumulation in plasma.

Anorectic toxicity. Anorexia (reduction of food consumption and body weight) was observed in all rats with three drugs after multiple im administration at 25, 50, and 100 mg/kg dose levels. Figure 1 shows the mean kinetic values of food ingestion and body weight for the control and treatment groups during the 50 mg/kg of seven daily multiple dose trials. Food consumption was 17.5 ± 4.2 (mean ± SD) g every day in each control rat (blank and vehicle-treated) during the 8-hour feeding sessions. The food intake was significantly reduced following multiple im administrations of AM and AE in dose dependence, which was the same observation as our previous data.[20]

Since food consumption was reduced, similar reductions of body weight have been observed during the study for all three drugs. All control group rats had a body weight increase of 4–7 g (approximately 2% of baseline every day). After treatment with DQHS, AM, and AE at a dose of 25 mg/kg with different vehicle formulations, the magnitude of body weight reduction (and also food intake reduction) from most to least was DQHS with DMAC/oil > DQHS with cremophore > AE with sesame oil > AM with sesame oil > AE with cremophore

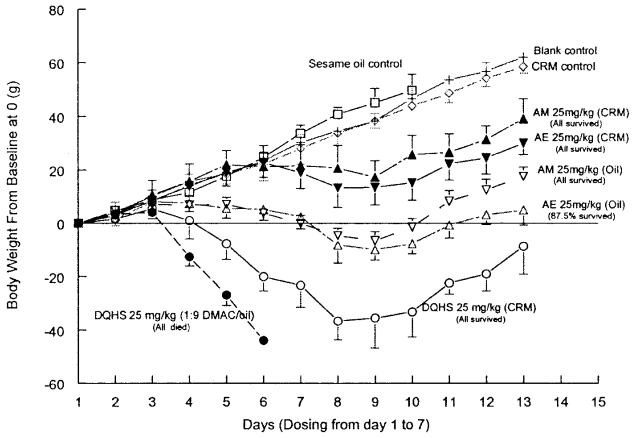

FIGURE 2. Effects of arteether (AE), artemether (AM), and dihydroartemisinin (DQHS) with sesame oil or dimethylacetamide (DMAC)/sesame oil and cremophore vehicles on body weight following a 25 mg/kg/day intramuscular injection for seven days in rats during 14 days clinical observation (n = 8). Bars show the mean and SD.

> AM with cremophore > any control group (Figure 2). This indicated that the anorectic toxicity (decreasing body weight) of DQHS was greater than with AE and AM, and the toxicity of each drug with sesame oil was greater than with cremophore. An anorectic index is shown in Table 1. In this study, the anorectic index is key in the evaluation of anorectic toxicity for these artemisinin drugs.

Severe anorectic toxicity of AE and AM was found after increasing the dose to 50 and 100 mg/kg. Rats administered AE with sesame oil showed 13% mortality at 25 mg/kg, and 100% mortality at 50 and 100 mg/kg. Rats administered AM with sesame oil had 100% survival at 25 mg/kg, only 17% survival at 50 mg/kg, and all rats died at 100 mg/kg. However, animals treated with the cremophore vehicle had survival at all dose levels with AE and AM (only 33% died at 100 mg/kg AECM) during the two weeks of clinical observation (Table 1). In all control animals, anorectic index ranged from 1.57 to 1.94; if the index for treated rats decreased below this range, it was interpreted as anorectic toxicity. For rats treated with AE and AM, the anorectic index was in the range of –0.28 to 0.75 at 25 mg/kg, –2.31 to 0.65 at 50 mg/kg, and –2.83 to –0.34 at 100 mg/kg daily for seven days. When compared with the three drugs, the anorectic index range for DQHS (–1.79 to –2.86) appeared to show much more toxicity than AE (0.59 to –0.44) and AM (0.75 to –0.28) at the same 25 mg/kg dose level with various vehicle formulations. The anorectic index of DQHS at 25 mg/kg exhibited a close range with AE and AM at 100 mg/kg (Table 1).

Compared with the different vehicle formulations for the same drug and same dose level, the anorectic toxicity of DQHS, AE, and AM with oil (sesame oil or 1:9 DMAC/sesame oil) was significantly higher than with cremophore (1:2 cremophore/saline or full cremophore) in rats ($P < 0.001$; Table 1), indicating that the cremophore formulation could reduce the anorectic toxicity from sesame oil with all three artemisinin drugs.

