Efficacy comparison of intravenous artelinate and artesunate in *Plasmodium berghei*-infected Sprague-Dawley rats

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SUMMARY

This paper reports the comparative antimalarial efficacy of intravenous artelinate and artesunate in rats. Prior to efficacy experiments, a *Plasmodium berghei*–Sprague-Dawley rat model of malaria was developed, in which the clearance effects of intravenous drugs could be readily compared. In efficacy experiments, groups of *P. berghei*-infected rats were given 3 daily intravenous treatments of artelinate or artesunate at molar equivalent dose rates (total of 0–191·2 μmoles/kg). Artelinate was superior to artesunate in terms of clearance (100% clearance dose of 95·6 μmoles/kg (40 mg/kg) versus 191·2 μmoles/kg for AS (73·4 mg/kg)) and parasite clearance time (1·7 $\pm$ 0·5 days for AL versus 2·7 $\pm$ 0·5 days for AS at a dose rate of 191·2 μmoles/kg, $P < 0{\cdot}01$). No frank clinical toxicity was observed, though both artesunate and artelinate induced dose-related vascular necrosis at the site of injection. The necrosis was less severe and reversible when the drugs were administered via femoral, rather than tail/foot veins. The data suggest that the *P. berghei*–7-week-old Sprague-Dawley rat model of malaria is reproducible and useful for assessing the efficacy of antimalarials and that artelinate is at least as potent, and safe, as artesunate, the leading clinical treatment for severe malaria.

Key words: artesunate, artelinate, antimalarials, *Plasmodium berghei*, rat malaria model.

INTRODUCTION

Efficacy studies from China and elsewhere have unambiguously demonstrated the activity of artemisinin derivatives against various *Plasmodium* species *in vitro* and *in vivo* (Chinese Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, 1982*a*, *b*; Jiang, Li & Guo, 1982; Li, Arnold & Guo, 1984; Li, 1990). Indeed, artemether, arteether (AE) and artesunate (AS), due to their rapidity of action, are amongst the most useful treatments for severe, drug-resistant falciparum malaria, and are in widespread use around the world (Warrell, 1999). Whilst AS has been registered in Europe and elsewhere, this is not the case in the United States. The US Army, through the Walter Reed Army Institute of Research, is currently developing either AS or another derivative, artelinate (AL), as potential treatments for severe malaria and eventual FDA registration. Selection of one of these compounds will be based on the results of parallel toxicity and efficacy studies currently being conducted in rats and Rhesus monkeys.

AS, given intravenously, is considered by many clinicians to be the most effective treatment for severe malaria and thus represents the 'gold standard' against which any new candidate artemisinin derivative should be compared (de Vries & Dien, 1996). AL is generally considered to be less potent *in vitro* against *Plasmodium falciparum* than either AS, or its metabolite dihydroartemisinin, but has comparable or superior efficacy against *P. berghei* in mice (Lin, Klayman & Milhous, 1987; Shmuklarsky *et al*. 1993; Bustos, Guy & Diquet, 1994; Milhous *et al*. 1996). This may be because AL has greater stability, achieves higher plasma concentrations, has longer plasma residence times and is more easily transferred across red cell membranes (Lin *et al*. 1987; Li *et al*. 1998*a*, *b*; Grace, Skanchy & Aguilar, 1999). The gastrointestinal and neuro-toxicity of AL and other artemisinins have been extensively documented in laboratory animals, particularly rats (Li *et al*. 1999; Petras *et al*. 2000; Li & Peggins, 2001). However, the *in vivo* efficacy and toxicity of AL and AS have never been systematically compared in the same species. Thus, the comparative therapeutic indices of the two compounds are unknown.

As the first part of a 2-phase efficacy/toxicity study, we investigated the efficacy of AS and AL in *P. berghei*-infected, 7-week-old Sprague-Dawley (SD) rats. This required the development of an appropriate malaria model. Mouse malaria models are the normal choice for antimalarial drug screening (refer to discussion by Dow, Reynoldson & Thompson, 1998, 1999). However, weanling rats are also used, as they are also susceptible to experimental infection by *P. berghei* (Smalley, 1975; Bhatia & Vinayak, 1984; Dow *et al*. 1999). However, as it is our eventual goal to conduct parallel pharmacokinetic experiments, we

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required animals at least 7 weeks in age (around 200 g). Larger animals are required in pharmacokinetic studies as they allow (i) the taking of larger blood samples thereby improving HPLC assay sensitivity and (ii) serial blood sampling. However, older animals are rarely used in efficacy experiments as they are less susceptible to infection, and the course of infection observed may be more variable (Smalley, 1975; Bhatia & Vinayak, 1984; Dow *et al*. 1998, 1999). Also, we anticipate that the clinical role of i.v. AS or AL would be as a treatment to rapidly clear parasitaemia prior to administration of oral medications. Therefore, it was necessary that the malaria model developed was one in which the parasite clearance effects of AL and AS could be directly compared, a condition not normally required when rat or mouse malaria models are used for the routine screening of antimalarials.

