Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 2761–2768

BIOORGANIC & MEDICINAL CHEMISTRY

Antimalarial and Antiproliferative Evaluation of Bis-Steroidal Tetraoxanes

Dejan Opsenica,[a] Goran Angelovski,[b,†] Gabriella Pocsfalvi,[c] Zorica Juranić,[d] Željko Žižak,[d] Dennis Kyle,[e] Wilbur K. Milhous[e] and Bogdan A. Šolaja[b,*]

[a]*Institute of Chemistry, Technology and Metallurgy, Belgrade, Yugoslavia*
[b]*Faculty of Chemistry, University of Belgrade, Studentski trg 16, PO Box 158, YU-11001 Belgrade, Yugoslavia*
[c]*Centro di Spettrometria di Massa Proteomica e Biomolecolare, Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, Avellino, Italy*
[d]*National Cancer Research Institute, Belgrade, Yugoslavia*
[e]*Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA*

Received 31 January 2003; accepted 1 April 2003

Abstract—Several *cis* and *trans* bis-steroidal 1,2,4,5-tetraoxanes possessing amide terminus were synthesised and evaluated as antimalarials and antiproliferatives. The compounds exhibited submicromolar antimalarial activity against *Plasmodium falciparum* D6 and W2 strains. The existence of HN–C(O) moiety was found necessary for pronounced antimalarial and antiproliferative activity. In antiproliferative screen, the *trans* tetraoxane **6** was found to exhibit a pronounced cytotoxicity on 14 cancer cell lines. In addition, tetraoxanes **11** and **12** exhibited significant cytotoxic activity too; microscopic examination of treated HeLa cells showed morphological appearance reminiscent for apoptosis (condensed and/or fragmented nuclei).

Introduction

Malaria is one of the most serious diseases affecting more than 500 million people worldwide.[1] Rapid rise of resistance of currently available drugs in many strains of parasite causing more than one million deaths per year, prompted WHO to establish the Roll Back Malaria as one of its highest priority programs.

1,2,4,5-Tetraoxacyclohexane (tetraoxane) moiety became a highly interesting pharmacophore since its antimalarial activity is very similar to that of 1,2,4-trioxanes[2] such as naturally occurring artemisinin and its semisynthetic derivatives. Therefore, efforts have been directed towards the synthesis of 1,2,4,5-tetraoxanes and association of the antimalarial activity thereof, both in vitro and in vivo.[3]

Our own investigation is focused on the synthesis of steroidal tetraoxanes and the evaluation of the antimalarial activity thereof against *Plasmodium falciparum* chloroquine-susceptible and chloroquine-resistant clones.[4,5] We are interested in the evaluation of cholic acid-derived carriers of tetraoxane pharmacophore differing in the C(24) substituent. To that purpose two series, *cis* and *trans*, of bis-steroidal tetraoxanes have been synthesized, and the importance of the amide moiety on high antimalarial activity has been demonstrated.[4b] The cholic acid-derived carrier is envisaged to render solubility under physiological conditions, and to enhance a cell membrane permeability due to its amphiphilic character. Primary and secondary amide derivatives are not ionised under physiological conditions (as could be envisaged for carboxylic acids), thus the possibility of cell membrane lysis is not expected.[5a] To explore the influence of the amide substitution pattern on antimalarial activity, we now present the synthesis the C(24) secondary and tertiary amides of both bis-steroidal tetraoxane series and discuss their in vitro antimalarial and antiproliferative screening results.

Chemistry

In our previous paper, we reported the preparation of cholic acid-derived bis-steroidal tetraoxanes by direct

*Corresponding author. Tel.: +381-11-63-86-06; fax: +381-11-63-60-61; e-mail: bsolaja@chem.bg.ac.yu
†Present address: Department of Chemistry, University of Dortmund.

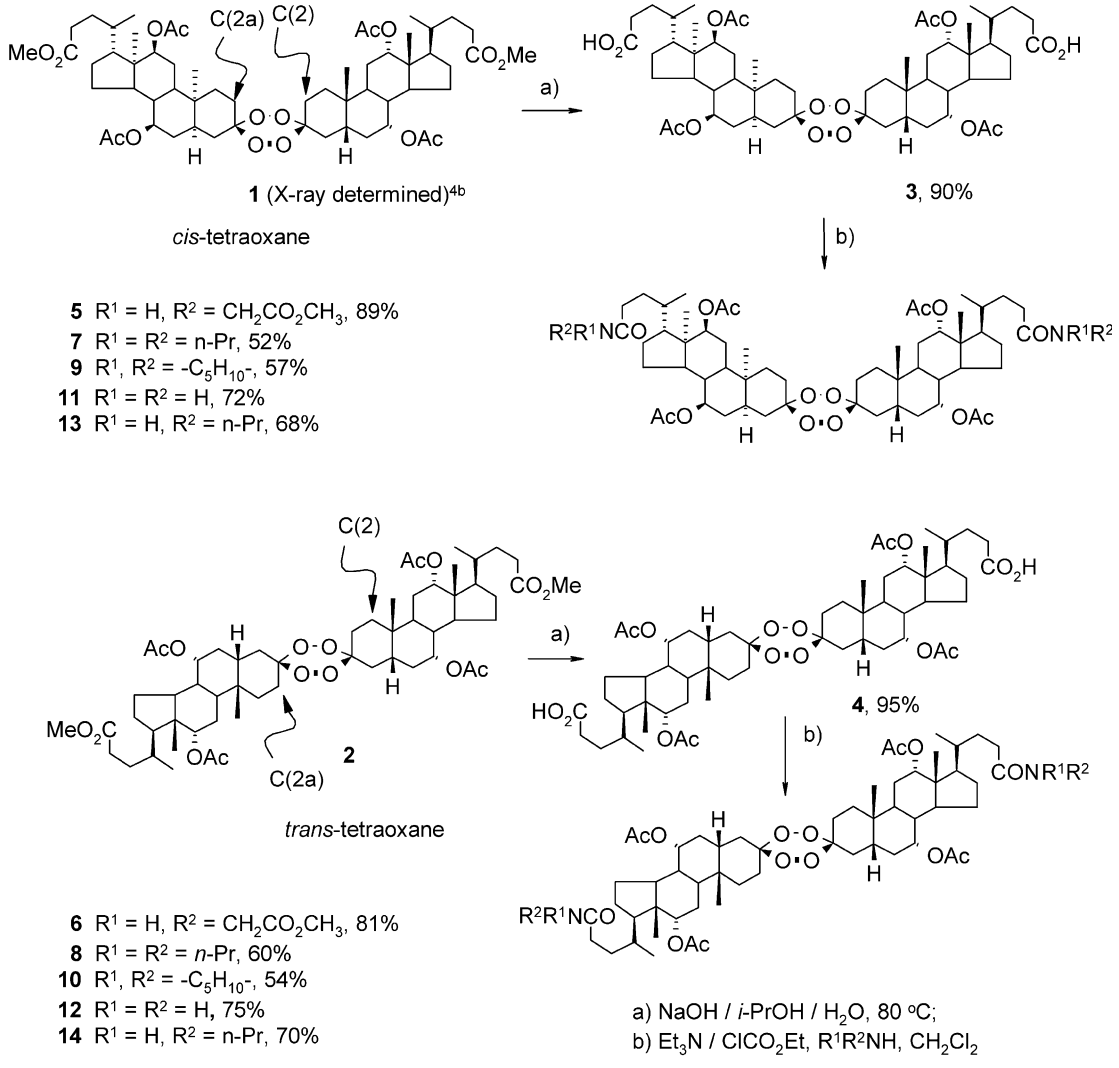
0968-0896/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0968-0896(03)00224-4

