Antimalarial Agents

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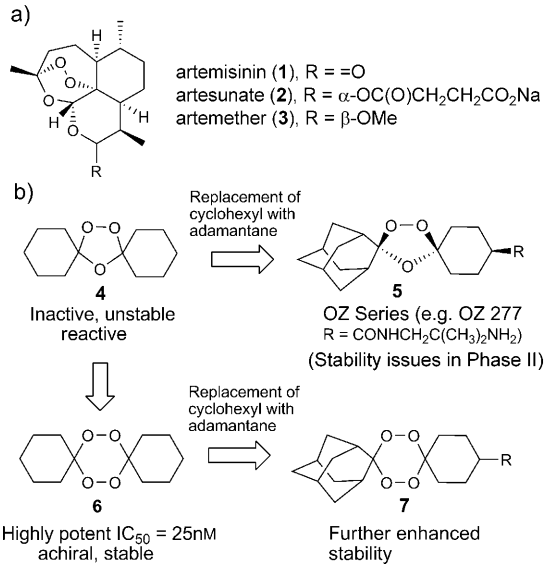
Identification of a 1,2,4,5-Tetraoxane Antimalarial Drug-Development Candidate (RKA 182) with Superior Properties to the Semisynthetic Artemisinins

Paul M. O'Neill, Richard K. Amewu, Gemma L. Nixon, Fatima Bousejra ElGarah, Mathirut Mungthin, James Chadwick, Alison E. Shone, Livia Vivas, Hollie Lander, Victoria Barton, Sant Muangnoicharoen, Patrick G. Bray, Jill Davies, B. Kevin Park, Sergio Wittlin, Reto Brun, Michael Preschel, Kesheng Zhang,* and *Stephen A. Ward*

Artemisinin (**1**) is an extract of the Chinese wormwood *Artemisia annua* and has been used since ancient times to treat malaria.[1] Today, semisynthetic derivatives artesunate (**2**) and artemether (**3**) are used clinically in drug combinations (ACT; artemisinin-based combination therapy).[2] However, first-generation analogues (e.g. **2** and **3**) have a limited availability,[3] high cost,[4] and poor oral bioavailability (Scheme 1 a).[5] In addition to these drawbacks there have been recent reports of high failure rates associated with ACTs suggesting the possibility of clinical artemisinin resistance along the Thai–Cambodian border.[6] In the light of these observations there is an urgent need to develop alternative endoperoxide-based therapies.[7]

The crucial structural functionality within artemisinin and synthetic 1,2,4-trioxanes[8] is the endoperoxide bridge. Recently a series of molecules based on an ozonide structure were developed from which the candidate OZ277[9] was shown to have impressive antimalarial activity profiles in vitro and in rodent models of malaria. However, the recent development of OZ277 has been hampered as this molecule was found to be unstable in the plasma of malaria patients during a phase II dose-ranging study.[10a,b]



Scheme 1. a) Artemisinin and its semisynthetic analogues. b) Comparison of tetraoxanes with trioxolane-based antimalarials.

In our hands, studies of endoperoxide stability have shown that 1,2,4,5-tetraoxanes[11a] (e.g. **6**) have significantly higher stability[11b] than their 1,2,4-trioxolane (**4**) or 1,2,4-trioxane counterparts.[11b] To exemplify further the chemical and biological differences between these two heterocycles it has been noted that the simple dispiro-1,2,4-trioxolane **4** is antimalarially inactive and unstable whereas the close chemically stable tetraoxane analogue **6** expresses antimalarial activity in the nanomolar range ($IC_{50} = 25$ nM) (Scheme 1 b). For good levels of antimalarial activity in the ozonide series, fusion of the 1,2,4-trioxolane ring system to an adamantane core (see for example **5**, Scheme 1) was found to be essential.[9] Given the foregoing observations we reasoned that similar substitution of the cyclohexyl group in antimalarially active analogue **6** would generate a new series of molecules with improved stability profiles and improved oral and pharmacokinetic profiles through optimization of the side chain in the generic structure **7** (R = polar water-solubilizing

