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New artesunic acid homodimers: Potent reversal agents of multidrug resistance in leukemia cells

Christoph Reiter [a], Astrid Herrmann [a], Aysun Çapci [a], Thomas Efferth [b,*], Svetlana B. Tsogoeva [a,*]

[a] Institute of Organic Chemistry I, University of Erlangen-Nuremberg, Henkestrasse 42, 91054 Erlangen, Germany
[b] Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, University of Mainz, Staudinger Weg 5, 55128 Mainz, Germany

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ABSTRACT

To evade the problem of multidrug resistance, hybridization of natural products in dimers is considered as an effective method. After the successful synthesis of three artesunic acid homodimers connected by different types of chemical linkers, we analyzed their activity against human CCRF-CEM and multidrug-resistant P-glycoprotein-overexpressing CEM/ADR 5000 leukemia cells and observed, that multidrug resistant cells were not cross-resistant to the new compounds. Collateral sensitivity was observed for artesunic acid homodimer **2**. The obtained results deliver valuable information about the linker's structure which is required for homodimers to be highly cytotoxic.

1. Introduction

For thousands of years *Artemisia annua*, which is a plant source for natural trioxane artemisinin, has been used in Chinese herbal medicine to treat malaria.[1] Artemisinin and its derivative artesunate are also found to be active against cancer.[2–6] There are different pathways which are involved in the explanation for the antitumor activity of artemisinin and artesunate. One of these relates to the phenomenon of apoptosis.[7] As tumor cells contain more Fe^{2+} ions than cells of healthy tissue, oxidative cleavage of the endoperoxide bonds of artemisinin is triggered more facially and reactive oxygen species (ROS) are formed as well as carbon-centered free radicals.[8] This leads to the intrinsic death pathway. Inhibition of angiogenesis is the second important pathway to illustrate the antitumor activity of artemisinin.[9] If the tumor reaches a defined size, diffusion is not sufficient to supply it with oxygen and nutrients. Hence the cancer tissue requires its own blood vessels.[10] As a consequence, inhibitors of angiogenesis are of a significant value in the anti-cancer therapy.

In clinical studies against metastatic uveal melanoma it has become apparent that the combination of artesunate with standard chemotherapeutic agents is an efficient alternative to inoperative standard medication.[11] This result is one example for the basic principle, that new artemisinin derivatives could own new and improved features. A relatively new approach is the hybridization of two (or even more) natural product fragments to obtain novel structures (dimers, trimers etc.) with improved properties compared to their parent compounds.[12–17]

In literature, several artemisinin dimers can be encountered.[18–22] In most cases, homodimers were synthesized and the linkers vary from aromatic[18,20] to alcoholic derivates[19] and phosphoric or sulfuric varieties.[21] In contrast, the research area of artesunate dimers is still widely unexplored and these dimers provide excellent opportunities as possible anti-cancer lead structures. In 2010, our groups published different hybrid molecules-including an artesunate homodimer that contains a propane linker with two amide bonds.[23] This homodimer is much more potent against cancer than its parent compound artesunate. Motivated by this outcome, we decided to synthesize further artesunic acid homodimers, three of which are presented here. By the choice of three completely different linkers (alcoholic, aliphatic and aromatic) we expected to find out which linker system gives the homodimer most active towards sensitive CCRF-CEM and multidrug resistant P-glycoprotein-overexpressing CEM/ADR 5000 leukemia cells.

2. Results and discussion

2.1. Synthesis

Based on the results from our groups in 2010,[23] we decided to synthesize homodimers of artesunic acid that are linked via two

* Corresponding authors. Tel.: +49 6131 39 25751; fax: +49 6131 39 23752 (T.E.); tel.: +49 9131 85 22541; fax: +49 9131 85 22865 (S.B.T.).
E-mail addresses: efferth@uni-mainz.de (T. Efferth), tsogoeva@chemie.uni-erlangen.de (S.B. Tsogoeva).