coupling of respective keto-esters or keto-amides, followed by separation of the resulting mixtures to the respective *cis* and *trans* isomers.[4b] During the research in the tetraoxane field, we now developed a route for transforming the tetraoxane C(24) esters via acids directly into the corresponding amides (Scheme 1). It was found that the stability of 1,2,4,5-tetraoxane moiety at elevated temperature enables smooth ester hydrolysis and further transformation of the obtained acids. This approach facilitates straightforward structure assignment and avoids the structure assignment based solely on correlation of the physical and spectroscopic data with previously synthesized compounds. Finally, this procedure simplifies the synthesis of various amides without the need for the preparation of respective keto-amides.

Cis and *trans* tetraoxane methyl esters (**1** and **2**, respectively) were selectively hydrolyzed at C(24) in high yield into the corresponding acids **3** and **4**. These products were identical in all respects to those obtained earlier (physical, spectroscopic and antiproliferative activity).[4b] Each acid was further transformed using the mixed anhydride method into the corresponding secondary and tertiary amide using methyl glycinate, di-*n*-propyl amine, and piperidine. The primary amides **11** and **12**, as well as the *n*-propyl amides **13** and **14**, were also prepared using the same procedure and were shown to be identical in all respect to those obtained earlier.[4b]

It is interesting to note that the regularity detected previously is observed with this series as well: *cis* series [Scheme 1: *cis*-C(2), C(2a), **5**, **7**, **9**] having in all cases higher mp and specific rotations than *trans* series (**6**, **8**, **10**); the two isomeric series can also be clearly distinguished by their ^{1}H NMR pattern (see Table 1 and ref 4b).

Molecular masses of the synthesized tetraoxanes were confirmed by using single-stage electrospray ionization mass spectrometry (ESI-MS) in positive ion mode. Under these conditions all the compounds yield abundant molecular ion peaks by coordinating hydrogen, ammonium, sodium, and potassium ions ($[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and/or $[M+K]^+$, respectively).



Scheme 1.

Table 1. Physical and selected spectral data of tetraoxanes **5**–**10**

Compd	Mp (°C)	$[\alpha]_D^{20}$	NMR ($CDCl_3$)		MS [method][a]
			^{1}H acetate methyls	^{13}C C(3) carbons	
5	249–251	+64	2.10	108.64	1193.74 ($[M+K]^+$) 1177.61 ($[M+Na]^+$) [ESI]
6	170–172	+38	2.12 2.08	108.64	1193.69 ($[M+K]^+$) 1177.70 ($[M+Na]^+$) [ESI]
7	222–227	+65	2.10	108.66	1179.87 ($[M]^+$), 1181.88 ($[M+2H]^+$), [ESI]
8	173–175	+49	2.12 2.07	108.66	1201.90 ($[M+Na]^+$) [ESI]
9	229–234	+69	2.10	108.64	1147.70 ($[M+H]^+$) [LSI]
10	182–183	+32	2.12 2.08	108.66	1185.79 ($[M+K]^+$), 1169.81 ($[M+Na]^+$), 1147.83 ($[M+H]^+$), [LSI]

[a]See Experimental for conditions.

Biological Activity and Discussion

The in vitro antimalarial activity of synthesized tetraoxanes **5**–**10** against *P. falciparum* D6 and W2 clones is given in Table 2. D6 is a clone from the Sierra I/UNC isolates and is susceptible to chloroquine and pyrimethamine, but has reduced susceptibilities to mefloquine and halofantrine. W2 is a clone of the Indochina I isolate and is resistant to chloroquine and pyrimethamine, but susceptible to mefloquine. In addition, the activities of primary and n-propyl amides (**11**–**14**) are given for comparison.

As shown in Table 2, the tetraoxanes **5**, **6**, **9**, **10** exert the antimalarial activity on submicromolar scale. It is apparent that tertiary amides **9**, **10** with piperidine-introduced rigidity are significantly more active than the respective open-chain congeners **7** and **8**. It should be also noted that methyl glycinates **5** and **6** were several times less potent in comparison to the secondary *n*-propyl amides **13** and **14**, although it was envisioned that hydrolysis to the corresponding natural glycocholic acid derivatives[3a] would render the solubility under physiological conditions and enhance the activity.[2h] Presented results indicate that for pronounced antimalarial activity in both series, *cis* and *trans*, the existence of hydrogen-bond donor H–NC(O) appears to be necessary (**13**, **14** vs **7**, **8** and **9**, **10**).

