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Mixed tetraoxanes containing the acetone subunit as antimalarials ☆

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ABSTRACT

Eleven new tetraoxanes possessing cholic acid-derived carrier and isopropylidene moiety were synthesized and were tested in vitro and in vivo. In vitro screening revealed that nine of them were more potent against CQ-resistant W2 than CQ-susceptible D6 strain and that two of them were equally or more potent than artemisinin and mefloquine against multi-drug resistant TM91C235 strain. Amine **8** cured all mice at the dose of 160 mg/kg/day, while the anilide **9** exhibited MCD $\leqslant$ 20 mg/kg/day. The diol **13** was most potent antiproliferative with GI_{50}, TGI, LC_{50} MG_MID 0.98 μM, 3.80 μM, 11.22 μM, respectively. All tested compounds showed no toxic effects.

1. Introduction

Malaria is one of the most deadly diseases of the developing world, affecting 300–500 million people annually with an estimated death toll of 1–3 million people. The most affected regions are tropical Africa, Southeast Asia, and the Amazon region. Malaria is caused by a one-celled parasite of the genus *Plasmodium*. Of the four species that infect humans, *Plasmodium falciparum* is the most dangerous, accounting for half of all clinical cases of malaria and 90% of deaths from the disease. Malaria kills one African child every 30 s, and the emergence of multi-drug resistant strains of plasmodia has rendered many affordable antimalarials such as chloroquine (CQ) much less effective in addressing the severe health issues in endemic regions.

Great efforts were recently expended to discover a new and efficacious endoperoxide-based antimalarial,[2] primarily because no resistance to this type of drugs has yet been observed.[3] Recently, MMV-financed research on trioxolanes afforded the first drug candidate for Phase II human trials[4] while researchers at WRAIR are rapidly advancing a GMP version of Artesunate through Phase II trials.

One part of our research in this field is focused on the development of a new type of peroxide antimalarial called a 1,2,4,5-tetraoxacyclohexane (tetraoxane) that utilizes a steroid carrier.[5] Use of this carrier is based on the expectation that the introduction of an amphiphilic lipid carrier that is spiro bound to a 1,2,4,5-tetraoxacyclohexane in the form of a mixed tetraoxane[6] will afford similar activity to the artemisinins,[2c] or to the 1,2,4-trioxolanes,[7] but with improved physicochemical properties. Although peroxide antimalarials have a rapid onset of action, their half-life is relatively short and recrudescence often occurs.[4] Therefore, the efforts to develop these new antimalarials are needed to sustain the future introduction of new drug candidates.

In this paper, we report on a series of new steroidal mixed tetraoxanes containing the acetone subunit and analyze their antimalarial activity. The idea was to introduce a simple subunit that had little or no effect on any other part of the spirotetraoxacyclohexane moiety, specifically, on the steroidal part, in order to examine the influence of a cholic acid-derived carrier on the antimalarial activity. A review of the literature revealed syntheses of several tetraoxane structures by other groups; however, none of the compounds had appreciable activity.[8,9]

2. Results and discussion

Mixed tetraoxanes **3–12** were prepared using the procedure previously described.[6a] *gem*-Dihydroperoxide **2** was directly coupled to acetone to give the parent methyl ester 3 (Scheme 1). Ester **3** was selectively hydrolyzed at C(24) into the corresponding acid **4**, which was further transformed into amides **5–12** using a mixed anhydride procedure. The overall yield starting from cholic acid was ca. 31% (calculated, e.g., to amide **9**).

☆ See Ref. 1.

