Preliminary Evaluation of Artemisinin-Cholesterol Conjugates as Potential Drugs for Treatment of Intractable Forms of Malaria and Tuberculosis

Mokhitli Morake[a], Dina Coertzen[b], Andile Ngwane[c], Johannes F. Wentzel[a], Ho Ning Wong[a], Frans J. Smit[a], Lyn-Marie Birkholtz[b], Ray-Dean Petersen[c], Bienyameen Baker[c], Ian Wiid[c], David D. N'Da*[a] and Richard K. Haynes*[a][d]

[a] Mr. M. Morake, Dr. H. N. Wong, Dr. F.J. Smit, Prof. D.D. N'Da, Prof. R.K. Haynes, Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom 2520, South Africa.
[b] Dr. D. Coertzen, Prof. L.-M. Birkholtz, Department of Biochemistry, Centre for Sustainable Malaria Control, University of Pretoria, Private Bag X20, Hatfield, 0028, South Africa.
[c] Dr. A Ngwane, Dr. R.- D. Petersen, Prof. B. Baker, Prof. I. Wiid, DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg 7505, South Africa.
[d] Department of Chemistry, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong.

Abstract: To evaluate the feasibility of developing drugs that may be active against both malaria and tuberculosis (TB) by utilizing in part putative cholesterol transporters in the causative pathogens and through enhancement of passive diffusion in granulomatous TB, artemisinin-cholesterol conjugates were synthesized by connecting the component molecules through various linkers. The compounds were screened *in vitro* against *Plasmodium falciparum* (*Pf*) and *Mycobacterium tuberculosis* (*Mtb*). Antimalarial activities (IC_{50}) against *Pf* drug sensitive NF54, and drug resistant K1 and W2 strains ranged from 0.03 – 2.6, 0.03 – 1.9 and 0.02 – 1.7 μM. Although the compounds are less active than the precursor artemisinin derivatives, the cholesterol moiety renders the compounds relatively insoluble in the culture medium, and variation in solubilities among the different compounds may reflect in the range of efficacies observed. Activities against *Mtb* H37Rv were assessed using a standardized CFU assay after 24 h pretreatment of cultures with each of the compounds. Percentage inhibition ranged from 3 – 38% and 18 – 52% at 10 and 80 μM, respectively. Thus, in contrast to the comparator drug artemether, the conjugates display enhanced activities. The immediate aims include the preparation of conjugates with enhanced aqueous solubilities, assays against malaria and TB *in vivo*, and for TB, assays using an infected macrophage model and assessment of granuloma influx.

Introduction

Malaria and tuberculosis (TB) are two of the most lethal infectious diseases of our time and continue to overburden the resource-limited regions of the world. For malaria, in 2014 alone, 214 million cases and 438,000 deaths are estimated to have occurred. The majority of cases (88%) and deaths (90%) occurred in the African region. In that same year, at least 306,000 children under the age of five died of malaria, of which 292,000 were in the African region.[1] Notwithstanding this, mortality appears to be decreasing given that in 2013 584,000 deaths were estimated compared to 438,000 in 2014.[2] TB has recently surpassed HIV as a leading cause of death due to infectious disease.[3] In 2015, 1.4 million deaths due to TB and 10.4 million new TB cases were estimated to have occurred on a global scale. Most of the cases were in the South-East Asian (61%) and African (26%) regions. The African region also accounted for most of the TB-HIV co-infections.[4] This region is also notorious for the relative number of TB cases with 288 TB cases recorded per 100,000 people compared to 133 TB cases per 100,000 people on a global basis in 2015.[5]

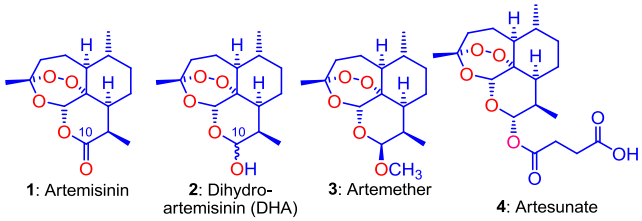


Figure 1. Artemisinin **1** and its current clinical derivatives, the hemiacetal dihydroartemisinin (DHA) **2**, the lactol ether artemether **3** and hemiester artesunate **4**.

Although the malaria parasite, in particular *P. falciparum* (*Pf*), has developed resistance to most antimalarial drugs,[6] reliance is now largely placed on the use of artemisinin and its derivatives (collectively called artemisinins) (Fig. 1) that are the most active antimalarial drugs currently available.[7] They are used in combination with other antimalarial drugs in artemisinin combination therapies (ACTs).[8] ACTs were developed so as to allow the artemisinins to reduce parasite biomass while the long acting partner drug continues clearing parasites once levels of the short-lived artemisinins had dropped below therapeutically-effective levels. Nonetheless, *Pf* resistance involving ACTs is now established in South-East Asia; if the resistant phenotype enters other endemic regions, malaria control programs will be seriously compromised. Whilst TB chemotherapy usually employs drug combinations comprising rifampicin, isoniazid, pyrazinamide and ethambutol,[9] multidrug-resistant TB (MDR-

TB) strains of *Mycobacterium tuberculosis* (*Mtb*) resistant to rifampicin and isoniazid have emerged. Extensively drug-resistant TB (XDR-TB) strains of *Mtb* additionally are resistant to fluoroquinolones and one or more aminoglycosides usually used as second-line drugs.[10] More alarming are recent reports of totally drug-resistant TB which is resistant to all second-line drugs.[11] The spread of these strains hamper progress made thus far to combat TB. Although newer drugs such as bedaquiline and delamanid have received conditional approval for use in the treatment of MDR-TB,[12] toxicity concerns are as yet unresolved and combination therapies are not yet available.[12,13] Overall, new TB drugs are urgently needed in order to eradicate these strains.

Artemisinin **1** appears to possess no activity against *Mtb*. However, in an elegant investigation, the dihydroartemisinin-mycobactin conjugate **5** (Fig. 2) was shown to be notably potent against *Mtb* including XDR-TB strains.[14] The lipophilic mycobactin moiety of the conjugate ligates Fe(III) which is then actively transported into the cell by the mycobactin transporter wherein the artemisinin exerts its cytotoxic effect; interestingly a strong ROS response was noted on administration, supporting the artemisinin oxidant hypothesis outlined below. Thus, providing that they can be transported into the cytosol, artemisinins are active against *Mtb*. Significantly, it has been shown that attachment of lipophilic steroidal groups to tetraoxanes provides compounds such as **6** that are also strikingly active against *Mtb* (Fig. 2).[15] However, the evident cytotoxicity of these compounds precludes their further development.

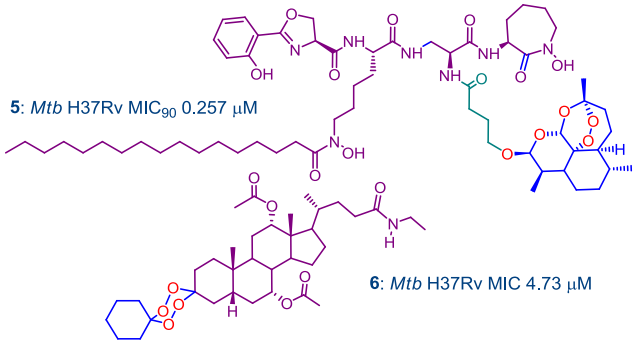


Figure 2. Artemisinins and analogues that are active against *Mtb in vitro*: the mycobactin-dihydroartemisinin conjugate **5** (ref. 14) and the steroidal tetraoxane **6** (ref. 15)

Under a programme designed to develop new triple drug combinations for the treatment of malaria, tuberculosis, and toxoplasmosis,[16] we are preparing and evaluating efficacies of drug combinations which have oxidant and redox properties respectively[17] coupled with a third partner with a different mode of action. The precept is that artemisinins and other peroxides behave as *oxidants* in the intracellular milieu by rapidly oxidizing reduced flavin cofactors such as $FADH_2$ of flavoenzyme disulfide reductases important for maintaining levels of GSH and other endogenous thiols required for intercepting reactive oxygen species (ROS).[17] *Pf* as an aerobic organism experiences abnormally high oxidative stress within an erythrocyte.[18,19] Thus, intraparasitic hexose mono-phosphate shunt (HMS) activity to generate NADPH required by flavin disulfide reductases glutathione reductase (GR), thioredoxin reductase (TrxR), lipoamide dehydrogenase and others is approximately 78 times higher than in normal erythrocytes.[18,20] *Pf* utilizes GR, TrxR and others for generation of GSH needed for detoxification of ROS. Thus, any drug that abruptly subverts redox homeostasis will have a cytotoxic effect. *Mtb* likewise is an aerobic organism and has to cope with high oxidative stress, particularly within alveolar macrophages.[21] *Mtb* utilizes mycothiol reductase (MR) whose substrate mycothiol (MSH) plays the same role in *Mtb* as GSH does in *Pf*.[22] In addition, ergothionine (EGT) is an important redox-modulating molecule for *Mtb*, and indeed adequate levels of EGT appear essential to survival.[22,23] Thus *Pf* and *Mtb* will be *susceptible* to oxidant-redox active drug combinations. *Pf* and *Mtb* also contain other flavoenzymes (diaphorases) including the essential respiratory enzymes Type II NADH:quinone oxidoreductases (NDH-2) that induce electron transfer from NADH to the cofactor FAD to menaquinone in the respiratory chain.[24] These are potentially susceptible to electron-scavenging by the *same* redox-oxidant drug combinations. Additional drug components added to the drug mixtures are also required on the basis of enhancing efficacy through enabling activity against a different target, and suppressing the emergence of resistant pathogens.

