Published in final edited form as:

Drug Dev Res. 2009 January 1; 71(1): 76–81. doi:10.1002/ddr.20350.

Antimalarial Preclinical Drug Development: A Single Oral Dose of A 5-Carbon-linked Trioxane Dimer Plus Mefloquine Cures Malaria-Infected Mice

Deuk Kyu Moon^a, Vandana Singhal^b, Nirbhay Kumar^b, Theresa A. Shapiro^{b,c}, and Gary H. Posner^{*,a,b}

^aDepartment of Chemistry, Johns Hopkins University, Baltimore, MD 21218

^bMalaria Research Institute, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205

^cDivision of Clinical Pharmacology, Johns Hopkins School of Medicine, Baltimore, MD 21205

Abstract

Three new 5-carbon-linked trioxane dimer carboxylate esters have been prepared from the natural trioxane, artemisinin in only 3-steps and 40–50% overall yields. Each one of these new chemical entities is at least as efficacious as the clinically used trioxane antimalarial drug artemether when combined with mefloquine hydrochloride in a low single oral dose cure.

Keywords

antimalarial chemotherapy; trioxane dimers; artemisinin combination therapy (ACT); single oral dose ACT cure

INTRODUCTION

Malaria parasites have developed widespread resistance to popular quinoline-based antimalarial drugs like chloroquine [Olliaro et al., 2000]. A new non-quinoline family of rapidly-acting antimalarial peroxides was discovered in China in the early 1970s and has become popular since then [Shizen, 2003; Klayman, 1985; O'Neill and Posner, 2004; Tang et al., 2004; Jefford, 2005; Haynes, 2006; Bégué and Bonnet-Delepon, 2007; Gelb, 2007]; natural trioxane artemisinin (**1**) and its semi-synthetic derivative trioxanes artemether (**2b**) and water-soluble sodium artesunate (**2c**) are now recommended by the World Health Organization (WHO) to be used in combination with a classical antimalarial amine drug for reliable chemotherapy of humans infected with malaria [WHO, 2006]. This artemisinin combination therapy (ACT) is now widely used in areas of the world where malaria is endemic [Ashley and White, 2005; Adjuik et al., 2004; Guttman et al., 2006; Myint et al., 2007; Sirima et al., 2009; de Pilla Varotti et al., 2008]. Typically, current ACT requires a repeated dose regimen, usually involving a total of 3–6 doses over several days [Sagara et al., 2008]. Patient compliance with adhering to such a repeated dose regimen, however, is often a serious challenge [Souares et al., 2009]. Therefore, a single dose cure is highly desirable. Toward this challenging and medically urgent goal, a series of trioxane dimers have been reported [Jung et al., 2003; Chadwick et al., 2009], including some that are able to cure malaria-infected mice after only a single subcutaneous dose [Posner et al., 2007], a

*corresponding author: gposner1@jhu.edu.

related series of trioxane dimers curative after three oral doses [Posner et al., 2007; Posner et al., 2008], and recently a trioxane dimer sulfone curative after only a single oral dose [Rosenthal et al., 2009]. We have described also a new monomeric trioxane fluoroanilide curative after only one single oral dose combined with mefloquine hydrochloride [Woodard et al., 2009].

Now we report an easily synthesized, new family of 5-carbon-linked, C-10 non-acetal trioxane dimer esters **6** (Fig 1) able to cure malaria-infected mice after only a single 7.1 mg/kg oral dose combined with mefloquine hydrochloride. These 5-carbon-linked trioxane dimer esters **6**, produced in only 3 steps and 40–50% overall yields from the natural compound **1**, complement our studies on 3-carbon-linked [Posner et al., 2003] and 4-carbon-linked [Paik et al., 2006] trioxane dimers.