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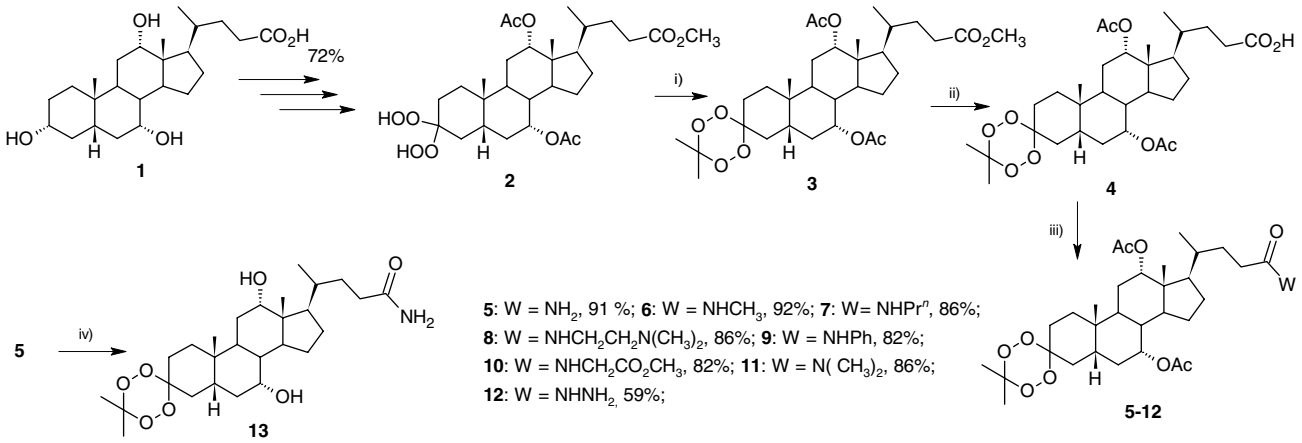

Scheme 1. Reagents and conditions: (i) acetone, H_2SO_4/CH_3CN, 53%; (ii) NaOH/*i*-PrOH/H_2O, 80 °C, 99%; (iii) $Et_3N/ClCO_2Et/CH_2Cl_2$, amine; (iv) NaOH/MeOH/H_2O, 70 °C, 96%.

Tetraoxanes were assayed in vitro against four *P. falciparum* strains: D6, W2, RCS, and TM91C235 (Tables 1 and 2). In general, tetraoxanes were more active against CQ-resistant strains (W2, RCS, and TM91C235) than the CQ-susceptible D6 strain. As noticed before,[5,6] amides exhibit better in vitro activity than the corresponding esters and acids.

Tetraoxanes **6**, **9**, and **11**–**13** were tested against a CQ-resistant *P. falciparum* isolate from South America (RCS) and were found to be more active against this strain than against the Southeast Asian strains, W2 and TM91C235. In addition, the *N*-(2-dimethylamino)ethyl and *N*-phenyl amides, **8** and **9**, respectively, were also potent against the latter two CQ-resistant strains. As it is believed that peroxide antimalarials exert their activity in the parasite food vacuole (FV),[10] which has a pH ~ 5.5, the *N*-(2-dimethylamino)ethyl derivative **8** was designed to potentially concentrate at the site of action through suppressed efflux of the protonated form through the FV membrane. Although tetraoxane **8** is less active than mefloquine against W2 strain, it exhibited an IC_{90}/IC_{50} ratio of 1.71 as compared to mefloquine's ratio of 2.72. Moreover, **8** is 2–3 times more active against the multi-drug resistant TM91C235 strain than mefloquine with a IC_{90}/IC_{50} ratio of about 1.5 times lower. *N*-Phenyl derivative **9** and mefloquine have similar activities against all tested strains; however, **9** exhibited a threefold better IC_{90}/IC_{50} ratio against TM91C235. The activity of both **8** and **9** is comparable to, or better than that of artemisinin (Tables 1 and 2).

Table 2
In vitro antimalarial activities of tetraoxanes **3**–**13** against *P. falciparum* RCS[a] and TM91C235[b] strains

Compound	W	IC_{50} (nM)		IC_{90} (nM)	
		RCS	TM91C235	RCS	TM91C235
3	OCH_3	ND[c]	25.99	ND	57.27
4	OH	ND	60.44	ND	231.16
6	$NHCH_3$	11.55	31.28	22.26	74.82
8	$NHCH_2CH_2N(CH_3)_2$	ND	7.16	ND	19.82
9	NHPh	1.89	15.75	4.45	20.89
11	$N(CH_3)_2$	7.19	25.13	14.00	50.93
12	$NHNH_2$	7.03	28.47	19.49	38.07
13	NH_2	38.70	227.70	167.17	498.31
Artemisinin[d]			13.04		17.40
Mefloquine[e]		2.33	16.44	5.00	65.71
Chloroquine[e]		192.45	144.90	397.09	268.76

[a] RCS is strain from Brazil, resistant to chloroquine, susceptible to mefloquine.
[b] TM91C235 (Thailand) is highly resistant to mefloquine, chloroquine, and quinine.
[c] ND, not determined.
[d] Average of >8 replicates.
[e] Control drugs.

