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Stereochemistry-Dependent Cytotoxicity of Some Artemisinin Derivatives

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We determined the cytotoxicity of some artemisinin derivatives against EN2 tumor cells using the MTT assay. Artemisinin (**1**) was clearly more cytotoxic than deoxyartemisinin (**2**), which lacks the endoperoxide bridge. Ether-linked dimers of dihydroartemisinin with defined stereochemistry were found to differ in the extent of cytotoxic effect on EN2 cells. The nonsymmetrical dimer (**3**) was more cytotoxic than the symmetrical dimer (**4**). The nonsymmetrical dimer of dihydrodeoxyartemisinin (**5**) lacking the endoperoxide bridges was also effective in the MTT assay, although less cytotoxic than **3** and **4**. Similarly, the symmetrical dimer (**6**) was less effective than **5**. Epoxides of artemisitene also showed that stereochemistry was an important factor for cytotoxicity. The results suggested that the endoperoxide bridge was not crucial for cytotoxicity to the tumor cells, but contributed to the cytotoxic effect apparently exerted by the ether linkage of the dimers. Flow cytometry data indicated that the dimers **3** and **4** caused an accumulation of the cells in the G_1-phase of the cell cycle. In contrast, artemisinin (**1**) caused a slight increase of S-phase cells.

Artemisinin (**1**), a sesquiterpene lactone endoperoxide isolated from the herb *Artemisia annua* L. (Asteraceae), is the parent compound for a novel class of antimalarials that are effective against multidrug-resistant *Plasmodium falciparum* strains.[1,2] Several authors have suggested that free radicals arising from the endoperoxide moiety were responsible for the death of malaria parasites inside red blood cells.[3−5] Recently, we have found that artemisinin and several derivatives were cytotoxic to tumor cells as well, after 2 h of incubation.[6,7]

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Table 1. IC_{50} Values (± Standard Deviation) of Artemisinin Derivatives Determined by the MTT Assay after 72 h of Incubation[a]

compound	IC_{50} (μM)
artemisinin (**1**)	0.98 ± 0.32
deoxyartemisinin (**2**)	111 ± 8.7
nonsymmetrical dimer of dihydroartemisinin (**3**)	0.11 ± 0.04
symmetrical dimer of dihydroartemisinin (**4**)	2.0 ± 0.16
nonsymmetrical dimer of dihydrodeoxyartemisinin (**5**)	8.9 ± 2.6
symmetrical dimer of dihydrodeoxyartemisinin (**6**)	99.8 ± 32
(11*R*)-epoxide of artemisitene (**7**)	12.7 ± 4.9
(11*S*)-epoxide of artemisitene (**8**)	>100[b]
cisplatin	0.18 ± 0.04

[a] $n \geq 3$. Cisplatin was used as a reference compound. [b] 100 μM resulted 36% growth inhibition.

Artemisinin (**1**, Chart 1) caused growth inhibition rather than cell killing of an Ehrlich ascites clone and HeLa tumor cells. Deoxyartemisinin (**2**) was not toxic at all, indicating that the endoperoxide was important for cytotoxicity. The nonsymmetrical dimer of dihydroartemisinin (**3**) was much more effective, causing more growth inhibition compared to **1**, but also causing actual cell killing at higher concentrations.[7] In the present study, more dimers (**4**−**6**) were tested for cytotoxicity to investigate the influence of the stereochemistry of the ether bridge linking the monomers. In addition, two isomeric epoxides of artemisitene were included (**7**, **8**). The effect of **1** and dimers **3** and **4** on the cell-cycle distribution was investigated to get more insight into the mode of action.