[*] Prof. P. M. O'Neill, Dr. R. K. Amewu, F. Bousejra ElGarah, Dr. J. Chadwick, Dr. V. Barton
Department of Chemistry, University of Liverpool
Liverpool, L69 7ZD (UK)
E-mail: pmoneill@liverpool.ac.uk
Prof. P. M. O'Neill, Dr. J. Chadwick, Prof. B. K. Park
MRC Centre for Drug Safety Science, Department of Pharmacology
University of Liverpool, Liverpool (UK)
M. Mungthin
Department of Parasitology
Phramongkutklao College of Medicine
Bangkok (Thailand)
Dr. G. L. Nixon, Dr. A. E. Shone, Dr. S. Muangnoicharoen, Dr. P. G. Bray, J. Davies, Prof. S. A. Ward
Liverpool School of Tropical Medicine, Liverpool (UK)
Dr. S. Wittlin, Prof. R. Brun
Swiss Tropical and Public Health Institute, Parasite Chemotherapy
Basel (Switzerland)
Dr. L. Vivas, H. Lander
Department of Infectious and Tropical Disease
London School of Hygiene and Tropical Medicine, London (UK)
Dr. M. Preschel, Dr. K. Zhang
Carbogen AMCIS, Hunzenschwil (Switzerland)

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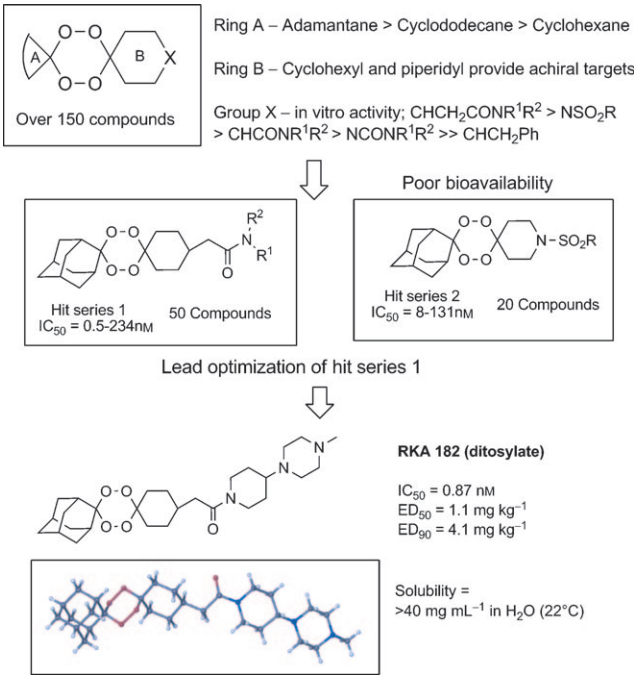
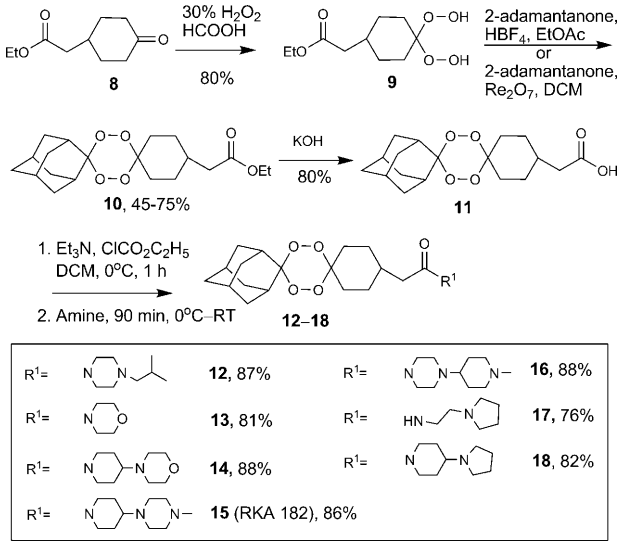
group). Identification of the optimal, metabolically stable polar side chain to counterbalance the lipophilicity of the adamantane functional group was the primary focus of the medicinal chemistry optimization.

In order to candidate-select a 1,2,4,5-tetraoxane we set a rigorous target product profile from the onset of our medicinal chemistry optimization, and for the candidate selection of RKA 182 (**15**) over 150 novel 1,2,4,5-tetraoxanes were synthesized and screened from two independent hit series (Scheme 2). After extensive in vitro, in vivo, and DMPK (drug metabolism and pharmacokinetics) studies on hit series 1 this template was selected over series 2 for lead optimization which ultimately led to the synthesis, profiling, and selection of RKA 182 (**15**; vide infra) as the development candidate.