Table 1
In vitro antimalarial activities of tetraoxanes **3**–**13** against *P. falciparum* D6[a] and W2[b] strains, and metabolic half-lives

Compound	W	IC_{50} (nM)		IC_{90} (nM)		Metabolic $t_{1/2}$ (min) (human)	Metabolic $t_{1/2}$ (min) (mouse)
		D6	W2	D6	W2		
3[c]	OCH_3	14.98	11.67	26.68	20.70	ND[d]	ND
4	OH	46.50	36.04	96.28	94.67	>60	>60
5	NH_2	12.21	9.40	ND	ND	>60	>60
6	$NHCH_3$	46.87	22.35	78.28	43.65	>60	>60
7	$NHPr^n$	7.40	18.14	16.16	29.25	24	25
8	$NHCH_2CH_2N(CH_3)_2$	11.60	9.79	26.24	16.76	>60	>60
9	NHPh	6.97	5.02	20.84	14.35	19	16
10	$NHCH_2CO_2CH_3$	14.28	5.98	ND	ND	70	32
11	$N(CH_3)_2$	35.54	17.29	71.47	31.62	49	17
12	$NHNH_2$	28.10	16.36	46.31	22.95	ND	ND
13	NH_2	215.25	153.31	346.51	233.95	>60	>60
Artemisinin[e]		9.0	6.7	12.8	11.5	—	—
Mefloquine[f]		10.52	3.09	21.65	8.41	—	—
Chloroquine[f]		13.72	349.35	17.63	491.53	—	—

[a] D6 is a clone from the Sierra I/UNC isolate and is susceptible to chloroquine and pyrimethamine, but has reduced susceptibilities to mefloquine and halofantrine.
[b] W2 is a clone of the Indochina I isolate and is resistant to chloroquine and pyrimethamine, but susceptible to mefloquine.
[c] Performed in duplicate, average reported.
[d] ND, not determined.
[e] Average of >8 replicates.
[f] Control drugs.

In attempt to increase the water solubility, we prepared tetraoxane **13** with free hydroxyl groups at C(7) and C(12). Unfortunately, despite much better calculated solubility (Advanced Chemistry Development, ACD/log*D* Solubility Suite, Version 8.0; 50 μg/mL, pH 2.0, log*P* 2.42) the in vitro activity of derivative **13** sharply decreased in comparison to parent tetraoxane **5** (calculated solubility 0.00354 μg/mL, pH 2.0, log*P* 4.21), and it is the least active compound in the series.

Derivatives **3**, **4, 8**, and **9** were screened in vivo (po) against the drug-sensitive *Plasmodium berghei* KBG 173 strain following the modified Thompson test.[6b,11] The data presented in Table 3 show that compounds **3** and **4** extended mouse survival with a minimum active dose (MAD) of 320 and 80 mg/kg/day, respectively. The *N*-phenyl amide **9** cured 2 mice on day 31 at all three administered doses; surviving mice showed complete clearance of parasitemia and surviving mice were considered cured even at the low dose of 20 mg/kg/day (0.035 mmol/kg/day). Compound **8**, administered orally at 320, 80, and 20 mg/kg, cured 4 of 5 mice at the high dose, had a minimum active dose of 80 mg/kg/day, and was inactive at the low dose. However, some observations merit further comment: out of 5 mice in the group receiving 320 mg/kg/day, all were parasite free from days 6–13. Parasite recrudesced in 2 mice on day 17. One of the two mice died on day 20; however, the second one had positive blood smears on days 17, 20, and 24 but negative blood smears on days 27 and 31. The other three mice had negative blood smears from days 6–30. Mean parasitemia results on days 17, 20, 24, 27, and 31 for that mouse were 2.36%, 1.9%, 1.4%, and 0%, respectively. The other three mice had negative blood smears on days 27 and 31. There was no indication of toxicity of the examined tetraoxanes on tested animals.