Results and Discussion

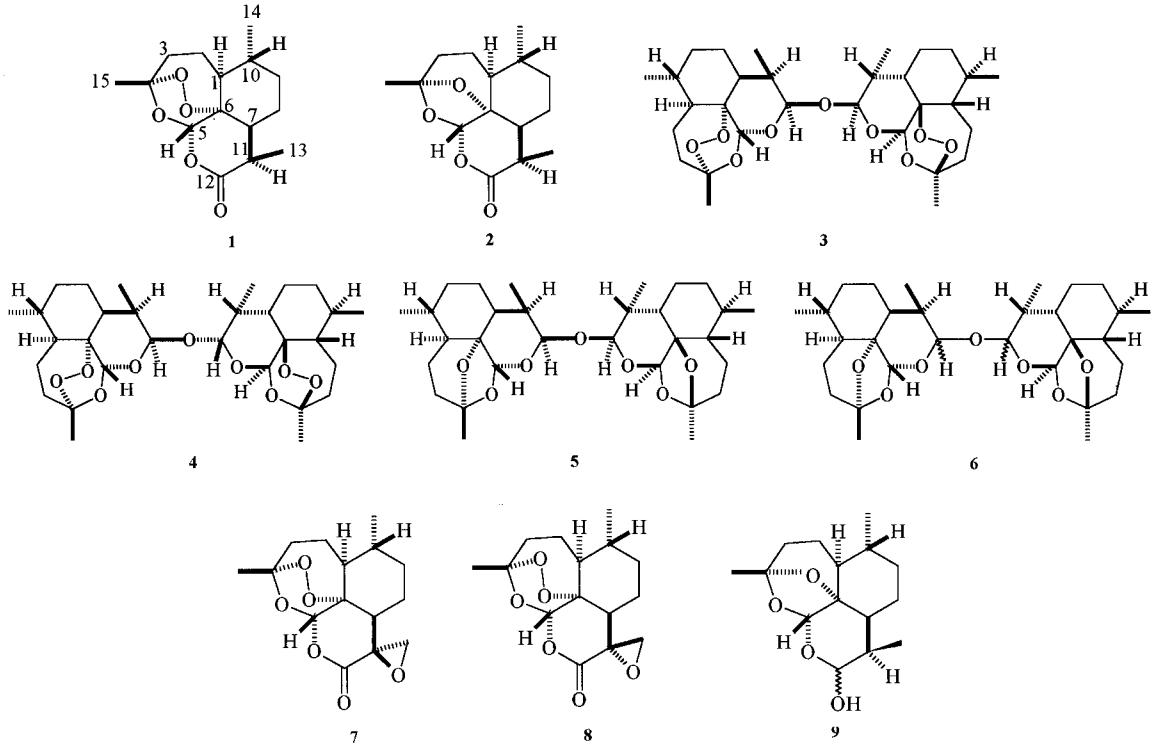
Table 1 shows IC_{50} values as determined by the MTT assay for 72 h of (continuous) incubation of EN2 tumor cells with the test compounds. Artemisinin (**1**) was 100-fold more cytotoxic than deoxyartemisinin (**2**). Therefore, it can be concluded that the endoperoxide group was an important determinant for cytotoxicity. Because an IC_{50} could be determined for **2**, the endoperoxide appeared not to be absolutely essential for cytotoxicity. The nonsymmetrical dimer **3** was 10-fold more cytotoxic than **1**, while the symmetrical dimer **4** was two times less cytotoxic than **1**. Compound **3** was 20 times more cytotoxic than **4**. Thus, small stereochemical differences at the ether bridge in **3** and **4** gave rise to large differences in cytotoxicity. Analogously, from the two dimers of dihydrodeoxyartemisinin the nonsymmetrical dimer **5** was more cytotoxic than the symmetrical dimer **6**. Unfortunately, the stereochemistry of the newly synthesized **6** at C-12 is uncertain. It is not yet known whether the ether bridge is α/α or β/β (see Experimental Section for details). As compared to dimers **3** and **4**, compound **5** was still relatively cytotoxic, despite the lack of endoperoxide bridges. Apparently, the ether linkage itself can also yield considerable cytotoxicity to EN2 cells, particularly when the linkage is nonsymmetrical. It would be interesting to test the α/α stereoisomer of the β/β symmetrical dimer **4** as well; however, the reaction of dimerization could not be controlled to yield the desired product.

