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Synthesis and evaluation of dihydroartemisinin and dihydroartemisitene acetal dimers showing anticancer and antiprotozoal activity

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ABSTRACT

Twelve artemisinin acetal dimers were synthesized and tested for antitumor activity in the National Cancer Institute (NCI) in vitro human tumor 60 cell line assay, producing a mean GI_{50} concentration between 8.7 (least active) and 0.019 μM (most active). The significant activity of the compounds in this preliminary screen led to additional in vitro antitumor and antiangiogenesis studies. Several active dimers were also evaluated in the in vivo NCI hollow fiber assay followed by a preliminary xenograft study. The title compounds were found to be active against solid tumor-derived cell lines and showed good correlation with other artemisinin-based molecules in the NCI database. The dimers were also evaluated for their antimalarial and antileishmanial activities. The antimalarial activity ranged from 0.3 to 32 nM (IC_{50}), compared to 9.9 nM for artemisinin.

1. Introduction

The occurrence of adverse effects and the development of resistance associated with the administration of conventional anticancer drugs[1] have shown the urgent need for development of new anticancer agents, especially those with novel and selective mechanisms of action. Although some of the promise of non-cytotoxic therapies is beginning to be realized (for example immunostimulants, growth factor antagonists and antisense therapy), the mainstay of the treatment of most cancers remains with cytotoxic drugs.

The natural product artemisinin (**1**) (Fig. 1) is a highly oxygenated sesquiterpene endoperoxide first isolated in 1971 from the Chinese plant *Artemisia annua* L.[2] The unusual endoperoxide structure and therapeutic significance of **1** as an antimalarial agent[3] against both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*[4] prompted wide-ranging research into its chemistry and pharmacology.[5] Artemisinin (**1**) and its derivatives caused membrane damage, alkylation, oxidation of proteins and fats, inhibition of protein and nucleic acid synthesis and interaction with cytochrome oxidase and the glutamine transport system in parasites.[6]

Despite the clinical significance of **1** in treating malaria, numerous drawbacks, for example, poor solubility in water and oil,[7] poor oral activity, short plasma half life,[8] and high rate of parasite recrudescence after treatment,[9] have limited its usefulness. Attempts to develop highly effective antimalarial agents with less adverse reactions led to the synthesis of a large number of artemisinin derivatives, for example, artemether, arteether, water soluble sodium artesunate and sodium artelinate,[10] 11-azaartemisinins,[11] fluorinated artemisinins,[12] artemisitene,[12] and dihydroartemisinin (DHA) (acetal and non-acetal) derivatives.[10]

In addition to its antimalarial activity, **1** had been reported to have cytotoxic effects against EN-2 (breast cancer), P-388 (leukemia), A549 (human lung carcinoma), HT-29 (colon cancer), MCF-7 (breast cancer) and KB (human malignant nasopharyngeal carcinoma) tumor cells.[13] As more analogs were evaluated for antitumor activity, the unsymmetrical DHA acetal dimer (**4**) (Fig. 1) was reported as being highly cytotoxic and more potent than cis-

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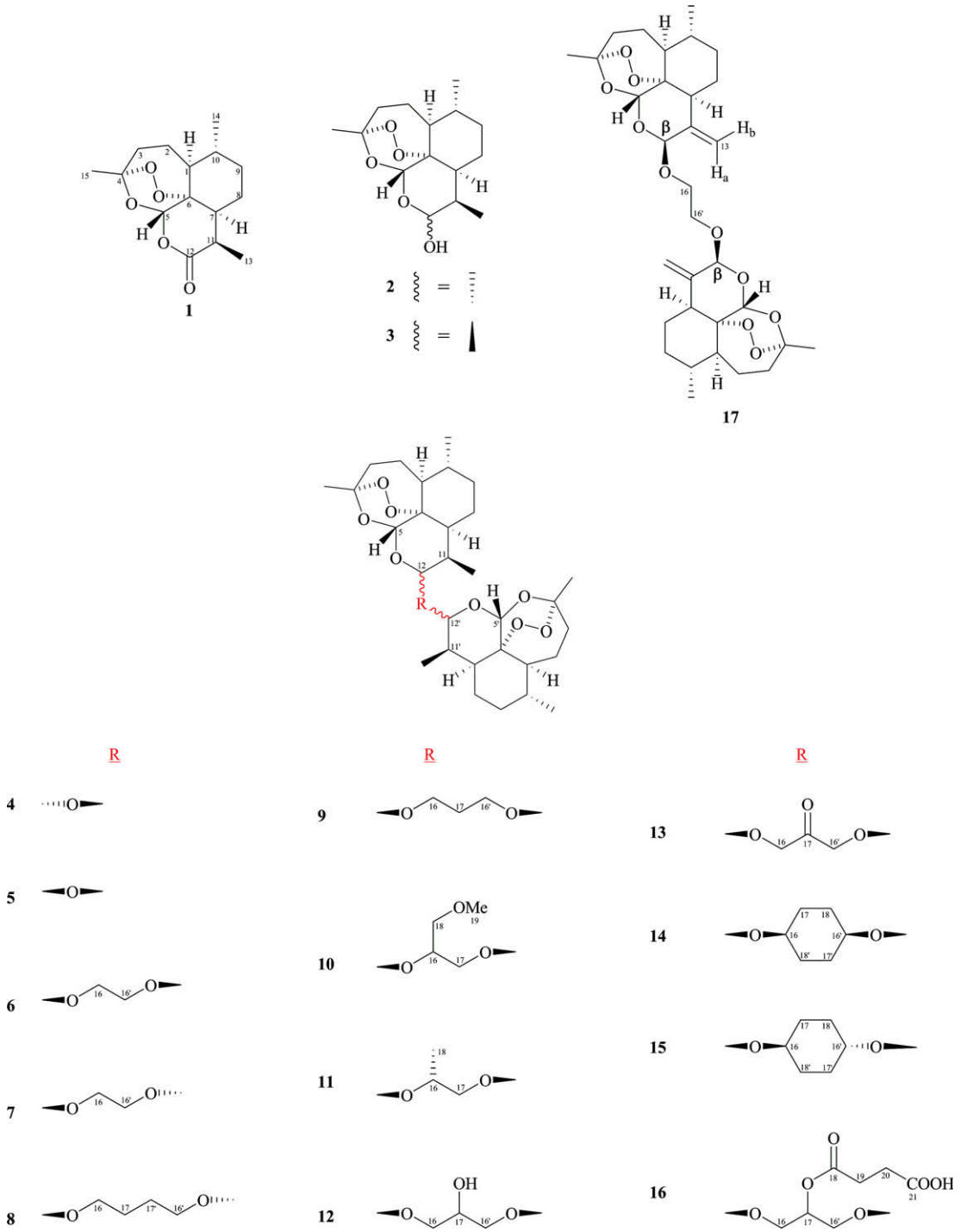


Figure 1. Artemisinin (**1**), dihydroartemisinin (DHA) (α-**2**, β-**3**), DHA acetal dimers (**4**–**16**) and dihydroartemisitene acetal dimer (**17**).

platin,[14] while the symmetrical DHA acetal dimer (**5**) (Fig. 1) also exhibited pronounced cytotoxic effects.[15] However, both dimers displayed bone marrow cytotoxicity.[16]

These findings stimulated an interest in preparing additional DHA acetal dimers with various linkers, for example, triethylene glycol [–O–$(CH_2)_2$–O–$(CH_2)_2$–O–$(CH_2)_2$–O–], 1,8-octanediol [–O–$(CH_2)_8$–O–] and hydroxyethyl disulfide [–O–$(CH_2)_2$–S–S–$(CH_2)_2$–O–].[17] A number of non-acetal dimers were also prepared. Several of these DHA acetal dimers exhibited antimalarial, anticancer and antiproliferative effects, while others showed activity comparable to that of calcitriol (1,25-dihydroxycholecalciferol), the active form of vitamin D, in a murine keratinocyte antiproliferative assay.[10]

This manuscript reports the synthesis and biological activity (anticancer and antiprotozoal) of twelve artemisinin acetal dimers with 1,2-, 1,3- and 1,4-diol derivative linkers (Fig. 1).[18,19] The primary goal of the work was to search for potential anticancer leads by evaluating the biological activities of these dimers in order to select appropriate candidates for further studies based on their efficacy, toxicity and stability. The anticancer (Fig. 2, Tables 1 and 2, Supplementary data), antiangiogenesis (Table 3), antimalarial

