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In vitro antiplasmodial activities of semisynthetic *N*,*N*′-spacer-linked oligomeric ergolines

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Abstract—Starting from three monomeric ergolines (terguride **1**, festuclavine **2**, pergolide **3**) *N*,*N*′-spacer-linked oligomeric derivatives were prepared using different aliphatic or arylalkyl spacers. The compounds have been evaluated for their in vitro antiplasmodial activity against the chloroquine-sensitive strain poW and the chloroquine-resistant clone Dd2 of *Plasmodium falciparum*. Additionally, the cytotoxic effects against mouse fibroblasts (NIH 3T3) in vitro, and human hepatocytes were evaluated. All monomers displayed only a weak antiplasmodial effect, but *N*-1,*N*-1′-spacer-linked dimerization substantially enhanced their antiplasmodial activity. The best activities were observed for compounds showing a distance of six carbon atoms between two monomers, which can be obtained by aliphatic or *p*-xylene linkers. The *N*-6,*N*-6′-spacer-linked depropylpergolide dimer **3i** exhibited the highest antiplasmodial activity of all compounds tested (IC_{50} values: 0.14 and 0.13 μM against poW and Dd2, respectively). Unfortunately, it displayed toxic effects against the mouse fibroblast cell line NIH 3T3 (IC_{50}: 0.1 ± 0.09 μM) and also against human hepatocytes at 100 μM (LDH-leakage: 15.58 ± 0.87 μkat/L; GSH-level: 8.15 ± 0.78 nmol/10^6 cells). However, the *N*-1,*N*-1′-spacer-linked trimer of festuclavine (**2f**), and also the *N*-1,*N*-1′-spacer-linked tetramer of terguride (**1g**) possessed remarkable antiplasmodial activities (IC_{50}: 0.54 and 1.53 μM, respectively, against Dd2) lacking cytotoxicity.

1. Introduction

Malaria still is the most dangerous parasitic disease, afflicting 40% of the world's population with an annual incidence of 300–500 million.[1] It is caused by protozoan parasites of the genus *Plasmodium*, of which *P. falciparum* is the most important one, producing the highest mortality. As standard antimalarials, for example chloroquine, are loosing efficacy due to spreading resistance of *P. falciparum* the search for new drugs is increasingly important.

Among compounds from natural sources ergolines are of paramount importance as ligands for serotonin (5-hydroxytryptamine, 5-HT) receptors, dopamine receptors, and adrenoceptors. The tetracyclic structure of the ergolines contains the essential features of the monoamine neurotransmitters 5-HT, dopamine, and noradrenaline. Thus, it is not surprising that many naturally occurring and semisynthetic ergolines have been shown to act as agonists, partial agonists or antagonists at receptors for these neurotransmitters. A number of ergolines has emerged as real agents for the treatment of vascular and neurological diseases and other disorders,[2] for example terguride (**1**), pergolide (**3**).

Certain natural ergolines, the clavine-type alkaloids agroclavine, festuclavine (**2**), and their semisynthetic derivatives revealed cytostatic activities. However, this effect did not involve the interaction with neurotransmitter receptors but seemed to be caused by a fundamentally new mechanism of action.[3]

First results with such monomeric ergolines introduced in our screening program for in vitro antiplasmodial activity[4] prompted us to investigate semisynthetic *N*,*N*-spacer-linked dimeric or oligomeric compounds including the two clinically used drugs **1** and **3** as well as the

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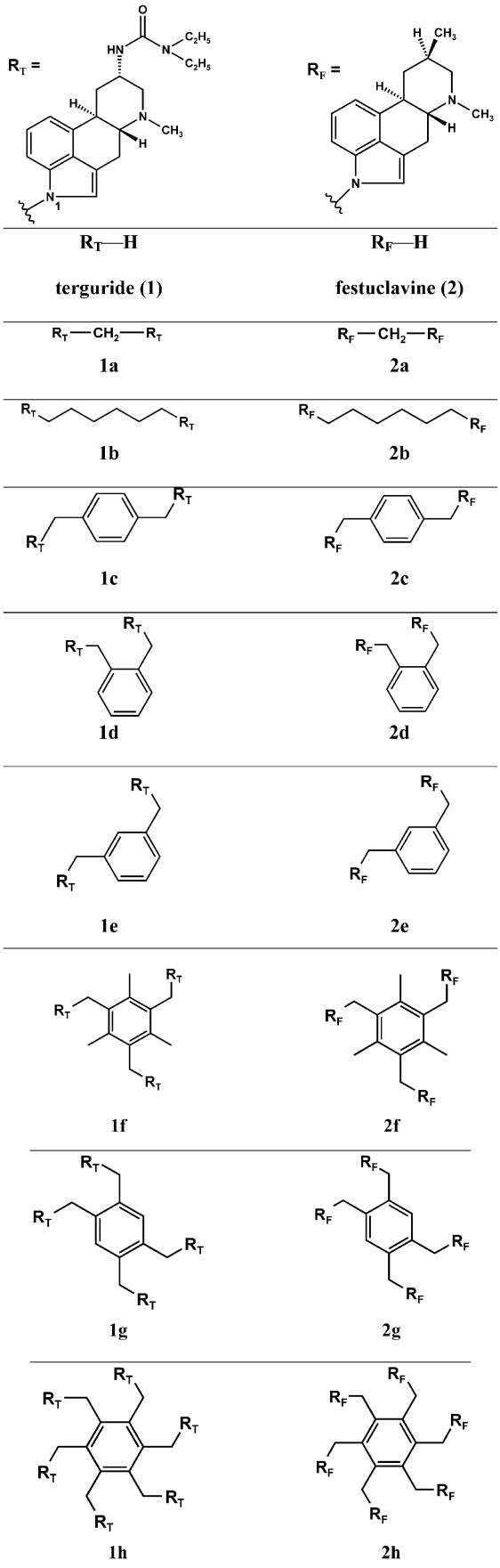


Figure 1. Chemical structures of *N*-1,*N*-1′-spacer-linked ergoline oligomers with terguride and festuclavine as monomers.

clavine **2** as monomers. They were linked by different spacers at different positions (*N*-1 and/or *N*-6) to yield dimeric up to hexameric compounds. Since toxic effects of the terguride derivatives **1a** and **1b** on lymphoid tumor cell lines in vitro were described previously,[5] we additionally evaluated the in vitro cytotoxic effects on primary cultured mouse fibroblasts (NIH 3T3) and human hepatocytes as well as the in vivo toxicity of one especially promising compound (**1g**) using Balb/c mice.