Neurotoxicity of AE. Neuropathologic examination of the target brain stem site (nucleus of the trapezoid body) was performed for AE formulated in sesame oil or cremophore at a dose of 25 mg/kg/day for seven days in male rats. The brains of rats treated intramuscularly had neuropathologic lesions with both incidence and grading reflecting a vehicle-effect relationship. At 25 mg/kg, all animals were affected and the lesions were prominent (Table 2). Neuropathologic results demonstrated that the neurotoxic changes in the rat brain treated with AESO (25 mg/kg im daily for seven days) were significantly more severe than those treated with AECM. The damage grades of AESO were moderate to severe (3.0–4.7), while AECM were mild to moderate (2.3–3.0) on rats killed on both day 7 and day 10 (Table 2). The characteristic changes in the affected neurons were chromatolysis and neuronal degeneration characterized by loss of Nissl substance, perikaryonal swelling, margination of the nucleus (nuclear eccentricity), nucleolar changes, and increased perikaryonal eosinophilia with occasional clumping of eosinophilic debris. More severe cases had bilateral lesions in other brain stem nuclear groups and a subjective reduction in the normal neuron numbers present compared with vehicle controls.

***In Vivo* efficacy in mice.** AE and AM showed comparable efficacy against *P. berghei* whether they were dissolved in

TABLE 1

Anorectic index and mortality (death/total) following 25 mg/kg daily intramuscular injections of DQHS, AE, and AM for seven days and clinical observation for 14 days in male rats (n = 6–8)*

Dose group	Controls (n = 6)	25 mg/kg (n = 8)	50 mg/kg (n = 6)	100 mg/kg (n = 6)
DQHS				
Blank	1.87 ± 0.15 (0/6)			
Full cremophore	1.71 ± 0.32 (0/6)	–1.79 ± 0.32 (3/8)		
1:9 DMAC/oil	1.57 ± 0.15 (0/6)	–2.86 ± 0.23 (8/8)		
t-test		$P < 0.001$		
AE				
1:2 CRM/saline	1.76 ± 0.17 (0/6)	0.59 ± 0.17 (0/8)	–0.66 ± 0.18 (0/6)	–1.59 ± 0.24 (2/6)
Sesame oil	1.94 ± 0.24 (0/6)	–0.44 ± 0.14 (1/8)	–2.31 ± 0.61 (6/6)	–2.83 ± 0.33 (6/6)
t-test		$P < 0.001$	$P < 0.001$	$P < 0.001$
AM				
1:2 CRM/saline	1.76 ± 0.17 (0/6)	0.75 ± 0.18 (0/8)	0.65 ± 0.43 (0/6)	–0.34 ± 0.11 (0/6)
Sesame oil	1.94 ± 0.24 (0/6)	–0.28 ± 0.09 (0/8)	–1.74 ± 0.16 (5/6)	–2.68 ± 0.36 (6/6)
t-test		$P < 0.001$	$P < 0.001$	$P < 0.001$

* The anorectic index is percentage of maximal change of body weight divided by the day the change occurred. Values are the mean ± SD. DQHS = dihydroartemisinin; AE = arteether; AM = artemether; DMAC = dimethylacetamide; oil = sesame oil, CRM = cremophore.

TABLE 2

Mean ± SD adverse effects in the rat brain stem after repeated arteether injections with vehicle sesame oil (AESO) or cremophore (AECM) by daily intramuscular dosing of 25 mg/kg for seven days (n = three/group)*

	Finding Vehicle	Control	AESO	AECM	*t*-test
Day 7	Neuronal chromatolysis	0	3.00 ± 1.00	2.33 ± 1.15	$P = 0.092$
Day 10	Neuronal chromatolysis	0	4.67 ± 0.58	3.00 ± 1.00	$P = 0.019$

* Findings were graded as 0 = no significant lesions; 1 = minimal; 2 = mild; 3 = moderate; 4 = marked; and 5 = severe.

sesame oil or cremophore following im treatment (Table 3). However, the results for the CD_{50} were much different for the two drugs with sesame oil than with cremophore. AE and AM with sesame oil had CD_{50} values of 34.1 (12.3–66.9) mg/kg and 35.6 (7.6–63.6) mg/kg, respectively. The drugs with cremophore had values of 14.2 (6.8–21.5) mg/kg for AE and 21.0 (18.5–23.4) mg/kg for AM (Table 3). The results showed that AE and AM formulated in cremophore had a significantly higher efficacy than the sesame oil formulation following the three daily multiple im injections.

