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Cytotoxicity of Artesunic Acid Homo- and Heterodimer Molecules toward Sensitive and Multidrug-Resistant CCRF-CEM Leukemia Cells

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A novel approach to circumvent multidrug resistance is hybridization of natural products in dimers. We analyzed homodimers of two artesunic acid molecules and heterohybrids of artesunic acid and betulin in human CCRF-CEM and multidrug-resistant P-glycoprotein-overexpressing CEM/ADR5000 leukemia cells. Multidrug-resistant cells were not cross-resistant to the novel compounds. Collateral sensitivity was observed for artesunic acid homodimer. Artesunic acid and artesunic acid homodimer induced G0/G1 cell cycle arrest, apoptosis, and formation of reactive oxygen species.

Introduction

The antimalarial artemisinin and artesunate are active toward tumor cells.[1a,b] The antitumor activity of artemisinins is mediated by several pathways, including inhibition of angiogenesis and induction of apoptosis.[2,3] Oxidative stress is involved in artesunate's activity toward *Plasmodia* and tumor cells.[4] Tumor cells contain more Fe^{2+} ions than normal cells, leading to oxidative cleavage of endoperoxide bonds and formation of reactive oxygen species (ROS[a]) and carbon-centered free radicals.[5] Artesunate also induces DNA breakage.[6]

Artesunate was used to treat two patients suffering from metastatic uveal melanoma after standard chemotherapy which did not show any benefits anymore.[7] The combination of artesunate with standard chemotherapeutic agents resulted in stabilization of one patient and in partial regression of the other patient. This raises the question whether novel artemisinin derivatives can be identified with improved features, e.g., by hybridization of artesunic acid with another cytotoxic natural product or by combining monomeric artesunic acid in dimers.

Hybridization of two (or more) natural products represents one of the most promising and fundamentally novel approaches for the design of new lead structures in medicinal chemistry.[8a−c] This idea is inspired by nature itself, since many known natural products are built of such fragments arising from different biosynthetic pathways.[8a,9]

Other bioactive natural products are betulin and betulinic acids. Betulin exhibited cytotoxic activity toward human lung cancer A549 cells by inducing apoptosis and specific changes in protein expression profiles.[10] Betulinic acid derivatives caused ROS production and concomitant dissipation of mitochondrial membrane potential resulting in cell apoptosis.[11]

The present work deals with artesunic acid hybrid molecules toward sensitive CCRF/CEM and multidrug-resistant CEM/ADR5000 leukemia cells.

Results and Discussion

Synthesis of Hybrid Molecules. Hybride molecules **3** and **5**−**7** were prepared by classical methods[12a−d] as summarized in Schemes 1 and 2. We first synthesized the active ester of **1**. As activator, PfpOH was used, which gave active ester **2** upon coupling with artesunic acid **1** by using DCC. This ester (2.0 equiv) was reacted with 1,3-diaminopropane (1.0 equiv) in DMF to give homodimer **3** (Scheme 1 and Supporting Information). For synthesis of hybrid molecule **5**, the selective protection of the primary alcohol of betulin was first carried out (Scheme 2 and Supporting Information). Betulin was treated with acetic anhydride in the presence of imidazole as activating reagent to yield the monoacetate **4**, which was subsequently reacted in a DCC/DMAP-mediated coupling with artesunic acid **1** to give the target hybrid **5**. Unprotected betulin (1 equiv) was directly coupled with **1** (1.0 and 2.0 equiv, respectively) employing DCC and EDCI as respective coupling reagents to yield hybrid molecules **6** and **7**.

Determination of Cytotoxicity by XTT Assay. CEM/ADR5000 cells displayed a significantly decreased sensitivity indicating resistance to the control drug doxorubicin (Figure 1a). Collateral sensitivities of CEM/ADR5000 cells were observed toward artemisinin and artesunic acid **1** (Figure 1b and c), but not toward betulin and compound **3** (Figure 1d and e). Multidrug-resistant CEM/ADR5000 cells exhibited collateral sensitivities toward **5**, **6**, and **7** (Figure 1f−h). The 50% inhibition concentrations (IC_{50}) are shown in Table 1. 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).

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[a]Abbreviations: 4-DMAP, 4-(dimethylamino)pyridine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; FCS, fetal calf serum; IC_{50}, 50% inhibition concentration; PfpOH, pentafluorophenol; PBS, phosphate buffered saline; ROS, reactive oxygen species.