2. Results

The in vitro antiplasmodial activities of the monomers terguride **1**, festuclavine **2**, pergolide **3**, and their spacer-linked oligomeric derivatives (Figs 1 and 2) are given in Table 1 in comparison to chloroquine as reference drug. These monomers displayed only a weak antiplasmodial effect. The *N*-6,*N*-6′-linked pergolide dimer **3i** exhibited the highest activities with IC_{50} values of 0.14 and 0.13 μM against poW and Dd2, respectively. The *N*-1,*N*-1′-linked dimers **1b**, **1c**, and **2b**, **2c** as well as the trimer **2f** also showed a good in vitro effect in our test systems.

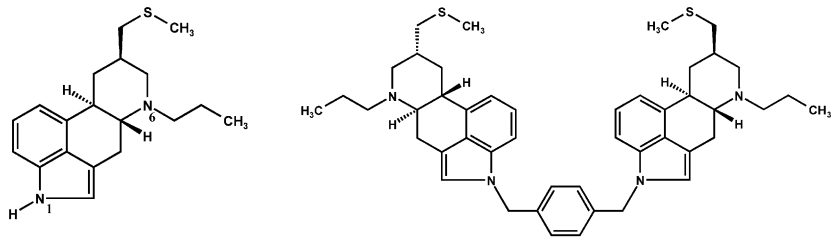
Permanent mouse fibroblasts (NIH 3T3 cell line) were used for the determination of the cytotoxicity against normal proliferative cells. Growth of fibroblasts assessed by the MTT assay was strongly inhibited by the series **1b**, **2b**–**2e**, and **3i**, and moderately inhibited by terguride (**1**), **1c**, and **1d**. However, festuclavine (**2**), pergolide (**3**), and the derivatives **1a**, **1f**–**1h**, **2f**, **2h**, and **3k** revealed no cytotoxic effect in this system (Table 1).

Primary cultures of human hepatocytes were used to assess toxic effects of parent compounds or their possible metabolites. After 24 h of exposition, the tested compounds were non-toxic in concentrations up to 10 μM in all parameters measured. At concentrations of 50 and 100 μM, a cytotoxic effect was displayed only by the compounds **2b** and **3i**. Addition of these compounds to human hepatocytes resulted in dose-dependent cell death as monitored by LDH leakage (i.e., increased plasma membrane permeability) and GSH depletion (Table 2) accompanied by destruction of the monolayer, with interruption of cellular contacts, a change in shape from polygonal to circular, and detachment of the cells.

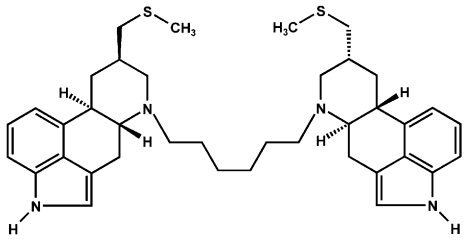
Acute treatment of Balb/c mice with a single dose of **1b**, **1c**, **1g**, and **1h** caused only marginal, non significant changes in liver and/or kidney parenchyma. After chronic treatment, all derivatives tested induced behavioral changes (anxiety, increased locomotor activity) that were most pronounced for compound **1h**.

3. Discussion

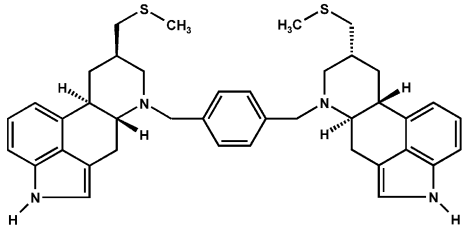
Our results show that a spacer-linked oligomerization of different ergoline monomers is able to enhance their antiplasmodial activity (Table 1). Regarding the *N*-1, *N*-1′-spacer-linked ergoline dimers (Fig. 1), a short aliphatic linker seems to be less favorable (**1a**, **2a**). The best activities are observed for compounds having a



3 3c



3i



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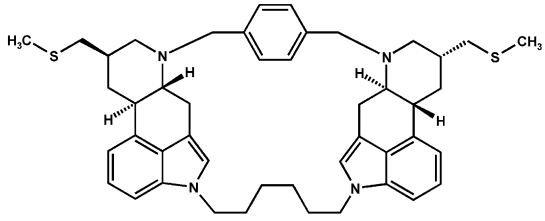

3k

Figure 2. Chemical structures of the pergolide dimer **3c** and the depropylpergolide dimers **3i**–**3k**.