Pharmacokinetic studies. The mean plasma concentration-time profile (n = 3) of AE formulated with sesame oil (AESO) and 1:2 cremophore/0.9% saline (AECM) after single iv and im doses is shown in Figure 3. AE with sesame oil and cremophore data showed a biphasic pattern of disposition and was fitted by a two-compartment open model after either iv or im injection. The concentration-time profiles of AE with the two vehicles are shown in Figure 4 after the seven daily im multiple administrations. The trough plasma concentrations increased significantly up to the last dosing in AESO-treated rats, but not in AECM-treated rats. Obviously, the drug accumulated (7.5-fold) in the plasma of animals administered with AESO (comparing the seventh dosing to the first dosing). Compared with the AECM-treated rats, only a slight AE level was increased (1.8-fold) on day 7 over that on day 1 following the multiple dosing.

Significant differences in pharmacokinetic parameters between AECM and AESO are shown in Table 4 after multiple im doses of 25 mg/kg. Plasma concentration on day 1 indicated that the mean ± SD Cmax of AECM (1,227 ± 171 ng/ml) was more than 13-fold higher than that of AESO (92.2 ± 19.9 ng/ml). The mean ± SD AUC of AECM (4,165 ± 676 ng·h/ml) was approximately four-fold higher than that of AESO (1,135 ± 277 ng·h/ml) on day 1. The total AUC of AECM (46,286 ± 2,061 ng·h/ml) during the seven-day dosing period was approximately 2.7-fold higher than that of AESO (16,922 ± 4,038 ng·h/ml). The mean ± SD elimination half-life was 7.0 ± 0.9 hours for AECM and 17.7 ± 2.7 hours for AESO on day 1. Based on the AUC data, the mean ± SD bioavailability was 74.5 ± 12.1% for AECM and 20.3 ± 5.0% for AESO after a single im injection. The data suggested that AECM, a less oily formulation, was more easily absorbed from the muscle with a higher Cmax and AUC, although it is rapidly eliminated with a much shorter half-life compared with AESO.

Greater drug accumulation in plasma was observed in the animals treated with 25 mg/kg of AESO per day for seven days. On day 7, the Cmax (311.9 ng/ml) and AUC (8,527 ng·h/ml) of AESO were 3.4-fold and 7.5 fold higher than the Cmax (92.2 ng/ml) and AUC (1,135 ng·h/ml) on day 1, respectively. Compared with AECM, the plasma concentration was not significantly accumulated in spite of giving the same dosage regimen as AESO in rats. With AECM, the Cmax and AUC were only 1.5-fold and 1.8-fold higher on day 7 than on day 1, respectively (Table 4). This data indicated that the plasma accumulation of AE has evidently been reduced when using the cremophore vehicle formulation.

TABLE 3

Antimalarial activity (50% cure dose [CD_{50}]) and corresponding 95% confidence interval (CI) of arteether and artemether given intramuscularly in a daily dose for three days against *Plasmodium berghei* in female mice (n = 8 for each dose level)*

	Arteether (CD_{50})		Artemether (CD_{50})	
Drug	mg/kg	95% CI	mg/kg	95% CI
Sesame oil formulation	34.1	12.3–66.9	35.6	7.6–63.6
Cremophore formulation	14.2	6.8–21.5	21.0	18.5–23.4

* When the mice survived and showed negative smears at 60 days, they were considered cured.

