

New Orally Active Artemisinin Dimer Antimalarials

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Abstract

Objective: To synthesize orally bioavailable artemisinin dimers and the evaluation of their in vivo antimalarial activity. **Methods:** Artemisinin dimers were synthesized and their antimalarial activity was determined in in vitro and in vivo studies (administered orally and IP). **Results:** Dimers 5 and 6 provided 100% suppression of parasitemia throughout the oral administration study, with all animals surviving up to day 28 (the last day of the study). **Conclusion:** Dimers 4-7 displayed markedly improved in vitro activity against *P. falciparum*, while the in vivo activity against *P. berghei* was highly encouraging, with 5 and 6 completely clearing parasitemia from the start of the drug treatment until the end of the study (day 28).

Keywords: Artemisinin, Artemisinin dimer, *Plasmodium berghei*, *Plasmodium falciparum*

INTRODUCTION

The natural product artemisinin (1) is a sesquiterpene endoperoxide first isolated in 1971 from the Chinese plant *Artemisia annua*.^[1] The compound was shown to have antimalarial activity against both chloroquine (CQ)-sensitive and CQ-resistant strains of *Plasmodium falciparum*. Artemisinin (1) is poorly soluble in water (<5 mg/L) and decomposes in other protic solvents, but is stable in most aprotic solvents.^[2,3] In an effort to increase stability when dissolved in oils or to enhance water solubility, numerous artemisinins have been synthesized to take advantage of their remarkable antimalarial activity.^[4,5] Because of the importance of the clinical effects of artemisinin in treating malaria, many derivatives were prepared to develop more effective and less toxic antimalarial agents.^[6-8] Initially, simple derivatives were prepared, for example, dihydroartemisinin (DHA, in which the lactone carbonyl is reduced resulting in a hemiacetal), artemether (the methyl ether of DHA), and several other ether and ester analogs.^[9,10] The sodium salt of the hemisuccinate (HS) ester (sodium artesunate) was one of these derivatives that showed more activity and less toxicity than artemether, the latter being more active than artemisinin itself. These artemisinin analogs in combination with other antimalarial drugs have been used in the artemisinin combination therapies (ACTs). ACTs are now the first line of treatments for *P. falciparum* malaria across the world.^[11,12] CQ though is recommended for

treatment of nonfalciparum malarias, namely, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*, ACTs are equivalent to CQ for treating non-falciparum malaria too.^[13,14] The utility of artemisinins has also been shown for reducing gametocytes carriage in uncomplicated malaria and prevention of malaria transmission.^[15]

Continued interest in the activity of artemisinin and DHA analogs later resulted in the preparation of artemisinin acetal dimers through reaction of DHA with boron trifluoride etherate. This finding stimulated interest in other types of DHA dimers. Artemisinin dimers linked with a polyethylene glycol spacer (3 units of ethylene glycol), an eight-carbon glycol, and a dithio-derivative, were prepared.^[16] They also prepared dimers of simpler trioxane dimers and DHA, where the linking units between the two molecules of DHA were dicarboxylic acids

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of different types.^[17] Zhang and Darbie^[18,19] proposed several DHA dimers to be linked through different coupling agents. The dimers of artemisinin and simpler trioxanes, besides prominent antimalarial potency, also exhibited significant anticancer and antiproliferative effects with some compounds being as active as calcitriol in an antiproliferative assay in murine keratinocytes. More recently, at our laboratory,^[20] we prepared a series of DHA dimers with 1,2- and 1,3-glycols, which were active in the anticancer screen carried out at the National Cancer Institute (NCI). The compounds showed promising selectivity in the 60-cell line anticancer screen, as well as activity in the antimalarial and antileishmanial screens. While these dimers had good activity in the anticancer and antiprotozoal screens, they showed limited water solubility, which imposed difficulties in the development of suitable formulations for these analogs. Additional new set of DHA dimers was prepared^[21] with functionality that would impart water-soluble characteristics. The compounds of this group represent a potential new class of antimalarial agents. The results on *in vitro* evaluation of these analogs, against CQ-susceptible and-resistant *P. falciparum* isolates and *in vivo* efficacy evaluation in *Plasmodium berghei*-mouse malaria model, are described.

MATERIALS AND METHODS

Preparation of compounds 4–7

The artemisinin dimers 4–7 were prepared according to the procedures outlined in US patents.^[20,21]

Preparation of the dimer glycerol hemisuccinate (4)

Compound 4 was prepared using dimer ketone 8 as the starting material. Dimer ketone (8) (1.94 g, 3.12 mmol) was dissolved in THF/water (225 mL, 2:1). To the stirred solution NaBH₄ (474 mg, 4 molar equivalent) was added in portions at room temperature over a 15 min period. The mixture was neutralized with HCl (2N), the solvent removed and the residue filtered, washed with water and air-dried to give dimer glycerol (9) (1.8 g, 92.5%). To a stirred solution of dimer glycerol (200 mg, 0.32 mmol) in dry methylene chloride (4 mL) were added triethylamine (0.14 mL, 1.3 equiv), dimethylaminopyridine (16 mg, 0.4 equiv), and succinic anhydride (92 mg, 3 equiv). The resulting solution was slowly stirred at room temperature for 16 h, followed by evaporation of the solvent and column chromatography eluting with hexanes/acetone (6:4) to afford 4 (156 mg, 67.2%).

¹H-NMR for one of the two symmetrical units (acetone-d₆, 500 MHz): δ 5.44 (1H, s, H-5 or H-5'), 5.40 (1H, s, H-5 or H-5'), 5.18 (1H, t, *J* = 4.98 Hz, 4.96, H-17), 4.75 (1H, d, *J* = 3.38 Hz, H-12 or H-12'), 4.74 (1H, d, *J* = 3.40 Hz, H-12 or H-12'), 4.00 (1H, m, H-16 or H-16'), 3.98 (1H, m, H-16 or H-16'), 3.61 (1H, dd, *J* = 4.5 Hz, 4.5, H-16 or H-16'), 3.56 (1H, q, *J* = 5.19 Hz, H-16 or H-16'), 2.65 (4H, m, H-19, H-20), 2.55 (1H, m, H-11), 2.30 (1H, ddd, *J* = 3.9 Hz, 3.0, 3.0, H-3), 2.07 (1H, m, H-3), 1.89 (1H, m, H-2), 1.84 (1H, m, H-8), 1.79 (1H, m, H-8), 1.69 (1H, m, H-9), 1.52 (1H, m, H-2), 1.45 (1H, m, H-7), 1.41 (1H, m, H-10), 1.33 (3H, s, H-15),

1.21 (1H, m, H-1), 0.97 (3H, dd, *J* = 6.4 Hz, 0.6, H-14), 0.96 (1H, m, H-9), 0.95 (3H, d, *J* = 7.4 Hz, H-13); ¹³C NMR for one of the two symmetrical units (acetone-d₆, 125 MHz): δ 172.99 (s, CO-OH), 171.7 (S, -CO-O-), 103.9 (s, C-4), 102.46 (d, C-12), 102.32 (d, C-12'), 88.02 (d, C-5), 87.99 (d, C-5'), 80.97 (s, C-6), 71.92 (d, C-17), 66.74 (t, C-16), 66.59 (t, C-18), 53.09 (d, C-1), 44.89 (d, C-7), 37.63 (d, C-10), 36.70 (t, C-3), 34.98 (t, C-8), 31.33 (d, C-11), 31.29 (d, C-11'), 29.48 and 28.78 (t, methylenes of CO-CH₂-CH₂-CO), 25.72 (q, C-15), 24.98 (t, C-2), 24.7 (t, C-9), 20.23 (q, C-14), 12.81 (q, C-13). HRESIFTMS *m/z* 723.3627 [M-H]⁺ (calcd for C₃₇H₅₅O₁₄, 723.3592).

