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Highly potent artemisinin-derived dimers and trimers: Synthesis and evaluation of their antimalarial, antileukemia and antiviral activities

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ABSTRACT

New pharmaceutically active compounds can be obtained by modification of existing drugs to access more effective agents in the wake of drug resistance amongst others. To achieve this goal the concept of hybridization was established during the last decade. We employed this concept by coupling two artemisinin-derived precursors to obtain dimers or trimers with increased in vitro activity against *Plasmodium falciparum* 3D7 strain, leukemia cells (CCRF-CEM and multidrug-resistant subline CEM/ADR5000) and human cytomegalovirus (HCMV). Dimer **4** (IC_{50} of 2.6 nM) possess superior antimalarial activity compared with its parent compound artesunic acid (**3**) (IC_{50} of 9.0 nM). Dimer **5** and trimers **6** and **7** display superior potency against both leukemia cell lines (IC_{50} up to 0.002 μM for CCRF-CEM and IC_{50} up to 0.20 μM for CEM/ADR5000) and are even more active than clinically used doxorubicin (IC_{50} 1.61 μM for CEM/ADR5000). With respect to anti-HCMV activity, trimer **6** is the most efficient hybrid (IC_{50} 0.04 μM) outperforming ganciclovir (IC_{50} 2.6 μM), dihydroartemisinin (IC_{50} >10 μM) and artesunic acid (IC_{50} 3.8 μM).

1. Introduction

Artemisinin (**1**) (Fig. 1) is a natural 1,2,4-trioxane sesquiterpene and is known to possess impressive anticancer,[1–5] antimalarial[1,6–9] and antiviral activity.[10–13] Regarding the diseases malaria and cancer, its mode of action is believed to be due to the endoperoxide bridge.[8,14–17] In case of human cytomegalovirus (HCMV), a similar mechanism might be responsible for the antiviral activity considering the fact that reactive oxygen intermediates are regulators of the activity of cellular redox-sensitive factors, such as NF-κB.[18] The anti-malaria drug artesunate inhibits replication of cytomegalovirus in vitro and in vivo.[18]

To get access to more effective and/or robust compounds than artemisinin (**1**), its derivatives like dihydroartemisinin (DHA, **2**) and artesunic acid (**3**) (Fig. 1) are very versatile precursors. Connecting these precursors chemically with one or more other natural product fragments lead to the formation of hybrid compounds.[19–30] These hybrids often possess strikingly improved or new biological activities compared to their parent compounds. Although none of these hybrids have reached clinical application yet, there are many examples available in literature with already very promising in vivo results demonstrating the great potential of the hybridization concept.[28,29,31–36]

In this study, four artemisinin-derived hybrids **4**–**7** (Fig. 2) were synthesized and investigated for their potency against the malaria parasites *Plasmodium falciparum* 3D7 strain, the leukemia cell lines CCRF-CEM and CEM/ADR5000 as well as against HCMV. Dimer **4** is known from literature,[37] but was prepared only by degradation of artesunic acid (**3**) and up to now not investigated for its potential against cancer cells and HCMV. The two artesunic acid dimers **8** and **9**, previously reported by our group[38,39] were investigated for their antiviral potential in the present work.

2. Results and discussion

2.1. Synthesis

All hybrids presented in this study (Fig. 2) are connected either by an ester-, an ether- or an amide bond. Hybrids **4**, **5** and **6** were

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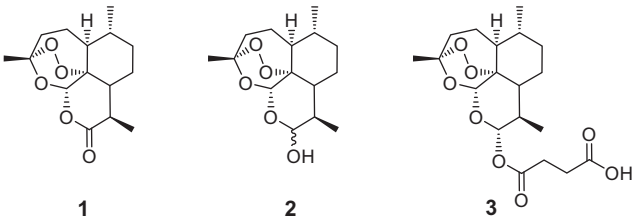


Figure 1. Artemisinin (**1**) and its derivatives dihydroartemisinin (**2**) and artesunic acid (**3**).

obtained by a Steglich esterification (DCM, DMAP and DCC at rt) of different artemisinin-derived precursors in yields ranging from 57% to quantitative (Scheme 1). The precursors DHA (**2**) and artesunic acid **3** are commercially available, artemisinin-derived acid **10**,[40] di-artemisinin-derived alcohol **11**[41,42] and artemisinin-derived alcohol **12**[43] were prepared by literature known procedures. A published reaction protocol (TMSOTf, $CHCl_3$, 0 °C)[44] yielded ether-hybrid **7** in 52% yield from dihydroartemisinin acetate **13** and alcohol **11**. In contrast to literature procedure[42] acetylated DHA **13** was obtained directly from DHA (**2**). Compound **13** was obtained as a mixture of α- and β-diastereomers.

