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STUDIES OF THE NEUROTOXICITY OF ORAL ARTEMISININ DERIVATIVES IN MICE

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Abstract. Intramuscular injections of high doses of the oil-soluble antimalarial artemisinin derivatives artemether and arteether produce an unusual pattern of selective damage to brain stem centers in experimental mammals, predominantly those involved in auditory processing and vestibular reflexes. We have shown recently in adult Swiss albino mice that parenteral artesunate, a water-soluble derivative, is significantly less neurotoxic than intramuscular artemether in this murine model. Using the same model, in which the drugs were administered daily for 28 days, the neurotoxic potential of the oral drugs was assessed and compared with the parenteral routes of administration. The dose causing neurotoxicity or death in 50% of animals (ED_{50}), was approximately 300 mg/kg/day of oral artemether and artesunate compared to 50 mg/kg/day of intramuscular artemether. Doses of intramuscular artemether > 100 mg/kg/day were uniformly lethal. When oral artemether was given in peanut oil there was an increase in neurotoxicity and mortality compared with the aqueous suspension ($P = 0.002$), and when the food pellets were coated with artemether in oil, giving relatively constant oral intake, neurotoxicity was further increased; ED_{50} = 150 mg/kg/day ($P = 0.017$). These data indicate that once-daily oral administration of artesunate or artemether is relatively safe, presumably because the central nervous system is exposed transiently, whereas constant exposure either from depot intramuscular injection of oil-based drug, or constant oral intake carries relatively greater neurotoxic potential.

INTRODUCTION

Artemisinin and its derivatives are the most rapidly effective of all antimalarial drugs. Their efficacy against multidrug-resistant falciparum malaria and their potential to delay antimalarial drug resistance when used in combinations have led to increasing use in recent years. The artemisinin derivatives have proved to be very well tolerated in clinical trials and in general use.[1] There have been remarkably few significant adverse effects documented in more than two million treated patients of whom several thousand were enrolled in detailed prospective studies.[1,2] Artemether, and the closely related compound arteether, are hydrophobic derivatives of dihydroartemisinin formulated for intramuscular injection in groundnut (peanut) and sesame oils, respectively. In rodents, dogs, and monkeys, intramuscular injections of artemether and arteether, in relatively high doses, have been associated with an unusual pattern of selective damage to certain areas of the brain stem (the red nucleus and nuclei caudal to that nucleus). These centers are concerned predominantly with the auditory and vestibular relays.[3–7] In a mouse model of neurotoxicity, we have shown recently that parenteral artesunate, a water-soluble derivative of dihydroartemisinin, is significantly less neurotoxic than intramuscular artemether.[8] Since both artesunate and artemether are metabolized *in vivo* back to the parent dihydroartemisinin, and as this is the most neurotoxic of all the compounds in cell culture systems,[9] these data suggested that pharmacokinetic rather than pharmacodynamic factors may be the more important in determining neurotoxic potential. We have therefore used this model to investigate the toxic potential of these same drugs when given orally.

MATERIALS AND METHODS

The method of evaluation used in this study was identical to that in a previous report,[8] except that only the most discriminating of the behavioral tests, that of balance and coordination, was used.

Animals. Adult female Swiss albino mice weighing 25–30 grams were kept for two weeks before starting the experiment. They were housed five to a cage with access to food and water *ad libitum.*

Drug administration. Artemether (80 mg/ml) suspended in groundnut (peanut) oil (Kunming Pharmaceutical Factory, Kunming, People's Republic of China) was dispensed in 1-ml ampules for intramuscular injection. Artesunate was dispensed as dry artesunic acid powder (60 mg) together with a 1-ml ampule of 5% sodium bicarbonate (Guilin No. 2 Pharmaceutical Factory, Guangxi, People's Republic of China), which were mixed together before injection. Intramuscular injections were given using a sterile 1-ml syringe (Terumo, Tokyo, Japan) and a sterile 25-gauge butterfly cannula (Abbott Ireland Ltd., Sligo, Ireland) attached to 90-cm sterile plastic tubing with an inner dimension of 0.3 mm. To give a precise dose, the tubing was marked at intervals corresponding to the required injectate volume. This allowed sequential injection of exact small volumes. The drug was drawn up into the butterfly and the needle tip was cleaned in 70% ethanol between each injection. An additional group in which artesunic acid (100 mg/kg/day) was suspended in peanut oil was also included. Injections were performed daily into alternate thighs.