Preparation of dimer oxime (5) starting from dimer ketone (8)

Dimer ketone 8 (100 mg, 0.16 mmol), sodium acetate (80 mg, 0.48 mmol), and hydroxylamine hydrochloride (14 mg, 0.1 mmol) were reacted in 10 mL of freshly distilled dichloromethane. The reaction mixture was then refluxed for 30 min under argon. Thin layer chromatography (TLC) indicated that the reaction was complete. The resulting reaction product was evaporated to dryness. The residue was dissolved in 6 ml of ethyl acetate, washed with water, dried over anhydrous sodium sulfate, and the solvent was evaporated to dryness.

The residue was chromatographed on silica gel column and eluted with hexane:ethyl acetate (90:10) with polarity increasing to 70:30. Fractions were collected and combined according to TLC similarities to give one major fraction of the desired product (76.0 mg). The spectral data were consistent with structure 5 provided in Figure 1 and ¹H-NMR (CDCl₃, 400 MHz) δ 5.42 (2H, s, H-5, and H-5'), 4.84 and 4.80 (1H each, d each, *J* = 2.4 Hz each, H-12 and H-12'), 4.65, 4.39, 4.37, and 4.16 (1H each, d each, *J* = 14.0, 11.6, 14.0, 12.0 Hz, respectively, H-16 and H-16'); 2.66 (2H, m, H-11 and H-11'); 2.38 and 2.03 (2H each, ddd and br d, respectively, *J* = 2.8 Hz each, H-3 and H-3'); 1.87 and 1.51 (2H each, m each, H-2 and H-2'); 1.74 (4H, m, H-9, and H-9'); 1.62 and 1.48 (4H each, m each, H-7 and H-7', H-8 and H-8', and H-10 and H-10'); 1.43 (6H, s, Me-15 and Me-15'); 1.26 (2H, m, H-1, and H-1'); 0.96-0.89 (12H, Me-13 and Me-13', and Me-14 and Me-14'). ¹³C-NMR (CDCl₃, 100 MHz) δ 155.21 (s, C = N); 104.16 and 104.13 (s, C-4 and C-4'); 102.48 and 101.05 (d, C-12 and C-12'); 87.93 (d, C-5 and C-5'); 81.02 and 80.98 (s, C-6 and C-6'); 64.42 and 60.24 (t, C-16 and C-16'); 52.55 (d, C-1 and C-1'); 44.38 (d, C-7 and C-7'); 37.44 and 37.39 (d, C-10 and C-10'); 36.43 (t, C-3 and C-3'); 34.66 (t, C-9 and C-9'); 30.87 and 30.72 (d, C-11 and C-11'); 26.05 (q, C-15 and C-15'); 24.64 (t, C-2 and C-2'); 24.52 (t, C-8 and C-8'); 20.32 (q, C-14 and C-14'); 13.06 and 12.96 (q, C-13 and C-13'); HRESIFTMS *m/z* 660.3343 [M + Na]⁺ (calculated for C₃₃H₅₁NNaO₁₁, 660.3360).

Preparation of dimer oxime-hemisuccinate (6)

Dimer oxime-HS was prepared by reacting dimer oxime 5 (100 mg), with succinic anhydride (1.1 molar equivalent)

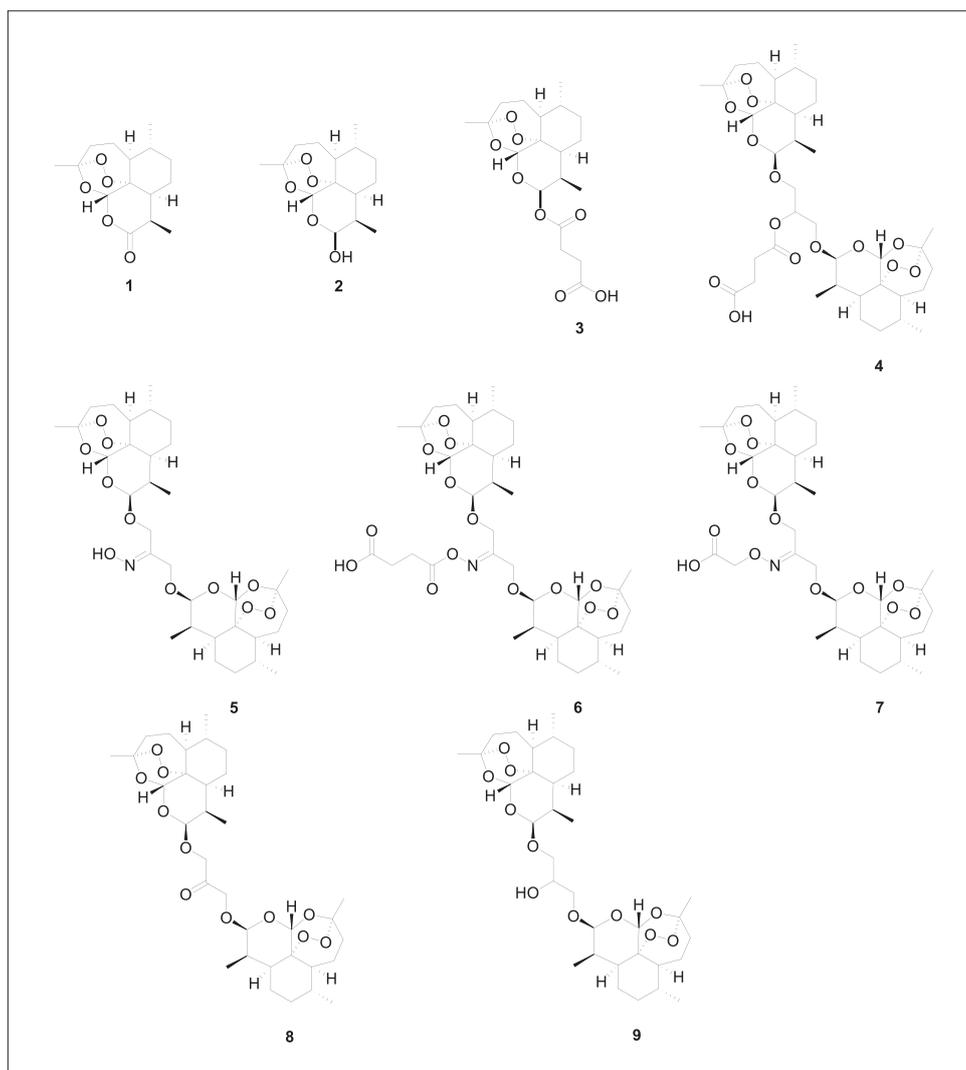


Figure 1: Structures of artemisinin (1) dihydroartemisinin (2) artesunic acid (3) and new artemisinin dimers (4–7)

in the presence of dimethylamino pyridine (DMAP, catalytic) and triethylamine (catalytic) in 3 ml of freshly distilled dichloromethane. The reaction mixture was allowed to stir at room temperature overnight, and TLC indicated a complete reaction.