Taking a closer look at the stereochemistry of the dimers **4** and **5** and trimers **6** and **7**, it can be deduced that compound **4** is an α,α-dimer as it was derived from purchased α-artesunic acid (**3**). Additionally, the coupling constant of 9.8 Hz for the proton at C-10 confirms the α-configuration. Dimer **5** possesses β,β-configuration as it was synthesized from its literature known precursors **10** and **12**, which both have β-configuration at C-10. Trimer **6** is a β,β,α-configured hybrid at the three C-10 positions of the artemisinin moieties due to the configuration of its precursors artesunic acid (**3**) and alcohol **11**.[42] The last hybrid **7** possesses a coupling constant of 3.3 Hz for the proton at C-10 of the DHA-moiety and therefore, compound **7** has β-configuration at all three C-10 positions of the artemisinin subunits.[42] The stability of the hybrids **4**–**7** was investigated by repeating the work of Posner and co-workers by heating the compounds at 60 °C for 20 h.[42] ^{1}H NMR analysis of the compounds show less than 5% decomposition suggesting the compounds tested were stable.

2.2. Biological evaluation and discussion

Hybrids **4**–**7** as well as their parent compounds DHA (**2**), artesunic acid (**3**) and the antimalarial drug chloroquine were evaluated for their in vitro activity against the drug-sensitive *P. falciparum* 3D7 parasite strain (Table 1).

All four hybrids possessed antimalarial activity in the nanomolar range with IC_{50} between 2.6 and 12.8 nM. The IC_{50} values of hybrids **4** (IC_{50} of 2.6 nM) and **5** (IC_{50} of 2.6 nM) were comparable to that of DHA (**2**) (IC_{50} of 2.5 nM) and more active than chloroquine (IC_{50} of 9.8 nM). While the activity of hybrid **4** towards *P. falciparum* was higher than its parent compound artesunic acid (**3**) (IC_{50} of 9.0 nM), it was lower when compared to the second parent compound DHA (**2**) (IC_{50} of 2.5 nM). Interestingly, the dimers with the two endoperoxide moieties (compounds **4** and **5**) were considerably more effective than the hybrids with three endoperoxide moieties (**6** and **7**), the latter exhibiting IC_{50} values of 12.8 nM. These were in the same concentration range as the one of the artemisinin-derived alcohol (**12**) (IC_{50} of 11.6 nM) with one endoperoxide moiety and slightly higher than that of artesunic acid (IC_{50} of 9.0 nM). These results imply that the addition of further peroxide groups does not necessarily increase antimalarial activity. Remarkably, hybrid **4**, which closely resembles artesunic acid (**3**), was one of the most active hybrids against malaria parasites in this study.

Antileukemic activity of hybrids **4**–**7** as well as of DHA (**2**) and artesunic acid (**3**) was investigated against wild-type CCRF-CEM as well as against multidrug-resistant P-glycoprotein over-expressing CEM/ADR5000 cells (Table 2). Contrary to the activity

