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ORIGINAL ARTICLE

A pharmacokinetic comparison of homodimer ARB-92 and heterodimer ARB-89: novel, potent antimalarial candidates derived from 7β-hydroxyartemisinin

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Abstract The ultimate goal of this study was to identify an orally active, affordable, potent and safe antimalarial drug based on the natural product artemisinin. During these efforts, a series of novel 7β-hydroxyartemisinin analogs were synthesized and characterized in vitro for their antimalarial activity against *Plasmodium falciparum*. Heterodimerization of 7β-hydroxyartemisinin provided the asymmetrical carbamate (ARB-89) while homodimerization provided the carbonate (ARB-92). These dimers were found to be highly active in vitro with an $IC_{50} \leq 0.50$ nM against *P. falciparum* infected human red blood cells (RBC). For further development as potential antimalarial agents, a battery of in vitro and in vivo pharmacokinetic experiments was performed to distinguish the fate of the discovery compounds ARB-89 and ARB-92. Two UPLC-MS methods were developed and validated for the analysis of the compounds. Both ARB-89 and ARB-92 exhibited moderate affinity (51 and 56%, respectively) to parasitized RBC, which is a perquisite for antimalarial activity. Following a single dose oral and intravenous pharmacokinetic study in rats, ARB-89 displayed a high clearance (92.8 ± 5.6 L/h kg), short elimination half-life ($t_{1/2}$, 1.2 ± 0.2 h) and moderate oral bioavailability (23.4%). ARB-89 was found to be excreted unchanged in feces, which may be due to its high lipophilicity, molecular weight and low oral exposure. In an attempt to identify a better lead antimalarial compound, ARB-92 was designed to be more water soluble than ARB-89 by incorporating a protonatable tertiary amine as part of the dimerizing ligand for 7*β*-hydroxyartemisinin. As anticipated, ARB-92 displayed a lower clearance (2.9 ± 0.7 L/h kg) and subsequently a longer $t_{1/2}$ (2.3 ± 0.2 h) compared to ARB-89. The oral bioavailability of ARB-92 was found to be 34% in rats, a value somewhat better than the marketed artemisinin derivatives artenimol (19.3%), artemether (19.7%) or artesunate (29.5%).

Keywords Malaria · Pharmacokinetics · Artemisinin dimers · DMPK · Protein binding

Introduction

According to the World Health Organization (WHO), malaria continues to be a major health problem in many areas around the globe and was reported to cause approximately 214 million illnesses and 438,000 deaths in 2015 (WHO 2015). Malaria is the most common tropical disease and is caused by protozoan parasites of the genus *Plasmodium*. Among all *Plasmodium* species that infect humans, *Plasmodium falciparum* and its resistant strains accounted for over 85% of deaths in humans due to malaria (Winstanley 2000). Traditionally, malaria has been treated with quinolines such as chloroquine, quinine, mefloquine, primaquine and with antifolates such as sulfadoxine and pyrimethamine (White 1992). Widespread resistance to

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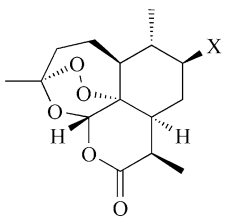
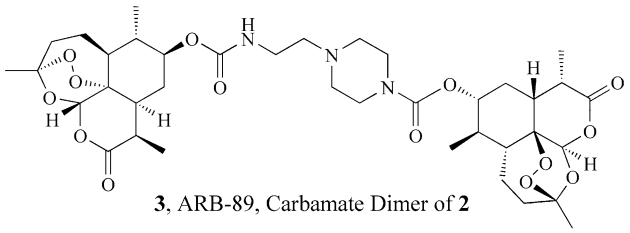
these commonly used drugs has resulted in an effort to synthesize new antimalarial compounds based on the natural product artemisinin (**1**, Fig. 1) (Meshnick 2002). Artemisinin is a sesquiterpene-lactone endoperoxide isolated from *Artemisia annua* L., a plant with a long history of medical use against malaria in China (Haynes and Krishna 2004; O'Neill and Posner 2004). The parent compound, as well as several of its semi-synthetic derivatives (artemether, arteether and artesunate) have been found to be effective as single agents or in artemisinin based combination therapies (ACT) against both chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* (WHO 2015). These compounds are noteworthy for both their rapid action against drug-resistant strains of malaria, as well as their unique effectiveness against cerebral malaria. Because of these features, ACT has entered into use as a first-line chemotherapy of malaria (Avery et al. 1999; Vroman et al. 1999). These first generation endoperoxides have some shortcomings, such as low oral bioavailability, high recrudescence and relatively short half-lives (Silamut et al. 2003; Gautam et al. 2009).