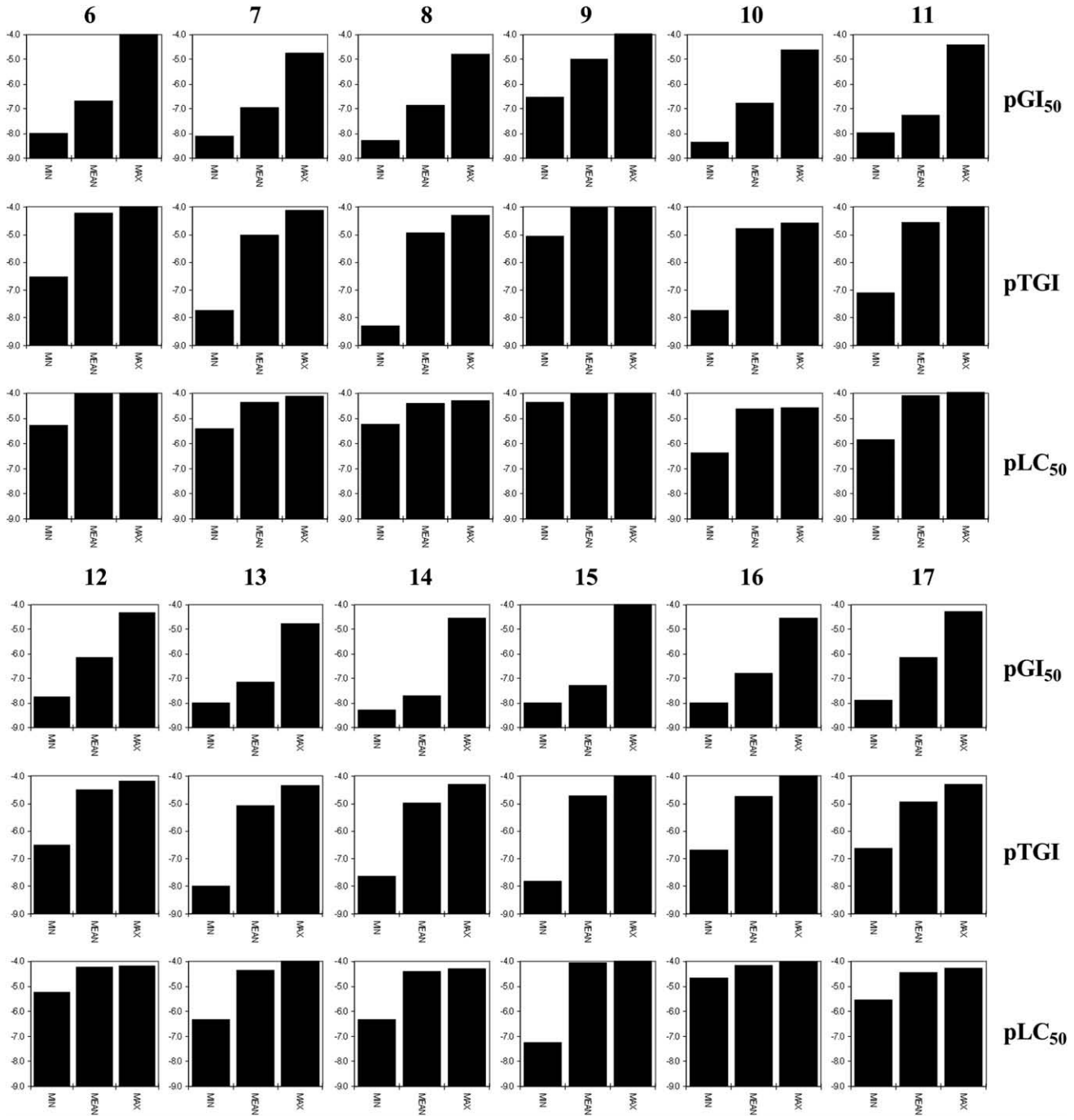


Figure 2. In vitro NCI 60 cell line anticancer assay data for **6**–**17**. The concentration ranges over which each dimer exhibited anticancer activity is depicted graphically. For each dimer the minimum, maximum and mean concentration required to achieve GI_{50}, TGI and LC_{50} activity is shown (see Supplementary data). $pGI_{50} = \log GI_{50}$. pTGI = log TGI. $pLC_{50} = \log LC_{50}$.

(Table 4) and antileishmanial (Table 5) activities of these dimers are presented here.

2. Results and discussion

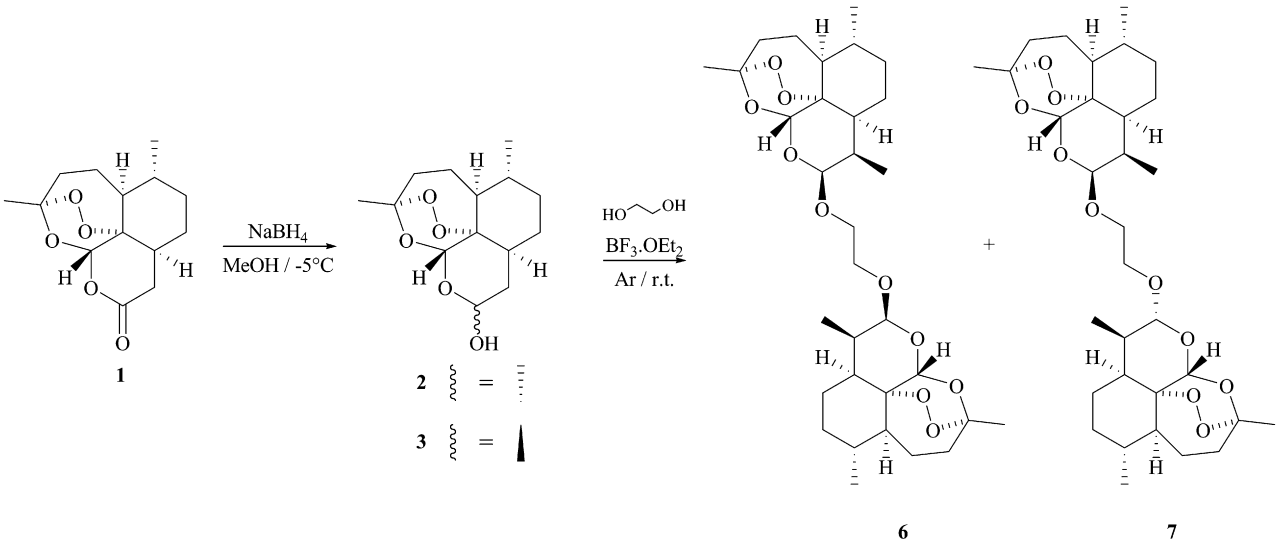
2.1. Chemistry

The dimers (**6**–**17**) (Fig. 1) were synthesized by reacting DHA (**2** and **3**) with a series of aliphatic and cycloaliphatic glycols under mild acidic conditions using borontrifluoride etherate ($BF_3{\cdot}OEt_2$), in dry ether, as a catalyst (Scheme 1). The reaction mixtures were chromatographed to isolate the desired products.

2.2. Biological activity

2.2.1. Anticancer activity

Dimers **6**–**17** were submitted to the NCI antitumor screening program and all were selected for evaluation in the in vitro 60 cell line screen against selected cell lines from leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer. The range of concentrations and mean concentration of each dimer producing GI_{50}, TGI and LC_{50} levels of anticancer activity occurred over a multiple log range of concentrations. Using the mean GI_{50} level as an index for comparing the relative activity of all agents, the cell lines



Scheme 1. Synthesis of **6** and **7**.

exhibit 50% net growth inhibition at concentrations between 8.7 μM ($pGI_{50} = -5.06$) for the least active dimer **9** and 0.019 μM ($pGI_{50} = -7.72$) for the most active dimer **14** (Fig. 2, Supplementary data).

Dimers **6**–**17** are far more active than a series of four artemisinin monomers evaluated in an earlier pilot experiment.[20] The mean GI_{50} levels of the monomers tested ranged from 9.33 μM ($pGI_{50} = -4.29$) to 51.3 μM ($pGI_{50} = -5.03$) with minimum GI_{50} levels ranging from 0.25 μM ($pGI_{50} = -6.60$) to 3.6 μM ($pGI_{50} = -5.44$). In contrast, the minimum GI_{50} levels for **6**–**17** ranged from 0.0044 μM ($pGI_{50} = -8.36$) to 0.295 μM ($pGI_{50} = -6.53$), indicating that on a molar basis, the dimers exhibit considerably better activity than the monomers in human cancer cell lines (Fig. 3).

The average mean graph of the GI_{50} activity for the dimers exhibit multi-log differential patterns of activity in the 60 cell line screen (Supplementary data). The data allows not only visualization of the 'pattern of activity' of each individual agent and overall pattern of activity of all agents, but also relates the sensitivity of each cell line to the mean sensitivity of all cell lines for each agent. It is therefore clear that **6**–**17** exhibit a potent and unique pattern of anticancer activity.

