

Activities of 11-Azaartemisinin and *N*-Sulfonyl Derivatives against Asexual and Transmissible Malaria Parasites

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Abstract

Dihydroartemisinin (DHA), either used in its own right or as the active drug generated in vivo from the other artemisinins in current clinical use—artemether and artesunate—induces quiescence in ring-stage parasites of *Plasmodium falciparum* (*Pf*). This induction of quiescence is linked to artemisinin resistance. Thus, we have turned to structurally disparate artemisinins that are incapable of providing DHA on metabolism. Accordingly, 11-azaartemisinin **5** and selected *N*-sulfonyl derivatives were screened against intraerythrocytic asexual stages of drug-sensitive *Pf* NF54 and drug-resistant K1 and W2 parasites. Most displayed appreciable activities against all three strains, with IC₅₀ values <10.5 nm. The *p*-trifluoromethylbenzenesulfonyl-11-azaartemisinin derivative **11** [(4'-trifluoromethyl)-benzenesulfonylazaartemisinin] was the most active, with IC₅₀ values between 2 and 3 nm. The compounds were screened against *Pf* NF54 early and transmissible late intraerythrocytic-stage gametocytes using luciferase and parasite lactate dehydrogenase (pLDH) assays. The 2'-thienylsulfonyl derivative **16** (2'-thiophenesulfonylazaartemisinin) was notably active against late-stage (IV–V) gametocytes with an IC₅₀ value of 8.7 nm. All compounds were relatively nontoxic to human fetal lung WI-38 fibroblasts, showing selectivity indices of >2000 toward asexual parasites. Overall, the readily accessible 11-azaartemisinin **5** and the sulfonyl derivatives **11** and **16** represent potential candidates for further development, in particular for transmission blocking of artemisinin-resistant parasites.

Keywords: artemisinin; azaartemisinin; gametocytes; malaria; transmission

Introduction

Malaria is caused by the protozoan parasite of the genus *Plasmodium* and is transmitted through the bite of an infected female *Anopheles* mosquito. Globally, an estimated 3.3 billion people in 97 countries are at risk of being infected with malaria, and 1.2 billion people are at high risk.¹ According to World Health Organization (WHO) estimates, 212 million cases of malaria occurred globally in 2015 resulting in 429 000 deaths. The malaria

burden is heaviest in Africa and is ranked amongst the top five causes of death in Africa. Drugs once used in standard chemotherapeutic regimens for treatment of malaria have become ineffective through development of resistance. The most notable example is of chloroquine (CQ), for which resistance was first reported in the late 1950s on the Thai–Cambodian border. The resistant phenotype was transmitted to Africa and other parts of the world, thus essentially nullifying efforts to eradicate the disease.² The introduction of artemisinin **1** and its derivatives dihydroartemisinin (DHA) **2**, the oil-soluble artemether **3** and water-soluble artesunate **4** (Figure 1), all first developed in China as antimalarial drugs,³ appeared in large measure to redress the ineffectiveness of CQ in providing a new drug class that was notably active against CQ-resistant parasites, and in particular, induced rapid killing of intraerythrocytic parasites. In light of earlier experience with antimalarial drugs vis-à-vis emergence of resistance, the WHO proscribed the use of the clinically used artemisinins **2–4** in monotherapy, and recommended artemisinin combination therapies (ACTs) employing a fixed-dose combination of the artemisinin with a longer half-life drug.⁴ However, in spite of this measure, greatly increased parasite clearance times in patients from the Thai–Cambodian border treated with ACTs began to be noted;⁵ the phenomenon is now known to translate into an artemisinin resistance associated with mutations in the *PfKelch* gene.⁶ For the latter, a single *P. falciparum* C580Y mutant alarmingly has become dominant, and has acquired resistance to piperazine that is commonly used as a partner in ACT therapy.⁷ Resistance as such is associated with induction of dormancy by the artemisinins in intraerythrocytic ring-stage parasites that thereby become inert to the artemisinin.⁸ Significantly, it is claimed that DHA **2**, used as a drug in its own right or that is active as the metabolite common to the most widely used clinical artemisinins artemether **3** and artesunate **4**, is key to inducing dormancy through binding to the kinase phosphatidylinositol-3-kinase *Pf*PI3K to prevent its ubiquitinylation.^{9–11} Interestingly, in silico modeling presents an optimum pose for DHA in the kinase binding site interacting with the key residues D1889 and Y1915. Structurally distinct artemisinins such as artelinate or artemisone are unable to adopt the DHA pose, and do not interact with these residues. Even though DHA is notably labile under physiological conditions, it is modeled intact into the *Pf*PKI3 target. Further, it is claimed that when the drug is “washed out”, activity of *Pf*PKI3 is restored.⁹ Yet it was not established if the drug was washed out; although the normal procedure of “washing out” was followed, no analyses for drug residues in the washings were carried out. Given the structural diversity of artemisinins and synthetic peroxides that elicit potent antimalarial activities, the presentation of a model invoking classical inhibition by binding into an endogenous receptor site is not convincing. According to precedent for this type of target involving redox-sensitive signal transduction pathways, the nature of the inhibition must equate with generation of reactive oxygen species (ROS) by the artemisinin and evidently, inhibition of PI3P by ROS.¹²

