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Original scientific paper

Tetraoxanes as inhibitors of apicomplexan parasites *Plasmodium falciparum* and *Toxoplasma gondii* growth and anti-cancer molecules

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Abstract: New cyclohexylidene 1,2,4,5-tetraoxanes with polar guanidine and urea based groups were synthesized and evaluated for their antimalarial activity against chloroquine resistant and susceptible *Plasmodium falciparum* strains. The derivatives showed moderate, nM range antimalarial activities and low cytotoxicity. The *N*-phenylurea derivative **24** exhibited the best resistance indices (RI_{W2} = 0.44, $RI_{TM91C235}$ = 0.80) and was not toxic against human normal peripheral blood mononuclear cells (IC_{50} > 200 μM). Seven derivatives were tested *in vitro* against four human cancer cell lines and they demonstrated high selectivity toward leukaemia K562 cells. One compound, derivative **21** with a primary amino group, was the first tetraoxane tested *in vivo* against *Toxoplasma gondii* as another apicomplexan parasite. Subcutaneous administration at a dose of 10 mg kg^{-1} day^{-1} for 8 days allowed the survival of 20 % of infected mice, thus demonstrating the high potential of tetraoxanes for the treatment of apicomplexan parasites.

Keywords: antimalarials; antiparasitic; peroxides; cancer; cytotoxicity.

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INTRODUCTION

Apicomplexan protozoa are single-celled parasites, with significant medical, veterinary and economic effects worldwide. From the aspect of human medicine, two apicomplexan infections are of major health concern – malaria, caused by *Plasmodium spp.*, and toxoplasmosis, caused by *Toxoplasma gondii*.

A potentially devastating disease, malaria represents an enormous public health problem in the majority of developing countries, further emphasized by the fact that nearly half of the world population is exposed to the infection.[1] Malaria may be caused by five *Plasmodium* species, *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae* and *P. knowlesi*, of which *P. falciparum* that causes cerebral malaria is the major threat. On a cellular level, all *Plasmodium* parasites contain acidic food vacuoles (FV), where digestion of haemoglobin occurs and it is generally accepted that the FV are the site of action for a number of quinoline-like drugs. The haem obtained from haemoglobin degradation is toxic to the parasite and is transformed into insoluble haemozoin pigment, while the globin is hydrolysed into individual amino acids. Antimalarial drugs active within FV appear to kill the parasite either by producing toxic free radicals[2] or by blocking haemozoin formation, as in the case of the 4-amino-7-chloroquinolines (ACQs).[3] The development of widespread drug-resistance to chloroquine (CQ), the most successful antimalarial drug, has resulted in severe health issues in malaria endemic regions. Although thorough investigation led to the realization that mutations in *P. falciparum* chloroquine resistant transporter (PfCRT), multidrug resistance protein 1 (PfMDR1) and multidrug resistance-associated protein (PfMRP) are responsible for the development of resistance of the malaria parasite against chloroquine and its analogues,[4] additional analysis appears necessary.[5] Therefore, significant focus has been placed on the synthesis of peroxide antimalarials active in FV[6] or interfering in NADPH balance acting as leuco-methylene blue (LMB) and $FADH_2$ oxidisers,[7] as well as on the development of new 4-aminoquinolines,[8] acridines[9] and other molecules that prevent haem polymerisation.[10]

T. gondii is an obligatory intracellular apicomplexan protozoan with worldwide distribution. Globally, it has been estimated that one in three persons is infected with *T. gondii*. In the United States, nearly 25 % of the adult population has been infected with this organism,[11] while most studies of European populations report 20−35 % seropositivity.[12] However, in South America, these rates can reach 50−75 %.[13] Individuals with a compromised immune system, such as HIV-infected persons and transplant recipients, are particularly prone to severe *T. gondii* induced disease, mainly as a result of reactivation of a previously latent infection.[14] When primary *T. gondii* infection occurs during pregnancy, it can be vertically transmitted, which may result in intrauterine death, birth defects, or to late sequellae, such as ocular disease and mental retardation.

As intracellular pathogens, *Plasmodium spp.* and *T. gondii* both rely on the invasion of host cells for survival and proliferation. Since both parasites belong to the same apicomplexan phylum, they have common metabolic pathways and thus may be susceptible to same drugs for prevention or treatment.[15–17] However, there are a number of differences between the two pathogens. During the asexual reproduction stage, *P. falciparum* exclusively targets human erythrocytes, whereas *T. gondii* can infect any cell in most vertebrates.[17] And, while malaria is transmitted only *via* the mosquito vector or blood exchange, infection with *T. gondii* occurs *via* ingestion of sporulated oocysts shed into the environment by infected cats, or by consuming bradyzoites in the form of tissue cysts from infected animals. Current therapies for *T. gondii* include drugs which inhibit folate metabolism, protein synthesis, or disrupt electron transport.[18,19] For the inhibition of folate metabolism, a combination of diaminopyrimidine antimalarials, such as trimethoprim **1** or pyrimethamine **2** (Fig. 1), and sulfonamides, such as sulfadiazine **3** (Fig. 1) or sulfamethoxazole, is the recommended therapy. This combination acts synergistically not only against *T. gondii*, but also against various bacterial and other parasitic microorganisms. In patients sensitive to sulfonamides, protein synthesis inhibitors such as macrolide and lincosamide antibiotics are the second line of drugs. Another antimalarial, atovaquone, an inhibitor of mitochondrial electron-transport processes, is the drug of choice as third line therapy.[20] In all these treatments, drug resistance, high cost, limited efficacy, and side effects[21] often result in discontinuation of therapy. Therefore, new agents with better activity and safety profiles and that are less expensive are

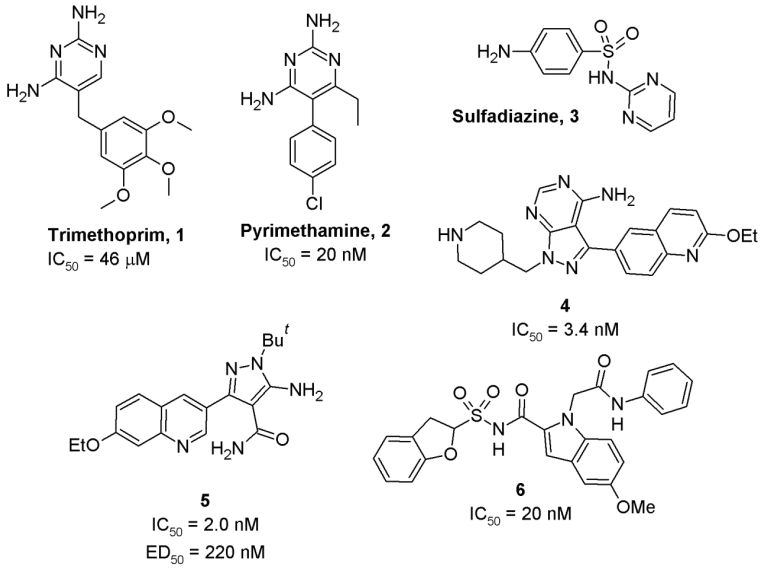


Fig. 1. Various inhibitors of the *T. gondii* parasite.

urgently needed.[19] A variety of *T. gondii* inhibitors designed for different targets have been reported in the last few years, such as inhibitors of Ca-dependent protein kinase-1 (TgCDPK1),[22,23] compounds which cause the disappearance of apicoplasts and plastid-like organelles,[16] phosphodiesterase,[17] purine nucleoside phosphorylase,[18] and biosynthesis of pantothenic acid.[24] Of these, outstanding activities were exhibited by TgCDPK1 inhibitors **4** and **5**,[22,23] and the inhibitor of the vitamin B5 biosynthetic pathway, derivative **6** (Fig. 1).[24]

Outstanding examples of drugs active against both *Plasmodium spp*. and *T. gondii* parasites include artemisinin (**7**, ART, Fig. 2) and its semi-synthetic derivatives. It was established that their main mechanism of action is interfering in calcium homeostasis and triggering of micronema.[25,26] ART and artemether **8** exhibited 100 times better *in vitro* activity against *T. gondii* than trimethoprim **1**, the current front-line drug.[19] Other semi-synthetic artemisinin derivatives, including **9**,[19] artemisone **10** or artemiside **11** have shown even better activities in the nM region (Fig. 2).[27] These derivatives inhibit multiple steps in the *T. gondii* cycle,[19,28] and the absence of activity of deoxyartemisinine confirmed the peroxide group as the pharmacophore.[25] *In vivo* results are rare and incomplete,[23] and only few studies are reliable. Thus, artemisinin showed complete clearance of parasitaemia after a 5-day treatment with 1.3 μg mL^{-1} day^{-1}.[29] An 8-day administration of artemiside at 10 mg kg^{-1} day^{-1} 3 days after sulfadiazine treatment prolonged survival in 80 % of the mice, without toxic side-effects.[24]

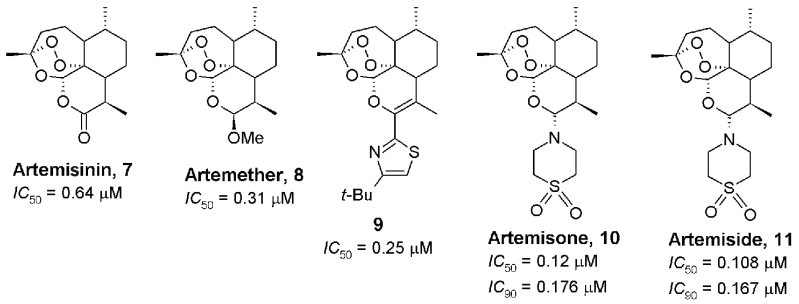


Fig. 2. Structures of artemisinin derivatives.