Our specific aim here is to enhance activity of the oxidant drug prior to combining it with the redox partner. Thus, we examine the effect of ligating the oxidant artemisinin to a group that assists in targeting selected stages of the malaria parasite, and in particular in the case of *Mtb* that assists in diffusion, either in a passive manner, or by utilizing a transporter in an active sense, across the mycolic acid barrier into the cytosol of the bacterium. In the latter case, the outer cell wall represents a formidable barrier to drug ingress, and the need to incorporate elements of structural design into drugs for penetration is well established.

Cholesterol is an important molecule for most organisms with roles such as acting as a carbon source, involvement in membrane or lipid raft formation, and in particular as a precursor for biosynthesis of vital molecules required by the organism.[25,26] In the human liver, the malaria parasite associates with the endoplasmic reticulum (ER) in hepatocytes so that it may salvage newly synthesized lipids. The parasite also continuously diverts cholesterol from low-density lipoproteins and hepatocytes during the liver stage.[27,28] During the intraerythrocytic stages, the cholesterol gradient is noted to decrease inwardly from the erythrocyte membrane to the parasitophorous vacuolar (PV) membrane of the parasite. Thus, the PV membrane contains cholesterol incorporated upon erythrocyte invasion. Overall, cholesterol plays a role in domain assemblies, lipid raft formation and protein trafficking in the malarial parasite.[29] *Mtb* utilizes cholesterol as a carbon source during its development.[26,30] Studies with murine TB models have identified the cholesterol transporter Mce4 which delivers cholesterol for metabolism into the bacterium. Cholesterol is initially acquired from the plasma membranes of the macrophages, and then from lipid-rich foamy macrophages. This molecule can also be acquired in insoluble crystal form from extracellular spaces.[30] One crucial issue is the ability of cholesterol to undergo passive diffusion through the granuloma into the core, wherein it is taken up by quiescent *Mtb* as an energy source via metabolic breakdown to methylmalonyl coenzyme A and eventual feed into the tricarboxylic acid cycle.[26,30,31] Overall, any drug which may penetrate a granuloma, and exert cytotoxic effects on quiescent bacteria is

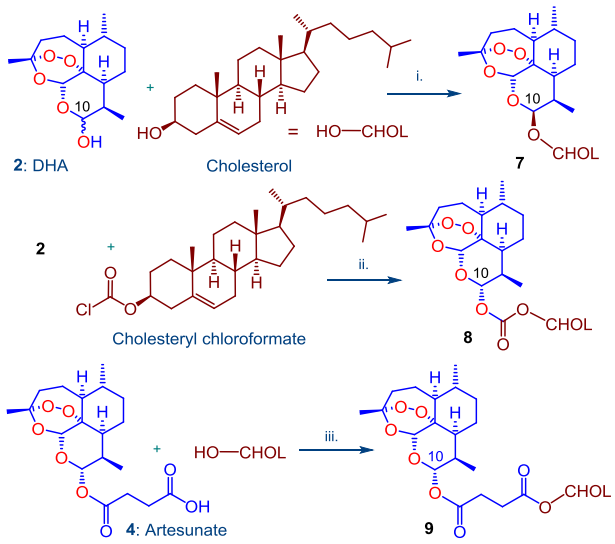
highly sought after, given the enormously aggregated epithelioid structure of the granuloma.

We describe here the preparation of artemisinin-cholesterol conjugates, and the results of preliminary screening *in vitro* against *Pf* and *Mtb*.

Results and Discussion

Chemistry

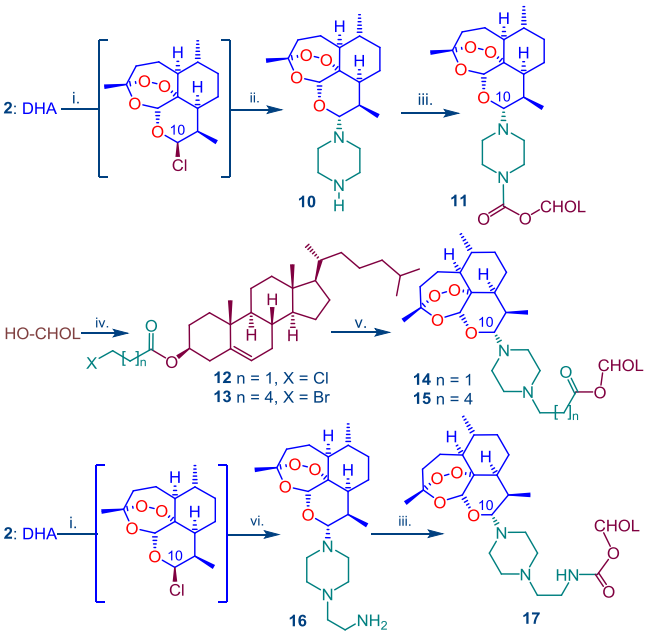
Three general coupling methods were used. Firstly, conjugates bearing an oxygen atom at C-10 of the artemisinin nucleus were prepared (Scheme 1). The cholesteryl lactol ether **7** was prepared by direct conjugation of cholesterol with DHA **2** according to the method used for preparing this compound and other acetals[32,33] (Scheme 1).



Scheme 1. C-10 O-linked artemisinin-cholesterol conjugates: i. Cholesterol (1.2 eq.), $BF_3{\cdot}OEt_2$ (0.9 eq.), CH_2Cl_2, 0 °C to room temperature, overnight; ii. Cholesteryl chloroformate (1.2 eq.), 4-*N*,*N*-dimethylaminopyridine (0.1 eq.), triethylamine (1.1 eq.), CH_2Cl_2, room temperature, 18 h; iii. Cholesteryl chloroformate (1 eq.), DCC (1 eq.), 4-*N*,*N*-dimethylaminopyridine (0.2 eq.), room temperature, 4 h.

The β-configuration at C-10 in **7** is secured through the ^{1}H NMR spectrum displaying a vicinal coupling of 5.1 Hz between H-9 and H-10 indicating syn axial-equatorial coupling.[33] The carbonate **8** was obtained in moderate yield (45%) following esterification of cholesterol chloroformate with **2** under standard basic conditions. Given that DHA acts as the nucleophile, the equilibrating mixture of DHA epimers react only via the α-epimer, as noted previously;[33] the α-configuration at C-10 is secured by the trans-diaxial coupling of 9.8 Hz between H-9 and H-10. The diester **9** was prepared in moderate yield (35%) by activation of the free carboxyl group of artesunate with dicyclohexyl carbodiimide and treatment with cholesterol. As in the foregoing case, the product has the α-configuration at C-10 ($J_{9,10}$ 9.9 Hz).

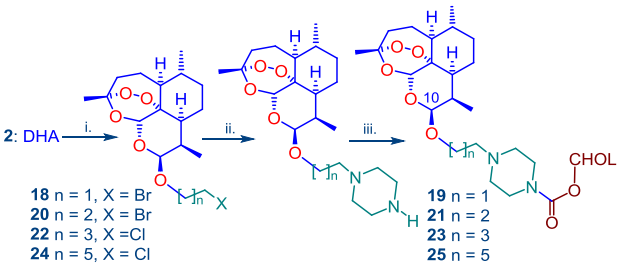
We have previously noted that artemisinin derivatives bearing an amino group at C-10 display superior antimalarial activities with respect to the O- or C-substituted counterparts. This is ascribed to facilitated hydride transfer from the reduced flavin cofactors to the peroxide of the artemisinin driven by protonation of the amino group at C-10 under intracellular conditions.[34] Accordingly, DHA **2** was converted into the piperazine derivative **10** by treatment with oxalyl chloride in the presence of DMSO followed by quenching of the reaction mixture by addition to an excess of piperazine (Scheme 2).[35] The reaction, which proceeds via the intermediate β-chloride works well with cyclic secondary amines. Notably, in attempts to vary the C-10 amino group attached to the DHA, it was found that the use of primary alkyl amines did not return aminated products. The α-configuration at C-10 is secured by the trans-diaxial coupling of 10.2 Hz between H-9 and H-10. Subsequent treatment of **10** with cholesteryl chloroformate provided the amide derivative **11** in a moderate yield (43%). Cholesterol was esterified with ω-haloalkyl acid chlorides to provide the haloesters **12** and **13**[36] that upon treatment with the artemisinin-piperazine derivative **10** gave the corresponding cholesterol conjugates **14** and **15** in 32 and 6% yields, respectively. The same idea was applied to the synthesis of compound **17** (Scheme 2) wherein 1-(2'-aminoethyl)-piperazine was used instead of piperazine to convert DHA into the artemisinin-piperazine derivative **16** in low yield (36%). Interestingly, the preference is for this bifunctional amine to react through the cyclic secondary amine site with the β-chloride formed *in situ*. The product arising from reaction through the exocyclic primary amino group was not observed. Treatment of **16** with cholesteryl chloroformate provided compound **17** in low overall yield (14%).



Scheme 2. C-10 N-linked artemisinin-cholesterol conjugates: i. DMSO (0.1 eq.), oxalyl chloride (1.13 eq.), toluene, room temperature, N_2 atmosphere; ii. Direct addition of reaction mixture from i. to piperazine (5 eq.) in CH_2Cl_2, overnight; iii. Cholesteryl chloroformate (1.2 eq.), triethylamine (1.2 eq.), CH_2Cl_2, overnight; iv. Cholesterol, ω-haloalkyl acid chloride (2 eq.), CH_2Cl_2, room temperature, 24 h; v. Compound **10** (1 eq.), triethylamine (1 eq.), THF, 65 °C reflux, 24 h; i. as above; vi. Direct addition of reaction mixture from i. to 1-(2'-aminoethyl)piperazine (5 eq.) in CH_2Cl_2, overnight; iii. as above.

