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Toxicity evaluation of artesunate and artelinate in *Plasmodium berghei*-infected and uninfected rats

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Summary A recent therapeutic index study in rats demonstrated that i.v. artesunate (AS) is safer than artelinate (AL). The present study of acute toxicity illustrated an LD_{50} of 177 mg/kg and 488 mg/kg for AL and AS, respectively, following daily i.v. injection for 3 days in *Plasmodium berghei*-infected rats. In uninfected rats, the LD_{50} values were 116 mg/kg and 351 mg/kg after a single dose of AL and AS, respectively. This study showed vascular necrosis in 50% of the animals at 13.5 mg/kg AL and at 42.8 mg/kg AS. Animals also showed moderate signs of renal failure at 40 mg/kg AL and 240 mg/kg AS (100 times higher than the therapeutic dose). Histopathological evaluation demonstrated mild to moderate tubular necrosis in uninfected rats treated with 40 mg/kg AL and 240 mg/kg AS; interestingly, fewer pathological lesions were observed in malaria-infected rats. Renal injury was reversible in all cases by Day 8 after cessation of dosing. No neurotoxicity was seen in any case with all i.v. regimens. In conclusion, AL and AS exhibit less toxic effects in *P. berghei*-infected rats than in uninfected rats. Both agents caused irreversible vascular irritation, reversible nephrotoxicity and no neurotoxicity at high doses. The data indicate that AS is three times safer than AL in rats.

1. Introduction

Studies conducted during the 1970s by Chinese investigators provided the first experimental information regarding the potential pharmacological effects of artesunate (AS), one of the artemisinin class of agents. Artemisinin compounds are in widespread use for the treatment of severe and complicated malaria in humans. AS is the most commonly used derivative and has been for more than 15 years; many clinicians feel that AS administered intravenously is the most effective treatment for severe malaria (Ashley and White, 2005). The effectiveness of AS in vivo has been attributed to its rapid and extensive conversion to dihydroartemisinin (DHA) (Batty et al., 1998; Li et al., 1998a, 1998b; McLean and Ward, 1998). DHA is 3—5-fold more potent in efficacy

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and toxicity than other artemisinin derivatives (Li et al., 1998c, 2002; McLean and Ward, 1998; Wesche et al., 1994) and can completely inhibit parasite growth within 2—4 h. DHA is the only artemisinin derivative with activity against all asexual blood-stage parasites (Skinner et al., 1996) in vitro. Recently, a therapeutic index study determined that AS is much safer than artelinate (AL) in malaria-infected rats (Xie et al., 2005).

AL is generally considered to be a potential antimalarial compound in vitro and in vivo (Bustos et al., 1994; Haynes et al., 2005; Li et al., 2003; Shmuklarsky et al., 1993; Tripathi et al., 1996), despite the fact that metabolic conversion of AL to DHA is limited (Grace et al., 1999; Li et al., 1998a; Titulaer et al., 1993), which may relate to the greater binding affinity of AL in erythrocytes, higher plasma concentrations and longer residence time of AL (Hartell et al., 2004; Li et al., 1998b; Xie et al., 2005). AL has lower neurotoxicity than arteether and artemether (Genovese et al., 2000) and greater stability in in vitro metabolic systems and biological fluids (Lin et al., 1987). In theory, AL should be safer, with lower toxicity and lower antimalarial efficacy than AS owing to the limited conversion to DHA (Li et al., 1998a, 1998b). However, experimental evidence has shown the AL is more toxic than AS in rats (Xie et al., 2005) and monkeys (our unpublished data). Compared with AS, the higher drug exposure levels (5-fold) and longer exposure times (20-fold) of AL in a rat pharmacokinetic study at equimolar doses may be the reason for its increased toxicity (Li et al., 2005).

A therapeutic index study of AL and AS was conducted in malaria-infected rats. The minimum suppressive effects of AL and AS were 2.3 mg/kg and 2.5 mg/kg, respectively, and the suppressive doses for half parasitaemia (SD_{50}) were 7.4 mg/kg and 8.6 mg/kg, respectively, indicating comparable efficacies for AL and AS. The maximum tolerated dose (MTD) for AS was 240 mg/kg with a therapeutic index of 32.6, and the MTD for AL was 80 mg/kg with a therapeutic index of 9.3. Taking into account the respective therapeutic indices, we have concluded that AS is a safer drug than AL (Xie et al., 2005). The higher toxic profile of AL is an unexpected finding.