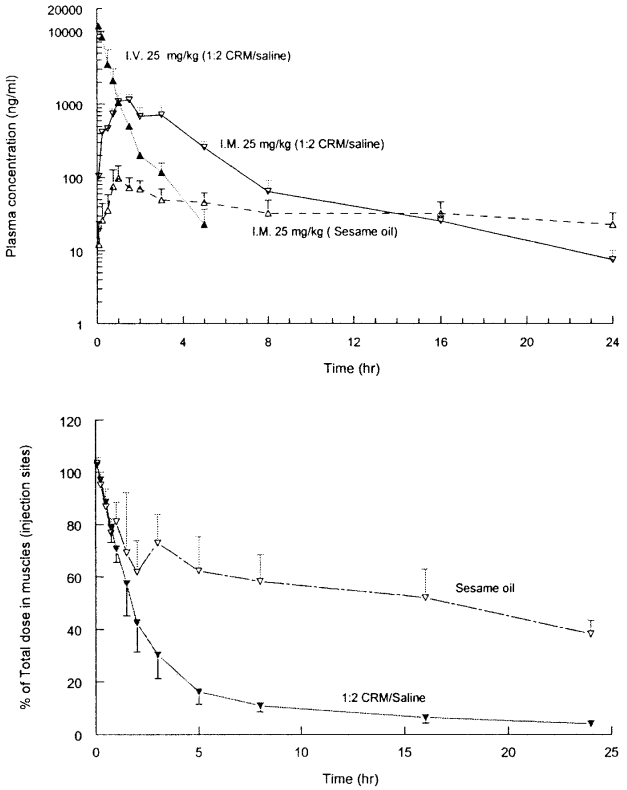

FIGURE 3. Plasma concentration-time profiles of arteether following a 25 mg/kg, single, intravenous (I.V.) and intramuscular (I.M.) injection in 1:2 cremophore/saline and a single I.M. injection in sesame oil **(top),** and the muscle (from I.M. injection sites) amounts-time profiles of arteether following a 25 mg/kg, single I.M. injection in 1:2 cremophore/0.9% saline (CRM) and sesame oil in male rats (**bottom,** n = 3). Bars show the mean and SD.

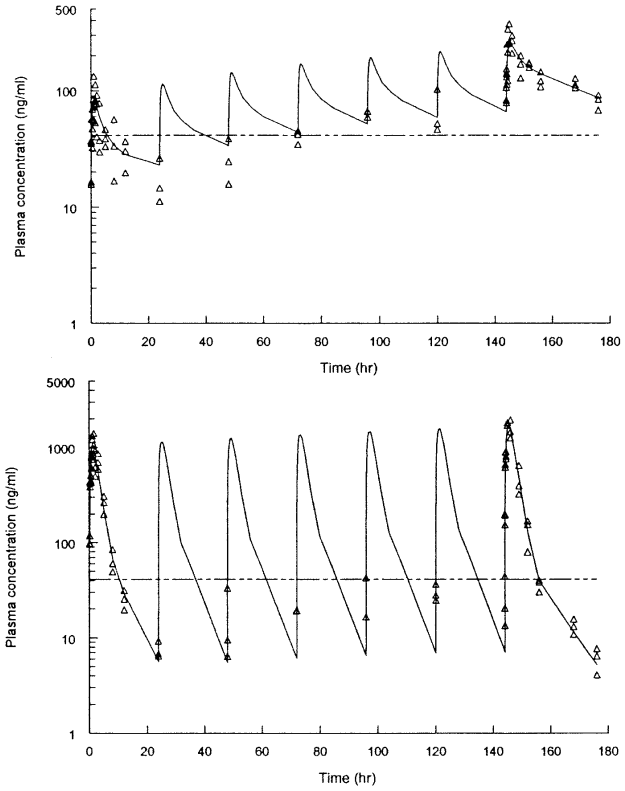

FIGURE 4. Plasma concentration-time profiles measured by high-performance liquid chromatography-with reductive electrochemical detector **(triangles)** and computer fitted curves by pharmacokinetic parameters **(solid line)** of arteether in sesame oil **(top)** and 1:2 cremophore/0.9% saline **(bottom)** following a 25 mg/kg, multiple, intramuscular injection once a day for seven days in male rats (n = 3). The minimal detected adverse effect level was estimated to be 41.32 ng/ml **(dashed line).**

The ratios of DQHS to AE were only 0.06–0.14 in all animals (Table 4) during the seven-day treatments, suggesting that the conversion of AE to DQHS was minimal after im injection in rats. The DQHS half-life was 4.7–6.6 hours for AECM-treated rats and 9.2–14.5 hours for AESO-treated rats. The total AUC of the AECM group during the seven-day dosing period was 5,346 ng·h/ml, which was 1.6-fold higher than with AESO (3,427 ng·h/ml).