Oral artesunate (Guilin No. 1 Pharmaceutical Factory, Guangxi, People's Republic of China) was prepared from 50–mg tablets, and oral artemether was used in two formulations; the injection preparation suspended in peanut oil (Kunming Pharmaceutical Factory) or a suspension in water of pure drug (kindly provided by Novartis, Basle, Switzerland). An additional control group of mice receiving no injections was also included (n = 18). Controls were also injected for 28 days with peanut oil alone (n = 10) using the same injection volume as drug administration.

Oral drug was administered once a day by intragastric gavage, or for one group precise quantities of the oil-based

TABLE 1
Intramuscular treatment groups, clinical findings, and outcome

Group	Treatment	No. of mice	No. of mice with abnormalities of balance and gait surviving to 120 days			No. of mice with normal balance and gait throughout 120 days	Deaths
			Reversible	Persistent	Total		
1	Artemether (150 mg/kg for 28 days)	20	0	0	0	0	20 (1 + 19*)
2	Artemether (200 mg/kg for 28 days)	20	0	0	0	0	20 (4 + 16*)
3	Artesunate (150 mg/kg for 28 days)	20	1	3	4	15	1
4	Artesunate (200 mg/kg for 28 days)	20	1	6	7	10	3 (2 + 1*)
5	Artesunate (250 mg/kg for 28 days)	20	3	2	5	11	4 (3 + 1*)
6	Artesunate in oil (100 mg/kg for 28 days)	20	0	0	0	18	2

* Death with preceding abnormal equilibrium.

artemether were coated on weighed food pellets. Each day the food was weighed again and the amount of artemether added was adjusted to ensure constant administration of the assigned dosage regimen.

Dose regimens. Animals were assigned randomly to receive intramuscular artemether or artesunate at the following doses: 150 mg/kg/day (n = 20 per group) and 200 mg/kg/day (n = 20), intramuscular artesunate (aqueous solution) was also given at 250 mg/kg/day (n = 20); intramuscular artesunic acid in peanut oil was given at 100 mg/kg/day (n = 20). Intramuscular injections of the two artemisinin derivatives were administered daily for 28 days to alternate thighs.

Oral artesunate and artemether in aqueous solutions were given at doses of 100, 150 (artemether only), 200, 250, and 300 mg/kg/day. Oral artemether in oil was given at doses of 100, 150 (n = 20 each group), and 200 mg/kg/day (n = 45; 25 were randomized initially and an additional 20 were studied subsequently). Peanut oil in the same volume was given as a control to 10 mice. Artemether (in oil) coated food pellets was given at doses adjusted daily to approximately 100, 150 (n = 20 each group), and 200 mg/kg/day (n = 25). Peanut-oil–coated food pellets were given to an additional 10 mice as controls.

Assessments. A standardized assessment of gait (balance and coordination) was recorded twice each week. After completion of the 28-day injection schedule, the mice were assessed weekly for their gait and fine coordination skills as described below. The mice were killed at the end of 120 days and perfusion fixation of the brains was performed to document any neuropathologic changes. The results of the neuropathology examinations will be reported separately.

Testing. The mouse was left to walk along the 9-mm wide 100-cm long horizontal edge of a wooden box for five steps and assessed for body equilibrium while walking. The following were noted: any abnormalities of gait, whether the mouse was unsteady, a body angle $> 5°$ from the vertical axis, stopping more than twice because of poor balance, hind legs slipping off the edge when walking for five steps, or whether the mouse fell off or could not walk at all. There were four outcomes: normal throughout, reversible neurologic abnormality, irreversible abnormality, or death. Mice that died either had a preceding neurologic abnormality or died suddenly without a preceding deficit.

Statistical analysis. The results were compared using the Wilcoxon rank sum test or Kruskal-Wallis tests, or chi square test, and correlations were assessed by the method of Spearman. Survival was evaluated by log rank analysis.

RESULTS

Parenteral administration. Artemether given intramuscularly was uniformly lethal at doses $\geq$ 150 mg/kg/day (Table 1). At doses $\geq$ 200 mg/kg/day, no mouse survived to the end of the injection schedule. In 35 of 40 mice, abnormalities of gait and balance preceded death. The interval from neurologic abnormality to death ranged from 0 to 21 days. Median (range) times to death were correlated negatively with dose, ranging from 23 (9–71) days at a dose of 100 mg/kg/day to 12 (4–21) days at a dose of 200 mg/kg/day. This gave a consistent cumulative dose which killed 50% of animals (LD_{50}) for intramuscular artemether of 2.3–2.4 g/kg. In contrast, intramuscular artesunate (aqueous solution) at a dose of 250 mg/kg/day was associated with no abnormalities in 11 of 20 mice, and only 4 deaths ($P \leq 0.0003$). Of the six mice with neurologic abnormalities, three recovered. At a dose of 100 mg/kg/day, 2 of the 20 mice receiving artesunate in oil died, compared with 2 of the 36 given aqueous artesunate (neither had preceding neurologic abnormalities); in our previous study of intramuscular oil alone, 1 of 18 died ($P > 0.2$).[8]