In previous efficacy and toxicity experiments, we noticed a marked decrease in urine output as well as observable haematuria and proteinuria in animals treated with AL. We also found that the two drugs, at higher dose levels, induced mild to severe vascular irritation around the tail vein injection site. Renal failure was demonstrated clinically and supported by histopathological examination in most of the rats treated with i.v. artelinic acid in $NaHCO_3$ (daily dose of 64—128 mg/kg for 3 days). Previously, renal failure had not been reported in animals or humans treated with artemisinin agents. Renal failure is usually associated with intravascular haemolysis or heavy parasitaemia (Boonpucknavig and Sitprija, 1979). Severe malaria with overall mortality of 1% affects 270 million people annually (Zinna et al., 1999) in tropical climates, and clinically significant renal failure commonly occurs with *Plasmodium falciparum* infections. Although the incidence of renal failure is <1% in malaria, mortality is reported to be as high as 45% in these cases.

High AL and AS drug dosing causing acute and subacute toxicity has not been systematically studied and compared in the same species. The present study utilises a rat-adapted model with and without *P. berghei* infection to study the cumulative nephrotoxic effects of multiple doses of i.v. AL and AS formulations. In this study, dose ranges for nephrotoxicity and vascular irritation were determined by pathological examination as well as blood and kidney profiles following single or multiple doses of i.v. AL and AS. The doses were selected at no effect, adverse effect and moderate adverse effect levels following the toxicological observations. This study sought to investigate whether a drug-related and reversible form of AL- and AS-induced nephrotoxicity occurs in malaria-infected and normal rats. In particular, the toxic potencies of AL and AS were compared.

2. Materials and methods

2.1. Chemicals

Artesunic acid (99.2% purity) and artelinic acid (98.0% purity) were manufactured as an L-lysine salt and obtained from the Walter Reed Chemical Inventory System. They were synthesised and manufactured by Knoll AG (Liestal, Switzerland). BASF Pharmaceuticals (Ludwigshafen, Germany) re-bottled the test articles obtained from the original company. AL (WR255663, BP 21847)/lysine and AS (WR256283, BM17174)/lysine salts were supplied through the Division of Experimental Therapeutics at the Walter Reed Army Institute of Research (Silver Spring, MD, USA). DL-Lysine and L-lysine monohydrochloride were obtained from Sigma Chemical Co. (St Louis, MO, USA) and 0.9% saline was purchased from Abbott Labs (Chicago, IL, USA). Pentobarbital, sodium citrate, heparin, D-glucose, glycerol and methanol were purchased from Sigma Chemical Co. Hema 3 stain was obtained from Fisher Scientific Co. (Barrington, IL, USA).

2.2. Animals and parasites

The ANKA strain of *P. berghei* used in this study was rat-adapted from a mouse strain by three successive 4-week passages through 7-week old rats (Charles River, NC, USA). Two pre-treatment films were taken from all animals for parasitaemia analysis. Animals with >5% parasitaemia (severe malaria) were selected for dose ranging and toxicity studies. Post-treatment films were obtained from each rat on a daily basis for 15 days. Seven-week-old (186—213 g body weight) Sprague-Dawley rats, uninfected or infected with *P. berghei*, were randomly assigned into study groups of three or eight animals. Research was approved by Institutional Animal Care and Use Committees, Walter Reed Army Institute of Research and conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and in adherence to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.3. Formulations of AL and AS

Lysine was chosen as the vehicle for both AS and AL in the present study instead of a basic solution (Na_2CO_3, $NaHCO_3$ or NaOH) to avoid vascular irritation induced by basic materials. Pre-formulated AL/lysine or AS/lysine salt was prepared

with 1:1 molecular weight of AL or AS with lysine. The salt weight was equivalent to the real weight of AL or AS for study use. The salt was dissolved in a solution containing 0.45% NaCl/0.1% L-lysine (w/v), which was prepared by mixing 0.9% NaCl with an equal volume of 0.2% L-lysine in sterile water. The dosing solutions were analysed using an HPLC-ECD system (Bioanalytical Systems, West Lafayette, IN, USA).

2.4. Acute toxicity studies

Dose ranging and lethal dose (LD_{50}) studies were conducted in *P. berghei*-infected and uninfected rats. In the infected group, four to six animals in each group received daily i.v. injections for 3 days of AL at doses ranging from 40 mg/kg to 320 mg/kg or of AS at doses ranging from 120 mg/kg to 720 mg/kg. The injections were limited to a total volume of 1—4 ml/kg via the femoral vein. Individual animal body weights were measured on a daily basis throughout the experiment and post mortem examinations were performed for animals found dead. Animals were observed for 2 weeks after dosing. In the uninfected group, each uninfected rat received a single i.v. injection of AL (in 0.1% lysine) at doses ranging from 40 mg/kg to 320 mg/kg or of AS at doses ranging from 60 mg/kg to 720 mg/kg. All animals were observed for 2 weeks after dosing. Following the observation period, all animals were humanely euthanized and submitted for post mortem examination.