MATERIALS

Dihydroartemisinin acetate (**3**), prepared from natural trioxane **1** in near quantitative yield [Posner et al., 2003], underwent tin-promoted dimerization [Posner et al., 2003] with bis-allylic silane **4** [Bigot and Breit, 2008] to form pure C-10 β ,C10 β -trioxane dimer alcohol **5** in 59% yield. X-ray crystallography of this crystalline ester **6a** confirmed the β -stereochemistry at C-10 and showed the two trioxane units to be distant from each other in the solid state (Fig. 2). The versatile secondary alcohol functional group in dimer **5** allowed diverse benzoate ester derivatives **6** to be prepared easily in only one additional step in 65–83% yields (Scheme 1). Each of these benzoate esters **6a–6c** in the absence of solvent is thermally stable at the elevated temperature of 60 °C for at least one week and at 70 °C for at least 24 hours, with less than 5% decomposition detected by ¹H NMR spectroscopy. As C-10 non-acetals, these trioxane dimers are much more hydrolytically stable than the clinically used C-10 acetal trioxane drugs **2b** and **2c**.

Preparation of 5-carbon-linked trioxane dimer alcohol **5**

A 10 mL round-bottomed flask was charged with dihydroartemisinin acetate **3** (270.0 mg, 0.87 mmol) and linker **4** (150.0 mg, 0.52 eq) in 3 mL of CH₂Cl₂ and then cooled to –78 °C. To the mixture was added a tin(IV) chloride solution (1M in CH₂Cl₂ 1.74 mL, 2 eq) dropwise over 10 min. After stirring for 50 min, the reaction mixture was quenched with NaHCO₃ (aq), extracted with CH₂Cl₂, dried over MgSO₄ and then concentrated. The crude product ($\beta\beta : \alpha\beta = 11 : 1$) was purified by silica gel chromatography (ethyl acetate/hexane 1/3) to give 165.0 mg (59% yield) of dimer alcohol **5** as a white solid. ¹H NMR : (400 MHz, CDCl₃) : 5.32 (dd, J=12Hz, 4Hz, 2H), 5.29 (s, 2H), 5.16 (d, J=12Hz, 2H), 4.71 (s, 1H), 4.50–4.47 (m, 1H), 4.41–4.36 (m, 1H), 2.68–2.65 (m, 2H), 2.34–2.25 (m, 3H), 2.21–2.12 (m, 2H), 2.07–1.99 (m, 3H), 1.91–1.88 (m, 2H), 1.79–1.75 (m, 2H), 1.66–1.59 (m, 4H), 1.46–1.35 (m, 8H including singlet 6H), 1.30–1.21 (m, 6H), 0.98–0.91 (m, 2H), 0.95 (d, J=4Hz, 6H), 0.87 (d, J=8Hz, 6H); ¹³C NMR : (100 MHz, CDCl₃) : 147.80, 147.61, 114.84, 112.32, 103.14, 103.01, 89.22, 89.12, 81.05, 81.01, 77.95, 75.53, 74.08, 52.21, 52.18, 44.24, 44.15, 37.48, 37.45, 36.61, 36.51, 34.45, 34.40, 32.39, 31.40, 30.52, 30.50, 29.67, 25.94, 25.89, 24.83, 24.76, 24.74, 24.69, 20.14, 13.05, 12.83; HRMS(FAB) calcd for C₃₇H₅₆O₉ [(M+H)⁺] 645.40026; found, 645.39919; [α]_D^{25.1} +105.1° (*c* = 0.9, CHCl₃); IR (thin film) 3457, 2937, 2874, 1450, 1378, 1206, 1124, 1090, 1052, 1007, 942, 877; mp : 51–59 °C.