Artemisinin and its derivatives,[30] artemisone[31] and other synthetic peroxides[32,33] also exert potent *in vitro* and *in vivo* anticancer activities affecting diverse signalling pathways that regulate cell cycle, differentiation, apoptosis, invasion and angiogenesis in cancer cells.[30] Artemisinins can functionally be clearly distinguished from the synthetic peroxides, in spite of the fact that they all share a common peroxide pharmacophore, which suggests that these molecules have multiple modes of action.[33] Furthermore, it was found that synthetic and semi-synthetic peroxides exhibited specific activities against cancer cells. Thus, steroidal mixed tetraoxanes show highly specific activity toward UO31 renal

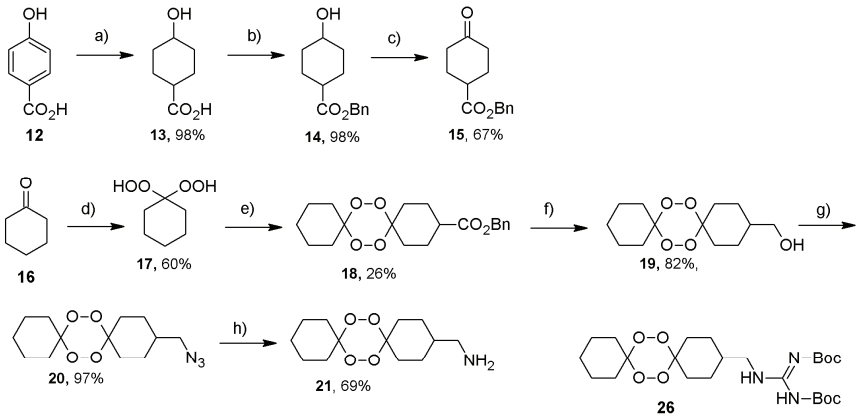
cancer cells,[2b] and artesunate against non-small cell lung cancer and colorectal cancer.[34]

The high antimalarial activities of dicyclohexylidene tetraoxanes that possess polar end groups attached to one of the cyclohexane units[35] encouraged us to explore the influence of modifications to the polar groups on their antimalarial and cytotoxic activities. For this, compound **21** was chosen since it showed the most promising *in vivo* activity against the malaria parasite.[35c]

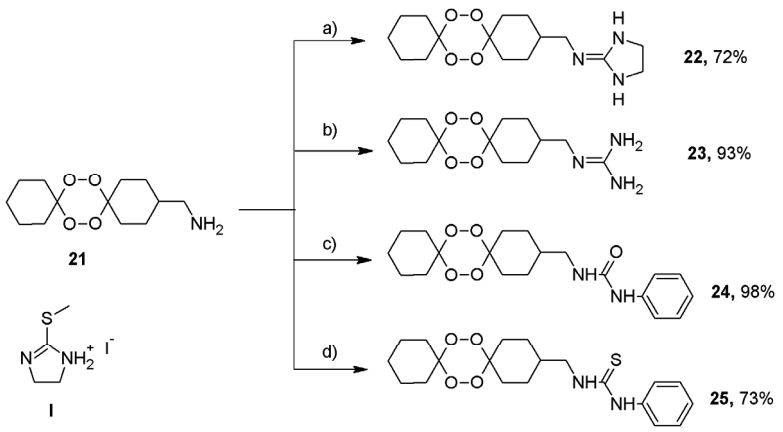
RESULTS AND DISCUSSIONS

Synthesis

Synthesis of targeted derivatives is presented in Schemes 1 and 2. The key intermediary tetraoxane amine **21** was prepared according to a previously described procedure (Scheme 1).[35c] In brief, cyclohexane was transformed into *gem*-dihydroperoxide **17** using 50 % H_2O_2 in the presence of Re_2O_7 as catalyst,[36] which was further coupled with benzyl 4-oxocyclohexanecarboxylate **15**[35a,37] producing tetraoxane benzyl ester **18**. The presence of the benzyl-group enabled a more efficacious purification of the crude product using a Biotage SP chromatography system. Reduction with $LiAlH_4$ produced alcohol **19**, which was transformed *via* the corresponding azide **20** into amine **21**. Final derivatives **22**–**25** (Scheme 2) were obtained from **21** and corresponding coupling reactants, in high to excellent yield (72–98 %). All derivatives were fully characterized and their purity, as determined by HPLC, was ≥95 %, with the exception of compound **23** that was 79–81 % pure. Full details are given in the Experimental and the Supplementary material to this paper.



Scheme 1. Reaction pathway for the synthesis of derivative **21**.



Scheme 2. Reaction pathway for the synthesis of derivatives **22**–**25**.

In the ^{1}H-NMR spectra, all derivatives showed a characteristic signal at around 3.0 ppm for the methylene groups bonded to the polar groups (imidazoline, guanidine, urea or thiourea), and characteristic signals for the introduced polar groups: singlet at 3.38 ppm for the ethylene group in **22** and multiplets in the region 7.50–6.80 ppm for the aromatic protons in **24** and **25**. In the ^{13}C-NMR spectra, all derivatives showed signals for peroxy-acetal carbons in 107–108 ppm region and signals in the 45–50 ppm region for the methylene groups bonded to the polar groups. Additionally, a signal at 160 ppm for sp^2 carbon in polar groups in **22**, **23** and **24**, and a signal at 180 ppm for **25** were present. Furthermore, aromatic carbons from the phenyl-groups in **24** and **25** appeared in the 117–140 ppm region.

Antimalarial activity

Compounds were screened *in vitro* against four *P. falciparum* strains: D6 (CQ susceptible (CQS) strain), W2 (CQ resistant (CQR) strain), TM91C235 (CQ and MFQ resistant) and TM90C2B (atovaquone resistant), following well-established protocols.[38] In brief, the Malaria SYBR Green I based fluorescence (MSF) assay is a microtiter plate drug sensitivity assay that uses the presence of malarial DNA as a measure of parasitic proliferation in the presence of antimalarial drugs or experimental compounds. The intercalation of SYBR Green I dye, and its resulting fluorescence, is relative to parasite growth, and a compound that inhibits the growth of the parasite will result in lower fluorescence intensities.

The antimalarial activities are presented in Table I. Tetraoxane **21**[35c] was also screened here in order to enable a more reliable comparison to be drawn

with the activities of the new derivatives. All derivatives, with the exception of **23**, showed low nanomolar activities against all four strains.

TABLE I. I*n vitro* antimalarial activities against *P. falciparum* strains and cytotoxicity (PBMC) of tetraoxanes **21**–**25**; n.t. – not tested

Compd.	*P. falciparum*, IC_{50} / nM				PBMC, IC_{50} / μM		*RI*[h]	*SI*[i]
	D6[a]	W2[b]	TM91C235[c]	TM90C2B[d]	(–)PHA[e,f]	(+)PHA[g]		
21	13.99	7.26	10.81	7.03	185.61	182.22	0.52/ 0.77	13265/25560/ 17165/26388
22	92.75	72.86	208.54	85.89	166.10	168.70	0.79/ 2.25	1791/2280/ 796/1934
23	243.84	494.37	1489.80	n.t.	>200	>200	2.03/ 6.11	820/400/ 134/–
24	36.50	15.97	29.14	21.40	>200	>200	0.44/ 0.80	5480/12525/ 6863/9343
25	16.30	9.94	28.28	n.t.	32.74	32.01	0.61/ 1.73	2008/3295/ 1158/–
ART[j]	6.66	3.79	16.90	6.23	n.t.	n.t.	0.57/ 2.54	
CQ	7.63	462.99	198.70	181.08	n.t.	n.t.	60.70/ 26.05	
MQ	14.69	6.52	80.28	11.60	n.t.	n.t.	0.44/ 5.47	