2.5. Vascular irritation scoring

Determination of the dose range was dependent on the identification of a no observed adverse effect level dose and a clear effect dose level. The top dose must show toxicity without mortality (moderate toxicity). This was set at one-half or one-quarter the single-dose LD_{50}. Intermediate doses were set to determine the dose—response. Vehicle control was also included. Four to five dose levels with five male rats per group were required. Groups of five to six randomised, 7-week-old rats were given AL or AS intravenously. For high doses (40—120 mg/kg for AL and 240—480 mg/kg for AS), 2—4 ml/kg of the appropriate drug dilution was administered. The general health status of the animals was monitored daily.

2.6. Renal and brain pathology

Plasmodium berghei-infected and uninfected Sprague-Dawley rats were randomly assigned into 15 groups of three to five animals. Rats received either i.v. AL at various doses of 20—120 mg/kg or i.v. AS at various doses of 30—240 mg/kg once daily through the femoral vein for 3 days. At Day 1 and Day 8 after the last dose, blood and urine samples were collected. On Day 8 the animals were euthanized by cervical dislocation and induced pneumothorax under isoflurane anaesthesia. The left and right kidneys were harvested, immersion fixed in 10% neutral-buffered formalin solution and routinely processed to produce paraffin-embedded blocks. The paraffin-embedded tissues were sectioned at 3—4 μm thickness, mounted on glass slides and stained with haematoxylin and eosin stains for light microscopic evaluation.

After harvesting the kidney, the pericardium was incised and a transcardiac perfusion through the left ventricle and aorta was performed using an 18-gauge cannula. The first perfusate used was chilled heparinised saline as a clearing solution. The right atrium was pierced to permit exsanguination and removal of perfusion fluids. After vascular clearing, perfusion fixation with chilled Bouin's fixative solution followed for 5—10 min. The head and cranium were carefully removed, avoiding pressure on the underlying brain. The brain remained in situ for several hours before removal from the skull to avoid the development of neuronal hyperchromatosis. Whole brains were removed and immersed in fresh Bouin's fixative overnight. Brains were blocked transversely at the caudal aspect of the pons for macroscopic analysis.

2.7. Data analysis

The MTD was defined as the dose that caused clinical toxicity in 100% of animals but did not cause death. LD_{50} and the dose causing vascular irritation in 50% of animals (VID_{50}) were calculated using the TableCurve 6.0 Program (Advanced Graphics Software, Inc., Encinitas, CA, USA). Vascular irritation on skin lesions was scored semi-quantitatively: 0, no lesion; 1, mild oedema at the injection site; 2, mild colour change (pink) in the tail; 3, moderate colour change (purple) in the tail; and 4, necrosis and severe colour change (dark purple). Means and SDs were calculated. Coefficients of variation were calculated as percentage of SD divided by mean value. Statistical analysis was conducted with Microsoft Excel (Microsoft Corporation) using Student's *t*-test for dependent samples to compare means of paired and unpaired samples between treatment groups.

3. Results

3.1. LD_{50} determinations

Toxicity dose ranging and LD_{50} studies were conducted in *P. berghei*-infected and uninfected rats. The infected rats received daily i.v. doses for 3 days and were observed for 2 weeks after dosing. Dose administration and death rates are shown in Table 1. Results showed the LD_{50} to be 177 mg/kg/day and 488 mg/kg/day for AL and AS injections, respectively, in infected rats; the majority of rats died on the first dosing day (Table 1). The acute toxicity study was also performed in uninfected rats following a single i.v. injection. After administration, all rats were observed for 2 weeks. The LD_{50} values in uninfected rats (single dose) were lower than those of malaria-infected animals (three daily doses), with values of 116 mg/kg and 351 mg/kg for AL and AS, respectively (Table 1).