Consequently, the objectives of this study were to optimize a malaria model utilizing older rats and to use it to compare the antimalarial efficacy of intravenous formulations of AL and AS.

MATERIALS AND METHODS

Parasite

P. berghei ANKA was maintained via weekly passage in mice or cryopreserved in liquid nitrogen. Details of the procedures used are outlined below.

Animals

Six-week-old male ICR mice (18–22 g) were used for passage of *P. berghei*. Four and 7-week-old male Sprague-Dawley rats (91–124 and 174–269 g respectively) were used for model optimization experiments. Seven-week-old male Sprague-Dawley (SD) rats were used for efficacy experiments. Four-week-old male SD rats were used as donor animals or for rat–rat passages. All animals were purchased from Charles River Laboratories, and quarantined (stabilization) for at least 7 days prior to infection. Food and water were provided *ad libitum*. All animal usage, care and handling conformed to 'Guide for the Care and Use of Laboratory Animals' (NIH, Publication No. 86-23, revised 1996). All procedures were reviewed and approved by the Institute's Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Chemicals

Artelinate, 4-(10′ dihydro-artemisinin-oxymethyl) benzoic acid hemihydrate (artelinate, WR255663), was manufactured as an L-lysine salt and obtained from the Walter Reed Chemical Inventory System. Artesunic acid and 5% sodium bicarbonate solution were obtained from Guilin Pharmaceutical II Factory Guangxi, China, and imported by Atlantic Pharmaceutical Co., Ltd, Bangkok, Thailand. L-lysine monohydrochloride was obtained from Sigma Chemical Co., 0·9% saline was purchased from Abbott Labs (Vhicage, IL) and pentobarbital, sodium citrate, heparin, D-glucose, glycerol and methanol were purchased from Sigma Chemical Co. Hema 3 stain was obtained from Fisher Scientific Co.

Cryopreservation and revival of P. berghei

When necessary, *P. berghei* was cryopreserved by mixing 5–6 drops of parasitized mouse or rat tail blood with 150 μl of Alsevier's solution (0·47% w/v NaCl, 2·1% w/v D-glucose in 8·7% glycerol) and snap-freezing the mixture in cyropreservation vials (Nunc) in liquid nitrogen. Blood was obtained from mice or rats with rising parasitaemias of 3·5–13 and 9–30% respectively, which had been infected with *P. berghei* 3–5 or 7 days earlier. When necessary, vials were thawed by immersion in water at room temperature for 5–10 min, and their contents injected i.p. into individual rats or mice using 27-gauge needles.

Serial passage of P. berghei *in mice and inoculation of donor rats*

Routine maintenance of *P. berghei* was conducted by serial passage through mice. Samples of 5–6 drops of parasitized blood were obtained from the tail vein of a mouse with a rising parasitaemia of 9–30%, which had been inoculated 3–5 days earlier with *P. berghei*. The blood was diluted in 0·3 ml of glucose citrate (GC) solution (3·0% w/v sodium citrate in 0·5% w/v D-glucose) and injected i.p. into mice using a 27-gauge needle. An identical procedure was used to infect donor rats with *P. berghei* prior to model optimization or efficacy experiments, except that the injection volume was 0·5 ml. For the multiple passage experiment, this method was also used for rat–rat passage. The rats from which the parasitized blood was obtained had rising parasitaemias of 3·5–13%.

Infection protocol for model optimization and efficacy experiments

In all model optimization experiments, donor rats were inoculated with *P. berghei* from various sources, as described above, 1 week prior to the experiment. The donor animals were anaesthetized using 5% isoflurane in oxygen. Anaesthesia was maintained using 2·5% isoflurane in oxygen. Blood was collected by cardiac puncture into 10 ml syringes containing 0·1 ml of 200 U/ml porcine heparin in normal saline. The needle was removed (to prevent shearing of red cells) and the heparinized blood was added to 5·0 ml of pre-warmed GC solution and incubated in a 37 °C

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water-bath for 10 min. During this incubation period the parasite concentration (parasitized red cells/ml) was calculated using the percentage parasitaemia of the donor rat (determined previously) and the red cell count (determined using a haemacytometer). The parasitized blood was then diluted to the desired concentration with pre-warmed GC. Rats were then injected i.p. with 0·5 ml of diluted parasites.