AE absorption from injection sites. The amount of AE remaining in the injection site (muscle) versus time with three different formulations is shown in Figure 3. The data indicated that at 24 hours after dosing, 37.6% of the single dose AESO still remained in the muscle, and only 4.7% of the AECM remained in the injection site. The amount of AE in the muscle rapidly decreased for 1–2 hours, and then exhibited a much slower decrease as two absorption phases for the two formulations. Following seven days of multiple dosing of AESO and AECM, 91.4% and 17.3%, respectively, of the single dose remained in the muscle (from seven injection sites) on day 8 (24 hours after last dosing) (Table 4). The muscle data supported the plasma results (Figure 3); a more complete absorption of AECM resulted in a higher bioavailability and a lower accumulation, and the prolonged absorption of AESO resulted in a higher accumulation in both muscle and plasma. The sesame oil with AE acted as a delayed release formulation.

DISCUSSION

AE and AM are strongly lipophilic and practically insoluble in water;[33] therefore, sesame oil is used extensively as a vehicle for pre-clinical and clinical, single or multiple, intramuscular injection studies and treatments.[34,35] This formulation, however, caused high levels of drug accumulation in plasma following multiple im injections,[6,7,9] which were associated with general toxicity and neurotoxicity.[5,6,11,12] In the present studies, a 1:2 cremophore/saline solution was used for comparison with sesame oil for evaluation of drug accumulation, toxicities, and efficacy related to toxicokinetic characteristics of AE and AM after multiple im doses in rats.

Drug accumulation in plasma of AE formulated with sesame oil was confirmed in this study with a 7.5-fold higher AUC on day 7 than on day 1, and consistent with our previous study. The absorption of AE from muscle (injection site) was incomplete in the first 48 hours after a single injection; at 24 hours and 48 hours after dosing, 38% and 22%, respectively, of the total dose of AE still remained in the injection site. The amount of AE in muscle rapidly decreased for 1–2 hours and then exhibited a much slower decrease. Half-lives for the fast and slow absorption phases in the muscle were 1.0 hour and 26.3 hours, respectively. Based on the half-life of the slow absorption phase, a single AESO dose (25 mg/kg) would be completely absorbed in 6–7 days. Acute toxicity data have shown that animals receiving a single high dose of AE in sesame oil died between days 5 and 11.[6] One would expect that the remaining dose amount would be absorbed later because an im total dose of AESO should be 100% absorbed in the absence of decomposition or metabolism at the injection site. The low bioavailability (20.3%) of AESO in these rats indicated incomplete muscle absorption on the first day, which was similar to the previous bioavailability results in rats of 23.4%.[6] The data suggest that the low bioavailability of AESO (normally calculated for the first 24 hours) in rats after im administration is due to the slow and prolonged absorption from the muscle.

Slight drug accumulation was found for AE formulated in 1:2 cremophore/0.9% saline with only a 1.8-fold higher AUC on the last day than on the first day. Muscle data with AECM demonstrated that at 24 hours after a single dose there was less than 5% of the total single dose left in the injection site, indicating that the formulation of AECM contributed to complete absorption from muscle. The bioavailability of AECM was 74.5%, which was 3.7 times higher than AESO. Due to better absorption from injection sites with AECM, the elimination half-life was shortened from 17.7–29.6 hours (AESO) to 7.0–9.1 hours (AECM) and the volume distribution was decreased from 54–150 liters (AESO) to 7–9 liters (AECM) during the seven-day treatment.

As we discussed previously,[6] the prolongation of half-lives and the increase in total volume of distribution of AESO was not only due to the accumulation in plasma and prolonged absorption from muscle, but also because of systemic toxicities in these animals. Only a pathophysiologic factor could change the slopes of distribution and elimination due to biotransformation, excretion, and protein or tissue bindings.[36,37] Therefore, the systemic toxicities (GI toxicity and neurotoxicity) encountered in this study should be considered an important factor of pathophysiologic and kinetic changes in these rats. It is clear that the systemic toxicity was related

TABLE 4

Pharmacokinetic parameters of arteether (AE) and dihydroartemisinin (DQHS), a metabolite of AE, following a single iv (25 mg/kg), and multiple im (25 mg/kg) administration with sesame oil and 1:2 cremophore (CRM)/0.9% saline daily for seven days in male rats (n = 3–7)*