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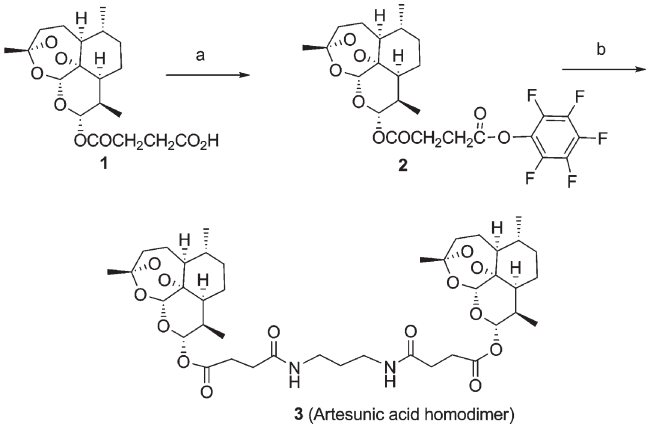
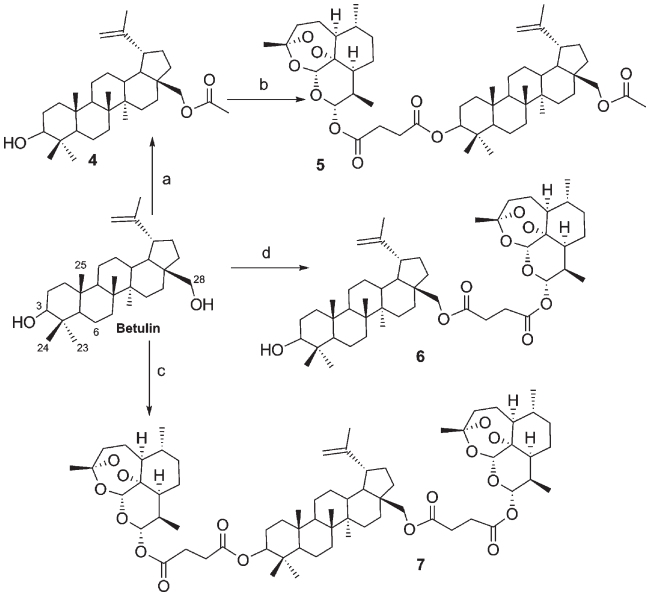
Scheme 1. Synthesis of **3**[a]



[a] Reagents and conditions: (a) PfpOH, DCC, EtOAc, −20 °C, 89%; (b) 1,3-diaminopropane, DMF, 20%.

Scheme 2. Synthesis of **5**−**7**[a]



[a] Reagents and conditions: (a) Ac_2O, imidazole, $CHCl_3$, 56%; (b) **1**, DCC, DMAP, CH_2Cl_2, 52%; (c) **1** (2 equiv), EDCI, DMAP, DMF, 61%; (d) **1** (1 equiv), DCC, DMAP, CH_2Cl_2, 54%.

Artesunic acid homodimer **3** was the most cytotoxic compound. Artesunic acid−betulin heterodimers **5**−**7** were less active than artesunic acid homodimer **3**. Interestingly, CEM/ADR5000 cells exhibited the highest collateral sensitivity toward artesunic acid homodimer **3**.

Analysis of Cell Cycle Distribution and Apoptosis by Flow Cytometry. Because of its effect on CEM/ADR5000 cells at low concentrations, artesunic acid was subjected to further analysis together with doxorubicin and artesunic acid **1** as control compounds. Cell cycle distribution and apoptosis were investigated using Nicoletti's assay. All compounds analyzed induced apoptosis (Figure 2). Artesunic acid homodimer **3** and artesunic acid **1** arrested cells in the G0/G1 phase of the cell cycle, and doxorubicin arrested cells in the G2/M phase.

Time and concentration kinetics were performed for artesunic acid **1**. Induction of apoptosis was observed in both cell lines upon exposure to artesunic acid **1**. Apoptotic CEM/ADR5000 cells appeared after shorter incubation periods and at lower concentrations of artesunic acid **1** compared to CCRF-CEM cells (Figure 3b). Additionally, the reaction was stronger in CEM/ADR5000 than in CCRF-CEM cells. G0/G1 phase arrest showed similar kinetics in both cell lines. After 24, 48, and 72 h of treatment, a dose-dependent increase of cells in G0/G1 phase was observed.

Corresponding results for artesunic acid homodimer **3** are shown in Figure 4. Apoptosis was induced by both cell lines in a time- and concentration-dependent fashion upon artesunic acid homodimer **3** exposure (Figure 4a and b). The arrest in G0/G1 phase was not as distinctive for artesunic acid homodimer **3** (Figures 5c and d) as it was for artesunic acid **1** (Figure 3c and d). After 48 h of incubation with 1 μg/mL artesunic acid homodimer **3**, both cell lines showed the most pronounced increase of cells in the G0/G1 phase.