It is important to note that the lowest concentration tested in the NCI 60 cell line screen was 10 nM = 10^{-8} M ($pGI_{50} = -8$) and that the most potent dimers conferred GI_{50} activity in a significant number of cell lines at even lower concentrations. Between 6 and 24 of the 60 cell lines were growth inhibited by **11**, **13**, **14**, **15** and **16** at concentrations below 10^{-8} M ($pGI_{50} \leqslant -8$) (Supplementary data). Not only did **6**–**17** impact leukemia cell lines, but some were also very active against one or more cell lines in each of the human cancer cell line panels. Such concentrations are readily achieved in the plasma of CD2F1 laboratory mice following parenteral administrations of **12**, **14** and **16** at well tolerated dosages.[20]

When the NCI COMPARE[21] analysis was run against the panel of standard chemotherapeutic agents, the cell sensitivity profile of these dimers was most similar to platinum probe compounds. However, when run against the entire database of open and proprietary compounds the greatest homologies were to each other and to other artemisinin-related compounds in the database with far less correlation to the platinum probe compounds. The COMPARE algorithm ranks an entire database in order of the similarity of the responses of the 60 cell lines to previously evaluated compounds compared to the responses of the probe compound. The results indicate that compounds high in this ranking may possess a mechanism of action similar to that of the probe compound. Correlations on micro-array data for the 60 cell lines indicate that cells most sensitive to these dimers contain lower levels of the mRNAs encoding proteins involved in integrin and hypoxia signaling. Lower levels of expression of these proteins may result in enhanced sensitivity either because these proteins are direct targets or because their reduced expression reflects a condition within the cell (for example redox potential) that augments sensitivity. A manuscript describing the mechanism of action of **6**–**17** has been submitted for publication.[22]

2.2.2. Antitumor activity

Dimers **12**, **14**, **15** and **16** were additionally assessed via the NCI in vivo hollow fiber assay against a standard panel of twelve human cancer cell lines[23] with all four compounds meeting the criteria for activity used by the NCI (Table 1). This assay has shown the ability to provide qualitative initial indications of in vivo drug efficacy and is used as a first-step in vivo screening assay for agents found to have reproducible activity in the in vitro anticancer drug screen. Compound **14** proved to be the most active with a total score of 36, which is equivalent to the value for the positive control, paclitaxel (total score 32 ± 4). Based upon the positive results found in the hollow fiber assay, **12**, **14** and **16** were further evaluated in a subcutaneous (s.c.) xenograft model of the human leukemia cell line, HL-60. All three compounds are considered active with optimal% T/C less than 40% (Table 2).

2.2.3. Antiangiogenesis activity

Angiogenesis is the phenomenon by which new blood vessels are generated in vivo. The newly generated blood vessels are needed for the growth of cancer cells at the tumor site.[24]

Human umbilical vein endothelial cells (HUVEC) were plated in growth factor rich medium in the presence of various concentrations of **6**–**17**.[25] Compounds **12**, **13**, **14** and **16** displayed growth inhibition of HUVEC in a time- and concentration-dependent manner at low nanomolar concentrations (IC_{50} 42–77 nM) in a 72 h exposure (Table 3). Furthermore, these compounds inhibited the migration of HUVEC in response to VEGF in a dose-dependent manner. The cell migration IC_{50} values for **12** and **14** were 1.8 and 3.3 μM, respectively, whereas the IC_{50} values for **13** and **16**

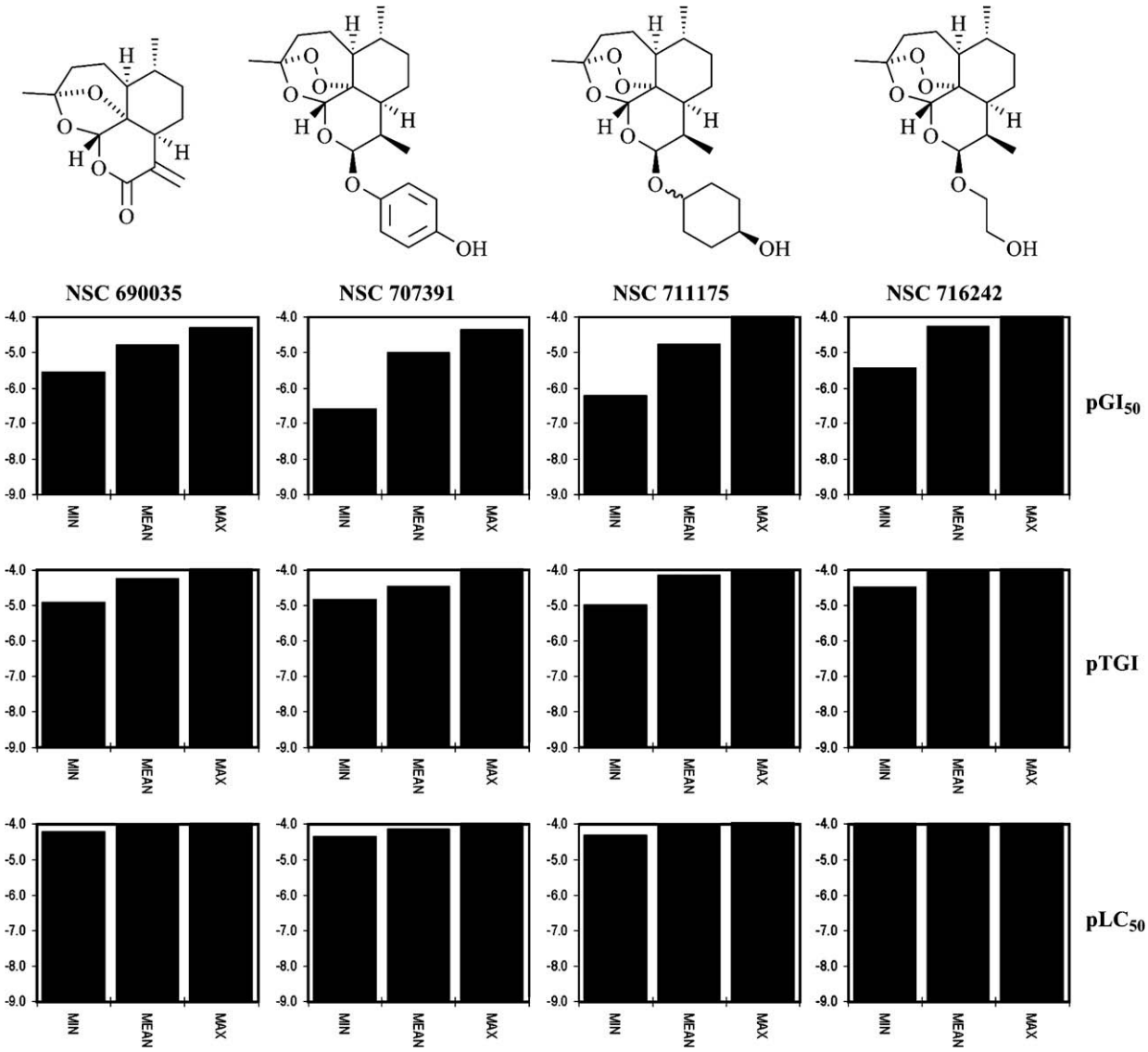


Figure 3. The concentration ranges over which each of four artemisinin monomers exhibited anticancer activity in the 60 cell line panel. $pGI_{50} = \log GI_{50}$. pTGI = logTGI. $pLC_{50} = \log LC_{50}$.

Table 1
In vivo hollow fiber assay standard protocol scores for **12**, **14**, **15** and **16**

Compound	i.p. score	s.c. score	Total score	Net cell kill
12	12	2	14	Yes
14	26	10	36	No
15	18	6	24	No
16	22	4	26	Yes

i.p.: Intraperitoneal. s.c.: Subcutaneous.

were higher at 28.5 and 45.3 μM, respectively. In addition, these compounds were tested for endothelial cord formation, which is demonstrated by the alignment of endothelial cells in capillary-like structures. HUVEC were plated on a three-dimensional layer of Matrigel® where they align, forming cords, which were evident a few hours after plating. Treatment of endothelial cells with **12**, **13**, **14** and **16** inhibited alignment of endothelial cells at higher concentrations (IC_{50} 30–50 μM) than those concentrations required for demonstrable activity in the proliferation and migration

Table 2
Impact of treatment with **12**, **14** and **16** on the growth of s.c. implanted HL-60 (TB) human leukemia xenografts

Compound	Dose (mg/kg/inj)	Optimal % T/C	Day of optimal % T/C	Growth delay in days	Toxicity related mortality	Max % relative mean weight loss
12	50	34%	14	34	0	5%
	25	68%	14	16	0	2%
14	50	34%	18	36	0	17%
	25	50%	18	8	0	3%
16	50	Toxic	Toxic	Toxic	8	Toxic
	25	23%	18	30	0	1%

All dosing was given once daily s.c. for 10 days (QD × 10).