Model optimization Exp. 1: effect of multiple passage and increased inoculums in 7-week-old rats

Groups of 5–9, seven-week-old male Sprague-Dawley rats were infected, as described above, with differing amounts of *P. berghei*-infected rat red blood cells from different sources. Rats in groups A, B and C were inoculated with $2{\cdot}0 \times 10^6$, $6{\cdot}7 \times 10^6$ and $2{\cdot}0 \times 10^7$ parasitized rat erythrocytes respectively. For these groups, *P. berghei* was passaged for 1 week in mice prior to inoculation of the donor rats. Rats in group D were inoculated with $2{\cdot}0 \times 10^7$ parasitized erythrocytes. However, for this group, *P. berghei* was passaged once through mice, and twice through rats prior to infection of the donor rats. Prior to infection of the Group D animals, individual 150 μl aliquots of parasitized blood were cryopreserved for later reinitiation of *P. berghei* infection in model optimization Exp. 2 and the efficacy experiments.

Model optimization Exp. 2: reproducibility and comparison of infection kinetics in 4 and 7-week-old rats

In the second model optimization experiment, 3 groups of 5–7, seven-week-old SD rats were infected with 2×10^7 *P. berghei*-parasitized erythrocytes. The parasitized erythrocytes were obtained from a donor rat infected 1 week earlier. The donor rats were infected using individual thawed lots of cryopreserved, parasitized blood collected from group D donor rats in the first model optimization experiment. The 3 groups of animals were infected with *P. berghei* on 3 separate occasions. The experiment was repeated in groups of 5, four-week-old, male SD rats; however, these rats were infected with $1{\cdot}2 \times 10^6$ parasitized erythrocytes due to their smaller size, as described elsewhere (Smalley, 1975). The second part of the experiment was conducted to compare *P. berghei* infection kinetics in 4 and 7-week-old rats under similar conditions.

Parameters used for assessment of severity and reproducibility of experimental P. berghei *infection*

The effects of multiple passage, age and inoculum were measured on 6 parameters: (i) patency (ii) frequency of delayed infection, (iii) level of mortality, (iv) peak mean parasitaemia, (v) day of peak parasitaemia and (vi) mean coefficient of variation of parasitaemia. Patency refers to the percentage of rats with confirmed infections. The incidence of delayed infection refers to the percentage of infected rats in which parasitaemias were undetectable prior to day 6. Delayed infections could conceivably skew drug efficacy data, and these animals were not included in efficacy studies. For each day on which parasitaemias were determined, the mean parasitaemia and standard deviation were determined for each group. Each day's coefficient of variation of parasitaemia (CV) was calculated by dividing the mean parasitaemia by the standard deviation. Mean coefficients of variation were calculated by averaging each day's coefficients of variation for each treatment group from days 6 through 17, or as indicated.

Design of efficacy experiments

Groups of 5–9, randomized, 7-week-old rats were inoculated with 2×10^7 parasitized erythrocytes, (and found to be infected with *P. berghei*, i.e. no negative rats or delayed infections), were given intravenous (i.v.) AL or AS (drug vehicle, 6, 12, 23·9, 47·8 and 95·6 μmoles drug/kg, 10 ml of drug solution/kg) on days 6, 7 and 8 post-infection. For high AL and AS doses (191·2 μmoles/kg), 2·0 ml/kg of the appropriate drug dilution (at concentrations of 95·6 μmoles drug/ml) were administered i.v. The composition and preparation of the drug vehicles used are described below. The general experimental design is similar to previously published efficacy studies using weanling rats (Dow *et al*. 1999, 2000). For the lower dose rates (0–47·8 μmoles/kg), drugs were administered via foot and tail veins. After both AL and AS were found to induce necrosis via this route, the site of injection was switched to the femoral vein. The general health of the animals was monitored daily. Except where indicated, parasitaemias were determined daily on days 6 through 12 post-infection and every second or third day after that. The experiments were terminated on day 21 (as by this time surviving rats have cleared their infections).

The highest AL solution was prepared by dissolving an appropriate amount of AL-lysine salt into 0·2% L-lysine in 0·45% saline. Lower AL concentrations were prepared by performing 2-fold dilutions in 0·2% L-lysine in 0·45% saline. The two AL drug vehicles used as controls were 0·2% L-lysine in 0·45% saline (AL VC 1) and 3% L-lysine in 0·45% saline (AL VC2, containing a higher concentration of L-lysine to allow for the extra L-lysine complexed to AL in the highest dosage group). The highest AS solution was prepared by dissolving an appropriate amount of artesunic acid in 5% sodium bicarbonate. Lower AS concentrations were prepared by performing 2-fold dilutions in 0·9% saline. The two AS drug vehicles used as controls were 5% sodium bicarbonate and 0·9% saline (AS VC 1 and AS VC2).