Analysis of ROS by Flow Cytometry. Aliquots of 5×10^5 cells/mL were incubated with different concentrations of each compound and analyzed by H_2DCFDA staining after 6, 24, 48, and 72 h of incubation. To illustrate the increase in intracellular ROS levels, histograms of nontreated controls and treated samples are shown in Figure 5. Control samples are displayed as black lines, whereas samples treated with compound are indicated by a gray background. The left column contains overlays obtained from CCRF-CEM cells. The right column displays overlays obtained from CEM/ADR5000 cells. The first row corresponds to artesunic acid **1** treatment (0.01 μg/mL, 72 h) (Figure 5a and b), the second row to artesunic acid homodimer **3** treatment (0.001 μg/mL, 24 h) (Figure 5c and d), and the third row corresponds to doxorubicin treatment (1 μg/mL, 6 h) (Figure 5e and f). Artesunic acid **1** and artesunic acid homodimer **3** caused significantly increased ROS levels. Only doxorubicin-treated CEM/ADR5000 cells did not reveal elevated ROS levels. The dose-dependency of ROS formation is shown in Figure 6.

Interestingly, multidrug-resistant CEM/ADR5000 cells were collateral sensitive to artemisinin, artesunic acid **1**, artesunic acid homodimer **3**, and compound **5**. The mechanistic basis of collateral sensitivity toward these compounds is unknown. Substances causing collateral sensitivity are of therapeutic interest, as they may be able to eradicate otherwise nonresponding tumors in a clinical setting. Artesunic acid homodimer **3** displayed the lowest IC_{50} values and best collateral sensitivity. Collateral sensitivity of CEM/ADR5000 cells toward artesunic acid **1** or artesunic acid homodimer **3** as measured by XTT assay was also found in the apoptosis assays.

The mechanisms of collateral sensitivity are still incompletely understood.[13] Compounds extruded by P-glycoprotein may consume ATP, and repletion of ATP from ADP by oxidative phosphorylation generates ROS leading to increased cell death.[14] Artesunic acid monomers and dimers produce ROS.[15] Hence, some of our dimers may produce more ROS than others, leading to higher degrees of collateral sensitivity. This hypothesis has to be investigated in future experiments and is beyond the scope of the present investigation. ROS production is associated with induction of G0/G1 cell cycle arrest.[16] Hence, there might be a direct relationship between the induction of ROS by artesunic acid dimers and G0/G1 arrest. Artesunic acid **1** and artesunic acid homodimer **3** treatment led to G0/G1 arrest. The cell cycle effects of artemisinin-type compounds are not uniform in the literature. Ovarian cancer cell lines underwent G2/M phase arrest.[17] G0/G1 blockage has been observed in cell