Table 3
In vitro antiangiogenesis activity of **12**, **13**, **14** and **16**

Compound	Growth inhibition IC_{50} (nM)	Cell migration IC_{50} (μM)	Cord formation IC_{50} (μM)
12	77	1.8	30.8
13	57	28.5	>50
14	42	3.3	38.8
16	56	45.3	45.8
Paclitaxel[a]	1.65	0.1	0.05
TNP-470[a]	3.16	0.5	1.00

[a] Control.

assays. These findings demonstrate the direct effect of **12**, **13**, **14** and **16** on angiogenic processes such as endothelial cell proliferation and migration, suggesting that they could be of value in modulating endothelial cells and tumor cell compartments. Further investigations are needed to completely delineate the molecular mechanisms linking endothelial growth inhibition and endothelial cell migration inhibition by these compounds.

2.2.4. Antimalarial activity

The in vitro antimalarial activity of **6**–**17** was evaluated against chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *P. falciparum* based on the determination of plasmodial LDH activity (Table 4).[26] The cytotoxicity of the compounds was assessed using Vero cells. Antimalarial activity is generally categorized as high (IC_{50} < 20 μM), moderate to weak (IC_{50} 20–350 μM) and inactive (IC_{50} > 350 μM) when compared to chloroquine [IC_{50} 29.7 (D6) and 744.1 (W2) nM] and artemisinin (**1**) [IC_{50} 9.9 (D6) and 15.9 (W2) nM]. The selectivity index (S.I.) is a measure of selective toxicity of the compound under investigation to malaria parasites, with S.I. ⩾ 10 indicating biological efficacy that is not due to in vitro cytotoxicity.[27] These guidelines suggest that **6**–**17** exhibit excellent antimalarial activity against both strains of the malaria parasite, with **6**, **11**, **12**, **13** and **16** being more active than chloroquine and artemisinin (**1**) against both the D6 and the W2 clones. The selectivity indices for **12** and **13** indicate selectivity for the D6 and the W2 clones, respectively (Table 4).

2.2.5. Antileishmanial activity

Compounds **12**–**15** were also assessed against the protozoan parasite *Leishmania donovani*, the causative agent of visceral leishmaniasis (kala-azar or black fever), with pentamidine[28] and amphotericin B[29] as controls (Table 5). The antileishmanial activity of **12**–**15** was comparable to that of the control drugs, which are currently used for the treatment of leishmaniasis. The toxicities of these known drugs have, however, limited their clinical use.

3. Conclusion

In summary, as part of our continued search for potential natural product-derived medicinal agents, a series of dihydroartemisinin acetal dimers were synthesized and assessed for their anticancer and antiprotozoal activity. It was found that these dimers were active against solid tumor-derived cell lines, exhibiting a potent and unique pattern of anticancer activity. In addition, the dimers were effective against angiogenic processes, for example, endothelial cell proliferation and migration. The antimalarial activity of some of the dimers were considerably higher than those of artemisinin and chloroquine against both D6 and W2 clones.

4. Experimental

4.1. General experimental conditions

All reactions were carried out in oven dried glassware. Diethyl ether (ether) was distilled under argon from sodium benzophenone ketyl prior to use. All chemicals were purchased from Sigma–Aldrich and used without further purification, except the diols, which were dried over grade I alumina prior to use.

Flash column chromatography was conducted with silica gel (particle size 230–400 mesh; Merck). Analytical thin-layer chromatography (TLC) was performed with silica gel 60 F_{254} plates (250 μm thickness; Merck) using hexanes/EtOAc mixtures as solvent systems. Visualization was accomplished by spraying with *p*-anisaldehyde spray reagent followed by heating with a hot-air gun. Melting points were recorded on an Electrothermal 9100 instrument. IR spectra were obtained using an AATI Mattson Gen-

Table 4
Antimalarial activity of **4**–**17**

Compound	*P. falciparum*				Cytotoxicity
	D6 Clone		W2 Clone		Vero cells TC_{50} (nM)
	IC_{50} (nM)	S.I.	IC_{50} (nM)	S.I.	
4[14,19]	23.6	>37	25.4	>34	>864
5[15,19]	11.8	>73	6.9	>125	>864
6	1.7	>48	1.2	>64	>80
7	31.9	>25	21.9	>37	>800
8	17.7	>43	14.1	>54	>764
9	14.0	21	8.5	35	296
10	17.2	>43	4.7	>159	>745
11	3.8	>21	5.1	>15	>78
12	0.3	>23,800	1.2	>6432	>7619
13	1.1	>6800	0.3	>23,800	>7644
14	4.6	>1587	8.0	>915	>7336
15	5.4	>1360	13.3	>553	>7336
16	1.4	ND	2.1	ND	ND
17	18.6	>43	5.1	>159	>806
Chloroquine[a]	29.7	>501	744.1	>20	>14,881
1[a]	9.9	>170	15.9	>106	>1686

IC_{50} = Concentration causing death to 50% of the cells.
S.I. = Selectivity index [TC_{50} (Vero cells)/IC_{50} (*P. falciparum*)].
TC_{50} = Concentration toxic to 50% of the cells.
ND = Not determined.
[a] Control.

Table 5
Antileishmanial activity of **12–15**

Compound	IC_{50} (μM)	IC_{90} (μM)
12	5.0	16.0
13	10.4	80.3
14	7.7	77.1
15	7.4	18.5
Pentamidine[a]	3.5	14.7
Amphotericin B[a]	0.1	0.2

IC_{50} = Concentration causing death to 50% of the cells.
IC_{90} = Concentration causing death to 90% of the cells.
[a] Control.

esis Series FTIR. Optical rotations were recorded at ambient temperature using a JASCO DIP-370 digital polarimeter. 1D and 2D NMR spectra were obtained on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C) or a Bruker DRX 400 spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C) using the solvent peak as internal standard. The spectra were recorded in $CDCl_3$ unless stated otherwise. The following abbreviations are used: singlet (s), doublet (d), triplet (t), quadruplet (c), quintuplet (q) and multiplet (m). Chemical shifts are reported in ppm and coupling constants (*J*) in Hertz. HRESIFTMS were obtained using a Bruker BioApex FTMS in ESI mode. Low resolution MS were measured on a ThermoQuest aQa LCMS.

4.2. Synthesis

See Supplementary data for IUPAC nomenclature of **1–17**.

4.2.1. Dihydroartemisinin (α-DHA, 2; β-DHA, 3)

Artemisinin (**1**) was isolated from *A. annua*[30] or obtained commercially.[31] DHA (mixture of **2** and **3**) was prepared by sodium borohydride reduction of **1** (Scheme 1).[32]

4.2.2. β,β-Symmetrical 1,2-ethanediol DHA acetal dimer (6) [NSC 698678]

To a stirred solution of DHA (1.24 g, 4.36 mmol) and dry 1,2-ethanediol (132.5 mg, 2.13 mmol) in dry ether (86 mL) was added $BF_3.OEt_2$ (187 μL) by hypodermic syringe (Scheme 1).[18] The reaction mixture was kept under argon during the course of the experiment. After 80 min additional $BF_3{\cdot}OEt_2$ (79 μL) was added while stirring was continued for a further 45 min. The reaction was quenched with 2% aqueous $NaHCO_3$ (50 mL), ether (150 mL) was added and the subsequent mixture extracted with ether (3 × 150 mL). The combined organic layer was washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to yield an oil. The crude product was crystallized from hexanes/EtOAc (1:3) to yield **6** (490 mg, 37.8%) and a mother liquor (270 mg).

Compound **6**: Cubic crystals; mp 195–196 °C, $[\alpha]_D$ +151.9 (*c* 0.052, $CHCl_3$); IR (KBr) ν_{max}: 2920, 2865, 1460, 1385, 1295, 1275, 1200, 1115, 1035, 990 cm^{-1}; ^{1}H NMR (500 MHz, for one of the two symmetrical units): δ 5.39 (1H, s, H-5), 4.80 (1H, d, *J* = 3.4, H-12), 3.95 (1H, ddd, *J* = 10.3, 3.2, 2.9, H-16α), 3.57 (1H, ddd, *J* = 10.1, 3.3, 3.0, H-16β), 2.62 (1H, m, H-11), 2.36 (1H, ddd, *J* = 14.1, 9.9, 4.0, H-3α), 2.03 (1H, m, H-3β), 1.88 (1H, m, H-2α), 1.78 (1H, m, H-8β), 1.74 (1H, m, H-8α), 1.61 (1H, m, H-9β), 1.48 (1H, m, H-2β), 1.46 (1H, m, H-7), 1.43 (3H, s, H-15), 1.31 (1H, m, H-10), 1.25 (1H, m, H-1), 0.95 (3H, d, *J* = 6.3, H-14), 0.90 (3H, d, *J* = 7.3, H-13), 0.89 (1H, m, H-9α); ^{13}C NMR (125 MHz, for one of the two symmetrical units): Table 6; HRESIFTMS [*m*/*z*] 617.3298 $[M+Na]^+$ (calcd for $C_{32}H_{50}O_{10}Na$, 617.3296).