[a]*P. falciparum* African D6 strain; [b]*P. falciparum* Indochina W2 strain; [c]*P. falciparum* TM91C235 strain; [d]*P. falciparum* TM90C2B strain; [e]non-stimulated with PHA; [f]PHA = phytohaemagglutinin, [g]stimulated with PHA; [h]resistance index (*RI*) is defined as the ratio of the IC_{50} values for the CQR *versus* CQS strain, W2/D6 and TM91C235/D6, respectively; [i]selectivity index (*SI*) is defined as the ratio of the IC_{50} values for PBMC (–)PHA/D6, PBMC (–)PHA/W2, PBMC (–)PHA/TM91C235 and PBMC (–)PHA/TM90C2B, respectively; [j]average of greater than eight replicates, CQ = chloroquine, MQ = mefloquine

Simultaneously, all derivatives except **23** were more active against the chloroquine-resistant (CQR) W2 strain than against the chloroquine-susceptible (CQS) D6 strain, which could be seen from the favourable *RI* values. The derivatives were somewhat less active against the multi-drug resistant TM91C235 (CQR and MQR strain) in comparison with the activities exhibited toward the W2 strain. In addition, the corresponding *RI* values were less favourable, with the exceptions **21** and **25**. However, three of the five derivatives were 7.4–2.8 times more active than MQ against the TM91C235 strain – **21**, IC_{50} = 10.81 nM, **24**, IC_{50} = 29.14 nM and **25**, IC_{50} = 28.28 *vs*. MQ, IC_{50} = 80.28 nM. The most active derivative was **21** that showed exceptional activities against all the resistant strains. Even against the atovaquone resistant strain TM90C2B, this amine showed activities comparable with those of MQ. Removing the basic character of the terminal amino-group attenuates the anti-malarial activity, as seen in the case of compounds **21**, **24** and **25**. Transformation of the primary amino-group into *N*-phenyl urea or thiourea reduced the antimalarial activity. However, **25** pre-

served high activity against the W2 strain, with an IC_{50} of 9.94 nM, which is comparable to those of MQ and **21** with values of 6.52 nM and 7.26 nM, respectively. On the other hand, increasing basic character of the terminal group did not increase the activity unconditionally. Introduction of more basic groups, such as imidazolidine or guanidine, led to a sharp decrease in activity. This was even more striking with the guanidine derivative **23**, which was 17, 68 and 138 times less active than **21** against D6, W2 and TM91C235 strains, respectively. Such sharp decreases in activity caused by more basic groups is in accordance to the behaviour observed with trioxolanes.[39] Derivative **23** was also less active than **22**, which makes it the least active compound within this series. Derivatives **22** and **23** have same pK_a values (Table S-I of the Supplementary material to this paper), which are two orders of magnitude higher than the pK_a for **21**, and correlations between pK_a or log *P* values (Table S-I) with antimalarial activity could not be established.[40] The derivatives were in general less active than ART against all four strains. Tetraoxane **21** was the only one with activities comparable with those of ART. However, two tetraoxanes **21** and **25** had better *RI* values compared to that of ART against multi-drug resistant strain TM91C235 – **21**, *RI* = 0.77 and **24**, *RI* = 0.80 *vs.* ART, *RI* = 2.54.

The toxicity of the compounds was estimated using human normal peripheral blood mononuclear cells (PBMC), non-stimulated (PBMC (–)PHA) and stimulated (PBMC (+) PHA) with PHA (Table I). In general, the PBMC assay for cytotoxicity estimation revealed that all compounds are well tolerated by normal immunocompetent cells, possessing $IC_{50} \geq 32$ μM. In addition, high selectivity indices (*SI*) were obtained for all compounds toward all *P. falciparum* strains. Derivative **21** had the best SI indices, which were in the range 13000–26000, depending on the strain. The second best SI was exhibited by the urea derivative **24**, which had the lowest cytotoxicity >200 μM and moderate antimalarial activities in the range 16–36 nM.

In vitro *cytotoxic activity*

Cytotoxic activities of the new tetraoxanes and the previous compounds **21** and **20**[35c] were evaluated against four human cancer cell lines: cervix adenocarcinoma HeLa, melanoma Fem-x, breast adenocarcinoma MDA-MB-361 and chronic myelogenous leukaemia K562 cells.

The intensities of the cytotoxic activities are given in Table II and Fig. 3. First, it should be emphasized that the tested tetraoxanes showed the strongest cytotoxic activities against myelogenous leukaemia K562 cells. The highest cytotoxicity against K562 cells was exerted by derivatives **25** with an IC_{50} of 6.15 μM, **24** with 13.23 μM, and **18**, **20** and **22** with an IC_{50} of *ca*. 18.5 μM.

TABLE II. *In vitro*[a] activities (IC_{50} / μM) of tetraoxanes against cancer cell lines (Fem-x, human melanoma cell line; HeLa, human cervical adenocarcinoma cell line; K562, myelogenous leukaemia cell line; MDA-MB-361, human breast adenocarcinoma cell line) and peripheral blood mononuclear cells (PBMC), non-stimulated or stimulated with PHA (phytohaemagglutinin)

Compd.	Fem-x	HeLa	K562	MDA-MB-361	PBMC (–)PHA	PBMC (+)PHA	$SI_{\text{Fem-x}}$[a]	SI_{HeLa}	SI_{K562}	$SI_{\text{MDA-MB-361}}$
21	79.75	63.56	33.68	>100	185.61	182.22	2	3	6	2
18	31.86	27.01	18.84	73.92	>200	>200	6	7	11	3
20	98.82	37.67	18.43	94.7	>200	>200	2	5	11	2
22	43.84	34.56	18.38	80.29	166.10	168.70	4	5	9	2
23	>100	75.66	81.49	90.91	>200	>200	2	3	2	2
24	52.68	39.85	13.23	96.85	>200	>200	4	5	15	2
25	15.67	10.51	6.15	41.13	32.74	32.01	2	3	5	1
cis-Pt	8.10	7.89	5.64	>33.34	>66.67	>66.67	8	8	12	2

[a]The selectivity index (*SI*) is defined as the ratio of the IC_{50} for PBMC (–)PHA / cancer cell line

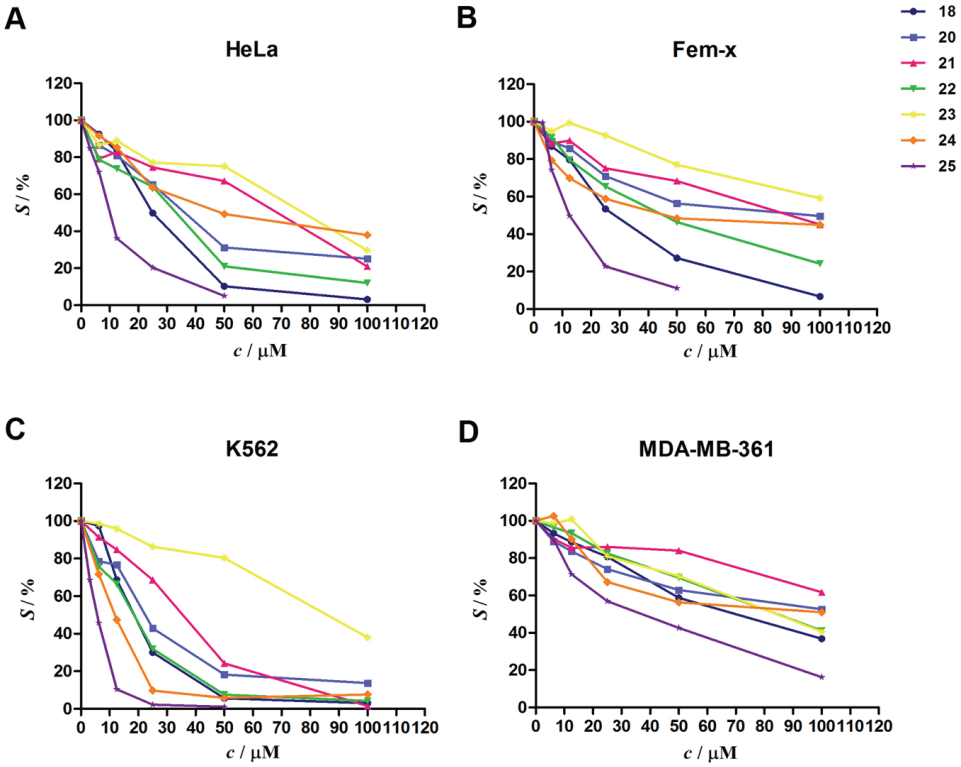


Fig. 3. Survival (*S*%) of: A) cervical adenocarcinoma HeLa, B) melanoma Fem-x, C) myelogenous leukaemia K562 and D) breast adenocarcinoma MDA-MB-361 cells grown for 72h in the presence of increasing concentrations of the investigated tetraoxanes, determined by MTT test.