The importance of stereochemistry for cytotoxicity was also demonstrated by epoxides of artemisitene (**7**, **8**). The (11*R*)-epoxide **7** was more cytotoxic than its isomer **8**. Similarly, the antimalarial activity of **7** was also found to be higher than that of **8**.[8] Previously, we showed that (11*R*)-OH artemisinin was more cytotoxic than its *S*-isomer.[6] These findings suggest that the oxygen has to be in a specific spatial position for the greatest biological effect. The epoxides were prepared in an attempt to increase the cytotoxicity to tumor cells. An epoxide is an electrophilic group that can be attacked by nucleophiles producing cytotoxicity to DNA and proteins (e.g., aflatoxin B1[9]). Surprisingly, **7** was almost 13-fold less cytotoxic than **1**. In addition, the antimalarial activity of **7** was also clearly lower.[8] We suggest that the epoxide moiety may have reacted with water or other medium components prior to the addition of cells leading to lower concentrations of active compound and to higher IC_{50} values.

Figure 1 shows the MTT IC_{50} values after different incubation times for five compounds. The cytotoxicity after 72 h for compounds **2**, **6**, and **8** was too low to establish this relationship. The compounds became less effective in inhibiting cell growth when the incubation period was shortened, inasmuch as all IC_{50} values increased compared to 72 h of incubation. It should be noted that the duration of the MTT assay was always 72 h. Thus, short incubations were followed by a long period of post-incubation growth. Because the MTT assay measures cell growth, the assay cannot discriminate between the action of the test compound and the recovery of cells. When the cells are allowed to recover for a long time, the cytotoxicity may be underestimated. The extremely high IC_{50} value of compound **1** after short incubation might be caused by recovery growth. The order of cytotoxicity of the compounds after each incubation period was more or less the same. The cytotoxicity of **3** and **4**, as determined by the MTT assay, did not decrease when incubation times shorter than 2 h were applied (*viz.* 5, 15, 60 min, data not shown). Apparently, the onset of the cytotoxic effect was achieved very rapidly.

Flow cytometry was applied to investigate a possible relation between the growth inhibition and changes in the cell-cycle distribution of EN2 cells in response to **1**, **3**, and **4**. These three compounds were chosen because **1** is the parent compound and **3** and **4** were both very active in the MTT assay. Compound **1** yielded a small dose-dependent accumulation of the cells in the S-phase after 24 h of incubation (Figure 2A). This effect was only temporary, since 48-h incubation yielded a normal distribution (data not shown). In contrast, the dimers of dihydroartemisinin (**3**, **4**) gave an accumulation of G_1-phase cells of about two-fold at the expense of S-phase cells after 24 h of incubation. The percentage of cells in G_2/M remained more or less constant (Figure 2B,C). These effects on the cell-cycle distribution were not caused by initial cell loss, the cell concentration after 24 h of incubation with the compounds was never lower than that at the beginning of the experiment (data not shown). The effect of **3** and **4** on the accumulation of cells in G_1 reached a plateau with increasing concentrations of the compound. The reason for this plateau might be that cells in a particular phase of the cell cycle were most susceptible to the compound. At a certain concentration all of these cells may be accumulated in the G_1-phase. This possibility may be investigated by synchronizing the cells. Another explanation for the plateau may be that a particular system is maximally

Chart 1



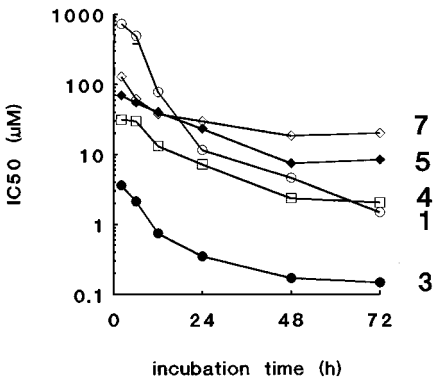


Figure 1. Cytotoxicity of compounds **1**, **3**, **4**, **5**, and **7** expressed as IC_{50} values (μM) in response to different incubation periods. The mean ± SEM is given for each point. Error bars fell inside the points.

inhibited at a given concentration of the compound, as is known for enzymes, receptors, and carrier systems.