Preparation of nitrobenzoate ester **6a**

A 10 mL round-bottomed flask was sequentially charged with dimer alcohol **5** (46.0 mg, 0.071 mmol), *p*-nitrobenzoyl chloride (67.0 mg, 5 eq) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in 1 mL of CH₂Cl₂ at room temperature. Finally, triethylamine (0.1 mL, 10 eq) was added to the reaction mixture. The reaction mixture was

stirred for 4h and then quenched with NH_4Cl (aq), extracted with EtOAc, dried over MgSO_4 and then concentrated. The crude product was purified by column chromatography (ethyl acetate/Hexane 1/8) to give 48.0 mg (85% yield) of ester **6a** as a white solid. Trituration with Et_2O gave crystalline ester **6a** for x-ray crystallography. ^1H NMR : (400 MHz, CDCl_3) : 8.29-8.22 (m, 5H), 6.02 (s, 1H), 5.38 (s, 2H), 5.35 (s, 1H), 5.34 (s, 1H), 5.30-5.28 (m, 2H), 4.65-4.53 (m, 2H), 2.67-2.57 (m, 2H), 2.39-2.25 (m, 4H), 2.19-2.15 (m, 2H), 2.01-1.97 (m, 2H), 1.91-1.88 (m, 2H), 1.78-1.75 (m, 2H), 1.65-1.62 (m, 4H), 1.42-1.35 (m, 8H including two singlets 6H), 1.28-1.21 (m, 6H), 0.98-0.91 (m, 2H), 0.94 (d, $J=8\text{Hz}$, 6H), 0.87 (d, $J=4\text{Hz}$, 3H), 0.85 (d, $J=8\text{Hz}$, 3H); ^{13}C NMR : (100 MHz, CDCl_3) : 163.1, 150.4, 142.45, 142.25, 135.9, 130.7, 123.5, 115.5, 114.5, 102.84, 102.81, 89.54, 89.36, 81.01, 80.88, 72.48, 71.59, 52.06, 51.99, 44.03, 43.97, 37.44 37.42, 36.64, 36.58, 34.35, 32.34, 31.11, 30.44, 30.42, 25.88, 25.84, 24.81, 24.73, 20.07, 20.04, 12.70, 12.60. $[\alpha]_{\text{D}}^{23.4} +43.50^\circ$ ($c = 1.6$, CHCl_3); IR (thin film) 2939, 1728, 1528, 1454, 1376, 1346, 1269, 1101, 1008, 877.; HRMS(ESI) m/z calcd for $\text{C}_{44}\text{H}_{59}\text{NO}_{12}\text{Na}$ ($\text{M}+\text{Na}$)+ 816.3929; found 816.3914; mp : 137–144 $^\circ\text{C}$.

Preparation of dinitrobenzoate ester **6b**

A 10 mL round-bottomed flask was charged with dimer alcohol **5** (20.0 mg, 0.031 mmol), 3,4-dinitrobenzoic acid (13.0 mg, 2 eq), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 12.0 mg, 2 eq) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in 1 mL of CH_2Cl_2 at room temperature. The reaction mixture was stirred for 2 h and then quenched with H_2O , extracted with EtOAc, dried over MgSO_4 and concentrated. The crude product was purified by column chromatography (ethyl acetate/hexane 1/8) to give 17.0 mg (65% yield) of dinitrobenzoate ester **6b** as an amorphous solid. ^1H NMR : (400 MHz, CDCl_3) : 8.62 (d, $J=4\text{Hz}$, 1H), 8.45 (d, $J=8\text{Hz}$, 1H), 7.97 (d, $J=8\text{Hz}$, 1H), 6.08 (s, 1H), 5.39-5.36 (m, 4H), 5.30 (s, 1H), 5.24 (s, 1H), 4.67-4.54 (m, 2H), 2.66-2.51 (m, 2H), 2.35-2.25 (m, 4H), 2.19-2.15 (m, 2H), 2.02-1.96 (m, 2H), 1.93-1.87 (m, 2H), 1.81-1.73 (m, 2H), 1.68-1.61 (m, 4H), 1.36 (s, 3H), 1.34 (s, 3H), 1.29-1.19 (m, 10H), 0.97-0.92 (m, 2H), 0.96 (d, $J=4\text{Hz}$, 6H), 0.89 (d, $J=4\text{Hz}$, 3H), 0.87 (d, $J=4\text{Hz}$, 3H); ^{13}C NMR : (100 MHz, CDCl_3) : 161.27, 142.28, 142.04, 135.61, 134.55, 126.41, 125.17, 116.1, 115.4, 102.84, 102.77, 89.67, 89.53, 81.78, 81.10, 81.05, 72.58, 71.72, 52.03, 51.92, 43.94, 43.84, 37.51 37.49, 36.59, 34.36, 32.48, 30.55, 30.50, 25.84, 24.88, 24.75, 20.09, 20.04, 12.63, 12.50.; $[\alpha]_{\text{D}}^{23.6} +35.72^\circ$ ($c = 1.3$, CHCl_3); IR (thin film) 2927, 1732, 1549, 1454, 1374, 1280, 1110, 1053, 1007, 845.; HRMS(ESI) m/z calcd for $\text{C}_{44}\text{H}_{58}\text{N}_2\text{O}_{14}\text{Na}$ ($\text{M}+\text{Na}$)+ 861.3780; found 861.3761.