The resulting reaction product was evaporated to dryness, and the residue was chromatographed on a silica gel column and eluted with hexane:ethyl acetate (90:10) with polarity increasing to 60:40. Fractions were collected and combined according to TLC similarities to give one major fraction of the desired product (96.0 mg). The mass of the compound was consistent with structure 6 provided in Figure 1, structure 6. ^1H NMR ($\text{DMSO-}d_6$, 500 MHz) δ 12.16, 11.23 (2H, each, broad singlet, OH), 5.34 and 5.32 (1H each, s each, H-5 and H-5'); 4.70 and 4.69 (1H each, d each, $J = 3.2$ Hz each, H-12 and H-12'); 4.65 and 4.38 (2H each, br d each, $J = 14.8$ and 15.6 Hz, respectively, H-16 and H-16'); 2.63, (2H, br m, H-11 and H-11'); 2.41 (4H br t, H-19 and H-20), 2.34 and 2.01 (2H each, br t and br d, respectively, $J = 13.6$ and 14.4 Hz, respectively, H-3 and H-3'); 1.85 and 1.50 (2H each, m each, H-2 and H-2');

1.73 (4H, br t, $J = 11.2$ Hz, H-9 and H-9'); 1.61 and 1.46 (4H each, m each, H-7 and H-7', H-8 and H-8', and H-10 and H-10'); 1.41 (6H, s, Me-15 and Me-15'); 1.22 (2H, m, H-1 and H-1'); 0.94-0.82 (12H, Me-13 and Me-13', and Me-14 and Me-14'); ^{13}C NMR ($\text{DMSO-}d_6$, 125 MHz) δ 173.64 (s, C = O), 153.10 (s, C = N), 103.43 (s, C-4), 103.41 (s, C-4'), 100.96 (d, C-12), 99.37 (d, C-12'), 87.09 (d, C-5), 87.05 (d, C-5'), 80.48 (s, C-6), 80.45 (s, C-6'), 64.84 and 59.58 (t, C-16 and C-18), 52.05 (d, C-1), 43.80 and 43.78 (d, C-7 and C-7'), 36.78 and 36.75 (d, C-10 and C-10'), 36.08 (t, C-3), 34.20 and 34.16 (t, C-9 and C-9'), 30.49 and 30.32 (d, C-11 and C-11'), 28.85 (t, C-19 and C-20), 25.67 (q, C-15 and C-15'), 24.20 (t, C-2), 24.04 (t, C-8), 20.21 (q, C-14), 12.75 and 12.68 (q, C-13 and C-13'); +ESI-MS m/z , 738.4 [$\text{M} + \text{H}$] $^+$, 760.6 [$\text{M} + \text{Na}$] $^+$.

Preparation of dimer oxime acid (7)

Dimer ketone 8 acetone (50 mg, 0.08 mmol), sodium acetate (40 mg, 0.48 mmol), and aminoxyacetic acid (9.1 mg, 0.1 mmol) were reacted in 5 ml of freshly distilled dichloromethane. The reaction mixture was refluxed for 4 h under argon then TLC indicated that the reaction was complete.

The resulting reaction product was dried, the residue dissolved in 6 ml of ethyl acetate, washed with water, dried again over anhydrous sodium sulfate and then evaporated to dryness. The residue (300 mg) was chromatographed on a silica gel column and eluted with chloroform followed by a chloroform: Methanol mixture with polarity increasing to chloroform: Methanol (90:10). Fractions were collected and combined according to TLC similarities to give one major fraction of the desired product (41.1 mg).

The spectral data were consistent with structure of 7 provided in Figure 1; ¹H-NMR (CDCl₃, 400 MHz) δ 8.23 (1H, br s, OH); 5.41 and 5.38 (1H each, s each, H-5 and H-5'); 4.85 and 4.81 (1H each, d each, *J* = 3.2 Hz each, H-12 and H-12'); 4.65 and 4.38 (2H each, br d each, *J* = 14.8 and 15.6 Hz, respectively, H-16 and H-16'); 4.62 (2H, s, H-18); 2.63 (2H, br m, H-11, and H-11'); 2.34 and 2.01 (2H each, br t and br d, respectively, *J* = 13.6 and 14.4 Hz, respectively, H-3 and H-3'); 1.85 and 1.50 (2H each, m each, H-2 and H-2'); 1.73 (4H, br t, *J* = 11.2 Hz, H-9 and H-9'); 1.61 and 1.46 (4H each, m each, H-7 and H-7', H-8 and H-8' and H-10 and H-10'); 1.41 (6H, s, Me-15 and Me-15'); 1.22 (2H, m, H-1 and H-1'); 0.94-0.82 (12H, Me-13 and Me-13', and Me-14 and Me-14'). ¹³C-NMR (CDCl₃, 100 MHz) δ 174.10 (s, C = O), 156.78 (s, C = N), 104.20 and 104.14 (s, C-4 and C-4'), 102.34 and 100.73 (d, C-12 and C-12'), 87.97 (d, C-5 and C-5'), 81.04 and 80.96 (s, C-6 and C-6'), 70.35 (t, C-18), 64.69 and 61.59 (t, C-16 and C-16'), 52.47 (d, C-1 and C-1'), 44.29 (d, C-7 and C-7'), 37.42 (d, C-10 and C-10'), 36.36 (t, C-3 and C-3'), 34.59 (t, C-9 and C-9'), 30.80 (d, C-11 and C-11'), 26.06 and 26.03 (q, C-15 and C-15'), 24.46 (t, C-2 and C-2'), 24.41 (t, C-8 and C-8'), 20.36 (q, C-14 and C-14'), 12.96 (q, C-13 and C-13'); HRESIFTMS *m/z* 718.3190 [M + Na]⁺ (calculated for C₃₃H₅₃NNaO₁₃, 718.3415).