Oral administration. Oral artemether in aqueous suspension was considerably less neurotoxic than intramuscular administration of the same drug. At doses of 150 and 200 mg/kg, only one of 20 mice in each group had persistent neurologic deficits and none died. At a dose of 250 mg/kg, there were 5 deaths and at a dose of 300 mg/kg, there were nine deaths and more neurologic deficits (Table 2). The results with oral artesunate were not significantly different: 17 (28%) of 60 mice that received $\geq$ 200 mg/kg died. Peanut oil alone given orally was not associated with any abnormalities.

Oral artemether in oil was more toxic than in aqueous solution. At a dose in oil of 200 mg/kg/day, 12 (27%) of 45

TABLE 2
Oral treatment groups, clinical findings, and outcome

Group	Treatment	No. of mice	No. of mice with abnormalities of balance and gait			No. of mice with normal balance and gait*	Deaths
			Reversible	Persistent	Total		
1	Artemether in oil (100 mg/kg for 28 days)	20	1	2	3	17	0
2	Artemether in oil (150 mg/kg for 28 days)	20	2	3	5	15	0
3	Artemether in oil (200 mg/kg for 28 days)	45	6	4	10	23	12 (9 + 3*)
4	Artemether in powder (100 mg/kg for 28 days)	20	1	0	1	19	0
5	Artemether in powder (150 mg/kg for 28 days)	20	0	1	1	19	0
6	Artemether in powder (200 mg/kg for 28 days)	20	0	1	1	19	0
7	Artemether in powder (250 mg/kg for 28 days)	20	0	3	3	12	5
8	Artemether in powder (300 mg/kg for 28 days)	20	0	2	2	9	9 (7 + 2*)
9	Artemether-coated pellet (100 mg/kg for 28 days)	20	1	3	4	16	0
10	Artemether-coated pellet (150 mg/kg for 28 days)	20	9	3	12	8	0
11	Artemether-coated pellet (200 mg/kg for 28 days)	25	10	10	20	5	0
12	Peanut oil–coated pellets for 28 days	10	0	0	0	10	0
13	Artesunate in water (100 mg/kg for 28 days)	20	0	0	0	20	0
14	Artesunate (200 mg/kg for 28 days)	20	1	0	1	19	3
15	Artesunate (250 mg/kg for 28 days)	20	0	1	1	12	7 (6 + 1*)
16	Artesunate (300 mg/kg for 28 days)	20	0	5	5	8	7 (5 + 2*)
17	Peanut oil by gavage for 28 days	10	0	0	0	10	0

* Death with preceding abnormal equilibrium.

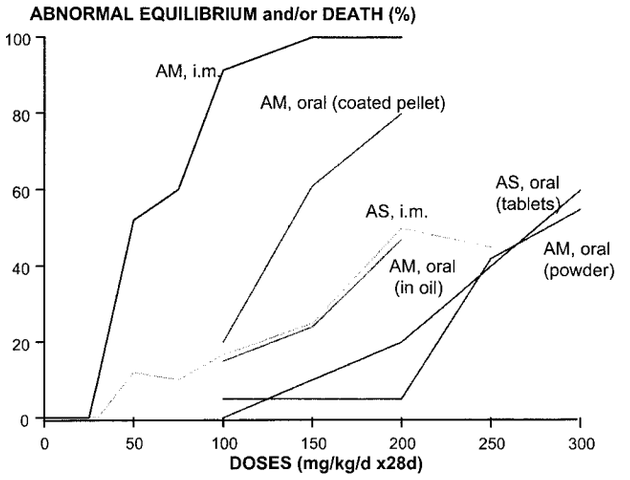

FIGURE 1. Proportion of mice (%) receiving artesunate (AS) and artemether (AM) daily for 28 days that survived and remained neurologically normal. Data from the earlier study[8] with parenteral doses of artesunate ≤ 100 mg/kg/day are also included. i.m. = intramuscular; d = day.