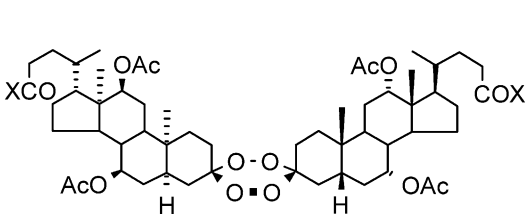
The presented structure–activity information is very important for our ongoing QSAR studies, the results of which will be published soon.

Encouraged by the discovery of antiproliferative activity of steroidal tetraoxanes **11**–**14** (Table 2 and ref. 4b) we submitted new tetraoxanes **5**–**10** to detailed antiproliferative screening against human melanoma Fem-X, human cervix carcinoma HeLa, and myelogenous leukemia (K562) cell lines (Table 3), as well as against a cell lines panel at National Cancer Institute (NIH-NCI; Table 4).[7]

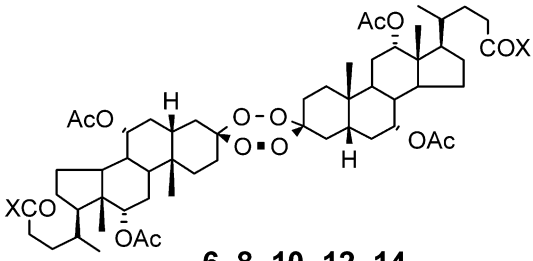
All new compounds except *trans* bis-tetraoxane **6** were active only at high concentrations in all cell lines shown in Table 3. In addition, compounds **5**, **7**–**10** did not pass the standard initial evaluation panel at NIH-NCI: 3-cell line, one dose primary anticancer assay (lung: H-460; breast: MCF7; CNS: SF-268; 44–85% cell growth at 1×10^{-4} M). However, it is interesting to note that *trans* bis-steroidal tetraoxane **6** is found to be at least 20–40 times more active than its *cis* isomer **5** against three cell lines tested. At NIH-NCI (Development Therapeutics Program) tetraoxane **6** (NSC 720388) has also shown to possess strong growth inhibiting properties against several types of cancer: non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, and renal cancer (GI50, TGI, LC50, Table 4). Submicromolar values have been measured for ten cell lines (GI50), while total growth inhibition (TGI) is reached at concentration as low as <2 μM. The tetraoxane **6** is also efficient in killing the cancer cells in vitro: the LC50 (lethal concentration) values are <5 μM (except for Renal cancer TK-10 cell line; LC50 = 8.06 μM) (Table 4). Its high cytostatic (GI50, TGI) and cytotoxic (LC50) activity[8] indicate that further structure development could afford an interesting new chemotherapeutic lead.

Compounds **11** and **12** also showed significant cytotoxicity to Fem-X and HeLa cells, indicating that H–NC(O) group in the investigated tetraoxanes could be important for their activity.

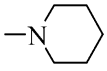
Table 2. In vitro antimalarial activity of tetraoxanes **5–14**



5, 7, 9, 11, 13



6, 8, 10, 12, 14

X	*cis*-C(2)C(2a)			*trans*-C(2)C(2a)		
	Compd	*P. falciparum* IC_{50} (μM)		Compd	*P. falciparum* IC_{50} (μM)	
		D6	W2		D6	W2
NH_2	**11**	0.024	0.019	**12**	0.13	0.06
$NHPr^n$	**13**	0.009	0.06	**14**	0.02	0.03
$NHCH_2CO_2CH_3$	**5**	0.14	0.13	**6**	0.14	0.15
$N(Pr^n)_2$	**7**	> 4.2	> 4.2	**8**	> 4.2	> 4.2
	**9**	0.77	0.36	**10**	0.22	0.11
Artemisinin		0.0086[a]	0.0073[a]	Chloroquine[b]	0.014	0.19
				Mefloquine[b]	0.028	0.005

[a]Ref 6.
[b]Control drug.

Table 3. In vitro antiproliferative activity of tetraoxanes **5–14**

Compound	IC_{50} (μM)[a,b] 72 h		Compound	IC_{50} (μM)[a] 72 h		
	Fem-X	HeLa		Fem-X	HeLa	K562
11	3.1	3.7	**5**	108	> 100	138.0
12	6.2	6.0	**6**	4.67	3.67	3.5
13	23	33	**7**	48	105	40.0
14	94	166	**8**	85	> 100	110,0
			9	80	90	72.0
			10	102	120	76.0

[a]IC_{50}, concentration of a drug needed to inhibit cell survival by 50%, compared with vehicle-treated control.
[b]Taken from ref 4b.

Table 4. In vitro antiproliferative activity of bis-tetraoxane **6** (NIH-NCI DTP), selected data

Panel	Cell line	Activity (μM)		
		GI50[a]	TGI[b]	LC50[c]
Non-small cell lung cancer	EKVX	0.99	1.78	3.19
	HOP-62	0.75	1.52	3.09
Colon cancer	HCC-2998	0.94	1.86	3.67
CNS cancer	SF-539	0.96	1.80	3.37
Melanoma	MALME-3M	0.78	1.93	4.78
	SK-MEL-2	0.76	1.65	3.59
Ovarian cancer	IGROV1	0.84	1.91	4.37
Renal cancer	786-0	0.76	1.42	2.67
	ACHN	0.84	1.57	2.96
	TK-10	1.18	2.55	8.06
	UO-31	0.83	1.51	2.75

[a]GI50, 50% growth inhibitory activity.
[b]TGI, total growth inhibition.
[c]LC50, concentration of the compound at which 50% of the cells are killed.

In order to determine the mode of HeLa cell death induced by the tetraoxanes **11** and **12**, morphological analysis by microscopic examination of acridine orange and ethidium bromide or Giemsa stained cells was performed. Figure 1 (B, C, F and G) shows typical morphological features of the late apoptosis (condensed and/or fragmented nuclei) induced by the treatment of HeLa cells with 8 μM of investigated tetraoxanes for 48 h. In addition, tetraoxane **11** induced considerable detachment of cells from the monolayer and the majority of the floating cells were apoptotic [Fig. 1 (D and H)].