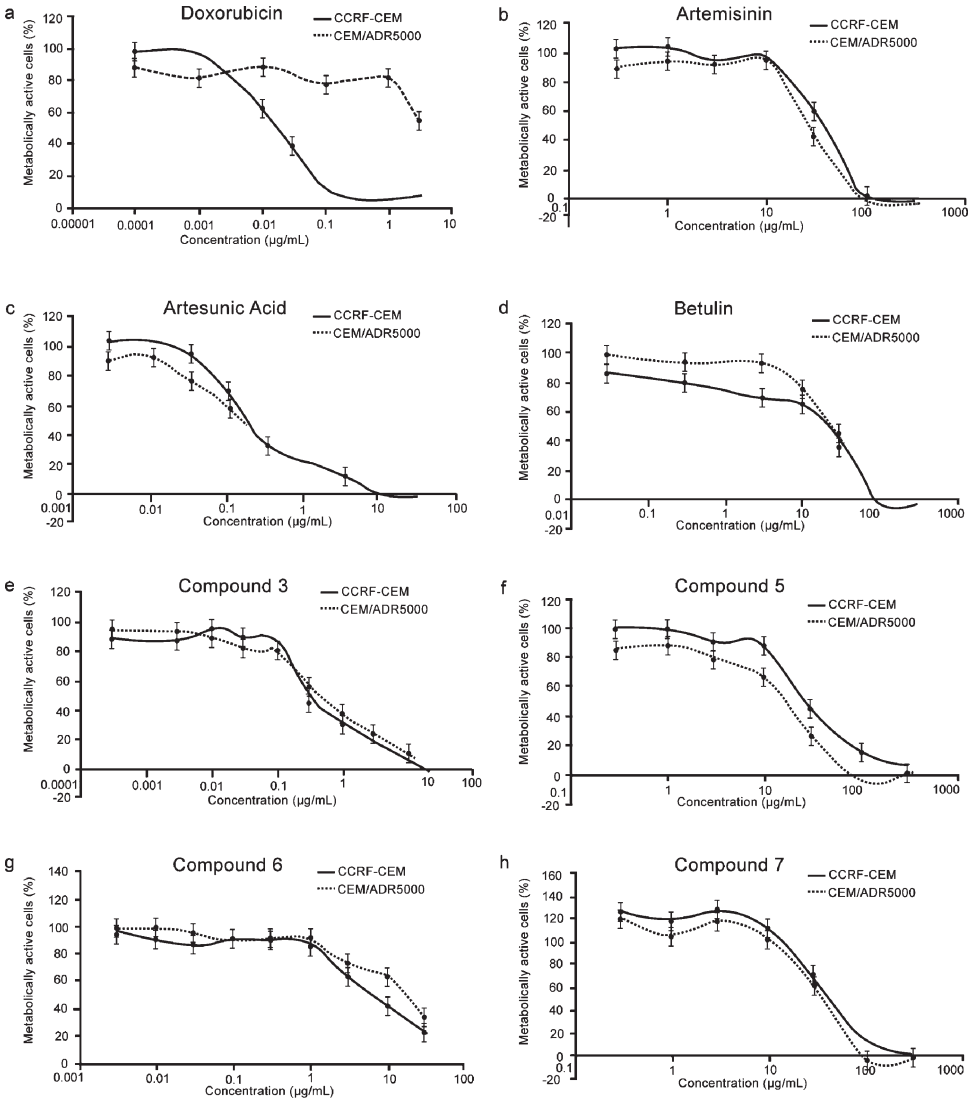


Figure 1. Cytotoxicity of CCRF-CEM and multidrug-resistant CEM/ADR5000 human leukemia cells toward artesunic acid homo- and heterodimers, artemisinin, and doxorubicin. Each experiment was independently performed two or three times, leading to nine values for each concentration. Mean values (±SEM) are plotted as a function of concentration for each compound for both cell lines. Errors were calculated by Gaussian error propagation: (a) doxorubicin; (b) artemisinin; (c) artesunic acid **1**; (d) betulin; (e) artesunic acid homodimer **3**; (f) compound **5**; (g) compound **6**; (h) compound **7**.

Table 1. IC_{50} for Artesunic Acid Homo- And Heterodimers, Artemisinin, and Doxorubicin in Sensitive CCRF-CEM and Multidrug-Resistant CEM/ADR5000 Human Leukemia Cells[a]

compound	molecular weight	IC_{50}		degree of resistance
		CCRF-CEM	CEM/ADR5000	
doxorubicin	579.98	36.9 ± 7.2 nM	> 5.2 μM	> 140[b]
(1) artemisinin	282.14	36.9 ± 6.9 μM	26.9 ± 4.4 μM	0.73
(2) **1** (artesunic acid)	384.42	1.8 ± 1.2 μM	1.2 ± 0.7 μM	0.67
(3) betulin	442.72	50.8 ± 16.0 μM	56.5 ± 8.6 μM	1.11
(4) **3** (artesunic acid homodimer)	806.94	1.2 ± 0.1 μM	0.2 ± 0.03 μM	0.17
(5) **5**	851.16	31.9 ± 8.6 μM	20.8 ± 3.5 μM	0.65
(6) **6**	809.12	9.7 ±1.3 μM	11.9 ±4.1 μM	1.23
(7) **7**	1175.53	42.9 ± 6.9 μM	42.1 ± 5.9 μM	0.98

[a] Dose response curves obtained by XTT assays were used to calculate IC_{50} values (mean ± SD) of two to three independent experiments with three parallel measurements. [b] IC_{50} value of doxorubicin for CEM/ADR5000 cells cannot be determined because of autoabsorbance of doxorubicin.

lines from various other tumor types.[18] G0/G1 arrests by artesunate may be mediated by down-regulation of the CDC25A protein, which is involved in the transition from G1 to S phase.[1b]

ROS formation was found upon treatment with artesunic acid **1**, artesunic acid homodimer **3**, and doxorubicin. However, in doxorubicin-treated CEM/ADR5000, only slightly increased ROS levels were measured because of