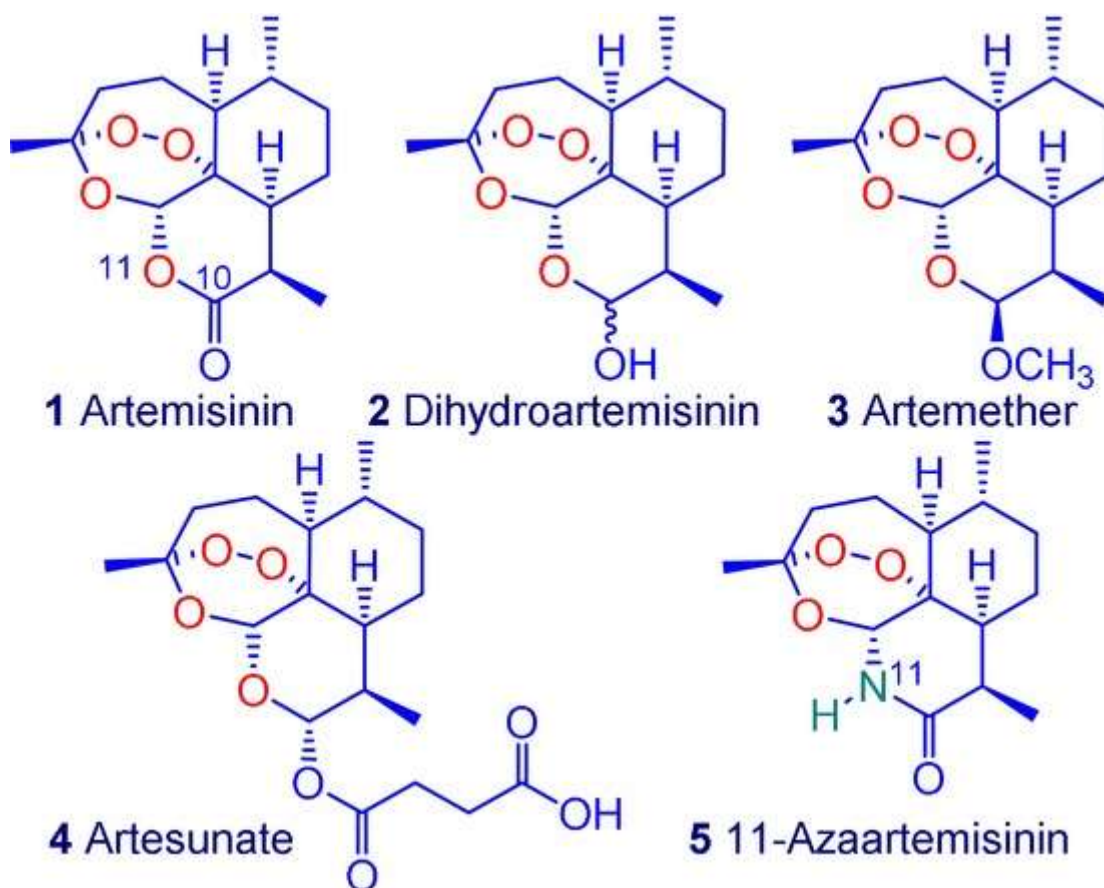


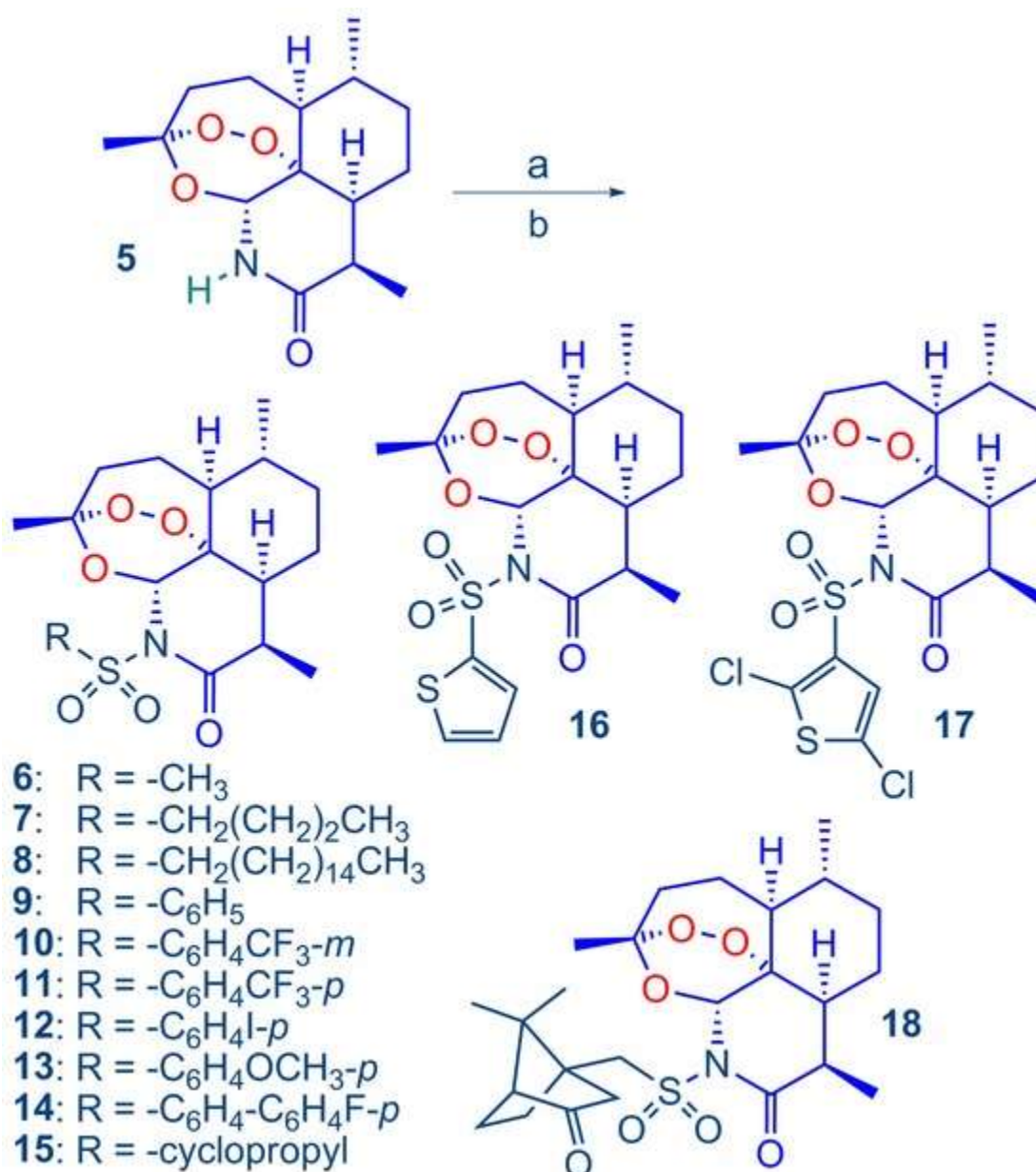
Figure 1. Structures of artemisinin **1** and its current clinical derivatives, the hemiacetal dihydroartemisinin (DHA) **2**, the lactol ether artemether **3**, and hemiester artesunate **4**. The latter two drugs essentially act as prodrugs for DHA via facile metabolism or hydrolysis, respectively. Azaartemisinin **5**, with the lactam replacing the lactone of artemisinin, is expected to be more stable at physiological pH, and is chemically incapable of providing DHA by hydrolysis or metabolism.

Overall, irrespective of the discrete mechanism that DHA exerts in induction of resistance, an examination of the efficacies of artemisinins that are unable to provide DHA on metabolism is mandated. Further, as emphasis must be placed on completely blocking transmission of the resistant phenotypes,¹³ those stages of intraerythrocytic parasites transmitted from host to vector, namely late-stage gametocytes, must also be susceptible to the new drug. However, the current clinical artemisinins either alone or incorporated into ACTs display moderate transmission blocking activities: they are at best moderately active against late-stage mature gametocytes,^{14, 15} Thus, our aim here is to develop newer artemisinins that do not provide DHA on metabolism or hydrolysis, and are active in particular against late-stage gametocytes.

11-Azaartemisinin **5** (Figure 1) is readily obtained from artemisinin and incorporates a lactam unit which typically is more stable under acidic and basic conditions than is the lactone of artemisinin.¹⁶ It is therefore expected that azaartemisinin and its derivatives will be more stable than artemisinin either at pH 7.4 or under the more acidic conditions of the stomach.^{17, 18} Further, azaartemisinin cannot provide DHA either by hydrolysis or metabolism as it is at a higher oxidation level than is DHA. Therefore, azaartemisinin and its derivatives fulfil the criterion of not providing DHA and are potentially attractive as follow

up drugs to the current clinical artemisinins. We have already demonstrated that certain azaartemisinin derivatives possess greatly enhanced thermal stabilities¹⁹ and several have good antimalarial activities.^{17, 20}

We describe here the preparation of selected *N*-alkane- and arenesulfonyl-11-azaartemisinins that are screened against asexual and sexual blood stages of *P. falciparum*. The antimalarial activities are compared with cytotoxicities of the azaartemisinins against a non-proliferating mammalian cell line. In the accompanying paper, activities of compounds selected from those described here are assessed against another apicomplexan parasite, namely *Neospora caninum*, and a set of proliferating cell lines.²¹



Scheme 1. Preparation of *N*-sulfonyl-azaartemisinin derivatives: a) LDA (1.5 equiv), THF, N₂, -78 °C, 3 h; b) RSO₂Cl (1.5 equiv), -78 °C, 3 h, silica gel chromatography, ethyl acetate/hexane (4:6).

Results and Discussion

Preparation of N-sulfonylalkyl and -aryl azaartemisinins

The parent compound 11-azaartemisinin **5** was obtained in good yield (73 %) from artemisinin and aqueous ammonia in a tetrahydrofuran/methanol mixture below 0 °C according to the literature method.¹⁹ It was deprotonated under aprotic conditions with lithium *N,N*-diisopropylamide (LDA) as previously described and then treated with the sulfonyl chloride to yield the known alkanesulfonyl azaartemisinins **6** and **8**,¹⁹ and the new *N*-sulfonyl-11-azaartemisinin derivatives **7** and **9–18** (Scheme 2).