Apart from potent in vitro and in vivo activity, an ideal antimalarial drug should exhibit acceptable oral bioavailability, long plasma concentration where the area under the curve (AUC) is above the minimum inhibitory concentration (MIC), high partitioning into parasitized erythrocytes, as well as a long half-life. These desired properties would reduce the dosing frequency of the drug and would lead to successful treatment of the disease. With this goal in mind, the M.A. Avery group, in the Division of Medicinal Chemistry at the University of Mississippi, synthesized a series of novel artemisinin dimers using the microbial metabolite of artemisinin, 7β-hydroxyartemisinin, furnished by the co-Divisional J.S. Williamson group. These rationally selected analogs were then tested in vitro against the W2 and D6 clones of *P. falciparum*. Many analogs demonstrated high in vitro activity, and several were evaluated for their in vivo activity. The best of these compounds was the carbamate

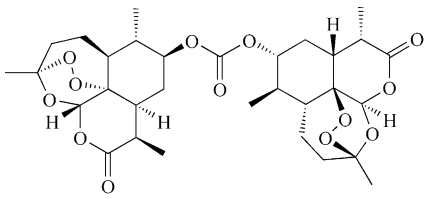


1, X = H, Artemisinin
2, X = OH, 7β-Hydroxyartemisinin

Fig. 1 Chemical structures of *R*(+)-artemisinin (**1**) and its microbial metabolite, 7β-hydroxyartemisinin (**2**)



3, ARB-89, Carbamate Dimer of **2**



4, ARB-92, Carbonate Dimer of **2**

Fig. 2 Chemical structures of dimers of 7β-hydroxyartemisinin: carbamate heterodimer ARB-89 (**3**) and carbonate homodimer ARB-92 (**4**)

(ARB-89, **3**) and carbonate (ARB-92, **4**), shown in Fig. 2. These two compounds exhibited potent antimalarial activity in vitro and the concentrations to inhibit the growth of 50% (IC_{50}) of the parasite *P. falciparum* were 0.50 nM (ARB-89) and 0.32 nM (ARB-92) (Table 1). Both compounds were also tested against *Plasmodium berghei* N in male CD1 mice (~20 g). The carbamate (ARB-89) and carbonate (ARB-92) demonstrated parasitemia suppression of >96 and >99% at a dose of 3.3 mg/kg as compared to 94% suppression shown by artesunate at the same dose. No signs of obvious cytotoxicity were observed with these compounds (Avery 2009). In order to expedite the selection of ARB-89 versus ARB-92 as a candidate drug, a series of pharmacokinetic studies were performed and discussed in this paper. The methods for preclinical ADME screening have evolved in this study to provide an efficient pipeline for new derivatives. Consequently, structure pharmacokinetic relationships can be derived that will be useful in the advanced design of plausible antimalarial drug candidates, reducing or eliminating expensive, undirected chemical synthesis.

Table 1 Physiochemical properties of ARB-89 and ARB-92

Compound	Molecular weight	IC_{50} (nM)[a]	cLog*P*	Solubility (μg/mL)
ARB-89	777.5	0.50	2.6	6.7
ARB-92	622.4	0.32	2.5	7.3

[a] IC_{50} Concentration of drug required to inhbit the growth of 50% of *Plasmodium falciparum* in vitro

Materials and methods

Chemicals and reagents

ARB-89 (7β-hydroxyartemisinin carbamate, **3**) and ARB-92 (7β-hydroxyartemisinin carbonate, **4**) (Fig. 2) with purities ≥99% were synthesized from 7β-hydroxyartemisinin (**2**, Fig. 1) by the M.A. Avery's research group, Division of Medicinal Chemistry, University of Mississippi. The preparation of 7β-hydroxyartemisinin was accomplished in a co-Divisional effort conducted by the J.S. Williamson's research group using preparative microbial metabolism of artemisinin (purchased in multi-Kg quantities, >99% purity from Mediplantex, Hanoi, Vietnam). Artemisinin (internal standard, IS) was purchased from Sigma-Aldrich (Milwaukee, WI, USA; purity >99%) and HPLC grade acetonitrile, methanol, chloroform, tertiary-butyl methyl ether (TBME), formic acid and water were purchased from Fisher (Fair Lawn, NJ, USA). Dulbecco's phosphate buffered saline (DPBS) and dimethyldichlorosilane (DMDS) were obtained from Sigma (St. Louis, MO, USA). HPLC vials were supplied by Waters (Milford, MA, USA). Drug-free rat plasma was furnished by Innovative Research Inc. (Southfield, MI, USA), and male *Sprague Dawley* rats were procured from Harlan Inc. (Indianapolis, IN, USA). Rat liver microsomes were distributed by CellZdirect Inc. (Pittsboro, NC, USA), and human type A^+ packed blood cells were obtained from Mississippi Blood Services (Jackson, MS, USA).