The nonsymmetrical dimer (**3**) was effective at lower concentrations than was the symmetrical dimer (**4**). Thus, similar to MTT cytotoxicity, the difference in stereochemistry was important for the effect on cell-cycle distribution. However, the same plateau value was reached at high concentrations of either dimer. Preliminary data on the deoxyartemisinin dimer (**5**) also revealed a G_1 accumulation, but at concentrations 10 times higher than that of **4** (data not shown). This suggests that the endoperoxide moiety was not absolutely necessary for the effect on the cell-cycle distribution. As mentioned above, **1**, which contains an endoperoxide, did not yield the G_1 accumulation at all. Instead, the ether linkage between the monomers was likely to play a role in the obvious accumulation of G_1-phase cells. The endoperoxide moiety is essential for the killing of the malaria parasite. Therefore, other mechanisms are likely to be involved in cytotoxicity to tumor cells.

To investigate the kinetics of the effect on the cell-cycle distribution, we chose a concentration for each dimer that caused the maximal effect on the distribution after 24 h of incubation and also a concentration exerting no or only a minor effect. The G_1 accumulation of the concentrations of the dimers exerting a maximal effect at 24 h gradually increased with the incubation time (Figure 3). At 12 h, 0.4 μM of compound **3** already caused a significant increase of the percentage of G_1 cells. At 48 h, the control percentage of G_1 cells increased as well, which indicated that the cell density became too high for optimal growth. From 24 to 48 h of incubation, there was no increase of the percentage of cells in the G_1-phase compared to their controls.

To investigate the difference between short- and long-term incubation, dimer **3** was also incubated for only 2 h, followed by 24 h of post-incubation growth. This also yielded a concentration-dependent G_1 accumulation (Figure 4). Directly after the incubation period of 2 h, the cell-cycle distribution was still normal (data not shown). Obviously, the cells need time to display the cytotoxic effect of **3** on the cell cycle. Compared to 24 h of continuous incubation, higher concentrations of **3** were necessary to reach the same maximal effect of almost 60% G_1-phase cells. This finding suggests that the same mechanism of action may be responsible for the short- and the long-term incubations with this compound.

The difference in efficacy between compounds **3** and **4** with respect to their cytotoxicity as determined by the MTT assay was also shown by the flow cytometry data. To further investigate the relationship between the cell-cycle effects and cytotoxicity, the flow cytometry data were compared with the MTT assay and two other methods for cytotoxicity, namely the clonogenic and the cell-count assay (Table 2). As a parameter for cytotoxic effect we choose the IC_{50} value. The incubation time with the compounds was 2 h or 24 h. It should be noted