4.2.3. α,β-Unsymmetrical 1,2-ethanediol DHA acetal dimer (7) [NSC 716243]

The mother liquor from crystallization of **6** was concentrated to leave a gummy residue (270 mg).[18] The residue was chromatographed on a silica gel column (45 g) and eluted with hexanes/EtOAc (9:1 to 7:3). Fractions were combined based on TLC analysis yielding **7** (50 mg, 3.9%).

Compound **7**: Oil; $[\alpha]_D$ +73.5 (*c* 0.034, $CHCl_3$); IR (KBr) ν_{max}: 2925, 2873, 1450, 1376, 1279, 1156, 1105, 1027, 877 cm^{-1}; ^{1}H NMR (500 MHz): δ 5.42 (1H, s, H-5), 5.33 (1H, s, H-5′), 4.82 (1H, d, *J* = 3.3, H-12), 4.47 (1H, d, *J* = 9.2, H-12′), 4.05 (1H, m, H-16β′), 4.01 (1H, m, H-16α), 3.66 (1H, m, H-16α′), 3.56 (1H, m, H-16β), 2.62 (1H, m, H-11), 2.40 (2H, m, H-3α′, H-11′), 2.37 (1H, m, H-3α), 2.04 (1H, m, H-3β), 2.01 (1H, m, H-3β′), 1.88 (3H, m, H-2α, H-2α′, H-8α′), 1.74 (1H, m, H-8β), 1.71 (2H, m, H-8α, H-9α′), 1.57 (1H, m, H-9β), 1.55 (1H, m, H-7′), 1.51 (2H, m, H-2β, H-2β′), 1.46 (1H, m, H-7), 1.44 (3H, s, H-15′), 1.43 (3H, s, H-15), 1.33 (1H, m, H-10), 1.29 (1H, m, H-10′), 1.28 (2H, m, H-1, H-8β′), 1.23 (1H, m, H-1′), 1.00 (1H, m, H-9β′), 0.96 (3H, d, *J* = 6.1, H-14′), 0.94 (3H, d, *J* = 6.3, H-14), 0.91 (3H, d, *J* = 7.3, H-13), 0.90 (1H, m, H-9α), 0.88 (3H, d, *J* = 7.2, H-13′); ^{13}C NMR (125 MHz): Table 6; HRESIFTMS [*m*/*z*] 617.3289 $[M+Na]^+$ (calcd for $C_{32}H_{50}O_{10}Na$, 617.3296).

4.2.4. α,β-Unsymmetrical 1,4-butanediol DHA acetal dimer (8) [NSC 709308]

To a stirred solution of DHA (140 mg, 0.49 mmol) in ether (20 mL) was added dry 1,4-butanediol (115 mg, 1.28 mmol) and $BF_3{\cdot}OEt_2$ (41 μL).[18] The mixture was stirred for 90 min followed by the usual work up. The crude product (195 mg, gum) was loaded on a silica gel column and eluted with hexanes/EtOAc, yielding the monomeric product. This monomeric product was dried, dissolved in ether (25 mL) and re-reacted with DHA (160 mg) and $BF_3{\cdot}OEt_2$ (45 μL). The reaction mixture was stirred for 85 min followed by the usual work up and silica gel purification (25 g, flash silica) to yield **8** (35.7 mg, 9.1%).

Compound **8**: Oil; $[\alpha]_D$ +117.9 (*c* 0.028, $CHCl_3$); IR (KBr) ν_{max}: 2924, 2873, 2361, 1450, 1376, 1174, 1102, 1029 cm^{-1}; ^{1}H NMR (500 MHz): δ 5.36 (1H, s, H-5), 5.31 (1H, s, H-5′), 4.76 (1H, d, *J* = 3.69, H-12), 4.40 (1H, d, *J* = 9.22, H-12′), 3.97 (1H, m, H-16β′), 3.84 (1H, m, H-16α), 3.41 (1H, m, H-16α′), 3.37 (1H, m, H-16β), 2.59 (1H, br m, H-11), 2.39 (1H, m, H-11′), 2.35 (2H, m, H-3α, H-3α′), 2.03 (2H, m, H-3β, H-3β′), 1.87 (2H, m, H-2α, H-2α′), 1.75 (3H, m, H-8α, H-8β, H-8α′), 1.68 (1H, m, H-9α′), 1.63 (4H, m, H-17α, H-17β, H-17α′, H-17β′), 1.62 (1H, m, H-9β), 1.55 (1H, m, H-7′), 1.50 (2H, m, H-2β, H-2β′), 1.47 (1H, m, H-7), 1.42 (6H, s, H-15, H-15′), 1.31 (1H, m, H-10), 1.31 (1H, m, H-10′), 1.29 (1H, m, H-8β′), 1.27 (1H, m, H-1), 1.24 (1H, m, H-1′), 0.98 (1H, m, H-9β′), 0.94 (6H, m, H-14, H-14′), 0.91 (1H, m, H-9α), 0.88 (3H, d, *J* = 8.08, H-13), 0.86 (3H, d, *J* = 7.82, H-13′); ^{13}C NMR (125 MHz): Table 6; HRESIFTMS [*m*/*z*] 645.3634 $[M+Na]^+$ (calcd for $C_{34}H_{54}O_{10}Na$, 645.3615).

4.2.5. β,β-Symmetrical 1,3-propanediol DHA acetal dimer (9) [NSC 705974]

To a stirred solution of DHA (216 mg, 0.76 mmol) in dry ether (28 mL) was added dry 1,3-propanediol (25 mg, 0.33 mmol) and $BF_3{\cdot}OEt_2$ (98 μL).[18] The mixture was stirred for 60 min followed by the usual work up. The crude product (200 mg) was purified by crystallization from hexanes/EtOAc to afford **9** (90 mg, 45.0%).

Compound **9**: Colorless powder; $[\alpha]_D$ +153.6 (*c* 0.044, $CHCl_3$); IR (film) ν_{max}: 2936, 2876, 2359, 2341, 1450, 1376, 1226, 1158, 1102, 1025, 991 cm^{-1}; ^{1}H NMR (500 MHz, for one of the two symmetrical units): δ 5.35 (1H, s, H-5), 4.75 (1H, d, *J* = 3.4, H-12), 3.90 (1H, m, H-16α), 3.41 (1H, m, H-16β), 2.59 (1H, m, H-11), 2.34 (1H, ddd, *J* = 14.0, 4.0, 3.1, H-3α), 1.99 (1H, m, H-3β), 1.84 (1H, m, H-2α), 1.80 (2H, m, H-17α, H-17β), 1.74 (1H, m, H-8β), 1.72 (1H, m, H-

8α), 1.61 (1H, m, H-9β), 1.50 (1H, m, H-2β), 1.43 (1H, m, H-7), 1.40 (3H, s, H-15), 1.34 (1H, m, H-10), 1.21 (1H, m, H-1), 0.92 (3H, d, J = 6.4, H-14), 0.87 (3H, d, J = 7.4, H-13), 0.89 (1H, m, H-9α); ^{13}C NMR (125 MHz, for one of the two symmetrical units): Table 6; HRESIFTMS [m/z] 631.3485 [M+Na]$^+$ (calcd for $C_{33}H_{52}O_{10}Na$, 631.3452).

4.2.6. β,β-Symmetrical 1-methoxymethyl-1,2-ethanediol DHA acetal dimer (10) [NSC 718560]

To a stirred solution of DHA (50 mg, 0.18 mmol) and (±)-1-methoxymethyl-1,2-ethanediol (18 mg, 0.20 mmol) in dry ether (5 mL) was added $BF_3{\cdot}OEt_2$ (266 μL) at intervals.[19] The stirring was continued for 60 min followed by the usual workup to give a gummy residue (70 mg). The crude product was purified by column chromatography (7.2 g, flash silica) using hexanes/EtOAc (80:20) for elution. Fractions (15 mL each) were collected and combined based on TLC similarities. Fractions 7–16 (16 mg) contained **10** and subsequent repeated chromatography using $CHCl_3$/MeCN (80:20) as eluent yielded **10** (9.0 mg, 16.4%).

Compound **10**: Oil; $[\alpha]_D$ +121.9 (c 0.032, CH_2Cl_2); IR (film) ν_{max}: 2949, 2874, 1448, 1377, 1195, 1104, 1030, 989, 876 cm^{-1}; ^{1}H NMR (500 MHz, two diastereoisomers present): δ 5.59, 5.46 (1H, s, H-5), 5.42, 5.38, (1H, s, H-5′), 5.04, 4.97 (1H, d, J = 3.41, 3.30, H-12), 4.81, 4.78, (1H, d, J = 3.28, 3.26, H-12′), 4.08, 4.02 (1H, m, H-16), 3.96/3.53, 3.99/3.38 (2H, m, H-17), 3.50/3.50, 3.52/3.44 (2H, m, H-18), 3.37, 3.33 (3H, s, H-19), 2.64 (2H, m, H-11, H-11′), 2.38 (2H, m, H-3α, H-3α′), 2.05 (2H, m, H-3β, H-3β′), 1.90 (2H, m, H-2α, H-2α′), 1.80 (2H, m, H-8β, H-8β′), 1.77 (2H, m, H-8α, H-8α′), 1.65 (2H, m, H-9β, H-9β′), 1.55 (2H, m, H-2β, H-2β′), 1.51 (2H, m, H-7, H-7′), 1.45 (6H, s, H-15, H-15′), 1.35 (2H, m, H-10, H-10′), 1.27 (2H, m, H-1, H-1′), 0.97 (6H, m, H-14, H-14′), 0.92 (6H, s, H-13, H-13′), 0.90 (2H, m, H-9α, H-9α′); ^{13}C NMR (125 MHz): Table 6; HRESIFTMS [m/z] 661.3609 [M+Na]$^+$ (calcd for $C_{34}H_{54}O_{11}$, 661.3582).