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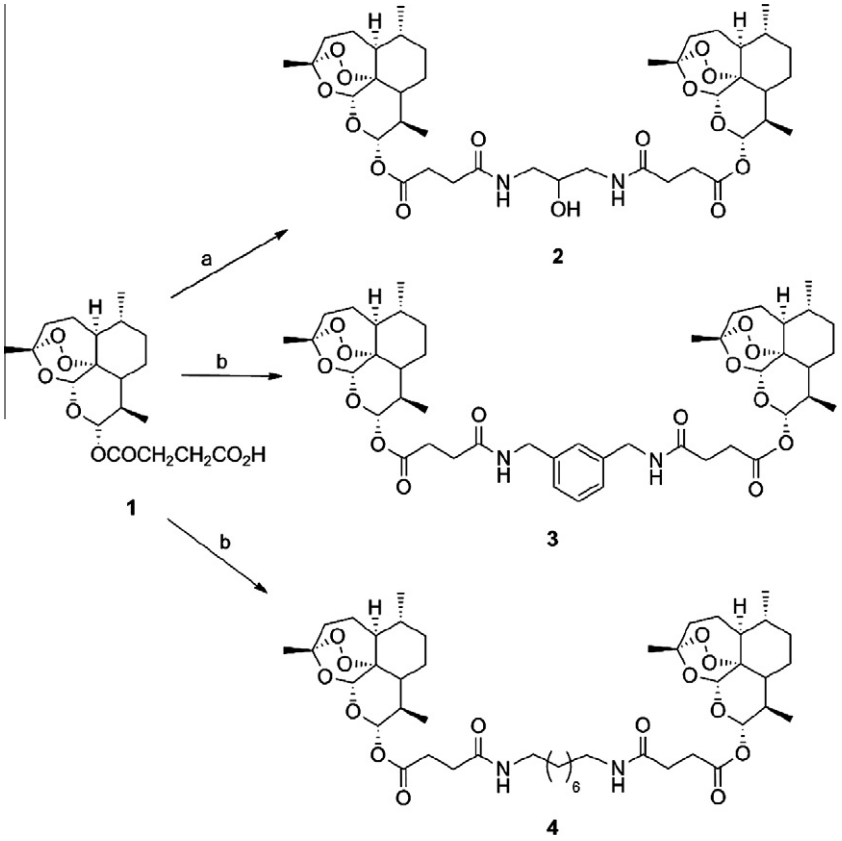

Scheme 1. Synthesis of **2**–**4**. Reagents and conditions: (a) 1,3-diamino-2-propanol, HOBt, EDCI, DMF, 83%; (b) *meta*-xylylenediamine, HOBt, EDCI, DMF, 86%; (c) 1,8-diaminooctane, HOBt, EDCI, DMF, 88%.

amide bonds. Referring to their chemical structure, three totally different linkers (aliphatic, aromatic and alcoholic) were chosen with the expectation to ascertain the biologically most active linker and thereby gain more knowledge about the artesunic acid homodimers' mode of action.

Hybrid molecules **2**–**4** were synthesized as displayed in Scheme 1. The amide bonds have been formed between the carboxylic acid function of artesunic acid and the primary amine function of the corresponding linker molecules.

Artesunic acid **1** (2.0 equiv) was coupled with the respective amine (1.0 equiv) employing EDCI (2.0 equiv) to form two amide bonds. To obtain an active ester and thereby promote the reaction, HOBt (1.0 equiv) was used. EDCI was preferred over DCC due to the easier work-up of the reaction mixture since no dicyclohexylurea was precipitating as a byproduct which often is not easy to get rid of.

All three homodimers could be obtained in good yields: 83% for the alcoholic dimer **2**, 86% for the aromatic dimer **3** and 88% for the aliphatic dimer **4** in one step. Elemental analysis demonstrated a purity of over 95% after purification with column chromatography and reprecipitation for all three dimers.

2.2. Determination of cell viability after treatment with artesunic acid homodimers

The dependence of the viability of the two cancer cell lines CCRF-CEM and CEM/ADR5000 with respect to the concentration of the respective homodimer **2**–**4** is displayed in Figure 1.