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Table 1. Mean peak parasitaemia, day of peak parasitaemia, CV, patency, delayed infection and mortality data for *Plasmodium berghei*-infected rats in model optimization experiments 1 and 2, and for various mouse and rat malaria models described in the literature

(Citation abbreviated to first author and year. Inoculums are expressed as millions of parasitized erythrocytes/rat. S.D., Standard deviation; N.R., not reported; N.C., not calculated or calculable. Literature CV values estimated from available data. * Statistically different from Group A rats. ** Statistically different from 4-week-old rats.)

	Age (Weeks)	Patency (%)	Delayed infections (%)	Mean CV in parasitaemia (%±S.D.)	Peak parasitaemia (%±S.D.)	Day of peak parasitaemia (days±S.D.)	Mortality (%)
Present study (inoculum, sample size)							
Optimization experiment 1							
Group A (2·0, 9)	7	78	22	210±18	41±29	13±1·4	0
Group B (6·7, 6)	7	83	17	110±24*	31±29	14±1·0	17
Group C (20, 5)	7	100	20	76±23*	43±23	14±0·89	20
Group D (20, 5)	7	100	0	48±9*	32±16	11±1·3*	24
Optimization experiment 2							
Pooled 4-week-old rats (20, 15)	4	100	0	30±15	61±26	12±1·5	33
Pooled 7-week-old rats (20, 17)	7	100	5.9	44±19**	38±26**	12±1·7	24
Literature citation (inoculum, strain)							
Rats							
Bhatia 84 (10, Wistar)	4	N.R.	N.R.	N.C.	50	14	12
Smalley 75 (4, Sprague-Dawley)	4	N.R.	N.R.	N.C.	46	13	67
Dow 99 (2·0, LEW/SSN)	5	N.R.	N.R.	98	52	14	56
Holloway 95 (1·0, Wistar)	6–8	N.R.	N.R.	N.C.	45	15	95
Smalley 75 (8, Sprague-Dawley)	7	N.R.	N.R.	N.C.	3·2	5	4·5
Bhatia 84 (10, Wistar)	10	N.R.	N.R.	N.C.	20	10	<12
Bhatia 84 (10, Wistar)	12	N.R.	N.R.	N.C.	17	12	<12
Dow 98 (2·3, Wistar)	13	N.R.	N.R.	231	1·3	6	0
Bhatia 84 (10, Wistar)	18	N.R.	N.R.	N.C.	7·5	9	<12
Mice							
Dow 98	6	N.R.	N.R.	39	>23	>6	100

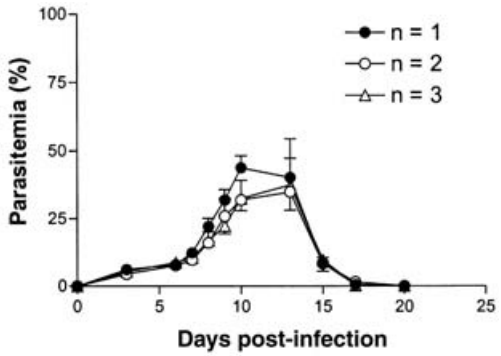

Fig. 1. Mean parasitaemia–time curves for 3 groups of 5 to 7, seven-week-old male Sprague-Dawley rats experimentally infected with *Plasmodium berghei* on independent occasions. Bars represent standard errors.

Determination of parasitaemia

Thin smears were prepared from tail blood, air dried, fixed with methanol, stained with Hema 3 according to the manufacturer's instructions, then examined under oil at 1000× magnification. One thousand red cells were examined, and parasitaemia was calculated using the following formula. Parasitaemia (%)=number of infected red blood cells/(number of infected red blood cells+number of uninfected red blood cells)×100. If the percentage parasitaemia was lower than 0·1%, ten thousand red cells were counted following the same procedures. Using this approach, a negative smear was one in which parasitaemia was <0·01%.