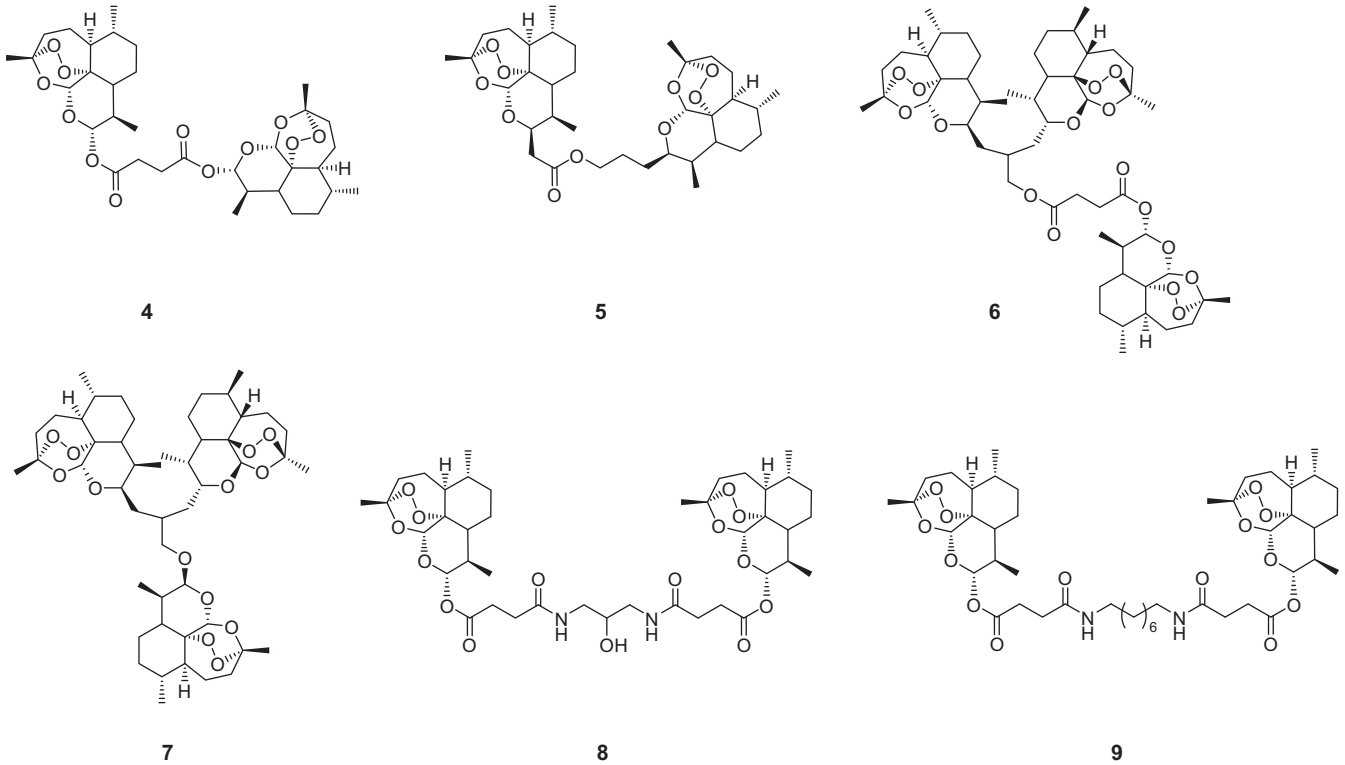
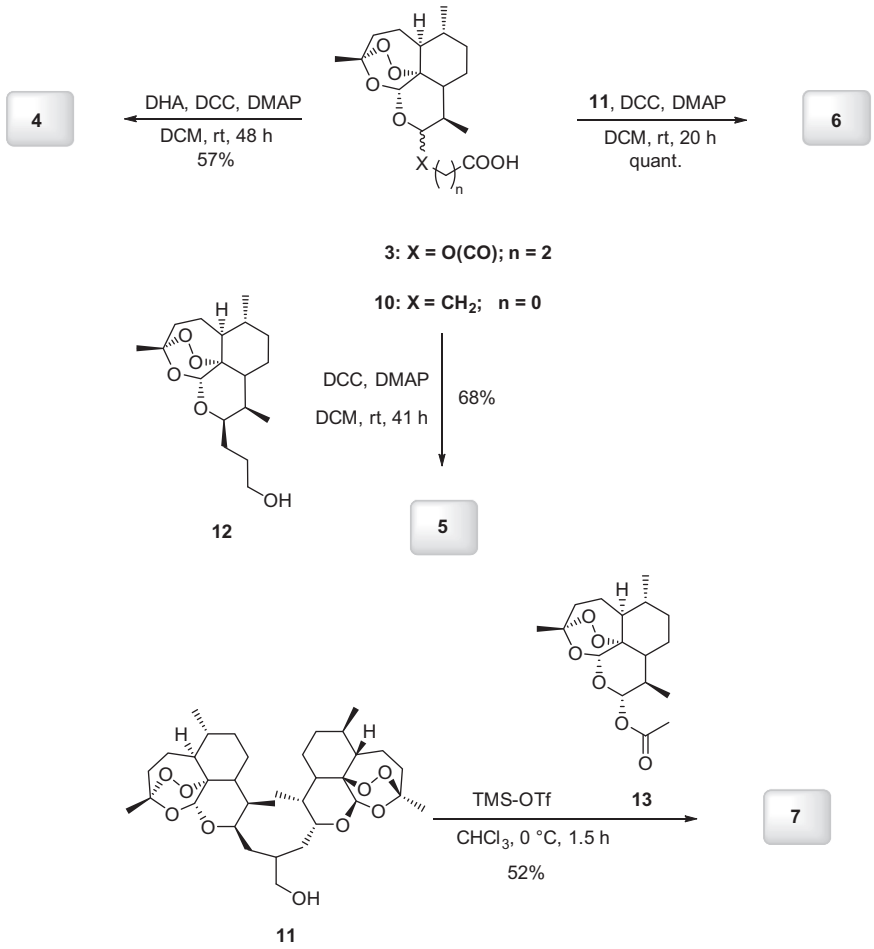


Figure 2. Hybrids **4**–**9** applied for biological tests against *P. falciparum* 3D7 parasites, CCRF-CEM, CEM/ADR5000 cells and HCMV in this work.



Scheme 1. Synthesis of artemisinin-derived hybrids **4**–**7**.

Table 1
IC_{50} values for chloroquine, DHA (**2**), artesunic acid (**3**) artemisinin-derived alcohol **12**, di-artemisinin-derived alcohol **11** and hybrids **4**–**7** against *P. falciparum* 3D7 parasites

Compound	Molecular weight	3D7 IC_{50} (nM)
Chloroquine	319.87	9.8 ± 2.8
DHA (**2**)	284.35	2.5 ± 0.5
Artesunic acid (**3**)	384.42	9.0 ± 1.5
Artemisinin-derived alcohol **12**	326.43	11.6 ± 0.7
Di-artemisinin-derived alcohol **11**[a]	606.79	1.1 ± 0.5
4	650.75	2.6 ± 0.4
5	634.80	2.6 ± 0.5
6	973.19	12.8 ± 1.2
7	873.12	12.8 ± 1.1

[a] This compound was investigated for its activity against *P. falciparum* NF54 by Posner and co-workers before and exhibited an IC_{50} of 0.87 nM.[42]