Lastly, ethers **18**, **20**, **22** and **24** were prepared through BF_3-catalyzed condensation of DHA **2** with halohydrins following the procedure generally used for coupling DHA with

alcohols.[32,33,37] These products were treated with excess piperazine and the crude amines so obtained were coupled with cholesteryl chloroformate in the next step. These reactions provided the carbamates **19** (40%)**, 21** (16%)**, 23** (7%) and **25** (10%) (Scheme 3). All the compounds were obtained as stereochemically pure isomers; although the intermediate **22** appeared to be a mixture according to its ^{1}H and ^{13}C NMR spectra, the final product obtained after reaction of the derived amine with cholesteryl chloroformate and purification by chromatography was the β-isomer **23**. The vicinal coupling ranging from 3.2 – 3.5 Hz between H-9 and H-10 in the ^{1}H NMR spectra indicates the β-configuration at C-10 was secured for all the compounds.



Scheme 3. C-10 O-linked artemisinin cholesteryl carbamates: i. Halohydrin (2 eq.), $BF_3{\cdot}Et_2O$ (0.9 eq.), CH_2Cl_2, 0 °C – room temperature, overnight; ii. Piperazine (5 eq.), tetrahydrofuran, DMF (0.1 eq.), 65 °C reflux, 24 h; iii. Cholesteryl chloroformate (1.2 eq.), triethylamine (1.2 eq.), CH_2Cl_2, 0 °C-room temperature, overnight.

Biological activities

Malaria parasite proliferation was directly monitored by detecting and monitoring intraparasitic DNA replication using the SYBR Green I assay. Activities *in vitro* were first assessed with a dual point assay by adding compounds at concentrations of 5 and 1 μM to asexual blood stages of the drug sensitive *Pf* NF54 strain. Those compounds which showed >70% inhibition at 5 μM and >50% inhibition at 1 μM (Figure S1, Supporting Information) were carried forward for IC_{50} determination against *Pf* NF54, and drug-resistant *Pf* K1 and W2 strains. Compounds **8**, **11, 14, 15, 17** and **21** were thereby carried forward for screening using artemether **3** and artesunate **4** (Fig. 1) as comparator compounds. Despite showing significantly decreased activity compared to compounds **3** and **4** (P<0.0001), compounds **14** and **15** were the most active against all three strains with IC_{50} values ranging from 0.024 to 0.078 μM respectively (Table 1). Compound **21** was the least active with IC_{50} values >1 against all three strains. Compounds **8**, **11** and **17** had IC_{50} values of 0.32, 0.16 and 0.20 μM against K1 and 0.36, 0.20 and 0.18 μM against W2 respectively. Resistance index (RI) values between the strains varied but all compounds displayed values less than 1 between drug-sensitive NF54, and drug-resistant K1 and W2 (Table 1); that is, these compounds show no cross-resistance. Cytotoxicity of the compounds was determined *in vitro* against the HEK293 cell line; these were generally insignificant as reflected in the high selectivity indices towards the parasite (Table 1). Compound **21** showed a low selectivity index (SI), implying that this compound may have exerted its antimalarial activity through non-selective toxicity. Overall, activities were some threefold less than artesunate against the drug resistant *Pf*

Table 1. Activities against asexual blood stage *Plasmodium falciparum* NF54, K1 and W2 and cytotoxicities against HEK293 cells.

Compound	CLog P[a]	Antimalarial activity[b] IC_{50}±SEM, μM			Resistance Index RI		Cytotoxicity IC_{50}, μM[e]	SI[f]
		NF54	K1	W2	RI[c]	RI[d]	HEK293	
3 Artemether	3.07	0.002 ± 0.00	0.009 ± 0.00	0.007 ± 0.00	4.8	3.8	nd	nd
4 Artesunate	2.94	0.003 ± 0.00	0.004 ± 0.00	0.002 ± 0.00	1.3	0.8	nd	nd
8	13.9	0.429 ± 0.04	0.315 ± 0.037	0.364 ± 0.05	0.73	0.85	83.3	194
11	14.6	0.213 ± 0.004	0.164 ± 0.065	0.209 ± 0.04	0.77	0.98	243	1141
14	14.7	0.031 ± 0.005	0.029 ± 0.005	0.024 ± 0.04	0.92	0.78	121	3903
15	15.3	0.078 ± 0.003	0.052 ± 0.008	0.070 ± 0.01	0.67	0.89	142	1821
17	14.3	0.267 ± 0.01	0.201 ± 0.010	0.176 ± 0.077	0.75	0.66	82.6	309
21	13.7	2.569 ± 0.046	1.946 ± 0.047	1.680 ± 0.657	0.76	0.65	74.2	28.9

[a] Values calculated with ACD/ChemSketch Version 14.02; [b] determined using the SYBR Green I assay; data averaged from n=3 independent biological repeats performed in technical triplicates, ± SEM.; [c] RI = IC_{50} K1/IC_{50} NF54; [d] RI = IC_{50} W2/IC_{50} NF54; [e] Cytotoxicities determined on HEK293 normal human embryonic kidney cell line using SRB assay; data from at least three independent biological replicates, each performed in triplicate; nd = not determined; [f] Selectivity index SI = IC_{50} Hek293/IC_{50} NF54

K1 and W2 for the most active compound **14**. Nevertheless, the data is encouraging given that all the compounds, with the exception of **14**, had notably poor solubility at 10 mM concentration in 100% DMSO stored at 20 °C. Poor solubility may be the reason for compounds **7**, **9**, **19, 23** and **25** not being active even during the dual-point dilution assay as this lack of activity according to structure-activity considerations vis-à-vis compound **14** is not at all apparent. All compounds are highly lipophilic with CLogP values ranging from 13.7 to 15.3 (Table 1). The high lipophilicity likely accounts for solubility problems encountered when dissolving these compounds in the dipolar aprotic solvent DMSO. It is not uncommon to encounter solubility problems with new chemical entities and this affects the quality of both *in vitro* and *in vivo* biological assays and consequently the interpretation of results.[38] A compound may appear to be less active than it really is because of low solubility. Inability of DMSO to solvate the compounds is ascribed to lipophilicity as expressed in poorly solvent-accessible surface areas and rotatable bonds.[39] The compounds have high molecular weights and compounds **15** (6 carbon linker), **23** (4 carbon linker) and **25** (6 carbon linker) have highly flexible linkers. Overall, in conjunction with evolving the synthetic routes here to generate more soluble artemisinin-cholesterol conjugates, resort will also be made to generating lipophilic formulations of the current compounds in order to facilitate administration.[40]

The antimycobacterial activity of the compounds against *Mtb* H37Rv was assessed by treating cultures with 10 and 80 μM of the drug dissolved in DMSO followed by incubation for 24 hrs. Colony forming unit (CFU) enumeration for 12 – 15 days was carried out following plating of the cultures on agar. Cholesterol, the artemisinin artemether **3** (Fig. 1) and the DHA-piperazine derivative **10** (Scheme 1) were used as comparator compounds. Artemether **3** bears an oxygen atom at C-10 and incorporates the structural elements of the artemisinin in the conjugates of Schemes 1 and 3, and the DHA-piperazine derivative **10** is either progenitor to or related to the conjugates in Scheme 2 in bearing a nitrogen atom attached to C-10. Data are presented in Table 2.

Compared to artemether **3** (80 μM, 13% inhibition), the simple cholesteryl ether analogue **7**, the cholesteryl carbonate derivative **8** and the artesunate conjugate **9** (Scheme 1) elicit significantly greater inhibitory activities (80 μM, 46-51%), even though the cholesteryl conjugates, in contrast to artemether, are poorly soluble in DMSO. The most active O-linked compound at the lower drug concentration was **23** (10 μM, 31%); given that inhibitory activity at 80 μM is only 47%, the inference is that intrinsic activity at the higher concentration *is limited by poor solubility of the compound*. For the C-10 N-linked artemisinin conjugates, the best were compounds **11** and **15**. Significantly, the artemisinin-piperazine derivative **10** (Scheme 2) is soluble in DMSO, and was also moderately active with 23- and 43% inhibition at 10 μM and 80 μM respectively. However, the compound is less active than the N-linked artemisinin-cholesterol conjugate **15** (10 μM 38%, 80 μM 52%). Once again, the relative difference in inhibitory activities between the two concentrations tends to suggest poor solubility limits activity at the higher concentrations. Irrespective of this, through comparison of the activities of each of artemether **3** and the DHA-piperazine derivative **10**, it is apparent that 10-amino-artemisinins, as argued elsewhere, are rather more active than the O-linked counterparts.[34] In the malaria assays, the nature of the linker did not impact on the efficacy of the compounds. However, for TB, there is the initial impression that compounds with longer alkyl chain linkers have better activity than those with shorter linkers. Compound **15** with a 6 carbon linker and **23** with 4 carbon linker were the most active conjugates which may suggest that increased lipophilicity enhances the passage through the lipophilic wall of the *Mtb*. Compounds bearing linkers with <4 carbon atoms had less than 22% inhibition at low concentration (10 μM) although it varied at the high concentration (18 – 51% inhibition at 80 μM). Nevertheless, the enhanced lipophilicity also depresses solubility, and for drug uptake, adequate solubility is an essential pre-requisite. However, it is clear that as compared to artemether itself, the economic attachment of cholesterol via a variety of linkers to C-10 of the artemisinin results in compounds with enhanced activities, and work in the area must continue in terms of designing conjugates with more polar linkers.