3.2. Vascular irritation

Tail necrosis was observed after three AL injections in two of six animals at 10 mg/kg, eight of nine animals at 20 mg/kg and all animals at 40 mg/kg. In a previous study, tail necrosis was observed after a single AS injection with $NaHCO_3$ at

Table 1 Maximum tolerated dose (MTD), death and vascular irritation after artelinate/lysine (AL) and artesunate/lysine (AS) in *Plasmodium berghei*-infected and uninfected male rats ($n = 4–6$)

Group	Dose (mg/kg)	Deaths	Daily death during dosing period		
			Day 1	Day 2	Day 3
Control	0.2% lysine	0/6			
Infected rats (three i.v. doses)					
AL	80	0/6 (MTD)			
	160	2/6	1	1	
	320	4/6	4		
		LD_{50} = 177 mg/kg (95% CI 157–196 mg/kg)			
		VID_{50} = 13.5 mg/kg (95% CI 12.9–14.1 mg/kg)			
AS	240	0/6 (MTD)			
	480	2/5	2		
	720	5/5	4	1	
		LD_{50} = 488 mg/kg (95% CI 478–497 mg/kg)			
		VID_{50} = 42.8 mg/kg (95% CI 11.2–74.4 mg/kg)			
Uninfected rats (single i.v. dose)					
AL	40	0/4 (MTD)			
	80	2/4	2		
	160	3/4	2	1	
	320	4/4	4		
		LD_{50} = 116 mg/kg (95% CI 40–192 mg/kg)			
AS	120	0/4 (MTD)			
	240	1/4	1		
	480	2/4	2		
	720	4/4	4		
		LD_{50} = 351 mg/kg (95% CI 251–451 mg/kg)			

LD_{50}: dose causing death in 50% of the animals; VID_{50}: dose causing vascular irritation in 50% of the animals.
Note: Statistical comparison was done for MTD, LD_{50} and VID_{50}.

doses as high as 64 mg/kg (unpublished data). In the present study, 3-day i.v. AS administration caused tail necrosis in two of six animals at 30 mg/kg, five of six animals at 60 mg/kg and all animals at 120 mg/kg. However, the necrosis did not occur at higher doses if the injection site was switched to the femoral vein. Pathological and clinical observation data revealed VID_{50} values at the injection site (tail veins) of 13.5 mg/kg for AL and 42.8 mg/kg for AS (Table 1).

Tail inflammation and necrosis (lower scores of 1 and 2) at the low dose levels resolved after cessation of dosing. All rats treated with 10 mg/kg AL and 30 mg/kg AS recovered from the skin lesions. Once the tail necrosis became severe (score 3), the damage was irreversible and the animals were euthanized. Macroscopically, the tails with vascular irritation were necrotic and blue distal to the injection site. Microscopically, there was severe dermal haemorrhage, extensive epidermal necrosis and ulceration, neutrophilic inflammation with secondary bacterial infection, and multiple deep venous thrombi. Histological examination showed that the marked necrosuppurative inflammation was evident as a junctional separation between necrotic and normal tissue, a histological characteristic of gangrene. Thrombi were present in larger veins as well as smaller vessels, but were not present in the larger artery proximally. The tail lesion was associated with i.v. drug administration and was consistent with thrombosis, subsequent ischaemia and necrosis distal to the injection site.

3.3. Renal failure due to AL and AS

Malaria-infected animals treated with high-dose AL (>20 mg/kg) and AS (>60 mg/kg) exhibited haemolysis and haemoglobinuria. Moderate to high amounts of haemoglobin were measured in the plasma and urine of rats treated with 40 mg/kg AL and 240 mg/kg AS (Table 2). Reduction in urine output was observed in animals treated with AL. After the second day of dosing, the urinary output decreased by 49–73% from 19.9 ± 2.6 ml to 6.6 ± 3.2 ml in the infected rats. After the third day of dosing, urinary output was still inhibited by 49%. Similar inhibition (approximately 50% on Day 2) was also observed in uninfected rats.

Urinary output increased in AS-treated animals at low dose levels (our unpublished data). In the present study, at the higher dose of AS urine output significantly increased (35%) after the first i.v. injection at 240 mg/kg (Table 2). The urinary output rapidly decreased on Day 3 in the infected rats; the urinary output decreased by 26% (21.3 ± 4.7 ml prior to the first injection to 15.7 ± 4.5 ml). After the fourth day of dosing the urinary output was still inhibited by 10% (19.1 ml) compared with pre treatment (21.3 ml). Similar inhibition (approximately 29–37%) was observed in uninfected rats.