Table 2
IC_{50} values for doxorubicin, artemisinin (**1**), DHA (**2**), artesunic acid (**3**), artemisinin-derived alcohol **12**, di-artemisinin-derived alcohol **11** and hybrids **4**–**7** in sensitive wild-type CCCR–CEM and multidrug-resistant P-glycoprotein-overexpressing CEM/ADR5000 cells

Compound	Molecular weight	CCRF-CEM IC_{50} (μM)	CEM/ADR5000 IC_{50} (μM)	Degree of cross-resistance
Doxorubicin	579.98	0.003	1.61 ± 0.17	536.67
Artemisinin (**1**)	282.34	36.90 ± 6.90	26.90 ± 4.40	0.73
DHA (**2**)	284.35	0.09	0.27 ± 0.01	3.00
Artesunic acid (**3**)	384.42	0.07 ± 0.03	0.19	2.71
Artemisinin-derived alcohol **12**	326.43	173.90	n.a.[a]	—
Di-artemisinin-derived alcohol **11**[b]	606.79	0.02	0.05 ± 0.02	2.50
4	650.75	2.75 ± 0.02	2.79 ± 0.02	1.01
5	634.80	0.09 ± 0.01	0.20 ± 0.02	2.22
6	973.19	0.002	0.20 ± 0.01	100.00
7	873.12	0.002	0.49 ± 0.21	245.00

[a] n.a. = not active, even at a concentration of 400 μM.
[b] This compound was tested for its efficacy against prostate cancer cell lines by Posner and co-workers before.[45]

against malaria parasites, hybrid **4** was the least active compound against wild-type leukemia cells with an IC_{50} value of 2.75 μM as well as against the multidrug-resistant ones (IC_{50} value of 2.79 μM). Its parent compounds DHA (**2**) and artesunic acid (**3**) were more active against both cell lines (IC_{50} values $\leqslant$0.27 μM). Hybrid **5** with an IC_{50} of 0.09 μM against CCRF-CEM and IC_{50} of 0.20 μM against CEM/ADR5000 cells, shows similar activities as DHA (**2**) and artesunic acid (**3**). Compounds **5**, **6** and **7** (IC_{50} values $\leqslant$0.49 μM) proved to be versatile agents in the fight against the multidrug resistant cell line CEM/ADR5000 as all three were at least three times as active as doxorubicin (IC_{50} value of 1.61 μM). Interestingly, hybrids **6** and **7** (both with an IC_{50} value of 0.002 μM) were also more active than doxorubicin (IC_{50} of 0.003 μM) against CCRF-CEM cells. Regarding the antileukemic activity, it was observed that C-10 non-acetal dimers (e.g. hybrid **5**) were more active in vitro than C-10 acetal compounds (e.g. hybrid **4**). Additionally, the compounds with three endoperoxide

groups possess higher activity against CCRF-CEM cells compared with those of one or two endoperoxide groups.

The antiviral activity of compounds was determined for HCMV, strain AD169-GFP, that expresses quantifiable amounts of the green fluorescent protein (GFP) as a reporter of viral replication (Table 3). Infection experiments were performed with cultures of primary human foreskin fibroblasts (HFFs) and measurements of antiviral activity were carried out according to a protocol established previously.[46,47] As two reference compounds, ganciclovir (GCV) and artesunic acid (**3**), exerting anti-HCMV activity at IC_{50} values of 2.6 ± 0.5 μM and 3.8 ± 0.4 μM, respectively, were compared to the novel derivatives. Most of these novel compounds showed improved efficacy of anti-HCMV activity in this test system, that is, IC_{50} values of 1.04 ± 0.05 μM for hybrid **4**, 0.31 ± 0.02 μM for hybrid **5** and 2.07 ± 0.48 μM for compound **8** (whereas compound **9** showed very poor activity up to 10 μM, similar to the unmodified, parent compound artemisinin and its metabolite dihydroartemisinin as previously demonstrated[47]). Importantly, trimer **6**, exerted an extremely strong antiviral activity already at nanomolar concentrations (IC_{50} 0.04 ± 0.01 μM). This finding demonstrated an anti-HCMV in vitro efficacy of the trimer **6** approximately two log10 levels higher than GCV and artesunic acid (**3**). In addition, hybrid **6** proved to be stable regarding repeated freezing/thawing and even exposure to temperatures up to 56 °C (data not shown). No signs of cytotoxicity was detected on HFFs up to a concentration of 10 μM when routinely monitored by light and fluorescence microscopy, thereby strongly arguing for the specific antiviral effect of the compound (which stood in contrast to trimer **7** producing cytotoxicity towards HFFs at an identical range of concentrations up to 10 μM). Thus, these results demonstrate a substantial increase of the anti-HCMV potency of artemisinin-derived hybrids compared to reference compounds, most of all for the case of trimer **6**.