The 50% inhibition concentrations (IC_{50}) values were calculated from dose response curves of wild-type CCRF-CEM and multidrug-resistant P-glycoprotein overexpressing CEM/ADR5000 cells by CCK-8 assays. The IC_{50} values are shown in Table 1. These values are compared to the control drug doxorubicin, to artemisinin and to artesunic acid **1**. Additionally, the values for artesunic acid homodimer **5** (Fig. 2) are listed, which was synthesized by the Tsogoeva group in 2010.[23] By comparing the values of the recent three homodimers **2**–**4** to that of compound **5**, important knowledge about the linker's influence on the anti-cancer activity can be obtained.

The IC_{50} values of CEM/ADR5000 cells were divided by the corresponding values of CCRF-CEM cells, resulting in the degrees of resistance (Table 1). Multidrug-resistant CEM/ADR5000 cells did not display cross resistance to artesunic acid homodimers **3** and **4** compared to CCRF-CEM wild-type cells (Table 1). Collateral sensitivity of CEM/ADR5000 cells was observed towards artesunic acid homodimer **2**. According to the comparison, the CEM/ADR5000 cells revealed high degrees of resistance to the selecting drug, doxorubicin.[24]

The fact that we did not find cross-resistance to the artesunic acid homodimers of the multidrug-resistant CEM/ADR5000 is pleasing, since it indicates that these compounds are not transported by the P-glycoprotein drug transporters. It could be envisioned that these compounds might still be active against otherwise refractory clinical tumors. CEM/ADR5000 cells are known to display cross-resistance to a large panel of natural compounds and synthetic drugs.[24] We observed hypersensitivity of CEM/ADR5000 cells to artesunic acid homodimer **2**. This phenomenon is termed collateral sensitivity. It is well known that multidrug resistant cells exerted this phenomenon towards standard drugs such as methotrexate or alkylating agents.[25] The molecular modes of action of collateral sensitivity have not been fully understood. Among the hypothesis to explain this phenomenon was the generation of reactive oxygen species by the corresponding drugs.[25] Since artemisinin-type compounds are known to form