Against cervix adenocarcinoma HeLa cells, compounds **18**, **20**, **22**, **24** and **25** exerted pronounced cytotoxic actions with IC_{50} values in the range of 10.51 and 39.85 μM. The highest cytotoxic effect was exhibited by derivative **25** with IC_{50} = 10.51 μM. Against melanoma Fem-x cells, compound **25** was the most active with an IC_{50} of 15.67 μM; derivative **18** exerted pronounced cytotoxic effects with IC_{50} value of 31.86 μM. Compounds **22** and **24** exerted moderate cytotoxic action, while the cytotoxic effects of the other derivatives were lower, with derivative **23** being the least active, showing an IC_{50} value higher than 100 μM. All tested tetraoxanes exerted low cytotoxic actions against breast adenocarcinoma MDA-MB-361 cells with IC_{50} values >73.92 μM, except derivative **25** which had moderate activity against this cell line, with an IC_{50} value of 41.13 μM. Tetraoxane **23** with a guanidine group was the least active compound in this series, showing lowest activities towards all cell lines in comparison with other members of the series.

All tetraoxanes exerted selective concentration-dependent cytotoxic activity against specific malignant cell lines, which was especially high against myelogenous leukaemia K562 cells. It is noteworthy that the cytotoxic actions of compounds **21**, **18**, **22**, **24** and **25** were notably higher against K562, HeLa and Fem-x cells than against MDA-MB-361 breast cancer cells, indicating selectivity in their anticancer action against specific malignant cell types.

To further evaluate the anticancer potential of the tested tetraoxanes, their cytotoxic activities were examined against normal healthy peripheral blood mononuclear cells (PBMC), both resting and stimulated to proliferate by mitogen phytohaemagglutinin (PHA), Table II and Fig. 4. Each of the examined tetraoxanes exerted remarkably higher cytotoxic activities against K562, HeLa and Fem-x malignant cell lines than against both non-stimulated and stimulated PBMC, indicating a significant specificity in their anticancer action. The highest

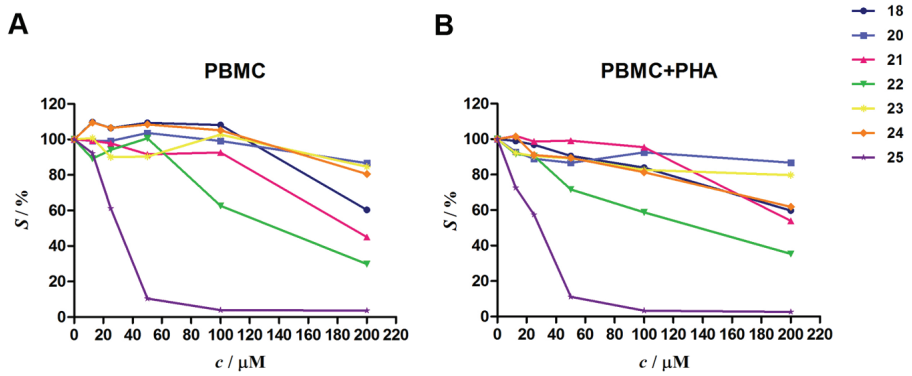


Fig. 4. Survival of (A) resting PBMC and (B) PBMC stimulated to proliferate by PHA grown for 72h in the presence of increasing concentrations of the investigated tetraoxanes, determined by the MTT test.

selectivity in antitumor action was observed against myelogenous leukaemia K562 cells in comparison with normal healthy PBMC with tetraoxanes **18**, **20** and **24**, which showed the highest SI values, 11, 11 and 15 respectively. In addition, the tested tetraoxanes showed good selectivity as follows: **18** and **24** against cervix adenocarcinoma HeLa and melanoma Fem-x cells, **20** against HeLa cells, and **22** against K562, HeLa and Fem-x cells. Low selectivity in the anticancer action of the tested tetraoxanes was observed against breast adenocarcinoma MDA-MB-361 cells, which showed the lowest sensitivity to the cytotoxic action of these compounds.

In contrast to antimalarial activity, it could not be concluded that activities depend on the presence of a specific functional group. Rather, the activity was specific towards particular cell lines. However, in general, the obtained results showed that derivatives **21**–**23** with basic groups have lower activities. Derivatives were less active than cisplatin with the exception of derivative **25**. The thiourea derivative **25** was the most active tetraoxane in this series, with IC_{50} values in the range 41–6.0 μM, which are the same as for cisplatin. However, the low *SI* of **25** for all tested cell lines makes this derivative less interesting, in spite of having good cytotoxic activities. Conversely, derivatives **18**, **20** and **24** had *SI* values for the K562 cell line in the same range as that for cisplatin, which makes them very interesting candidates for further examinations.

The mechanisms of cytotoxic action of **18**, **20** and **24** were examined by cell cycle analysis and morphological assessment of cell death modalities induced by these tetraoxanes using fluorescence microscopy. The tested compounds at IC_{50} concentrations induced time-dependent percentage increases of the target K562 cells in the subG1 phase, as could be seen in Fig. 5. It is noteworthy that compound **20** at the IC_{50} concentration caused the most remarkable increase in the percentage of K562 cells in the subG1 cell cycle phase after 48 h treatment. In addition, treatment with $2IC_{50}$ concentrations of three tetraoxanes resulted in an increase in the percentages of apoptotic subG1 K562 cells after 24 h exposure. After 48h, the percentages of subG1 K562 cells exposed to $2IC_{50}$ concentrations of **18**, **20** and **24** were approximately 12 times higher compared to the control cell sample, pointing to a strong proapoptotic activity of these compounds. Examination of changes in the morphological features confirmed that the tested tetraoxanes triggered apoptosis in K562 leukaemia cells after 24 h treatment. Typical hallmarks of apoptotic K562 cells induced by tetraoxanes, such as shrinkage of the nucleus, chromatin condensation and fragmentation of the nucleus in addition to orange–red stained cells in the final stages of apoptosis are presented in Fig. 6.

Prominent proapoptotic effects of tetraoxanes **18**, **20** and **24** in addition to high selectivity in their anticancer activity against myelogenous leukaemia K562 cells in comparison to normal immunocompetent PBMC, suggest significant anticancer potential of these compounds.

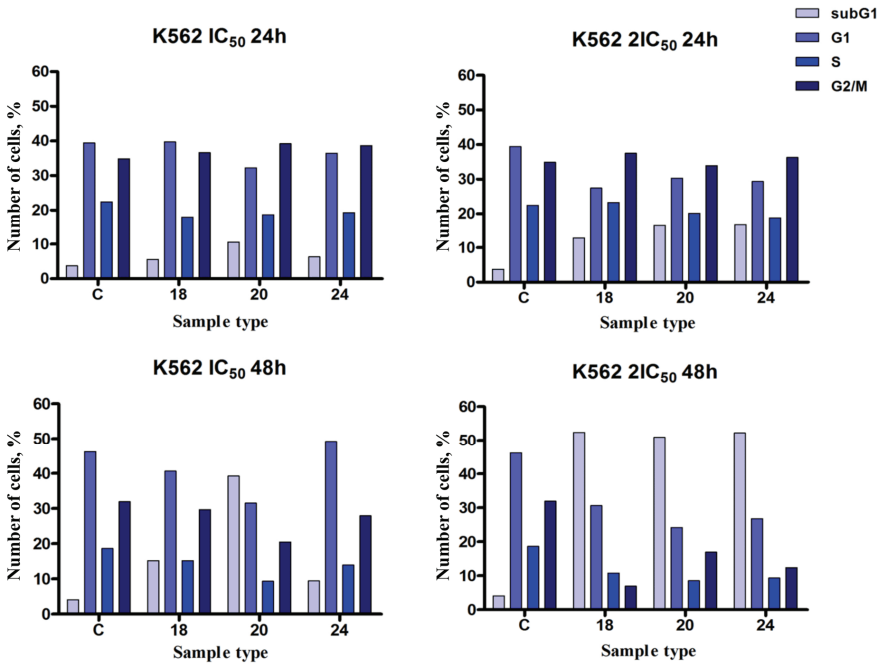


Fig. 5. Changes in the cell cycle phase distribution of K562 cells induced by the investigated tetraoxanes **18**, **20** and **24** (tested concentrations corresponded to IC_{50} and $2IC_{50}$ values). C: control K562 cell sample.