Table 3
IC_{50} values of anti-HCMV activity (AD169-GFP) displayed in virus-infected HFFs: DHA (**2**), artesunic acid (**3**) artemisinin-derived alcohol **12**, di-artemisinin-derived alcohol **11** and hybrids **4**–**9**

Compound	Molecular weight	HCMV IC_{50} (μM)
Ganciclovir (GCV)[a]	255.23	2.6 ± 0.5
Artesunic acid (**3**)[a]	384.42	3.8 ± 0.4
Artemisinin (**1**)[a]	282.34	>10
DHA (**2**)[a]	284.35	>10
4	650.75	1.04 ± 0.05
5	634.80	0.31 ± 0.02
6	973.19	0.04 ± 0.01
7	873.12	n.d.[*]
8	822.94	2.07 ± 0.48
9	877.07	>10

[a] IC_{50} values have been previously reported.[18]
[*] Not determined due to cytotoxicity.

3. Conclusion

In summary, a series of artemisinin-derived hybrids (two dimers and two trimers) were synthesized and evaluated for their in vitro potential as antimalarial, antileukemia, and antiviral agents. Regarding the activity against *P. falciparum* 3D7 parasites all four compounds proved to be active with IC_{50} values lower than 13 nM. Dimers **4** and **5** (IC_{50} value of 2.6 nM for both) were more active than trimers **6** and **7** (IC_{50} of 12.8 nM for both). These two trimers proved to be highly active agents against CCRF-CEM leukemia cells as their activity (IC_{50} value of 0.002 μM for both) was in the same range as the one of the applied drug doxorubicin (IC_{50} of 0.003 μM). All three hybrids **5**, **6** and **7** were 3- to 8-fold more active against the multidrug-resistant leukemia cell line CEM/ADR5000 and, therefore, could be regarded as potential drug alternatives to doxorubicin. Evaluated for their anti-HCMV potential, hybrids **4**, **5** and **6** (IC_{50} values 0.04–1.04 μM) showed higher efficacy than their parent compounds DHA (**2**) and artesunic acid (**3**) as well as the common applied drug GCV. This study clearly proves the power of hybrids consisting of two or three artemisinin-derived moieties in the fight against leukemia and viruses. Therefore, further investigations on this substance class are underway in our laboratories.

4. Experimental section

4.1. Chemistry

All reactions were performed in flame-dried glassware under a nitrogen atmosphere. After column chromatography all hybrids, besides hybrid **4**, were reprecipitated to yield a pure compound for elemental analysis and biological tests (solvent mixture given in the experimental procedures). DCM and $CHCl_3$ were dried initially over $CaCl_2$ and then distilled from P_2O_5. All other solvents were purified by distillation using rotary evaporation or were purchased in HPLC-quality. Reagents obtained from commercial sources were used without further purification. TLC chromatography was performed on precoated aluminium silica gel SIL G/UV254 plates (Macherey-Nagel & Co.). The detection occurred via fluorescence quenching or development in a phosphomolybdic acid solution (10% in EtOH). All products were dried in high-vacuum (10^{-3} mbar). ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature on a Bruker Avance spectrometer operating at 300 MHz. ESI Mass spectra were recorded on a Bruker micrOTOF II focus TOF MS-spectrometer. IR spectra were recorded on a Varian IR-660 apparatus. The Absorption is indicated in wave numbers [cm^{-1}]. Elemental analysis (C, H, N), carried out with an Euro EA 3000 (Euro Vector) machine and an Elementar vario MICRO cube machine, is within ±0.43% of the calculated values confirming a purity of >95%. DHA (**2**) and artesunic acid (**3**) was obtained from ABCR (Karlsruhe, Germany).

4.1.1. Procedure for α-dihydroartemisinin acetate 13

At −78 °C DHA (**2**) (1.50 g, 5.28 mmol, 1.0 equiv) was dissolved in dry DCM. Afterwards, pyridine (1.49 mL, 1.46 g, 18.5 mmol, 3.5 equiv) and DMAP (774 mg, 6.34 mmol, 1.2 equiv) were added to the reaction mixture. After addition of acetic anhydride (1.99 mL, 2.15 g, 21.1 mmol, 4.0 equiv), the solution was stirred at −78 °C for 3 h, then slowly warmed to rt and stirred o/n. The reaction was quenched by adding a saturated solution of NH_4Cl (30 mL). After phase separation the aqueous phase was extracted with DCM (3 × 20 mL). The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a colorless solid. The crude product was purified by column chromatography (hexanes/EtOAc 9:1) and ester **13** could be obtained as a colorless solid (1.50 g, 4.60 mmol, 87%, configuration: α 48%; β 52%).

Analysis for the α-isomer: R_f = 0.30 (PE/EtOAc 9:1, phosphomolybdic acid). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.83 (d, J = 7.2 Hz, 3 H), 0.94 (d, J = 6.0 Hz, 3 H), 0.97–1.07 (m, 1 H), 1.19–1.80 (m, 10 H), 1.82–1.92 (m, 1 H), 1.96–2.06 (m, 1 H), 2.11 (s, 3 H), 2.29–2.42 (m, 1 H), 2.47–2.61 (m, 1 H), 5.42 (s, 1 H), 5.78 (d, J = 9.9 Hz, 1 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.1, 20.2, 21.1, 22.0, 24.5, 26.0, 31.7, 34.0, 36.2, 37.2, 45.2, 51.5, 80.1, 91.5, 91.8, 104.5, 169.8 ppm.