In vitro antimalarial assay

Antimalarial activity was determined *in vitro* on CQ-sensitive (D6, Sierra Leone) and -resistant (W2, Indochina) strains of *P. falciparum*. The 96 well-microplate assay is based on an evaluation of the effect of the compounds on growth of asynchronous cultures of *P. falciparum*, determined by the assay of parasite lactate dehydrogenase (pLDH) activity as described earlier.^[22] The appropriate dilutions of the drugs were prepared in DMSO or RPMI-1640 medium and added to the cultures of *P. falciparum* (2% hematocrit, 2% parasitemia), set up in clear flat bottomed 96 well plates. The plates were placed into the humidified chamber and flushed with a gas mixture of 90% N₂, 5% CO₂ and 5% O₂. The cultures were incubated at 37°C for 48–120 h. The growth of the parasite in each well was determined by pLDH assay using Malstat[®] reagent as described earlier.^[23,24] The medium and RBC controls were also set up in each plate. The standard antimalarial agents, CQ and artemisinin, were tested as the positive controls, while DMSO was tested as the negative control. The antimalarial efficacy (expressed as IC₅₀) was determined by the dose-response analysis. All the analogs were also tested for mammalian cell cytotoxicity against the African green monkey kidney epithelial (vero) cells.^[25]

In vivo antimalarial assay

Berghei (NK65)

Mouse malaria model was used to evaluate the compounds for *in vivo* antimalarial activity. The antimalarial suppressive, survival and curative activities were monitored.^[22] Male albino mice (ND4 Swiss strain) weighing 15–20 g were procured from Harlen Laboratories USA. The standard drugs and test compounds were prepared in a vehicle composition containing sterile water/EtOH (200 proof)/Cremophor EL (8:1:1). The protocol for *in vivo* antimalarial assay was approved by the IACUC of the University of Mississippi.

Animal housing

Colony bred mice (15–20 g) were used for maintaining the parasite. The mice were kept in quarantine for 1 week and provided a standard diet and water *ad libitum*. The infected mice were housed in groups of five in solid bottom cages. The control and untreated mice were housed in separate cages in the same room. All the animal procedures were conducted under sterile conditions under a laminar flow.

Infection

Mice were inoculated intraperitoneally (IP) with *P. berghei*-infected mouse blood in groups of five animals. Parasitemia was monitored by preparation of blood smears from the tail snip and staining with Giemsa. Mice with parasitemia >30% were anesthetized by IP injection of ketamine/xylazine, followed by a collection of blood from the heart in sterile citrated saline. The blood count was adjusted to 4 × 10⁷ infected erythrocytes per 100 L by dilution with sterile saline. Mice used in the *in vivo* assay were inoculated IP with 4 × 10⁷ infected erythrocytes per 100 L in sterile citrated saline.

Drug treatment

The *P. berghei*-infected mice were divided into groups of five, housed in separate cages, and each group was treated with the standard antimalarial drug or the test compounds approximately 1 h postinoculation. The test compounds 1, 4, and 5 were administered IP once daily over a three days' course at three different dosages (3.3, 10, and 30 mg/kg) for each compound. An additional group of five mice was treated with the vehicle control. The test compounds 5, 6, and 7 were administered orally once daily over a three days' course at three different dosages (11.1, 33.3 and 100 mg/kg) for each compound. Two additional groups of five mice each were treated with the vehicle control and the standard antimalarial drug sodium artesunate (3) (30 mg/kg), respectively. The mice were closely monitored for a minimum of 1 h posttreatment. Body weight, movements and behavior were monitored once daily for 28 days postinfection or until death. Parasitemia was recorded on days 5, 7, 10, 14, 21, and 28 postinoculation. The levels of parasitemia and mean survival time (MST) were compared in treated and vehicle control groups. The mice free from the parasitemia up to 28 days postinoculation were considered as cured. The *in vivo* results were computed as suppression of parasitemia on day 5/7 postinoculation, MST of the animals and cure of the infection (no detectable parasitemia till day 28 postinoculation).^[22]

RESULTS

The structures of artemisinin dimers evaluated in the present study are shown in Figure 1 along with currently used artemisinin analogs. The artemisinin dimers 4–7 reported here, are water-soluble, stable at room temperature and can be readily synthesized. The pathways for chemical synthesis of these analogs are presented in Figure 2. All the analogs were checked for their purity by TLC and appeared to be pure. Later, an LC-MS/MS method was developed. A representative chromatogram is presented to show purity of the artemisinin dimer oxime [Figure 3]. Details on the column, the solvent system and other conditions used for HPLC and MS/MS analyses of the artemisinin dimers are presented in Tables 1 and 2.

In vitro antimalarial activity of artemisinin dimers

Compounds 4–7 displayed encouraging *in vitro* antimalarial activity against CQ-susceptible and resistant *P. falciparum* strains [Table 3], with appreciably enhanced IC_{50} values compared to artemisinin (1) and the CQ controls. *In vitro* antimalarial efficacy of the dimer analogs (4-7) was comparable to DHA (2). These results prompted us for evaluation of these compounds *in vivo* in *P. berghei*-mouse malaria model.

In vivo antimalarial activity of artemisinin dimers

Initially, the analogs 4 and 5 were tested *in vivo* through IP route [Table 3]. Treatment of *P. berghei*-infected mice with

dimers 4 and 5 ensured complete suppression (>99.9%) of parasitemia at the end of day five postinoculation and when administered IP at all doses, while mice treated with artemisinin (1) still retained low levels of parasitemia at 10 and

Table 1: Liquid chromatography-mass spectrometry/mass spectrometry conditions for the analysis of artemisinin dimer oxime

LC	Shimadzu Nexera dual pump
MS	AB Sciex QTrap 4500
MS source	ESI
LC column	Phenomenex Luna 5u C18 (2) 100 A 50 × 2.00 mm
Run time	16 min
Injection volume	10 μ L
LC flow rate	0.5 mL/min
LC Solvent A	Water with 0.1% formic acid
LC Solvent B	Acetonitrile with 0.1% formic acid
MS curtain gas	20
MS CAD	Medium
MS IS	5500
MS temperature	300
MS GS1	70
MS GS2	60

LC: Liquid chromatography, MS: Mass spectrometry, ESI: Electrospray ionization, CAD: collisionally activated dissociation, IS: TurbolonSpray Voltage, GS1: Nebulizer Gas, GS2: Heater Gas