In conclusion, we have synthesized new tetraoxanes possessing amide termini and found that lack of the H–NC(O) moiety significantly diminishes antimalarial and antiproliferative activity of steroidal tetraoxanes (e.g., **13**, **14** vs **7**, **8**; Tables 2 and 3). Extensive antiproliferative evaluation exposed a secondary amide **6** as potential new lead in search for efficacious chemotherapeutic compound, while the initial results on **11** and **12** reveal the apoptic nature of cell death induced by our compounds.

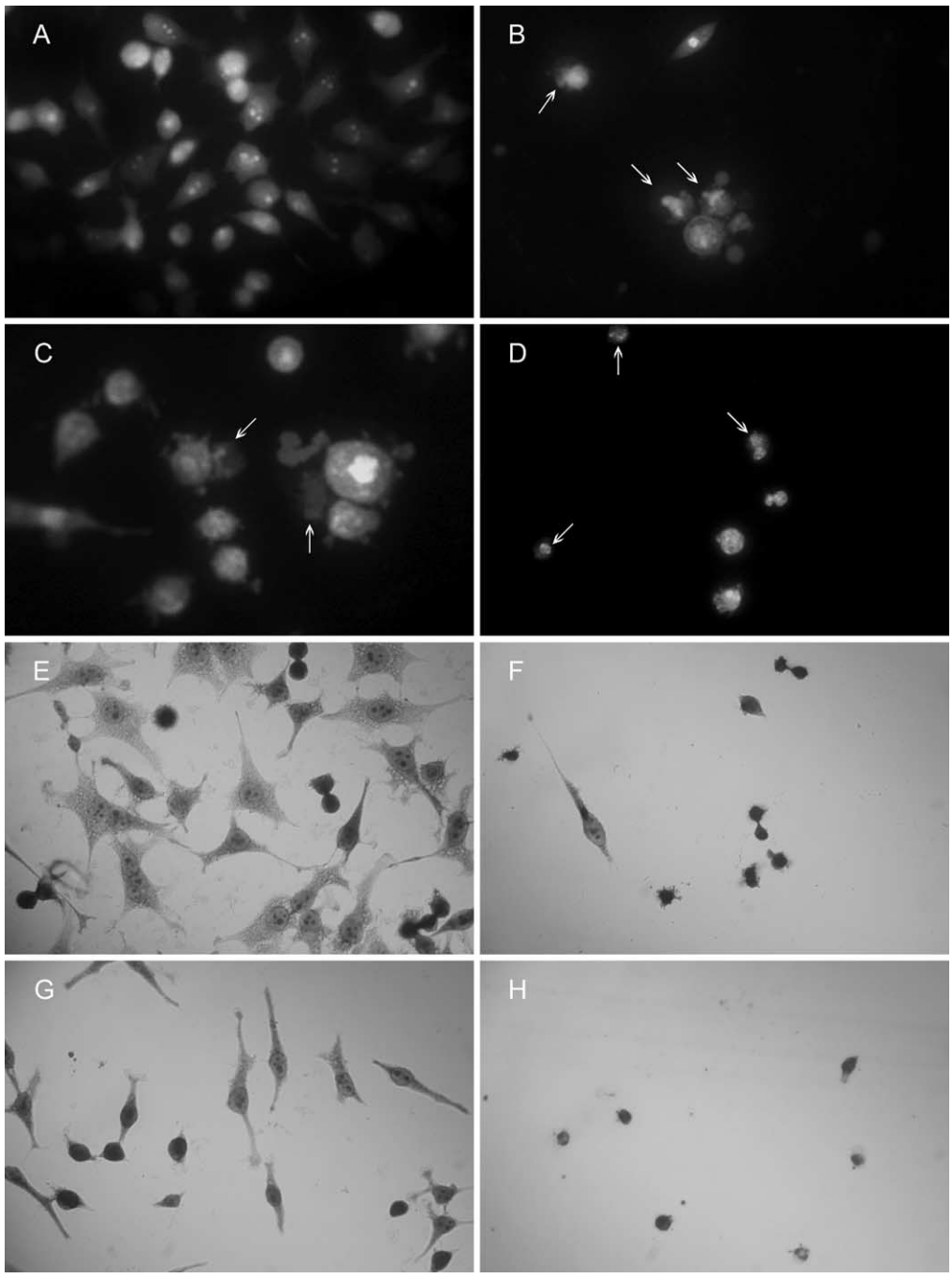


Figure 1. Acridine orange and ethidium bromide (A–D), or Giemsa (E–H) stained HeLa cells after treatment for 48 h with medium alone (A, E), or with 8 μM of compound **11** (B, F) or **12** (C, G), respectively. Detached cells from the same cultures treated with compound **11** (D, H) are pelleted by centrifugation, and stained in the same manner as adherent cells. Arrows indicate the cells in terminal stage of apoptosis (with condensed or fragmented nuclei). Magnification 400×.

Experimental

General

Melting points were determined on a Boetius PMHK apparatus and were not corrected. Specific rotations were determined on Perkin–Elmer 141-MC at the given temperatures. IR spectra were recorded on Perkin–Elmer spectrophotometer FT-IR 1725X. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 spectrometer (at 200 and 50 MHz, respectively) in the indicated solvent using TMS as internal standard. Chemical shifts are expressed in ppm (δ) values and coupling constants (*J*) in Hz. Mass spectra were obtained using a hybrid, quadrupole-orthogonal time of flight (QqQ-TOF), instrument (QStar Pulsar; Applied Biosystems). Samples were dissolved in pure acetonitrile (HPLC grade) at concentration 1.5 pmol/μL. A sample solution was introduced into the ion source using a built-in syringe pump. The following ion source parameters were applied: 5500 V accelerating voltage, 100 V orifice voltage and 40 V skimmer voltage. The resolution of the instrument in TOF-MS mode was measured