4.1.2. General procedure for hybrids 4 and 6

A solution of artesunic acid (**3**) and DMAP in dry DCM was cooled to 0 °C. The corresponding alcohol **2** or **11** and DCC was

added and the reaction mixture was slowly warmed to rt and stirred o/n (for hybrid **6**) or for 47 h (for hybrid **4**). The precipitated DCU was removed by filtration and the solvent was removed under reduced pressure. The residue was purified by column chromatography. Both hybrids were obtained as colorless solids.

*Hybrid **4**:*[37] artesunic acid (**3**) (150 mg, 0.39 mmol, 1.0 equiv), DMAP (14.3 mg, 0.12 mmol, 31 mol %), DCM (11.1 mL), DHA (**2**) (333 mg, 1.17 mmol, 3.0 equiv), DCC (88.6 mg, 0.43 mmol, 1.1 equiv). Column conditions: Hexanes/EtOAc 1:1. Yield: 144 mg, 0.22 mmol, 57%. R_f = 0.61 (Hexanes/EtOAc 1:1, phosphomolybdic acid). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.84 (d, J = 7.1 Hz, 6 H), 0.94 (d, J = 5.7 Hz, 6 H), 0.98–1.07 (m, 2 H), 1.19–1.53 (m, 14 H), 1.59–2.06 (m, 10 H), 2.35 (td, J = 13.9, 3.8 Hz, 2 H), 2.46–2.86 (m, 6 H), 5.41 (s, 2 H), 5.76 (d, J = 9.9 Hz, 2 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.1, 20.2, 22.0, 24.5, 25.9, 28.8, 31.7, 34.0, 36.2, 37.2, 45.2, 51.5, 80.1, 91.4, 92.1, 104.4, 171.1 ppm. Anal. Calcd for $C_{34}H_{50}O_{12}$: C, 62.75; H, 7.74; Found: C, 62.51; H, 7.75.

*Hybrid **6**:* artesunic acid (**3**) (66.3 mg, 0.17 mmol, 1.5 equiv), DMAP (4.21 mg, 0.04 mmol, 30 mol %), DCM (5.0 mL), alcohol (**11**) (70.0 mg, 0.12 mmol, 1.0 equiv), DCC (35.7 mg, 0.17 mmol, 1.5 equiv). Column conditions: Hexanes/EtOAc 4:1, 2:1. Yield: 112 mg, 0.12 mmol, quant. Reprecipitation: Et_2O/*n*-hexane. R_f = 0.54 (Hexanes/EtOAc 3:2, phosphomolybdic acid). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.74–1.06 (m, 21 H), 1.11–1.51 (m, 24 H), 1.51–1.79 (m, 10 H), 1.83–1.92 (m, 3 H), 1.92–2.05 (m, 3 H), 2.08–2.40 (m, 4 H), 2.47–2.75 (m, 7 H), 4.15–4.27 (m, 3 H), 4.27–4.36 (m, 1 H), 5.26 (s, 2 H) 5.39 (s, 1 H), 5.75 (d, J = 9.9 Hz, 1 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.0, 12.8, 13.2, 14.1, 20.1, 20.2 (2×), 20.3, 21.9, 22.6, 24.5, 24.7 (2×), 24.8, 25.9, 26.0 (2×), 28.9, 29.3, 29.8, 30.5, 31.5, 31.7, 34.0, 34.1, 34.4, 34.5, 36.2, 36.5, 36.6, 37.2, 37.4, 44.2, 44.5, 45.2, 51.5, 52.1, 52.4, 66.9, 71.5, 73.5, 80.0, 81.1 (2×), 88.7, 89.3, 91.4, 92.0, 102.8, 103.1, 104.4, 171.1, 172.0 ppm. MS (ESI): m/z = 996 $[M+Na]^+$; HRMS (ESI): m/z Calcd for $[C_{53}H_{80}O_{16}Na]^+$: 995.5339, found: 995.5318. FT-IR (ATR): $\tilde{\nu}$ = 2923 (w), 2871 (w), 1736 (m), 1450 (w), 1375 (w), 1312 (w), 1277 (w), 1250 (w), 1222 (w), 1183 (w), 1151 (m), 1130 (m), 1101 (m), 1037 (s), 1009 (s), 942 (m), 927 (m), 912 (w), 877 (m), 845 (m), 826 (m), 766 (w), 730 (w), 704 (w), 640 (w), 592 (w), 551 (w), 532 (w), 497 (w), 487 (w), 452 (w) cm^{-1}. Anal. Calcd for $C_{53}H_{80}O_{16}$: C, 65.41; H, 8.29; Found: C, 65.55; H, 8.50.