The synthesis of lead tetraoxanes (hit series 1) is depicted in Scheme 3. The synthetic route is high-yielding, comprises only five steps, and is divergent in the last step making expedient parallel synthesis possible (see the Supporting Information).[12a] In vitro and in vivo data of the most potent compounds synthesized in hit series 1 can be seen in Table 1. These molecules exhibit an IC_{50} of less than 6 nM against both chloroquine-sensitive 3D7 and chloroquine-resistant K1 strains of *P. falciparum* with the lowest IC_{50} being 0.8 nM (Table S1 in the Supporting Information).[14] Tetraoxane analogues also have ED_{50}/ED_{90} values of less than 3.5/9.5 mg kg^{-1}, with the lowest values being $ED_{50}/ED_{90} = 0.99/$



Scheme 2. Tetraoxane candidate development:[13] SAR trends of novel 1,2,4,5-tetraoxanes and candidate selection of RKA182.



Scheme 3. Synthesis of polar tetraoxane derivatives **12–18**.

Table 1: In vitro and in vivo[15] data for tetraoxanes **12–18** and pharmacokinetic parameters after a single intravenous (1 mg kg^{-1}) and single oral (10 mg kg^{-1}) administration in the rat.

Compound	IC_{50} [nM]		% Inhibition 30 mg kg^{-1}[a]	ED_{50} [mg kg^{-1}]	ED_{90} [mg kg^{-1}]	10 mg kg^{-1} (po) $t_{1/2}$ [h]	F[b] [%]
	3D7	K1					
12	1.4 ± 0.1	0.9 ± 0.2	100	3.47	5.40	1.64 ± 0.34	9
13	5.2 ± 0.5	0.8 ± 0.3	100	3.18	3.88	5.89 ± 1.12	11
14	2.5 ± 0.2	2.8 ± 1.2	99.7	3.02	9.25	1.72 ± 0.76	9
15	4.9 ± 1.21	1.9 ± 1.9	100	1.82	8.38	3.53 ± 1.12	24
15 (tosylate salt)	0.8 ± 0.2	1.1 ± 0.8	100	1.33	4.18	2.38 ± 0.90	38 (42)[d]
16	6.0 ± 1.2	1.5 ± 1.0	100	2.23	5.12	NC	36
17	1.2 ± 1.0	0.9 ± 0.7	100	0.99	1.41	1.08 ± 0.33	9
18	1.2 ± 0.8	0.9 ± 0.4	100	1.60	2.91	1.23 ± 0.45	23
artesunate	1.8 ± 0.6	1.6 ± 0.8	100	3.96	11.72	NC	NC
artemether	7.8 ± 0.9	3.2 ± 2.3	100	3.80	12.24	ND	1.4[c]
chloroquine[c]	12.5 ± 5.6	250.0 ± 20.2	100	2	4.5	–	–

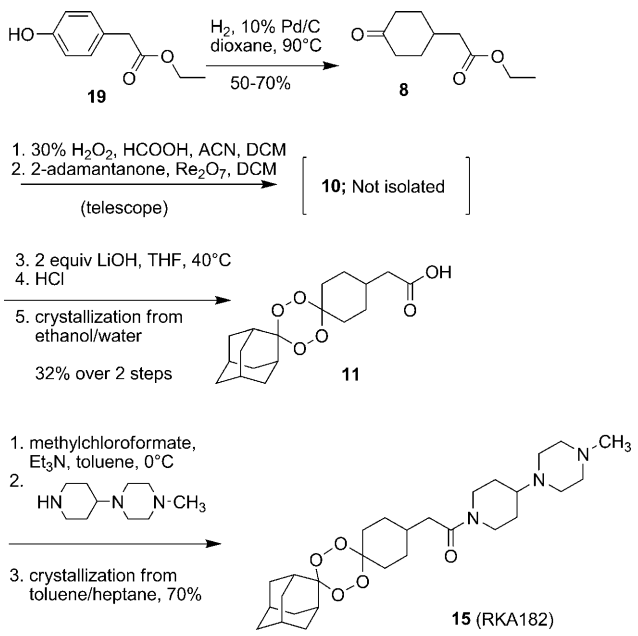
[a] Parasitemia was determined by microscopic examination of Giemsa stained blood films taken on day 4. Microscopic counts of blood films from each mouse were processed using spreadsheet (Microsoft Corp.) and expressed as percentage of inhibition from the arithmetic mean parasitemias of each group in relation to the untreated group. [b] Indicates F (oral bioavailability in rat) calculated using AUC_{0-t} and actual doses (see the Supporting Information). [c] Data taken from Vennerstrom et al.[9] NC = not calculable; ND = not determined. [d] Oral bioavailability (8 mg kg^{-1} oral/1 mg kg^{-1} iv) in mouse (average of two experiments).