Assessment of drug efficacy

The antimalarial efficacy of AL and AS was assessed in terms of the 100% clearance dose and parasite clearance time. Clearance was defined as observance of 2 negative smears, 1 day apart, prior to day 11 in drug-treated rats. The 100% clearance dose (CD_{100}) was defined as 100% clearance in 100% of rats with no frank clinical toxicity. The parasite clearance time was defined as the time between initiation of i.v. drug treatment (day 6) and clearance. The recrudescence rate and time to recrudescence were used as secondary measures of efficacy. Recrudescence occurred if parasites were observed on thin smears from rats for which clearance was previously noted. Recrudescence time was the time between the first, positive, post-clearance smear and the initiation of i.v. drug treatment on day 6. As self-cure occurs in

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Table 2. Effects of AL on various clinical and parasitological endpoints in 7-week-old, male, *Plasmodium berghei*-infected Sprague-Dawley rats following daily intravenous treatment for 3 days

(AL was administered daily for 3 days. VC1 and VC2 refer to AL vehicle control groups 1 and 2. The recrudescence rate was calculated only for those treatment groups and specific animals in/for which drug-induced clearance was observed prior to day 11 post-infection. PCT, refers to parasite clearance time and was calculated only for those treatment groups where drug-induced clearance was observed prior to day 11 post-infection. S.D., Standard deviation.)

	Daily dose of AL (μmoles/kg)							
	VC1	VC2	6·0	12·0	23·9	47·8	95·6	191·2
Number of animals	6	5	5	6	6	9	6	6
Clearance (%)	0	0	0	0	0	67	100	100
Recrudescence rate (%)						100	100	100
PCT (days ± S.D.)						2·8 ± 0·8	2·2 ± 0·4	1·7 ± 0·5
Time to recrudescence (days ± S.D.)						4·8 ± 0·8	7·0 ± 0·6	8·2 ± 1·0
Mortality (%)	17	0	0	0	0	0	0	0

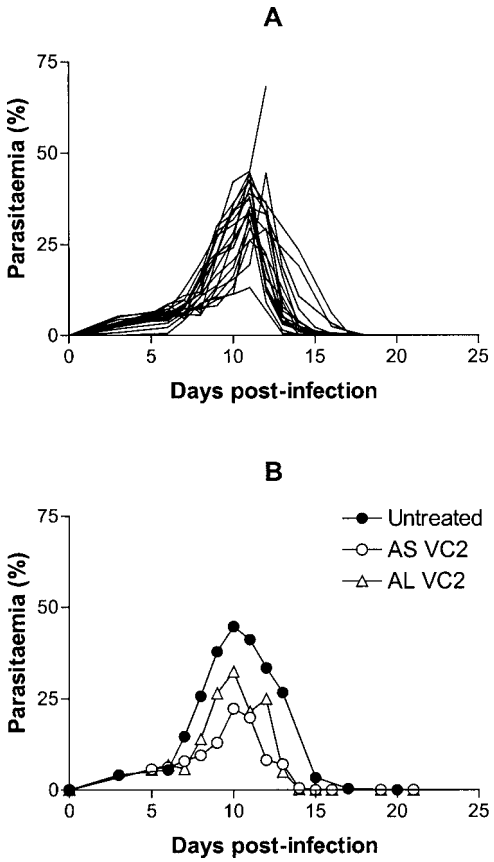

Fig. 2. (A) Individual parasitaemia–time curves for untreated 7-week-old, male, *Plasmodium berghei*-infected Sprague-Dawley rats and rats treated with AL or AS drug vehicles. In all these animals parasitaemias peaked on or after day 11 post-infection. (B) In 1 of 5 untreated, AS VC2 and AL VC2-treated rats, parasitaemias peaked on day 10, but declined only marginally or peaked again on days 11–12 post-infection.

most rats on or after day 13, drug-induced cure (i.e. complete clearance after drug treatment until termination of the experiment on day 21) was not a germane measurement of efficacy in the context of this study.

Statistical analyses

When comparing the parasite clearance times of equivalent AL and AS doses or mean CVs, parasitaemias and time to peak parasitaemias in 4 and 7-week-old rats, statistical significance was assessed using the Student's *t*-test. When comparing mean CVs, parasitaemias and time to peak parasitaemias across treatment groups in optimization Exp. 1, statistical significance was assessed using single factor ANOVA followed by Dunnett's test. *P* values $<0{\cdot}05$ were considered significant.

RESULTS

Model optimization Exp. 1

A 7-week-old male SD rat–*P. berghei* model of malaria was optimized by altering the inoculum and number of prior rat passages. Increasing the inoculum size resulted in a less variable course of infection as indicated by higher rates of patency and lower mean CVs (Table 1). Increased inoculum size also resulted in higher mortality, but had little effect on the number of delayed infections. Multiple passage of parasites prior to experimental infection resulted in the least variable course of infection, with the highest rates of patency and mortality and lowest mean CV and incidence of delayed infections (Table 1). Mean peak parasitaemias were not significantly altered when the inoculum size or number of prior passages were increased. However, the time to peak parasitaemias was significantly reduced when the number of prior passages was increased.