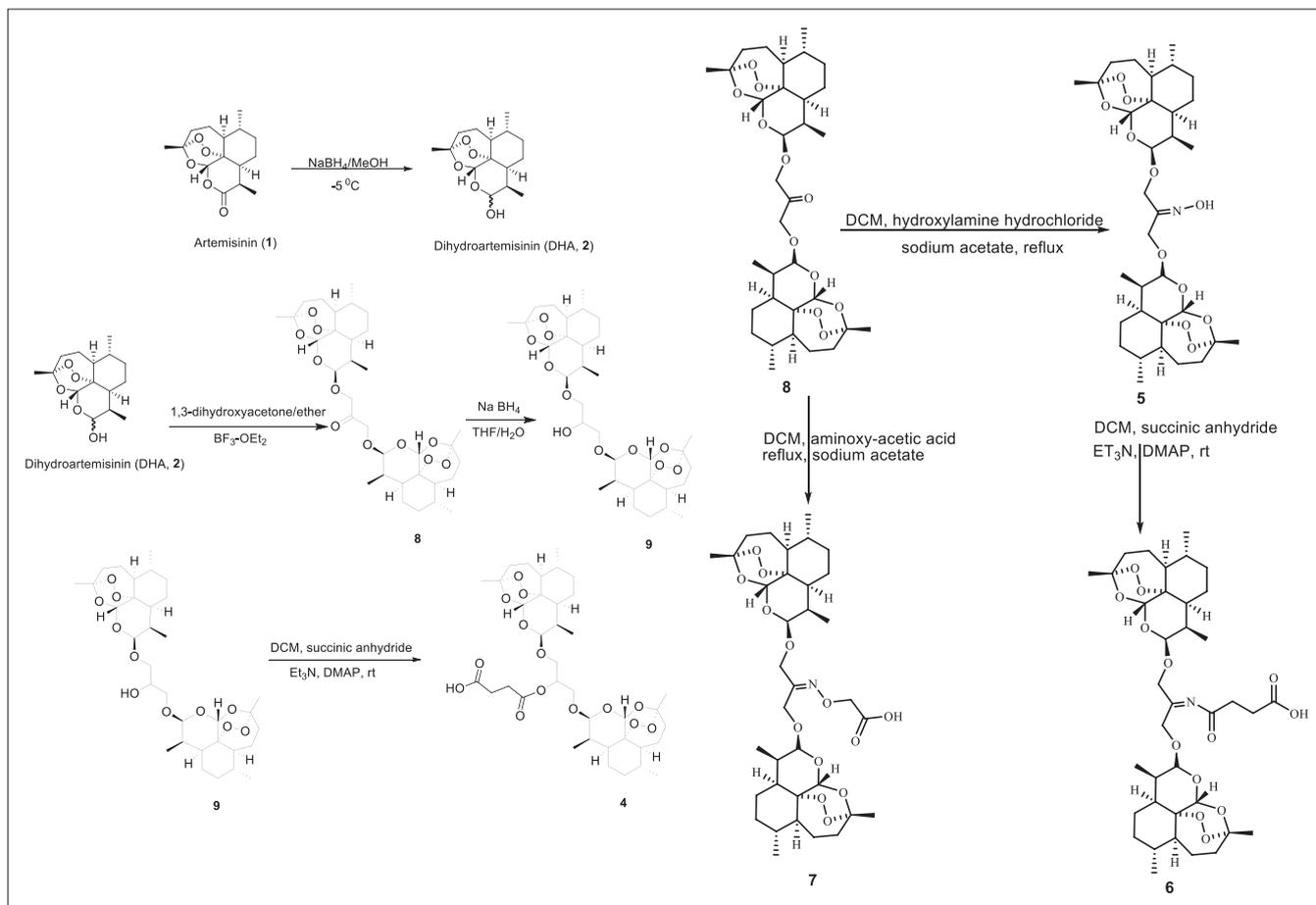


Figure 2: A general scheme for the synthesis of dimers 2–9

30 mg/kg doses [Table 4]. The parasitemia, however, gradually increased after day five in the group of mice treated with 1 [Figure 3a] and 4 [Figure 3b]. The increase in parasitemia in mice treated with 1 was comparable to the control untreated mice [Figure 4] with a just marginal increase in MST [Table 4 and Figure 4a]. The parasitemia in mice treated 30 mg/kg dose of 4 still remained significantly lower compared

to control untreated mice [Figure 4b] resulting in significant increase in MST [Figure 4b]. One (out of 5) mouse in this group remained clear from parasitemia (considered cured) until day 28 [Table 4 and Figure 4b]. All the mice (5/5) treated IP with 5 (10 and 30 mg/kg dose) remained clear until day 28 [Table 4 and Figure 4c]. While 4/5 mice treated with 3.3 mg/kg dose of 5 remained clear until day 28 [Table 4 and Figure 4c]. The dimer 5 was fully curative at 10 and 30 mg/kg dose, while at 3.3 mg/Kg dose cured 80% mice.

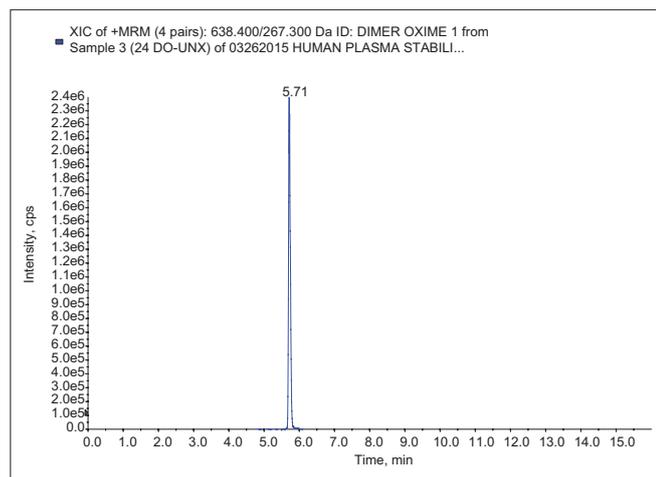


Figure 3: Liquid chromatography-mass spectrometry/mass spectrometry sample chromatogram for analysis of artemisinin dimer oxime

Table 2: Liquid chromatography-mass spectrometry/mass spectrometry time program for the analysis of artemisinin dimer oxime

LC-MS/MS time program			
Time	Module	Event	Parameter (%)
0.00	Pumps	Pump B Conc.	30
3.00	Pumps	Pump B Conc.	30
5.00	Pumps	Pump B Conc.	95
10.00	Pumps	Pump B Conc.	95
12.00	Pumps	Pump B Conc.	30
16.00	System controller	Stop	

LC: Liquid chromatography, MS: Mass spectrometry. Pump A is water with 0.1% formic acid and Pump B is Acetonitrile with 0.1% formic Acid

Promising results through IP route encouraged us to test the dimer analogs through oral route of administration. The analogs 5, 6, and 7 were tested orally (p.o.) [Table 5]. The compounds were formulated in a vehicle composition containing sterile water/EtOH (200 proof)/Cremophor EL (8:1:1). All the analogs were completely soluble in this vehicle formulation. Sodium artesunate (3), the water-soluble analog of artemisinin, and CQ were tested as positive controls in this batch of the experiment. Artesunic acid (3) and CQ produced almost complete suppression in parasitemia at the end of day 5 postinoculation and treatment [Table 3 and Figure 5a, b]. Even though, sodium artesunate (3), (tested at 30 mg/kg dose), and CQ (tested up to 100 mg/kg dose), significantly increased the MST [Table 3 and Figure 6a, b] and suppressed the parasitemia [Table 3 and Figure 5a, b], none of the standard antimalarial drugs produce cure.

Treatment of *P. berghei*-infected mice with dimers 5 (33.3 and 100 mg/kg), 6 (33.3 and 100 mg/kg), and 7 (100 mg/kg) ensured complete clearance of parasitemia at the end of day five postinoculation and treatment, when administered orally [Table 5 and Figure 6c-e]. Dimer 7, however, was found to be toxic at 100 mg/kg. Dimers 5 and 6 provided 100% parasitemia suppression at 100 mg/kg throughout the oral study, with all animals surviving up to day 28 (last day of the study) [Table 6 and Figure 7c].