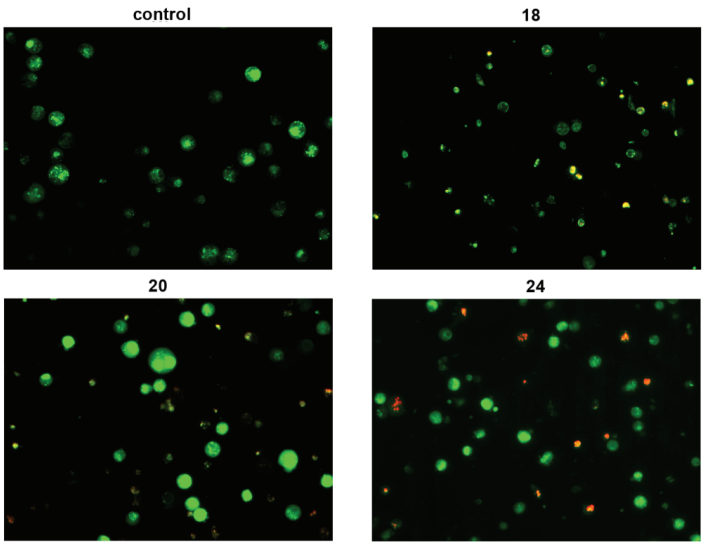


Fig. 6. Photomicrographs of acridine orange/ethidium bromide-stained control K562 cells and K562 cells treated with $2\times IC_{50}$ concentrations of **18**, **20** and **24**, for 24 h.

In vivo *activity against* T. gondii

Derivative **21** was chosen for examination of *in vivo* activity against *T. gondii.* The activity was examined in murine models of infection with tachyzoites of the highly virulent RH strain of *T. gondii* following a well-established protocol.[41] The compound exhibited no visible toxicity as judged by the quality of fur, gait and general activity of the uninfected treated mice. The activity of **21** was compared to that of ART.

The results obtained in the treated animals are presented in Fig. 7. In the infection model where infection was established with 10^2 per mL, all untreated mice died between day 6 and day 9 p.i. (mean 8 days). Most importantly, treatment with both **21** and ART in 10 mg kg^{-1} day^{-1} doses allowed survival of 20 % of infected treated mice after 15 days (Fig. 7a). Compared with the control mice, treatment with both drugs significantly prolonged survival, but the effect of **21** was more pronounced (P = 0.014) than that of ART (P = 0.0413), whereas the effects of the two compounds were mutually comparable (P = 0.9086).

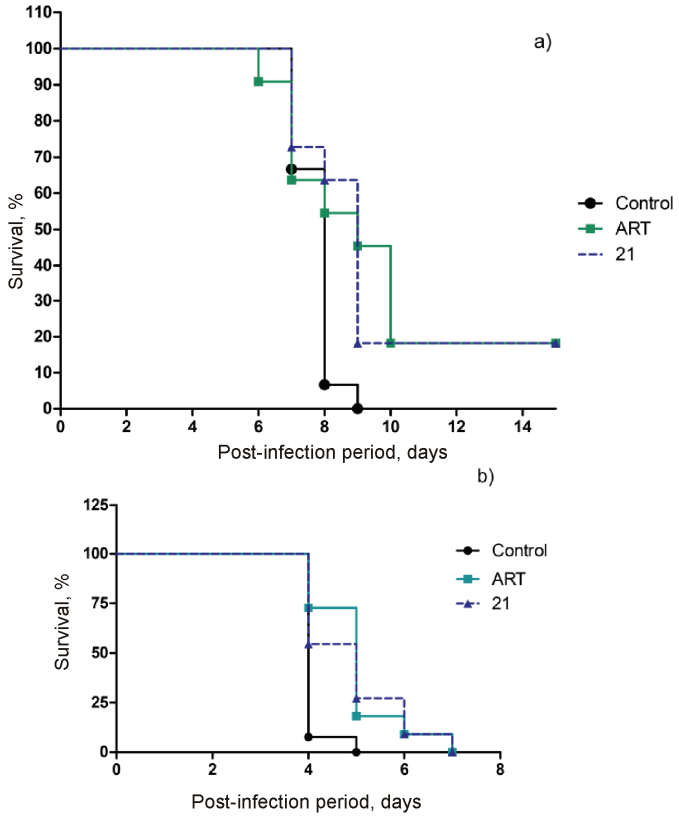


Fig. 7. Survival of infected mice after treatment with **21** or ART; parasite concentration: a) 10^2 and b) 10^6 mL^{-1}.

In mice infected with 10^6 mL^{-1}, all untreated mice died within 5 days. Compared to these, treatment with either **21** or ART significantly prolonged survival ($P = 0.0092$ and $P = 0.0013$, respectively) but did not afford complete protection. The antitoxoplasmatic activity of the two compounds was similar ($P = 0.8358$).

Pathohistological analysis was performed on all surviving infected treated mice (four treated with **21** and two treated with ART), as well as on representative controls (non-infected treated mice), which were all sacrificed day 24 p.i. No pathological changes other than extramedullary haematopoiesis (characteristic of these mice) and mild lymphocyte infiltration of the liver were revealed in both **21** and ART-treated mice, except for the finding of focal myocarditis in one ART-treated infected mouse. These results indicate no significant macroscopic or microscopic toxicity of the tested compounds, and moreover, show the potential of derivative **21** to clear *T. gondii* infection.

EXPERIMENTAL

Chemistry

Melting points were determined on a Boetius PMHK or a Mel-Temp apparatus and were not corrected. Optical rotations were measured on a Rudolph Research analytical automatic polarimeter, Autopol IV in dichloromethane (DCM) or methanol (MeOH) as solvent. The IR spectra were recorded on a Perkin-Elmer FT-IR 1725X spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Gemini-200 spectrometer (at 200 and 50 MHz, respectively), and on a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) employing the indicated solvents (Supplementary material) using TMS as the internal standard. Chemical shifts are expressed in ppm (δ) values and coupling constants (J) in Hz. The ESI-MS spectra were recorded on an Agilent Technologies 6210 Time-Of-Flight LC-MS instrument in the positive ion mode with CH_3CN/H_2O 1/1 with 0.2 % HCOOH as the carrying solvent solution. The samples were dissolved in CH_3CN or MeOH (HPLC grade purity). The selected values were as follows: capillary voltage, 4 kV; gas temperature, 350 °C; drying gas, 12.l min^{-1}; nebulizer pressure, 310 kPa and fragmentator voltage, 70 V. Elemental analyses were realized on the Vario EL III- C,H,N,S/O elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} and RP-18 F_{254} plates. Column chromatography was performed on Lobar LichroPrep Si 60 (40–63 μm), or RP-18 (40–63 μm) columns coupled to a Waters RI 401 detector, and on Biotage SP1 system with a UV detector and FLASH 12+, FLASH 25+ or FLASH 40+ columns charged with KP-SIL (40–63 μm, pore diameter 60 Å), or KP-C18-HS (40–63 μm, pore diameter 90 Å) as adsorbent. Compounds **18** and **20**–**25** were analyzed for purity (HPLC) using an Agilent 1200 HPLC system equipped with Quat pump (G1311B), injector (G1329B) 1260 ALS, TCC 1260 (G1316A) and a 1260 Infinity refractive index detector (RID) was. HPLC analyses were performed in two diverse systems. Method A: Zorbax Eclipse Plus C18, 4.6 mm×150 mm, 1.8 μm, S.N. USWKY01594 was used as the stationary phase. The compounds were eluted using an isocratic protocol and eluent was made from water/MeOH, 30/70 (*V*/*V*). The compounds were dissolved in methanol; the final concentrations were 0.5 mg mL^{-1}. Method B: Poroshell 120 EC-C18, 4.6 mm×50 mm, 2.7 μm, S.N. USCFU07797 was used as the stationary phase. The compounds were

eluted using an isocratic protocol and the eluent was made from water/MeOH, 30/70 (*V*/*V*). The compounds were dissolved in methanol; the final concentrations were 0.5 mg mL^{-1}.

Synthetic procedures

*Benzyl 7,8,15,16-tetraoxadispiro[5.2.5.2]hexadecane-3-carboxylate (**18**)*. Into ice-cold (ice–water mixture) solution of *gem*-dihydroperoxide **17** (412.7 mg, 2.53 mmol) and ketone **15** (374.8 mg, 2.53 mmol) in CH_2Cl_2 (5 mL), under intensive stirring, precooled freshly prepared solution of conc. H_2SO_4/CH_3CN (1/10, *V*/*V*, 1.82 mL) was added dropwise. After one hour stirring at the same temperature, the reaction was quenched with water and the layers were separated. Water layer was extracted with CH_2Cl_2 (3×50 mL). The combined organic layers were washed with sat. $NaHCO_3$ (3×20 mL), brine (2×10 mL) and dried over anh. Na_2SO_4. The solvent was removed under reduce pressure and the product was isolated after column chromatography purification (dry-flash, SiO_2-column, eluent hexane/EtOAc, gradient 97/3 → 9/1). Yield: 241.9 mg (26 %), as an amorphous powder.