distance of six carbon atoms between the monomeric moieties, which can be obtained as well by aliphatic as by *p*-xylene linkers. The *o*- or *m*-xylene linkers leading to a shorter distance between the ergoline moieties produce compounds that exhibit slightly lower activities (**1d**, **1e**, **2d**, **2e**). However,the trimers **1f**, **2f** and the tetramers **1g**, **2g** with 1,3,5- and 1,2,4,5-linked aromatic core, respectively, are still remarkably active, whereas the attachment of further monomers is not of advantage. The hexamers **1h**, **2h** might be too bulky to reach the antiplasmodial target. Additionally, the activities of three *N*-6,*N*-6′-linked ergoline dimers (Fig. 2) containing depropylpergolide as monomer were evaluated. In this series, **3i** seemed to be most promising, because it showed IC_{50} values of 0.14 and 0.13 μM, respectively, against the strain PoW and the clone Dd2 of *P. falciparum*. However, compound **3i**, displayed the highest toxicity against the NIH 3T3 cell line (Table 1) and proved to be even toxic to metabolic active hepatocytes although at a higher concentration (Table 2). An effect on the redox status of the cells (depletion of GSH) seems to play an important role in the toxicity induced by these compounds. This high toxicity is not very surprising since it had already been observed in previous studies of our group that a non-spacer-linked ergoline dimer, 2-(2,3-dihydrofestuclavin-2-yl)festuclavine, had shown similar toxicities in the L5178y mouse lymphoma cell system, thus surmounting all clavine type ergoline monomers.[3,6] Comparison of the IC_{50} values in the antiplasmodial and cytotoxic assays of certain other tested *N*,*N*′-spacer-linked ergolines (**1f**–**1h**, **2f**, **2h**) nevertheless revealed no correlation between both activities. From these findings, it can be concluded that different mechanisms of action are responsible for these

Table 1. In vitro antiplasmodial activities against *Plasmodium falciparum* and in vitro cytotoxic effect on mouse fibroblasts NIH 3T3 of spacer-linked oligomeric ergolines in comparison with their monomers; *N*-1,*N*-1′-linked: **1a**–**1h**, **2a**–**2h**, **3c**; *N*-6,*N*-6′-linked: **3i**, **3j**; both linkages: **3k**

Compd		Antiplasmodial activities IC_{50} (μM ± SD)		Cytotoxic effect IC_{50} (μM ± SD)
		PoW	Dd2	NIH 3T3
Terguride (monomer)	**1**	25.55 ± 12.84	30.45 ± 24.75	50.1 ± 1.8
Dimers, CH_2-linked	**1a**	3.90 ± 1.13	3.52 ± 1.19	98.2 ± 2.3
$(CH_2)_6$-linked	**1b**	0.43 ± 0.92	0.45 ± 0.11	2.2 ± 0.8
p-Xylene-linked	**1c**	0.51 ± 0.07	0.54 ± 0.08	25.3 ± 1.8
o-Xylene-linked	**1d**	1.98 ± 0.33	0.73 ± 0.28	45.3 ± 2.7
m-Xylene-linked	**1e**	5.72 ± 3.26	4.16 ± 2.82	15.7 ± 0.9
Trimer[a]	**1f**	1.18 ± 1.00	3.29 ± 1.87	> 100
Tetramer[b]	**1g**	0.84 ± 0.60	1.53 ± 1.37	> 100
Hexamer[c]	**1h**	14.90 ± 3.71	7.91 ± 2.52	> 100
Festuclavine (monomer)	**2**	8.80 ± 6.98	14.90 ± 9.74	> 100
Dimers, CH_2-linked	**2a**	1.89 ± 0.97	0.95 ± 0.23	1.5 ± 0.06
$(CH_2)_6$-linked	**2b**	0.67 ± 0.30	0.49 ± 0.23	0.5 ± 0.1
p-Xylene-linked	**2c**	0.57 ± 0.47	0.46 ± 0.11	0.2 ± 0.1
o-Xylene-linked	**2d**	1.59 ± 1.26	1.31 ± 1.35	1.2 ± 0.08
m-Xylene-linked	**2e**	1.10 ± 0.78	0.84 ± 0.51	1.8 ± 0.09
Trimer[a]	**2f**	0.75 ± 0.86	0.54 ± 0.42	> 100
Tetramer[b]	**2g**	1.64 ± 1.19	0.72 ± 0.12	n.d.[d]
Hexamer[c]	**2h**	2.65 ± 0.88	2.19 ± 0.55	> 100
Pergolide (monomer)	**3**	14.15 ± 5.61	9.11 ± 3.22	> 100
Dimer, *p*-xylene-linked	**3c**	1.57 ± 0.29	1.69 ± 0.74	2.5 ± 0.12
Depropylpergolide dimers, $(CH_2)_6$-linked	**3i**	0.14 ± 0.02	0.13 ± 0.03	0.1 ± 0.09
p-Xylene-linked	**3j**	1.50 ± 0.13	1.21 ± 0.21	2.7 ± 0.17
Twice linked	**3k**	7.09 ± 1.96	9.75 ± 2.13	> 100
Chloroquine × $2H_3PO_4$		0.011 ± 0.002	0.073 ± 0.012	67.2 ± 2.3

[a] 2,4,6-Trimethyl-mesitylene-linked.
[b] 3-Methyl-pseudocumene-linked.
[c] 4,5,6-Trimethyl-hemellitene-linked.
[d] n.d., not determined.

Table 2. Effect of compounds **2b** and **3i** on human hepatocytes after 24 h incubation

Compd	Concentration	LDH leakage	GSH depletion
	(μM)	(μkat)	(nmol/10^6cells)
2b	50	10.40 ± 1.15[a]	10.24 ± 1.12[a]
	100	17.52 ± 1.48[a]	2.82 ± 0.25[a]
3i	50	6.94 ± 0.92[a]	27.92 ± 1.58
	100	15.58 ± 0.87[a]	8.15 ± 0.78[a]
Control (DMSO)		2.53 ± 0.11	32.83 ± 2.94
Triton (1%)[b]		23.16 ± 1.5	2.74 ± 0.82

The data are given as mean ± SD, $n = 5$. All other compounds used in this study displayed no toxic effects at 100 μM.
[a] Values significantly different from the control ($p < 0.05$).
[b] Values for maximal damage of the hepatocytes.

activities. Thus, compounds like the trimer **2f** or the tetramer **1g** might be really promising as leads for antiplasmodial drugs since they show remarkable activities against *P. falciparum* and no cytotoxicity. The promising profile of **1g** could be supported also in vivo since this compound proved to be non-toxic to Balb/c mice given as one single dose and caused behavioral changes after chronic treatment only at the highest dose but no damages of liver, kidneys, and spleen.