It was calculated that tetraoxane **8** was appreciably more soluble than the other acetone-derived tetraoxanes (calculated solubility 5380 μg/mL, pH 2.0), and since it was metabolically quite stable with half-lives >60 min in human and mouse liver microsomes (Table 1), the activity of this compound was examined in sc screen. Tetraoxane **8** was administered sc at 10, 20, and 80 mg/kg twice per day at 12 h apart for 3 days. Necropsies were performed in all animals, and the eight mice that died prior to day 31 showed typical gross lesions typical of a fatal malaria infection, such as gray swollen liver, dark spleen, and pale emaciated carcass (similar to the control group mice). In the 160 mg/kg/day group, 5 of 5 mice survived through day 31 and had negative blood smears from days 6–31. All mice were considered to be cured, and no significant gross lesions were observed at necropsy. In the 40 mg/kg/day dose group, all mice had negative blood smears on days 6 and 10. One mouse was cured, and the other four mice recrudesced on day 13. Three of the 4 mice died by day 27: one on day 18, one on day 20, and the other on day 27, with all showing typical malaria gross lesions at necropsy. The other mouse maintained low positive blood smear results during days 13–20 and cleared from days 24–31. Mean parasitemia results on days 10, 13, 17, 20, 24, 27, and 31 were 0%, 0.004%, 15.48%, 3.9%, 12.53%, 0%, and 0%, respectively. No gross lesions were seen in this mouse at necropsy, which was consistent with the presence of negative blood smears from days 27 to 31. Finally, at the low dose of 20 mg/kg/day, a delay in patency was observed in all mice: four mice were negative on day 6 but parasite recrudesced on days 10 and 13. The other mouse had a very low positive parasitemia (0.002%) on day 6. All mice died between days 14 and 17 (1, 1, 1, and 2 mice on days 14, 15, 16, and 17, respectively). Mean parasitemia results on days 10, 13, and 17 were 0.032%, 8.72%, and 26.8%, respectively.

In vitro metabolism studies were performed on compounds **5–8, 10, 11**, and **13** to assess the bioavailability of possible drug candidates after oral administration. The metabolic stability assays were done using human and mouse liver microsomes.[6c] Stable compounds were defined as having half-lives >60 min and the relevant data are given in Table 1. The in vitro metabolic half-life can be used to approximate an in vivo half-life/hepatic clearance rate. The tetraoxanes demonstrated a wide range of stability with half-lives from 24 min to greater than 60 min. Metabolite ID was performed in order to identify differences in Phase I metabolism across species, as well as to aid in interpretation of future animal testing. The primary routes of metabolism for the tetraoxanes include hydroxylation, dihydroxylation, demethylation, and desaturation; species differences were sometimes observed (Table 4).

In trying to correlate the metabolic profile with the in vitro and in vivo efficacy data, no consistent or direct correlation could be made between the metabolic profile and in vitro and in vivo testing. Specifically, there did not appear to be a correlation between the hepatic clearance rate, as estimated by metabolic stability, and efficacy or toxicity. Even so, the ADME data collected are currently being used in conjunction with physicochemical and efficacy data to identify structural liabilities within the compounds that may be easily modified to improve drug-like properties without

Table 3
In vivo antimalarial activity of tetraoxanes **3**, **4**, **8**, and **9** after administration against drug-sensitive *P. berghei* (KBG 173 strain)

Compound		mg/kg/day	Mice dead/day died	Mice alive day 31/total	Survival time[a] (days)
3, po[b]		320	1/9, 1/16, 3/21	0/5	18
		80	3/9, 1/21	1/5	16
		20	1/7, 2/9, 2/21	0/5	13
4, po		320	2/14, 2/16, 1/25	0/5	17
		80	1/7, 1/12, 1/16, 2/21	0/5	15
		20	4/9, 1/12	0/5	10
8	po	320	1/20	4/5	29
		80[c]	1/10, 1/12, 1/14, 1/20	0/5	14
		20	5/7–9	0/5	8
	sc[d]	160		5/5	31
		40	1/18, 1/20, 1/27	2/5	25
		20	1/14, 1/15, 1/16, 2/17	0/5	16
9, po		320	1/13, 1/17, 1/21	2/5	23
		80	3/21	2/5	25
		20	1/14, 1/17, 1/21	2/5	23
Infected control group			5/7	0/5	7