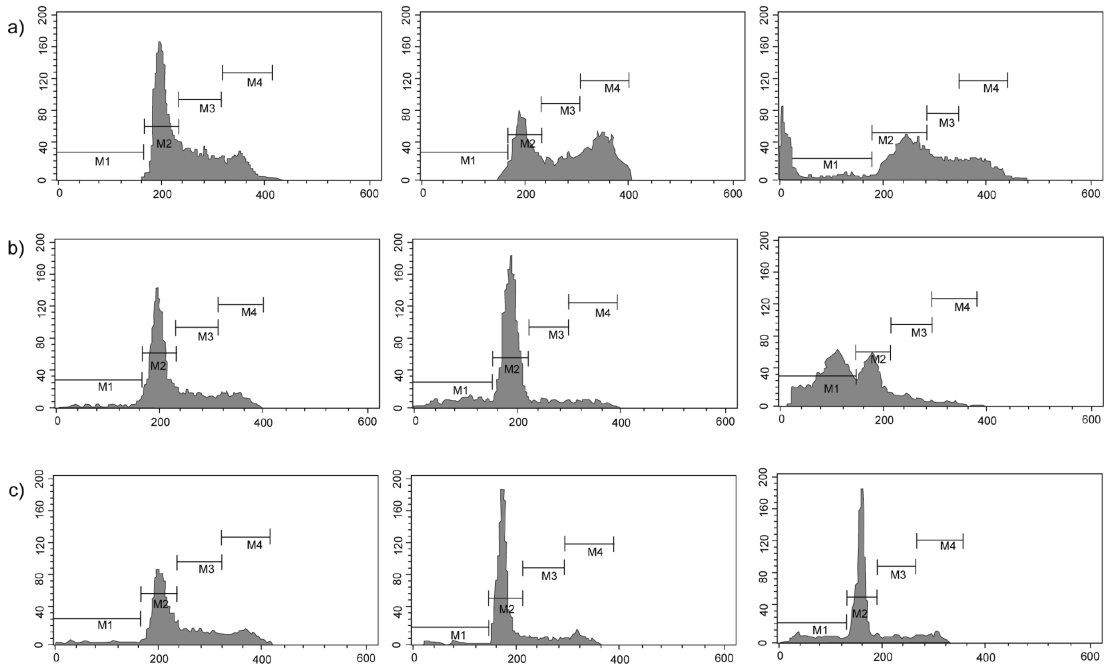


Figure 2. Cell cycle and apoptosis analysis by flow cytometry: (left column) nontreated control cells; (middle column) cell cycle effects; (right column) induction of apoptosis; (a) doxorubicin, G2/M phase arrest (0.01 μg/mL, 48 h), apoptosis induction (1 μg/mL, 24 h); (b) artesunic acid, G0/G1 phase arrest (10 μg/mL, 48 h), apoptosis induction (2 μg/mL, 48 h); (c) artesunic acid homodimer **3**, G0/G1 phase arrest (1 μg/mL, 48 h), apoptosis induction (2 μg/mL, 48 h).

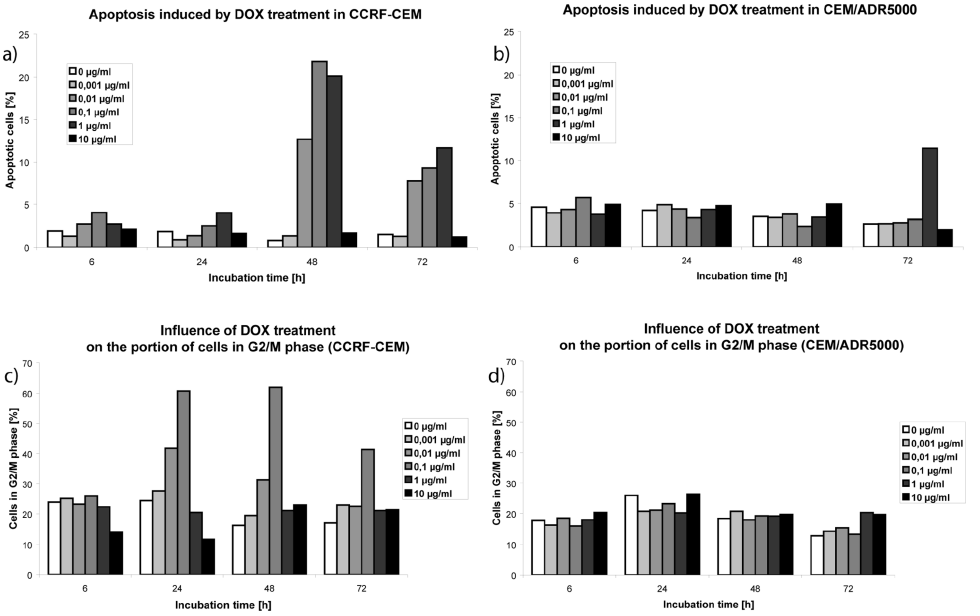


Figure 3. Time and concentration kinetics of apoptosis and cell cycle arrest upon treatment with artesunic acid **1**: apoptosis in (a) CCRF-CEM and (b) CEM/ADR5000 cells and cell cycle distribution in (c) CCRF-CEM and (d) CEM/ADR5000 cells.

overexpression of P-glycoprotein. It is concluded that the efflux of doxorubicin by P-glycoprotein prevented the doxorubicin-induced ROS generation in CEM/ADR5000 cells. Artesunic acid **1** and artesunic acid homodimer **3** led to ROS formation in CCRF-CEM and CEM/ADR5000 cell lines, since both compounds are presumably not transported by P-glycoprotein.