4. Experimental

4.1. General

Identity and purity of all compounds were deduced from NMR data. NMR spectra were measured on a Varian INOVA-400 spectrometer (399.91 MHz for ^1H, 100.57 MHz for ^{13}C) in $CDCl_3$ at 30 °C. Residual solvent signal (δ_H 7.265, δ_C 77.0) served as an internal reference. Asterisk (*) in proton spectra denotes HMQC readouts. Positive ion MALDI MS spectra were measured on a Bruker BIFLEX reflectron time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany) equipped with a multiprobe sample inlet, a gridles delayed extraction ion source and a nitrogen laser (337 nm). A saturated solution of α-cyano-4-hydroxycinnamic acid in aqueous 50% acetonitrile/0.1% TFA was used as a MALDI matrix. Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of α-cyano-4-hydroxycinnamic acid and a peptide standard (angiotensin II, Aldrich). TLC was performed on silica gel F_{254} plates (Merck), spots were visualized by UV light and by charring with 10% H_2SO_4 in ethanol. Compounds **1a**–**1h**, **3c**, and **3i**–**k** have been prepared as described previously.[5,7,8]

4.1.1. General procedure for the synthesis of spacer-linked ergolines 2a–h. Finely powdered KOH (370 mg, 6.6 mmol) was stirred with DMSO (2 mL) for 10 min. Festuclavine (**2**) (120 mg, 0.5 mmol) was added and the stirring was continued for another 30 min. After cooling to 10 °C halogenated spacer dissolved in 2 mL of DMSO was slowly added. The reaction was typically completed within 3 h as indicated by TLC ($CHCl_3$/MeOH 94.5:5.5) and was terminated by pouring in water (40 mL). The precipitated product was filtered off, washed with water until neutral reaction and after drying

was purified by flash chromatography (10% MeOH in $CHCl_3$ + 0.01% aq ammonia).

4.1.2. Di[6′-methyl-8β′-(methyl)ergolin-1′-yl-]methane (2a). Compound **2a** was synthesized according to the typical procedure with dichloromethane (0.7 mmol, 0.05 mL) as spacer. The reaction was terminated after 8 h. The yield of the product after chromatography was 54%. ^{1}H NMR ($CDCl_3$, 30 °C): 1.004 (d, J = 6.6 Hz, 2×8-CH_3, 6H), 1.086 (ddd, J = 12.0, 12.1, 13.0 Hz, 2×H-9a, 2H), 1.951 (t, J = 11.41 Hz, 2×H-7a, 2H), 2.106 (br s, 2×H-8, 2H), 2.178 (m, 2×H-5, 2H), 2.484 (s, 2×CH_3–N, 6H), 2.636 (dddd, J = 1.8, 3.8, 3.8, 13.0 Hz, 2×H-9b, 2H), 2.740 (br t, 2×H-4b, 2H), 3.013 (br d, 2×H-7b, 2H), 3.040 (m, 2×H-10, 2H), 3.332 (dd, J = 4.4, 14.9 Hz, 2×H-4b, 2H), 6.239 (s, CH_2, 2H), 6.862 (d, J = 1.5 Hz, 2×H-2, 2H), 6.945 (ddd, J = 0.8, 1.3, 7.0 Hz, 2×H-12, 2H), 7.210 (dd, J = 7.0, 8.2 Hz, 2×H-13, 2H), 7.267 (ddd, J = 0.8, 1.0, 8.2 Hz, 2×H-14, 2H); ^{13}C NMR ($CDCl_3$, 30 °C): 19.45 (2×8-CH_3), 26.30 (2×C-4), 29.99 (2×C-8), 36.13 (2×C-9), 40.11 (2×C-10), 42.64 (2×CH_3–N), 56.43 (CH_2), 64.84 (2×C-7), 66.88 (2×C-5), 106.97 (2×C-14), 112.32 (2×C-3), 113.89 (2×C-12), 120.77 (2×C-2), 123.61 (2×C-13), 126.96 (2×C-16), 133.45 (2×C-15), 133.65 (2×C-11); MS MALDI TOF $[M+H]^+$ (found 493.35, required 493.33 for $C_{33}H_{41}N_4$).

4.1.3. 1,6-Di[6′-methyl-8β′-(methyl)ergolin-1′-yl-]hexane (2b). Compound **2b** was synthesized according to the typical procedure with 1,6-dibromo-hexan (Aldrich) (0.25 mmol, 0.04 mL) as spacer. The reaction was terminated after 1.5 h. The yield of the product after chromatography was 62%. ^{1}H NMR ($CDCl_3$, 30 °C): 1.016 (d, J = 6.5 Hz, 2×8-CH_3, 6H), 1.113 (ddd, J = 11.9, 12.0, 13.0 Hz, 2×H-9a, 2H), 1.331 (m, 2×CH_2-3′, 4H), 1.791 (m, 2×CH_2-2′, 4H), 1.927 (dd, J = 11.1, 11.4 Hz, 2×H-7a, 2H), 2.071 (m, 2×H-8, 2H), 2.166 (ddd, J = 4.3, 9.7, 11.0 Hz, 2×H-5, 2H), 2.491 (s, 2×CH_3–N, 6H), 2.654 (dddd, J = 2.0, 3.8, 3.8, 13.0 Hz, 2×H-9b, 2H), 2.736 (ddd, J = 1.5, 11.0, 14.7 Hz, 2×H-4a, 2H), 2.994 (ddd, J = 1.9, 3.7, 11.1 Hz, 2×H-7b, 2H), 3.009 (m, 2×H-10, 2H), 3.386 (dd, J = 4.3, 14.7 Hz, 2×H-4b, 2H), 4.015 (dd, J = 6.9, 7.0 Hz, 2×CH_2-1′, 4H), 6.717 (d, J = 1.5 Hz, 2×H-2, 2H), 6.905 (ddd, J = 0.8, 1.4, 7.0 Hz, 2×H-12, 2H), 7.069 (ddd, J = 0.8, 0.8, 8.2 Hz, 2×H-14, 2H), 7.157 (dd, J = 7.0, 8.2 Hz, 2×H-13, 2H); ^{13}C NMR ($CDCl_3$, 30 °C): 19.52 (2×8-CH_3), 26.58, 26.61 (2×C-4, 2×CH_2-3′), 30.16 (2×CH_2-2′), 30.42 (2×C-8), 36.30 (2×C-9), 40.37 (2×C-10), 42.75 (2×CH_3-N), 46.31 (2×CH_2-1′), 65.07 (2×C-7), 67.16 (2×C-5), 106.96 (2×C-14), 110.31 (2×C-3), 112.63 (2×C-12), 121.41 (2×C-2), 122.60 (2×C-13), 126.55 (2×C-16), 133.30 (2×C-11), 133.80 (2×C-15); MS MALDI TOF $[M+H]^+$ (found 563.45, required 563.41 for $C_{38}H_{51}N_4$).