Table 5 shows the percentage parasitemia suppression (PS) based on 100% parasitemia in the control group. A 99% suppression means an undetectable parasite in the blood. It also shows the number of animals per group of 5, which survive up to day 28 per group. These surviving animals were euthanized

Table 3: In vitro antimalarial activity of new artemisinin dimers against chloroquine-resistant and susceptible Plasmodium falciparum

Drug/analog	<i>Plasmodium falciparum</i>				Vero cell cytotoxicity TC ₅₀ (nM)
	D6 clone		W2 clone		
	IC ₅₀ (nM)	SI	IC ₅₀ (nM)	SI	
Artemisinin (1)	32.9	>51.2	42.5	>39.7	>1686
DHA (2)	14.8	95.2	14.1	100.0	1407
Dimer glycerol HS (4)	17.9	11.5	12.4	16.7	207
Dimer oxime (5)	13.8	19.3	9.9	27.0	267
Oxime HS (6)	56.9	113.3	47.4	136.0	6451
Oxime acid (7)	11.1	15.6	12.6	13.6	172
CQ	41.6	>357.9	378.3	>39.3	>14,881

SI=TC₅₀ (cytotoxicity)/IC₅₀ (*Plasmodium falciparum*). IC₅₀: Concentration causing 50% growth inhibition, TC₅₀: Concentration toxic at 50% of the cells, CQ: Chloroquine, SI: Selectivity index, DHA: Dihydroartemisinin, HS: Hemisuccinate

Table 4: In vivo antimalarial activity of new artemisinin dimers in Plasmodium berghei - mouse malaria model through intraperitoneal administration

Drug/analog (IP)	Dosage (mg/kg)	Percentage PS ^a		Survival ^b	Day of death					MST ^c	Cure ^d
		Day 5	Day 7		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5		
Control	-	0.0	0.0	0	15	20	11	11	20	15.4	0/5
1	3.3	57.9	40.2	0	23	16	22	21	26	21.6	0/5
	10	96.7	55.4	0	25	25	21	13	26	22	0/5
	30	97.5	52.8	0	15	21	21	14	26	19.4	0/5
4	3.3	99.9	95.2	0	25	25	25	25	18	23.6	0/5
	10	99.9	99.2	0	27	>28	26	26	26	26.6	0/5
	30	99.9	99.9	3	27	>28	24	>28	>28	27	1/5
5	3.3	99.9	99.9	5	>28	>28	>28	>28	>28	>28	4/5
	10	99.9	99.9	5	>28	>28	>28	>28	>28	>28	5/5
	30	99.9	99.9	5	>28	>28	>28	>28	>28	>28	5/5

^aPercentage PS is based on 100% parasitemia in the control group. 99.9% suppression shows undetectable parasite in blood, ^bNumber of animals per group of 5 surviving up to day 28 per group. The surviving animals were euthanized on 28th day, the end of the experiment, ^cMean survival time in days, ^dNumber of mice per group of 5 without parasitemia until day 28. IP: Intraperitoneal, MST: Mean survival time, PS: Parasitemia suppression

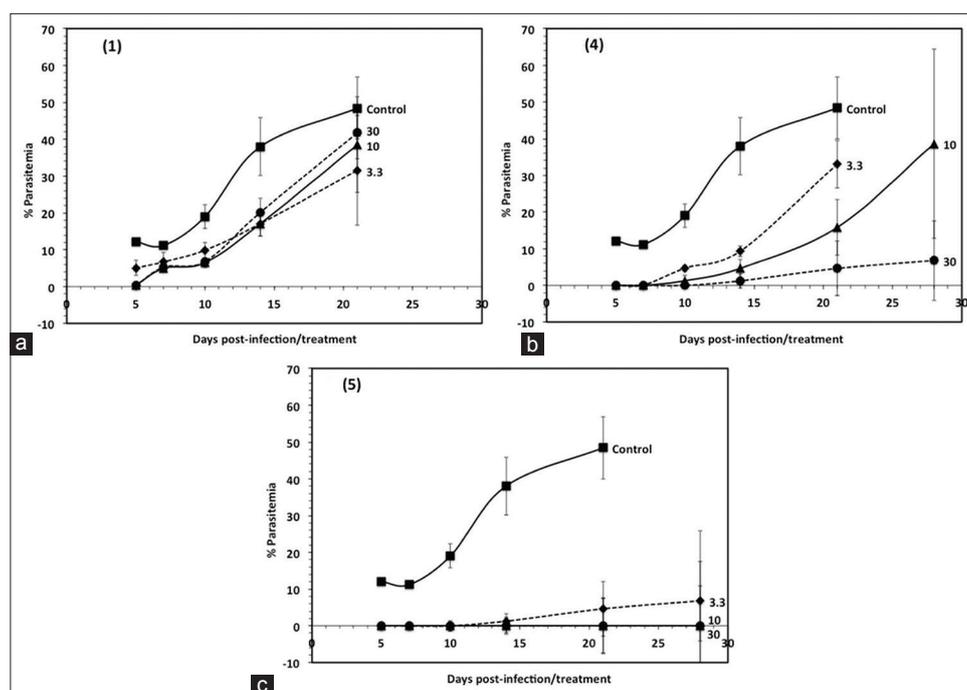


Figure 4: Progress of parasitemia in *Plasmodium berghei*-infected mice treated IP with (a) Artemisinin (1) (b) artemisinin dimer hemisuccinate (4) and (c) artemisinin dimer oxime (5). Each point values are mean \pm standard deviation of 5 or number of mice alive at the day of the analysis [Table 4]

on the 28th day, the end of the experiment. In addition, it shows the number of mice per group of 5 without parasitemia until day 28, with table 5 showing the mean survival time in days.

The body weight of the infected animals treated IP with 1 or 4 [Figure 1 and Table 4] and orally with 7 [Figure 2 and Table 5] generally decreased over the course of the study, while those treated with 5 (IP and orally) and 6 (orally) showed gain in body weight, suggesting no adverse effects for compounds 5 or 6. The gain of body weight as compared to an untreated control may be due to suppression or clearance of the parasitemia.

DISCUSSION

The proven activity of the endoperoxide artemisinin has stimulated the search for other endoperoxides, derivatives of artemisinin or totally synthetic trioxanes, with the better therapeutic profile.^[5,26] The promising therapeutic utility of artemisinin analogs has also been suggested beyond malaria, namely, antiviral, broad antiprotozoal, anthelmintic, antifungal, and more prominently anticancer actions.^[27-29] Early artemisinin derivatives prepared and evaluated have been DHA, arteether and artemether, and the HSeater of dihydroartemisinin, which are currently in clinical use. In addition, dimers of artemisinin