While artesunic acid−betulin hybrids **5** and **6** showed only moderate cell growth inhibitory activity (reduced with respect to that of artesunic acid **1**, albeit improved compared to betulin), artesunic acid homodimer **3** revealed considerable cytotoxicity. The mode of action of homodimer **3** is most probably the same as that of artesunic acid **1**. The improved activity of homodimer **3** may be attributed to the presence of two endoperoxide moieties. As a consequence, its capability to interact with its target molecules is amplified, resulting in an improved growth inhibitory capability. Other dimer and trimer molecules of artemisinin and artesunate have been described in the literature.[19a,b]

Intriguingly, hybrid **7**, which also contains two artesunic acid moieties linked via betulin, was considerably less active

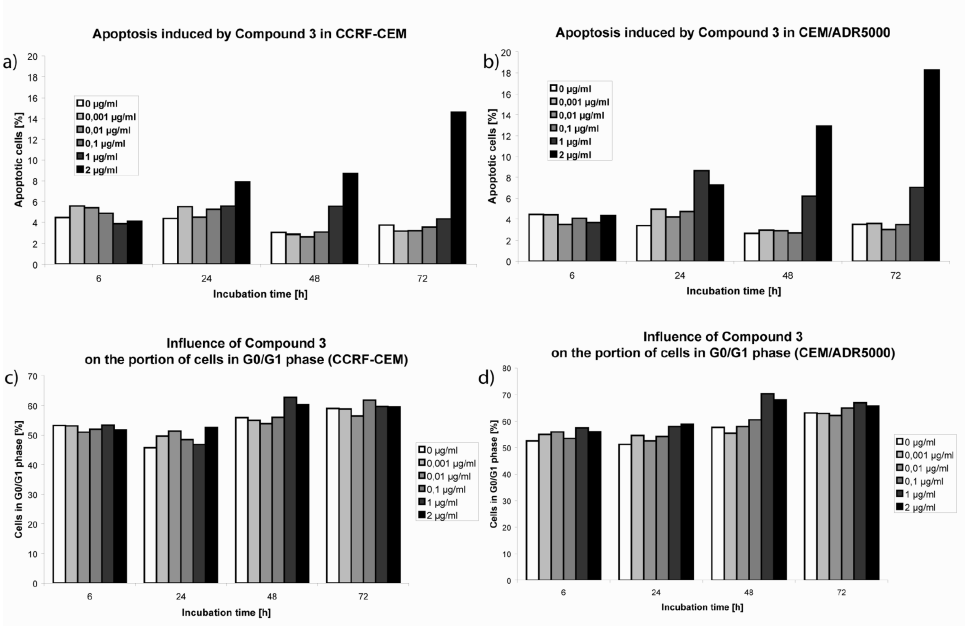


Figure 4. Time and concentration kinetics of apoptosis and cell cycle arrest upon treatment with artesunic acid homodimer **3**: apoptosis in (a) CCRF-CEM and (b) CEM/ADR5000 cells and cell cycle distribution in (c) CCRF-CEM and (d) CEM/ADR5000 cells.

