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ORIGINAL PAPER

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Selection and reversal of *Plasmodium berghei* resistance in the mouse model following repeated high doses of artemether

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Abstract Artemether, a derivative of artemisinin, is effectively used for the treatment of malaria without any clinically relevant resistance to date. Artemether has also been developed as an antischistosomal agent, exhibiting highest activity against immature parasites. Here, we employ a rodent model and investigate whether the proposed artemether treatment schedule to prevent schistosome-attributable morbidity might select for *Plasmodium berghei* resistance. Mice infected with an ANKA strain of *P. berghei* were treated with artemether at either 47 mg/kg or 300 mg/kg. Once every 7–10 days, parasitized erythrocytes were passed to the next group of mice, receiving the same doses of artemether, for 50 passages. Resistance development was slow but increased considerably over the final ten passages. At the higher dose of artemether, the indices of resistance were 4.8 and 8.8 after 40 and 50 passages, respectively. Importantly, resistance was unstable, since sensitivity reverted to near-normal after five passages without drug pressure. A moderate index of *P. berghei* resistance and no apparent reversibility was found in comparative experiments employing pyronaridine. In conclusion, the pace of resistance development in *P. berghei* to repeated high doses of artemether is slow and reversible.

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Introduction

Malaria is the most important parasitic disease in the world, causing >300 million clinical attacks and >1 million deaths annually; and it disproportionately affects children under 5 years of age in sub-Saharan Africa (Breman 2001). Early diagnosis and prompt treatment has become the backbone of control. For several decades, chloroquine was the drug of choice, due to its high efficacy, good tolerability, ease of administration, low cost, and the slow pace at which resistance developed (Wellems and Plowe 2001; Ridley 2002). However, high levels of chloroquine resistance are now common throughout Africa, prompting more than ten countries to replace it with sulfadoxine-pyrimethamine (SP) as the first-line antimalarial drug (Wongsrichanalai et al. 2002). Unfortunately, resistance to SP soon developed, with efficacy lasting <5 years in many settings (Hastings et al. 2002a; Wongsrichanalai et al. 2002). Epidemiologists argue that the rapid spread of drug-resistant strains of *Plasmodium falciparum* is the primary obstacle to malaria control and that, without new drugs, the disease burden might double over the next 20 years (Breman 2001; Nosten and Brasseur 2002).

Against this background, the development of artemisinin and several semisynthetic derivatives (arteether, artemether, artesunate, their active metabolite dihydroartemisinin) as antimalarial drugs was of great importance (Klayman 1985; Li and Wu 2003). The evidence base is now compelling that artemisinins are safe and can clear malaria-related symptoms and parasitemia more promptly than any other antimalarial drug; and there is no report of clinically relevant resistance thus far (for a recent review, see Meshnick 2002). However, in mice infected with *P. berghei* and repeatedly treated either with artemisinin, artemether or artesunate at increasing doses, resistant malaria parasites could be

established (Li et al. 1986; Liu and Ren 1987; Cheng et al. 1988; Chen et al. 2002). Importantly, these phenotypes were unstable, since resistance indices reverted to normal values after the removal of drug pressure.

More recently, artemether also became recognized as an antischistosomal drug. It displays potent activity against the immature stages of schistosomes and primarily targets the worm tegument (Utzinger et al. 2001; Xiao et al. 2002a). The effects of artemether on biochemical metabolism (Xiao et al. 2000) and antioxidant systems (Xiao et al. 2002c) have been assessed, its possible long-term toxicity has been investigated (Xiao et al. 2002b), and progress has been made to elucidate its possible mechanism of action against schistosomes (Xiao et al. 2001, 2003). Laboratory studies and preliminary clinical trials have demonstrated that combination chemotherapy with praziquantel and artemisinins is beneficial over praziquantel monotherapy (Utzinger et al. 2003b). Consequently, artemether exhibits potential within an integrated schistosomiasis control approach (Utzinger et al. 2001, 2003a; Xiao et al. 2002a; N'Goran et al. 2003).

The purpose of this paper is to investigate whether the proposed artemether treatment schedule to prevent schistosome-attributable morbidity might select for resistant *P. berghei* in the mouse model. For comparison, the antimalarial drug pyronaridine (Dutta et al. 2000) was also tested.