to be 8500 at 50% valley definition. Spectrum acquisition was made in positive ion mode in the mass range of *m*/*z* 300–1500. For exact mass measurements an internal standard (kind gift from Applied Biosystems) containing cesium iodide with a known synthetic peptide giving well resolved peaks at *m*/z 132.90543 and 829.5398) was used. Thin-layer chromatography (TLC) has been performed on precoated Merck silica gel 60 F_{254} plates, and Lobar LichroPrep Si 60 (40–63 μm) columns coupled to Waters RI 401 detector were used for column chromatography. Methyl esters **1** and **2** were hydrolyzed using 3 equiv NaOH in *i*-PrOH/H_2O (3/1, v/v; reflux, 15 min) affording the acids **3** and **4**, respectively, being identical in all respects to those obtained earlier.[4b] Amides **11**, **12**, **13**, and **14** were prepared according to the procedure below given for **5**, and were identical in all respects to those obtained earlier.[4b]

Bis(*N*-(methyl ethanoate-2-yl)-3-dioxy-7α,12α-diacetoxy-5β-cholan-24-amide) 5. A solution of **3** (498 mg, 0.49 mmol), in dry CH_2Cl_2 (50 mL), with added Et_3N (136 μL, 0.98 mmol) and $ClCO_2Et$ (94 μL, 0.98 mmol) was stirred for 60 min at 0 °C. Then $NH_2CH_2CO_2Me$•HCl (377 mg, 3.0 mmol) and Et_3N (416 μL, 3.0 mmol) in dry CH_2Cl_2 (20 mL) were added, and after 30 min of stirring the reaction mixture was warmed to rt. After 90 min, it was diluted with H_2O, the layers were separated and the reaction mixture was worked-up in the usual manner.[4] The crude product was purified by column chromatography (Lobar A, LichroPrep Si 60, eluent EtOAc) affording tetraoxane **5** (507 mg, 89%). Mp = 249–251 °C (colorless powder, CH_2Cl_2/*i*-Pr_2O). $[\alpha]_D^{20}$ +64 (*c* 1.00, $CHCl_3$). IR (KBr): 3416 m, 2957 m, 1744 s, 1658 m, 1551 w, 1443 w, 1386 m, 1257 s, 1235 s, 1085 w, 1034 m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 6.05–5.95 (m, 2×H–N), 5.09 (bs, 2×H–C(12)), 4.92 (bs, 2×H–C(7), 4.04 (d, *J* = 5 Hz, 2×H_2C–N), 3.77 (s, 2×CH_3O_2C(26), 2.10 (bs, 4×CH_3COO–), 0.94 (s, 2×H_3C(10)), 0.82 (d, *J* = 5.8 Hz, 2×H_3C–C(20)), 0.73 (s, 2×H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 173.39, 170.58, 170.53, 108.64, 75.23, 70.56, 52.39, 47.38, 44.98, 43.23, 41.12, 37.58, 34.56, 33.01, 31.23, 30.59, 28.35, 27.11, 25.60, 22.71, 22.05, 21.58, 21.31, 17.50, 12.17. ESI-MS (*m*/*z* (%)): 1193.74 ($[M+K]^+$, 33), 1178.79 ($[M+Na+H]^+$, 35), 1177.61 ($[M+Na]^+$, 40), 1177.50 (20), 989.72 (12), 963.70 (15), 909.65 (15), 797.42 (15), 796.46 (40), 795.40 (100), 795.24 (20), 710.28 (20), 701.48 (25), 655.35 (35), 608.86 (50), 608.36(75), 600.35 (80), 523.19 (65), 463.35 (20). Anal. calcd for $C_{62}H_{94}N_2O_{19}$•H_2O (1173.46): C 63.46, H 8.25, found: C 63.76, H 8.60.

Bis(*N*-(methyl ethanoate-2-yl)-3-dioxy-7α,12α-diacetoxy-5β-cholan-24-amide) 6. According to the above given procedure for **5**, the acid **4** (498 mg, 0.49 mmol) was transformed into **6** (460 mg, 81%) using a suspension of 6 equiv of $NH_2CH_2CO_2Me$•HCl/6 equiv Et_3N in dry CH_2Cl_2 (20 mL). Column chromatography (Lobar B, LichroPrep Si 60, eluent: EtOAc); mp = 170–172 °C (colorless powder, CH_2Cl_2/*i*-Pr_2O). $[\alpha]_D^{20}$ = +38 (*c* 1.00, $CHCl_3$). IR (KBr): 3415 m, 2960 m, 1745 s, 1660 w, 1539 w, 1447 w, 1383 m, 1248 s, 127 m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 6.00–5.90 (m, 2×H–N), 5.09 (bs, 2×H–C(12)), 4.92 (bs, 2×H–C(7), 4.04 (d, *J* = 5.4 Hz, 2×H_2C–N), 3.77 (s, 2×CH_3O_2C(26), 2.12 (bs, 2×CH_3COO–), 2.08 (bs, 2×CH_3COO–), 0.94 (s, 2×H_3C(10)), 0.82 (d, *J* = 5.8 Hz, 2×H_3C–C(20)), 0.73 (s, 2×H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 173.39, 170.60, 170.51, 170.31, 108.64, 75.27, 70.61, 68.28, 52.35, 47.41, 45.01, 43.24, 41.13, 37.60, 34.60, 33.05, 31.25, 30.52, 28.33, 27.13, 25.64, 22.80, 22.03, 21.51, 21.34, 17.52, 12.18. ESI-MS (*m*/*z* (%)): 1193.69 ($[M+K]^+$, 15), 1177.70 ($[M+Na]^+$, 20), 702.48 (5), 701.46 (10), 637.32 (5), 635.48 (5), 629.35 (5), 617.84 (5), 617.32 (15), 616.85 (22), 616.33 (30), 610.35 (5), 609.86 (12), 609.35 (30), 608.85 (70), 608.35 (100), 605.54 (5), 601.89 (10), 601.37 (25), 600,86 (65), 600.36 (90), 591.46 (5). Anal. calcd for $C_{62}H_{94}N_2O_{19}$ (1155.44): C 64.45, H 8.20, found: C 64.51, H 8.53.