Ultra performance liquid chromatography-mass spectrometry (UPLC-MS) analysis

Quantification of ARB-89 and ARB-92 in rat plasma, feces, urine and human erythrocyte supernatant was accomplished using a Waters Micromass Quattro Micro™ system (Manchester, UK) coupled with an Acquity UPLC™ (Waters, Milford, MA, USA). A modified method reported by Pabbisetty et al. (2012) was used for the quantification of ARB-89 in the biological matrices. In addition, a UPLC-MS procedure was developed and validated for the quantification of ARB-92 using electrospray ionization in the positive mode. The *m*/*z* 778.4, 623.3 and 283.4 were selected for analysis using selected ion recording (SIR) of ARB-89, ARB-92 and the IS, respectively. For both methods, capillary voltage, extractor voltage, source temperature and desolvation temperature were 3.1 KV, 0.4 V, 100 and 350 °C. The UPLC-MS system was controlled by MassLynx software (Version 4.0, Waters, Milford, MA, USA).

Chromatographic separations were achieved on a Acquity BEH C18 Column (1.7 µm, 2.1 × 50 mm, Waters, Milford, MA, USA). The mobile phases consisted of acetonitrile and 0.3%v/v aqueous formic acid (60:40, %v/v) for ARB-89 and acetonitrile and 0.1%v/v aqueous formic acid (60:40, %v/v) for ARB-92. The UPLC flow rate was 0.3 mL/min, sample injection volume was 10 µL, and total analysis time was 3 min for both methods. Rat plasma samples of ARB-89, including the blank, the blank with the IS, the calibration standards, the QC standards and test samples were extracted using a simple protein precipitation technique. For this technique, an aliquot (90 µL) of rat plasma sample was spiked with 10 µL of the IS solution and the resultant mixture was quenched with 300 µL of acetonitrile. Samples were vortex mixed for 10 min followed by centrifugation at 3000×*g* for 10 min. The supernatant was collected and injected for analysis using UPLC-MS. The carbonate, ARB-92, was extracted from rat plasma samples using a liquid–liquid extraction technique. For this technique, an aliquot (90 µL) of the rat plasma was spiked with 10 µL of the IS solution, and then placed in a microtube. The extraction solvent, TBME (1 mL), was added, and the samples were vortexed for 10 min. Following centrifugation at 3000×*g* for 10 min, the supernatant (≈900 µL) was collected and evaporated to dryness under a vacuum at 35 °C. The residues were reconstituted with 100 µL of acetonitrile and injected into the UPLC-MS for analysis.

Both methods were validated in rat plasma according to the Food and Drug Administration (FDA) guidelines for selectivity, recovery, matrix effect, linearity, precision, accuracy and stability. The bioanalytical methods were also partially validated in rat feces, urine and human erythrocyte supernatants (FDA 2001).

Lipophilicity and solubility

The octanol–water partition coefficient (clog*P*) of the compounds was calculated using ACD/log*P* (version 10, Advanced Chemistry Development, Inc., Toronto, ON, Canada, http://www.acdlabs.com, Nov. 2006). Thermodynamic aqueous solubility of ARB-89 and ARB-92 were estimated using a shake flask method at room temperature (Glomme et al. 2005). Approximately 1 mg (N = 3) of the compound was shaken with 10 mL of water in a glass vial at 500 RPM for 24 h at room temperature. Samples were taken at 4, 8, 12 and 24 h and analyzed for compound using the validated UPLC-MS method.

Uptake by non-parasitized and parasitized erythrocytes

A method previously described by Shah et al. (2009) was used for erythrocyte uptake studies of compounds ARB-89 and ARB-92. In brief, fresh non-parasitized packed A^+ human blood cells were washed with DPBS twice and subjected to centrifugation at 10,000×*g* for 5 min. The white blood cells and platelets were removed and discarded using a glass transfer pipette. Parasitized RBC (erythrocytes cultivated with the D6 strain of *P. falciparum*) were also