Materials and methods

Mice, parasites, and drugs

Male and female mice of a Kunming strain, weighing 20 ± 2 g and purchased from the Shanghai Animal Center of the Chinese Academy of Sciences (Shanghai, China), were used throughout. Mice were fed commercial rodent food and water ad libitum in the animal care facility of the Institute of Parasitic Diseases (IPD), Chinese Center for Disease Control and Prevention (Shanghai, China).

An ANKA strain of *P. berghei*, maintained at IPD over the past 47 years, was employed. Erythrocytes parasitized with *P. berghei* were collected from donor mice and, according to the level of parasitemia, blood was diluted with physiological saline to reach approximately 10^7 *P. berghei*-parasitized donor erythrocytes per 0.2 ml.

Artemether was obtained from the Kunming Pharmaceutical Corp. (lot 97080; Kunming, China). The drug was suspended in 1% tragacanth at final concentrations of either 4.7 g/l or 30 g/l. The Department of Pharmaceutical Chemistry at IPD synthesized pyronaridine phosphate, which was dissolved in distilled water. It was used at a concentration of 0.47 g/l, calculated by pyronaridine free bases.

Development of resistance

Fifteen mice were intraperitoneally infected in random order, each with approximately 10^7 *P. berghei*-parasitized erythrocytes. The day of infection was designated D_0. On D_3, the parasitemia of each infected mouse was assessed and, when it reached 3–5%, three groups of five mice were formed. Each mouse in the first group was treated orally with artemether at a dose of 47 mg/kg (corresponding to about 50% CD_{50} for *P. berghei* in mice). The second group of mice was each given artemether at a 6.4-fold higher dose (300 mg/kg). Doses of 300–400 mg/kg have previously been used for the prevention of patent schistosome infections in the murine model (Utzinger et al. 2001); and a dose of 300 mg/kg corresponds to about $3\times$ CD_{50} for *P. berghei* in mice. The third group of mice received oral pyronaridine at a dose of 4.7 mg/kg ($2\times$ CD_{50}). Blood was examined on D_6, D_8, D_{10}, D_{13}, D_{15}, D_{17}, etc. When the parasitemia reached 3–5% in any of the groups, mice were selected for blood donation for subsequent passages. At each passage, the same dose of the drugs was given as on D_3. In all three groups, *P. berghei* were passed for a total of 50 passages.

Determination of resistant *P. berghei*

The sensitivity of *P. berghei* to two different dose levels of artemether and pyronaridine was determined with a standard 4-day test (Peters et al. 1975). After every fifth to tenth passage, groups of ten mice were infected with 10^7 *P. berghei*-parasitized donor erythrocytes. Infected mice were then treated daily for four consecutive days, commencing 3 h post-infection with either artemether (at two different doses) or pyronaridine. Parasitemia was assessed by Giemsa-stained thin smears prepared from tail blood after the final dose. The reduction in mean parasite counts in any of the treatment groups was calculated as a percentage of those quantified in the untreated control groups. The 90% effective level (ED_{90}) was calculated using a linear regression method and an index of resistance (I_{90}) was calculated from the ratio of ED_{90} of the resistance line to that of the parent line (for an example, see Peters et al. 1975). The I_{90} values were grouped into four categories, according to Merkli and Richle (1980): (1) $I_{90} = 1.0$, sensitive, (2) $I_{90} = 1.01$–10.0, slight resistance, (3) $I_{90} = 10.01$–100.0, moderate resistance, and (4) $I_{90} > 100.0$, high resistance.

Results

Intervals among passages

When mice infected with *P. berghei* and treated orally with artemether at the lower dose of 47 mg/kg at 3 days post-infection, the parasitemia reached 3–5% in some of the mice within 3 days. Blood collection for obtaining parasitized erythrocytes was therefore carried out on D_7 and employed for subsequent infections. Following each passage, artemether was administered at the same dose as at 3 days post-infection. Throughout the experiments, the same interval of 7 days was adhered to between subsequent passages, up to the final 50th passage. The total duration of this series of experiments was 16 months.

Infected mice that received oral artemether at the higher dose of 300 mg/kg displayed parasitemia levels of 3–5% at D_8. The intervals between subsequent passes of *P. berghei*-parasitized erythrocytes to mice was once every 10 days throughout the experiments, up to the final passage. Consequently, this series of experiments took 24 months.