1.41 mg kg^{-1} (tetraoxane **17**) when tested in mice infected with the *P. berghei* ANKA parasite. The activity of **17**, in terms of ED_{50} and ED_{90} in the four-day test,[15] surpasses that of any synthetic endoperoxide reported in the literature to date after oral administration. Piperidinyl piperazine-functionalized tetraoxane **15**, when formulated as a tosylate salt, has outstanding in vitro activity (< 1 nM) and in vivo activity with an ED_{50}/ED_{90} of 1.33/4.18 mg kg^{-1} which is superior to artemether and artesunate and comparable to artemisone, the leading next-generation semisynthetic artemisinin which is currently undergoing phase II clinical trials.[16] In studies conducted on mouse survival, mice treated at 3×10 mg kg^{-1} per day with **15** survived 22 days in comparison with only 9 days for artesunate (see the Supporting Information).

Pharmacokinetic parameters and oral bioavailability were determined after intravenous (1 mg kg^{-1}) and oral administration (10 mg kg^{-1}) in Sprague–Dawley rats (Table 1). The most significant observation from this study is that tetraoxanes **15**, **16**, and **18** have oral bioavailabilities of greater than 20% (Tables S2 and S3 in the Supporting Information). Taking this information together with the antimalarial activity data it was clear that although the activity of **12** and **17** in the four-day test was very good, the poor bioavailability of **17**'s and **12**'s complicated in vivo metabolic profile meant that these molecules were not considered further. Compounds **13** and **18** were ruled out at this point due to relatively poor bioavailability for the former and a very short half-life predicted for the latter tetraoxane. Although **16** was more potent in the mouse survival studies this tetraoxane was also ruled out due to lower maximum tolerated dose (MTD) in rats (100 mg kg^{-1} for **16** versus 400 mg kg^{-1} for **15**) and lower overall exposure (AUC) than **15** in rat pharmacokinetic studies. Based on the outstanding in vitro and in vivo antimalarial activity and pharmacokinetic profile **15** was selected as the lead candidate. Formulation work on **15** delivered the compound as a ditosylate salt which had improved oral bioavailability of 38% in the rat (42% in the mouse; Table 1 and Table S4 in the Supporting Infomration).

Having selected **15** as the drug candidate a scalable, industrial synthesis was sought. The industrial synthesis (Carbogen AMCIS) of **15** comprises only four steps (due to the synthesis of **8** in one step from direct hydrogenation of phenol ester **19**) involving a single chromatography step and has a projected low cost of goods (Scheme 4). The key step in the scale-up of this potentially hazardous chemistry involved the in situ generation of the keto ester **10** without isolation of the *gem*-dihydroperoxide **9** followed by hydrolysis (of the ester function) to provide the acid **11** in acceptable yields on a 1.2 kg scale. The application of the Dussault procedure (employing Re_2O_7 as a mild Lewis acid)[12b] for the key tetraoxane ring-forming step was also crucial to the scale-up of this chemistry.

As a part of our preclinical development **15** was also tested against eleven different southeast asian isolates from patients who had failed ACT combination chemotherapy (Figure 1), and RKA 182 (**15**) clearly demonstrated superiority over mefloquine, artesunate, and artemisinin with all measured IC_{50} values below 5 nM. (Whilst the five- to tenfold increase in potency versus artemisinins for **15** is reassuring,



Scheme 4. Industrial synthesis of tetraoxane **15** (RKA 182).