[a] Including cured mice.
[b] Groups of five *P. berghei* (KBG 173 strain) infected CD-1 mice were treated on days 3, 4, and 5 postinfection with tetraoxanes suspended in 0.5% hydroxyethylcellulose–0.1% Tween 80. Mice alive on day 31 with no parasites in a blood film are considered cured.
[c] One mouse excluded.
[d] Drugs were suspended in sesame oil, and administered beginning on day 3 postinfection two times a day.

Table 4
Metabolites tentatively identified by LC–MS/MS in human and mouse microsomes

Compound	Human metabolite formation[a]			Mouse metabolite formation		
	$[MH+16]^+$	$[MH-2]^+$	$[MH+2]^+$	$[MH+16]^+$	$[MH-2]^+$	$[MH+2]^+$
4	·			·		
5	·	·		·		
6	·			·		
7	·	·		·	·	
8	·			·		
9[b]	·			·		
10	·		·			
11	·		·	·		·
13	·			·		

[a] Tentative assignment: $[MH+16]^+$ = hydroxylation; $[MH-2]^+$ = desaturation; $[MH+2]^+$ = demethylation + hydroxylation.
[b] Undergoes dihydroxylation in both species.

Table 5
Inhibitory and lethal concentration in μM calculated from MG_MID point values

Compound	**4**	**5**	**6**	**8**	**10**	**11**	**13**
GI_{50}[a]	15.49	8.71	4.47	3.39	8.51	16.98	0.98
TGI[b]	37.15	34.67	18.20	10.23	33.88	41.69	3.80
LC_{50}[c]	75.86	79.43	47.86	22.39	77.62	75.86	11.22

[a] GI_{50}, the concentration of the drug that inhibits percentage growth by 50%.
[b] TGI, total growth inhibition (concentration of the drug to achieve cytostasis).
[c] LC_{50}, concentration of the compound at which 50% of the cells are killed.

sacrificing compound potency. Since the in vitro activity of these compounds is comparable to or better than artemisinin and mefloquine, continuing efforts will focus on strategies to improve in vivo potency. Future work will continue to explore this promising class of antimalarial tetraoxane derivatives.

Although the in vivo tested compounds showed no toxicity on tested animals, based on previous results,[5b,c] it was of interest to examine present tetraoxanes' cytotoxicity against diverse cancer cell lines. Seven compounds were tested in vitro by the National Cancer Institute (NCI), Bethesda, in a panel of 60 human tumor cell lines. Selected results are given as in Table 5. In general, the tested tetraoxanes exhibit good total growth inhibitory activity, and low overall cytotoxicity against cancer cell lines as indicated by MG_MID (meangraph midpoint) values (Table 5).

The most active compound was the primary amide **13** that has free hydroxyl groups at C(7) and C(12) and exhibits strong antiproliferative activity against the renal cancer cell line A498 (GI_{50} = 0.092 μM, TGI = 0.71 μM, and LC_{50} = 11.50 μM). It is more potent in inhibiting the cell growth than in cell-killing, that is, its GI_{50} value is 125 times lower than respective LC_{50}. It is interesting to note that the compound being the least active against malaria parasites is the most active against cancer cell lines, suggesting that, in this case, the structural features disfavoring antimalarial activity stimulate the antiproliferative activity.

3. Conclusion

In this work, we reported the results of our study on mixed tetraoxanes possessing cholic acid-derived carrier and 'open chain' isopropylidene moiety originating from acetone. The obtained results indicate that the compounds are efficient antimalarials in vitro and in vivo. Acetone-derived tetraoxanes are more potent against CQ-resistant W2 and multi-drug resistant TM91C235 strains, than against CQ-susceptible D6 strain. These results, taken together with the ease of the compounds' synthesis (amide derivatives were obtained in ca. 44% from cholic acid), their in vivo potential, and established non-toxicity, reveals this class of tetraoxanes as promising alternative to other peroxide antimalarials.