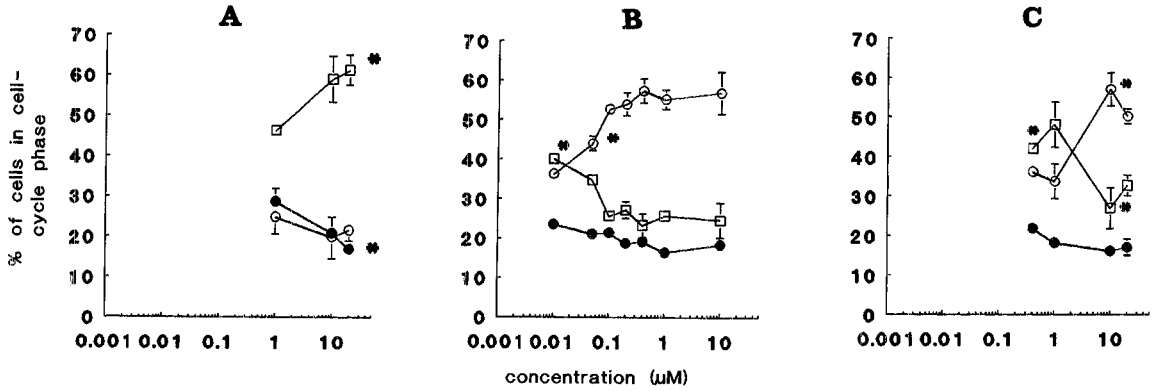


Figure 2. Dose-dependent effects on the cell-cycle distribution in response to compounds **1** (A), **3** (B), and **4** (C). The incubation period was 24 h. The percentage of cells in a certain phase of the cell cycle (G_1 phase: ○, S-phase: □, G_2/M-phase: ●) is plotted against the concentration of the compound (μM). The mean ± SEM is given for each point. The control values were: %G_1: 31.73 ± 2.11; %S: 47.91 ± 1.95; %G_2/M: 20.36 ± 1.44. The other values were compared with these control values. Statistical significance is indicated by an asterisk ($p < 0.05$). For clarity only the first value in a row is labeled, which significantly differs from the control.

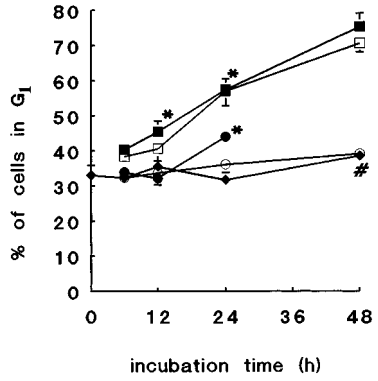


Figure 3. Time-dependency of the G_1 accumulation caused by **3** and **4**. These compounds were tested in two concentrations: 0.05 (●) and 0.4 μM (■) for **3**; 0.4 (○) and 10 μM (□) for **4**. The mean ± SEM is given for each point. Values were compared to the control $t = 0$ value. Statistical significance is indicated by an asterisk ($p < 0.05$). For clarity only the first value in a row is labeled, which significantly differs from the control. The control (♦) at 48 h is significantly (#) increased compared to that at 24 h.

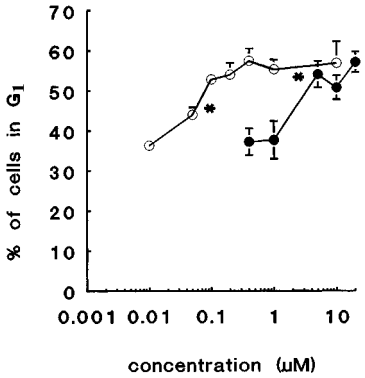


Figure 4. Comparison of the G_1 accumulation caused by compound **3** after 24 h of incubation (○) and 2 h of incubation followed by 24-h post-incubation (●). The mean ± SEM is given for each point. Control values were 31.73 ± 2.11 (24-h incubation) and 32.23 ± 1.26 (2-h incubation). The other values were compared with these control values. Statistical significance is indicated by an asterisk ($p < 0.05$). For clarity only the first value in a row is labeled, which significantly differs from the control.

that the durations of the assays were not the same. The flow cytometry and cell-count assays were stopped at 24 h, while the MTT and the clonogenic assay were evaluated, respectively, two and six days later. According to each method applied, **3** was more effective than **4** and **1**. The test compound concentration range needed to reach the plateau value of the percentage of G_1 or S cells was lower or equal to the IC_{50} values of the MTT and the cell-count assay. These findings prompted us to speculate that the growth inhibitory effect of the dimers is due to an accumulation of the cells in the G_1-phase of the cell cycle and the concomitant depletion of S-phase cells. In contrast, the growth inhibitory effect of **1** seemed to correlate with an increase of the percentage of S-phase cells. Apparently, these cells were arrested in the S-phase, while the dimers possibly prevented entry into the S-phase.