N-*(7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadec-3-ylmethyl)-4,5-dihydro-1*H-*imidazol-2--amine* (**22**). A mixture of **21** (100.0 mg, 0.39 mmol), **I** (142.2 mg, 0.58 mmol) and Et_3N (0.5 mL) in methanol (3 mL) was stirred under an inert atmosphere (Ar) for 3 days at 70 °C. The solvent was evaporated, the residue dissolved in CH_2Cl_2, 10 % NaOH solution added and the mixture intensively shaken. The layers were separated, the organic layer was washed once with water, once with brine and dried over anh. Na_2SO_4. The solvent was removed under reduce pressure and the product isolated after column chromatography purification (Lobar, SiO_2-column A, eluent CH_2Cl_2/CH_3OH sat. NH_3 = 1/1). Yield: 91 mg (72 %).

*1-(7,8,15,16-Tetraoxadispiro[5.2.5.2]hexadec-3-ylmethyl)guanidine (**23**)*. A mixture of **21** (100.0 mg, 0.39 mmol), *S*-methylthiourea sulphate (162.0 mg, 0.58 mmol) and Et_3N (0.5 mL) in methanol (3 mL) was stirred at 70 °C under an inert atmosphere (Ar) for 3 days. Into the warm solution, methanol was added (10 mL) and the warm mixture was filtered, the solvent removed under reduce pressure and product isolated after column chromatography purification (dry-flash, SiO_2-column, gradient CH_2Cl_2/MeOH, = 9/1 → 1/1) as a pale yellow oil that became solid with time. Yield: 108.6 mg (93 %).

*1-Phenyl-3-(7,8,15,16-tetraoxadispiro[5.2.5.2]hexadec-3-ylmethyl)urea (**24**)*. A mixture of **21** (150.0 mg, 0.58 mmol) and phenyl-isocyanate (63.4 μL, 0.58 mL) in CH_2Cl_2 (10 mL), was stirred at r.t. under an inert atmosphere (Ar) for 45 min. The solvent was evaporated and product isolated after column chromatography purification (dry-flash, SiO_2-column, gradient hexane/EtOAc = 1/1 → EtOAc → EtOAc/MeOH = 7/3). Yield: 215 mg (98 %).

*1-Phenyl-3-(7,8,15,16-tetraoxadispiro[5.2.5.2]hexadec-3-ylmethyl)thiourea (**25**)*. A mixture of **21** (78.2 mg, 0.30 mmol) and phenyl-isothiocyanate (35.57 μL, 0.30 mL) in CH_2Cl_2 (15 mL) was stirred under an inert atmosphere at r.t. for 2 h. The solvent was removed under reduce pressure and the product isolated after column chromatography purification (dry-flash, SiO_2-column, gradient hexane/EtOAc = 9/1 → EtOAc). Yield: 87.3 mg (73 %).

In vitro *antimalarial activity*

The i*n vitro* antimalarial drug susceptibility screen is a modification of the procedures first published by Desjardins *et al*.[42] with modifications developed by Milhous *et al*., the details of which are given in ref. 8b. All the synthesized aminoquinolines were screened *in vitro* against the *P. falciparum* strains CQ and MFQ, as well as the susceptible strain D6 (clone of the Sierra I/UNC isolate), the CQ resistant but MFQ susceptible strain W2 (clone of the Indochina I isolate), and the CQ and MFQ resistant strain TM91C235 (clone of the South–East Asian isolate), and TM90C2B (clone of the Thailand isolate).

Cytotoxic activity

Reagents. Stock solutions of investigated tetraoxanes were prepared in dimethyl sulphoxide (DMSO) at concentrations of 10 mM and subsequently diluted with complete nutrient medium (RPMI-1640 without phenol red) supplemented with 3 mmol L^{-1} L-glutamine, 100 μg mL^{-1} streptomycin, 100 IU mL^{-1} penicillin, 10 % heat inactivated foetal bovine serum (FBS), and 25 mM HEPES, and adjusted to pH 7.2 with bicarbonate solution. RPMI-1640, FBS, HEPES, and L-glutamine were purchased from Sigma–Aldrich, St. Louis, MO, USA.

Cell cultures. Human cervical adenocarcinoma HeLa, human melanoma Fem-x and human breast adenocarcinoma MDA-MB-361 cells were cultured as a monolayer, while human chronic myelogenous leukaemia K562 cells were grown in a suspension in the complete nutrient medium, at 37 °C in a humidified air atmosphere with 5 % CO_2, as previously described.[43]

Preparation of peripheral blood mononuclear cells (PBMC). PBMC were separated from whole heparinised blood (obtained from two healthy volunteers) by Histopaque®-1077 (Sigma–Aldrich) density gradient centrifugation. Interface cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{2+}, 145 mM Cl^- and 35 g L^{-1} gelatine polymers, pH 7.4), were counted and suspended in nutrient medium with 10 % FBS.[43]

Treatment of human malignant cell lines. HeLa (2,000 cells per well), Fem-x (5,000 cells per well) and MDA-MB-361 cells (10,000 cells per well) were seeded into 96-well microtiter plates. Twenty hours later, after cell adherence, five different concentrations of the investigated compounds in complete nutrient medium were added to the cells (range 6.25–100 μM or 3.125–50 μM), except for the control cells to which nutrient medium only was added. K562 cells (5,000 cells per well) were seeded two hours before addition of the investigated compounds to obtain final concentrations within the above-mentioned range. All experiments were realised in triplicate. Cisplatin was used as a positive control.

Treatment of PBMC from healthy donors. PBMC cells were seeded at a density of 150,000 cells per well in nutrient medium only, or in the nutrient medium enriched with 5 μg mL^{-1} of phytohaemagglutinin (PHA – INEP, Belgrade, Serbia) in 96-well microtiter plates. Two hours later, five different concentrations of the investigated tetraoxanes were added to the wells with non-stimulated and PHA-stimulated PBMC (in order to obtain five final concentrations within the range of 12.5–200 μM). All experiments were realised in triplicate. Cisplatin was used as a positive control.

Determination of cell survival. Cell survival 72 h after addition of the drug was determined by the MTT test according to the method of Mosmann[44] as modified by Ohno and Abe.[45] Briefly, 10 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg mL^{-1} in phosphate buffered saline) was added to each well.[43] The samples were incubated for a further four hours at 37 °C in a humidified atmosphere with 5 % CO_2. Then, 100 μL of 10 % SDS solution was added to the wells. The absorbance was measured at 570 nm the next day. To obtain cell survival (S / %), the absorbance at 570 nm of a sample with cells grown in the presence of various concentrations of agent was divided by the absorbance of the control sample (the absorbance of cells grown only in nutrient medium), implying that the absorbance of the blank was always subtracted from the absorbance of a corresponding sample with target cells. The IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50 %, compared to the corresponding control.

Cell cycle analysis. K562 cells were exposed to two different concentrations of the examined compounds **18**, **20** and **24** (corresponding to the IC_{50} and $2IC_{50}$ values determined

after 72 h treatment) for 24 and 48 h. After incubation, the target cells were collected, washed and fixed in 70 % ethanol on ice. The cell samples were stored at −20 °C for at least one week before staining. The cells were collected by centrifugation, washed, resuspended in PBS containing RNase A at a final concentration of 200 μg mL^{-1} and incubated for 30 min at 37 °C. Subsequently, the propidium iodide solution was added to the cells at a final concentration of 40 μg mL^{-1}.[43]

The phase distribution of the cell cycle was determined using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data (10,000 events collected for each sample) were analysed using CELLQuest software (BD Biosciences).

Morphological evaluation of K562 cell death. To examine the mode of death of human chronic myelogenous leukaemia K562 cells induced by the investigated tetraoxanes, morphological analysis by microscopic examination of acridine orange/ethidium bromide-stained target cells was performed, as already described.[43] The K562 cells were seeded in 6-well plates (200,000 cells per well) in complete nutrient medium. After 2 h, the cells were treated with the investigated compounds for 24 h at concentrations corresponding to double the IC_{50} values obtained after 72 h treatments. After this period, the target cells were collected by centrifugation and stained with 20 μL of a mixture of acridine orange and ethidium bromide (3 μg mL^{-1} AO and 10 μg mL^{-1} EB in PBS) dyes, and visualized under a fluorescence microscope – Carl Zeiss PALM MicroBeam with Axio Observer.Z1 using AxioCam MRm (filters Alexa 488 and Alexa 568).

Anti-toxoplasmatic activity

Mice. Female Swiss Webster mice (Medical Military Academy Animal Research Facility, Belgrade, Serbia) weighing 18 to 20 g at the beginning of each experiment were used. The mice were housed six to a cage and offered drinking water *ad libitum*.

T. gondii. Tachyzoites of the virulent RH strain maintained through serial intraperitoneal (i.p.) passages were used. For the experimental infections, tachyzoites were harvested from mouse peritoneal fluids 72 h post infection and purified by centrifugation, cotton wool filtration, and needle extraction. The parasites were counted in a haemocytometer, and their numbers were adjusted to 2×10^6 mL^{-1} with saline. The suspensions were also serially 10-fold diluted, and 0.5-mL aliquots of 2×10^2 and 2×10^6 mL^{-1} dilutions were inoculated i.p. into fresh mice.