4. Experimental

4.1. General

Melting points were determined on a Boetius PMHK apparatus and were not corrected. Optical rotation measurements were performed on a Perkin-Elmer 341 polarimeter, at the given temperatures. Concentrations are expressed in g/100 mL. IR spectra were recorded on Perkin-Elmer spectrophotometer FT-IR 1725X. ^1H 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. ESI mass spectra were acquired on a TSQ (Thermo Quest; Finnigan) quadrupole instrument. Samples were dissolved in HPLC grade acetonitrile. Positive ions: capillary temperature 250 °C; ESI spray voltage 4.5 kV; multiplier: 1500 V; loop injection into a solution of H_2O/CH_3CN/HCOOH (1%) = 18:80:2 (v/v/v); flow rate: 0.3 mL/min. FAB mass spectra were recorded with a JEOL JMS-SX 102 A spectrometer and on a VG-ZAB-T instrument equipped with Cs ion gun. Accelerating voltage was set to 8 kV using MNBA as matrix. Probe accurate mass measurements were performed in the presence of PEG internal calibrant at 5000 resolution. Metabolic stability experiments: ThermoFinnigan TSQ triple quadrupole mass spectrometer coupled to a ThermoQuest Surveyor MS pump LC system using Xcalibur software, Version 1.3. ThermoFinnigan LTQ ion trap mass spectrometer coupled to Agilent 1100 LC system using Xcalibur software, Version 1.4. ESI positive ion. 0.1% formic acid, acetonitrile, water. Waters XTerra MS C18 column, 2.1 × 50 mm, 3.5 μm particle size with 2.1 × 10 mm corresponding guard column. Gradient 95:5 formic:ACN to 25:75 over 6 min, returning to 95:5 at 6.01 at equilibrating for 3 min. Flow rate 300 μL/min. ESI negative ion. Ten millimolar ammonium acetate (pH 9.2), acetonitrile, water. Waters symmetry C8 column, 2.1 × 50 mm, 5 μm particle size. Gradient 95:5 Am acetate:ACN to 5:95 over 3 min, hold for 5 min, returning to 95:5 at 8.10 min and equilibrating for 4 min. Flow rate 300 μL/min, equilibration at 350 μL/min. Thin-layer chromatography (TLC) has been performed on precoated Merck silica gel 60 F_{254} plates, using *N*,*N*-dimethyl-*p*-phenylene-diammonium dichloride peroxide reagent for peroxide moiety detection,[12] and Lobar LichroPrep Si 60 (40–63 μm) columns coupled to Waters RI 401 detector were used for column chromatography. Where appropriate, the compounds are listed according to their elution order.

gem-Dihydroperoxide (**2**) and tetraoxanes **3**–**12** were synthesized according to procedure described earlier.[6a]

4.1.1. Methyl 7α,12α-diacetoxy-5β-cholan-24-oate-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (3)

To the cold solution (ice-bath) of dihydroperoxide **2** (500.0 mg, 0.9 mmol) in dichloromethane (12.5 mL), acetone (132 μL, 1.80 mmol) was added and after 30 min, 650 μL of ice-bath cooled (H_2SO_4/CH_3CN) mixture (1:10, v/v) was added dropwise. The reaction mixture was stirred at 0 °C for 15 min, and after usual work-up the crude mixture was purified by column chromatography (Lobar, LichroPrep RP-18, eluent MeOH/H_2O = 8:2) to afford tetraoxane **3**. Yield 284 mg (53%). Colorless foam, softening at 77–80 °C. $[\alpha]_D^{20}$ +48.50 (*c* 1.04, $CHCl_3$). IR(KBr): 2953s, 2872s, 1738s, 1606w, 1440s, 1377s, 1240s, 1165s, 1126s, 1103s, 1076m, 1026s, 966w, 943w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 5.09 (br s, H–C(12)), 4.95–4.90 (m, H–C(7)), 3.66 (s, $CH_3O_2C(24)$), 2.13 (br s, CH_3COO–), 2.08 (br s, CH_3COO–), 0.95 (H_3C–C(10)), 0.81 (d, J = 6.0 Hz, H_3C–C(20)), 0.74 (H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 174.44, 170.51, 108.15, 107.82, 75.18, 70.57, 51.40, 47.21, 44.96, 43.23, 37.55, 34.58, 34.45, 32.03, 30.72, 30.61, 28.37, 27.02, 25.60, 22.65, 22.02, 21.49, 21.31, 20.58, 17.37, 12.09. FAB-MS (*m*/*z*): 1189.8