Antimalarial activities and cytotoxicities

All compounds, with the exception of the hexadecanesulfonyl azaartemisinin **8** that turned out to be poorly soluble in the culture medium, were screened in vitro against intraerythrocytic *P. falciparum* (*Pf*) drug-sensitive NF54 and drug resistant K1 and W2. Malaria parasite proliferation was directly monitored in their intraerythrocytic environment through detecting and monitoring intraparasitic DNA replication using the SYBR Green I assay. SYBR Green I is a fluorescent dye that intercalates with DNA, and therefore a correlation between DNA content (SYBR Green I signal) and parasitemia can be used to monitor decrease in parasitemia as a measurement of inhibition of parasite proliferation.

The IC₅₀ values are summarized in Table 1. Overall the compounds were active against all three strains with the most active displaying IC₅₀ values of <10 nm. It is noteworthy that 11-azaartemisinin **5** elicited relatively very good activities (6–10.5 nm) against all three strains; in terms of cost-of-goods, the data is attractive, given the ease of accessibility of **5** from artemisinin. The *p*-trifluoromethylbenzenesulfonyl derivative **11** is the most active of the series screened here with IC₅₀ values ranging between 2.04–3.05 nm, activities similar to those of artemether **3** and artesunate **4**. Surprisingly, the *m*-trifluoromethylbenzenesulfonyl derivative **10** was the least active with IC₅₀ values ranging between 41.3–64.5 nm. The *p*-iodobenzenesulfonyl derivative **12** also exhibited poor activities (IC₅₀ 27.30–58.14 nm), and it was some twofold less active against the drug resistant K1 and W2 strains. The known methanesulfonyl derivative **6** elicited approximately the same order of efficacy with the SYBR Green I assay as recorded using the tritiated hypoxanthine assay (IC₅₀ 11.78 nm vs. 4.5 nm respectively).¹⁹ All compounds were screened against human fetal lung fibroblasts WI-38 cell line (HFLF) to gauge the relative activities toward mammalian cells with respect to asexual stages of the malaria parasite. Emetine²² was used as a standard (Table 1). 11-Azaartemisinin and the derivatives possessed several thousand-fold lower cytotoxicities.

Table 1. In vitro activities of clinically used artemisinins, azaartemisinin **5**, and *N*-sulfonyl derivatives against asexual *P. falciparum* parasites and mammalian WI-38 cells.

Compd	ClogP ^[a]	Antimalarial activity IC ₅₀ [nm] ^[b]			RI _{K1} ^[c]	RI _{W2} ^[d]	IC ₅₀ [μm]	SI ^[f]
		NF54	K1	W2				
2	2.71	0.57±0.01	0.8±0.5	0.37±0.11	1.40	0.65	ND	ND
3	3.05	1.86±0.17	2.64±0.18	6.7±0.6	1.41	3.60	ND	ND
4	2.93	3±0.29	3.26±0.2	2.4±0.2	1.09	0.8	ND	ND
5	2.63	10.48±1.36	9.44±2.93	6.02±1.81	0.90	0.57	>100	>9542
6	2.77	14.51±1.64	11.78±1.17	8.27±2.41	0.81	0.57	>100	>6892
7	4.36	4.39±0.83	5.29±1.52	4.36±0.77	1.20	0.99	>100	>22 779
9	4.73	5.17±0.44	5.26±1.77	4.18±0.65	1.02	0.81	>100	>19 342
10	5.61	41.34±13.93	60.74±7.10	64.50±7.65	1.47	1.56	>100	>2419
11	5.61	2.04±0.59	3.15±1.26	2.39±0.50	1.55	1.17	>100	>49 020
12	5.85	27.30±4.89	51.89±5.26	58.41±12.06	1.90	2.14	>100	>3663
13	4.97	27.57±8.04	40.51±12.13	30.31±2.50	1.47	1.10	>100	>3627
14	6.76	26.37±2.23	19.93±9.27	21.30±3.56	0.76	0.81	>100	>3792
15	3.35	10.15±1.05	6.19±2.40	8.89±2.80	0.61	0.88	>100	>9852
16	4.47	5.68±0.53	6.27±2.31	4.26±0.76	1.10	0.75	>100	>17 606
17	5.92	3.74±0.95	5.11±2.11	2.82±0.37	1.37	0.75	43.20	11 551
18	5.07	13.23±2.93	9.01±5.14	4.45±0.39	0.68	0.34	>100	>7559
Em ^[g]	4.36	ND	ND	ND	ND	ND	0.05	ND

[a] Calculated log*P* values using ChemBioDraw 2014. [b] Results are the mean±SEM of three independent biological replicates, each performed as technical triplicates. [c] Resistance index: IC₅₀ K1/IC₅₀ NF54. [d] Resistance index: IC₅₀ W2/IC₅₀ NF54. [e] Human fetal lung fibroblast WI-38 cell line (HFLF). [f] Selectivity index: IC₅₀ WI-38/IC₅₀ NF45. [g] Emetine. ND: not determined.

The in vitro gametocytocidal activity was determined using two different assay methods. The luciferase assay was used to determine effect of the compounds on early (>90 % stages I–III) and late-stage (>90 % stages IV–V) *Pf* NF54 gametocytes. Two transgenic parasite lines are employed in the luciferase assays: NF54-*Pf*S16-GFP-Luc which expresses the reporter gene in the early stages and NF54-Mal8p1.16-GFP-Luc which expresses the reporter gene in the late stages. The second assay method involves measurement of parasite lactate dehydrogenase (pLDH) to measure gametocyte drug sensitivity.²³ 11-Azaartemisinin **5** and the more active derivatives against asexual intraerythrocytic parasites were selected for assessment of gametocytocidal activity with the luciferase assay. However, at a 72 h incubation period with the luciferase assay, unfortunately no meaningful IC₅₀ values could be obtained against late-stage gametocytes. However, the longer incubation period (72+72 h) of the pLDH assay yielded meaningful drug activities. Thus, in those cases, where the assay did not provide meaningful results, their IC₅₀ values were determined using the pLDH assay (Table 2). Notably, 11-azaartemisinin **5** and other derivatives showed good activities (IC₅₀<1 μm) against early- and late-stage gametocytes. Compounds **16**, **17** and **18** showed preference toward late-stage gametocytes as determined with the pLDH assay. All the other compounds had preference for early-stage gametocytes. Compound **11**, which demonstrated the best activity against asexual NF54 (IC₅₀ 2.04 nm, Table 1) was also active against early- and late-stage gametocytes. Although compound **18** was specific for late-

stage gametocytes (IC₅₀ 25.3 nm), it was less active against asexual NF54 (IC₅₀ 13.23 nm, Table 1). The standout compounds were **16** and **17** with activities against asexual NF54 (IC₅₀ 5.68 and 3.74 nm, respectively, Table 1) and specific toward late-stage gametocytes (IC₅₀ 8.7 and 11.9 nm respectively, Table 2). The comparator compounds artemether **3** and artesunate **4** were active against early-stage gametocytes, but do not display the stage specificity of compound **16**. Once again, based on comparative cost-of-goods, 11-azaartemisinin **5** also presents itself as a convenient compound incapable of providing dihydroartemisinin for development into a transmission blocking drug.

Table 2. In vitro gametocytocidal data for artemisinins, azaartemisinin **6**, and *N*-sulfonyl derivatives on early-stage (EG; I–III) and late-stage (LG; IV–V) gametocytes.^[a]

Compd	EG IC ₅₀ [nm] ^[a]		LG IC ₅₀ [nm] ^[b]		Specificity ^[c] (IC ₅₀ pLDH/IC ₅₀ NF54)
	Luciferase		pLDH		
	48 h	72 h	72+72 h		
3	37.74±2.08	223.9	ND	5.9	
4	62.83±3.14	171.0	ND	2.7	
5	170.4±19.3	166.1	622.2	3.7	
6	233.1±18.0	22.0	655.9	2.8	
7	169.3±27.8	ND	564.2	3.3	
9	186.1±49.5	ND	175.1	0.9	
11	356.9±78.6	ND	85.1	0.2	
16	165.7±25.9	ND	8.7	0.05	
17	421.6±148.2	ND	11.9	0.02	
18	464.8±111.2	ND	25.3	0.06	

[a] Results against the early stages are the mean±SEM of three independent biological replicates, performed as technical triplicates (*n*=3). [b] Data for the late stages are from a single independent biological replicate (*n*=1), performed as technical triplicates. [c] Stage specificity: IC₅₀ LG/IC₅₀ EG calculated using IC₅₀ values obtained for compounds **3** and **4** as determined by luciferase assay, and IC₅₀ values obtained for all other compounds by pLDH assay; a value of >1 indicates specificity for early stages, and <1 specificity for late stages. ND: not determined.