Those mice that were infected with *P. berghei* and received an initial oral dose of 4.7 mg/kg pyronaridine reached a parasitemia of 3–5% only at D_{18}. Parasitized erythrocytes were therefore passed once every 20 days. However, following the third passage, the same level of

parasitemia was reached after a considerably shorter interval, namely 12 days. Consequently, *P. berghei* parasites were passed once every 14 days, up to the eighth passage. Intervals between subsequent passages were further shortened to 10 days, up to the 18th passage, and to 7 days until the end of the experiments (50th passage). Overall, this series of experiments lasted for 22 months.

Development of resistance to the drugs

Table 1 summarizes the ED_{90} and I_{90} values measured in *P. berghei*-infected mice that were repeatedly treated with either artemether or pyronaridine. Both at the lower and at the higher doses of artemether, the development of resistance by the rodent parasite *P. berghei* to this drug was slow, since only slight resistance levels of 2.4–2.5 were quantified after 20–25 passages. The I_{90} gradually increased following the next 20 passages, to reach levels of 4.5–4.8 after the 40th or 45th passage. After completion of the 50th passage, the indices of resistance had increased to 7.1 at the lower (47 mg/kg) and 8.8 at the higher (300 mg/kg) dose of artemether. These values still indicate a low level of resistance. Moreover, the resistance phenotype was unstable, since the sensitivities of the malaria parasites to artemether reverted significantly when the parasites derived from the 50th passage were transferred through mice without drug pressure for five passages. The lower dose of artemether showed an I_{90} value of 1.6, while the corresponding I_{90} at the higher dose of artemether was 2.2. At the higher dose of artemether, an additional experiment was carried out by passing *P. berghei* parasites derived from the 30th passage through mice without drug pressure for five passages. This resulted in an I_{90} of 1.1.

The development of resistance in the *P. berghei* parasite to pyronaridine, repeatedly administered at a dose of 4.7 mg/kg, was considerably more rapid and more pronounced when compared with artemether. After the 15th passage, the malaria parasites showed an I_{90} of 5.4, which was higher than the I_{90} values obtained after 40–45 passages of artemether, even when administered at a high dose. When pyronaridine was employed for 25 sequential passages, a moderate index of resistance of 16.6 was found. This I_{90} did not increase further following subsequent passages, but fluctuated between 13.3 and 16.8 between the 35th and 50th passages. However, and in sharp contrast to artemether, the sensitivity of *P. berghei* parasites derived from the 50th passage exhibited no apparent recovery to pyronaridine after they were transferred through mice without drug pressure for five passages.

Table 1 Development and reversal of resistance to artemether (repeatedly administered at either low or high doses) and pyronaridine by an ANKA strain of *Plasmodium berghei* in mice (I_{90} = ED_{90}resistance strain/ED_{90} parent strain; see Materials and methods). Malaria parasites derived from the 30th passage were transferred through mice without drug pressure for five passages; and the sensitivity of the parasites to artemether increased significantly, with I_{90} (3.6/3.3) = 1.1.*n.d.* Not determined

Number of passages	Artemether				Pyronaridine	
	47 mg/kg		300 mg/kg		4.7 mg/kg	
	ED_{90}	I_{90}	ED_{90}	I_{90}	ED_{90}	I_{90}
Parental line	3.3	–	3.3	–	0.8	–
5	4.0	1.2	n.d.	n.d.	0.8	1.0
10	n.d.	n.d.	8.1	2.5	n.d.	n.d.
15	5.8	1.8	n.d.	n.d.	4.4	5.4
20	n.d.	n.d.	8.1	2.5	n.d.	n.d.
25	7.7	2.4	n.d.	n.d.	13.6	16.6
30	n.d.	n.d.	13.8	4.2	n.d.	n.d.
35	8.1	2.5	n.d.	n.d.	13.8	16.8
40	n.d.	n.d.	16.0	4.8	n.d.	n.d.
45	14.8	4.5	n.d.	n.d.	12.3	15.0
50	23.3	7.1	29.1	8.8	10.9	13.3
After removal of drug pressure						
5	5.3	1.6	7.1	2.2	10.0	12.1