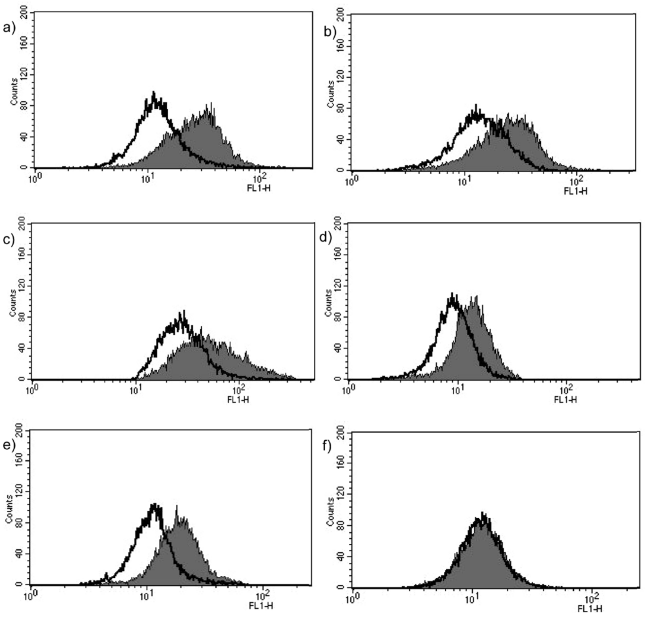


Figure 5. Determination of ROS upon treatment with artesunic acid **1**, artesunic acid homodimer **3**, or doxorubicin by flow cytometry. Untreated control samples are displayed as black lines. Samples treated with compound are highlighted in gray: artesunic acid **1** treatment of (a) CCRF-CEM (0.01 μg/mL, 72 h) and (b) CEM/ADR5000 cells (0.01 μg/mL, 72 h), artesunic acid homodimer **3** treatment of (c) CCRF-CEM (0.001 μg/mL, 24 h) and (d) CEM/ADR5000 cells (0.001 μg/mL, 24 h), and doxorubicin treatment of (e) CCRF-CEM (1 μg/mL, 6 h) and (f) CEM/ADR5000 cells (1 μg/mL, 6 h).

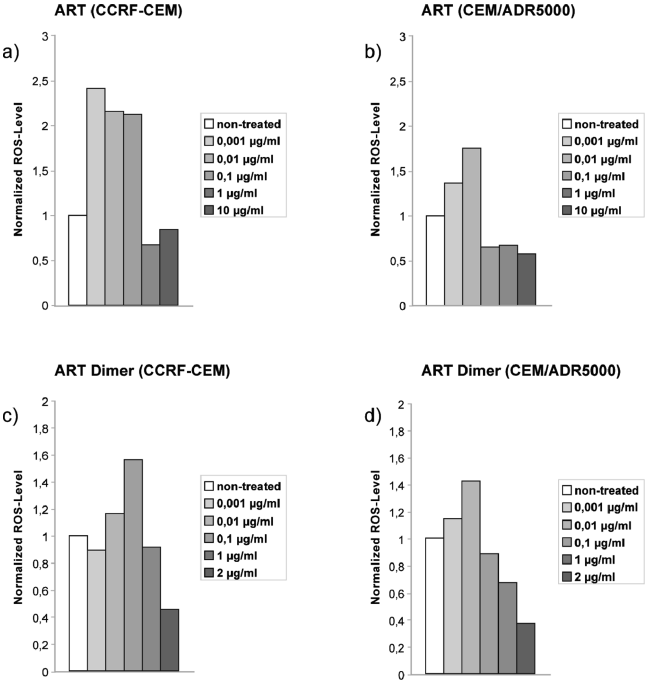


Figure 6. Time and concentration kinetics of ROS generation upon treatment with artesunic acid **1**, artesunic acid homodimer **3**, or doxorubicin. ROS levels were normalized using the median of the three nontreated controls: artesunic acid **1** treatment of (a) CCRF-CEM and (b) CEM/ADR5000 cells and artesunic acid homodimer **3** treatment of (c) CCRF-CEM and (d) CEM/ADR5000 cells.

than artesunic acid homodimer **3**. It seems that betulin serves in **7** solely as a linker, which is not flexible and is much more sterically demanding compared to the diaminopropane linker. We assume that the interaction of the individual endoperoxide moiety with its respective target may be impaired.

Conclusions

We have generated homodimers of artesunic acid molecules and heterohybrids of artesunic acid and betulin. The activity of these substances has been analyzed in a human leukemia cell line (CCRF-CEM) and its multidrug-resistant subline, CEM/ADR5000, and compared to doxorubicin. Multidrug-resistant cells were not cross-resistant to the novel compounds, some of

which were even collateral sensitive. The highest degree of collateral sensitivity was observed for artesunic acid homodimer **3**. Furthermore, induction of G0/G1 cell cycle arrest and apoptosis and ROS formation were observed for both artesunic acid **1** and artesunic acid homodimer **3**.