mice died compared with none of 20 mice receiving the aqueous suspension ($P = 0.03$). Since only 3 of the 12 deaths were associated with preceding neurotoxicity, some of these deaths may be attributed to a different etiology than in the 35 (with preceding neurotoxicity) of 40 deaths resulting from parenteral administration of artemether ($P < 0.0001$). Even if these sudden deaths are excluded, there was still significantly more neurotoxicity with oral artemether in oil compared with the aqueous solution. However, when artemether–coated food pellets were given, there was a further significant increase in neurotoxicity compared with once-a-day oral administration either in aqueous suspension ($P < 0.0001$) or in oil ($P = 0.017$), although there were no deaths (Figure 1). At a dose of 200 mg/kg/day in coated food pellets, 20 of 25 mice had evidence of neurologic deficits that were permanent in 10 (50%). At this dose, the median (range) onset of abnormal equilibrium was 24 (13–44) days after starting treatment compared with 9 days when the same dose was given by intramuscular injection ($P < 0.001$). Again the peanut oil vehicle, given continuously with food, was not associated with any abnormalities.

DISCUSSION

In clinical practice, the artemisinin derivatives are usually administered once a day, either parentally for severe malaria or orally for uncomplicated malaria. Artemether and artesunate, the most widely used drugs of this class, are used for both indications. Courses of treatment seldom exceed seven days, and in many cases are no more than three days (particularly if used in combination with other antimalarial drugs). No evidence of neurotoxicity has emerged in extensive clinical studies of the artemisinin derivatives, which have included detailed neurologic testing, and specific evaluation of auditory-evoked potentials.[10] This extensive clinical experience has given rise to increased confidence in the safety of this important class of antimalarial drugs.

The results of this laboratory study in a murine model of

artemisinin neurotoxicity indicate that there are significant differences in the neurotoxic potential of these drugs depending on the route of administration. Intramuscular artemether dissolved in peanut oil is considerably more neurotoxic than the same drug given orally or as a water-soluble analogue given parenterally. At a dose of 150 mg/kg/day, intramuscular artemether was uniformly lethal, whereas the same dose of drug given by aqueous suspension orally induced neurologic abnormalities in only 1 of 20 mice tested and no deaths resulted. Oral artemether was given once a day in a formulation that was comparable with the current method of administration in the treatment of falciparum malaria. There are no data on the pharmacokinetic properties of these drugs in mice, but in dogs and humans oral administration is associated with greater bioavailability than the intramuscular oil-based injections.[11] However, the injections result in considerably prolonged absorption and thus long-lasting blood concentration profiles.[11,12] With daily administration this would result in continuous central nervous system exposure compared with intermittent exposure (albeit at higher blood levels) with oral administration or with parenteral artesunate. Following oral administration of aqueous solutions in this study, the incidence of clinically evident neurotoxicity exceeded 5% only at doses of 250 mg/kg/day; a total dose of 7 g/kg. Without allometric correction this is more than 500 times greater than the usual total antimalarial dose in humans, and suggests a considerable margin of safety for the oral drugs. In aqueous suspension, oral artemether was no more neurotoxic than oral artesunate. However, when oral artemether was given in peanut oil, there was significantly greater neurotoxicity. This suggests that the oral absorption, and perhaps disposition, of artemether is different when presented in oil. To test the hypothesis that neurotoxicity resulted from sustained blood concentrations and thus sustained central nervous system exposure, the oil-based formulation was coated on food pellets, which the mice ate frequently. Neurotoxicity increased significantly compared with once-a-day oral administration of the same oil formulation with $>$ 50% of the mice receiving 150 mg/kg/day having neurologic abnormalities. These data suggest that continuous high level exposure of the central nervous system to artemether, or the metabolite dihydroartemesinin, causes neuronal damage.

The therapeutic ratio is considerably greater for intermittent once-a-day administration of rapidly absorbed oral or parenteral drugs. The original observations of neurotoxicity with intramuscular arteether and artemether have highlighted an important area for study with this class of drugs. It appears that antimalarial and neurotoxic potential cannot be dissociated suggesting a common mode of action. However, whereas neurotoxicity requires sustained exposure of the brain to these drugs, the parasiticidal effect is obtained with brief exposure. Thus pharmacokinetics rather than pharmacodynamics determine the neurotoxic potential of the artemisinin derivatives and this is exploited in oral antimalarial treatment. Current once-a-day treatment regimens minimize the potential for neurotoxicity. However, the therapeutic ratio with continuous use, either if these drugs were used in prophylaxis or incorporated in herbal tonics, may be much narrower.

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