Ideally, an antimalarial drug or drug combinations should target both the asexual blood stages and the transmissible stages such as late-stage gametocytes so as to hinder transmission of resistant parasites from an infected individual. Most antimalarial drugs target the asexual blood stage, that may cure a malaria patient but also allowing further transmission weeks after the clearance of the asexual parasites.²⁴ There is also evidence of enhanced rates of transmission of drug resistant parasites from drug-treated individuals.²⁵ Although it has been ascertained that the current clinical artemisinins can decrease gametocyte carriage,^{26, 27} in general these are not regarded as transmission blocking drugs. Thus, that compounds in the current series such as the *p*-trifluoromethylbenzenesulfonyl derivative **11** and the 2-thienylsulfonyl derivative **16** are relatively active against both asexual- and late-stage gametocytes represents an important event in the development of transmission blocking antimalarial drugs. Not least, the potent activities recorded herein for late-stage gametocytes by these artemisinins have to call into question the oft-cited, yet surely questionable hypothesis that artemisinins require activation by heme to exert

antimalarial activities,²⁸ given especially that late-stage gametocytes are not heme-dependent organisms.²⁹

The next stage of the work is to screen the best compounds in vitro against artemisinin-resistant isolates taken from patients who have not responded to malaria chemotherapy with one or more ACTs, and to develop more economic methods for the synthesis of the most active compounds. Results of work in this area will be reported separately.

Conclusions

A series of *N*-sulfonylazaartemisinin derivatives have been prepared, and most are shown to be significantly active against blood-stage asexual parasites, and against early and late-stage gametocytes. Overall the compounds are relatively non-toxic and displayed good selectivity values toward the malaria parasite vis-à-vis normal mammalian cells. 11-Azaartemisinin, and the sulfonyl derivatives **11** and **16** represent hit compounds that are to be taken forward for pharmacokinetic studies and further screening studies in vivo.

Experimental Section

Materials and methods

All reagents were of analytical grade, and were obtained from Sigma–Aldrich (South Africa) and used as supplied. Solvents were purchased from Associated Chemical Enterprises (ACE, South Africa). Tetrahydrofuran (THF; Sigma–Aldrich) was dried by storing over sodium and benzophenone under a nitrogen atmosphere and distilled prior to use. Lithium diisopropylamide (LDA) was generated in situ under a nitrogen atmosphere using *n*-butyllithium, *N,N*-diisopropylamide, and 2,2'-bipyridine as endpoint indicator. Artemisinin was purchased from Changzhou Kaixuan Chemical Co. (Chunjiang, China).

¹H and ¹³C NMR spectra were recorded on a Bruker Avance™ III spectrometer as solutions in CDCl₃. Chemical shifts (δ) are reported in parts per million (ppm), and the ¹H chemical shifts are reported downfield of tetramethylsilane (TMS) with internal reference to the residual proton in CDCl₃ (δ 7.25 ppm). ¹³C chemical shifts were internally referenced to the CDCl₃ resonances (δ 77.00 ppm). The splitting patterns are abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), and m (multiplet). Spectra were analyzed with MestReNova Software, version 5.3.2-4936. Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument in conjunction with the attenuated total reflectance (ATR) sampling technique. High-resolution mass spectrometry (HRMS) was performed on a Bruker MicroTOF Q II mass spectrometer, equipped with an ESI source set at 180 °C using Bruker Compass DataAnalysis 4.0 software. A full scan from *m/z* 50 to 1500 was performed at a capillary voltage of 4500 V, an end plate offset voltage of –500 V, with the nebulizer set at 0.4 Bar, r, and a collision cell RF voltage of 100 Vpp. Melting points (mp) were determined with a Büchi melting point B-545 instrument and were uncorrected. Column chromatography was performed using high-purity grade silica gel (pore size 60 Å, 70–230 mesh, 63–200 μ m) from Sigma–Aldrich and thin-layer chromatography was performed with silica gel plates (60F₂₅₄) from Merck.

Syntheses

11-Azaartemisinin 5: A solution of artemisinin **1** (10.0 g, 35.42 mmol) in THF (200 mL) and methanol (60 mL) was cooled down to $-15\text{ }^{\circ}\text{C}$ and treated with NH_4OH (33 % aqueous, 100 mL). The resulting mixture was left to stir for 10 h at this temperature, during which time the color changed to a very pale yellow. The solution was evaporated under reduced pressure, without heating, to leave a pale-yellow foam. This was dissolved in CH_2Cl_2 (250 mL) and treated with *p*-toluenesulfonic acid monohydrate (6.8 g, 35.84 mmol) at room temperature. The resulting mixture was left to stir for 12 h after which it was washed with 5 % aqueous sodium bicarbonate (400 mL) and water (500 mL). The organic layer was separated and dried over anhydrous MgSO_4 . The MgSO_4 was removed via filtration and the filtrate was evaporated under reduced pressure to leave the residue as a pale foam. The foam was crystallized using ethyl acetate/hexane (4:6) to yield 11-azaartemisinin **5** (7.2 g, 73 %) as colorless needles; yield: 7.2 g (73 %); mp: $143\text{--}144.5\text{ }^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3): $\delta=6.25$ (s, 1 H, H-11), 5.39 (s, 1 H, H-12), 3.27–3.19 (m, 1 H, H-9), 2.44–2.35 (m, 1 H, H-4 α), 2.04–1.96 (m, 2 H), 1.84–1.67 (m, 3 H), 1.49–1.30 (m, 6 H), 1.13 (d, $J=7.4$ Hz, 3 H, H-15), 1.04–0.95 ppm (m, 5 H); ^{13}C NMR (151 MHz, CDCl_3): $\delta=173.48$ (C10), 104.96 (C3), 79.97 (C12a), 75.70 (C12), 51.14 (C5a), 46.03 (C8a), 37.75 (C9), 36.66 (C25), 33.89 (C7), 32.88 (C9), 25.71 (C1'), 25.25 (C13), 23.16 (C5), 19.91 (C14), 12.25 ppm (C15); IR (ATR): $\tilde{\nu}_{\text{max}}=3298, 2964, 2884, 1656, 1444, 1133, 830, 730, 690\text{ cm}^{-1}$; MS (m/z): calcd for $\text{C}_{15}\text{H}_{24}\text{NO}_4^+$: 282.1705 [$M+H$] $^+$, found: 282.1700.