Table 2. IC_{50} Values[a] (μM ± Standard Deviation) of Artemisinin (**1**), the Nonsymmetrical (**3**) and Symmetrical (**4**) Dimer of Dihydroartemisinin Determined by the MTT Test, the Clonogenic Assay, and Cell Counts

method	compd	IC_{50} or plateau value (μM), at 2-h incubation and 24-h postincubation time	IC_{50} or plateau values (μM) at 24-h incubation
MTT assay	**1**		11.5 ± 1.8
	3	3.6 ± 0.35	0.35 ± 0.07
	4		7.2 ± 1.7
clonogenic assay	**1**		>50[b]
	3	55.6 ± 1.5[c]	1.0 ± 0.26
	4		>50[d]
cell-count assay	**1**		30 ± 16.1
	3	9.3 ± 5.2	0.54 ± 0.07
	4		8.9 ± 2.9
flow-cytometry−plateau	**1**		1−10
	3	1−5	0.05−0.2
	4		1−10

[a] The IC_{50} value is the concentration of the compound in μM exerting 50% effect compared to the control. [b] 50 μM of **1** resulted in 20% cell kill. [c] See Beekman *et al.*[7] [d] 50 μM of **4** resulted in 30% cell kill.

The nonsymmetrical dimer of dihydroartemisinin (**3**) clearly caused clonogenic cell death, which was determined by the clonogenic assay (Table 2). However, a direct relationship between the cell-cycle distribution changes and cell death is not likely, since after 2 h of incubation with concentrations of **3** far below 55.6 μM with no cell-killing potency, cells accumulated in G_1 24 h later. In addition, **4** and **1** yielded only marginal cell death despite obvious growth-inhibitory effects.

Further investigation is needed to establish the molecular mechanism by which the dimers are able to

cause the G_1 accumulation. Normal cell-cycle kinetics are controlled by the so-called cell-cycle checkpoints. Tumor cells' growth, however, is less controlled and not strictly regulated by these checkpoints. This may be caused by an increase of cyclin/cyclin-dependent kinase complexes and/or mutation or deletion of proteins playing a role in inhibiting cell-cycle progression. Still, tumor cells depend on numerous regulatory proteins of the cell cycle for their growth.[10,11] The EN2 cells, which were used for the experiments in this study, were cloned from the wild-type Ehrlich ascites tumor cell line. There are no literature data on the status of the regulators of the cell cycle in these cell lines. In our laboratory the parent cell line was subjected to karyo characterization (not published). Many chromosomal aberrations were found, which implied that the G_1/S checkpoint was not functioning normally. This is the case for many tumor cells. Apparently, the dimers accumulated the cells in the G_1-phase by cell-cycle control systems, which were still operational in the EN2 tumor cells leading to inhibition of cell growth. Based on the importance of stereochemistry for the degree of cytotoxicity caused by the dimers, we suggest that a specific target is responsible for the cell-cycle effects of these compounds. The quick onset of the toxicity (5 min) is an observation favoring this suggestion. A variety of factors noxious for the cell, such as DNA damage, deprivation of nutrients, serum starvation, and inhibition of biochemical processes, may lead to G_1 accumulation in normal and tumor cells. The inhibitors of the cell cycle, the cyclin-dependent kinase inhibitors, play an important role in the response to these events.[12] Therefore, investigations on the levels of these inhibitors may give further insight into the mechanism of the cell-cycle effects caused by the dimers **3** and **4**.

Experimental Section

Cell Line. The murine EN2, a cloned Ehrlich ascites tumor cell line, was grown in suspension culture in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) plus 0.2 mg/mL of streptomycin and 200 IU/mL of penicillin G, at 37 °C in a shaking incubator. The doubling time of the cells was ca. 12 h. Exponentially growing cells were used for all experiments. In all experiments more than 95% of the cells excluded trypan blue.