Dosing Parameters	iv single Vehicle: CRM/saline	im multiple			
		Sesame oil (AESO)		1:2 Cremophore/saline (AECM)	
		Day 1	Day 7	Day 1	Day 7
Arteether					
Cmax (ng/ml)	14482 ± 1023	92.2 ± 19.9	311.9 ± 54.6	1227 ± 171	1826 ± 118
Tmax (min)	0	1.42 ± 0.63	1.67 ± 0.58	1.33 ± 0.29	1.33 ± 0.58
AUC (ng·h/ml)	5593 ± 591	1135 ± 277	8527 ± 1006	4165 ± 676	7312 ± 588
AUC_{D1-8} (ng·h/ml)			16922 ± 4038		46286 ± 2061
Vss (liter)	0.98 ± 0.25	150.2 ± 33.7	54.1 ± 7.8	8.70 ± 1.21	5.06 ± 0.27
Cl (ml/min/kg)	71.3 ± 9.11	73.7 ± 2.8	147.9 ± 6.19	76.3 ± 2.9	76.0 ± 1.9
$t_{1/2}$ distribution (h)	0.22 ± 0.04	0.71 ± 0.47	1.08 ± 0.49	1.00 ± 0.04	1.18 ± 0.14
$t_{1/2}$ elimination (h)	0.82 ± 0.05	17.66 ± 2.68	29.57 ± 2.07	6.96 ± 0.93	9.06 ± 1.69
MAT (h)	–	0.76 ± 0.40	0.87 ± 0.39	1.04 ± 0.03	1.07 ± 0.10
MRT (h)	0.74 ± 0.18	23.56 ± 3.42	41.51 ± 2.84	6.21 ± 0.66	5.83 ± 0.09
Bioavailability (%)		20.3 ± 5.0	152.4 ± 17.9	74.5 ± 12.1	130.7 ± 10.5
% of single daily dose in muscle		37.6 ± 8.2	91.4 ± 11.6	4.7 ± 1.8	17.3 ± 3.3
Dihydroartemisinin					
Cmax (ng/ml)	815.8 ± 164.2	16.02 ± 4.05	123.8 ± 28.9	171.1 ± 79.4	188.4 ± 91.0
Tmax (min)	0	0.92 ± 0.14	0.86 ± 0.35	0.92 ± 0.14	1.33 ± 0.29
AUC (ng·h/ml)	350.0 ± 97.1	117.1 ± 33.0	790.3 ± 220.6	607.7 ± 101.5	708.2 ± 29.2
AUC_{D1-8} (ng·h/ml)			3427 ± 759		5346 ± 831
$t_{1/2}$ elimination (h)	1.08 ± 0.16	9.17 ± 2.54	14.47 ± 2.17	4.71 ± 0.71	6.64 ± 1.25
DQHS:AE ratio	0.06 ± 0.02	0.09 ± 0.03	0.08 ± 0.03	0.14 ± 0.03	0.11 ± 0.01

* On day 1 the amount of dose in the muscle was measured at 24 hours after the first dosing, and on day 7 the amount of dose was measured at 24 hours after the last dosing. Values are the mean ± SD. iv = intravenous; im = intramuscular; CRM/saline = 1:2 cremophore/0.9% saline; AESO = AE with sesame oil; AECM = AE with cremophore; AUC = area under the curve; Vss = volume of distribution at steady state; Cl = mean clearance; MAT = mean arrival time; MRT = mean residence time.

to the plasma drug accumulation, which was extended and consistent with our previous data.[6] Actually, more severe anorectic toxicity and neurotoxicity were observed in AESO-treated rats than in AECM-treated rats.

Gastrointestinal toxicity (reductions of food consumption and body weight) of AE and AM was confirmed in each dose level in the treated animals. This toxicity may have resulted from an inhibition of gastric motility due to neurotoxicity.[20] Our previous data showed that gastric motility (gastric retention and transit) was influenced by multiple doses of artemisinin drugs and that these changes were reflected in decreases in body weight and body weight gain.

Artemisinin drug-induced GI toxicity was dose dependent on reductions in food intake, water intake, and body weight gain in rats. To evaluate the anorectic toxicity, a 50% diet inhibition of day (ID_{50}) has been previously used with data of the food and water consumption.[20] In this study, we found that body weight was more sensitive, more objective, and more accurate in reflecting GI toxicity than food and water consumption in rats. Sometimes, the food and/or water intakes were completely inhibited (100%) at early periods in the high-dose treatment and cannot indicate the anorexia; the body weight was still a trusted marker in monitoring the anorectic toxicity until death of the animals. Indeed, a 20% reduction of body weight was selected as an emergence sign for us to discontinue the multiple dosing and to killed the animal for pathologic evaluation. In addition, possible error was always inevitable during collection of the food and water samples.