Experimental Section

General. All reactions were performed in flame-dried glassware under an argon atmosphere. Solvents were dried and purified by standard procedures and distilled prior to use. Reagents obtained from commercial sources were used without further purification. TLC chromatography was performed on precoated aluminum silica gel SIL G/UV$_{254}$ plates (Marcherey, Nagel & Co.) or silica gel 60-F$_{254}$ precoated glass plates (Merck). ^{1}H NMR spectra were recorded with Varian Unity 300 (300 MHz). ^{13}C NMR spectra were recorded on a Unity Inova-600 (150 MHz) instrument. ESI mass spectra were recorded with a LCQ Finnigan spectrometer. High-resolution mass spectra were measured with a Bruker APEX IV 7 T FT-ICR instrument. A Perkin-Elmer 241 polarimeter was used for optical rotation measurements. Elemental analysis (C, H, N) was carried out with an EA 1110 CHNS machine from CE Instruments. On the basis of ^{1}H NMR, ^{13}C NMR, HR-MS (ESI), and elemental analysis (Supporting Information), compounds **3** and **5**−**7** were at least >95% pure. Doxorubicin, artemisinin, and betulin obtained from Sigma-Aldrich (Taufkirchen, Germany) and artesunic acid obtained from Saokim Ltd. (Hanoi, Vietnam) were at least >98% pure.

Artesunic Acid Pentafluorophenyl Ester 2. A cooled solution of *N*,*N*-dicyclohexylcarbodiimide (DCC) (315 mg, 1.52 mmol, 1.03 equiv) in dry EtOAc (5 mL) was added dropwise to a stirred solution of **1** (564 mg, 1.47 mmol, 1 equiv) and pentafluorophenol (PfpOH) (315 mg, 1.71 mmol, 1.16 equiv) in dry $CHCl_3$ (5 mL) at −20 °C. The reaction mixture was warmed to 0 °C and stirred overnight. The urea which precipitated was removed by filtration and the solvent was subsequently removed under vacuum. The residue was purified by column chromatography on SiO_2 to afford 717 mg (89%) of **2**. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 0.77 (d, J = 6.9 Hz, 3 H, CH_3), 0.89 (d, J = 6.0 Hz, 3 H, CH_3), 0.90−1.12 (m, 1 H), 1.17−1.19 (m, 1 H), 1.29 (s, 3 H, CH_3), 1.30−1.64 (m, 6 H), 1.78−1.85 (m, 1 H), 1.96−2.02 (m, 1 H), 2.06−2.38 (m, 2 H), 2.84 (dd, J = 4.9, 7.6 Hz, 2 H), 3.08 (dd, J = 4.9, 7.6 Hz, 2 H), 5.57 (s, 1 H), 5.70 (d, J = 9.6 Hz, 1 H) ppm.

Artesunic Acid Homodimer 3. To a stirred solution of **2** (320 mg, 0.58 mmol, 2.02 equiv) in dimethylformamide (DMF) (1 mL) was added dropwise a solution of 1,3-diaminopropane (20.20 mg, 0.27 mmol, 1 equiv) in DMF (1 mL), and the resulting mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography on SiO_2 ($CHCl_3$/MeOH 9:1) to give the product **3** as a white solid (20%). R_f = 0.33 (hexane/EtOAc 1:1). $[\alpha]^{20}_D$ +18.6° (c 0.145, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.82 (d, J = 6.9 Hz, 6 H, 2 CH_3), 0.93 (d, J = 6.0 Hz, 6 H, 2 CH_3), 0.98−1.08 (m, 2H), 1.22−1.42 (m, 14 H), 1.54−1.94 (m, 10 H), 1.94−2.01 (m, 2H), 2.26−2.60 (m, 8 H), 2.70−2.79 (m, 4 H), 3.20−3.34 (m, 4 H, 2 CH_2), 5.40 (s, 2 H, 2 CH), 5.72 (d, J = 9.9 Hz, 2 H), 6.72 (t, J = 5.3 Hz, 2H, 2 NH) ppm.

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/mL doxorubicin every other week for 3 days to maintain overexpression of P-glycoprotein. The multidrug resistance profile of CEM/ADR5000 has been reported.[20]

Cytotoxicity Assay (XTT Assay). The cytotoxicity of compounds was determined by Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany), which is based on cleavage of the yellow tetrazolium salt XTT by ubiquitous dehydrogenases leading to the formation of an orange formazan dye. The amount of dye is commensurate to the number of metabolic active cells. By means of an ELISA plate reader, the formazan dye can be quantified. The test has been described elsewhere.[21]

Analysis of Cell Cycle Distribution and Apoptosis. The development of cell cycle distribution and apoptosis was investigated by propidium iodide staining and flow cytometry (Nicoletti's assay). The assay has been described elsewhere.[22] Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). For each sample 10 000 cells were counted.

Analysis of ROS. The formation of ROS was determined by flow cytometry and staining with 2′,7′-dichlorodihydrofluoresceine diacetate (H_2DCFDA, Sigma München, Germany). The assay has been described elsewhere.[22]

Supporting Information Available: Experimental procedures, ^{1}H and ^{13}C NMR spectra, and MS (ESI) data for target compounds **3** and **5**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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