N-Methanesulfonylazaartemisinin 6: 11-azaartemisinin **5** (1 g, 3.55 mmol) in dry THF (10 mL) was added to a stirred solution of LDA (5.35 mmol, 1.5 equiv) in dry THF (15 mL) at $-78\text{ }^{\circ}\text{C}$. The resulting mixture was left to stir for 3 h under a nitrogen atmosphere at $-78\text{ }^{\circ}\text{C}$ after which methanesulfonyl chloride (0.5 mL, 6.46 mmol, 1.5 equiv) was added to the reaction mixture. The solution was left to stir for another 3 h at $-78\text{ }^{\circ}\text{C}$ and then for another 30 min at room temperature. The mixture was quenched with saturated aqueous ammonium chloride (20 mL), diluted with water (10 mL) and extracted with ethyl acetate (3 \times 30 mL). The extracts were washed with brine (30 mL), dried (MgSO_4) and then filtered. The filtrate was evaporated under reduced pressure to leave a white solid, which was submitted to flash column chromatography with ethyl acetate/hexane (4:6). The eluent was evaporated to dryness to leave **6** as a pale-yellow powder (481 mg, 38 %), mp: $245\text{ }^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3): $\delta=6.04$ (s, 1 H, H-12), 3.43–3.29 (m, 4 H, H-1' and H-9), 2.44–2.34 (m, 1 H, H-4 α), 2.03–1.95 (m, 2 H), 1.84–1.67 (m, 3 H), 1.50–1.30 (m, 2 H), 1.43–1.33 (m, 4 H), 1.26–1.20 (m, 4 H), 1.06–0.98 ppm (m, 4 H); ^{13}C NMR (151 MHz, CDCl_3): $\delta=174.09$ (C10), 105.37 (C3), 80.37 (C12a), 78.83 (C12), 51.45 (C5a), 44.43 (C8a), 44.23 (C1'), 37.34 (C1'), 36.51 (C9), 36.15 (C4), 33.71 (CH_2), 25.45 (C13), 24.88 (CH_2), 22.40 (CH_2), 19.66 (C14), 13.79 ppm (C15); IR (ATR): $\tilde{\nu}_{\text{max}}=2978, 1709, 1446, 1349, 1165, 963, 519, 485\text{ cm}^{-1}$; MS (m/z): calcd for $\text{C}_{16}\text{H}_{26}\text{NO}_6\text{S}^+$: 360.1480 [$M+H$] $^+$, found: 360.1439. The data are in agreement with those reported in the literature.¹⁹

1'-Butanesulfonylazaartemisinin 7: Compound **7** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 1-butanesulfonyl chloride (0.7 mL, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) as pale-yellow powder (484 mg, 34 %), mp: $120\text{ }^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3): $\delta=6.03$ (s, 1 H, H-12), 3.71–3.61 (m, 1 H, H-1' α), 3.52–3.44 (m, 1 H, H-1' β), 3.40–3.32 (m, 1 H, H-9), 2.43–2.35 (m, 1 H, H-4 α), 2.08–1.99 (m, 2 H), 1.96–1.85 (m, 2 H,

(CH₂), 1.81–1.66 (m, 3 H), 1.55–1.45 (m, 4 H), 1.44–1.30 (m, 4 H, H-13 and H-5a), 1.23–1.17 (m, 4 H), 1.06–0.99 (m, 4 H), 0.95 ppm (t, *J*=7.4 Hz, 3 H, H-4'); ¹³C NMR (151 MHz, CDCl₃): δ=174.19 (C10), 105.39 (C3), 80.38 (C12a), 78.34 (C12), 56.76 (C1'), 51.54 (C5a), 44.82 (C8a), 37.29 (CH), 36.59 (CH₂), 35.90 (C9), 33.66 (CH₂), 25.41 (C13), 24.85 (CH₂), 24.59 (CH₂), 22.33 (CH₂), 21.67 (CH₂), 19.65 (C14), 13.67 (C15), 13.52 ppm (C4'); IR (ATR): $\tilde{\nu}_{\max}$ =2931, 2879, 1700, 1344, 1201, 1146, 1023, 946, 648, 534, 487 cm⁻¹; MS (*m/z*): calcd for C₁₉H₃₂NO₆S⁺: 402.1950 [*M*+H]⁺, found: 402.1930.

1'-Hexadecanesulfonylzaartemisinin 8: Compound **8** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 1-hexadecanesulfonyl chloride (1.73 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) as a bright-yellow oil (498 mg, 25 %); ¹H NMR (600 MHz, DMSO): δ=5.88 (s, 1 H, H-12), 3.61 (ddd, *J*=14.1, 10.0, 5.6 Hz, 1 H, H-1'α), 3.45 (ddd, *J*=14.0, 10.1, 5.7 Hz, 1 H, H-1'β), 3.30–3.25 (m, 1 H, H-2'α), 3.18–3.13 (m, 1 H, H-9), 2.28–2.20 (m, 1 H, H-4α), 2.08–2.02 (m, 1 H), 2.00–1.95 (m, 1 H), 1.80–1.76 (m, 1 H), 1.75–1.61 (m, 6 H), 1.38–1.33 (m, 9 H), 1.28–1.24 (m, 14 H), 1.08 (d, *J*=7.5 Hz, 3 H, H-15), 1.01 (t, *J*=8.6 Hz, 2 H), 0.95 (d, *J*=6.2 Hz, 3 H, H-14), 0.90–0.81 ppm (m, 9 H); ¹³C NMR (151 MHz, DMSO): δ=173.30 (C10), 104.74 (C3), 80.08 (C12a), 77.72 (C12), 55.59 (C1'), 50.55 (C5a), 43.30 (CH), 36.73 (CH), 35.97 (CH₂), 35.43 (C9), 32.85 (CH₂), 31.31 (CH₂), 29.11–28.21 (C13'–4'), 27.28 (C3'), 24.84 (C13), 22.28 (CH₂), 22.11 (CH₂), 19.30 (C14), 13.97 (C15), 13.33 ppm (C16'); IR (ATR): $\tilde{\nu}_{\max}$ =2921, 2852, 1707, 1571, 1377, 1366, 1306, 1040, 645, 487 cm⁻¹; MS (*m/z*): calcd for C₃₁H₅₆NO₆S⁺: 570.3828 [*M*+H]⁺, found: 570.3819. The data are in agreement with those reported in the literature.¹⁹

Benzenesulfonylzaartemisinin 9: Compound **9** was obtained from **5** (1.0 g, 3.55 mmol), LDA and benzenesulfonyl chloride (0.7 mL, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **9** as off-white crystals; yield: 400 mg (27 %); mp: 147 °C; ¹H NMR (600 MHz, CDCl₃): δ=8.19 (d, *J*=7.5 Hz, 2 H, H-2' and H-6'), 7.59 (t, *J*=7.4 Hz, 1 H, H-4'), 7.50 (t, *J*=7.8 Hz, 2 H, H-3' and H-5'), 6.21 (s, 1 H, H-12), 3.33–3.26 (m, 1 H, H-9), 2.35 (td, *J*=14.5, 3.9 Hz, 1 H, H-4α), 2.06–1.99 (m, 2 H), 1.84–1.76 (m, 2 H), 1.72–1.64 (m, 1 H, H-8a), 1.61–1.52 (m, 2 H), 1.36 (dt, *J*=11.1, 7.3 Hz, 1 H, H-5a), 1.28–1.14 (m, 7 H), 1.08–0.96 ppm (m, 4 H); ¹³C NMR (151 MHz, CDCl₃): δ=172.98 (C10), 140.22 (C1'), 133.68 (C4'), 129.21 (C2' and C6'), 128.53 (C3' and C5'), 105.34 (C3), 80.42 (C12a), 78.70 (C12), 51.54 (C5a), 44.93 (C8a), 37.36 (CH), 36.62 (CH₂), 35.92 (C9), 33.70 (CH₂), 24.88 (CH₂), 22.42 (CH₂), 19.67 (C14), 13.46 ppm (C15); IR (ATR): $\tilde{\nu}_{\max}$ =2921, 1702, 1413, 1199, 881, 828, 560 cm⁻¹; MS (*m/z*): calcd for C₂₁H₂₈NO₆S⁺: 422.1637 [*M*+H]⁺, found: 422.1612.