Test Compounds. The sesquiterpene lactone artemisinin (**1**) isolated from the herb *A. annua* L. (Asteraceae) was kindly provided by ACF Beheer BV (Maarssen, The Netherlands). Deoxyartemisinin (**2**) was prepared by hydrogenation of (**1**) using $Pd/CaCO_3$ as a catalyst, as described in the literature.[13] The dimers of artemisinin (**3**, **4**) were prepared as reported previously.[6] The two epoxides of artemisitene were prepared by epoxidizing artemisitene by dimethyloxirane using a described procedure.[9] The two deoxy dimers **5** and **6** were prepared from dihydrodeoxyartemisinin (**9**) by treatment with BF_3 etherate. The nonsymmetrical dimer **5** was previously described,[14] while **6** was obtained as colorless crystals from CH_2Cl_2−MeCN: mp 162−165 °C; $[\alpha]_D$ −264.4° (*c*, 0.1%, EtOAc); IR (KBr) (cm^{-1}) ν_{max} 2920, 1450, 1380, 1260, 1205, 1140, 1110, 1030, 1000; ^{1}H NMR (CDCl3) δ 5.28 (2H, s, H-5 and H-5′), 4.85 (2H, d, J = 6.2 Hz, H-12 and H-12′), 2.43 (2H, m, H-11 and H-11′), 1.49 (6H, s, C-15 and C-15′), 1.04 (6H, d, J = 7.2 Hz, C-13 and C-13′), 0.89 (6H, d, J = 5.5 Hz, M-14 and M-14′); ^{13}C NMR ($CDCl_3$) δ 45.4 (1) (C-1, C-1′), 22.1 (2) (C-2, C-2′), 34.8 (2) (C-3, C-3′), 107.4 (0) (C-4, C-4′), 95.9 (1) (C-5, C-5′), 81.6 (0) (C-6, C-6′), 41.1 (1) (C-7, C-7′), 22.6 (2) (C-8, C-8′), 34.2 (2) (C-9, C-9′), 35.3 (1) (C-10, C-10′), 31.2 (1) (C-11, C-11′), 98.1 (1) (C-12, C-12′), 14.8 (3) (C-13, C-13′), 18.7 (3) (C-14, C-14′), 23.8 (3) (C-15, C-15′); CIMS (NH_3) m/z $[M]^+$ 536 (25) with the base peak at 251.

The stereochemistry at C-12 of dimer **6** is uncertain. In **4**, whose structure was confirmed by X-ray crystallography, $J11\alpha$, 12α = 3.3 Hz, while $J11\alpha$, 12β = 8.2 Hz, thus suggesting an α stereochemistry for C-12 in **6**, as its $J11,12$ = 6.2 Hz (see above). However, this is yet to be unambiguously proven. The identity of all test compounds was checked using various spectroscopic methods. Spectral data were in agreement with literature values. All compounds were dissolved as concentrated stock solution in 100% DMSO (Merck, Darmstadt, Germany) and stored at −20 °C. The concentration of DMSO used in our experiments never exceeded 1%, which was found to be not cytotoxic to cells. Cisplatin (Aldrich, Milwaukee, WI) was used as a reference compound.

Cytotoxicity Assays. The MTT assay is based on the reduction of the soluble MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into a blue-purple formazan product, mainly by mitochondrial reductase activity inside living cells.[15] The number of cells was found to be proportional to the extent of formazan production for the cell line used in this study. To 50 μL of the compounds diluted with medium 50 μL containing 800 cells (EN2) was added into 96-well microtiter plates (Nunc, Roskilde, Denmark). The incubation periods were 2, 6, 12, 24, or 48 h, or lasting the entire experiment (72 h) at 37 °C in a humidified incubator with 5% CO_2. After exposure periods shorter than 72 h, the test compounds were washed away in several steps always leaving behind the cells in 50 μL of medium. Between the washing steps, the plates were centrifuged (10 min, 20 °C, 210 *g*). Then, the cells were further incubated, completing the 72 h at 37 °C in a humidified incubator with 5% CO_2. Control experiments demonstrated that the residual test compound present after washing did not cause additional cytotoxicity. Then, 20 μL of a 5 mg/mL stock solution of MTT (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS: 1.6 mM KH_2PO_4, 6.5 mM $Na_2HPO_4 \cdot 12\ H_2O$, 0.137 mM NaCl, 2.7 mM KCl, pH 7.4) was added, and the plates were incubated for a further 3 h 45 min. The medium was removed after centrifugation (15 min, 20 °C, 210 *g*), leaving behind the formazan product, which was subsequently dissolved in 200 μL of 100% DMSO. After mixing, the absorbance (A) was measured at 550 nm using a spectrophotometer (Titertek Multiscan, Flow Laboratories, Irvine, Scotland). Cell growth was calculated using the formula