Table 2. Antimycobacterial activities against *Mycobacterium tuberculosis* H37Rv determined by CFU enumeration.[a]

Compound	[b]Av CFU/mL ± SD (10 μM)	% inhibition	[b]Av CFU/mL ± SD (80 μM)	% inhibition
Cholesterol	$1.48x10^5$ ±1.2	4	$1.3x10^5$±1.5	16
Artemether **3**	$1.5x10^5$ ±1.5	3	$1.35x10^5$±1.5	13
7	$1.18x10^8$ ±4.4	22	$1.3 x10^8$±3.2	51
8	$1.15x10^8$±2.1	14	$1.48x10^8$ ± 5.1	44
9	$1.62x10^8$±3.3	15	$1.43x10^8$ ± 2.2	46
10	$1.92x10^8$±5	23	$1.52x10^8$ ± 3.9	43
11	$1.52x10^8$±3.1	19	$1.3x10^8$ ± 3.2	51
14	$1.42x10^8$±2.4	13	$1.62x10^8$ ± 2.8	39
15	$1.55x10^8$ ± 1.4	38	$1.28x10^8$ ± 4.1	52
17	$1.55x10^8$ ± 2.3	18	$1.77x10^8$ ± 3.3	33
19	$1.97x10^8$ ± 2.4	3	$2.18x10^8$ ± 4.5	18
21	$1.13x10^8$ ± 2.5	14	$1.92x10^8$ ± 5	28
23	$1.50x10^8$ ± 2.5	31	$1.42x10^8$ ± 4.6	47
25	$1.82x10^8$ ± 3.7	27	$1.63x10^8$ ± 4.9	38
DMSO	$2.65x10^8$ ± 4.4	0	$2.48x10^8$ ± 3.2	0

[a] *Mtb* H37Rv cultures were treated with the compound at 10 and 80 μM for 24 h following which 200 μL was centrifuged, re-suspended and washed twice in Middlebrook 7H9 broth and for CFU assay were plated on 7H11 agar. CFUs were counted after 12-15 days; [b] Av CFU/mL ± SD = average colony forming units/mL ± SD.

Conclusion

Conjugation of dihydroartemisinin via either O- or N-linked functional groups to cholesterol generated ether, ester and carbamate linked artemisinin-cholesterol conjugates. Antimalarial

activities against *Pf* NF54, K1 and W2 strains were less than those of artemether and artesunate, but results are encouraging in the context of the future evaluation of the cholesteryl moiety in enhancing targeting of parasite stages in an *in vivo* situation, in particular against liver stage parasites. Compounds **14** and **15** were the most active compounds. These represent hit compounds that will not only be used as a platform to design newer conjugates with enhanced polarity so as to improve solubility, but also in the development of lipophilic formulations employing liposomes and others to enhance activities.

For the antimycobacterial screens, the device of attaching the readily available cholesterol to the artemisinin results in enhancement of activities with respect to the parent artemisinin. However, the implication that the low solubility limits expression of the intrinsic activities of these compounds encourages us to proceed with formulation studies involving the current conjugates, and with the design and synthesis of newer conjugates with enhanced polarities so as to improve solubility and permeability. In the interim, compounds **15** and **23** will be taken forward further screening both in macrophages, where uptake also will be assessed, and through alternative *in vitro* screens, employing for example the mutant H37RvMA*::gfp* strain. If these compounds retain activities in these screens, their ability to target quiescent *Mtb* and to penetrate the granuloma will also be determined.

Experimental Section

General

Reagents were purchased from Sigma Aldrich, Johannesburg, South Africa and used as supplied. Bulk solvents, magnesium sulfate and sodium hydrogen carbonate were purchased from ACE Chemicals, Johannesburg, South Africa. Dichloromethane was distilled, dried over calcium carbonate and stored over 3 Å molecular sieves. Diethyl ether and tetrahydrofuran (THF) were dried over sodium, and distilled before use. Dihydroartemisinin was purchased from Changzhou Kaixuan Chemical Co, Chunjiang, China.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance^{TM} III spectrometers as solutions in chloroform-d ($CDCl_3$). Chemical shifts (δ) are reported in parts per million (ppm) and the ^{1}H chemical shifts are reported downfield of tetramethylsilane (TMS) with internal reference to the residual proton in $CDCl_3$ (δ 7.25 ppm). ^{13}C chemical shifts were internally referenced to the $CDCl_3$ 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). The coupling constant *J* are reported in Hz. Spectra were analysed with MestReNova Software, version 5.3.2-4936. High resolution mass spectrometry (HRMS) was recorded 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. Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument. Melting points (mp) were determined with a Büchi melting point B-545 instrument and are uncorrected. Column chromatography was performed using high-purity grade silica gel (pore size 60 Å, 70-230 mesh, 63-200 μm) from Sigma Aldrich and thin layer chromatography was performed with silica gel plates ($60F_{254}$) from Merck.

Syntheses

Preparation of C-10 O-linked artemisinin-cholesterol conjugates (Scheme1)

*Compound **7***. A solution of dihydroartemisinin (DHA, **2**) (0.5 g, 1.76 mmol) and cholesterol (1360 mg, 3.52 mmol, 2 eq.) was prepared in dichloromethane (20 mL) and cooled to 0 °C in an ice-bath. To this solution $BF_3{\cdot}OEt_2$ (200 µL, 0.9 eq.) was added dropwise and the reaction mixture was left to stir in the dark for 4 h as the temperature increased from 0 °C to room temperature. The reaction mixture was quenched with saturated aqueous $NaHCO_3$ (20 mL), extracted with dichloromethane (3 x 20 mL) and the extracts were combined and dried over $MgSO_4$. Following filtration to remove $MgSO_4$, the filtrate was evaporated under reduced pressure and the residue was submitted to column chromatography on silica gel. Elution with ethyl acetate-hexane (1:9 v/v) gave the product that was obtained after evaporation of the eluate as a white powder. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.41 (s, 1H, H-12), 5.30 (d, 1H, *J* = 5.1 Hz, H-10), 4.88 (t, 1H, *J* = 5.1 Hz, H-6'), 3.48 - 3.56 (m, 1H, 3'-H), 2.56 - 2.53 (m, 1H, H-4'), 1.40 (s, 3H, H-13), 0.96 (s, 3H, H-19'), 0.91 (d, *J* = 6.5 Hz, 3H, H-14), 0.87 (d, *J* = 6.5 Hz, 3H, H-15), 0.84 (s, 3H, 21'-H), 0.82 (dd, *J* = 6.5, 2.7 Hz, 6H, H-26', H-27'), 0.63 (s, 3H, H-18'); ^{13}C NMR (151 MHz, $CDCl_3$): 141.04 (C-5'), 122.21 (C-6'), 104.21 (C-3), 99.84 (C-10), 88.52 (C-12), 81.60 (C-12a), 76.64 (C-3'), 57.05 (C-14'), 56.49 (C-17'), 52.97 (C-5a), 50.44 (C-9'), 44.87 (C-8a), 42.65 (C-13'), 23.18 (C-26',27'), 14.48 (C-14), 13.56 (C-18'); IR ν_{max} cm^{-1}: 2936, 2868, 2850, 1464, 1439, 1375, 1362, 1137, 1099, in agreement with the data reported in the literature.[33]

*Compound **8***. A solution of DHA (1000 mg, 3.52 mmol) in dichloromethane (7.5 mL) containing a catalytic amount of 4-*N*,*N*-dimethylaminopyridine (100 mg, 0.81 mmol, 0.1 eq) and triethylamine (539 µL, 3.87 mmol, 1.1 eq.) was treated with cholesteryl chloroformate (1630 mg, 3.87 mmol, 1.1 eq.) in dichloromethane (7.5 mL). The mixture was stirred for 18 h at room temperature, and then quenched with saturated $NaHCO_3$ (15 mL) and extracted with dichloromethane (3 x 15 mL). The extracts were combined, and dried over $MgSO_4$. After filtration, the solvent was evaporated under reduced pressure to leave a residue that was submitted to chromatography on silica gel. Elution with ethyl acetate-hexane (3:7, v/v) gave after evaporation of the eluate the product as a white solid (1109 mg, 45%), that started melting at 160 °C and was completely molten at 185 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.56 (d, *J* = 9.8 Hz, 1H, H-10), 5.42 (s, 1H, H-12), 5.36 (d, *J* = 4.8 Hz, 1H, H-6"), 4.46 – 4.51 (m, 1H, H-3"), 2.59 – 2.53 (m, 1H, H-8a), 2.35 (d, *J* = 2.9 Hz, 2H, H-4"), 1.41 (s, 3H, H-13), 1.40 (s, 3H, H-15), 0.98 (s, 3H, H-14), 0.93 (d, *J* = 6.1 Hz, 3H, H-19"), 0.88 (d, *J* = 1.8 Hz, 3H, H-21"), 0.84 (dd, *J* = 6.6 Hz, 2.6 Hz, 6H, H-26", H-27"), 0.65 (s, 3H, H-18"); ^{13}C NMR (151 MHz, $CDCl_3$): 153.56 (C-1'), 139.37 (C-5") , 122.88 (C-6"), 104.43 (C-3), 95.53 (C-10), 91.43 (C-12), 79.90 (C-12a), 78.20 (C-3") , 56.66 (C-14"), 56.08 (C-17") , 51.47 (C-5a), 49.94 (C-9"), 45.19 (C-8a), 42.28 (C-13"), 39.67 (C-24"), 22.80 (C-25", C-26"), 19.25 (C-19"), 18.68 (C-21"), 12.09 (C-15), 11.83 (C-18"); IR ν_{max} cm^{-1}: 2934, 2867, 1750, 1456, 1375, 1250, 1204, 1182, 1170; MS: *m/z*: calcd for $C_{43}H_{68}O_7Na^+$: 719.4863 $[M+Na]^+$; found: 719.4774.