Model optimization Exp. 2

The results of the first model optimization experiment indicated that the most severe, and least variable, course of infection was observed when *P. berghei* was passaged multiple times through rats prior to an experiment. Therefore, *P. berghei*-parasitized blood

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Table 3. Effects of AS on various clinical and parasitological endpoints in 7-week-old, male, *Plasmodium berghei*-infected Sprague-Dawley rats following daily intravenous treatment for 3 days

(AS was administered daily for 3 days. VC1 and VC2 refer to AS vehicle control groups 1 and 2. The recrudescence rate was calculated only for those treatment groups and specific animals in/for which drug-induced clearance was observed prior to day 11 post-infection. PCT, refers to parasite clearance time and was calculated only for those treatment groups where drug-induced clearance was observed prior to day 11 post-infection.)

	Daily dose of AS (μmoles/kg)							
	VC1	VC2	6·0	12·0	23·9	47·8	95·6	191·2
Number of animals	4	5	4	6	6	6	6	6
Clearance (%)	0	0	0	0	0	67	67	100
Recrudescence rate (%)						100	100	100
PCT (days ± S.D.)						2·0 ± 0·0	2·5 ± 0·6	2·7 ± 0·5
Time to recrudescence (days ± S.D.)						5·0 ± 0·8	5·8 ± 1·3	8·2 ± 1·3
Mortality (%)	25	0	0	0	0	0	0	0

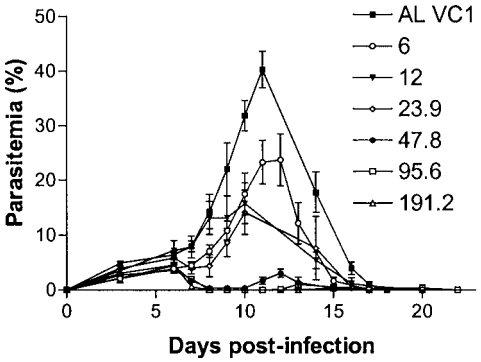

Fig. 3. Mean parasitaemia–time curves for 7-week-old, male, *Plasmodium berghei*-infected Sprague-Dawley rats treated with AL VC1 or 6–191·2 μmoles/kg AL daily on days 6, 7 and 8 post-infection. Note that data for the vehicle control group was collected from 2 groups of rats infected on different occasions, and for which parasitaemias were determined on different days. For clarity, the data from only 1 of these groups (containing 3 animals) is presented. Bars represent standard errors.

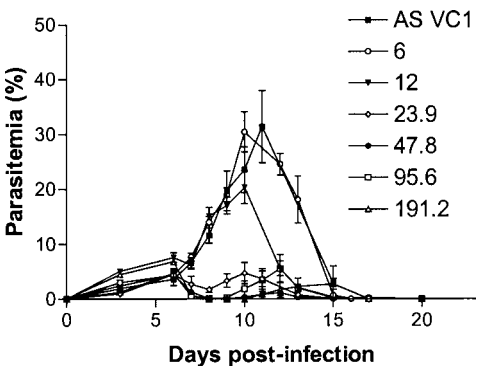

Fig. 4. Parasitaemia–time curves for 7-week-old, male, *Plasmodium berghei*-infected Sprague-Dawley rats treated with AS VC1 or 6–191·2 μmoles/kg AS daily on days 6, 7 and 8 post-infection. Bars represent standard errors.

was collected from the donor rats of group D and cryopreserved for later reinitiation of experimental infections in the second model optimization and efficacy experiments. The second model optimization experiment was conducted to determine whether reinitiation of experimental infection using cryopreserved parasites resulted in reproducible courses of parasitaemia in 4 and 7-week-old rats. The course of infection in 7-week-old rats was reproducible in 3 independently replicated experiments (Fig. 1). These results were pooled and compared with the corresponding data for the younger rats. Overall infection kinetics were similar, although mean parasitaemias were significantly higher and CV values were significantly lower, in the younger animals (Table 1). However, mean peak parasitaemias and coefficients of variation were within the range of those reported for mouse and rat malaria models used previously for efficacy experiments (Table 1).

Infection kinetics and proposed measures of efficacy

Parasitaemia-time plots for individual control, AS and AL-vehicle treated rats are presented in Fig. 2A. In 18 of 20 vehicle-dosed and 4 of 5 untreated animals, parasitaemias peaked on or after day 11. In the remaining 3 animals, parasitaemias peaked on day 10, but declined only marginally or peaked again on days 11–12 post-infection (Fig. 2B). However, all untreated and vehicle-dosed control rats self-cleared their infections on or after day 13 post-infection. Therefore in AL or AS-treated rats, any clearance observed prior to day 11 is clearly a consequence of a drug effect, rather than a host immune response.