Bis(*N*,*N*-*di*(*n*-Propyl)-3-dioxy-7α,12α-diacetoxy-5β-cholan-24-amide) 7. According to the above given procedure for **5**, the acid **3** (290 mg, 0.29 mmol) was transformed into **7** (176 mg, 52%). Column chromatography (Lobar B, LichroPrep Si 60, eluent: heptane/EtOAc = 3/7); mp = 222–227 °C (colorless powder, ether/*n*-hexane). $[\alpha]_D^{20}$ = +65 (*c* 1.00, $CHCl_3$). IR (KBr): 3458 m, 2968 s, 2882 m, 1745 s, 1653 s, 1461 m, 1383 m, 1248 s, 1084 m, 1034 m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): $CDCl_3$): 5.10 (bs, 2×H–C(12)), 4.92 (bs, 2×H–C(7), 3.30–3.10 (m, 4×$CH_3CH_2CH_2$–N), 2.10 (bs, 4×CH_3COO–), 1.80–1.40 (m, 4×$CH_3CH_2CH_2$–N), 1.00–0.80 (m, 2×H_3C(10), 4×$CH_3CH_2CH_2$–N, 2×H_3C–C(20)), 0.73 (s, 2×H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$):172.89, 170.56, 108.66, 75.29, 70.61, 49.62, 47.71, 47.73, 45.03, 43.23, 37.62, 34.91, 34.60, 31.34, 30.14, 28.39, 27.17, 25.66, 22.78, 22.27, 22.07, 21.58, 21.29, 20.87, 17.70, 12.19, 11.33, 11.22. ESI-MS [*m*/*z* (%)]: 1181.88 ($[M+2H]^+$, 10), 1179.87 ($[M]^+$, 20), 663.48 (10), 592.44 (5), 591.95 (10), 591.43 (35), 590.93 (75), 590.43 (100), 560.42 (10), 299.31 (5), 297.29 (10), 271.28 (20), 265.27 (5). Anal. calcd for $C_{68}H_{110}N_2O_{14}$•H_2O (1197.66): C 68.20, H 9.43, found: C 68.20, H 8.91.

Bis(*N*,*N*-*di*(*n*-Propyl)-3-dioxy-7α,12α-diacetoxy-5β-cholan-24-amide) 8. According to the above given procedure for **5**, the acid **4** (300 mg, 0.3 mmol) was transformed into **8** (211 mg, 60%). Column chromatography (Lobar B, LichroPrep Si 60, eluent: heptane/EtOAc = 3/7); mp = 173–175 °C (colorless powder, ether/*n*-hexane). $[\alpha]_D^{20}$ = +49 (*c* 1.00, $CHCl_3$). IR (KBr): 3472 s, 2960 s, 2939 m, 2875 w, 1745 s, 1653 s, 1468 m, 1440 m, 1383 m, 1255 s, 1091 w, 1027 m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 5.10 (bs, 2×H-C(12)), 4.92 (bs, 2×H-C(7), 3.40–3.10 (m, 4×$CH_3CH_2CH_2$–N), 2.12 (bs, 2×CH_3COO–), 2.07 (bs, 2×CH_3COO–), 1.70–1.40 (m, 4×$CH_3CH_2CH_2$–N), 1.00–0.80 (m, 2×H_3C(10), 4×$CH_3CH_2CH_2$–N, 2×H_3C–C(20)), 0.74 (s, 2×H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 172.86, 170.54, 170.33, 108.66, 75.32, 70.63, 49.60, 47.72, 47.41, 45.03, 43.24, 37.60, 34.92, 34.60, 31.32, 30.14, 28.35, 27.17, 25.64, 22.78, 22.27, 22.03, 21.51, 21.34, 20.87, 17.70, 12.19, 11.33, 11.24. ESI-MS [*m*/*z* (%)]: 1201.90 ($[M+Na]^+$, 30), 795.42 (5), 702.53 (10), 685.51 (20). Anal. calcd for $C_{68}H_{110}N_2O_{14}$ (1179.64): C 69.24, H 9.40, found: C 69.45, H 9.17.

Bis(3-dioxy-7α,12α-diacetoxy-5β-cholan-24-piperidin-24-on) 9. According to the above given procedure for **5**, the acid **3** (297 mg, 0.29 mmol) was transformed into **9** (192 mg, 57%). Column chromatography (Lobar B, LichroPrep Si 60, eluent: EtOAc); mp = 229–234 °C (colorless powder, CH_2Cl_2/*i*-Pr_2O). $[\alpha]_D^{20} = +69$ (*c* 1.00, $CHCl_3$). IR (KBr): 3458 w, 2946 m, 2875 w, 1738 s, 1653 m, 1447 w, 1383 m, 1255 s, 1084 w, 1034 m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 5.10 (bs, 2×H–C(12)), 4.92 (bs, 2×H–C(7), 3.60–3.45 (m, 2×CH_2–N), 3.40–3.30 (m, 2×–CH_2–N), 2.10 (bs, 4×CH_3COO–), 1.70–1.45 (m, 2×$CH_2CH_2CH_2$), 0.94 (s, 2×H_3C(10)), 0.83 (d, $J = 6.00$ Hz, 2×H_3C–C(20)), 0.73 (s, 2×H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 171.62, 170.56, 108.64, 75.25, 70.57, 47.58, 46.65, 44.99, 43.21, 42.54, 37.60, 34.98, 34.58, 31.28, 30.45, 28.37, 27.17, 26.50, 25.48, 24.49, 22.76, 22.07, 21.62, 21.31, 17.65, 12.17. LSI-MS [*m*/*z* (%)]: 1147.70 ($[M+H]^+$, 27), 906.08 (3), 574.36 (100). Anal. calcd for $C_{66}H_{102}N_2O_{14}$ (1147.55): C 69.08, H 8.96, found: C 69.24, H 8.84.