*Compound **9***. To a solution of artesunate (1000 mg, 2.60 mmol) in dichloromethane (10 mL) was added *N*,*N*'-dicyclohexylcarbodiimide (540 mg, 2.60 mmol, 1 eq.) and 4-*N*,*N*-dimethylaminopyridine (64 mg, 0.52 mmol, 0.2 eq.). To this mixture was then added a solution of cholesterol (1010 mg, 2.60 mmol, 1 eq.) in dichloromethane (10 mL) and the resulting mixture was stirred at room temperature for 4 h. The white precipitate that formed was removed by filtration, and the filtrate was washed with deionized water (20 mL) and the organic phase was dried over $MgSO_4$. Following filtration of $MgSO_4$ the solvent was evaporated from the filtrate under reduced pressure. The residue was submitted to column chromatography over silica gel. Evaporation of the eluate obtained with ethyl acetate-hexane (3:7 v/v) left a white powder (689 mg, 35%), that started melting at 152 °C and was completely molten at 185 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.77 (d, *J* = 9.9 Hz, 1H, H-10), 5.41 (s, 1H, H-12), 5.34 (d, *J* = 4.1 Hz, 1H, H-6"), 4.62 - 4.57 (m, 1H, H-

3"), 2.74 – 2.50 (m, 4H, H-2', H-3'), 2.37 - 2.32 (m, *J* = 3.2 Hz, 1H, H-14"), 2.29 - 2.28 (m, *J* = 7.1 Hz, 1H, H-8a), 1.41 (s, 3H, H-13), 0.98 (s, 3H, H-19"), 0.94 (d, *J* = 6.1 Hz, 3H, H-14), 0.88 (d, *J* = 6.5 Hz, 3H, H-15), 0.85 (d, *J* = 1.8 Hz, 3H, H-21"), 0.82 (dd, *J* = 2.6, 2.9 Hz, 6H, H-26", H-27"), 0.65 (s, 1H, H-18"); ^{13}C NMR (151 MHz, $CDCl_3$): 171.47 (C-4'), 171.19 (C-1'), 139.57 (C-5"), 122.65 (C-6"), 104.44 (C-3), 92.08 (C-10), 91.47 (C-12), 80.10 (C-12a), 74.33 (C-3"), 51.53 (C-5a), 49.97 (C-9"), 45.20 (C-8a), 42.27 (C-13"), 39.69 (C-24"), 31.86 (C-6), 18.68 (C-21"), 12.06 (C-15), 11.82 (C18"); IR ν_{max} cm^{-1}: 2949, 2895, 2867, 2847, 1761, 1714, 1467, 1458, 1374; MS: *m/z*: calcd for $C_{46}H_{72}O_8Na^+$: 774.5047 $[M+Na]^+$; found: 774.5047.

Preparation of C-10 N-linked artemisinin-cholesterol conjugates (Scheme 2)

*Compound **10***. To a suspension of DHA (1000 mg, 3.52 mmol) in toluene (10 mL) under a nitrogen atmosphere was added DMSO (25.1 µL, 0.35 mmol, 0.1 eq.) and this was followed by dropwise addition of oxalyl chloride (350 µL, 1.13 eq.). The reaction mixture was left to stir for 1 h at room temperature and then was added directly to a solution of piperazine (1500 mg, 5 eq.) in dichloromethane (10 mL). The resulting mixture was stirred overnight at room temperature under nitrogen, and then quenched with saturated aqueous $NaHCO_3$ (20 mL), and extracted with ethyl acetate (3 x 20 mL). The extracts were combined and dried over $MgSO_4$. After filtration, the solvent was removed by evaporation under reduced pressure to leave the crude product that was submitted to column chromatography. Elution with methanol-dichloromethane (1:9 v/v) and evaporation of the eluate gave the intermediate **10** as cream white solid (743 mg, 60%), mp 163.6 – 163.7 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.20 (s, 1H, H-12), 3.92 (d, *J* = 10.2 Hz, 1H, H-10), 3.07 - 2.80 (m, 4H, H-5', H-3'), 2.68 - 2.61 (m, 4H, H-6', H-2'), 2.55 - 2.46 (m, 1H, H-8a), 2.27 (td, *J* = 14.0, 3.9 Hz, 2H, H-4), 1.98 - 1.89 (m, 2H, H-7), 1.32 (s, 3H, H-13), 0.88 (d, *J* = 6.3 Hz, 3H, H-14), 0.73 (d, *J* = 7.3 Hz, 3H, H-15); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 103.96 (C-3), 91.64 (C-10), 91.06 (C-12), 80.34 (C-12a), 51.74 (C-5a), 50.70 (C-6', C-2'), 45.84 - 45.69 (C-5', C-3'), 26.02 (C-13), 24.78 (C-5), 21.67 (C-7), 20.31 (C-14), 13.45 (C-15); IR ν_{max} cm^{-1}: 3261, 2926, 2869, 2839, 1738, 1453, 1408, 1375, 1349; MS: *m/z*: calcd for $C_{19}H_{33}N_2O_4^+$: 353.2440 $[M+H]^+$; found: 353.2469.

*Compound **11***. To a stirred solution of **10** (740 mg, 2.09 mmol) and triethylamine (344 µl, 2.72 mmol, 1.2 eq.) in dichloromethane (10 mL) under a nitrogen atmosphere was added a solution of cholesteryl chloroformate (1030 mg, 2.72 mmol, 1.2 eq.) in dichloromethane (5 mL). The reaction mixture was stirred overnight and was then quenched with saturated aqueous $NaHCO_3$ (20 mL). The crude mixture was extracted with dichloromethane (3 x 20 mL) and the extracts were combined and dried over $MgSO_4$. After filtration to remove $MgSO_4$ the filtrate was concentrated under reduced pressure to leave the crude product that was submitted to column chromatography over silica gel. Elution with ethyl acetate-hexane (2:8 v/v) gave the product as a white powder (688 mg, 43%), mp 169 – 172 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.34 (d, *J* = 2.3 Hz, 1H, H-6"), 5.25 (s, 1H, H-12), 4.45 – 4.48 (m, 1H, H-3"), 4.00 (d, *J* = 8.5 Hz, 1H, H-10), 3.44 - 3.40 (m, 2H, H-5'), 2.88 – 2.96 (m, 2H, H-3'), 2.63 – 2.52 (m, 4H, H-6', H-2'), 1.62 (s, 3H, H-13), 1.36 (s, 3H, H-15), 0.99 (s, 3H, H-19"), 0.92 (d, *J* = 6.1 Hz, 3H, H-14), 0.89 (d, *J* = 6.5 Hz, 3H, H-21"), 0.84 (dd, *J* = 6.6, 2.8 Hz, 6H, H-26", H-27"), 0.65 (s, 3H, H-18"); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 154.97 (C-7'), 140.07 (C-5"), 122.36 (C-6"), 103.84 (C-3), 91.79 (C-10), 90.75 (C-12), 80.26 (C-12a), 74.64 (C-3"), 56.65 (C-14"), 56.08 (C-17"), 45.80 (C-5', C-3'), 44.05 (C-8a), 42.28 (C-13"), 20.25 (C-14), 19.34 (C-19"), 18.68 (C-21"), 13.46 (C-15), 11.82 (C-18"); IR (ATR) ν_{max} cm^{-1}: 2930, 2864, 1690, 1429, 1379, 1246, 1195, 1127, 1110; MS: *m/z*: calcd for $C_{47}H_{77}N_2O_6^+$: 765.5782 $[M+H]^+$; found: 765.5678.

Next, cholesterol was converted into the acid chlorides **12** and **13** as follows. Cholesterol (1000 mg, 2.59 mmol) was dissolved in dichloromethane (20 mL) and triethylamine (541 µL, 5.18 mmol, 2 eq.). To this mixture the acid chloride (5.18 mmol, 2 eq.) was added and the reaction mixture was left to stir at room temperature overnight. The reaction mixture was quenched with saturated $NaHCO_3$ (20 mL) and the crude mixture extracted with dichloromethane (3 x 20 mL). The extracts were combined and dried over $MgSO_4$. After filtration to remove $MgSO_4$ the filtrate was concentrated under reduced pressure to leave the crude product. To a solution of **10** (1050 mg, 2.98 mmol) in THF (10 mL) under reflux (65 °C) was added triethylamine (286 µL, 2.05 mmol, 1.3 eq.) followed by a solution of the crude product from the previous step in THF (10 mL). The reaction mixture was left to stir for 24 h and was quenched with saturated aqueous $NaHCO_3$ (20 mL). The crude mixture was extracted with diethyl ether (3 x 20 mL) and combined extracts were dried over $MgSO_4$. Following filtration to remove $MgSO_4$ the filtrate was concentrated under reduced pressure and submitted to column chromatography over silica gel. Elution with ethyl acetate-hexane (v/v) column chromatography gave pure product. This method was used for synthesis of the conjugates **14**, **15** and **17** as described below.