Discussion

In view of widespread *P. falciparum* resistance to chloroquine, the rapidly increasing extent of resistance to other antimalarials, and a growing number of epidemiological settings with multi-drug resistant falciparum malaria, artemisinin and its derivatives have become critically important for the treatment and control of this disease. There are at least four features of the artemisinins worth discussing. First, no cross-resistance between artemisinins and other artimalarial drugs has been observed, with the possible exception of mefloquine (Noedl et al. 2001). Second, owing to their very short elimination half-lives, the chances of developing resistance appear to be low (Hastings et al. 2002b). Indeed, there have been no reports to date of clinically relevant resistance among this group of antimalarials, although these drugs have been used widely, particularly in Southeast Asia (White 1999; Price 2000; Walker et al. 2000; Haynes 2001; Hyde 2002; Meshnick 2002; Ittarat et al. 2003). Third, there is high recrudescence following a single dose. Therefore, treatment courses of at least three consecutive days are required when artemisinins are used alone, or more appropriately in combination with other antimalarials (Hastings et al. 2002b; Ridley 2002; Ittarat et al. 2003; White and Pongtavornpinyo 2003). Fourth, there is limited evidence from animal models that sustained high parenteral doses of certain artemisinin derivatives can result in some unique and selective brain stem neurotoxicity (Dayan 1998; Genovese et al. 2000; Li et al. 2002). Fortunately, there is no evidence for similar reactions in humans.

While the short half-lives of the artemisinins necessitate repeated drug administration or combination chemotherapy, it is probably the most crucial pharmacokinetic feature for delaying the development of resistance (White 1999; Hastings et al. 2002b; White and Pongtavornpinyo 2003). However, it is important

to note that resistant lines of *P. berghei* have been generated in the mouse model. For example, the administration of artemisinin at stepwise-increasing doses to mice infected with an ANKA strain of *P. berghei* revealed an I_{50} of 53.4 after 58 passages (Li et al. 1986). In another study, an I_{50} of 18.4 was found after 60 passages (Chen et al. 2002). Similarly, lines of *P. berghei* have been established resistant to artemether with an I_{90} of 16 after 70 passages (Cheng et al. 1988) and to artesunate with an I_{90} of 29.3 after 21 passages (Liu and Ren 1987). Importantly, these resistant phenotypes were unstable, since the sensitivities progressed toward normal values after several passages without drug pressure. Recently, a resistant strain of *P. yoelii* was also obtained in the murine model (Peters and Robinson 1999). Resistance was unstable and appeared to be influenced by multiple factors, e.g. the accumulation of significantly less drug than in sensitive parasites and an alteration in the translationally controlled tumor protein homologue, which is a possible drug target (Walker et al. 2000).

The experimental work presented here focused on artemether and exhibits two unique aspects over previous studies. First, instead of stepwise increases in the dose of artemether, the drug was administered at the same dose throughout. Second, there were constant intervals of 7 days or 10 days between subsequent treatments. Adhering to this protocol and administering artemether at two different doses resulted in slight indices of resistance after completion of the 50th passage, namely 7.1–8.8. This is considerably lower than the I_{90} of 16, which was found previously by another group of researchers after 70 passages (Cheng et al. 1988). Comparing these two studies reveals that the pace of resistance development was slower when artemether was administered at the same dose throughout, as opposed to stepwise increases. Reassuringly, resistance development appears to be unstable and reversible, since the sensitivities at the two different artemether concentrations approached normal levels after only five passages after the removal of drug pressure. Therefore, different ways of exposing rodent malaria parasites to artemether seem to select a common phenotype of resistance that is reversible. These observations are consistent with unstable artemisinin resistance in *P. yoelii* and the causes are likely to be multifactorial.

Finally, although it is speculative to extrapolate resistance mechanisms across species and hosts, our results might be of practical importance. Our experimental design mirrored the proposed treatment schedule of artemether for the prevention of patent schistosome infections in humans. Namely, repeated administration once every 2–4 weeks during transmission periods (Utzinger et al. 2001; Xiao et al. 2002a; N'Goran et al. 2003). In view of the possibility to select artemisinin-resistant *P. falciparum*, at least in culture (Inselburg 1985), for the time being artemisinins should not be recommended against schistosomiasis in areas where malaria co-exists. However, as demonstrated here and confirming previous results, the risk of resistance development in the malarial parasite appears to be low.

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