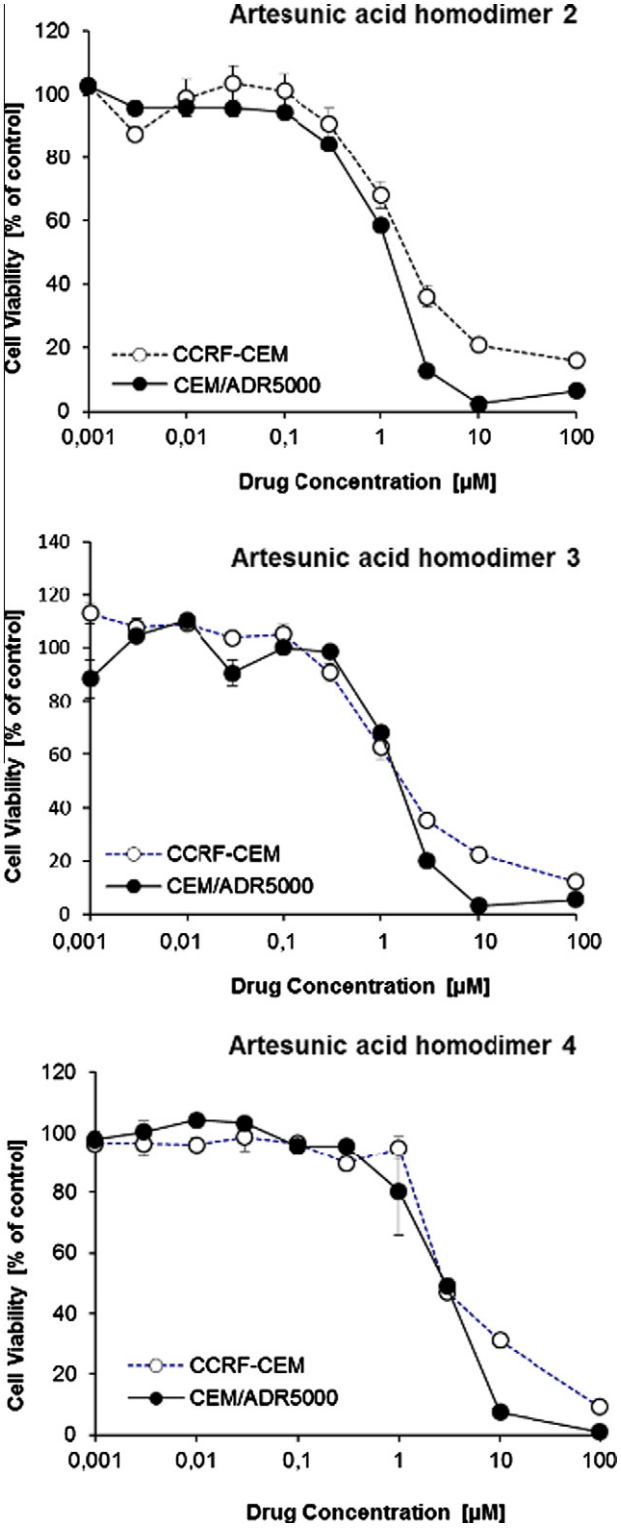

Figure 1. Cytotoxicity of artesunic acid homodimers **2**–**4** toward CCRF-CEM and multidrug-resistant CEM/ADR5000 human leukemia cells. Each experiment was independently performed at least two times with each 6 parallel measurements, leading to 12 values per concentration. Mean values (±SEM) are plotted as a function of concentration for each compound for both cell lines: artesunic acid homodimer **2**; artesunic acid homodimer **3**; artesunic acid homodimer **4**.

ROS and carbon-centered molecules by breaking the endoperoxide bridge in the molecule, it is possible that this is the explanation for collateral sensitivity of CEM/ADR5000 cells towards artesunic acid homodimer **2**.

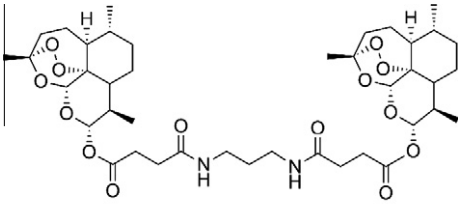

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Figure 2. Structure of artesunic acid homodimer **5.**

3. Conclusions

We have synthesized three new artesunic acid homodimers (using different types of chemical linkers), whose significant advantage is that they are easily prepared and can be obtained in good yields. The cytotoxic activity of the three homodimers has been analyzed in a human leukemia cell line (CCRF-CEM) and its multidrug-resistant subline, CEM/ADR5000, and compared to doxorubicin. The multidrug-resistant cells were not cross-resistant to any of the dimers and for artesunic acid homodimer **2** even collateral sensitivity was observed.

It seems, that the nature and length of the linker in a dimer drug is crucial and might also be partially responsible for the biological effect and could be involved in overcoming cross resistance. These results provide further insights in the development of new potent artesunic acid homo- and hetero-dimers. This work is in progress in our laboratory.

4. Experimental section

4.1. General

Synthesis of Hybrid Molecules: All reactions were performed in flame-dried glassware under a nitrogen atmosphere. After column chromatography, the dimers **2–4** were reprecipitated from CH_2Cl_2 in hexane to yield a pure compound for Elemental Analysis and biological tests. All 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 an anisaldehyde solution (6.00 g anisaldehyde, 2.50 m L concn H_2SO_4, 250 m L EtOH). All products were dried in high-vacuum (10^{-3} mbar). ^1H NMR spectra were recorded with a BRUKER Avance 300 (300 MHz). ^{13}C NMR spectra were recorded either with a BRUKER Avance 400 (100 MHz) or a BRUKER Avance 300 (75 MHz). Maldi Mass spectra were recorded with a SHIMADZU BIOTECH AXIMA Confidence. A PERKIN ELMER polarimeter (Model 341) was used for optical rotation. IR spectra were recorded with a BRUKER Tensor 27. Elemental Analysis (C, H, N), carried out with an Euro EA 3000 (Euro Vector) machine and an EA 1119 CHNS, CE machine, is within ±0.37 % of the calculated values confirming a purity of >95%. Artesunic acid obtained from ABCR (Karlsruhe, Germany) and 1,3-Diamino-2-propanol, *meta*-Xylylenediamine and 1,8-Diaminooctane obtained from Sigma Aldrich (Taufkirchen, Germany) were at least 95% pure.