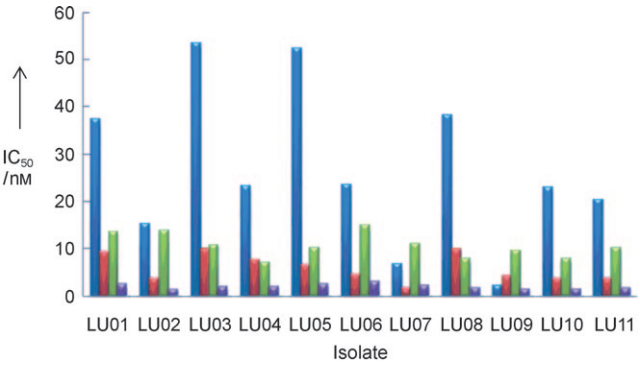


Figure 1. IC_{50} data against Cambodia–Thai patient isolates for **15** compared with other antimalarials. Blue: mefloquine; red: artesunate; green: artemisinin; purple: RKA 182.

the true test of activity versus artemisinin-resistant strains can only be assessed in a dynamic human model of malaria due to lack of stable inherent resistance phenotype in vitro.) To assess the speed of action a single oral dose of 30 mg kg^{-1} **15** was administered to mice infected with 4×10^6 *P. berghei* ANKA infected red cells two days postinfection (Figure 2). Parasitemias rapidly decreased to undetectable levels 24 h after treatment with **15**, whereas treatment with the same dose of artesunate (ASN) reduced parasitemias up to 5% 8 h posttreatment increasing rapidly thereafter.

To demonstrate the remarkable stability of tetraoxane **15** in both noninfected and infected red blood cells, in vitro studies were carried out to assess the percentage recovery of drug at set time points in noninfected and infected blood (Figure 3). It can be seen from this data that OZ277 rapidly degrades in infected blood cells giving no recovery of drug after only 35 min. In sharp contrast, RKA 182 (**15**) shows 79% recovery after 4 h in infected blood. In vivo pharmacokinetic analysis was also performed to demonstrate the impact of

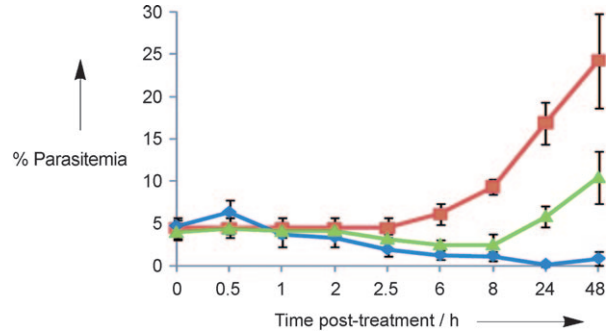
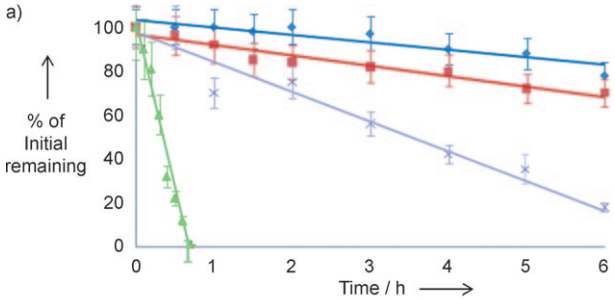


Figure 2. Speed of action of **15** (RKA182) following a single oral dose of 30 mg kg^{-1}. Red: no drug; green: ASN 30 mg kg^{-1}; blue: RKA182 30 mg kg^{-1}.

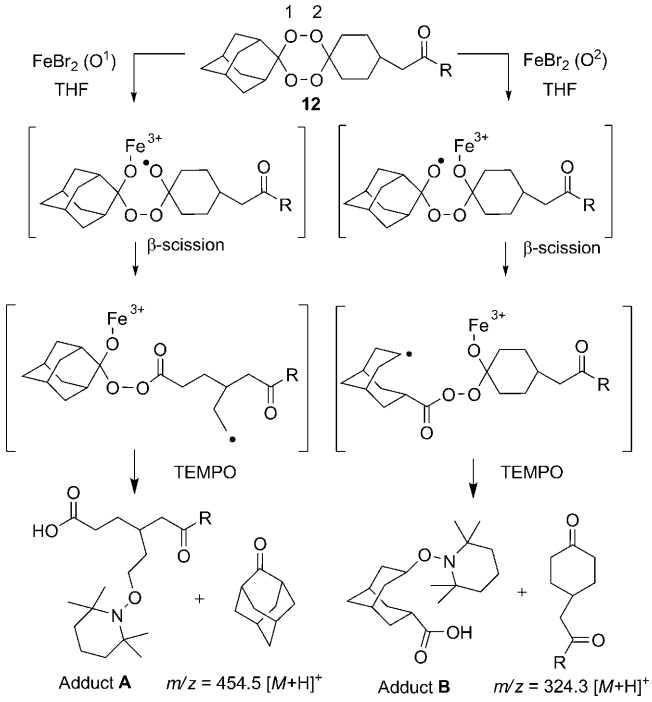


b)