$$\frac{[A_{550}\,(\text{treated cells}) - A_{550}\,(\text{culture medium})]\ 100}{[A_{550}\,(\text{control cells}) - A_{550}\,(\text{culture medium})]}$$

The concentration resulting in 50% growth inhibition (IC_{50}) was determined and used as a parameter for cytotoxicity.

The principle of the clonogenic assay (CA) is to investigate the ability of an individual cell to form a

colony on a soft-agar plate after treatment with a test compound.[16] Cells not able to form a colony are considered to be clonogenically dead. Samples of 10^5 cells/mL were obtained from the incubations for flow cytometry after 24 h of incubation (see below). The cell suspensions were washed with medium, diluted, and mixed with 10^5 feeder cells/mL to obtain about 100 colonies per plate. The feeder cells were EN2 cells supra-lethally irradiated with 180 Gy of γ-rays. Subsequently, 0.1 mL cell suspension was plated in Petri dishes containing 0.5% agar (Difco, Detroit, MI) in RPMI 1640 supplemented with 10% newborn calf serum (Gibco) plus 0.2 mg/mL of streptomycin and 200 IU/mL of penicillin G. To correct for possible cell loss during the washing procedure, the treated cells that had remained in the test tube were counted. Cells were allowed to grow for 1 week until colonies could be counted. Colonies containing more than 50 cells were counted. The concentration of a test compound resulting in 50% of the control colonies was denoted as the IC_{50} value and was used as a parameter for cytotoxicity.

Flow Cytometry. This method was adapted from Dolbeare *et al.*[17] Cells (10^5/mL) were incubated in a shaking incubator at 37 °C in 100-mL bottles. Test compounds were added for continuous incubation, and after 6, 12, 24, and 48 h samples were taken for flow cytometry or the clonogenic assay. For a short-term incubation, cells were washed twice with fresh medium after 2 h and grown for an additional 24 h. Each cell sample was counted for the cell-count assay with a Bürker counting chamber. Subsequently, 10^6 cells were centrifuged (5 min, 200 *g*). The cells were washed (×3) with PBS at 4 °C. Then, cells were fixed with 70% EtOH and stored at 4 °C. One day before the flow cytometry assay, cells were centrifuged and incubated with 2 mL of 2.7 N HCl−Triton X-100 0.5% (v/v) for 20 min. Then, cells were washed (×3) with PBS/Tween-80 0.5% (v/v). Between the washing steps the cells were centrifuged for 5 min at 200 *g*. The pellet was dissolved in 0.2 mL of a solution containing 0.01 mg/mL propidium iodide (Sigma, St. Louis, MO, from a 1-mg/mL stock solution in H_2O). The propidium iodide is a fluorescent dye that intercalates between the DNA strands. The samples were stored at 4 °C. Just before the flow cytometry, the cells were pipetted up and down to avoid aggregates. The DNA content of 10^4 cells was measured with a Becton Dickinson FACstar flowcytometer, with a 488-nm argon laser. The data were analyzed with ModFit 5.2 yielding the cell-cycle distributions. Dividing cells duplicate their DNA in the S-phase; the actual mitosis takes place in the M-phase. These phases are seperated by two G (gap)-phases, G_1 and G_2. Thus, in the G_2/M-phase cells have twice as much DNA as in the G_1-phase. In the S-phase the DNA is increased gradually. The method does not distinguish between the G_0- and G_1-phases or between the G_2- and M-phases. Some cells could be in G_0, but it is known that tumor cells rarely shift into this non-cycling state. Therefore, we just use the term G_1. The Student's *t*-test was used for statistical analysis of the data.

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