Highly correlative coefficient values were fitted to be positive between the body weight, food intake ($r = 0.92 \pm 0.03$), and water intake ($r = 0.95 \pm 0.02$) at the three dose levels (25, 50, and 100 mg/kg) of AE and AM in the seven daily dose treatments. This means that the rats have about 2% of body weight (5–7 g) gain every day that will be dependent on the intake of 14–21 g of food and 25–32 ml of water daily. Therefore, the body weight could indicate the consumption of food and water in evaluating the anorectic toxicity alone.[38–40] In this study, the decrease of body weight was calculated as a sole index of the anorectic toxicity to evaluate the GI toxicity of the artemisinin drugs in rats (Table 1). The index results successfully showed that 1) the anorectic toxicity was of dose dependence for AE and AM; 2) the cremophore formulation significantly reduced the GI toxicity of the three drugs at any dose level; and 3) the GI toxicity of DQHS was approximately four-fold higher than with AE and AM in rats.

Histopathologic results demonstrated neurotoxic changes in the brain; lesions in rats treated with AESO (25 mg/kg im for seven days) were significantly more severe than in rats treated with AECM. The damage grades were moderate to severe for AESO, while they were mild to moderate for AECM on rats killed on both day 7 and day 10. Therefore, the cremophore vehicle formulation (AECM) results in substantially less neurotoxicity than the traditional sesame oil vehicle formulation (AESO). This was similar to the anorectic toxicity data described earlier. The neurotoxicity in this study consists of a selective neuronopathy with degenerate or chromatolytic neurons in the CNS, especially in the brain stem. In the rat, the target brain stem nucleus or group of neurons consistently and most severely affected is the nucleus of the trapezoid body.[41,42] With the artemisinin derivatives reported to cause neurotoxicity in laboratory animals, there is a selective brain stem neurotoxicity at the lowest adverse effect doses progressing to more widespread CNS (brain and spinal cord) lesions at higher doses. There is a dose response with more severe neurotoxicity (chromatolysis and neuronal necrosis) at higher doses. Since the AECM had reduced plasma accumulation, it was very clear that the toxicities of AESO were related to drug formulation and plasma accumulation.[5,6,11,12]

The *in vivo* efficacy model showed that AE and AM had comparable activity against *P. berghei* in mice whether they were dissolved in sesame oil or in cremophore following multiple im treatments (Table 3). The CD_{50} demonstrated that AE and AM formulated with cremophore had significantly higher efficacy than that with sesame oil. This finding is very important for clinical development of the two oil-soluble drugs. First, AE and AM formulated with sesame oil have already been shown to have highly efficacy in patients with malaria,[9,35,43,44] and an additional increase in efficacy with a vehicle formulation change should provide further support for the two drugs in clinical use. Second, since the fatal neurotoxicity of the two drugs seems to be caused by the drug accumulation or by the sesame oil formulation in our studies, it appears that the parent drugs are not too toxic as we had envisioned. AE formulated with cremophore not only increased the efficacy, but also reduced the neurotoxicity in rats, indicating that new formulations could improve clinical treatment by the two drugs. Finally, the pharmacokinetic data of AE with cremophore showed a much different profile from AE with sesame oil in this study, and the PK parameters would guide the drug development to the proper intramuscular formulation and dosage regimen for clinical treatment.

Plasma concentration showed that the Cmax and AUC of AECM were approximately 13-fold and five-fold, respectively, higher than with AESO on day 1. Even the total AUC of AECM (46,286 ng·h/ml) during the seven-day period was still 2.7-fold higher than with AESO (16,922 ng·h/ml). The much higher level of AE exposure in the animals using a cremophore vehicle truly contributed to the antimalarial efficacy, and without a doubt, the antimalarial efficacy is drug concentration dependent.[45–48] It was anticipated that the toxicities would also increase with the higher AE level in the rats treated with cremophore. However, the reverse was observed; the GI toxicity and neurotoxicity were significantly reduced in this study when rats were treated with AECM compared with AESO.