Infected non-treated mice served as infection controls. Mice were randomly assigned into experimental groups and treated with **21** or ART. Both drugs were administered at a dose of 0.2 mg per mouse per day (10 mg kg^{-1} day^{-1}), subcutaneously (s.c.) for 8 consecutive days starting from the day of infection (day 0). Survival of the mice was monitored for another week after the end of treatment, meaning a total follow-up period of 15 days post infection (p.i.). To control for drug side effects (toxicity), separate groups of non-infected animals were given **21** (10 mg kg^{-1} day^{-1}) and ART (10 mg kg^{-1} day^{-1}) in the same manner and duration as in the experimental groups.

Statistical analysis

The rates of survival in particular treatment groups were estimated by the Kaplan–Meier product limit method and compared by the log-rank test. The level of statistical significance was 0.05.

CONCLUSIONS

Herein, the synthesis and biological activity of new cyclohexylidene mixed 1,2,4,5-tetraoxanes containing polar guanidine and urea based groups were reported. Four new tetraoxanes were tested *in vitro* against *P. falciparum* CQR and CQS strains. The derivatives showed moderate nanomolar antimalarial activities, and differences in activities were clearly influenced by changes in the structures of the introduced polar groups. The *N*-phenylurea derivative **24** showed the best resistance indices (RI_{W2} = = 0.44, $RI_{TM91C235}$ = 0.80), the highest *SI* score against all four tested *P. falciparum* strains and the lowest toxicity against PBMC (IC_{50} > 200 μM). Seven tetraoxanes were tested *in vitro* against four human cancer cell lines and five of them showed pronounced cytotoxic effects against myelogenous leukaemia K562 cells in 6.15–18.84 μM concentrations with high SI_{K562} index. The amino-tetraoxane **21** was the first one evaluated for its anti *T. gondii* effect *in vivo*, in a murine model of acute toxoplasmosis. An 8-day treatment at a dose of 10 mg kg^{-1} day^{-1} allowed survival of 20 % of the infected mice. The obtained results clearly showed the activity of the investigated tetraoxanes against acute murine toxoplasmosis, suggesting the potential of synthetic organic peroxides in *T. gondii* treatment.

SUPPLEMENTARY MATERIAL

Calculated pK_a and log *P* values for derivatives **21**–**23**, synthetic procedures for derivatives **13**–**17** and **19**–21 and analytical data for derivatives **13**–**25** and HPLC chromatograms for determination of the purity of tested compounds are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

ТЕТРАОКСАНИ КАО ИНХИБИТОРИ АПИКОМПЛЕКСНИХ ПАРАЗИТА *Plasmodium falciparum* И *Toxoplasma gondii* И АНТИ-КАНЦЕРСКИ МОЛЕКУЛИ

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Синтетисана је група нових циклохексилиденских 1,2,4,5-тетраоксана који имају гванидино и уреидо поларне групе и испитана је њихова антималаријска активност према хлорокивин-резистентним и осетљивим сојевима *Plasmodium falciparum*. Деривати

показују умерене активности у nM опсегу и ниску цитотоксичност. Дериват *N*-фенил--урее **24** има најбољи индекс резистенције (RI_{W2} = 0,44; $RI_{TM91C235}$ = 0,80) и није токсичан према хуманим нормалним мононуклеарним ћелијама периферне крви (IC_{50} > 200 μM). Седам деривата је тестирано *in vitro* према четири хумане малигне ћелијске линије и показало је високу селективност према K562 ћелијама леукемије. Дериват **21** који има примарну амино групу је први тетраоксан тестиран *in vivo* према још једном паразиту из филума Apicomplexa, *Toxoplasma gondii*. Приликом супкутаног администрирања, дневна доза од 10 mg kg^{-1} омогућила је преживљавање 20 % инфицираних мишева, што показује висок потенцијал тетраоксана за терапију инфекција изазваних апикомплексним паразитима.

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REFERENCES

1. S. I. Hay, C. A. Guerra, A. J. Tatem, A. M. Noor, R. W. Snow, *Lancet*, **4** (2004) 327
2. a) D. M. Opsenica, B. A. Šolaja, *J. Serb. Chem. Soc.* **74** (2009) 1155, and references cited therein; b) I. Opsenica, N. Terzić, D. Opsenica, G. Angelovski, M. Lehnig, P. Eilbracht, B. Tinant, Z. Juranić, K. S. Smith, Z. S. Yang, D. S. Diaz, P. L. Smith, W. K. Milhous, D. Đoković, B. A. Šolaja, *J. Med. Chem.* **49** (2006) 3790
3. P. M. O'Neill, V. E. Barton, S. A. Ward, J. Chadwick, *4-Aminoquinoline: Chloroquine, Amodiaquine and Next-Generation Analogues*, in *Treatment and Prevention of Malaria: Antimalarial Drug Chemistry, Action and Use*, H. M. Staines, S. Krishna, Eds., Springer, Basel, 2012, p. 19
4. A. Ecker, A. M. Lehane, D. A. Fidock, *Molecular Markers of Plasmodium Resistance to Antimalarials*, in *Treatment and Prevention of Malaria: Antimalarial Drug Chemistry, Action and Use*, H. M. Staines, S. Krishna, Eds., Springer, Basel, 2012, p. 249
5. P. D. Roepe, *Biochemistry* **50** (2011) 163
6. a) D. M. Opsenica, B. A. Šolaja, *Second-Generation Peroxides: The OZs and Artemisone*, in *Treatment and Prevention of Malaria: Antimalarial Drug Chemistry, Action and Use*, H. M. Staines, S. Krishna, Eds., Springer, Basel, 2012, p. 211; b) R. D. Slack, A. M. Jacobine, G. H. Posner, *Med. Chem. Commun.* **3** (2012) 281; c) N. Kumar, R. Singh, D. S. Rawat, *Med. Res. Rev.* **32** (2012) 581; d) J. L. Vennerstrom, S. Arbe-Barnes, R. Brun, S. A. Charman, F. C. K. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. T. Santo, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin, W. N. Charman, *Nature* **430** (2004) 900
7. a) R. K. Haynes, W.-C. Chan, H.-N. Wong, K.-Y. Li, W.-K. Wu, K.-M. Fan, H. H. Y. Sung, I. D. Williams, D. Prosperi, S. Melato, P. Coghi, D. Monti, *ChemMedChem* **5** (2010) 1282; b) R. K. Haynes, K.-W. Cheu, M. M.-K. Tang, M.-J. Chen, Z.-F. Guo, Z.-H. Guo, P. Coghi, D. Monti, *ChemMedChem* **6** (2011) 279
8. a) M. Videnović, D. M. Opsenica, J. C. Burnett, L. Gomba, J. E. Nuss, Ž. Selaković, J. Konstantinović, M. Krstić, S. Šegan, M. Zlatović, R. J. Sciotti, S. Bavari, B. A. Šolaja, *J. Med. Chem.* **57** (2014) 4134; b) I. M. Opsenica, M. Tot, L. Gomba, J. E. Nuss, R. J. Sciotti, S. Bavari, J. C. Burnett, B. A. Šolaja, *J. Med. Chem.* **56** (2013) 5860; c) I. M. Opsenica, K. K. Smith, L. Gerena, S. Gaica, B. A. Šolaja, *J. Serb. Chem. Soc.* **73** (2008) 1021
9. M. Tot, D. M. Opsenica, M. Mitrić, J. C. Burnett, L. Gomba, S. Bavari, B. A. Šolaja, *J. Serb. Chem. Soc.* **78** (2013) 1847, and references cited therein
10. a) K. Kaur, M. Jain, R. P. Reddy, R. Jain, *Eur. J. Med. Chem.* **45** (2010) 3245; b) E. Milner, W. McCalmont, J. Bhonsle, D. Caridha, J. Cobar, S. Gardner, L. Gerena, D.