4.1.4. 1,4-Di[6′-methyl-8β′-(methyl)ergolin-1′-yl-methyl-]-benzene (2c). Compound **2c** was synthesized according to the typical procedure with *p*-bis(bromomethyl)benzene[5] (0.25 mmol, 66 mg) as spacer. The reaction was terminated after 1.5 h. The yield of the product was 61%. ^{1}H NMR ($CDCl_3$, 30 °C): 1.015 (d, J = 1.5 Hz, 2×8-CH_3, 6H), 1.108 (ddd, J = 12.0, 12.1, 12.9 Hz, 2×H-9a, 2H), 1.911 (dd, J = 11.2, 11.3 Hz, 2×H-7a, 2H), 2.057 (m, 2×H-8, 2H), 2.151 (ddd, J = 4.3, 9.7, 11.2 Hz, 2×H-5, 2H), 2.469 (s, 2×CH_3–N, 6H), 2.647 (m, 2×H-9b, 2H), 2.713 (ddd, J = 1.5, 11.2, 14.7 Hz, 2×H-4a, 2H), 2.982 (ddd, J = 1.5, 3.7, 11.2 Hz, 2×H-7b, 2H), 3.009 (m, 2×H-10, 2H), 3.374 (dd, J = 4.3, 14.7 Hz, 2×H-4b, 2H), 5.197 (s, 2×CH_2, 4H), 6.742 (d, J = 1.5 Hz, 2×H-2, 2H), 6.917 (ddd, J = 0.7, 0.9, 7.0 Hz, 2×H-12, 2H), 7.034 (ddd, J = 0.8, 0.8, 8.2 Hz, 2×H-14, 2H), 7.058 (br s, H-2′/6′ and H-3′/5′, 4H), 7.135 (dd, J = 7.0, 8.2 Hz, 2×H-13, 2H); ^{13}C NMR ($CDCl_3$, 30 °C): 19.63(2×8-CH_3), 26.84 (2×C-4), 30.51 (2×C-8), 36.42 (2×C-9), 40.71 (2×C-10), 43.06 (2×CH_3-N), 49.79 (2×CH_2), 65.37 (2×C-7), 67.04 (2×C-5), 107.01 (2×C-14), 111.52 (2×C-3), 112.92 (2×C-12), 121.61 (2×C-2), 122.83 (2×C-13), 126.78 (2×C-16), 127.28 (4×C-2′), 133.81 (2×C-11), 134.05 (2×C-15), 137.36 (2×C-1′); MS MALDI TOF $[M+H]^+$ (found 583.41, required 583.37 for $C_{40}H_{47}N_4$).

4.1.5. 1,2-Di[6′-methyl-8β′-(methyl)ergolin-1′-yl-methyl-]-benzene (2d). Compound **2d** was synthesized according to the typical procedure with *o*-bis(bromomethyl)-benzene[5] (0.25 mmol, 66 mg) as spacer. The reaction was terminated after 1.5 h. The yield of the product was 64%. ^{1}H NMR ($CDCl_3$, 30 °C): 1.029 (d, J = 6.6 Hz, 2×8-CH_3, 6H), 1.142 (ddd, J = 12.0, 12.0, 13.0 Hz, 2×H-9a, 2H), 2.010 (dd, J = 11.3, 11.4 Hz, 2×H-7a, 2H), 2.159 (m, 2×H-8, 2H), 2.287 (m, 2×H-5, 2H), 2.548 (s, 2×CH_3-N, 6H), 2.676 (dddd, J = 1.9, 3.7, 3.7, 13.0 Hz, 2×H-9b, 2H), 2.839 (m, 2×H-4a, 2H), 3.060 (ddd, J = 1.9, 3.6, 11.4 Hz, 2×H-7b, 2H), 3.120 (m, 2×H-10, 2H), 3.404 (dd, J = 4.3, 14.8 Hz, 2×H-4b, 2H), 5.186 (s, 2×CH_2, 4H), 6.749 (d, J = 1.5 Hz, 2×H-2, 2H), 6.926 (ddd, J = 0.7, 1.3, 7.0 Hz, 2×H-12, 2H), 7.002 (m, 2×H-*ortho*, H-*meta*, 3H), 7.016 (ddd, J = 0.7, 0.9, 8.2 Hz, 2×H-14, 2H), 7.138 (dd, J = 7.0, 8.2 Hz, 2×H-13, 2H), 7.203 (m, H-*meta*, 1H); ^{13}C NMR ($CDCl_3$, 30 °C): 19.52 (2×8-CH_3), 26.52 (2×C-4), 30.13 (2×C-8), 36.24 (2×C-9), 40.30 (2×C-10), 42.74 (2×CH_3-N), 50.08 (2×CH_2), 64.99 (2×C-7), 67.10 (2×C-5), 107.24 (2×C-14), 111.03 (2×C-3), 113.03 (2×C-12), 121.77 (2×C-2), 122.92 (2×C-13), 125.59 (C-*meta*), 126.23 (2×C-*ortho*), 126.70 (2×C-16), 129.20 (C-*meta*), 133.27 (2×C-11), 134.09 (2×C-15), 138.49 (2×C-*ipso*). Three signals of spacer =C–H (intensity ratio 1:2:1) are due to local asymmetry caused by two *ortho*-disposed bulky groups; MS MALDI TOF $[M+H]^+$ (found 583.43, required 583.37 for $C_{40}H_{47}N_4$).