centrifuged, and the culture medium was discarded. All glassware was pre-silanized with 5%v/v DMDS in toluene to minimize binding of the compounds to the glassware. The RBC hematocrit was adjusted to 33% for both the parasitized and non-parasitized erythrocytes. Working solutions of ARB-89 and ARB-92 were made by diluting stock solutions (100 µg/mL), resulting in a final concentration of 1.42 and 3.55 µM that were used for the uptake studies in the parasitized and non-parasitized erythrocytes. The concentration of compounds were selected on the basis of our previous studies (Vyas et al. 2002; Shah et al. 2009). At these concentrations, most of the carrier proteins on erythrocyte membranes become saturated. Due to saturation of surface proteins, any further increase in analyte concentration will lead to a decline in the percent uptake of the compound (Shah et al. 2009). All the contents were mixed using a shaker water bath at 37 °C for 2 h at 75 shakes/min. At the end of the incubation period, the samples were transferred into tubes and centrifuged at 10,000×*g* for 5 min to separate the supernatant and cell pellets. The supernatant was carefully withdrawn, and the RBC pellets were discarded. The supernatant was then analyzed using the UPLC-MS method described *vide supra*.

Plasma protein binding

The protein binding of ARB-89 and ARB-92 was determined using an ultrafiltration technique. Relevant biological concentrations (100 ng/mL to 1 µg/mL) of the analyte were achieved in rat plasma. After these samples were incubated at 37 °C for 15 min, they were pipetted into centrifuge tubes (Centrifree Amicon, Schwalbach, Germany) and centrifuged at 1500×*g* for 10 min (Ackerman et al. 1988). To 90 µL of the ultrafiltrates that were collected from the bottom of the centrifuge tube, 10 µL of the IS was added, and the resultant samples were processed and analyzed using UPLC-MS. Nonspecific binding of the compounds to the centrifuge tubes was also determined and incorporated in the calculation for the protein binding values.

Metabolism study using rat liver microsomes

The metabolic stability of ARB-89 and ARB-92 was evaluated in pooled rat liver microsomes by observing the disappearance of these compounds over an incubation period of 60 min. Incubation mixtures consisted of liver microsomes (1 mg microsomal protein/mL), test compound (5 µM) and ammonium acetate buffer (50 mM, pH 7.4). The reaction was initiated with the addition of 25 µL of nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system to the incubation mixtures. The NADPH regenerating system consisted of NADP (1 mM, pH 7.4), glucose-6-phosphate (1 mM, pH 7.4), magnesium chloride (3 mM) and glucose-6-phosphate dehydrogenase (1 unit/mL). The samples were incubated at 37 °C, and the reaction was terminated at time points 0, 15, 30, 45 and 60 min using the addition of three volumes of ice-cold acetonitrile. The zero-time point product served as the 100% concentration sample. The test samples were centrifuged at 3000×*g* for 10 min at 4 °C, and the supernatants were analyzed using UPLC-MS.

Pharmacokinetic studies in rats

Right jugular vein cannulated male *Sprague Dawley* rats (225 ± 25 g) were used for the pharmacokinetic studies. The rats were housed in metabolic cages and allowed free movement and access to standard rodent diet and water. Before the pharmacokinetic study, the animals were fasted overnight but provided water ad libitum throughout the experiment. Standard rodent diet was provided 2 h after the dosing of the animals. For intravenous formulations of ARB-89 and ARB-92, 1 mg of each compound was dissolved in 1 mL of 10% Tween-80, 10% ethanol and 80% normal saline. The intravenous formulations were pre-filtered through 0.2 µm syringe filters (Corning, New York, USA). The composition for the oral formulations of both compounds consisted of 25 mg of each compound in 1 mL of a mixture containing 10% Tween-80, 10% peanut oil, 20% ethanol and 60% normal saline. Both formulations were quantified for drug content using UPLC-MS.

The rats were divided into four groups (N = 6 each). The first group was treated with a single oral dose (100 mg/kg) of ARB-89, the second group was given a single intravenous dose (5 mg/kg) of ARB-89. A third group was treated with a single oral dose (100 mg/kg) of ARB-92 and the fourth group was dosed with a single intravenous administration (5 mg/kg) of ARB-92. A serial sampling technique was employed in which blood samples (~225 µL) were collected at 0, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24 h and 0, 1, 2, 4, 6, 8, 9, 10, 12, 24, 48 h post-dose for intravenous and oral pharmacokinetic studies, respectively. The plasma samples were separated by centrifugation (3000×*g* for 10 min, 4 °C) and stored at −80 °C until analyzed. Urine and fecal samples were also collected at 0, 2, 4, 8, 12, 24 and 48 h after compound administration.