4.2.7. β,β-Symmetrical (*R*)-methyl-1,2-ethanediol DHA acetal dimer (11) [NSC 704825]

To a stirred solution of DHA (50 mg, 0.18 mmol) and (−)-(*R*)-methyl-1,2-ethanediol (7.0 mg, 0.092 mmol) in dry ether (5 mL) was added $BF_3{\cdot}OEt_2$ (266 μl) at intervals.[19] The stirring was continued for 60 min followed by the usual work up to give a gummy residue (49 mg). The crude product was purified by column chromatography (6.0 g, flash silica) using $CHCl_3$/MeCN (98:2) for elution. Fractions (2 mL each) were collected and combined based on TLC similarities. Fractions 9–12 afforded **11** (11.5 mg, 21.5%).

Compound **11**: Oil; $[\alpha]_D$ +541.7 (c 0.024, MeOH); IR (film) ν_{max}: 2940, 2922, 2872, 1450, 1376, 1194, 1104, 1029 cm^{-1}; ^{1}H NMR (500 MHz): δ 5.37 (1H, s, H-5), 5.45 (1H, s, H-5′), 4.96 (1H, d, J = 3.32, H-12), 4.77 (1H, d, J = 3.21, H-12′), 4.00 (1H, m, H-16β), 3.80 (1H, dd, J = 10.00, 4.17, H-17β), 3.31 (1H, dd, J = 10.10, 6.01, H-17α), 2.63 (2H, m, H-11, H-11′), 2.40 (2H, ddd, J = 10.9, 3.4, 2.9, H-3α, H-3α′), 2.04 (2H, m, H-3β, H-3β′), 1.89 (2H, m, H-2α, H-2α′), 1.82 (2H, m, H-8β, H-8β′), 1.75 (2H, m, H-8α, H-8α′), 1.64 (2H, m, H-9β, H-9β′), 1.51 (2H, m, H-2β, H-2β′), 1.48 (1H, m, H-7 or H-7′), 1.45 (6H, s, H-15, H-15′), 1.42 (1H, m, H-7 or H-7′), 1.32 (2H, m, H-10, H-10′), 1.26 (2H, m, H-1, H-1′), 1.24 (3H, d, J = 6.34, H-18), 0.97 (3H, d, J = 6.25, H-14 or H-14′), 0.96 (3H, d, J = 5.95, H-14 or H-14′), 0.94 (2H, m, H-9α, H-9α′), 0.92 (3H, d, J = 7.15, H-13 or H-13′), 0.91 (3H, d, J = 7.00, H-13′); ^{13}C NMR (125 MHz): Table 6; HRESIFTMS [m/z] 631.3482 [M+Na]$^+$ (calcd for $C_{33}H_{52}O_{10}Na$, 631.3452).

4.2.8. β,β-Symmetrical 2-hydroxy-1,3-propanediol DHA acetal dimer (12) [NSC 716240]

To a stirred solution of DHA (160 mg, 0.56 mmol) and dry 2-hydroxy-1,3-propanediol (26 mg, 0.28 mmol) in dry ether (10 mL) was added $BF_3{\cdot}OEt_2$ (267 μL).[19] The mixture was stirred under argon for 70 min followed by the usual work up to leave a gummy residue (199 mg). Upon crystallization from ether, it yielded **12** (52 mg, 29.6%).

Alternatively, ketone **13** (1.94 g, 3.12 mmol) was dissolved in THF/water (225 mL, 2:1). To the stirred solution $NaBH_4$ (474 mg, 4 equiv) was added in portions at room temperature over a 15 min period. The mixture was neutralized with 2 N HCl, the solvent removed and the residue filtered, washed with water and air dried to give **12** (1.8 g, 92.5%).

Compound **12**: Cubic crystals; $[\alpha]_D$ +173 (c 0.022, $CHCl_3$); IR (film) ν_{max}: 3525 (OH), 2953, 2933, 2881, 1449, 1376, 1194, 1176, 1134, 1107, 1027, 991 cm^{-1}; ^{1}H NMR (500 MHz, for one of the two symmetrical units): δ 5.35 (1H, s, H-5), 4.77 (1H, t, J = 3.8, H-12), 3.84 (2H, m, H-16α, H-17), 3.47 (1H, d, J = 10.27, 4.30, H-16β or H-16β′), 3.40 (1H, d, J = 9.80, 5.40, H-16β or H-16β′), 2.61 (1H, m, H-11), 2.38 (1H, br s, O*H*), 2.33 (1H, ddd, J = 14.0, 4.0, 3.9, H-3α), 1.98 (1H, m, H-3β), 1.83 (1H, m, H-2α), 1.71 (1H, m, H-8β), 1.67 (1H, m, H-8α), 1.61 (1H, m, H-9β), 1.48 (1H, m, H-2β), 1.45 (1H, m, H-7), 1.38 (3H, s, H-15), 1.32 (1H, m, H-10), 1.21 (1H, m, H-1), 0.91 (3H, d, J = 6.3, H-14), 0.88 (3H, d, J = 7.4, H-13), 0.88 (1H, m, H-9α); ^{13}C NMR (125 MHz, for one of the two symmetrical units): Table 6; HRESIFTMS [m/z] 625.3512 [M+H]$^+$ (calcd for $C_{33}H_{53}O_{11}$, 625.3582).

4.2.9. β,β-Symmetrical 2-keto-1,3-propanediol DHA acetal dimer (13) [NSC 722593]

DHA (284 mg, 1.00 mmol) and 2-keto-1,3-propanediol (45 mg, 0.25 mmol) were suspended in dry ether (10 mL) under argon at 5 °C.[19] To the mixture was added $BF_3{\cdot}Et_2O$ (0.25 mmol, 31 μL, 35.5 mg), followed by stirring at 5 °C for 20 min and at room temperature for 1 h. Work up was done as usual to give a residue (319 mg) and subsequent column chromatography (30 g) eluting with hexanes/EtOAc (95:5 to 80:20) afforded four major fractions. The most polar fraction was identified as **13** (140.2 mg, 45.1%).

Compound **13**: White powder; $[\alpha]_D$ +191.7 (c 0.024, $CHCl_3$); IR (film) ν_{max}: 2924, 2875, 2248, 1739.5, 1450, 1377, 1194, 1108, 1030, 989 cm^{-1}; ^{1}H NMR (500 MHz, for one of the two symmetrical units): δ 5.44 (1H, s, H-5), 4.80 (1H, t, J = 3.20, H-12), 4.46 (1H, d, J = 17.20, H-16α), 4.28 (1H, d, J = 17.60, H-16β), 2.67 (1H, m, H-11), 2.36 (1H, ddd, J = 27.80, 13.95, 3.73, H-3α), 2.02 (1H, m, H-3β), 1.89 (1H, m, H-2α), 1.86 (1H, m, H-8β), 1.81 (1H, m, H-8α), 1.67 (1H, m, H-9β), 1.48 (1H, m, H-2β), 1.48 (1H, m, H-7), 1.43 (3H, s, H-15), 1.38 (1H, m, H-10), 1.25 (1H, m, H-1), 0.98 (3H, d, J = 7.20, H-14), 0.95 (3H, d, J = 6.40, H-13), 0.91 (1H, m, H-9α); ^{13}C NMR (125 MHz): Table 6; HRESIFTMS [m/z] 645.3240 [M+Na]$^+$ (calcd for $C_{33}H_{50}O_{11}Na$, 645.3245).

4.2.10. β,β-Symmetrical *cis*-1,4-cyclohexanediol DHA acetal dimer (14) [NSC 716239] and β,β-symmetrical *trans*-1,4-cyclohexanediol DHA acetal dimer (15) [NSC 721015]

A mixture of DHA (744 mg, 2.62 mmol) and 1,4-cyclohexanediol (149 mg, 1.28 mmol, *cis*/*trans* mixture) in dry ether (20 mL) was stirred at 5 °C.[19] $BF_3{\cdot}OEt_2$ (83 μL) was added to the mixture while stirring continued at room temperature for 1 h, followed by addition of a second portion of $BF_3{\cdot}OEt_2$ (83 μL) and additional stirring for 1 h. Usual work up afforded an oily residue (890 mg) and subsequent column chromatography (32 g) eluting with hexanes/ether (92:2, 96:4, 94:6 and 90:10) yielded **14** (238 mg, 28.0%) and **15** (184 mg, 21.7%).