Efficacy of AL and AS

In the efficacy experiments, the courses of infection in untreated rats or rats given the AL and AS drug vehicles were similar to those of the model optimization experiments. At a dose of 95·6 μmoles/kg AL, the CD_{100} was achieved, as all rats were cleared, and no obvious toxicity was observed (Table 2, Fig. 3). In contrast, the CD_{100} of AS was only achieved when the dose rate was increased to

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Table 4. Vascular necrotic effects of artelinate (AL) and artesunate (AS) at variable dose levels following daily i.v. administration for 3 days in *Plasmodium berghei*-infected 7-week-old male Sprague-Dawley rats (number of necrotic rats/number of test rats)

(Necrosis was reversible in all cases except for 1 afflicted rat treated with 47·6 μmoles/kg AL and 4 afflicted animals treated with 191·2 μmoles/kg AS via the tail vein route.)

Dosage (μmoles/kg)	23·9	47·8	95·6	191·2
AL (tail and foot veins)	3/6	6/9		
AL (femoral vein)			0/6	2/6
AS (tail and foot veins)	0/6	0/6	1/6	5/6
AS (femoral vein)				0/6

191·2 μmoles/kg (Table 3, Fig. 4). At this dose level the parasite clearance time of AL was also significantly shorter (1·7 and 2·7 days respectively, $P<0·05$, unpaired *t*-test, Tables 2 and 3). In all other respects measured, AL and AS were identical (Tables 2 and 3). The clearance data suggest that AL is approximately 2-fold more potent and is a more rapidly acting antimalarial than AS.

Toxicity of AL and AS

No animal treated with AL or AS at any dose rate died or exhibited frank symptoms of toxicity. However, at the site of injection, both AL and AS induced dose-related vascular necrosis, which was not observed in vehicle-dose rats (Table 4). At lower dose rates, the necrosis was reversible, except in the case of 1 of 6 afflicted rats treated with 47·6 μmoles/kg AL, and 4/5 afflicted rats treated with 191·2 μmoles/kg AS (Table 4). These rats were euthanased. However, when the injection site was switched to the femoral vein, the incidence of vascular necrosis decreased sharply and was reversible in all cases (Table 4).

DISCUSSION

Mice are the normal choice of model in pre-clinical drug development programmes for malaria because they are highly susceptible to infection by rodent plasmodia and develop fulminating infections with high parasitaemias and mortality (discussed by Dow *et al*. 1998, 1999). These traits enable convenient assessment of the blood schizonticidal and curative effects of test drugs. However, while there is no restriction on the rodent species which may be used for the standardized tests necessary for the development of most pharmaceuticals, rats, not mice, are the more commonly used species (FDA Centre for Veterinary Medicine, 1994; FDA Committee for Drug Evaluation and Research, 1996). In order to obtain a good indication of both the efficacy and therapeutic index of a new antimalarial, it is imperative to investigate in parallel, the efficacy and toxicity of candidate antimalarials in a rat model of malaria.

However, in future, we anticipate conducting pharmacokinetic experiments in parallel with these, for which serial sampling of sufficiently large volumes of blood (for HPLC analyses) will be required. For this purpose we required animals at least 7 weeks of age. However, animals of this age have never been utilized in efficacy experiments of the type described here, and there are conflicting reports in the literature regarding their susceptibility to *P. berghei* infection. Holloway *et al*. (1995) described a rat model utilizing 6 to 8-week-old rats, in which peak parasitaemias and mortality were in excess of 45 and 90%, respectively. In contrast, Smalley (1975) described peak parasitaemias and mortality of 3·2 and 4·5%, respectively for *P. berghei*-infected 7-week-old rats despite using a much higher inoculum. Thus it was critical to ensure, that, in our hands, the course of *P. berghei* infection in 7-week-old rats was sufficiently similar to the weanling rat and mouse malaria models described in earlier efficacy studies (Dow *et al*. 1998, 1999).

Using the mouse malaria model for comparison, an ideal rat model would be one characterized by (i) high parasitaemias (>20%), (ii) high and consistent mortality (100%) and (iii) a reproducible course of infection (a CV in parasitaemia as low as possible). Previous work has shown that high parasitaemias and a reproducible course of infection are possible in rats. However, experimental *P. berghei* infections in rats are generally self-limiting and the mortality rate is variable, and it has proved difficult to achieve 100% mortality, even in studies where the reported mortality rate was high. In this study, mortality rates were relatively low in both 4 and 7-week-old animals, and this parameter was not considered useful for selecting which model to use in subsequent efficacy experiments. Thus, high parasitaemias and reproducible courses of infection are the most important criteria to consider. Also, we considered a high rate of patency (close to 100%) and low incidence of delayed infections (close to 0%) important, as these characteristics make efficacy experiments easier to design.