No reduction in urinary output or significant changes in renal chemistry profiles were noted in animals treated with AL at $\leq$20 mg/kg or AS at $\leq$120 mg/kg. The ratio of

Table 2 Mean urinary output, haemoglobinuria and blood urea nitrogen (BUN) in animals treated with artelinate (AL) at 40 mg/kg (96 μmoles/kg) or artesunate (AS) at 240 mg/kg (624 μmoles/kg) in *Plasmodium berghei*-infected and uninfected rats

Study group	Urinary output			Haemoglobinuria[a]		BUN	
	VC	AL	AS	AL	AS	AL	AS
Infected rats ($n = 4–7$)							
Prior dosing	20.5 ± 3.5	19.9 ± 2.6	21.3 ± 4.7	0	0	14.7 ± 4.5	14.0 ± 4.6
Day 1	21.2 ± 3.1	16.4 ± 5.1	28.8 ± 3.8	+++	+		
Day 2	17.8 ± 4.5	6.6 ± 3.2[c]	18.8 ± 3.0	++	+		
Day 3	18.0 ± 2.9	10.1 ± 2.7[b]	15.7 ± 4.5[b]	++	0		
Day 4	19.7 ± 2.8	13.4 ± 1.6	19.1 ± 2.4	+	0	18.7 ± 4.9[b]	33.5 ± 3.4[c]
Day 11	19.2 ± 1.7	18.6 ± 2.9	22.4 ± 1.9	0	0	14.8 ± 3.5	13.8 ± 4.6
Uninfected rats ($n = 4–8$)							
Prior dosing	18.8 ± 2.2	21.9 ± 3.1	23.1 ± 4.1	0	0	15.1 ± 3.4	14.8 ± 4.4
Day 1	19.6 ± 3.7	17.9 ± 3.6	31.1 ± 4.2	++	+		
Day 2	18.0 ± 1.8	9.7 ± 1.9[b]	19.0 ± 5.2	+++	+		
Day 3	20.3 ± 2.4	12.0 ± 2.6[b]	14.4 ± 4.9[c]	+	+		
Day 4	18.7 ± 3.1	15.6 ± 2.5	16.3 ± 3.2[b]	0	0	19.8 ± 1.7[b]	23.5 ± 3.6[b]
Day 11	22.3 ± 4.2	20.5 ± 3.0	22.6 ± 2.4	0	0	14.2 ± 2.6	14.3 ± 3.7

VC: vehicle (lysine) control.
Note: Three daily i.v. injections were given on Days 1, 2 and 3. Day 11 is 8 days after administration of the last dose. Data are presented as arithmetic mean ± SD.
[a] Haemoglobinuria was scored in terms of severity: 0, haemolysed trace; +, small amount; ++, moderate amount; +++, large amount.
[b] $P < 0.05$ compared with prior dosing sample.
[c] $P < 0.01$ compared with prior dosing sample.

blood urea nitrogen (BUN) and creatinine was within normal parameters both for infected and uninfected groups for both drugs at these dose levels. However, when the dose was increased to 40 mg/kg for AL and to 240 mg/kg for AS a marked increase in BUN concentration and a decrease in total protein level was seen compared with the control group (Table 2; $P < 0.05$).

3.4. Renal and brain histopathology due to AL and AS

The primary changes observed in renal histopathology were acute tubular degeneration, necrosis and early tubule regeneration. Mild to moderate tubular necrosis and regeneration were observed both in infected and uninfected rats treated with high-dose AL and AS (Table 3). The morphological changes in the kidney are consistent with the clinical pathological indications of renal failure. One of four infected animals dosed with AL 40 mg/kg showed minimal multifocal tubular single cell necrosis and degeneration after 3 days of injections. Two of three infected rats dosed with 120 mg/kg AL had severe tubular necrosis and regeneration with prominent tubular ectasia and protein and granular casts.

More severe renal damage was present in uninfected animals than in the malaria-infected rats receiving the same AL dosage. Two of four uninfected rats treated with 20 mg/kg AL and all animals treated with 40 mg/kg AL had mild acute tubular necrosis and collecting duct epithelial degeneration and necrosis. Mild to moderate multifocal, acute tubular necrosis and collecting duct epithelial degeneration and necrosis were observed in uninfected rats dosed with 80 mg/kg AL (Figure 1C). In AS-treated animals, renal damage was only seen at the highest dose of 240 mg/kg. No significant lesions were noticed either in infected or uninfected animals dosed with ≤120 mg/kg AS (Figure 1A). Two of the three infected rats treated with 240 mg/kg AS revealed moderate multifocal tubular necrosis and regeneration. Similar renal injury was also noticed in all uninfected rats treated with 240 mg/kg AS daily for 3 days (Figure 1B).

On Day 8 after the last dose of treatment with 40 mg/kg AL and 240 mg/kg AS, neuronal damage was not found in any animals following the single or multiple i.v. injections. Similar to the vehicle alone, the rats did not show histopathological abnormality on Day 1 after the same treatments of AL and AS.