The concentration–time data of ARB-89 and ARB-92 were subjected to non-compartmental analysis using WinNonlin (Version 6.4, Pharsight Corporation, Mountain View, CA). Peak plasma concentration (C_{max}) and the time associated with C_{max} (T_{max}) were obtained from the visual inspection of the observed data. The AUC was calculated using the linear trapezoidal rule from time 0 to infinity. The first order terminal phase rate constant, λ_z, was estimated by linear regression of time versus the natural logarithm of the concentration, and the $t_{1/2}$ was calculated using the equation $t_{1/2} = 0.693/\lambda_z$. Plasma clearance (CL) was calculated

using the equation CL = Dose/AUC after intravenous and oral administration and the apparent volume of distribution (V_d) after intravenous and oral administration was calculated using the equation $V_d = \text{Dose}/(\lambda_z \times \text{AUC})$. The percent oral bioavailability was calculated using the equation $\%F = (AUC_{oral} \times Intravenous_{dose})/(AUC_{intravenous} \times Oral_{dose}) \times 100$. For the selection of the better drug candidate, concentration–time profiles of both compounds were compared and the specific pharmacokinetics parameters (C_{max}, T_{max}, AUC, $t_{1/2}$, CL and V_d) were subjected to Student's t test using GraphPad Prism, version 5.01 (GraphPad Software Inc, CA, USA). Statistical analysis was preformed separately for oral and intravenous route of administration.

Results

UPLC-MS analysis

Direct infusion of ARB-89, ARB-92 and IS (200 ng/mL) in both positive and negative electrospray ionization modes into the mass spectrometer at a constant flow rate of 10 µL/min using a Harvard Pump (Harvard Apparatus, Holliston, USA) was used to tune the compounds. In SIR positive ionization mode, the protonated molecular ions *m/z* 778.4, 623.3 and 283.4 were selected for the analysis of ARB-89, ARB-92 and the IS, respectively. Due to structural similarities between ARB-89 and ARB-92, artemisinin was used as the IS for both methods. Source and compound MS parameters were also optimized to achieve the maximum response for the compounds. Various mobile phase compositions (acetonitrile and methanol with aqueous buffers or pH modifiers *i.e.*, formic acid, ammonium formate, ammonium acetate and acetic acid), columns (C18, C8, Cyano and HILIC) and flow rates (0.2–0.4 mL/min) were tested. However, chromatographic separation, acceptable peak shape and short retention time were achieved using an Acquity BEH C18 Column (1.7 µm, 2.1 × 50 mm) with mobile phases consisted of 60% acetonitrile in aqueous formic acid (0.3%v/v) and 60% acetonitrile in aqueous formic acid (0.1%v/v) for ARB-89 and ARB-92, respectively. Retention times for analyte and the IS were 1.18 and 2.02 min for ARB-89 and 1.36 and 1.95 min for ARB-92, respectively (Figs. S1, S2). Both protein precipitation (methanol and acetonitrile) and liquid–liquid extraction techniques (chloroform, *n*-hexane, ethyl acetate and TBME) were used for the extraction of ARB-89 and ARB-92 from the bio-matrices. Protein precipitation using acetonitrile and liquid–liquid extraction using TBME resulted in a concentration independent, consistent, precise and reproducible recovery of ARB-89 (94.0–96.3%) and ARB-92 (94.6–98.0%), respectively (Tables S1, S2). Linearity range for both methods in rat plasma was 1–10,000 ng/mL, and correlation coefficients were always ≥0.99. Both methods were validated following FDA guidelines for accuracy, linearity, matrix effect, precision, selectivity and stability (FDA 2001). After successful validation in rat plasma (Tables S1, S2), these methods were applied for the analysis of in vitro and in vivo pharmacokinetic studies of ARB-89 and ARB-92 test samples.

Lipophilicity and solubility

It is generally preferable for an oral drug candidate to have acceptable aqueous solubility, this lessens formulation problems, improves solubility in gastric fluids and enhances in vitro testing reproducibility (Lipinski 2000). The aqueous solubility (Table 1) of ARB-89 and ARB-92 at room temperature was found to be 6.7 and 7.3 µg/mL, respectively. The calculated clog*P* for ARB-89 and ARB-92 was 2.6 and 2.5, which indicated a desired lipophilic nature of both compounds. Although there are no strict cutoff values for solubility and lipophilicity, it is generally advantageous for a drug candidate to have high enough water solubility to dissolve in body fluids at therapeutic concentration and sufficient lipophilicity to cross the biological membranes.