4.2. Synthesis

4.2.1. Artesunic acid homodimer 2

In an evacuated flask, 41.0 mg (0.46 mmol, 1.0 equiv) of 1,3-diamino-2-propanol were dissolved in anhydrous DMF (1.70 m L)

Table 1
IC_{50} values for Doxorubicin, Artemisinin, Artesunic Acid **1**, Artesunic Acid homodimers **2**–**5** in sensitive wild-type CCRF-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	0.037	>5.20	>140[a]
Artemisinin	282.14	36.90 (±6.90)	26.90 (±4.40)	0.73
Artesunic acid **1**	384.42	1.80 (±1.20)	1.20 (±0.70)	0.67
2	822.94	2.16 (±0.99)	1.21 (±0.15)	0.56
3	869.01	1.60 (±0.70)	1.51 (±0.11)	0.94
4	877.07	2.94 (±0.33)	3.04 (±0.44)	1.03
5	806.94	1.20 (±0.10)	0.20 (±0.03)	0.17

[a] IC_{50} value of doxorubicin for CEM/ADR 5000 cells cannot be determined because of autoabsorbance of doxorubin.

under N_2. At 0 °C a solution of 62.0 mg (0.46 mmol, 1.0 equiv) HOBt and 350 mg (0.91 mmol, 2.0 equiv) of artesunic acid in anhydrous DMF (1.80 m L) was added and the reaction mixture was stirred for 5 min at 0 °C. At 0 °C and under N_2 174.0 mg (0.91 mmol, 2.0 equiv) of EDCI were added and the colourless solution was stirred for 4 d. After the addition of 15 m L EtOAc and 20 m L H_2O, the two phases were separated and the water phase was extracted with 4 × 15 m L EtOAc. The combined organic layers were washed with 3 × 15 mL H_2O and 4 × 15 mL brine. It was dried over $MgSO_4$ and the solvent was removed under reduced pressure. Chromatography ($CHCl_3$/MeOH 12:1) yielded product **2** as a colourless solid (313 mg, 0.38 mmol, 83%). R_f = 0.51 ($CHCl_3$/MeOH 12:1, anisaldehyde); ^{1}H NMR (300 MHz, $CDCl_3$):δ = 0.82 (d, J = 7.1 Hz, 6H, 2 × CH_3), 0.93 (d, J = 5.7 Hz, 6H, 2 × CH_3), 0.97–1.06 (m, 2H), 1.17–1.45 (m, 14H), 1.53–1.64 (m, 2H), 1.64–1.80 (m, 4H), 1.80–1.92 (m, 2H), 1.92–2.10 (m, 2H), 2.24–2.61 (m, 8H), 2.64–2.84 (m, 4H), 3.17–3.47 (m, 4H, 2 × CH_2), 3.77 (t, J = 4.8 Hz, 1H, C*H*OH), 3.84–4.10 (bs, 1H, CHO*H*), 5.40 (s, 2H, 2 × CH), 5.73 (d, J = 9.8, 2H, 2 × CH), 6.61 (dt, J = 17.7, 6.0 Hz, 2H, 2 × NH) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.2, 20.3, 22.1, 24.7, 26.0, 29.8 (2×), 30.7, 30.8, 31.9, 34.2, 36.3, 37.4, 43.0, 45.3, 51.6, 70.2, 80.3, 91.6, 92.4, 104.6, 172.0, 172.1, 172.9, 173.1 ppm; FT–IR (ATR): $\tilde{\nu}$ = 3297 (m), 2926 (m), 2871 (w), 1747 (m), 1654 (m), 1541 (w), 1455 (w), 1376 (w), 1200 (w), 1164 (w), 1131 (w), 1100 (w), 1035 (s), 1015 (s), 927 (w), 877 (w), 847 (w), 825 (w) cm^{-1}; MS (MALDI-TOF, dhb-Matrix): m/z = 845 ($[M+Na]^+$), 861 ($[M+K]^+$); Anal. Calcd for $C_{41}H_{62}N_2O_{15}$: C, 59.84; H, 7.59; N, 3.40. Found: C, 59.71; H, 7.81; N, 3.31; $[\alpha]_D^{20}$ = +7° (c = 0.1, CH_2Cl_2).