4.1.6. 1,3-Di[6′-methyl-8β′-(methyl)ergolin-1′-yl-methyl-]-benzene (2e). Compound **2e** was synthesized according to the typical procedure with *m*-bis(bromomethyl)benzene[5] (0.25 mmol, 66 mg) as spacer. The reaction was terminated after 1.5 h. The yield of the product was 69%. ^{1}H NMR ($CDCl_3$, 30 °C): 1.027 (d, J = 6.6 Hz, 2×8-CH_3, 6H), 1.130 (ddd, J = 12.0, 12.0, 12.9 Hz, 2×H-9a, 2H), 1.939 (dd, J = 11.2, 11.3 Hz, 2×H-7a, 2H), 2.085 (m, 2×H-8, 2H), 2.187 (ddd, J = 4.4, 9.8, 11.1 Hz, 2×H-5, 2H), 2.498 (s, 2×CH_3–N, 6H), 2.666 (m, 2×H-9b, 2H), 2.751 (ddd, J = 1.5, 11.1, 14.7 Hz, 2×H-4a, 2H), 3.005 (ddd, J = 2.0, 3.8, 11.2 Hz, 2×H-7b, 2H), 3.032 (m,

2×H-10, 2H), 3.397 (dd, J = 4.4, 14.7 Hz, 2×H-4b, 2H), 5.185 (s, 2×CH_2, 4H), 6.746 (d, J = 1.5 Hz, 2×H-2, 2H), 6.936 (ddd, J = 0.7, 1.3, 7.1 Hz, 2×H-12, 2H), 7.01* (m, H-2′, H-4′, 2H), 7.018 (ddd, J = 0.7, 0.9, 8.2 Hz, 2×H-14, 2H), 7.03* (br s, H-6′), 7.145 (dd, J = 7.1, 8.2 Hz, 2×H-13, 2H), 7.204 (m, H-3′, 1H); ^{13}C NMR ($CDCl_3$, 30 °C): 19.62 (2×8-CH_3), 26.80 (2×C-4), 30.45 (2×C-8), 36.40 (2×C-9), 40.64 (2×C-10), 43.01 (2×CH_3–N), 50.07 (2×CH_2), 65.31 (2×C-7), 67.08 (2×C-5), 107.12 (2×C-14), 111.44 (2×C-3), 112.97 (2×C-12), 121.67 (2×C-2), 122.88 (2×C-13), 125.64 (C-6′), 126.23 (C-2′, C-4′), 126.79 (2×C-16), 129.18 (C-3′), 133.70 (2×C-11), 134.09 (2×C-15), 138.52 (C-1′, C-5′); MS MALDI TOF $[M+H]^+$ (found 583.31, required 583.37 for $C_{40}H_{47}N_4$).

4.1.7. 1,3,5-Tri[6′-methyl-8β′-(methyl)ergolin-1′-yl-methyl]-2,4,6-trimethylbenzene (2f). Compound **2f** was synthesized according to the typical procedure with 1,3,5-trimethyl-2,4,6-tris(bromomethyl)benzene[5] (0.167 mmol, 66.5 mg) as spacer. The reaction was terminated after 3 h. The yield of the product was 48%. ^{1}H NMR ($CDCl_3$, 30 °C): 1.017 (d, J = 6.6 Hz, 3×8-CH_3, 9H), 1.123 (ddd, J = 12.1, 12.1, 12.8 Hz, 3×H-9a, 3H), 1.960 (dd, J = 11.2, 11.4 Hz, 3×H-7a, 3H), 2.099 (m, 3×H-8, 3H), 2.213 (m, 3×H-5, 3H), 2.305 (s, 3×CH_3, 9H), 2.494 (s, 3×CH_3–N, 9H), 2.663 (m, 3×H-9b, 3H), 2.732 (m, 3×H-4a, 3H), 3.018 (m, 3×H-7b, 3H), 3.048 (m, 3×H-10, 3H), 3.333 (m, 3×H-4b, 3H), 5.290 (d, J = 14.0 Hz, 3×CH_2a, 3H), 5.331 (d, J = 14.0 Hz, 3×CH_2b, 3H), 6.414 (br s, 3×H-2, 3H), 6.947 (ddd, J = 1.2, 1.3, 6.7 Hz, 3×H-12, 3H), 7.153 (ddd, J = 0.9, 1.0, 8.2 Hz, 3×H-14, 3H), 7.195 (dd, J = 6.7, 8.2 Hz, 3×H-13, 3H); ^{13}C NMR ($CDCl_3$, 30 °C): 16.35 (3×CH_3), 19.56 (3×8-CH_3), 26.71 (3×C-4), 30.28 (3×C-8), 36.33 (3×C-9), 40.47 (3×C-10), 42.86 (3×CH_3-N), 45.03 (3×CH_2), 65.13 (3×C-7), 67.10 (3×C-5), 106.88 (3×C-14), 110.89 (3×C-3), 113.17 (3×C-12), 119.57 (3×C-2), 122.87 (3×C-13), 126.87 (3×C-16), 131.96 (3×C-1′ or 3×C-2′), 133.57 (3×C-11), 134.08 (3×C-15), 139.07 (3×C-1′ or 3×C-2′); MS MALDI TOF $[M+H]^+$ (found 877.50, required 877.59 for $C_{60}H_{73}N_6$).