Compound **14**: Amorphous foam; mp 93–97 °C; R_f 0.33 (hexanes/EtOAc, 7:3); $[\alpha]_D$ +142 (c 0.036, MeOH); IR (film) ν_{max}: 2938, 2872, 1448, 1375, 1227, 1194, 1122, 1099, 1029 cm^{-1}; ^{1}H NMR (500 MHz, for one of the two symmetrical units): δ 5.42 (1H, s, H-5), 4.90 (1H, d, J = 3.3, H-12), 3.78 (2H, br s, H-16$_{eq}$, H-16′$_{ax}$), 2.62 (1H, m, H-11), 2.36 (1H, ddd, J = 14.0, 12.4, 3.8, H-

3α), 2.02 (1H, m, H-3β), 1.88 (1H, m, H-2α), 1.78 (1H, m, H-8β), 1.75 (1H, m, H-8α), 1.70 (2H, m, H-17_{ax}, H-$18'_{ax}$), 1.62 (1H, m, H-9β), 1.61 (4H, m, H-17_{eq}, H-18_{eq}, H-$17'_{eq}$, H-$18'_{eq}$), 1.56 (2H, m, H-18_{ax}, H-$17'_{ax}$), 1.52 (1H, m, H-2β), 1.45 (1H, m, H-7), 1.43 (3H, s, H-15), 1.31 (1H, m, H-10), 1.25 (1H, m, H-1), 0.95 (3H, d, J = 6.2, H-14), 0.92 (1H, m, H-9α), 0.90 (3H, d, J = 7.2, H-13); ^{13}C NMR (125 MHz, for one of the two symmetrical units): Table 6; HRESIFTMS [m/z] 687.3445 $[M-1]^-$ (calcd for $C_{36}H_{55}O_{10}$, 687.3510).

Compound **15**: Cubic crystals; mp 146–148 °C, R_f 0.42 (hexanes/EtOAc, 8:2); $[\alpha]_D$ +114 (c 0.042, $CHCl_3$); IR (film) no OH absorption; ^{1}H NMR (400 MHz): δ 5.37 (2H, s, H-5, H-5′), 4.84 (2H, d, J = 3.32, H-12, H-12′), 3.71 (2H, br s, H-16_{eq}, H-$16'_{eq}$), 2.56 (2H, m, H-11, H-11′), 2.32 (2H, d, J = 14.1, 14.0, 13.9, H-3α, H-3α′), 1.98 (2H, m, H-3β, H-3β′), 1.87 (2H, m, H-18, H-17′), 1.76 (4H, m, H-2α, H-2α′, H-17, H-18′), 1.70 (2H, m, H-8β, H-8β′), 1.67 (2H, m, H-8α, H-8α′), 1.59 (2H, m, H-9β, H-9β′), 1.45 (2H, m, H-2β, H-2β′), 1.44 (2H, m, H-18, H-17′), 1.41 (2H, m, H-7, H-7′), 1.39 (6H, s, H-15, H-15′), 1.26 (2H, m, H-10, H-10′), 1.25 (2H, m, H-17, H-18′), 1.19 (2H, m, H-1, H-1′), 0.91 (6H, d, J = 6.3, H-14, H-14′), 0.85 (2H, m, H-9α, H-9α′), 0.83 (6H, d, J = 7.21, H-13, H-13′); ^{13}C NMR (100 MHz): Table 6; HRESIFTMS [m/z] 671.3772 $[M+Na]^+$ (calcd for $C_{36}H_{56}O_{10}Na$, 671.3765).

4.2.11. β,β-Symmetrical 1,3-dihydroxy-2-propyl succinate DHA acetal dimer (succinate derivative of 12) (16) [NSC 724910]

To a stirred solution of **12** (200 mg, 0.32 mmol) in dry methylene chloride (4 mL) were added triethylamine (0.14 mL, 1.3 equiv), 4-dimethylaminopyridine (16 mg, 0.4 equiv) and succinic anhydride (92 mg, 3 equiv).[19] The resulting solution was slowly stirred at room temperature for 16 h, followed by evaporation of the solvent. Column chromatography using hexanes/acetone (6:4) afforded **16** (156 mg, 67.2%).

Compound **16**: White amorphous solid; R_f 0.68 (hexanes/acetone, 1:1); $[\alpha]_D$ +110.6 (c 0.044, MeOH); IR (film) ν_{max}: 1685.7, 1786.7 cm^{-1}; ^{1}H NMR (acetone-d_6, 500 MHz, for one of the two symmetrical units): δ 5.44 (1H, s, H-5 or H-5′), 5.40 (1H, s, H-5 or H-5′), 5.18 (1H, t, J = 4.98, 4.96, H-17), 4.75 (1H, d, J = 3.38, H-12 or H-12′), 4.74 (1H, d, J = 3.40, H-12 or H-12′), 4.00 (1H, m, H-16 or H-16′), 3.98 (1H, m, H-16 or H-16′), 3.61 (1H, dd, J = 4.5, 4.5, H-16 or H-16′), 3.56 (1H, q, J = 5.19, H-16 or H-16′), 2.65 (4H, m, H-19, H-20), 2.55 (1H, m, H-11), 2.30 (1H, ddd, J = 3.9, 3.0, 3.0, H-3α), 2.07 (1H, m, H-3β), 1.89 (1H, m, H-2α), 1.84 (1H, m, H-8β), 1.79 (1H, m, H-8α), 1.69 (1H, m, H-9β), 1.52 (1H, m, H-2β), 1.45 (1H, m, H-7), 1.41 (1H, m, H-10), 1.33 (3H, s, H-15), 1.21 (1H, m, H-1), 0.97 (3H, dd, J = 6.4, 0.6, H-14), 0.96 (1H, m, H-9α), 0.95 (3H, d, J = 7.4, H-13); ^{13}C NMR (acetone-d_6, 125 MHz, for one of the two symmetrical units): Table 6; HRESIFTMS [m/z] 723.3627 $[M-H]^+$ (calcd for $C_{37}H_{55}O_{14}$, 723.3592).

4.2.12. β,β-Symmetrical 1,2-ethanediol dihydroartemisitene acetal dimer (17) [NSC 720877]

To a stirred solution of dihydroartemisitene[33] (75 mg, 0.027 mmol) and 1,2-ethanediol (52 mg, 0.84 mmol) in dry ether (15 mL) was added $BF_3{\cdot}OEt_2$ (18 μL), followed by stirring for 24 h and work up as usual.[19] Column chromatography of the residue eluting with hexanes/EtOAc (80:20 to 50:50) afforded **17** as a gum (7.0 mg, 8.9%).

Compound **17**: Gum; $[\alpha]_D$ +181 (c 0.022, MeOH); IR (film) ν_{max}: 2937, 2875, 1681, 1449, 1376, 1191, 1102, 987 cm^{-1}; ^{1}H NMR (400 MHz, for one of the two symmetrical units): δ 5.78 (1H, s, H-5), 5.27 (1H, s, H-12), 4.98 (1H, s, H-13a), 4.86 (1H, s, H-13b), 3.79 (1H, d, J = 9.0, H-16α), 3.47 (1H, d, J = 8.6, H-16β), 2.31 (1H, m, H-3α), 2.20 (1H, m, H-7), 2.16 (1H, m, H-8β), 1.73 (1H, m, H-3β), 1.71 (1H, m, H-8α), 1.56 (1H, m, H-2α), 1.43 (1H, m, H-2β), 1.39 (1H, m, H-9β), 1.33 (3H, s, H-15), 1.26 (1H, m, H-1), 1.16 (1H, br s, H-10), 0.84 (1H, m, H-9α), 0.76 (3H, d, J = 6.2, H-14); ^{13}C NMR (100 MHz, for one of the two symmetrical units): Table 6; HRESIFTMS [m/z] 613.2943 $[M+Na]^+$ (calcd for $C_{32}H_{46}O_{10}Na$, 613.2983).