Preparation of sulfonylbenzoate ester **6c**

A 10 mL round-bottomed flask was charged with dimer alcohol **5** (20.0 mg, 0.031 mmol), 4-(methylsulfonyl)benzoic acid (12.4 mg, 2 eq), EDC (12 mg, 2 eq) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in 1 mL of CH_2Cl_2 at room temperature. The reaction mixture was stirred for 2 h and then quenched with H_2O , extracted with EtOAc, dried over MgSO_4 and concentrated. The crude product was purified by column chromatography (ethyl acetate/hexane 1/2) to give 19.7 mg (78% yield) of sulfonylbenzoate **6c** as an amorphous solid. ^1H NMR : (400 MHz, CDCl_3) : 8.26 (d, $J=8\text{Hz}$, 2H), 8.02 (d, $J=8\text{Hz}$, 2H), 6.02 (s, 1H), 5.38 (s, 2H), 5.35 (d, $J=4\text{Hz}$, 2H), 5.31 (d, $J=4\text{Hz}$, 2H), 4.62-4.60 (m, 1H), 4.58-4.56 (m, 1H), 3.07 (s, 3H), 2.68-2.58 (m, 2H), 2.37-2.26 (m, 4H), 2.19-2.15 (m, 2H), 2.02-1.98 (m, 2H), 1.93-1.88 (m, 2H), 1.79-1.75 (m, 2H), 1.66-1.62 (m, 4H), 1.39-1.37 (m, 2H), 1.39 (s, 3H), 1.36 (s, 3H), 1.30-1.22 (m, 6H), 0.96-0.90 (m, 2H), 0.95 (d, $J=4\text{Hz}$, 6H), 0.87 (d, $J=8\text{Hz}$, 3H), 0.86 (d, $J=4\text{Hz}$, 3H); ^{13}C NMR : (100 MHz, CDCl_3) 163.42, 144.10, 142.52, 142.32, 135.37, 130.62, 127.48, 115.56, 114.42, 102.91, 102.86, 89.58, 89.39, 81.07, 80.83, 72.60, 71.65, 52.12, 52.05, 44.35, 44.02, 37.49 37.47, 36.68, 36.63, 34.39, 32.37, 31.08, 30.47, 25.92, 25.89, 24.85, 24.77, 20.12, 20.09, 12.77, 12.65.; $[\alpha]_{\text{D}}^{24.5} +45.01^\circ$ ($c = 0.95$,

CHCl₃); IR (thin film) 2939, 2875, 1728, 1646, 1455, 1321, 1269, 1176, 1155, 753.; HRMS(ESI) m/z calcd for C₄₅H₆₂O₁₂S Na (M+Na)⁺ 849.3854; found 849.3831.