Uptake by non-parasitized and parasitized erythrocytes

Artemisinin analogs are shizonticidal agents, working on RBC infected by the malarial shizont. Therefore, it is essential for antimalarial compounds to have an affinity for parasitized RBC membranes, in the absence of specific transporters, for desired in vivo effects (Meshnick et al. 1991). The percentage uptake of ARB-89 and ARB-92 into normal RBC ranged from 28 to 33%. During uptake studies in parasitized RBC, ARB-89 and ARB-92 exhibited an uptake of 51 and 56%, respectively. Artemisinin was used as a control, and it exhibited a 59% uptake into *P. falciparum* infected RBC. Altered membrane permeability and surface proteins can be attributed to the increased uptake of artemisinin and its analogs in infected RBC as opposed to non-parasitized RBC. Both ARB-89 and ARB-92 have essential structural features for the improved uptake in parasitized RBCs. However, we also need some uptake of the compounds in normal RBC to prevent malaria infection.

Plasma protein binding

Protein binding is a critical parameter for the pharmacokinetic-pharmacodynamic correlation of any medicinally active compound, because only the 'unbound' fraction of the compound is pharmacologically active. To study the effect of analyte concentration on plasma protein binding, five concentrations (0.1, 0.5, 1.0, 5.0 and 10.0 µg/mL) of ARB-89 and ARB-92 were selected and their plasma protein binding fractions were found to be 86.3 ± 1.0 and 85.2 ± 0.5%,

respectively. Furthermore, the protein binding was also found to be concentration independent (Table 2).

Metabolism study using rat liver microsomes

Metabolic stability studies using liver microsome or S9 fractions are generally evaluated before in vivo dosing is performed. It is considered to be an important parameter because metabolic stability is directly related to the exposure, bioavailability, in vivo half-life and dosage regimen of a compound (Smith and van de Waterbeemd 1999). In vitro half-life and intrinsic clearance were calculated using the data collected from the metabolic stability studies to estimate in vivo drug pharmacokinetics and half-life. In most of the preclinical pharmacokinetic and ADME evaluation studies, the cutoff for acceptable metabolic stability is that more than 30% of the parent compound should remain unchanged after 60 min of incubation (White 2000). Both ARB-89 and ARB-92 exhibited acceptable metabolic stability with more than 50% of the compounds remained after 60 min of incubation. Based on the in vitro pharmacokinetic studies, these compounds were selected for further pharmacokinetic evaluation in rats.

Table 2 Protein binding of ARB-89 and ARB-92 in rat plasma

Concentration (μg/mL)	%Plasma protein binding	
	ARB-89	ARB-92
0.1	86.6 ± 1.0	85.9 ± 0.5
0.5	86.0 ± 0.5	85.5 ± 0.3
1.0	86.0 ± 0.3	85.5 ± 0.6
5.0	86.1 ± 0.1	85.9 ± 0.2
10.0	86.7 ± 0.6	86.6 ± 0.2
Mean	86.3 ± 1.0	85.2 ± 0.5

Each value represents N = 3 each concentration, mean ± SD

Pharmacokinetic studies in rats

After intravenous and oral administration of ARB-89 and ARB-92, close and continuous visual monitoring of the animals revealed that there were no severe acute toxicity responses to the compounds, as none of the animals showed any signs of behavioral or neurological toxicity during the entire study period. The plasma concentration–time profile following a single oral or intravenous dose of ARB-89 and ARB-92 is shown in Fig. 3. Following the intravenous administration, ARB-89 and ARB-92 were quantified up to 4 and 6 h, because the analyte concentrations were below LLOQ after 12 and 24 h post intravenous dose, respectively. A non-compartmental approach was applied for the calculation of pharmacokinetic parameters. Pharmacokinetic parameters are shown in Table 3. Following the oral dose (100 mg/kg), both ARB-89 and ARB-92 were absorbed very slowly, and C_{max} was observed around 8 h post dosing. ARB-92 showed a 36-fold higher C_{max} (1457.5 ± 30.9 ng/mL) than ARB-89 (40.3 ± 1.7 ng/mL). For ARB-89 and ARB-92, V_d (187.2 ± 26.5 and 9.9 ± 0.7 L/kg) is larger than the total blood volume (0.054 L/kg; Davies and Morris 1993) of the rats indicating the extra-vascular distribution. Extensive accumulation of both compounds in body tissues may be responsible for the long T_{max} (≥7.5 h).

The carbonate dimer of artemisinin, ARB-92, was found to has better oral exposure than the carbamate analog, ARB-89, as the AUC and $t_{1/2}$ were 41.6- and 1.4-fold higher than that of ARB-89 for the same oral dose (Table 3). The plasma CL of ARB-89 and ARB-92 was found to be 92.8 ± 5.6 and 2.9 ± 0.7 L/h kg, and both compounds were eliminated unchanged through urine and feces (Fig. 4). The absolute oral bioavailability of ARB-89 and ARB-92 was 23.4 and 34.0%, respectively.