4.1.3. Synthesis of hybrid 5

To DMAP (9.40 mg, 0.08 mmol, 30 mol %) and 1,2,4-trioxane acid **10** (84.0 mg, 0.26 mmol, 1.0 equiv) dry DCM (3.6 mL) was added and the resulting solution was cooled to 0 °C. After the consecutive additions of a solution of alcohol **12** (92.4 mg, 0.28 mmol, 1.1 equiv) in dry DCM (3.7 mL) and DCC (58.4 mg, 0.28 mmol, 1.1 equiv), the reaction mixture was slowly warmed to rt and stirred for 40.5 h. Subsequently, the precipitated DCU was filtered off and the solvent was removed under reduced pressure. The yellow oil was purified by column chromatography and hybrid **5** was obtained as a colorless solid.

Column conditions: Hexanes/EtOAc 1:1. Yield: 111 mg, 0.17 mmol, 68%. Reprecipitation: MeOH/Et_2O/*n*-hexane. R_f = 0.64 (Hexanes/EtOAc 1:1, phosphomolybdic acid). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85 (d, J = 7.4 Hz, 6 H), 0.94 (d, J = 5.8 Hz, 6 H), 0.95–1.06 (m, 2 H), 1.20–1.49 (m, 16 H), 1.59–2.05 (m, 12 H), 2.23–2.36 (m, 2 H), 2.40–2.49 (m, 1 H), 2.58–2.81 (m, 3 H), 4.05–4.21 (m, 3 H), 4.71–4.82 (m, 1 H), 5.28 (s, 1 H), 5.29 (s, 1 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.0, 13.1, 20.2 (2×), 24.6, 24.7, 24.9, 26.0, 26.1, 26.7, 29.7, 30.3, 34.4, 35.9, 36.5 (2×), 37.4, 44.2, 44.3, 52.2, 52.3, 64.7, 71.7, 74.9, 80.8, 81.1, 89.0 (2×), 103.1, 103.2, 171.7 ppm. MS (ESI): m/z = 657 $[M+Na]^+$; HRMS (ESI): m/z Calcd for $[C_{35}H_{54}NaO_{10}]^+$: 657.3609, found: 657.3628. FT-IR (ATR): $\tilde{\nu}$ = 2937 (s), 2874 (s), 2359 (m), 2334 (w), 2183 (w), 2118 (w), 2020 (w), 1733 (s), 1450 (m), 1375 (s), 1311 (w), 1279 (m), 1252 (m), 1176 (m), 1101 (s), 1053 (s), 1008 (s), 941 (m), 877 (s), 845 (m), 825 (m), 767 (w), 728 (w), 635 (w), 549 (m), 485 (m), 439 (m) cm^{-1}. Anal. Calcd for $C_{35}H_{54}O_{10}$: C, 66.22; H, 8.57; Found: C, 66.33; H, 8.69; N, 0.29.

4.1.4. Synthesis of hybrid 7

Dihydroartemisinin acetate **13** (64.5 mg, 0.20 mmol, 1.0 equiv) and alcohol **11** (120 mg, 0.20 mmol, 1.0 equiv) were dissolved in dry $CHCl_3$ (2.0 mL) and cooled to 0 °C. Subsequently, TMSOTf (3.58 μL, 4.40 mg, 0.02 mmol, 0.1 equiv) was added. The resulting reaction mixture was stirred at 0 °C for 1.5 h and quenched with sat. Na_2CO_3 (0.5 mL). Additional H_2O was added and the aqueous phase extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (20 mL), dried over $NaSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography and hybrid **7** was received as a colorless solid.

Column conditions: Hexanes/EtOAc 4:1. Yield: 90.0 mg, 0.10 mmol, 52%. Reprecipitation: Et_2O/*n*-hexane. R_f = 0.18 (Hexanes/EtOAc 4:1, phosphomolybdic acid). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.75–0.98 (m, 21 H), 1.11–1.66 (m, 28 H), 1.66–1.92 (m, 9 H), 1.92–2.06 (m, 4 H), 2.20–2.39 (m, 3 H), 2.47–2.74 (m, 3 H), 3.57 (dd, J = 9.8, 4.8 Hz, 1 H), 3.89 (dd, J = 9.8, 4.5 Hz, 1 H), 4.12–4.21 (m, 1 H), 4.26–4.37 (m, 1 H), 4.83 (d, J = 3.3 Hz, 1 H), 5.25 (s, 1 H), 5.27 (s, 1 H), 5.39 (s, 1 H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 12.9, 13.2, 13.4, 14.1, 20.1, 20.3, 20.4, 22.6, 24.5, 24.6, 24.7, 24.8 (2×), 26.2, 30.2, 30.3, 30.5, 30.6, 31.1, 31.5, 34.5 (2×), 34.7, 35.8, 36.5, 36.6, 36.7, 37.2, 37.4 (2×), 44.4, 44.5, 44.7, 52.2, 52.5, 52.6, 70.2, 72.0, 74.7, 81.0, 81.1, 87.6, 88.4, 89.2, 102.2, 102.9, 103.2, 103.8 ppm. MS (ESI): m/z = 896 $[M+Na]^+$; HRMS (ESI): m/z Calcd for $[C_{49}H_{76}NaO_{13}]^+$: 895.5178, found: 895.5177. FT-IR (ATR): $\tilde{\nu}$ = 2921 (w), 2870 (w), 1449 (w), 1374 (w), 1278 (w), 1250 (w), 1222 (w), 1193 (w), 1178 (w), 1100 (m), 1051 (m), 1037 (m), 1008 (s), 960 (m), 936 (m), 907 (w), 877 (m), 849 (m), 825 (m), 764 (w), 728 (w), 673 (w), 651 (w), 593 (w), 548 (w), 496 (w), 484 (w), 449 (w), 436 (w) cm^{-1}. Anal. Calcd for $C_{49}H_{76}O_{13}$: C, 67.40; H, 8.77; Found: C, 67.83; H, 9.04.