3.5. Reversibility study

Selected data on the reversibility of observed nephrotoxicity are presented in Table 3. On Day 1 after the final dose (non-recovery group), rats treated with 40 mg/kg or 80 mg/kg AL or with 120 mg/kg or 240 mg/kg AS showed dose-related histopathological changes in the total number of affected tubules and the corresponding severity scores. Tubules affected scores were high for AL at 80 mg/kg and AS at 240 mg/kg, with average scores of 2.18 ± 0.45 and 2.60 ± 0.23, respectively (Table 3). Overall, >40% of the tubules were affected in these two groups. The severity scores were mild to moderate for AL at 80 mg/kg (1.35 ± 0.18) and AS at 240 mg/kg (1.52 ± 0.21).

Animals treated with AL and AS at the same dose levels were also sampled on Day 8 post dosing (Table 3). Both scores were significantly lower in recovery animals treated with AL 40 mg/kg ($P < 0.01$) or 80 mg/kg ($P < 0.001$) and AS 240 mg/kg ($P < 0.001$) compared with the animals sacrificed on Day 1

Table 3 Renal histological effects in all animals and recovery study in uninfected male Sprague-Dawley rats dosed with artelinate/lysine (AL) 40—120 mg/kg or artesunate/lysine (AS) 120—240 mg/kg intravenously. Animals were sacrificed at 24 h (1 day) and 192 h (8 days) after the last dose ($n = 3$—5)

Group	Drug/dose	Number of rats with changes/total number	Tubules affected score[a]	Severity score[b]
Infected rats				
	VC	0/5	0	0
	AL 40 mg/kg	1/4	1.00 ± 0.35	0.46 ± 0.18
	AL 120 mg/kg	2/3	3.13 ± 0.68	2.77 ± 0.82
	AS 120 mg/kg	0/3	0.05 ± 0.02	0.03 ± 0.01
	AS 240 mg/kg	2/3	2.21 ± 0.56	1.44 ± 0.52
Uninfected rats				
Non-recovery group (Day 1 post dosing)	VC	0/4	0.09 ± 0.08	0.08 ± 0.06
	AL 40 mg/kg	3/3	1.26 ± 0.22	0.83 ± 0.09
	AL 80 mg/kg	3/3	2.18 ± 0.45	1.35 ± 0.18
	AS 120 mg/kg	1/4	0.03 ± 0.01	0.02 ± 0.0
	AS 240 mg/kg	4/4	2.60 ± 0.23	1.52 ± 0.21
Recovery group (Day 8 post dosing)	VC	0/4	0.01 ± 0.01	0.01 ± 0.01
	AL 40 mg/kg	1/5	0.07 ± 0.02[d]	0.05 ± 0.01[c]
	AL 80 mg/kg	3/5	0.24 ± 0.29[d]	0.16 ± 0.17[d]
	AS 120 mg/kg	0/4	0	0
	AS 240 mg/kg	4/4	0.38 ± 0.21[d]	0.31 ± 0.18[d]

VC: vehicle (lysine) control.
Note: Statistical comparison was done for tubules affected score and severity score. Data are presented as arithmetic mean ± SD.
[a] Tubules affected scoring: 0, normal; 0.5, <5% tubules affected; 1, 5—20% affected; 2, 21—40% affected; and 3, >40% affected. Overall scores are the mean of 20 randomly selected 400× magnification fields within the renal cortex of each rat.
[b] Severity scoring: 0, normal; 0.5, minimal; 1, mild; 2, moderate; and 3, severe.
[c] $P < 0.01$ compared with non-recovery at same dose level.
[d] $P < 0.001$ compared with non-recovery at same dose level.

post dosing (non-recovery). The severity scores were 0.05 and 0.16 for 40 mg/kg and 80 mg/kg AL, respectively, on Day 8 compared with 0.83 and 1.35 on Day 1 (Figure 1D). On Day 8 the severity score was 0.31 for AS at 240 mg/kg compared with the Day 1 severity score of 1.52. Furthermore, no renal damage was found either in infected or uninfected rats dosed with 120 mg/kg AS. These results indicate that renal tubules affected were reduced from >40% (score for non-recovery group) to <5% both for AS and AL groups in the recovery study. The severity of the lesions also decreased from mild—moderate to minimal.