Since toxicity is dependent on chemical/drug exposure level and time,[49,50] the higher exposure concentration of AECM should have increased the toxicity in this study. However, the toxicity was not increased in this study because the exposure time of AECM had been shortened. In fact, the PK data confirmed that AE concentration during the seven daily treatments showed a large interval curve in AECM-treated rats compared with AESO-treated rats, which displayed gradual accumulation and no interval curve (Figure 4). Genovese and others[51] estimated that a minimal daily dose of AE of 6.25 mg/kg for seven days is a no detectable pathologic effect dose (NDPED) in causing the neurotoxicity in rats. Statistically significant neuropathology in brain stem nuclei was observed in the group of rats (6 of 6 affected) treated with 12.5 mg/kg. These results demonstrate that AE-induced brainstem neuropathology in rats can occur at the relatively high dose of 12.5 mg/kg (doubled NDPED dose) for seven days. The minimal plasma AE concentration with a 12.5 mg/kg dose has been simulated by our previous studies,[6] and 41.32 ng/ml was calculated as the minimal detected neurotoxicity level (MDNL) in plasma that should risk causing neurotoxicity.[52,53]

The mean ± SD exposure time of AE at over plasma concentration of 41.32 ng/ml was 164.28 ± 7.91 hours in this seven-day (192 hours) study after daily 25 mg/kg im injections of AESO (Figure 4). When the exposure time of AESO to AECM (102.96 ± 5.26 hours) was compared, 37% less exposure time was found in the cremophore animals at the same concentration level. Therefore, the shorter exposure time of AECM was a key reason in reduced toxicities in this study. The data were similar to those of some drug exposure investigations that found continued exposure over time (drug exposure time) rather than a high concentration (drug exposure level) over a short time (interval time) contributes to the toxicity.[49,50,54,55]

It is well known that AE was variably converted to the active metabolite, DQHS, which is 3–5 times more active and toxic than AE in animal species.[30,56,57] Thus, evaluating the conversion rates of various artemisinin drugs is very important in assessing the risk of neurotoxicity and evaluating the efficacy of AE. In the present study, two formulations showed a very low and similar conversion rate of AE to DQHS with 0.06–0.14 DQHS:AE ratios in rats. A high DQHS level (1.6-fold) was found in the AECM-treated rats that did not contribute to neurotoxicity but to efficacy. The half-life of DQHS in AESO-treated rats was two times longer than in AECM-treated rats, suggesting that drug exposure time is much more important than drug exposure level to effect neurotoxicity in those animals.

Clinical trials data of AE with sesame oil following five-day treatment in humans demonstrated a similar high level accumulation and low drug concentration in blood, as showed in this animal study.[9,58] The results contributed to a low therapeutic potential of intramuscular AE when compared with oral DQHS, oral and intravenous artesunate (AS), intramuscular AM, and even intramuscular α/β-AE formulated with peanut oil in humans.[9,59–61] Although far fewer undesirable side effects were reported in these trials, the lower efficacy limited the use of the drug in patients infected with *P. falciparum* malarias. Previous PK data demonstrated that the AM plasma concentration was more than three-fold higher than that of AE in rats at a same dose level and with the same sesame oil formulation.[20] It may be that AM has a less lipophilic property than AE that favors absorption from muscle. Better efficacy of α/β-AE was found in a peanut oil formulation in India,[59–61] suggesting that peanut oil may also facilitate better absorption of this drug in humans. Therefore, further investigation on new intramuscular formulations needs to be done to increase efficacy and decrease toxicity of the oil-soluble antimalarial drug AE.

In conclusion, cremophore as an im vehicle greatly increased plasma AE concentration, but not accumulation, and decreased by more than one-third the exposure time to a high neurotoxic risk level. Obviously, AECM showed increased efficacy in mice and decreased GI toxicity and neurotoxicity in rats compared with AESO after the multiple im administrations. DQHS, a more active and toxic metabolite of AE, showed similar pharmacologic properties as its parent drug. The higher drug exposure levels of AE and DQHS at short exposure times showed a greater association with antimalarial efficacy, and the longer drug exposure time (accumulation) at a low plasma concentration showed a greater association with toxicity.

Authors' addresses: Q. G. Li, Y. Z. Si, D. E. Kyle, and W. K. Milhous, Division Experimental Therapeutics, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500, Telephone: 301-319-9351, Fax: 301-319-9449, E-mail: qigui.li@na.

amedd.army.mil. S. R. Mog, Division of Pathology, Naval Medical Research Center, 503 Robert Grant Ave., Silver Spring, MD 20910-7500. M. Gettayacamin, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand.

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