(3'-Trifluoromethyl)benzenesulfonylzaartemisinin 10: Compound **10** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 3-(trifluoromethyl)benzenesulfonyl chloride (0.85 mL, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **10** as white crystals; yield: 16 mg (1 %); mp: 167 °C; ¹H NMR (600 MHz, CDCl₃): δ=8.47 (d, *J*=8.0 Hz, 1 H, H-6'), 8.42–8.34 (s, 1 H, H-2'), 7.86 (d, *J*=7.8 Hz, 1 H, H-4'), 7.68 (t, *J*=7.9 Hz, 1 H, H-5'), 6.21 (s, 1 H, H-12), 3.33–3.26 (m, 1 H, H-9), 2.37–2.30 (m, 1 H, H-4α), 2.07–2.00 (m, 2 H), 1.85–1.78 (m, 2 H), 1.72–1.65 (m, 1 H, H-8a), 1.59–1.50 (m, 2 H), 1.38–1.33 (m, 1 H), 1.26–1.22 (m, 1 H), 1.20 (d, *J*=7.5 Hz, 3 H, H-15), 1.13 (s, 3 H, H-13), 1.10–1.01 ppm (m, 4 H); ¹³C NMR (151 MHz, CDCl₃): δ=173.08 (C10), 141.07 (C1'), 132.78 (C6'), 130.97 (C7'), 130.20 (C4'), 129.26 (C5'), 126.13 (C2'), 105.29 (C3), 80.13 (C12a), 78.67 (C12), 51.33 (C5a), 44.73 (C8a), 37.21 (CH), 36.38 (CH₂), 35.84 (C9), 33.47 (CH₂), 24.70 (C13), 24.59 (CH₂), 22.22 (CH₂), 19.48 (C14),

13.29 ppm (C15); IR (ATR): $\tilde{\nu}_{\max}$ =2972, 2831, 2718, 2488, 1702, 1467, 1397, 1152, 1103, 509 cm^{-1} ; MS (m/z): calcd for $\text{C}_{22}\text{H}_{27}\text{F}_3\text{NO}_6\text{S}^+$: 490.1511 [$M+H$] $^+$, found: 490.1499.

(4'-Trifluoromethyl)benzenesulfonylazaartemisinin 11: Compound **11** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-(trifluoromethyl)benzenesulfonyl chloride (1.3 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **11** as an off-white powder; yield: 306 mg (18 %); mp: 168.1 °C; ^1H NMR (600 MHz, CDCl_3): δ =8.32 (d, J =8.3 Hz, 2 H, H-2' and H-6'), 7.77 (d, J =8.4 Hz, 2 H, H-3' and H-5'), 6.21 (s, 1 H, H-12), 3.34–3.26 (m, 1 H, H-9), 2.41–2.33 (m, 1 H, H-4 α), 2.08–2.00 (m, 2 H), 1.86–1.79 (m, 2 H), 1.73–1.67 (m, 1 H, H-8a), 1.60–1.51 (m, 2 H), 1.40–1.34 (m, 1 H, H-5a), 1.27–1.21 (m, 1 H), 1.20–1.13 (m, 6 H, H-15 and H-13), 1.11–0.99 ppm (m, 4 H); ^{13}C NMR (151 MHz, CDCl_3): δ =173.19 (C10), 143.62 (C1'), 135.35 (C4'), 129.83 (C2'), 129.79 (C6'), 125.72 (C3'), 125.69 (C5'), 124.21 (C7'), 105.44 (C3), 80.35 (C12a), 78.89 (C12), 51.50 (C5a), 44.89 (C8a), 37.39 (CH), 36.57 (CH₂), 35.99 (C9), 33.65 (CH₂), 24.88 (C13), 24.87 (CH₂), 22.43 (CH₂), 19.65 (C14), 13.44 ppm (C15); IR (ATR): $\tilde{\nu}_{\max}$ =2984, 2855, 1705, 1366, 1166, 711, 554, 429 cm^{-1} ; MS (m/z): calcd for $\text{C}_{22}\text{H}_{27}\text{F}_3\text{NO}_6\text{S}^+$: 490.1511 [$M+H$] $^+$, found: 490.1497.

(4'-Iodobenzene)sulfonyl-azaartemisinin 12: Compound **12** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-iodobenzenesulfonyl chloride (1.61 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **12** as a pale-pink powder; yield: 431 mg (22 %); mp: 182.8 °C; ^1H NMR (600 MHz, CDCl_3): δ =7.90–7.85 (m, 4 H, H-2', H-3, H-5' and H-6'), 6.18 (s, 1 H, H-12), 3.32–3.27 (m, 1 H, H-9), 2.36 (dt, J =14.5, 3.8 Hz, 1 H, H-4 α), 2.07–2.00 (m, 2 H), 1.84–1.78 (m, 2 H), 1.70–1.66 (m, 1 H, H-8a), 1.58–1.50 (m, 2 H), 1.36 (td, J =11.0, 6.4 Hz, 1 H, H-5a), 1.25–1.16 (m, 7 H), 1.06–0.96 ppm (m, 4 H); ^{13}C NMR (151 MHz, CDCl_3): δ =172.93 (C10), 139.69 (C1'), 137.66 (C2' and C6'), 130.50 (C3' and C5'), 105.23 (C3), 101.49 (C4'), 80.18 (C12a), 78.59 (C12), 51.36 (C5a), 44.78 (C8a), 37.20 (CH), 36.44 (CH₂), 35.75 (C9), 33.49 (CH₂), 24.82 (C13), 24.71 (CH₂), 22.24 (CH₂), 19.49 (C14), 13.26 ppm (C15); IR (ATR): $\tilde{\nu}_{\max}$ =2934, 2854, 1704, 1567, 1385, 1235, 1054, 1018, 895, 601, 561 cm^{-1} ; MS (m/z): calcd for $\text{C}_{21}\text{H}_{27}\text{INO}_6\text{S}^+$: 548.0603 [$M+H$] $^+$, found: 548.0586.

(4'-Methoxybenzene)sulfonyl-azaartemisinin 13: Compound **13** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-methoxybenzenesulfonyl chloride (1.1 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **13** as off-white crystals; yield: 19 mg (12 %); mp: 159 °C; ^1H NMR (600 MHz, CDCl_3): δ =8.11 (d, J =9.0 Hz, 2 H, H-2' and H-6'), 6.95 (d, J =9.0 Hz, 2 H, H-3' and H-5'), 6.19 (s, 1 H, H-12), 3.86 (s, 3 H, H-8'; OCH₃), 3.32–3.25 (m, 1 H, H-9), 2.38–2.29 (m, 1 H, H-4 α), 2.07–2.00 (m, 2 H), 1.83–1.73 (m, 2 H), 1.70–1.55 (m, 3 H), 1.36 (dt, J =11.0, 7.5 Hz, 1 H, H-5a), 1.25–1.20 (m, 4 H), 1.17 (d, J =7.4 Hz, 3 H, H-15), 1.05–0.95 ppm (m, 4 H); ^{13}C NMR (151 MHz, CDCl_3): δ =172.79 (C10), 163.59 (C4'), 131.54 (C1'), 131.45 (C2' and C6'), 113.48 (C3' and C5'), 105.12 (C3), 80.23 (C12a), 78.40 (C12), 55.64 (C8'), 51.43 (C5a), 44.87 (C8a), 37.18 (CH), 36.50 (CH₂), 35.66 (C9), 33.54 (CH₂), 24.94 (C13), 24.73 (CH₂), 22.23 (CH₂), 19.51 (C14), 13.26 ppm (C15); IR (ATR): $\tilde{\nu}_{\max}$ =2965, 2828, 2717, 2487, 1702, 1397, 1313, 1152, 1026, 695, 508 cm^{-1} ; MS (m/z): calcd for $\text{C}_{22}\text{H}_{30}\text{NO}_7\text{S}^+$: 452.1742 [$M+H$] $^+$, found: 452.1714.