Goodine, C. Lanteri, V. Melendez, N. Roncal, J. Sousa, P. Wipf, G. S. Dow, *Malaria J.* **9** (2010), doi:10.1186/1475-2875-**9**-51; c) C. A. Lanteri, J. D. Johnson, N. C. Waters, *Recent Pat. Antiinfect. Drug Discov.* **2** (2007) 95
11. J. G. Montoya, O. Liesenfeld, *Lancet* **363** (2004) 1965
12. a) F. Berger, V. Goulet, Y. Le Strat, J. C. Desenclos, *Bull. Epidemiol. Hebd. (Paris)* **14** (2008) 117; b) B. Bobić, A. Nikolić, I. Klun, O. Djurković-Djaković, *Wien Klin. Wochenschr.* **123** (2011) Suppl. 1 and 2
13. a) E. A. Figueiró-Filho, F. R. Senefonte, A. H. Lopes, O. O. de Morais, V. G. Souza Júnior, T. L. Maia, G. Duarte, *Rev. Soc. Bras. Med. Trop.* **40** (2007) 181; b) B. Carme, F. Bissuel, D. Ajzenberg, R. Bouyne, C. Aznar, M. Demar, S. Bichat, D. Louvel, A. M. Bourbigot, C. Peneau, P. Neron, M. L. Darde, *J. Clin. Microbiol.* **40** (2002) 4037
14. O. Djurković-Djaković, *Srp. Arh. Celok Lek.* **126** (1998) 197
15. E. Moine, C. Denevault-Sabourin, F. Debierre-Grockiego, L. Silpa, O. Gorgette, J.-C. Barale, P. Jacquiet, F. Brossier, A. Gueiffier, I. Dimier-Poisson, C. Enguehard-Gueiffier, *Eur. J. Med. Chem*. **89** (2015) 386e400, doi 0.1016/j.ejmech.2014.10.057
16. D. Kadri, A. K. Crater, H. Lee, V. R. Solomon, S. Ananvoranich, *Exp. Parasitol.* **145** (2014) 135
17. B. L. Howard, K. L. Harvey, R. Stewart, M. F. Azevedo, B. S. Crabb, I. G. Jennings, P. R. Sanders, D. T. Manallack, P. E. Thompson, C. J. Tonkin, P. R. Gilson, *ACS Chem. Biol.* **10** (2015) 1145
18. K. Dzitko, A. Paneth, T. Plech, J. Pawełczyk, L. Węglińska, P. Paneth, *Antimicrob. Agents Chemother.* **58** (2014) 7583
19. C. P. Hencken, L. Jones-Brando, C. Bordon, R. Stohler, B. T. Mott, R. Yolken, G. H. Posner, L. E. Woodard, *J. Med. Chem.* **53** (2010) 3594
20. A. E. Vercesi, C. O. Rodrigues, S. A. Uyemura, L. Zhong, S. N. J. Moreno, *J. Biol. Chem.* **273** (1998) 31040
21. a) J. McAuley, K. M. Boyer, D. Patel, M. Mets, C. Swisher, N. Roizen, C. Wolters, L. Stein, M. Stein, W. Schey, J. Remington, P. Meier, D. Johnson, P. Heydeman, E. Holfels, S. Withers, D. Mack, C. Brown, D. Patton, R. McLeod, *Clin. Infect. Dis.* **18** (1994) 38; b) A. B. Foot, Y. J. Garin, P. Ribaud, A. Devergie, F. Derouin, E. Gluckman, *Bone Marrow Transpl.* **14** (1994) 241
22. S. M. Johnson, R. C. Murphy, J. A. Geiger, A. E. DeRocher, Z. Zhang, K. K. Ojo, E. T. Larson, B. G. K. Perera, E. J. Dale, P. He, M. C. Reid, A. M. W. Fox, N. R. Mueller, E. A. Merritt, E. Fan, M. Parsons, W. C. Van Voorhis, D. J. Maly, *J. Med. Chem.* **55** (2012) 2416
23. Z. Zhang, K. K. Ojo, R. S. R. Vidadala, W. Huang, J. A. Geiger, S. Scheele, R. Choi, M. C. Reid, K. R. Keyloun, K. Rivas, L. K. Siddaramaiah, K. M. Comess, K. P. Robinson, P. J. Merta, L. Kifle, W. G. J. Hol, M. Parsons, E. A. Merritt, D. J. Maly, C. L. M. J. Verlinde, W. C. Van Voorhis, E. Fan, *ACS Med. Chem. Lett*. **5** (2014) 40
24. S. N. Mageed, F. Cunningham, A. W. Hung, H. L. Silvestre, S. Wen, T. L. Blundell, C. Abell, G. A. McConkey, *Antimicrob. Agents Chemother.* **58** (2014) 6345
25. K. Nagamune, S. N. J. Moreno, L. D. Sibley, *Antimicrob. Agents Chemother.* **51** (2007) 3816
26. K. Nagamune, W. L. Beatty, L. D. Sibley, *Eukaryot. Cell* **6** (2007) 2147
27. I. R. Dunay, W. C. Chan, R. K. Haynes, L. D. Sibley, *Antimicrob. Agents Chemother.* **51** (2007) 2147
28. J. G. D'Angelo, C. Bordón, G. H. Posner, R. Yolken, L. Jones-Brando, *J. Antimicrob. Chemother.* **63** (2009) 146

29. K. Ou-Yang, E. C. Krug, J. J. Marr, R. L. Berens, *Antimicrob. Agents Chemother.* **34** (1990) 1961
30. a) G. L. Firestone, S. N. Sundar, *Expert. Rev. Mol. Med.* **11** (2009) e32; b) M. P. Crespo-Ortiz, M. Q. Wei, *J. Biomed. Biotechnol.* **2012** (2012) 247597, and references cited therein
31. A. M. Gravett, W. M. Liu, S. Krishna, W.-C. Chan, R. K. Haynes, N. L. Wilson, A. G. Dalgleish, *Cancer Chemother. Pharmacol.* **67** (2011) 569
32. I. N. Cvijetić, Ž. P. Žižak, T. P. Stanojković, Z. D. Juranić, N. Terzić, I. M. Opsenica, D. M. Opsenica, I. O. Juranić, B. J. Drakulić, *Eur. J. Med. Chem.* **45** (2010) 4570
33. R. H. van Huijsduijnen, R. K. Guy, K. Chibale, R. K. Haynes, I. Peitz, G. Kelter, M. A. Phillips, J. L. Vennerstrom, Y. Yuthavong, T. N. C. Wells, *PLoS One* **8** (2013) e82962, doi:10.1371/journal.pone.0082962
34. Z. Y. Zhang, S. Q. Yu, L. Y. Miao, X. Y. Huang, X. P. Zhang, Y. P. Zhu, X. H. Xia, D. Q. Li, *Chin. J. Integr. Med.* **6** (2008) 134; b) S. Krishna, S. Ganapathi, I. C. Ster, M. E. M. Saeed, M. Cowan, C. Finlayson, H. Kovacsevics, H. Jansen, P. G. Kremsner, T. Efferth, D. A. Kumar, *EBioMedicine* **2** (2015) 82
35. a) I. Opsenica, N. Terzić, D. Opsenica, W. K. Milhous, B. Šolaja, *J. Serb. Chem. Soc.* **69** (2004) 919; b) I. Opsenica, D. Opsenica, M. Jadranin, K. Smith, W. K. Milhous, M. Stratakis, B. Šolaja, *J. Serb. Chem. Soc.* **72** (2007) 1181; c) I. Opsenica, D. Opsenica, K. S. Smith, W. K. Milhous, B. A. Šolaja, *J. Med. Chem.* **51** (2008) 2261
36. P. Ghorai, P. H. Dussault, *Org. Lett.* **10** (2008) 4577
37. a) S. D. Kuduk, R. K. Chang, R. M. Di Pardo, C. N. Di Marco, K. L. Murphy, R. W. Ransom, D. R. Reiss, C. Tang, T. Prueksaritanont, D. J. Pettibone, M. G. Bock, *Bioorg. Med. Chem. Lett.* **18** (2008) 5107; b) A. Bahadoor, A. C. Castro, L. K. Chan, F. G. Keaney, M. Nevalainen, V. Nevalainen, S. Peluso, D. A. Snyder, T. T. Tibbitts, WO 2011/140190 Al
38. W. K. Milhous, N. F. Weatherly, J. H. Bowdre, R. E. Desjardins, *Antimicrob. Agents Chemother.* **27** (1985) 525
39. Y. Tang, Y. Dong, S. Wittlin, S. A. Charman, J. Chollet, F. C. K. Chiu, W. N. Charman, H. Matile, H. Urwyler, A. Dorn, S. Bajpai, X. Wang, M. Padmanilayam, J. M. Karle, R. Brun, J. L. Vennerstrom, *Bioorg. Med. Chem. Lett.* **17** (2007) 1260
40. For pK_a calculations, Epik, version 2.9, Schrödinger, LLC, New York, NY, 2014 and for log P calculations, QikProp, version 4.1, Schrödinger, LLC, New York, NY, 2014 were used.
41. O. Djurković-Djaković, T. Nikolić, F. Robert-Gangneux, B. Bobić, A. Nikolić, *Antimicrob. Agents Chemother.* **43** (1999) 2240
42. R. E. Desjardins, C. J. Canfield, D. E. Haynes, J. D. Chulay, *Antimicrob. Agents Chemother.* **16** (1979) 710
43. N. M. Krstić, I. Z. Matić, Z. D. Juranić, I. T. Novaković, D. M. Sladić, *J. Steroid. Biochem. Mol. Biol.* **143** (2014) 365
44. T. Mosmann, *J. Immunol. Methods* **65** (1983) 55
45. M. Ohno, T Abe, *J. Immunol. Methods* **145** (1991) 199.