4.2.2. Artesunic acid homodimer 3

In an evacuated flask, 152 μl (156 mg, 1.15 mmol, 1.0 equiv) of *meta*-xylylenediamine were dissolved in anhydrous DMF (4.00 mL) under N_2. At 0 °C a solution of 155 mg (1.15 mmol, 1.0 equiv) HOBt and 885 mg (2.30 mmol, 2.0 equiv) of artesunic acid in anhydrous DMF (4.90 mL) was added and the reaction mixture was stirred for 5 min at 0 °C. At 0 °C and under N_2, 441 mg (2.30 mmol, 2.0 equiv) of EDCI were added. The solution was allowed to warm up to rt and was stirred at rt for 4 d. After the addition of 140 mL EtOAc and 100 m L H_2O, the two phases were separated and the water phase was extracted with 3 × 100 mL EtOAc. The combined organic layers were washed with 3 × 100 mL H_2O and 3 × 100 mL brine. It was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The residue was purified by column chromatography ($CHCl_3$/MeOH 29:1 and Et_2O/PE 15:1) to obtain homodimer **3** as a colourless solid (860 mg, 0.99 mmol, 86%). R_f = 0.30 ($CHCl_3$/MeOH 29:1, anisaldehyde); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.80 (d, J = 7.1 Hz, 6H, 2 × CH_3), 0.93 (d, J = 5.8 Hz, 6H, 2 × CH_3), 0.98–1.06 (m, 2H), 1.19–1.50 (m, 12H), 1.52–1.63 (m, 2H), 1.63–1.79 (m, 6H), 1.80–1.92 (m, 2H), 1.93–2.06 (m, 2H), 2.35 (dt, J = 13.9, 3.9 Hz, 2H, CH), 2.41–2.61 (m, 6H), 2.62–2.86 (m, 4H), 4.41 (ddd, J = 26.7, 15.1, 6.0 Hz, 4H, 2 × CH_2–Ar), 5.39 (s, 2H, 2 × CH), 5.71 (d, J = 9.8 Hz, 2H, 2 × CH), 6.38 (t, J = 6.0 Hz, 2H, 2 × NH), 7.13 (d, J = 6.8 Hz, 2H, 2 × Ar–H), 7.21–7.28 (m, 2H, 2 × Ar–H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$): δ = 12.3, 20.5, 22.1, 24.7, 25.9, 29.8, 30.8, 31.9, 34.1, 36.2, 37.3, 43.4, 45.3, 51.6, 80.2, 91.5, 92.4, 104.5, 126.3, 126.7, 128.8, 138.8, 171.2, 172.1 ppm; FT–IR (ATR): $\tilde{\nu}$ = 2926 (m), 1749 (m), 1656 (m), 1539 (m), 1450 (w), 1377 (w), 1162 (w), 1131 (w), 1099 (w), 1034 (m), 1016 (m), 925 (w), 876 (w), 729 (w) cm^{-1}; MS (MALDI-TOF, sin-Matrix): m/z = 891 ($[M+Na]^+$), 907 ($[M+K]^+$); Anal. calcd for $C_{46}H_{64}N_2O_{14}$: C, 63.58; H, 7.42; N, 3.32. Found: C, 63.63; H, 7.53; N, 2.98; $[\alpha]_D^{20}$ = +6 (c = 0.1, CH_2Cl_2)