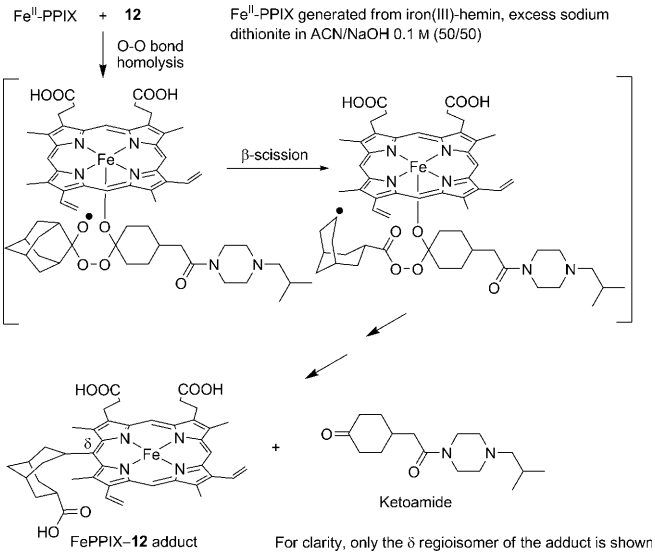
Mouse model[a]	C_{max} [ng mL^{-1}]	AUC_{tot} (h)$^+$ [ng mL^{-1}]
uninfected control	106.11	245.97
P. berghei infected	76.80	290.55

[a] Mice dosed orally with 0.75 mg RKA182 (salt) in water (16 mg kg^{-1})

Figure 3. a) Stability of **15** in noninfected and infected red blood cells in comparison with OZ277; blue diamonds: RKA182 noninfected RBC; red squares: RKA182 2% infected RBC; light gray ×: OZ277 noninfected RBC; green triangles: OZ277 1% infected RBC. b) Pharmacokinetic parameters in infected versus noninfected mice.



Scheme 5. TEMPO spin-trapping of C-radical intermediates generated following **12**–Fe^{II} activation. R = N(CH$_2$CH$_2$)$_2$NCH$_2$*i*Pr.



Scheme 6. Heme alkylation by tetraoxane **12**.

infection on the exposure profile of **15** and the data (Figure 3b) clearly demonstrates equivalent exposure (AUC) in both infected and noninfected mice. Taken together, these data suggests **15** is more stable than OZ277 in which malaria infection was associated with a significant reduction in drug plasma concentrations in phase II trials.[17]

In order to characterize the potential mediators of the antimalarial activity of RKA182 we performed mechanistic studies with iron(II) bromide in THF in the presence of the spin-trapping agent 2,2,6,6-tetramethyl-1-piperidine-1-oxyl (TEMPO). From these studies, we were able to intercept both the primary and secondary carbon centered radicals to produce two TEMPO adducts **A** and **B** (Scheme 5).

The behavior of the tetraoxanes reported here is distinct from 1,2,4-trioxolanes since only the secondary carbon centered radical species has been characterized from OZ277 and other 1,2,4-trioxolanes.[9] Since heme alkylation is believed to play a vital role in the mechanism of action of endoperoxide antimalarials[17] (Scheme 6) we examined the reactivity of **12** with ferrous heme. LC–MS analysis confirmed an *m*/*z* 782.3 Da for three adducts (maximum absorption of the Soret band at 430 nm) that result from the covalent bonding of the tetraoxane-derived secondary carbon centered radical and the heme porphyrin. This process may play an important role in the molecular mechanism of action of these derivatives.

In conclusion, we have identified the first water-soluble 1,2,4,5-tetraoxane drug candidate that has outstanding antimalarial activity, stability, low toxicity (see the Supporting

Information), and ADME properties (absorption, distribution, metabolism, and excretion) that overcome most of the problems encountered previously with the synthetic and semisynthetic antimalarial endoperoxide drugs that have progressed into preclinical development. This work firmly establishes the potential of the tetraoxane pharmacophore to provide the next generation of synthetic drugs for deployment in the control and eradication of malaria as a component of combination chemotherapy.

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