*Compound **14***. The product obtained from the reaction of chloroacetyl chloride with cholesterol, compound **12**, was treated with compound **10** according to the method described above. The product was purified by chromatography with ethyl acetate-hexane (2.5:7.5, v/v) to give a light-purple solid (743 mg, 32.0%), that started to melt at 145 °C and was completely molten at 203 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.34 (d, *J* = 3.7 Hz, 1H, H-6"), 5.24 (s, 1H, H-12), 4.64 (s, 1H, H-3"), 4.03 (d, *J* = 9.9 Hz, 1H, H-10), 3.29 (s, 2H, H-6'), 3.21 (s, 2H, H-7'), 3.15 – 3.05 (m, 2H, H-5', H-3'), 2.90 – 2.73 (m, 2H, H-2'), 1.34 (s, 3H, H-13), 0.98 (s, 3H, H-19"), 0.91 (d, *J* = 6.2 Hz, 3H, H-14), 0.88 (d, *J* = 6.5 Hz, 3H, H-15), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26", H-27"), 0.79 (d, *J* = 6.6 Hz, 3H, H-21"), 0.64 (s, 3H, H-18"); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 139.37 (C-5"), 122.84 (C-6"), 103.94 (C-3), 91.51 (C-10), 90.41 (C-12), 80.25 (C-12a), 74.82 (C-3"), 56.63 (C-14"), 56.08 (C-17"), 51.62 (C-5a), 49.94 (C-6', C-2'), 45.79 (C-13"), 23.79 (C-23"), 22.79 (C-26", C-27"), 20.26 (C-14), 19.27 (C-19"), 18.68 (C-21"), 13.39 (C-15), 11.82 (C-18"); IR (ATR) ν_{max} cm^{-1}: 3422, 2931, 2867, 1743, 1450, 1376, 1208, 1176, 1128; MS: *m/z*: calcd for $C_{48}H_{79}N_2O_6^+$: 779.5938 $[M+H]^+$; found: 779.5897.

*Compound **15***. The product obtained from the reaction of bromohexanoyl chloride with cholesterol, compound **13**, was treated with compound **10** as described above. The crude product was purified by chromatography with ethyl acetate-hexane (1:9, v/v) to give a cream-white solid (144 mg, 5.79%) that started softening at 121 °C and was completely molten at 171 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.34 (d, *J* = 4.0 Hz, 1H, H-6"'), 5.24 (s, 1H, H-12), 4.61 – 4.54 (m, 1H, H-3"'), 4.06 (d, *J* = 9.4 Hz, 1H, H-10), 2.54 – 2.51 (m, 2H, H-1"), 2.33 – 2.24 (m, 2H, H-5"), 1.33 (s, 3H, H-13), 1.25 – 1.20 (m, 2H, H-8), 0.99 (s, 3H, H-15), 0.92 (d, *J* = 6.3 Hz, 3H, H-14), 0.88 (d, *J* = 6.5 Hz, 3H, H-19"'), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26"', H-27"'), 0.76 (d, *J* = 7.1 Hz, 3H, H-21"'), 0.65 (s, 3H, H-18"'); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 139.64 (C-5"'), 122.59 (C-6"'), 103.95 (C-3), 91.40 (C-10), 80.28 (C-12a), 73.83 (C-3"'), 56.65 (C-1'), 56.08 (C-17"'), 51.58 (C-5a), 49.98 (C-5'), 45.72 (C-8a), 42.27 (C-13"'), 22.53 (C-26"', C-27"'), 20.25 (C-14), 19.29 (C-19"'), 18.68 (C-15), 13.39 (C-21"'), 11.82 (C-18"'); IR (ATR) ν_{max} cm^{-1}: 2927, 2864, 1732, 1460, 1444, 1375, 1322, 1236, 1188, 1173; MS: *m/z*: calcd for $C_{52}H_{87}N_2O_6^+$: 835.6564 $[M+H]^+$; found: 835.6573.

*Compound **17***. DMSO (25.1 µL, 0.35 mmol, 0.1 eq.) was added to a suspension of DHA (1000 mg, 3.52 mmol) in toluene (10 mL) under an inert atmosphere at room temperature. Oxalyl chloride (350 µL, 1.13 eq.) was added dropwise to the resulting solution. The reaction mixture was left to stir for 1 h at room temperature and then transferred into a solution of 1-(2-aminoethyl)piperazine (1500 mg, 5 eq. of **2**) in dichloromethane (10 mL). The reaction mixture was stirred overnight at room temperature under nitrogen, and then quenched with saturated aqueous $NaHCO_3$ (20 mL). The crude mixture was extracted with ethyl acetate (3 x 20 mL) and the combined extracts were dried over $MgSO_4$. After filtration to remove $MgSO_4$, the filtrate was concentrated by evaporation under reduced pressure to give a yellow oil. This was dissolved in dichloromethane (5 mL) without further purification and triethylamine (588 µL, 4.2 mmol, 1.2

eq. to **2**) was added. This was followed by addition of a solution of cholesteryl chloroformate (1900 mg, 4.2 mmol, 1.2 eq. to **2**) in dichloromethane (5 mL). The reaction was stirred overnight at room temperature and quenched with saturated $NaHCO_3$ (20 mL). The crude mixture was extracted with dichloromethane (3 x 20 mL) and dried on $MgSO_4$. $MgSO_4$ was removed by filtration and the filtrate was submitted to column chromatography on silica gel. Elution with ethyl acetate-hexane (3:7, v/v) column chromatography gave product as a cream-white solid (141 mg, 4.96%) that started softening at 150 °C and was completely molten at 170 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.33 (d, *J* = 4.8 Hz, 1H, H-6'''), 5.25 (s, 1H, H-12), 4.45 (s, 1H, H-3'''), 4.09 (d, *J* = 7.2 Hz, 1H, H-10), 3.52 – 3.43 (m, 2H, H-8"), 2.55 – 2.49 (m, 1H, H-8a), 2.35 – 2.28 (m, 4H, H-6', H-2'), 1.35 (s, 3H, H-13), 0.92 (d, *J* = 6.3 Hz, 3H, H-15), 0.89 (d, *J* = 6.5 Hz, 3H, H-14), 0.84 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26''', H-27'''), 0.76 (d, *J* = 7.1 Hz, 3H, H-21'''), 0.65 (s, 3H, H-18'''); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 156.49 (C-10"), 122.39 (C-6'''), 103.86 (C-3), 91.42 (C-12), 80.27 (C-12a), 74.53 (C-3'''), 56.69 (C-7"), 56.10 (C-17'''), 50.00 (C-2'), 42.28 (C-13'''), 27.98 (C-9''), 22.80 (C-26''', C-27'''), 20.24 (C-14), 19.29 (C-19'''), 18.68 (C-15), 13.39 (C-21'''), 11.82 (C-18'''); IR (ATR) ν_{max} cm^{-1}: 3344, 2931, 2866, 1701, 1460, 1434, 1376, 1333, 1303; MS: *m/z*: calcd for $C_{49}H_{82}N_3O_6^+$: 808.6204 $[M+H]^+$; found: 808.6201.

Preparation of C-10 O-linked artemisinin cholesteryl carbamates (Scheme 3).

A solution of DHA (1000 mg, 3.52 mmol) was prepared in dichloro-methane (20 mL) and the halohydrin (7.04 mmol, 2 eq.) was added at 0 °C in an ice-bath. To this solution $BF_3{\cdot}EtO_2$ (390.7 µL, 3.17 mmol, 0.9 eq.) was added and the reaction flask was covered with aluminum foil. The reaction mixture was stirred overnight with a gradual increase of temperature from 0 °C to room temperature and then quenched with saturated aqueous $NaHCO_3$ (20 mL), followed by extraction with dichloromethane (3 x 20 mL). The extracts were combined and dried over $MgSO_4$. The $MgSO_4$ was removed by filtration and the filtrate was concentrated by evaporation under reduced pressure. The resulting concentrate was either submitted to column chromatography on silica gel to give the intermediates **22** and **24** or direct recrystallization to give **18** and **20**. To the solution of piperazine (5 eq.) and triethylamine (5 eq.) in THF (8 mL) was added the appropriate intermediate in THF (8 mL). This mixture was stirred for 24 h under reflux at 65 °C and was then washed with deionized water (16 mL). The solution was dried over $MgSO_4$. Following filtration to remove $MgSO_4$, the filtrate was concentrated under reduced pressure and the crude product was used in the next step without further purification. To a cooled solution of the crude product in dichloromethane (10 mL) was added triethylamine (1.2 eq.) and then a solution of cholesteryl chloroformate (1.2 eq.) in dichloromethane (10 mL). The reaction mixture was stirred overnight as the temperature increased from 0 °C and then washed with deionized water (3 x 20 mL), the crude mixture was extracted with diethyl ether (3 x 20 mL) and the combined extracts were dried over $MgSO_4$. After removing the $MgSO_4$ by filtration, the filtrate was concentrated under reduced pressure and the products **19**, **21**, **23** and **25** were obtained by column chromatography and elution with ethyl acetate-hexane (v/v).