Using these criteria as a benchmark, we optimized a 7-week-old SD rat malaria model by altering the inoculum size and number of prior passages, and compared it to a model utilizing 4-week-old animals. We did not consider i.v. administration of parasitized erythrocytes, as it was our intention to administer test agents via this route. We also did not consider the possibility of using immunocompromised rats, as these, too, may not be appropriate for parallel toxicity tests. In both 4 and 7-week-old rats, appropriately high levels of patency and low incidences of delayed infections were observed. Mean parasitaemias were significantly higher in the younger animals, but were within the range described for mouse models in both groups of rats. There was significantly less variation in parasitaemias for the

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younger rats. However, the mean CV in parasitaemia for the 7-week-old rats was nearly as low as that reported for a mouse malaria model, and lower than that reported for a weanling rat model of malaria. Also, the overall course of infection was similar in both 4 and 7-week-old rats and reproducible in independent experiments for the 7-week-old rats. Thus, whilst the 4-week-old rat model would be the ideal choice, our 7-week-old rat model met most of the requirements necessary for efficacy studies, and was therefore an adequate substitute.

The proposed use of intravenous AL or AS is in the context of severe malaria, in which the desired clinical outcome is rapid clearance of parasitaemia and reversal of coma and/or neurological symptoms, such that patients are able to tolerate the administration of oral antimalarial medications. Thus we anticipate a short course of intravenous AL or AS would be followed by oral administration of mefloquine (or other antimalarials). In considering parasitological endpoints, then, our goal was clearance, not cure, as would normally be the case if one were using a mouse model to assess the effectiveness of new test agents. Clearance was very deliberately defined as consecutive negative thin smears 24 h apart prior to day 11. The day 11 restriction was necessary, as *P. berghei* infections are cleared naturally in SD rats beyond this point. In comparing the efficacy of AS and AL, we used 2 parameters; (i) the CD_{100} or the dose of drug resulting in clearance of all animals with no toxicity, and (ii) parasite clearance time. We also made note of recrudescence rates and times but did not consider these parameters critical for comparing the efficacy of AL and AS, given the stated clinical indications for their use.

The effects of AL and AS on *P. berghei* malaria were similar to those observed clinically with artemisinin derivatives; that is, parasitaemias were rapidly cleared but recrudesced several days after treatment (de Vries & Dien, 1996). However, the degree of their antimalarial effect was different. The 100% clearance doses of AL and AS were 95·6 and 191·2 μmoles/kg respectively, suggesting that AL was a 2-fold more potent antimalarial *in vivo*. The antimalarial effect of AL was also more rapid, as parasite clearance times at a dose level of 191·2 μmoles/kg were shorter in rats treated with AL (1·7 days) than those treated with AS (2·7 days). AL is therefore at least as (if not more) potent than one of the current treatments of choice for severe malaria (Warrell, 1999).

In the present study, it was also found that both AL and AS induce vascular necrosis at the site of injection, but the effect is diminished and reversible if the injection site is changed. Also, similar necrotic effects are not observed in humans after intravenous administration of artesunate (Barradell & Fitton, 1995). It is possible that the necrotic effect is simply due to high localized concentrations of the drug at the injection site or the smaller size of rat tail veins, and that the effect is diminished by superior dissolution of drug when injected via the femoral route. The absence of necrotic effects in humans after intravenous AS is administered may simply reflect the larger vein size and the lower dose and reduced concentration of the drug in the injection solution (Wilairatana *et al*. 1997). Thus, though AL induced greater vascular necrosis than AS, this observation need not factor into the drug selection process.

AS has a good clinical safety record (Warrell, 1999). However, a number of artemisinin derivatives, in particular AE, are neurotoxic at supertherapeutic dose rates in laboratory animals (Brewer *et al*. 1994), which has obvious implications for their eventual registration in the US. Recent studies indicate that neither AL nor AS, when administered i.m. as single molecular equivalent doses, are as neurotoxic as AE (Li *et al*. 1999). When given intramuscularly AL has a higher 50% lethal dose than AS (Li *et al*. 1999). While these data may not be directly comparable to the present study as the number of doses and route of administration used were different, they suggest that AL may be at least as safe as AS. We are currently testing this hypothesis in a comparative toxicity study of i.v. AL and AS in Sprague-Dawley rats.

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