Bis(3-dioxy-7α,12α-diacetoxy-5β-cholan-24-piperidin-24-on) 10. According to the above given procedure for **13**, the acid **4** (299 mg, 0.30 mmol) was transformed into amide **10** (183 mg, 54%). Column chromatography (Lobar B, LichroPrep Si 60, eluent: EtOAc); mp = 182–183 °C colorless powder, CH_2Cl_2/*i*-Pr_2O). $[\alpha]_D^{20} = +32$ (*c* 1.00, $CHCl_3$). IR (KBr): 3451 w, 2939 m, 2875 w, 1738 s, 1646 m, 1454 m, 1383 m, 1248 s, 1091 w, 1034 m cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 5.10 (bs, 2×H–C(12)), 4.92 (bs, 2×H–C(7), 3.60–3.45 (m, 2×CH_2–N), 3.40–3.30 (m, 2×–CH_2–N), 2.12 (bs, 2×CH_3COO–), 2.08 (bs, 2×CH_3COO–), 1.70–1.45 (m, 2×$CH_2CH_2CH_2$), 0.94 (s, 2×H_3C(10)), 0.83 (d, $J = 6.00$ Hz, 2×H_3C–C(20)), 0.74 (s, 2×H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 171.64, 170.54, 108.66, 75.29, 70.63, 47.60, 46.67, 45.03, 43.24, 42.55, 37.60, 35.00, 34.60, 31.28, 30.46, 28.35, 27.19, 26.51, 25.49, 24.51, 22.80, 22.03, 21.51, 21.34, 17.66, 12.18. LSI-MS [*m*/*z* (%)]: 1185.79 ($[M+K]^+$, 18), 1169.81 ($[M+Na]^+$, 100), 1159.32 (36), 1147.83 ($[M+H]^+$, 68), 1137.34 (2). Anal. calcd for $C_{66}H_{102}N_2O_{14}$•$1.5H_2O$ (1174.58): C 67.49, H 9.01, found: C 67.55, H 9.04.

Antimalarial activity

The in vitro antimalarial drug susceptibility screen is a modification of the procedures first published by Desjardins et al.,[9] with modifications developed by Milhous et al.[10] In brief, the assay relies on the incorporation of radiolabeled hypoxanthine by the parasites and inhibition of isotope incorporation is attributed to activity of known or candidate antimalarial drugs. For each assay, proven antimalarials, are used as controls. The incubation period is 66 h and the starting parasitemia is 0.2% with a 1% hematocrit. The media used is an RPMI-1640 culture media with no folate or *p*-aminobenzoic acid (PABA) and 10% normal heat inactivated human plasma. For quantitative in vitro drug susceptibility testing, two well-characterized *P. falciparum* malaria clones are normally used, W2 and D6.[11] W2 is a clone of the Indochina I isolate and is resistant to chloroquine and pyrimethamine, but susceptible to mefloquine. D6 is a clone from the Sierra I/UNC isolates and is susceptible to chloroquine and pyrimethamine, but has reduced susceptibilities to mefloquine and halofantrine.

Drugs were dissolved directly in dimethylsulfoxide (DMSO) and diluted 400-fold with complete culture media. The compounds are then diluted 2-fold, 11 times, to give a concentration range of 1048-fold. These dilutions are performed automatically by a Biomek 1000 or 2000 Liquid Handling System into 96-well microtiter plates. The diluted drugs are then transferred (25 μL) to test plates, 200 μL of parasitized erythrocytes (0.2% parasitemia and 1% hematocrit) are added, and incubated at 37 °C in a controlled environment of 5% CO_2, 5% O_2 and 90% N_2. After 42 h, 25 μL of 3H-hypoxanthine is added and the plates incubated for an additional 24 h. At the end of the 66-h incubation period, the plates are frozen at −70 °C to lyse the red cells and later thawed and harvested onto glass fibre filter mats by using a 96-well cell harvester. The filter mats are then counted in a scintillation counter and the data downloaded with the custom, automated analysis software developed at WRAIR. For each drug, the concentration response profile is determined and 50% inhibitory concentrations (IC_{50}) are determined by using a non-linear, logistic dose response analysis program.

Antiproliferative activity

Materials and methods. Stock solutions of investigated compounds were prepared in DMSO at a concentration of 8 mM, and afterwards diluted by nutrient medium (RPMI 1640 medium supplemented with L-glutamine (3 mmol/L), streptomycin 100 μg/mL and penicillin 100 IU/mL, 10% heat inactivated fetal bovine serum, FBS and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution) to various final concentrations. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemicals) was dissolved, 5 mg/mL in phosphate buffer saline pH 7.2, and filtered through Millipore filter, 0.22 μm, before use. RPMI 1640 cells culture medium and fetal bovine serum, (FBS) were products of Sigma Chemicals.

Cells culture. Human malignant melanoma Fem-X cells, and human cervix carcinoma HeLa cells were maintained as a monolayer culture, and myelogenous leukaemia K562 cells as a suspension culture in the same nutrient medium. The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere by twice weekly subculture.

Treatment of Fem-X, HeLa and K562 cells. Target cells were seeded, in triplicate (2000 cells per well), into 96-well microtiter flat bottomed plates and 20 h later, five different concentrations of investigated compounds were added to the wells to various final concentrations, except to the control wells where a nutrient medium with correspondent concentration of DMSO only was added to the cells. All analyses were done in triplicate. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank, in triplicate too.

Morphological analysis of HeLa cells death. HeLa cells were seeded on coverslips (5×10^4 cells) in 2 mL of complete medium, and after 24 h, were treated with 8 μM of tetraoxanes **11** and **12** for 48 h. After this time, the cells were stained with 15 μL of acridine orange/ethidium bromide (3 μg/mL AO and 10 μg/mL EB in PBS) and visualized under a fluorescence microscope using blue filter. Alternatively, the cells were fixed with methanol at room temperature, stained with Giemsa stain, and examined by light microscopy. Detached cells from representative cultures of tetraoxane **11** treated cells were harvested by aspirating supernatants, pelleted by centrifugation ($300\times g$, 5 min at room temperature), and stained in a same way as adherent cells.