4.3. Biological assays

4.3.1. In vitro anticancer assay

Dimers **6**–**17** were tested in the NCI human tumor 60 cell line assay.[34] The compounds are solubilized in dimethyl sulfoxide (DMSO) at 200X and diluted into RPMI-1640 containing 5% fetal bovine serum (FBS). Serial 10-fold dilutions are prepared for a total of 5 concentrations (10^{-4} to 10^{-8} M). The compounds are added to 24 h old cultures of each of the 60 cell lines used in the panel. Following 48 h incubation, the medium is removed, the cells are fixed and stained with sulforhodamine B and the total stain quantitated by optical density determinations. The cell growth for each cell line can be determined by using a time zero cell control, allowing calculations of the 50% growth inhibitory concentration (GI_{50}), the total growth inhibition (TGI) and the 50% lethal concentration (LC_{50}). The data is plotted as mean bar graphs and/or as dose-response curves. COMPARE analyses were performed as a means to identify similarities with other known or experimental chemotherapeutic agents.[21]

4.3.2. In vivo antitumor assay

Compounds **12**, **14**, **15** and **16** were assessed for activity in an in vivo hollow fiber antitumor efficacy assay.[23] Each of twelve cancer cell lines were loaded into biocompatible polyvinylidene fluoride hollow fibers at inoculation densities that provide cell replication for a period of 7–8 days. Two sets of 3 fibers containing distinct cell lines are implanted into mice intraperitoneally (i.p.) and subcutaneously (s.c.), respectively. Each mouse, therefore, carries 3 fibers i.p. and 3 fibers s.c. representing 3 distinct cell lines. The mice are treated i.p. with the experimental agent once daily for 4 days, the fibers are collected 24 h after the last treatment and the viable cell mass in each fiber is determined using a stable endpoint MTT assay.[23] The percent net growths for the experimentally treated and vehicle control samples are calculated by comparison to the viable cell mass at the time of implantation (time zero). A point system is used to assess the activity of the compounds, with a value of 2 assigned for each compound dose that results in a 50% or greater reduction in viable cell mass in either i.p. or s.c. implanted fibers. A combined i.p. and s.c. score of 20 or higher, an s.c. score of 8 or higher, or cell kill of one or more cell lines are considered as indicators of potential activity.

4.3.3. In vivo subcutaneous xenograft model

Compounds **12**, **14** and **16** were assessed for in vivo efficacy in a subcutaneous xenograft model. HL-60 human leukemia tumors were injected s.c. into female athymic nude mice. The mice were monitored for tumor growth and randomized to one of 6 treatment groups (n = 10) or the control group (n = 20) when the tumors reached a size of 100 ± 25 mg. The tumors were monitored with twice weekly caliper measurements and the tumor mass was calculated as:

$$\text{wt in mg} = 1/2 \times (\text{tumor length}) \times (\text{tumor width})^2$$

The experiment was terminated when the tumor reached a size of 2000 mg. The median tumor weights were calculated for each group and the indices of activity were calculated.[35]

4.3.4. In vitro antiangiogenesis assay

Compounds **12**, **13**, **14** and **16** were subjected to the NCI antiangiogenesis assays according to their standard protocol for HUVEC

Table 6
^{13}C NMR data for compounds **1–3** and **6–17**

Position	**1**[41]	α-**2**[42]	β-**3**[42]	**6**[a,c]	**7**[a,c]	**8**[a,c]	**9**[a,c]	**10**[a,c]	**11**[a,c]	**12**[a,c]	**13**[a,c]	**14**[a,c]	**15**[a,d]	**16**[b,c]	**17**[a,d]
1,1′	49.90	51.53	52.47	52.60	52.99, 52.07	53.00, 52.07	53.00	53.04/52.96/52.92	52.99/52.95	52.94	52.52	53.04	53.03	53.09	52.12
2,2′	24.79	24.64	24.64	24.71	25.08/25.06	25.10/25.06	24.99	25.09	25.07, 25.07	24.99	24.64	25.06	25.08	24.98	24.64
3,3′	35.77	36.26	36.32	36.46	36.85/36.73	36.85/36.73	36.91	36.84	36.86/36.81	36.86	36.39	36.86	36.88	36.70	34.36
4,4′	105.22	104.34	104.03	104.07	104.37, 104.62	104.61, 104.62	104.38	104.47/104.43/104.35	104.48, 104.43	104.50	104.14	104.39	104.40	103.92	103.52
5,5′	93.62	91.19	87.65	87.93	88.26, 91.56	88.28, 91.58	88.25	88.44/88.37/88.24	88.24, 88.47	88.32	88.12	88.42	88.44	88.03/87.99	88.05
6,6′	79.38	80.34	81.11	81.09	81.51, 80.67	81.53, 80.72	81.49	81.52/81.45	81.43, 81.51	81.40	80.97	81.52	81.55	80.97	80.80
7,7′	44.80	45.35	44.44	44.50	44.90, 45.71	44.89, 45.72	44.88	44.92/44.81	44.93/44.83	44.75	44.29	44.94	44.92	44.89	48.33
8,8′	23.32	22.07	24.49	24.44	24.77, 22.60	24.88, 22.59	24.93	24.87/24.79	24.87/24.82	24.99	24.35	24.87	24.87	24.74	31.37
9,9′	33.45	34.19	34.71	34.70	35.08, 34.65	35.06, 34.65	35.06	35.18/35.05	35.09/35.03	34.99	34.61	35.15	35.10	34.99	34.36
10,10′	37.42	37.30	37.40	37.48	37.76/37.73	37.83, 37.76	37.74	37.94/37.87/37.80/37.78	37.93/37.90	37.77	37.36	37.92	37.91	37.63	37.01
11,11′	32.78	34.77	30.76	30.86	33.27, 32.99	31.34, 33.00	31.32	31.30/31.26/31.20	31.30/31.27	31.29	30.74	31.20	31.22	31.33/31.29	143.08
12,12′	171.92	94.69	96.21	102.44	102.44, 100.23	102.37, 100.49	102.60	101.88/101.38,103.16/102.68	102.56,102.15	103.15, 103.08	102.49	100.02	100.44	102.46/102.32	101.23
13,13′	12.47	12.70	13.13	12.99	13.41, 12.83	13.41, 12.97	13.47	13.53/13.48/13.38/13.34	13.54, 13.54	13.45	13.01	13.52	13.49	12.81	114.52
14,14′	19.74	20.18	20.32	20.37	20.72, 20.64	20.73, 20.65	20.80	20.76/20.71	20.75, 20.75	20.76	20.28	20.73	20.74	20.23	20.01
15,15′	25.10	25.81	25.95	26.18	26.55/26.39	26.58/26.43	26.60	26.59	26.61/26.58	26.53	26.07	26.58	26.60	25.72	25.85
16,16′	–	–	–	67.98	67.67, 68.06	68.61, 69.14	65.51	75.16/74.64	73.36	70.31, 70.16	71.65	72.72	73.87	66.74/66.59	66.57
17,17′	–	–	–	–	–	26.95/26.76	30.49	69.30/68.29	72.49	70.07	204.38	29.70	27.51	71.92	–
18,18′	–	–	–	–	–	–	–	73.48/72.67	19.43	–	–	27.41	30.41	171.70	–
19,20	–	–	–	–	–	–	–	59.40/59.33	–	–	–	–	–	29.48/28.87	–
21		–	–	–	–	–	–	–	–	–	–	–	–	172.99	–

[a] $CDCl_3$.
[b] Acetone-d_6.
[c] 125 MHz.
[d] 100 MHz.

initial in vitro testing, that is, growth inhibition, cord formation and cell migration (chemotaxis).[34] Compounds are compared to the positive control agents TNP-470 [*O*-(chloroacetylcarbamoyl) fumagillol][36] and paclitaxel.[37]

4.3.5. In vitro antimalarial assay

The assay is based on the determination of plasmodial LDH activity.[26] A suspension of red blood cells infected with D6 or W2 strains of *P. falciparum* was added to a 96-well plate containing 10 μL of test samples diluted in medium at various concentrations. The plate was placed in a modular incubation chamber flushed with $N_2/O_2/CO_2$ (90:5:5) and incubated at 37 °C for 72 h. Parasitic LDH activity was determined by using Malstat™ reagent.[38] IC_{50} values were computed from the dose-response curves by plotting percent growth versus test concentration. Artemisinin (**1**) and chloroquine were included in each assay as drug controls.

4.3.6. In vitro antileishmanial assay

The in vitro antileishmanial activity was evaluated against a culture of *L. donovani* promastigotes.[26] A three-day-old culture was diluted to 5×10^5 promastigotes/mL. Drug dilutions (50–3.1 μg/mL) were prepared directly in cell suspension in a 96-well plate, followed by incubation at 26 °C for 48 h. Growth of leishmanial promastigotes was determined by the Alamar blue assay.[39] Standard fluorescence was measured by a Fluostar Galaxy plate reader at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Pentamidine and amphotericin B were used as the control drugs. Percent growth was calculated and plotted against the tested concentrations in order to determine the IC_{50} and IC_{90} values.

4.3.7. Cytotoxicity assay

The in vitro cytotoxicity was determined against Vero cells (monkey kidney fibroblasts). The assay was performed in a 96-well tissue culture-treated plate.[26,40] Cells were seeded to the wells of a 96-well plate (25,000 cells/well) and incubated for 24 h. Samples at different concentrations were added and the plate was incubated for 48 h. The number of viable cells was determined by the Neutral Red assay. IC_{50} values were determined from dose curves of percent growth versus test concentrations. Doxorubicin was used as a positive control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.11.050.

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