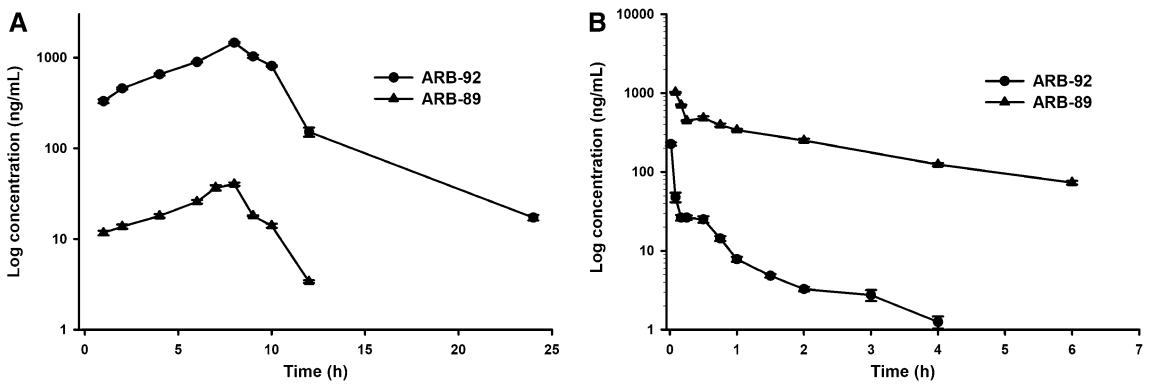


Fig. 3 Mean plasma concentration–time profiles of ARB-89 and ARB-92 after single **A** oral (100 mg/kg) and **B** intravenous administration (5 mg/kg) in male *Sprague Dawley* rats (N = 6). The graphical bar represents the SD

Table 3 Pharmacokinetic parameters of ARB-89 and ARB-92 after 100 mg/kg oral and 5 mg/kg intravenous routes of administration in male *Sprague Dawley* rats

Parameters	Oral		Intravenous	
	ARB-89 (**3**)	ARB-92 (**4**)	ARB-89 (**3**)	ARB-92 (**4**)
C_{max} (ng/mL)	40.3 ± 1.7	1457.5 ± 30.9*	–	–
T_{max} (h)	7.8 ± 0.5	8.0 ± 0.0	–	–
AUC (ng h/mL)	251.8 ± 8.3	10702.0 ± 143.5*	53.8 ± 2.2	1572.1 ± 45.3*
$t_{1/2}$ (h)	1.2 ± 0.2	2.7 ± 0.2*	1.2 ± 0.2	2.3 ± 0.2*
V_d (L/kg)	160.1 ± 8.3	11.3 ± 0.5*	187.2 ± 26.5	9.9 ± 0.7*
Clearance (L/h kg)	92.8 ± 4.2	2.9 ± 0.2*	92.8 ± 5.6	2.9 ± 0.7*
Bioavailability (%)	23.4	34.0	–	–

Each value represents N = 6, mean ± SD

AUC area under the plasma concentration–time curve, C_{max} plasma peak concentration, T_{max} time to C_{max}, $t_{1/2}$ elimination half-life, V_d volume of distribution

*$p < 0.0001$

Discussion

Both compounds, ARB-89 and ARB-92, exhibited potent antimalarial activity, adequate metabolic stability and acceptable exposure to erythrocytes during the in vitro studies. However, following the in vivo pharmacokinetic studies, ARB-89 displayed a high CL and a short $t_{1/2}$ and resulted in a low oral bioavailability (23%). Poor aqueous solubility and a high molecular weight (MW = 777.5) might be the major reasons behind the incomplete absorption and low bioavailability of ARB-89. The excretion studies showed that 24% of the dosed compound ARB-89 was excreted unchanged through the feces, while only 3.7% was estimated through the urine. Following the oral dose of ARB-89 (100 mg/kg), the C_{max} was found to be 40.3 ± 1.7 ng/mL. This low level can be ascribed to the large V_d of ARB-89 that was extensively distributed in the body with a V_d of 187.2 ± 26.5 and 160.1 ± 8.3 L/kg after oral and intravenous administration, respectively. The possibility exists that the majority of the compound exists outside the blood by accumulation in various tissues.

ARB-92 displayed a lower CL (2.9 ± 0.7 L/h kg) and longer $t_{1/2}$ (2.3 ± 0.2 h) when compared to ARB-89. The oral bioavailability of ARB-92 was found to be 34%, which is 1.45-fold higher than that of ARB-89. Following oral dosing, the V_d (11.3 ± 0.5 L/kg) of ARB-92 was found to be smaller than ARB-89 (160.1 ± 8.3 L/kg); thus, the C_{max} was found to be over 36.2-fold higher for ARB-92 (1457 ± 30.9 ng/mL) than ARB-89 (40.3 ± 1.7 ng/mL).