4.2. Biological activity studies

Cytotoxicity studies against CCRF-CEM and CEM/ADR5000 leukemia cells:

4.2.1. Cell Culture

Human leukemic CCRF-CEM and the P-glycoprotein expressing CEM/ADR5000 cells were obtained from the University of Jena (Department for Pediatrics, University of Jena, Germany) and were cultivated in RPMI 1640 medium supplemented with 10% (v/v) inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin at 37 °C with 5% CO_2 in humidified atmosphere (95% relative humidity). CEM/ADR5000 cells were treated with 5000 ng/mL doxorubicin once per week to keep them resistant.[48] The multidrug-resistance profile of CEM/ADR5000 has been reported.[49,50] Cells were passaged twice a week and used for experiments in the logarithmic phase.

4.2.2. Resazurin viability assay

CCRF-CEM or CEM/ADR5000 cells were seeded in appropriate density (10000 cells/well) in a 96-well plate with a total volume of 200 μL. Compounds were added in varying concentration [0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 and 100 μM]. Each concentration was tested six times within each experiment and each experiment was repeated three times. Additionally, CEM/ADR5000 cells were tested with doxorubicin alone and in combination with both, three derivatives (10 μM) and verapamil [0.1, 0.3, 1, 3, 10 and 100 μM]. After 72 h at 37 °C and 5% CO_2

20 μL resazurin 0.01% w/v in ddH_2O was added to each well and there was a further incubation of the plates for 4 h. The plates were measured using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The test compound concentrations required to inhibit 50% of cell proliferation were represented by IC_{50} values, calculated from dose response curves.

4.2.3. HCMV GFP-based replication assay

HCMV GFP-based replication assays were carried out over a duration of seven days (multi-round infection) with primary human foreskin fibroblasts (HFFs) infected with a GFP-expressing recombinant human cytomegalovirus (HCMV AD169-GFP) as described before.[46,47] All data represent mean values of determinations in quadruplicate (HCMV infections performed in duplicate, GFP measurements of total cell lysates performed in duplicate). Processing and evaluation of data was performed by the use of Excel (means and standard deviations).

4.2.4. Cytotoxicity studies against *P. falciparum* 3D7 strains

4.2.4.1. *P. falciparum* culture. *P. falciparum* 3D7 parasites were cultured in type A-positive human erythrocytes at a hematocrit of 5% in RPMI 1640 supplemented with 25 mM HEPES, 0.1 mM hypoxanthine, 50 μg/ml gentamycin and 0.5% albumax I. Cultures were incubated at 37 °C under controlled atmospheric conditions of 5% O_2, 3% CO_2, and 92% N_2 at 95% relative humidity.

4.2.4.2. In vitro antimalarial activity assay. Cultures used in cell proliferation assays were synchronized by sorbitol treatment.[51] Concentrations to inhibit parasite growth by 50% (IC_{50}) were determined using the SYBR Green I malaria drug sensitivity assay.[52] 100 μl aliquots of a cell suspension containing ring stages at a parasitemia of 0.2% and a hematocrit of 2% were added to the wells of 96-well microtiter plates. Plates were incubated for 72 h in the presence of different drug concentrations. Subsequently, cells of each well were lysed with 100 μl lysis buffer (40 mM Tris, pH 7.5, 10 mM EDTA, 0.02% saponin, 0.08% Triton X-100) containing 8.3 μM SYBR green. Plates were incubated for 1 h in the dark at room temperature under constant mixing before fluorescence (excitation wavelength 485 nm; emission wavelength >520 nm) was determined using a microtiter plate fluorescence reader (Victor X4; Perkin Elmer). Drugs were serially diluted 1:3 with initial drug concentrations being 243 nM for chloroquine, artesunic acid (**3**) and its derivates and 81 nM for dihydroartemisinin (**2**) and it derivates. Each drug concentration was examined in triplicate and repeated at least three times. Uninfected erythrocytes (hematocrit 2%) and infected erythrocytes without drug served as controls and were investigated in parallel. Percent growth was calculated as described by Beez.[53] Data were analyzed using the SigmaPlot (version 12.0; Hill function, three parameters) and Sigma Stat programs.

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Supplementary data

Supplementary data (^{1}H NMR, ^{13}C NMR and mass spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.07.048.

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