4. Discussion

This dose ranging study showed the LD_{50} of AL and AS (177 mg/kg and 488 mg/kg, respectively) in *P. berghei*-infected rats was higher than those in uninfected animals (116 mg/kg and 351 mg/kg), indicating that infected rats were able to tolerate more drug than uninfected animals. The drugs were more toxic in uninfected animals than in malaria-infected rats even though the infected animals received three multiple administrations. One possible explanation may be an enhanced uptake of the drug by parasites and the reduced free drug in blood. *Plasmodium falciparum*-infected erythrocytes take up and concentrate [^{3}H] DHA (Gu et al., 1984) and [^{14}C] artemisinin (Kamchonwongpaisam et al., 1994; Meshnick et al., 1991) at 100—300-fold higher concentrations than uninfected erythrocytes. The maximum uptake of the drug occurred after 1—2 h. The uptake was reversible, saturable and appeared to be at least partially dependent on metabolic energy (Gu et al., 1984; Kamchonwongpaisam et al., 1994).

LD_{50} values in uninfected and infected AL-treated animals were 116 mg/kg and 177 mg/kg, respectively, and they were 351 mg/kg and 488 mg/kg, respectively, in AS-treated animals. These data indicate that AL toxicity appeared to be three times higher than that of AS. Similar results were seen in the vascular irritation study; the VID_{50} of infected rats was 13.5 mg/kg for AL-treated rats and 42.8 mg/kg for AS-treated rats. Our therapeutic index studies showed similar findings, with 2—3-fold higher toxic potency for AL than for AS (Xie et al., 2005).

Both drugs caused renal failure, indicated by haemolysis, haemoglobinuria, altered urine output (oliguria, polyuria), elevated BUN or serum creatinine levels, and renal tubular damage in rats at high dosages. Drug-induced mild to moderate nephrotoxicity was seen in rats treated with 40 mg/kg AL and with 240 mg/kg AS. Similar to the findings of the LD_{50} and VID_{50} studies, the toxic potential of AL is three times higher than that of AS. The diuretic effect of AS (Campos et al., 2001; Seguro and Campos, 2002) was confirmed in our animals at dose levels from 30 mg/kg to 120 mg/kg, even on the first day of dosing of 240 mg/kg, which was reversible in the present study.

The antimalarial and toxic properties of effective artemisinin agents are dependent on the presence of an endoperoxide bridge; artemisinin agents lacking this bridge,