Acknowledgements

The authors from Belgrade thank the Ministry for Science, Technology and Development of Serbia, (projects 1579 and 1614) for financing this work. We are indebted to the National Cancer Institute, Bethesda, MD, USA, for evaluation of our tetraoxanes.

References and Notes

1. (a) Malaria Foundation International, http://www.malaria.org/, and the sites given therein. (b) Wahlgren, M.; Bejarano, M. T. *Nature* **1999**, *400*, 506.
2. Selected references: (a) Lin, A. J.; Klayman, D. L.; Milhous, W. K. *J. Med. Chem.* **1987**, *30*, 2147. (b) Nga, T. T.; Menage, C.; Beque, J.-P.; Bonnet-Delpon, D.; Gantier, J.-C.; Pradines, B.; Doury, J.-C.; Thac, T. D. *J. Med. Chem.* **1998**, *41*, 4101. (c) Lin, A. J.; Miller, R. E. *J. Med. Chem.* **1995**, *38*, 764. (d) Posner, G. H.; Parker, M. H.; Northorp, J.; Elias, J. S.; Ploypradith, P.; Xie, S.; Shapiro, T. A. *J. Med. Chem.* **1999**, *42*, 300. (e) Posner, G. H.; Ploypradith, P.; Parker, M. H.; O'Dowd, H.; Woo, S.-W.; Northrop, J.; Krasavin, M.; Dolan, P.; Kensler, T. W.; Xie, S.; Shapiro, T. A. *J. Med. Chem.* **1999**, *42*, 4275. (f) Mekonnen, B.; Weiss, E.; Katz, E.; Ma, J.; Ziffer, H.; Kyle, D. E. *Bioorg. Med. Chem.* **2000**, *8*, 1111. (g) O'Neill, P. M.; Miller, A.; Bishop, P. D.; Hindley, S.; Maggs, J. L.; Ward, S. A.; Roberts, S. M.; Scheinmann, F.; Stachulski, A. V.; Posner, G. H.; Park, B. K. *J. Med. Chem.* **2001**, *44*, 58. (h) Posner, G. H.; Jeon, H. B.; Parker, M. H.; Krasavin, M.; Paik, I.-H.; Shapiro, T. A. *J. Med. Chem.* **2001**, *44*, 3054.
3. (a) Dong, Y.; Matile, H.; Chollet, J.; Kaminsky, R.; Wood, J. K.; Vennerstrom, J. L. *J. Med. Chem.* **1999**, *42*, 1477. (b) McCullogh, K. J.; Wood, J. K.; Bhattacharjee, A. K.; Dong, Y.; Kyle, D. E.; Milhous, W. K.; Vennerstrom, J. L. *J. Med. Chem.* **2000**, *43*, 1246. (c) Vennerstrom, J. L.; Dong, Y.; Andersen, S. L.; Ager, A. L., Jr.; Fu, H.-N.; Miller, R. E.; Wesche, D. L.; Kyle, D. E.; Gerena, L.; Walters, S. M.; Wood, J. K.; Edwards, G.; Holme, A. D.; McLean, W. G.; Milhous, W. K. *J. Med. Chem.* **2000**, *43*, 2753 and references cited therein. (d) Vennerstrom, J. L.; Ager, A. L.; Andersen, S. L.; Grace, J. M.; Wongpanich, V.; Angerhofer, C. K.; Hu, J. K.; Wesche, D. L. *Am. J. Trop. Med. Hyg* **2000**, *62*, 573. (e) Jefford, C. W.; Rossier, J.-C.; Milhous, W. K. *Heterocycles* **2000**, *52*, 1345. (f) Kim, H.-S.; Nagai, Y.; Ono, K.; Begum, K.; Wataya, Y.; Hamada, Y.; Tsuchiya, K.; Masuyama, A.; Nojima, M.; McCullough, K. J. *J. Med. Chem.* **2001**, *44*, 2357.
4. (a) Todorović, N. M.; Stefanović, M.; Tinant, B.; Declercq, J.-P.; Makler, M. T.; Šolaja, B. A. *Steroids* **1996**, *61*, 688 and references cited therein. (b) Opsenica, D.; Pocsfalvi, G.; Juranić, Z; Tinant, B.; Declercq, J.-P.; Kyle, D. E.; Milhous, W. K.; Šolaja, B. A. *J. Med. Chem.* **2000**, *43*, 3274 and references cited therein.
5. (a) Šolaja, B. A.; Terzić, N.; Pocsfalvi, G.; Gerena, L.; Tinant, B.; Opsenica, D.; Milhous, W. K. *J. Med. Chem.* **2002**, *45*, 3331. (b) Opsenica, D.; Pocsfalvi, G.; Milhous, W. K.; Šolaja, B. A. *J. Serb. Chem. Soc.* **2002**, *67*, 465.
6. McCullough, K. J.; Wood, J. K.; Bhattacharjee, A. K.; Dong, Y.; Kyle, D. E.; Milhous, W. K.; Vennerstrom, J. L. *J. Med. Chem.* **2000**, *43*, 1246.
7. http://dtp.nci.nih.gov/branches/btb/ivclsp.html
8. For comparison, the average GI50, TGI and LC50 values of well-known cytostatic *cis*-platinum (NSC 119875) for cell lines given in Table 4 are 3.31, 26.26 and 424.56 (μM), respectively. For further details see: http://dtp.nci.nih.gov/docs/static_pages/compounds/119875.html
9. Desjardins, R. E.; Canfield, C. J.; Haynes, D. E.; Chulay, J. D. *Antimicrob. Agents Chemother.* **1979**, *16*, 710.
10. Milhous, W. K.; Weatherly, N. F.; Bowdre, J. H.; Desjardins, R. E. *Antimicrob. Agents Chemother.* **1985**, *27*, 525.
11. Oduola, A. M. J.; Milhous, W. K.; Weatherly, N. F.; Bowdre, J. H.; Desjardins, R. E. *Exp. Parasitol* **1988**, *67*, 354.