4'-(4''-Fluorophenyl)benzenesulfonylazaartemisinin 14: Compound **14** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 4-(4'-fluorophenyl)benzene-1-sulfonyl chloride (1.16 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **14** as pale-yellow

crystals; yield: 21 mg (1 %); mp: 142 °C; ^1H NMR (600 MHz, CDCl_3): δ =8.23 (d, J =8.5 Hz, 2 H, H-2' and H-6'), 7.65 (d, J =8.6 Hz, 2 H, H-3' and H-5'), 7.56 (dd, J =8.8, 5.2 Hz, 2 H, H-8' and H-12'), 7.18–7.13 (m, 2 H, H-9' and H-11'), 6.23 (s, 1 H, H-12), 3.34–3.28 (m, 1 H, H-9), 2.36 (dt, J =14.6, 3.8 Hz, 1 H, H-4 α), 2.06–2.02 (m, 2 H), 1.84–1.79 (m, 2 H), 1.71–1.67 (m, 1 H, H-8a), 1.61–1.53 (m, 2 H), 1.38 (dd, J =11.4, 5.9 Hz, 1 H, H-5a), 1.26–1.22 (m, 4 H), 1.19 (d, J =7.4 Hz, 3 H, H-15), 1.08–1.02 ppm (m, 4 H); ^{13}C NMR (151 MHz, CDCl_3): δ =172.97 (C10), 162.30 (C10'), 145.41 (C1'), 138.56 (C4'), 135.44 (C7'), 129.71 (C2' and C6'), 129.13 (C8' and C12'), 126.84 (C3' and C5'), 116.06 (C9' and C11'), 105.21 (C3), 80.25 (C12a), 78.52 (C12), 50.86 (C5a), 44.83 (C8a), 37.19 (CH), 36.47 (CH_2), 35.73 (C9), 33.51 (CH_2), 24.84 (C13), 24.71 (CH_2), 22.25 (CH_2), 19.51 (C14), 13.26 ppm (C15); IR (ATR): $\tilde{\nu}_{\text{max}}$ =3014, 2936, 2748, 2518, 1703, 1603, 1472, 1244, 1145, 1084, 680, 550, 508 cm^{-1} ; MS (m/z): calcd for $\text{C}_{27}\text{H}_{31}\text{FNO}_6\text{S}^+$: 516.1856 [$M+\text{H}$] $^+$, found: 516.1827.

Cyclopropanesulfonylazaartemisinin 15: Compound **15** was obtained from **5** (1.0 g, 3.55 mmol), LDA and cyclopropanesulfonyl chloride (0.55 mL, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **15** as a white powder; yield: 76 mg (6 %); mp: 133.1 °C; ^1H NMR (600 MHz, CDCl_3): δ =5.95 (s, 1 H, H-12), 3.43–3.35 (m, 1 H, H-9), 3.22–3.16 (m, 1 H, H-1'), 2.42–2.30 (m, 1 H, H-4 α), 2.07–1.98 (m, 2 H), 1.82–1.74 (m, 2 H), 1.72–1.66 (m, 1 H, H-8a), 1.58–1.46 (m, 3 H), 1.44–1.35 (m, 5 H), 1.25 (d, J =7.5 Hz, 3 H, H-15), 1.18–0.99 ppm (m, 7 H); ^{13}C NMR (151 MHz, CDCl_3): δ =173.52 (C10), 105.18 (C3), 80.12 (C12a), 77.94 (C12), 51.38 (C5a), 44.89 (C8a), 37.12 (CH), 36.42 (CH_2), 35.57 (C9), 33.56 (C1'), 33.49 (CH_2), 25.41 (C13), 24.69 (CH_2), 22.17 (CH_2), 19.49 (C14), 13.27 (C15), 6.73 (C3'), 6.42 ppm (C2'); IR (ATR): $\tilde{\nu}_{\text{max}}$ =2966, 2940, 1710, 1350, 1273, 1204, 1129, 975, 927, 707, 594, 492 cm^{-1} ; MS (m/z): calcd for $\text{C}_{18}\text{H}_{28}\text{NO}_6\text{S}^+$: 386.1637 [$M+\text{H}$] $^+$, found: 386.1619.

2'-Thiophenesulfonylazaartemisinin 16: Compound **16** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 2-thiophenesulfonyl chloride (0.97 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **16** as a brown powder; yield: 156 mg (10 %); mp: 150.1 °C; ^1H NMR (600 MHz, CDCl_3): δ =7.94 (d, J =3.7 Hz, 1 H, H-2'), 7.66 (d, J =4.9 Hz, 1 H, H-4'), 7.09–7.05 (m, 1 H, H-3'), 6.18 (s, 1 H, H-12), 3.38–3.27 (m, 1 H, H-9), 2.35 (td, J =14.6, 3.9 Hz, 1 H, H-4 α), 2.07–1.96 (m, 2 H), 1.83–1.75 (m, 2 H), 1.72–1.65 (m, 1 H, H-8a), 1.59–1.53 (m, 2 H), 1.40–1.32 (m, 1 H, H-5a), 1.26–1.19 (m, 4 H), 1.15 (s, 3 H, H-13), 1.07–0.99 ppm (m, 4 H); ^{13}C NMR (151 MHz, CDCl_3): δ =173.08 (C10), 140.47 (C1'), 135.33 (C2'), 133.68 (C4'), 126.61 (C3'), 105.21 (C3), 80.29 (C12a), 79.09 (C12), 51.32 (C5a), 44.53 (C8a), 37.22 (CH), 36.41 (CH_2), 35.94 (C9), 33.57 (CH_2), 24.74 (C13), 24.71 (CH_2), 22.27 (CH_2), 19.51 (C14), 13.47 ppm (C15); IR (ATR): $\tilde{\nu}_{\text{max}}$ =2976, 2879, 1706, 1365, 1142, 1128, 1013, 696, 670, 596, 551, 466 cm^{-1} ; MS (m/z): calcd for $\text{C}_{19}\text{H}_{26}\text{NO}_6\text{S}_2^+$: 428.1201 [$M+\text{H}$] $^+$, found: 428.1178.

2',5'-Dichlorothiophenesulfonyl-azaartemisinin 17: Compound **17** was obtained from **5** (1.0 g, 3.55 mmol), LDA and 2,5-dichlorothiophene-3-sulfonyl chloride (1.3 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **17** as a white powder; yield: 271 mg (15 %); mp: 168.3 °C; ^1H NMR (600 MHz, CDCl_3): δ =7.31 (s, 1 H, H-2'), 6.06 (s, 1 H, H-12), 3.33–3.24 (m, 1 H, H-9), 2.36–2.27 (m, 1 H, H-4 α), 2.00–1.91 (m, 2 H), 1.75–1.66 (m, 2 H), 1.65–1.60 (m, 1 H, H-8a), 1.53–1.45 (m, 2 H), 1.33–1.26 (m, 4 H), 1.10–1.03 (m, 4 H), 1.00–0.90 ppm (m, 4 H); ^{13}C NMR (151 MHz, CDCl_3): δ =172.68 (C10), 135.08 (C1'), 132.11 (C5'), 128.01 (C2'), 126.68 (C3'), 105.44 (C3), 80.32 (C12a), 79.26 (C12), 51.42 (C5a), 44.60 (C8a), 37.18 (CH), 36.37 (CH_2), 33.55 (C9), 24.91 (C13), 24.66 (CH_2), 22.24 (CH_2), 19.51 (C14),

13.25 ppm (C15); IR (ATR): $\tilde{\nu}_{\max}$ =2976, 2879, 1642, 1515, 1425, 1212, 1155, 1090, 1065, 646, 523, 483 cm^{-1} ; MS (m/z): calcd for $\text{C}_{19}\text{H}_{24}\text{Cl}_2\text{NO}_6\text{S}_2^+$: 496.0422 [$M+H$] $^+$, found: 496.0360.