4.1.8. 1,2,4,5-Tetra[6′-methyl-8β′-(methyl)ergolin-1′-yl-methyl-]benzene (2g). Compound **2g** was synthesized according to the typical procedure with 1,2,4,5-tetrakis(bromomethyl)benzene (Aldrich) (0.125 mmol, 56 mg) as spacer. The reaction was terminated after 2 h. The yield of the product was 45%. ^{1}H NMR ($CDCl_3$, 30 °C): 1.013 (d, J = 6.6 Hz, 4×8-CH_3, 12H), 1.100 (ddd, J = 12.1, 12.1, 12.7 Hz, 4×H-9a, 4H), 1.938 (t, J = 11.3 Hz, 4×H-7a, 4H), 2.090 (m, 4×H-8, 4H), 2.159 (ddd, J = 4.3, 9.8, 11.2 Hz, 4×H-5, 4H), 2.498 (s, 4×CH_3–N, 12H), 2.639 (m, 4×H-9b, 4H), 2.711 (ddd, J = 1.4, 11.2, 14.8 Hz, 4×H-4a, 4H), 3.003 (m, 4×H-7b, 4H), 3.01 (m, 4×H-10, 4H), 3.334 (dd, J = 4.3, 14.8 Hz, 4×H-4b, 4H), 5.031 (s, 4×CH_2, 8H), 6.487 (d, J = 1.4 Hz, 4×H-2, 4H), 6.741 (s, 2×H-2′, 2H), 6.758 (d, J = 8.2 Hz, 4×H-14, 4H), 6.909 (dd, J = 1.0, 7.2 Hz, 4×H-12, 4H), 7.070 (dd, J = 7.2, 8.2 Hz, 4×H-13, 4H); ^{13}C NMR ($CDCl_3$, 30 °C): 19.52 (4×8-CH_3), 26.58 (4×C-4), 30.22 (4×C-8), 36.23 (4×C-9), 40.37 (4×C-10), 42.84 (4×CH_3-N), 47.48 (4×CH_2), 65.03 (4×C-7), 67.02 (4×C-5), 107.06 (4×C-14), 111.48 (4×C-3), 113.32 (4×C-12), 121.30 (4×C-2), 123.08 (4×C-13), 126.66 (4×C-16), 129.86 (2×C-2′), 133.51 (4×C-11), 133.97 (4×C-15), 135.58 (4×C-1′); MS MALDI TOF $[M+H]^+$ (found 1087.60, required 1087.70 for $C_{74}H_{87}N_8$).

4.1.9. Hexa[6′-methyl-8β′-(methyl)ergolin-1′-yl-methyl-]-benzene (2h). Compound **2h** was synthesized according to the typical procedure with hexakis(bromomethyl) benzene[5] (0.083 mmol, 53 mg) as spacer. The reaction was terminated after 18 h. The yield of the product was 38%. ^{1}H NMR (399.90 MHz, $CDCl_3$, 30 °C): 1.019 (d, J = 6.5 Hz, 6×8-CH_3, 18H), 1.103 (ddd, J = 12.1, 12.1, 12.6 Hz, 6×H-9a, 6H), 1.928 (dd, J = 11.2, 11.4 Hz, 6×H-7a, 6H), 2.062 (m, 6×H-8, 6H), 2.141 (ddd, J = 4.2, 10.0, 11.0 Hz, 6×H-5, 6H), 2.498 (s, 6×CH_3-N, 18H), 2.640 (m, 6×H-9b, 6H), 2.682 (m, 6×H-4a, 6H), 2.997 (m, 6×H-10, 6H), 3.00* (m, 6×H-7b, 6H), 3.329 (dd, J = 4.2, 14.8 Hz, 6×H-4b, 6H), 5.281 (d, J = 14.3 Hz, 6×CH_2a, 6H), 5.328 (d, J = 14.3 Hz, 6×CH_2b, 6H), 6.408 (d, J = 1.4 Hz, 6×H-2, 6H), 6.706 (d, J = 8.3 Hz, 6×H-14, 6H), 6.940 (dd, J = 1.0, 7.2 Hz, 6×H-12, 6H), 7.052 (dd, J = 7.2, 8.3 Hz, 6×H-13, 6H); ^{13}C NMR ($CDCl_3$, 30 °C): 19.61 (6×8-CH_3), 26.80 (6×C-4), 30.45 (6×C-8), 36.38 (6×C-9), 40.62 (6×C-10), 43.08 (6×CH_3–N), 44.51 (6×CH_2), 65.27 (6×C-7), 66.84 (6×C-5), 107.36 (6×C-14), 112.65 (6×C-3), 113.77 (6×C-12), 119.51 (6×C-2), 123.32 (6×C-13), 127.09 (6×C-16), 134.09 (6×C-11, 6×C-15), 138.89 (6×C-*ipso*); MS MALDI TOF $[M+H]^+$ (found 1592.21, required 1592.03 for $C_{108}H_{127}N_{12}$).