4.2.3. Artesunic acid homodimer 4

In an evacuated flask, 47.0 mg (0.33 mmol, 1.0 equiv) of 1,8-diaminooctane were dissolved in anhydrous DMF (1.20 mL) under N_2. At 0 °C, a solution of 45.0 mg (0.33 mmol, 1.0 equiv) HOBt and 250 mg (0.65 mmol, 2.0 equiv) of artesunic acid in anhydrous DMF (1.30 mL) were added and the reaction mixture was stirred for 5 min at 0 °C. At 0 °C and under N_2, 125 mg (0.65 mmol, 2.0 equiv) of EDCI were added and the colourless solution was stirred at rt for 4 d. After the addition of 15 mL EtOAc and 20 mL H_2O, the two phases were separated and the water phase was extracted with 4 × 15 mL EtOAc. The combined organic layers were washed with 3 × 15 mL H_2O and 4 × 15 ml brine. It was dried over $MgSO_4$ and the solvent was removed under reduced pressure. Chromatography ($CHCl_3$/MeOH 30:1) afforded compound **4** as a colourless solid (257 mg, 0.29 mmol, 88 %). R_f = 0.80 ($CHCl_3$/MeOH 9:1, anisaldehyde); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.82 (d, J = 7.0 Hz, 6H, 2 × CH_3), 0.93 (d, J = 6.0 Hz, 6H, 2 × CH_3), 0.98–1.07 (m, 2 H), 1.21– .54 (m, 26H), 1.54–1.79 (m, 8H), 1.81–1.94 (m, 2H), 1.95–2.04 (m, 2H), 2.28–2.58 (m, 8H), 2.63–2.82 (m, 4H), 3.15–3.28 (m, 4H), 5.40 (s, 2H, 2 × CH), 5.74 (d, J = 9.9 Hz, 2 H, 2 × CH) ppm; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 12.3, 20.5, 22.2., 24.8, 26.3, 27.0, 29.3, 29.7, 30.0, 31.2, 32.0, 34.3, 36.5, 37.5, 39.8, 45.5, 51.8, 80.3, 91.7, 92.4, 104.8, 171.3, 172.0 ppm; FT–IR (ATR): $\tilde{\nu}$ = 3308 (m), 2927 (s), 1750 (m), 1651 (m), 1542 (m), 1454 (w), 1376 (w), 1201 (w), 1159 (w), 1131 (m), 1099 (w), 1009 (s), 911 (w), 876 (w), 825 (w), 727 (m) cm^{-1}; MS (MALDI-TOF, sin-Matrix): m/z = 900 ($[M+Na]^+$), 916 ($[M+K]^+$); Anal. Calcd for $C_{46}H_{72}N_2O_{14}$: C, 62.99; H, 8.27; N, 3.19. Found: C, 63.36; H, 8.54; N, 3.05; $[\alpha]_D^{20}$ = +4° (c = 0.1, CH_2Cl_2)

4.2.4. Cell culture

Cells 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/m L doxorubicin every other week for three days to maintain overexpression of P-glycoprotein.[26] The multidrug resistance profile of CEM/ADR5000 has been reported.[24,27]

Cell viability assay: CCRF-CEM or CEM/ADR5000 cells were plated in 96-well plates and maintained in RPMI1640 medium. Cell viability was determined by a commercially Cell Counting Kit

(CCK-8, Dojindo Laboratories, Munich, Germany). Ten microliters of CCK-8 reagent were added to each well and incubated at 37 °C for 3 h, until the media turned yellow. Absorbance was measured at 450 nm in a spectrophotometer. Each experiment was performed in 6 parallel measurements and repeated at least two times.

Supplementary data

Supplementary data (^{1}H- and ^{13}C NMR spectra of products) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.07.015.

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