10-Camphorsulfonylzaartemisinin 18: Compound **18** was obtained from **5** (1.0 g, 3.55 mmol), LDA and (1S)-(+)-10-camphorsulfonyl chloride (1.3 g, 5.33 mmol, 1.5 equiv), and chromatography (ethyl acetate/hexane, 4:6) to give **18** as a white powder; yield: 484 mg (27 %); mp: 186 °C; ^1H NMR (600 MHz, CDCl_3): δ = 6.06 (s, 1 H, H-12), 4.27 (d, J =14.7 Hz, 1 H, H-1' α), 3.40–3.33 (m, 2 H), 2.60–2.53 (m, 1 H, H-6' α), 2.43–2.36 (m, 2 H, H-4 α), 2.10 (t, J =4.5 Hz, 1 H, H-7'), 2.07–1.98 (m, 3 H), 1.94 (d, J =18.4 Hz, 1 H, H-3' β), 1.81–1.75 (m, 2 H), 1.74–1.66 (m, 2 H), 1.57–1.48 (m, 2 H), 1.44 (s, 3 H, H-13), 1.43–1.34 (m, 2 H), 1.26–1.20 (m, 7 H), 1.04–0.98 (m, 4 H), 0.94 ppm (s, 3 H, H-9'); ^{13}C NMR (151 MHz, CDCl_3): δ =214.15 (C4'), 173.60 (C10), 105.22 (C3), 80.31 (C12a), 78.62 (C12), 59.26 (C2'), 54.27 (C1'), 51.37 (C5a), 47.44 (C8'), 44.35 (C8a), 43.24 (C7'), 42.46 (C3'), 37.18 (CH), 36.39 (CH₂), 35.94 (C9), 33.61 (CH₂), 26.81 (C5'), 25.86 (C6'), 25.37 (C13), 24.65 (CH₂), 22.23 (CH₂), 20.19 (C9'), 19.76 (C10'), 19.50 (C14), 13.59 ppm (C15); IR (ATR): $\tilde{\nu}_{\max}$ =2935, 1743, 1705, 1571, 1405, 1356, 1014, 855, 645, 621 cm^{-1} ; MS (m/z): calcd for $\text{C}_{25}\text{H}_{38}\text{NO}_7\text{S}^+$: 496.2369 [$M+H$] $^+$, found: 496.2383.

Biological screening

P. falciparum parasites were maintained at 37 °C in human erythrocytes (O^+) suspended in complete culture medium (RPMI 1640 medium supplemented with 25 mM HEPES, 20 mM d-glucose, 200 μM hypoxanthine, 0.2 % sodium bicarbonate, 24 $\mu\text{g mL}^{-1}$ gentamycin and 0.5 % AlbuMAX II) in a gaseous environment of 90 % N_2 , 5 % O_2 , and 5 % CO_2 according to the method established by Verlinden and co-workers.³⁰ In vitro ring-stage intraerythrocytic *P. falciparum* parasite cultures (1 % hematocrit, 1 % parasitemia) were treated with the compounds; drug-sensitive NF54 and drug-resistant K1 (CQ, pyrimethamine, mefloquine and cycloguanil) and W2 (CQ, quinine, pyrimethamine and cycloguanil) strains were used. The controls for this assay included CQ disulfate (1 μM , as positive control) and complete RPMI media (as negative control), grown for 96 h at 37 °C under a 90 % N_2 , 5 % O_2 , and 5 % CO_2 gas mixture in 96-well plates. At the conclusion of the 96 h growth period, equal volumes (100 μL each) of the *P. falciparum* parasite cultures were combined with SYBR Green I lysis buffer (0.2 $\mu\text{L mL}^{-1}$ 10 000 \times SYBR Green I, Invitrogen; 20 mM Tris, pH 7.5; 5 mM EDTA; 0.008 % (w/v) saponin; 0.08 % (v/v) Triton X-100). The samples were incubated at 37 °C for 1 h after which the fluorescence was measured using a Fluoroskan Ascent FL microplate fluorimeter (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The 'background' fluorescence (i.e., that measured in the samples derived from CQ-treated red blood cell samples in which parasite proliferation was completely inhibited) was subtracted from the total fluorescence measured for each sample to provide a measure of parasite proliferation.^{31, 32} Data obtained were analyzed in Excel 2007 and sigmoidal dose–response curves were plotted using GraphPad 5.0. Results are from three biological replicates, performed in technical triplicates.

Gametocytes were produced according to a published method.³³ The luciferase reporter assay was established to enable accurate, reliable and quantifiable investigations of the stage-specific action of gametocytocidal compounds for each of the early- and late-stage gametocyte marker cell lines; NF54-PfS16-GFP-Luc and NF54-Mal8p1.16-GFP-Luc. Drug assays were set up on days 5 and 10 (representing >90 % of either early-stage I/II/III or

mature-stage IV/V gametocytes, respectively). In each instance, assays were set up using a 2–3 % gametocytemia, 1.5 % hematocrit culture and 48 h for early-stage gametocytes and 72 h for late-stage gametocytes drug pressure in a gas chamber (90 % N₂, 5 % O₂, and 5 % CO₂) at 37 °C. Luciferase activity was determined in 20 µL parasite lysates by adding 50 µL luciferin substrate (Promega Luciferase Assay System) at room temperature and detection of resultant bioluminescence at an integration constant of 10 s with the GloMax®-Multi+ Detection System with Instinct® Software. Results are from three biological replicates for early-stage gametocytes and a single replicate for late-stage gametocytes, performed in technical triplicates.

The pLDH assay was set up on day 10 late-stage gametocytes (>90 % mature-stage IV/V gametocytes). The assays were set up using a 1 % gametocytemia and 2 % hematocrit culture and 72 h drug pressure in a gas chamber (90 % N₂, 5 % O₂, and 5 % CO₂) at 37 °C, after which the media was replaced with fresh media and the culture incubated for another 72 h. Gametocyte viability was measured by lysing 20 µL of the parasite suspension with 100 µL of a Malstat reagent [0.21 % v/v Triton X-100; 222 mm l-(+)-lactic acid; 54.5 mm Tris; 0.166 mm 3-acetylpyridine adenine dinucleotide (APAD; Sigma–Aldrich); adjusted to pH 9 with 1 m NaOH] followed by addition of 25 µL PES/NBT [1.96 mm nitro blue tetrazolium chloride NBT; 0.239 mm phenazine ethosulfate (PES)]. Absorbance was measured with a Multiskan Ascent 354 multiplate scanner (Thermo Labsystems, Finland) at 620 nm. Results are from a single replicate, performed in technical triplicates.

Cytotoxicity

The cytotoxic effects of the compounds were tested by sulforhodamine B (SRB) assay on the WI-38 cell line. The SRB assay was developed³⁴ to measure drug-induced cytotoxicity and cell proliferation. Its principle is based on the ability of the protein dye sulforhodamine B (Acid Red 52) to bind electrostatically in a pH-dependent manner to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the fixed cellular protein, while under mild basic conditions it can be extracted from cells and solubilized for measurement. The SRB assay is performed at the CSIR in accordance with the protocol of the Drug Evaluation Branch, NCI, and the assay has been adopted for this screen. The WI-38 cell line—normal Human Fetal Lung Fibroblast (HFLF) from ECACC—was routinely maintained as a monolayer cell culture at 37 °C, 5 % CO₂, 95 % air and 100 % relative humidity in EMEM containing 10 % fetal bovine serum, 2 mm l-glutamine and 50 µg mL⁻¹ gentamicin. For the screening experiment, the cells (21–50 passages) were inoculated in a 96-well microtiter plates at plating densities of 10 000 cells per well and were incubated for 24 h. After 24 h the cells were treated with the synthesized compounds which were previously dissolved in DMSO and diluted in medium to produce five concentrations. Cells without any drug addition served as control. The blank contains complete medium without cells. Emetine was used as a standard. The plates were incubated for 48 h after addition of the synthesized compounds. Viable cells were fixed to the bottom of each well with cold 50 % trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10 mm Tris base for optical density determination at the wavelength 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software for determination of 50 % of cell growth inhibition (IC₅₀).

Disclaimer

Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s), and the NRF does not accept any liability in this regard thereto.

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Conflict of interest

The authors declare no conflict of interest.

Supporting Information

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