*Compound **18***. 1-Bromoethanol was used and the pure product was obtained as a fine white solid by recrystallization from methanol (867 mg, 63%); the solid started softening at 160 °C and was completely molten at 169 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.51 (s, 1H, H-12), 4.86 (d, *J* = 3.4 Hz, 1H, H-10), 4.19 – 4.09 (m, 1H, H-19), 3.80 (t, *J* = 5.5 Hz, 2H, H-2'), 3.58 – 3.50 (m, 2H, H-1''), 2.70 – 2.63 (m, 1H, H-8a), 1.45 (s, 3H, H-13), 0.97 (d, *J* = 6.4 Hz, 3H, H-15), 0.95 (d, *J* = 7.4 Hz, 3H, H-14); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.14 (C-3), 102.07 (C-10), 88.17 (C-12), 81.12 (C-12a), 68.20 (C-1'), 52.60 (C-5a), 44.38 (C-8a), 37.42 (C-9), 36.42 (C-4), 34.69 (C-7), 31.46 (C-2'), 30.92 (C-6), 26.18 (C-13), 24.67 (C-5), 24.39 (C-8), 20.40 (C-15), 13.01 (C-14); IR (ATR) ν_{max} cm^{-1}: 2953, 2921, 2889, 2868, 1464, 1422, 1373, 1343, 1270 in agreement with the literature.[41]

*Compound **19***. The product **18** (850 mg, 2.17 mmol) from the previous step was used and the final product was obtained by column chromatography and eluting with ethyl acetate-hexane (6:4, v/v) as white solid (695 mg, 39.5%) that started softening at 120 °C and was completely molten at 175 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.41 (s, 1H, H-12), 5.35 (t, *J* = 4.8 Hz, 1H, H-6''), 4.78 (d, *J* = 3.3 Hz, 1H, H-10), 4.52 – 4.46 (m, 1H, H-3''), 4.00 (s, 2H, H-1'), 3.64 – 3.45 (m, 4H, H-7', H-5'), 2.65 – 2.57 (m, 4H, H-8', H-4'), 2.36 – 2.25 (m, 2H, H-4''), 1.40 (s, 3H, H-13), 0.99 (s, 3H, H-19''), 0.93 (d, *J* = 6.3 Hz, 3H, H-15), 0.88 (d, *J* = 7.0 Hz, 3H, H-14), 0.86 (s, 3H, H-21''), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26'',H-27''), 0.65 (s, 3H, H-18''); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 139.89 (C-5''), 122.55 (C-6''), 104.10 (C-3), 102.00 (C-10), 87.90 (C-12), 80.99 (C-12a), 56.63 (C-23), 56.08 (C-17''), 52.96 (C-2', C-8'), 52.50 (C-5a), 49.95 (C-1', C-7'), 44.27 (C-8a), 42.27 (C-13''), 31.87 (C-6), 28.17 (C-16''), 24.67 (C-13), 22.79 (C-26'', C-27''), 20.34 (C-14), 19.33 (C-19''), 18.68 (C-21''), 13.06 (C-15), 11.82 (C-18''); IR (ATR) ν_{max} cm^{-1}: 2934, 2866, 1699, 1459, 1431, 1376, 1235; MS: *m/z*: calcd for $C_{49}H_{81}N_2O_7^+$: 809.6044 $[M+H]^+$; found: 809.6034.

*Compound **20***. 3-Bromo-1-propanol was used and the pure product was obtained by recrystallization from methanol as a white solid (576 mg, 40%), mp 88 – 91 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.44 (s, 1H, H-12), 4.82 (d, *J* = 3.5 Hz, 1H, H-10), 4.02 (ddd, *J* = 10.2, 6.2, 5.4 Hz, 2H, H-1'), 3.55 – 3.47 (m, 2H, H-3'), 2.66 (dt, *J* = 7.7, 3.5 Hz, 1H, H-8a), 2.39 (td, *J* = 14.0, 4.0 Hz, 2H, H-2'), 1.46 (s, 3H, H-13), 0.97 (d, *J* = 6.4 Hz, 3H, H-15), 0.93 (d, *J* = 7.3 Hz, 3H, H-14); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.13 (C-3), 102.12 (C-10), 87.95 (C-12), 81.07 (C-12a), 65.71 (C-1'), 52.29 (C-5a), 44.40 (C-8a), 37.47 (C-9), 36.44 (C-4), 34.65 (C-7), 32.56 (C-2'), 30.91 (C-6), 26.21 (C-3'), 24.68 (C-13), 24.54 (C-8), 20.39 (C-15), 13.00 (C-14); ν_{max} cm^{-1}: 2990, 2945, 2928, 2865, 1480, 1452, 1384, 1373, 1361, 1250, 1011 in agreement with the literature.[33]

*Compound **21***. The product **20** (550 mg, 1.36 mmol) from the previous step was used and the final product was obtained by column chromatography and eluting with ethyl acetate-hexane (8:2, v/v) as a light yellow powder (181 mg, 16%) that started softening at 142 °C and was completely molten at 184 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.34 (s, 1H, H-6''), 5.27 (s, 1H, H-12), 4.74 (d, *J* = 3.3 Hz, 1H, H-10), 4.52 – 4.46 (m, 1H, H-3''), 3.88 (dd, *J* = 15.9, 5.9 Hz, 2H, H-8'), 3.62 (s, 2H, H-1'), 3.40 (dd, *J* = 16.0, 6.1 Hz, 2H, H-6'), 2.63 – 2.58 (m, 4H, H-9', H-5'), 2.31 - 2.25 (m, 5H, H-8a, H-3', H-4'), 1.40 (s, 3H, H-13), 0.99 (s, 3H, H-19''), 0.93 (d, *J* = 6.3 Hz, 3H, H-14), 0.88 (d, *J* = 6.5 Hz, 3H, H-15), 0.86 (d, *J* = 7.4 Hz, 3H, H-21''), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26'', H-27'), 0.65 (s, 3H, H-18''); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 139.68 (C-5''), 122.62 (C-6''), 104.12 (C-3), 102.06 (C-10), 87.88 (C-12), 80.99 (C-12a), 75.32 (C-3''), 66.02 (C-1'), 53.41 (C-9', C-5'), 52.48 (C-5a, C-3'), 44.29 (C-8a, C-8', C-6'), 30.81 (C-2'), 24.64 (C-13), 22.80 (C-26'', C-27''), 20.32 (C-14), 19.32 (C-19''), 18.68 (C-21''), 13.02 (C-15), 11.82 (C-18''); IR (ATR) ν_{max} cm^{-1}: 2935, 2866, 1699, 1461, 1431, 1376, 1230; MS: *m/z*: calcd for $C_{50}H_{83}N_2O_7^+$: 823.6200 $[M+H]^+$; found: 823.6207.

*Compound **22***. 4-Chloro-1-butanol was used and the final product was obtained by chromatography and eluting with ethyl acetate-hexane (1:9, v/v) as a yellow oil (748 mg, 57%). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.40 (s, 1H, H-12A), 5.35 (s, 1H, H-12B), 4.79 (dd, *J* = 16.6, 5.9 Hz, 1H, H-10A), 4.45 (t, *J* = 9.4 Hz, 1H, H-10B), 3.95 – 3.86 (m, 1H, H-1'), 3.60 (dt, *J* = 13.0, 4.9 Hz, 4H, H-3', H-4'), 3.43 (ddd, *J* = 9.8, 8.0, 3.1 Hz, 2H, H-2'), 2.69 – 2.60 (m, 1H, H-8a), 2.53 – 2.27 (m, 2H, H-8), 1.46 (s, 3H, H-13), 0.97 (d, *J* = 6.4 Hz, 3H, H-15), 0.92 (d, *J* = 7.4 Hz, 3H, H-14); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.28 (C-3A), 104.12 (C-3B), 102.03 (C-10A), 100.07 (C-10B), 91.21 (C-12B), 87.93 (C-12A), 81.12 (C-12aA), 80.36 (C-12aB), 67.96 (C-1'A), 67.61 (C-1'B), 52.59 (C-5aA), 51.67 (C-5aB), 45.34 (C-8aA), 45.02 (C-8aB), 20.39 (C-15A), 20.31 (C-15B), 13.05 (C-14A), 12.66 (C-14B); IR (ATR) ν_{max} cm^{-1}: 2922, 2871, 1447, 1375, 1279, 1251, 1226, 1193, 1175, 1155.

*Compound **23***. **22** (700 mg, 1.87 mmol) was used and the final product was obtained by chromatography and elution with ethyl acetate-hexane

(8:2, v/v) as cream-white solid (109 mg, 7%) that started softening at 117 °C and was completely molten at 191 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.35 (s, 1H, H-12), 5.28 (s, 1H, H-6''), 4.74 (d, *J* = 3.3 Hz, 1H, H-10), 4.49 (s, 1H, H-3''), 3.83 (dt, *J* = 9.9, 6.1 Hz, 4H, H-1', H-7'), 3.65 (s, 2H, H-9'), 3.37 (t, *J* = 6.2 Hz, 2H, H-4'), 2.63 – 2.58 (m, 4H, H-10', H-6'), 2.31 (dq, *J* = 26.5, 13.8 Hz, 1H, H-8''), 1.41 (s, 3H, H-13), 0.99 (s, 3H, H-19''), 0.93 (d, *J* = 6.3 Hz, 3H, H-14), 0.88 (d, *J* = 7.2 Hz, 3H, H-15), 0.87 (d, *J* = 7.4 Hz, 3H, H-21''), 0.84 (dd, *J* = 6.6, 2.8 Hz, 6H, H-26'', H-27''), 0.65 (s, 3H, H-18''); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 122.68 (C-6''), 104.11 (C-3), 102.03 (C-10), 95.27 (C-12), 87.89 (C-12a), 81.04 (C-3''), 67.74 (C-1'), 56.63 (C-4'), 56.08 (C-17''), 49.95 (C-9''), 44.32 (C-7', C-9'), 24.65 (C-13), 22.80 (C-26'', C-27''), 22.54 (C-8), 21.01 (C-11''), 20.33 (C-14), 19.32 (C-19''), 18.68 (C-21''), 13.08 (C-15), 11.82 (C-18''); IR (ATR) ν_{max} cm^{-1}: 2936, 2869, 2851, 1695, 1463, 1432, 1415, 1376; MS: *m/z*: calcd for $C_{51}H_{85}N_2O_7^+$: 837.6357 $[M+H]^+$; found: 837.6288.