Even though the carbamate analog contains inherent amine group susceptible to form ammonium species for the improved aqueous solubility, the carbonate bridge in ARB-92 was found to have increased aqueous solubility, thereby decreasing the V_d. This indicates that more of the compound was present in the central compartment (blood) than in the peripheral compartments (tissues). Following a single oral dose the total ARB-89 and ARB-92 excreted unchanged through urine was 3.7 and 16.9%, respectively. The urinary excretion of ARB-92 was 4.6-fold higher than ARB-89. This indicates that ARB-92 is the better absorbed systematically (Fig. 4A).

Upon erythrocyte infection by *P. falciparum* shizonts, the cell's permeability is dramatically modified because of the alterations in phospholipid distribution between inner and outer sheaths of the RBC. Parasitized RBC also harbor parasite-produced proteins and pores that function as transporters for drugs like artemisinin. As a result parasitized RBC have greater permeability than non-parasitized RBC towards the artemisinin-class of drugs (Shah et al. 2009). This phenomenon is confirmed by the selective accumulation of antimalarial compounds in parasitized RBC as compared to non-parasitized RBC.

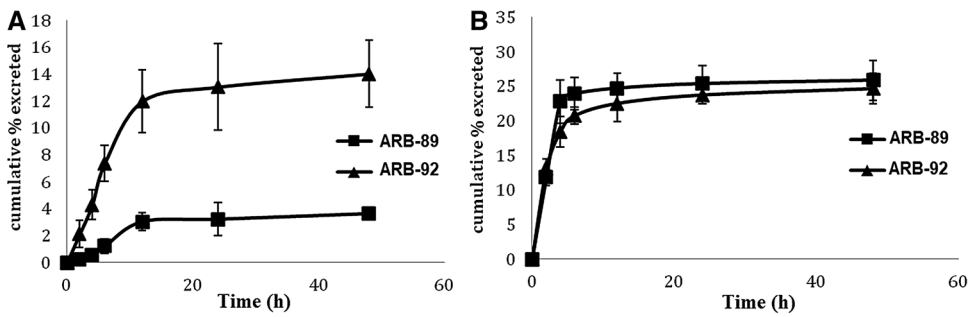


Fig. 4 Mean **A** urine and **B** feces excretion profiles of ARB-89 and ARB-92 after single oral dose administration (100 mg/kg) to male *Sprague Dawley* rats (N = 6). The *graphical bar* represents the SD

Similar observations were reported by Asawamahasakda et al. (1994) in which dihydroartemisinin was taken up selectively by parasitized RBC membranes but not by non-parasitized RBC. Both ARB-89 and ARB-92 (MW > 600) exhibited a RBC uptake of 51–56%, and artemisinin exhibited a similar value of 59%. The decrease in uptake of the dimers is minor compared to artemisinin, but it might be due to the higher molecular weight of the compounds and the linkage at C7 position. Both ARB-89 and ARB-92 have high molecular weight and poor aqueous solubility at pH 7, however despite deviating from the Lipinski's rule of five ARB-92 was found to exhibit an acceptable oral bioavailability level of 34%. This was almost equivalent to marketed antimalarial artemisinin derivatives such as arteether (35.4%) and better than some of the derivatives such as artenimol or dihydroartemisinin (19.3%), artemether (19.7%) and artesunate (29.5%) (Li et al. 1998). The half-life of ARB-92 (2.7 h) and ARB-89 (<2 h), were better than some of the marketed antimalarial artemisinin derivatives, supporting their potential evolution as antimalarial leads (Nosten et al. 1994; Li et al. 1998).

Conclusion

In this study, preclinical pharmacokinetic properties of the artemisinin-based lead compounds (ARB-89 and ARB-92) were assessed, and the parameters that play an important role in increasing oral bioavailability of antimalarial compounds were also identified. A series of ADME experiments, including bioanalytical method development, plasma protein binding, rat liver microsomal stability, RBC (both normal and parasitized) to plasma partitioning and in vivo pharmacokinetics studies in rats were performed. The studies demonstrated that ARB-92 might be a better candidate for oral antimalarial therapy than ARB-89, because it exhibited higher in vitro and in vivo activity, superior metabolic stability, longer $t_{1/2}$ and greater accumulation in RBC than ARB-89. The newly identified artemisinin based carbonate, ARB-92, showed acceptable pharmacokinetic and pharmacodynamic potential, and this study delivers a useful acumen in its further development as an antimalarial candidate drug.

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