4.2. Antiplasmodial assay

The chloroquine-sensitive strain PoW and the chloroquine-resistant clone Dd2 of *P. falciparum* were maintained in continuous culture in human red blood cells (A^+) diluted to 5% haematocrit in RPMI 1640 medium supplemented with 25 mM HEPES, 30 mM $NaHCO_3$, and 10% human A^+ serum.[9] Compounds were dissolved in DMSO (20 mg/mL) and diluted with medium to a final concentration of 10 μg/mL. Antiplasmodial activity tests were performed in 96-well culture plates as described by Desjardins et al.[10] Briefly, aliquots of 150 μL of parasitized culture (2.5% haematocrit, 0.5% parasitaemia) were exposed to two-fold dilutions of test substances. After incubation in a candle jar for 24 h, 0.5 μCi [^{3}H]-hypoxanthine (1mCi/mL, ICN) was added to each well and the plates incubated for further 18 h. Cells were harvested onto glass fiber filters with a cell harvester (Inotech) and incorporated radioactivity was determined by a liquid scintillation counter (1450 Microbeta plus). Antiplasmodial effects were expressed as 50% inhibitory concentrations (IC_{50}s), that is as the concentration of a compound, which caused a 50% inhibition of growth in comparison to identical cultures without this compound. The percentage of growth inhibition was calculated as: (1−[cpm in drug treated cultures/cpm in untreated cultures])×100 and the IC_{50}s were estimated by interpolation. All substances were tested in triplicate in three independent experiments. Chloroquine×2H_3PO_4 was used as standard drug for positive control.

4.3. Hepatocyte isolation and cultivation

Human liver tissue was obtained from multiorgan donors, with the approval of the Czech Ethical Committee. The hepatocytes were isolated from the HTK pre-washed liver using two-step collagenase perfusion.[11] Yield and viability of cells were assessed by Trypan blue exclusion test. Hepatocytes were resuspended in ISOM medium, consisting of a 1:1 mixture of Ham F12 and William's E, supplemented with additives as follows: glucose (7 mM), glutamine (2.4 mM), penicillin (100 U/mL), streptomycin (10 μM), sodium pyruvate (0.4 mM), dexamethasone (1.8 μM), holo-transferrin (5 mg/L), ethanolamine (1 μM), insulin (350 nM), glucagon (0.2 mg/L), linolic acid (11 μg/L), ascorbic acid (15 mg/L), amphotericin B (1.4 mg/L), pH 7.2.[12] Cells were seeded on collagen-coated six-well plates (area 9.4 cm^2) with a density of 1.25×10^5 cells/cm^2. For the first 4 h, culture medium was enriched with 5% of fetal calf serum to improve cell attachment. Following the stabilization period of 24–48 h, hepatocytes were treated for 24 h with test compounds (final concentrations 1, 5, 10, 50, 100 μM) and/or with vehicle (DMSO) for control. The cultivation and exposures were carried out under sterile conditions, using a humidified incubator at 37 °C under an atmosphere containing 5% CO_2. Integrity of the cell membrane was determined by the leakage of lactate dehydrogenase (LDH) into the medium. Depletion of reduced glutathione (GSH) was estimated by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). Cultures were routinely checked under an Olympus CK-2 microscope.[13]

4.4. LDH assay

Enzyme activity was measured in 50 μL samples by determining the decrease in absorbance at 340 nm for 3 min in a reaction mixture of 1.22 mM pyruvate (1 mL) in 50 mM phosphate buffer (pH 7.5) + 20 μL NADH (6.2 mg/mL) and expressed in μkat/L.

4.5. GSH level

Hepatocytes were washed and harvested in 0.5 mL of PBS with 0.1% of Triton-X100. An aliquot of the obtained lysate (0.36 mL) was denatured with 0.04 mL cold trichloroacetic acid (25%). After 10 min of incubation on ice, the mixture was centrifuged ($3000\times g$, 10 min, 4 °C) and 0.3 mL of the supernatant were mixed with 1.0 mL of Tris-base (0.8 M)-EDTA (0.02 M) buffer, pH 8.9. Following the addition of 0.1 mL of DTNB (0.01 M) in methanol, absorbance of the sample was measured at 412 nm against DTNB blank. The level of GSH (nmol/10^6 cells) was calculated using absorptivity of 13,600 M cm^{-1}. The standard solution of GSH (1.0 mM dissolved in deionised water) was used for the control of the recovery and reproducibility of the measurement.

4.6. Cultivation of fibroblasts

The mouse fibroblast NIH 3T3 cell line was obtained from the European Collection of Cell Cultures (CAMR, Salisbury, UK). The cells were cultured in a 5% CO_2 atmosphere at 37 °C in Dulbecco's medium D-5921 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin. 10^4 cells/well were placed into 96-well microculture plates and grown for 24 h (attachment time). Thereafter, cells were exposed to tested compounds (final concentration 0.1, 0.5, 1, 10, 25, 50, 75, 100 μM) for 24 h. Cytotoxicity was assessed by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

4.7. MTT assay

After the treatment, culture medium was replaced with fresh medium and 100 μL of MTT reagent (5 mg/mL in PBS) was added. Three hours later, the medium was removed, cells washed with PBS and lysed for 5 min with 1 mL of DMSO containing 1% ammonia. The lysate was diluted 20 times with DMSO (+1% ammonia) and absorbance at 540 nm was measured against blank (DMSO + 1% ammonia). Results were normalized to the control value (i.e., $100\times A_{sample}/A_{control}$) and expressed as percentage of control. IC_{50} values were determined from the concentration–activity curve.

4.8. In vivo toxicity studies

In vivo toxicity studies were performed with single female Balb/c mice weighing 30 g maintained on standard diet. Age and sex matched mice (6–7 animals per group) were injected intra-peritoneally 1.7×10^{-4}mol/kg (body weight), 3.3×10^{-5} mol/kg, 3.3×10^{-6} mol/kg, 3.3×10^{-7} mol/kg, 1.7×10^{-9} mol/kg in 0.2 mL of saline once a week for 4 weeks (chronic toxicity). The control animals received the equivalent volume of saline. The animals were evaluated concerning behavioral disturbances and killed by decapitation under ether anesthesia 24 h after the last injection in order to evaluate macroscopically the liver, kidneys, and spleen. All procedures were conducted in accordance with the European Convention for the Care and Use of Laboratory Animals as approved by the Czech Animal Care and Use Committee.

4.9. Statistical analyses

Data were analyzed by ANOVA, followed by Dunnett's test. Results are presented as mean ± SD. A p value of <0.05 was considered to be statistically significant.

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