*Compound **24**.* 6-Chloro-1-hexanol was used and the final product was obtained by chromatography and elution with ethyl acetate-hexane (3:7, v/v) as a colourless oil (573 mg, 40%). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.40 (s, 1H, H-12), 4.79 (d, *J* = 3.4 Hz, 1H, H-10), 3.86 (dt, *J* = 9.6, 6.5 Hz, 2H, H-1'), 3.84 – 3.52 (m, 2H, H-6'), 3.43 – 3.35 (m, 6H, H-2', H-3' H-5'), 2.80 – 2.59 (m, 1H, H-8a), 2.49 – 2.34 (m, 2H, H-4), 2.08 – 1.90 (m, 2H, H-8), 1.46 (s, 3H, H-13), 0.97 (d, *J* = 6.4 Hz, 3H, H-15), 0.92 (d, *J* = 7.4 Hz, 3H, H-14); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 104.08 (C-3), 102.00 (C-10), 87.93 (C-12), 81.16 (C-12a), 68.26 (C-1'), 52.61 (C-5a), 45.03 (C-6'), 44.49 (C-8a), 37.52 (C-9), 36.47 (C-4), 34.69 (C-7), 32.57 (C-6), 30.96 (C-2', C-5'), 29.53 (C-4'), 26.25 (C-3'), 25.60 (C-13), 20.40 (C-15), 13.06 (C-14); IR (ATR) ν_{max} cm^{-1}: 2923, 2865, 1448, 1375, 1343, 1251, 1226, 1193, 1176.

*Compound **25**.* The compound **24** (500 mg, 1.24 mmol) was used and the final product was obtained by chromatography and elution with ethyl acetate-hexane (4:6 v/v) as cream-white powder (54.0 mg, 9.5%) that started softening at 126 °C and was completely molten at 162 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.34 (d, *J* = 6.7 Hz, 1H, H-12), 5.34 (s, 1H, H-6''), 4.74 (d, *J* = 3.2 Hz, 1H, H-10), 4.52 – 4.45 (m, 1H, H-3''), 3.79 (dd, *J* = 16.1, 6.5 Hz, 2H, H-1'), 3.58 (s, 2H, H-9'), 3.33 (dd, *J* = 16.1, 6.5 Hz, 2H, H-11'), 2.61 – 2.56 (m, 2H, H-12'), 2.34 (dt, *J* = 14.1, 3.8 Hz, 4H, H-6', H-8'), 2.26 (d, *J* = 12.3 Hz, 2H, H-4''), 2.02 – 1.92 (m, 2H, H-8), 1.41 (s, 3H, H-13), 0.99 (s, 3H, H-19''), 0.93 (d, *J* = 6.3 Hz, 3H, H-14), 0.88 (d, *J* = 6.6 Hz, 3H, H-15), 0.86 (d, *J* = 7.4 Hz, 3H, H-21''), 0.83 (dd, *J* = 6.6, 2.7 Hz, 6H, H-26'', H-27''), 0.64 (s, 3H, H-18'').; ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 154.77 (C-13'), 139.72 (C-5''), 122.55 (C-6''), 104.03 (C-3), 101.94 (C-10), 87.87 (C-12), 81.10 (C-12a), 75.05 (C-3''), 68.25 (C-1'), 58.41 (C-14''), 56.63 (C-6'), 52.54 (C-8', C-12'), 49.94 (C-9''), 44.42 (C-9', C-11'), 30.89 (C-2'), 27.98 (C-4'), 27.09 (C-5'), 26.20 (C-3'), 26.03 (C-13), 22.79 (C-26'', C-27''), 22.53 (C-11''), 20.36 (C-14), 19.32 (C-19''), 18.68 (C-21''), 13.01 (C-15), 11.82 (C-18''); IR (ATR) ν_{max} cm^{-1} 2927, 2851, 1698, 1431, 1234, 1225; MS: *m/z*: calcd for $C_{53}H_{89}N_2O_7^+$: 864.6670 $[M+H]^+$; found: 865.6639.

Antimalarial assays

Malaria parasite proliferation can be directly monitored in their intraerythrocytic environment through detecting and monitoring DNA replication in the absence of interference from erythrocytes, which lack DNA.[42] SYBR Green I is a fluorescent dye that intercalates in the DNA minor groove. The correlation between DNA content (SYBR Green I signal) and parasitaemia can be used to monitor the decrease in parasitaemia as a measurement of inhibition of parasite proliferation.[43] *P. falciparum* (*Pf*) parasites were maintained at 37 °C in human erythrocytes (O^+) suspended in complete culture medium RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 200 µM hypoxanthine (Sigma-Aldrich), 0.2% sodium bicarbonate, 24 µg/ml gentamycin (Sigma-Aldrich) and 0.5% AlbuMAX II in an atmosphere consisting of a 90% N_2, 5% O_2, and 5% CO_2.[44] *In vitro* ring stage intraerythrocytic *Pf* parasite cultures genotyped according to drug sensitive or resistant strains; W2 (chloroquine, quinine, pyrimethamine and cycloguanil resistant), K1 (chloroquine, pyrimethamine, mefloquine and cycloguanil resistant) and NF54 (drug sensitive) (1% haematocrit, 1% parasitaemia). The parasite suspensions were treated with the compounds, firstly at 5-, 1µM, 500-100 nM for dual point analysis and secondly, based on the activity obtained with dual points, through a two-fold serial dilution to obtain a full dose-response. Results were expressed as the inhibitory compound concentration at 50% parasite viability/proliferation (IC_{50}). The controls for this assay included chloroquine disulfate (1 µM, as positive control) and complete RPMI media (as negative control) and grown for 96 h at 37 °C under the 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 µL/mL 10000 x 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 chloroquine-treated iRBC 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. Data obtained were analysed in Excel and sigmoidal dose-response curves were plotted using GraphPad 5.0. Experiments were performed in technical triplicates, for three independent biological replicates.

Cytotoxicity assays

The non-malignant human embryonic kidney cell line HEK293 (ATCC® CRL-1573™) were cultured in Dulbecco's modified essential medium (DMEM; Hyclone, GE healthcare, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 200 mM L-glutamine and 1% non-essential amino acids (Lonza, Basel, Switzerland). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For compound treatment, cells were seeded into a 96 well plate and cultured until 80-90% confluent. Stock solutions of the compounds were prepared in ethanol preheated to 40 °C. All subsequent dilutions were prepared in serum free DMEM and vehicle controls were included in all experiments.

Following this, *in vitro* cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich). The HEK293 cells were seeded in a 96-well plate and incubated until cells were ~90% confluent. After 24 hours exposure to the compounds (12 -1800 µM) the growth medium was removed, cells rinsed twice with 1 x phosphate buffered saline (PBS) and 100 µL fresh serum free medium containing 5 mg/mL MTT solution was added. Cells were then incubated for 4 hours at 37 °C, after which the MTT was carefully removed and replaced with 100 µL DMSO. After 1 hour of incubation at 37 °C, cell viability was determined using a microplate reader (SpextraMac Paradigm) at an excitation wavelength of 550 nm and emission wavelength of 630 nm with DMSO measured as a blank. Cell viability is expressed as a percentage relative to the untreated control, which is assumed to be 100 % viable. As a positive control, cells were treated with 0.01% Triton-X 100 (Sigma-Aldrich, St Louis, MO, USA) for 4 hours. Using the MTT assay data, IC_{50} values were calculated using GraphPad Prism 5. In brief, data were normalized to the negative controls (presumed to be 100% viable), followed by the log-transformation of the concentration values. The curve was fitted using the log (inhibitor) vs. response function and the IC_{50} values calculated. Experiments were performed at least in triplicate.

Antituberculosis assay

Mtb H37Rv from stock cultures was initially inoculated for 1:10 dilution in a 25 cm^2 tissue flask for 4 days and the OD_{600} reached 0.6. These cultures were sub-cultured at 1:5 dilutions into 75 cm^2 until OD_{600} of 0.3 was reached for the experiment. Aliquots of 10 mL from a single culture

were transferred into 25 cm^2 tissue flasks. Each culture flask was treated with the desired amount of drug (80 and 10 µM) for 24 hrs and one control flask with no drugs. Two hundred µL of the untreated culture flasks were used to count CFU/ml at the time of treatment (T-zero). After 24 hrs of incubation at 37 °C, aliquots of 200 µL from each culture flask was centrifuged, re-suspended and washed twice in 7H9 and CFU were plated on 7H11 agar. CFUs were counted after 12-15 days.

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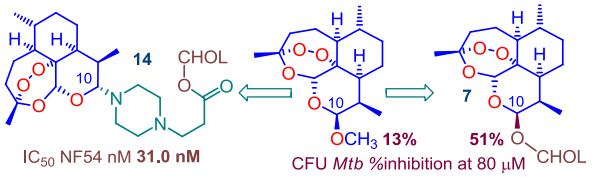
Disclaimer: Any opinion, finding and conclusion or recommendation expressed in this material is that of the authors and the NRF does not accept any liability in this regard.

Keywords: Malaria, tuberculosis, artemisinins, cholesterol, conjugates.

Entry for the Table of Contents



Because of the potential of initiating active transport of substrates containing a peroxide pharmacophore via way of a putative cholesterol transporter, a series of artemisinin-cholesterol conjugates were prepared, and whilst less active than the parent artemisinin against *P. falciparum*, were appreciably more active against *M. tuberculosis in vitro*. Poor solubility appears to limit activity of the conjugates.

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