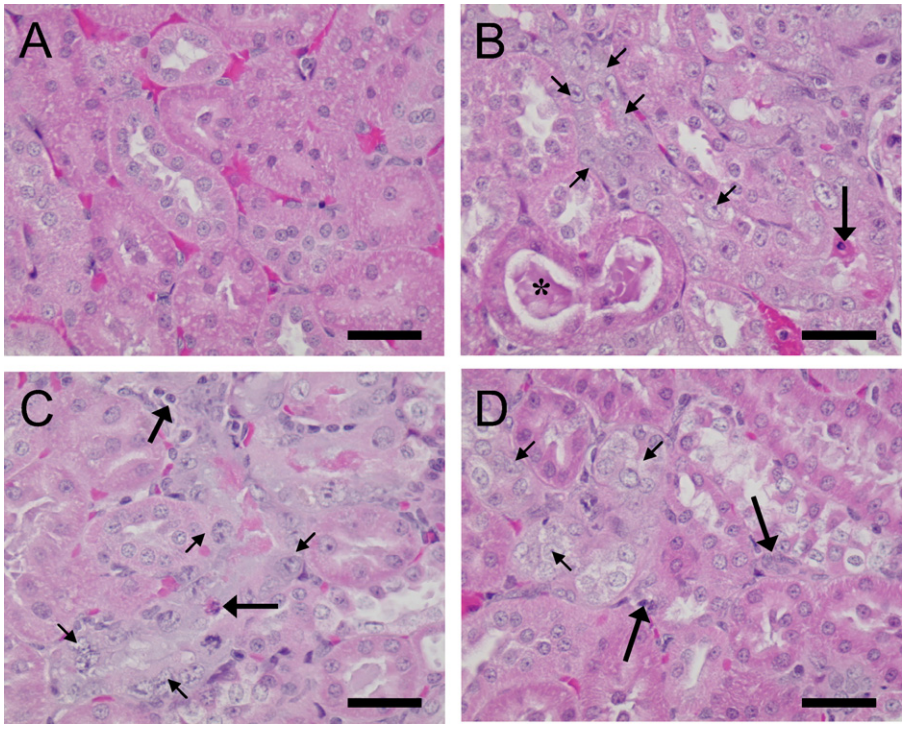

Figure 1 Kidney sections from rats treated with artelinate (AL) or artesunate (AS) once daily for 3 days. (A) No significant lesions on Day 1 following the last dose of 120 mg/kg AS. (B) Tubular dilation with intratubular cellular debris (asterisk), tubular epithelial degeneration and necrosis (large arrow) and cellular nuclear hypertrophy and early tubular regeneration (small arrows) on Day 1 post dosing with 240 mg/kg AS both in infected and uninfected rats. (C) Tubular epithelial degeneration and necrosis (large arrows) and cellular nuclear hypertrophy and early tubular regeneration (small arrows) on Day 1 following the last dose of 80 mg/kg AL in uninfected animals. (D) Tubular collapse and loss (large arrows) and tubular epithelial nuclear hypertrophy and regeneration (small arrows) on Day 8 post dosing with 80 mg/kg AL. Bar = 75 μm.

a known source of oxygen free radicals, are devoid of antimalarial and toxic activities (Brossi et al., 1988; Klayman, 1985). Most free radical-generating drugs cause 'oxidant damage' by producing oxygen free radicals such as superoxide, which then cause indiscriminate damage to the cell (Halliwell and Gutteridge, 1999). Artemisinin derivatives were at first thought to act in this manner (Krungkrai and Yuthavong, 1987; Meshnick et al., 1989). Artesunate is shown to induce lipid peroxidation (Meshnick et al., 1989) both in infected and uninfected erythrocytes, as well as membrane protein thiol oxidation in isolated erythrocyte membranes.

Artemisinin agents do not act like a typical oxidant drug that causes promiscuous damage to proteins, nucleic acids and lipids (Halliwell and Gutteridge, 1999). Unlike most other oxidant drugs, artemisinin cannot be cyclically oxidised and reduced (Zhang et al., 1992), only one free radical can result from one drug molecule, and all of the oxidant end products observed experimentally were observed at very high drug concentrations (>100 μM or >3800 ng/ml) (Berman and Adams, 1997; Meshnick, 2002; Scott et al., 1989). Numerous in vitro and in vivo studies have demonstrated that reactive oxygen metabolites including superoxide (free radicals) and hydroxyl radicals are implicated in drug-induced acute renal failure (Baliga et al., 1999; Du and Yang, 1994; Walker et al., 1999). Limited reports (Karbwang et al., 1998; Taylor and White, 2004) indicated that drug-induced renal failure is not easily diagnosed in humans because the interference from severe malaria or the therapy dose, which is one-hundredth of the toxic dose to cause renal failure, never caused nephrotoxicity in humans.

Toxicokinetic evaluation demonstrated that an immediate and complete hydrolysis occurred for AS (5.26 ratio of DHA/AS) but not for AL (0.01 ratio of DHA/AL) in *P. berghei*-infected rats. At equimolar dosages, the much higher plasma concentration (area under the curve $(AUC)_{1-3\ Day}$, 84.4 μg h/ml) and longer half-life ($t_{1/2}$ = 7.1 h) of AL were attributed to the poor conversion rate of AL to DHA and the enterohepatic circulation of AL compared with AS and its active metabolite DHA (total $AUC_{1-3\ Day}$, 15.7 μg h/ml; $t_{1/2}$, 0.36—0.72 h). The high drug exposure level and the long exposure time of AL in rats may bring about its more severe toxicities (LD_{50}, vascular irritation and nephrotoxicity) and also greater efficacy (Li et al., 2003) compared with AS (Li et al., 2005).

In the present study, vascular irritation, intravascular haemolysis, haemoglobinuria and renal failure may be attributed to free radicals from AL and AS causing oxidant and lipid peroxidation, especially at high dose levels. The clinical manifestations of renal failure were consistent with acute tubular necrosis in rats treated with AL (≥40 mg/kg) and AS (≥240 mg/kg) resulting in blood concentrations above the published toxic artemisinin molar concentration of 100 μM (Berman and Adams, 1997; Dong and Vennerstrom, 2003; Meshnick et al., 1996). Toxicities were found both in infected and uninfected animals. Therefore, the drugs are considered to cause toxicity unrelated to the

P. berghei parasite infection. In conclusion, both drugs showed a greater level of toxicity in uninfected animals than in malaria-infected rats. Both agents caused renal damage, but significant nephrotoxicity was found only at high dose levels that far (100-fold) exceed the therapeutic dosage. Furthermore, renal damage was reversible following cessation of treatment. In addition, neurotoxicity was not detected in any case with all i.v. regimens in this study. Cumulatively, the LD_{50}, vascular irritation and nephrotoxicity data show the toxic effect of AL to be 3-fold higher than that of AS in rats.

Conflicts of interest statement
The authors have no conflicts of interest concerning the work reported in this paper.

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