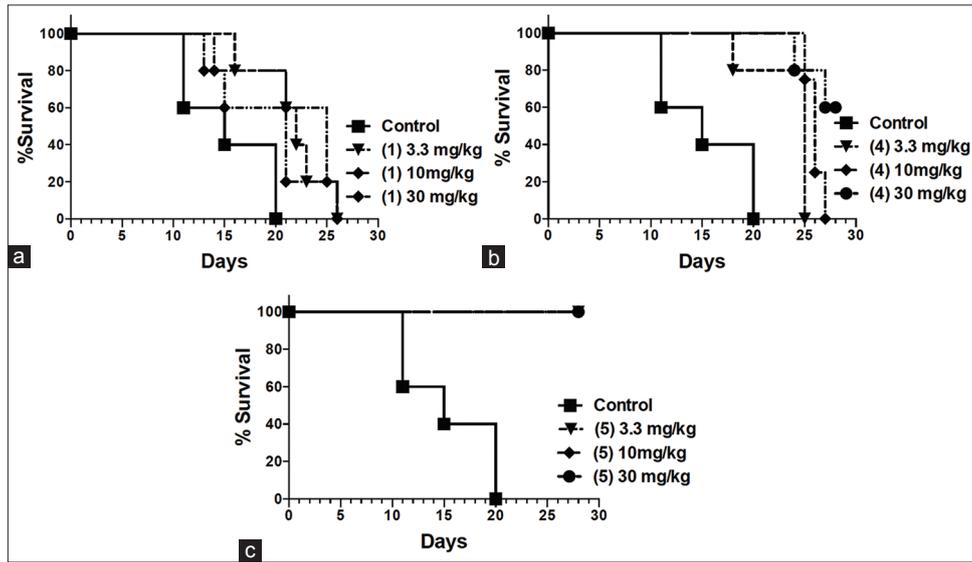


Figure 5: Survival pattern of *Plasmodium berghei*-infected mice treated IP with (a) Artemisinin (1) (b) Artemisinin dimer hemisuccinate (4) and (c) artemisinin dimer oxime (5)

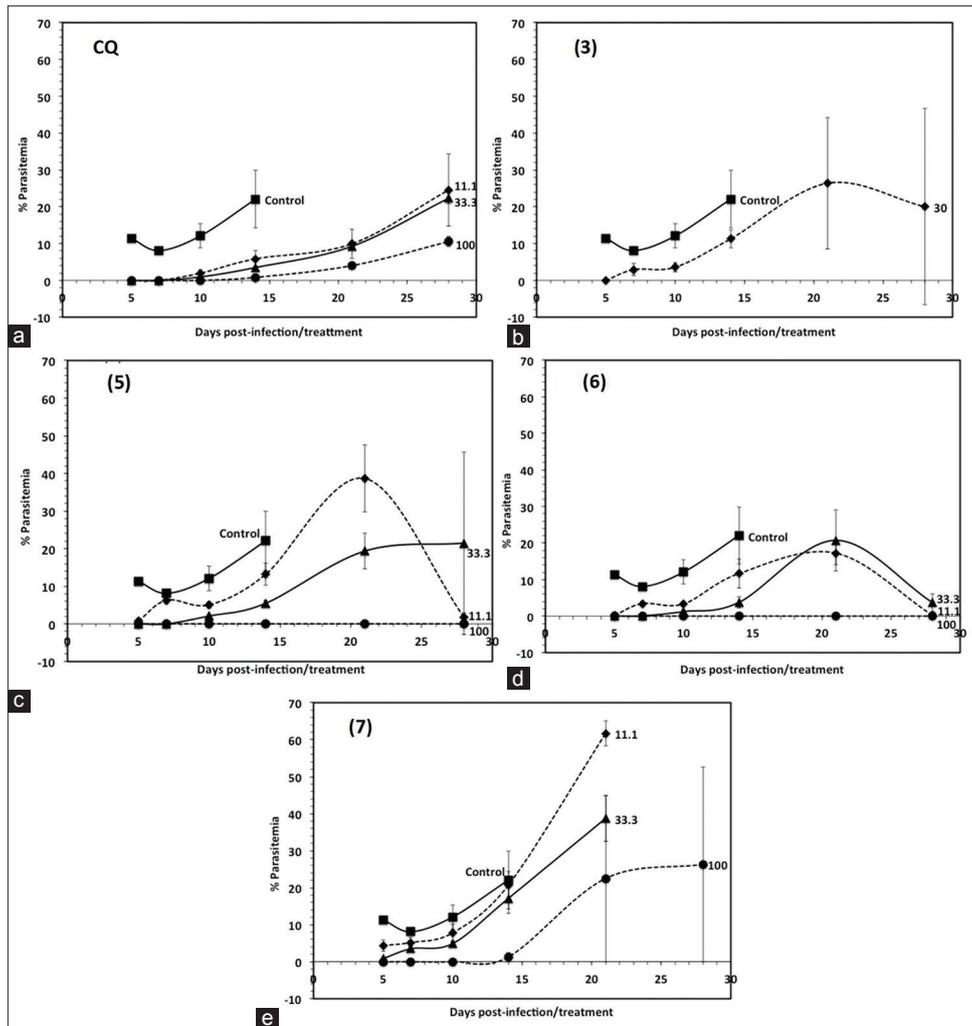


Figure 6: Progress of parasitemia in *Plasmodium berghei* infected mice treated orally with (a) chloroquine (b) sodium artesunate (3) and (c) Artemisinin dimer oxime (5), (d) artemisinin dimer oxime hemisuccinate (6) and (e) artemisinin dimer oxime acid (7). Each point values are mean \pm standard deviation of 5 or number of mice alive at the day of the analysis [Table 5]

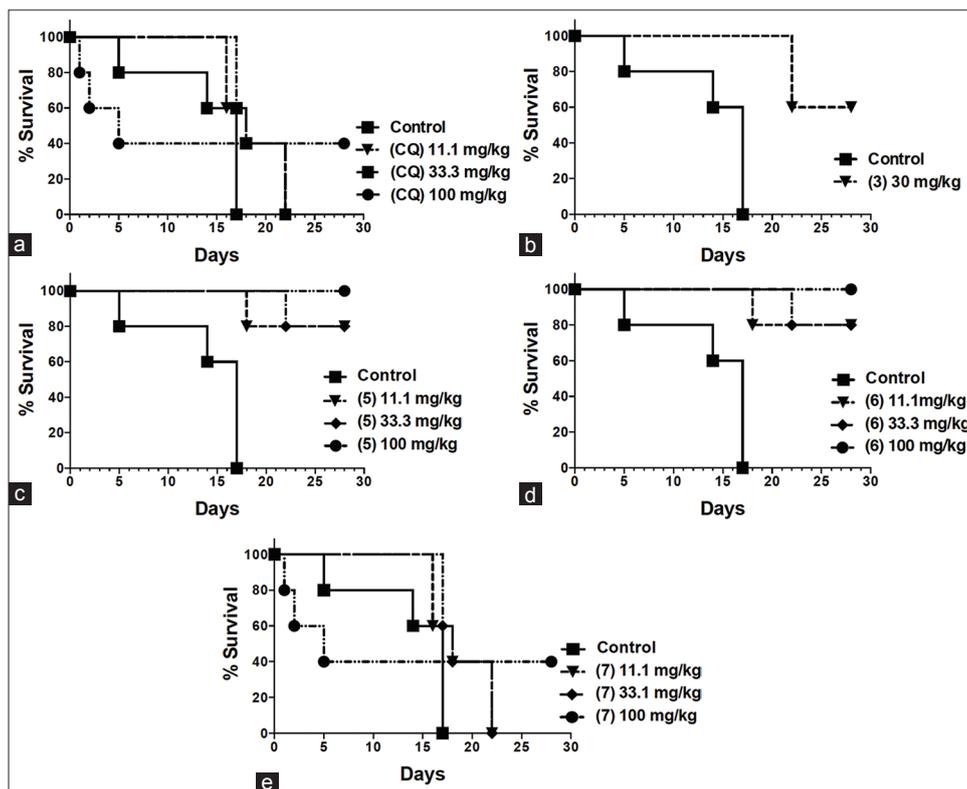


Figure 7: Survival pattern of *Plasmodium berghei*-infected mice treated orally with (a) chloroquine (b) sodium artesunate (3) and (c) artemisinin dimer oxime (5), (d) Artemisinin dimer oxime hemisuccinate (6) and (e) artemisinin dimer oxime acid (7) [Table 3]