ANTIMALARIAL EFFICACY

Each trioxane dimer **6a–6c** (0.90 mg) was dissolved in 0.11 mL of 7:3 Tween 80:ethanol and then diluted with 1.10 mL of distilled water for oral administration to 5-week old C57BL/6J male mice (Jackson Laboratory) weighing about 21 grams that were infected ip on day 0 with the *Plasmodium berghei*, ANKA malaria strain (2×10^7 parasitized erythrocytes) [Chen et al., 2006]. Each of 5 mice in a group was treated orally 24 hours post infection with a single dose of 0.20 mL (0.20 mL/1.21 mL $\times$ 0.9 mg = 0.15 mg) of diluted compound solution, corresponding to a dose of 7.1 mg/kg, combined with 21 mg/kg of mefloquine hydrochloride. Determining blood parasitemia levels as well as monitoring the duration of animal survival compared to survival time of infected animals receiving no drug are both widely accepted as measures of drug efficacy in antimalarial drug development. Three days after infection, an average of 8% blood parasitemia (microscopy after staining with giemsa) was observed in the control (no drug) group, and by day 10 post infection this control group showed substantial (approximately 25%) weight loss. The average survival time of the animals receiving no drug was 15.8 days post infection. All of the mice in the study receiving trioxane drug **2b** or trioxane dimers **6a–6c** plus mefloquine hydrochloride survived 30 days after infection and showed substantial (approximately 10–20%) weight gain. With mefloquine hydrochloride alone at a single oral dose of 21 mg/kg, one mouse died on day 30 and the remaining 4 mice on day 30 had an average of 25% parasitemia. Widely accepted indications of complete cure (*i.e.* 100% efficacy) are survival of animals to day 30 post infection with no detectable malaria parasites in the animal's blood at that time. Experimental results are summarized in Table 1.

RESULTS AND DISCUSSION

It is apparent from the data in Table 1 that the dimeric trioxane nitrobenzoate ester **6a**, at a single oral dose of only 7.1 mg/kg plus 21 mg/kg of mefloquine hydrochloride, is similar in antimalarial efficacy to trioxane drug **2b** and that both dinitrobenzoate ester **6b** and sulfonylbenzoate ester **6c** are fully efficacious at curing the malaria-infected mice; all five mice in this 30-day surviving group were completely cured (no parasites in their blood on day 30 post infection). All of the benzoate esters **6a–6c**, as well as trioxane drug **2b**, caused at least 99.5% suppression of parasitemia on day 3 post-infection. The parent alcohol **5** was less efficacious than benzoate esters **6a–6c** (data not shown). Neither overt toxicity nor behavioral change attributable to trioxane drug administration was observed in any of the malaria-infected animals cured by trioxane benzoate esters **6a–6c** plus mefloquine hydrochloride combination.

In conclusion, easy 3-step syntheses of dimeric trioxane benzoate esters **6** were achieved in good overall yields from the natural trioxane artemisinin (**1**); scale-up synthesis to kilogram quantities of these thermally and hydrolytically stable new chemical entities is expected to be straightforward. The single oral dose antimalarial efficacy of dimeric benzoate esters **6b–6c** combined with mefloquine hydrochloride is at least as good as that of the popular clinically-used monomeric trioxane drug **2b**. Investigation of the preclinical pharmacology of dimeric trioxane benzoate esters **6a–6c** will allow a fuller comparison of the chemotherapeutic value of these semi-synthetic endoperoxides versus that of the popular antimalarial trioxane drug **2b** [Sagara et al., 2009; Gautam et al., 2009].

Acknowledgments

We thank the NIH (AI 34885 to G.H.P.), the Johns Hopkins Malaria Research Institute, and the Bloomberg Family Foundation for financial support (to G.H.P. and to N.K.).

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Fig 1.

Figure 2.
X-ray structure of nitrobenzoate **6a**

Table 1

Antimalarial efficacy using a single oral dose (7.1 mg/kg) of trioxane combined with mefloquine hydrochloride (21 mg/kg) in *Plasmodium berghei*-infected mice

Trioxane	Number of mice parasite free (day 30)	% parasitemia on day 30	% suppression of parasitemia (day 3)
2b	4	1.6 (1 mouse)	> 99.5
6a	4	1.6 (1 mouse)	> 99.5
6b	5	0	> 99.5
6c	5	0	> 99.5
mefloquine alone	1	25 (4 mice)	91.5