Table 5: *In vivo* antimalarial activity of new artemisinin dimers in *Plasmodium berghei* - mouse malaria model through oral (p.o.) administration

Drug (oral)	Dosage (mg/kg)	Percentage PS		Survival	Day of death					MST	Cure	
		Day 5	Day 7		Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5			
Control	-	0.0	0.0	0	14	17	17	17	5	14	0/5	
CQ	11.1	99.9	99.9	4	>28	>28	>28	>28	>28	>28	0/4	
	33.3	99.9	99.9	5	>28	>28	>28	>28	>28	>28	0/5	
	100	99.9	99.9	5	>28	>28	>28	>28	>28	>28	0/5	
3	30	99.9	64.1	3	>28	>28	22	22	>28	25.6	0/5	
	5	11.1	93.6	22.2	4	18	>28	>28	>28	>28	26	0/5
		33.3	99.9	99.9	4	>28	22	>28	>28	>28	26	0/5
6	100	99.9	99.9	5	>28	>28	>28	>28	>28	>28	5/5	
	11.1	98.0	57.6	4	>28	>28	>28	>28	18	26	0/5	
	33.3	99.9	99.2	5	>28	>28	>28	>28	>28	>28	1/5	
7	100	99.9	99.9	5	>28	>28	>28	>28	>28	>28	5/5	
	11.1	61.3	36.0	0	16	22	18	22	16	18.8	0/5	
	33.3	92.9	57.0	0	22	22	17	18	17	19.2	0/5	
	100	99.9	99.9	2	2*	5*	>28	1*	>28	12.8	1/5	

^aPercentage PS is based on 100% parasitemia in the control group. 99.9% suppression shows undetectable parasite in blood, ^bNumber of animals per group of 5 surviving up to day 28 per group. The surviving animals were euthanized on 28th day, the end of the experiment, ^cMean survival time in days, ^dNumber of mice per group of 5 without parasitemia until day 28, *Death related to toxicity. PS: Parasitemia suppression, MST: Mean survival time, CQ: Chloroquine

prepared by joining two molecules of DHA together, either directly or through a linker moiety between the two DHA molecules.^[30,31] Most of these dimers were found to be more active as antimalarial agents than either artemisinin or DHA. Examples of those dimers include the α - and β -isomers of

dimers of DHA^[32] with no linkers in between. The asymmetric isomer was more active than the symmetric isomer, and both were more active than the corresponding monomer. Several dimers of DHA coupled through a linker were also prepared over time and tested for antimalarial activity.

Selected sets of artemisinin dimer analogs showed promising therapeutic activity and oral bioavailability as suggested by prominent antimalarial efficacy of these artemisinin dimers in *P. berghei* mouse malaria model through oral route of administration. A set of C-10 carba trioxane dimers starting from DHA-acetate were prepared.^[33] Some of these dimers were 10–20 times as active as artemisinin *in vitro*. These compounds were also active *in vivo* by both intravenous (i.v.) and oral routes at 10 mg/kg but without total suppression of parasitemia or cure of malaria infection. Subsequently, isonicotinate-N-oxide and the isobutyric acid dimer analogs were prepared from one of those dimers and their activities as antimalarial agents were compared with those of sodium artesunate.^[34] Both analogs were more active than sodium artesunate by both i.v. and oral routes. Subsequently, Chadwick *et al.*^[35] prepared another set of C-10 carba artemisinin dimers linked through a phosphate ester group. These dimers showed strong *in vitro* antimalarial activity, but no *in vivo* data were reported. More recently, Mazzone *et al.*^[36] prepared a series of artemisinin-derived dimer carbonates and thiocarbonates. These were tested *in vivo* in combination with mefloquine, which shared prolongation of the survival times of malaria-infected mice, over the clinically used artemether in combination with mefloquine. However, no data were presented on the activity of these dimers as antimalarial in a monotherapy protocol.

We report preparation and antimalarial efficacy evaluation of three types of artemisinin dimer analogs with ether type linkers and different functionalities. These functionalities were intended to improve aqueous solubility of these analogs, which may improve their oral bioavailability. The dimer analogs 4, 5, and 7 showed significantly improved antimalarial activity against both CQ-susceptible and-resistant strains of *P. falciparum* compared to artemisinin (1) and dihydroartesunate (2).

Initial evaluation of dimer analogs 4 and 5 through IP route in mouse-*P. berghei* malaria model confirmed promising *in vivo* antimalarial efficacy of the analogs, which was much superior compared to artemisinin. The analogs produced 100% cure at 10 and 30 mg/kg doses and 80% cure at a dose of 3.3 mg/kg. *In vivo* antimalarial efficacy of analog 5 was further established through oral route of administration, which confirmed significant oral bioavailability of this analog. This analog (5) produced >90% suppression in parasitemia at the 11.1 mg/kg dose. Only 1 out of 5 mice developed an early rise in parasitemia and died on day 18 postinfection. The remaining four mice showed delayed rise in parasitemia and remained alive till 28 days postinfection and treatment. The dose of 100 mg/kg produced a complete cure, i.e., all 5 mice in this group were free from parasitemia till day 28 postinfection/treatment. The dimer oxime hemisuccinate analog 6, though was >3-fold less active than 5 in *in vitro P. falciparum* antimalarial assay, showed significantly improved *in vivo* antimalarial efficacy, when administered orally, compared to the analog 5. This indicates better oral bioavailability of 6 compared to 5. The

analog 6 showed 20% cure at 33.3 mg/kg dose and 100% cure at 100 mg/kg dose. Even at 11.1 mg/kg dose analog 6 produced 98% suppression in parasitemia, and all 5/5 mice remained alive till 28 days postinfection. None of these artemisinin dimer analogs showed any apparent toxicity in mice up to the highest dose tested, i.e., 100 mg/kg/day for 3 days.

CONCLUSION

Dimers 4–7 displayed markedly improved *in vitro* activity against *P. falciparum*, while the *in vivo* activity against *P. berghei* was highly encouraging, with 5 and 6 completely clearing parasitemia from start of the drug treatment till the end of the study (day 28). The improved antimalarial efficacies of 5 and 6 compared to clinically used artemisinin antimalarials 1 and 3 could be ascribed, in part, to the aqueous solubility and improved metabolic stability of these analogs. Current *in vivo* evaluations of the analogs were done with a prototype formulation. Oral bioavailability of the analogs can be further improved by optimization of the formulation. The data presented suggest the possibility of developing an effective oral malaria treatment using better pharmaceutical formulation of one of these dimers.

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Conflicts of interest

There are no conflicts of interest.

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