([2M]$^+$, 23), 733.5 (28), 663.5 (14), 613.2 ([M+NH$_4$]$^+$, 17), 595.4 ([M+H]$^+$, 100). Anal. Calcd for $C_{32}H_{50}O_{10}$+H_2O: C, 62.72; H, 8.55. Found: C, 62.29; H, 8.28. Note: in one attempt we obtained a sample with correct microanalytical data; however, it is difficult to obtain a H_2O free sample without significant heating in vacuo. Then, decomposition is probable.

4.1.2. 7α,12α-Diacetoxy-5β-cholan-24-oic acid-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (4)

Methyl ester **3** (300.0 mg, 0.5 mmol) was hydrolyzed at 80 °C with NaOH (30.26 mg, 0.7 mmol) in *i*-PrOH/H_2O mixture (120 mL, 3:1 v/v). After 15 min the reaction mixture was cooled and diluted with 50 mL H_2O and 100 mL CH_2Cl_2. Water layer was acidified to pH 2 with diluted HCl, and layers were separated. Water layer was further extracted with CH_2Cl_2 (3× 30 mL), combined organic layers were washed with water and brine, dried over anhyd Na_2SO_4 and evaporated to dryness. Yield 291.2 mg (99%). Mp = 195–197 °C (colorless prisms, diisopropylether). $[\alpha]_D^{20}$ +47.95 (*c* 1.20, $CHCl_3$). IR(KBr): 3453m, 2965s, 2869w, 1735s, 1702m, 1452w, 1378s, 1256s, 1245s, 1203m, 1182m, 1163w, 1128w, 1103w, 1075w, 1026m, 968w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): 5.09 (br s, H–C(12)), 4.95–4.90 (m, H–C(7)), 2.13 (br s, CH_3COO–), 2.09 (br s, CH_3COO–), 0.95 (H_3C–C(10)), 0.82 (d, *J* = 5.8 Hz, H_3C–C(20)), 0.74 (H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 179.72, 170.67, 108.22, 107.92, 75.27, 70.70, 47.27, 45.03, 43.30, 37.62, 34.63, 34.47, 30.74, 30.45, 28.42, 27.08, 25.68, 22.73, 22.07, 21.58, 21.40, 20.69, 17.41, 12.17. FAB-MS (*m*/*z*): 1161.68 (2M$^+$, 3), 603.28 ([M+Na]$^+$, 5), 580.70 (M$^+$, 4). Anal. Calcd for $C_{31}H_{48}O_{10}$+0.5H_2O: C, 63.14; H, 8.38. Found: C, 63.58; H, 8.29.

4.2. General procedure for preparation of amides 5–12

A solution of **4** (600.0 mg, 1.03 mmol), in dry CH_2Cl_2 (40 mL), with added Et_3N (143 μL, 1.03 mmol) and $ClCO_2Et$ (99 μL, 1.03 mmol) was stirred for 60 min at 0 °C. Given amount of amine was 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 a usual manner.[6a] Crude product was purified by column chromatography.

4.2.1. 7α,12α-Diacetoxy-5β-cholan-24-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (5)

Using a suspension of 10 equiv NH_4Cl and 10 equiv Et_3N in dry CH_2Cl_2 (20 mL), 541.7 mg (91%) of **5** was obtained. Column chromatography: Lobar B, LichroPrep Si 60, eluent EtOAc. Colorless foam, softening at 122–124 °C. $[\alpha]_D^{20}$ +42.12 (*c* 1.02, $CHCl_3$). IR(KBr): 3456m, 2957s, 2877m, 1736s, 1674s, 1618w, 1445m, 1378s, 1244s, 1204m, 1164w, 1127w, 1076w, 1027m, 965w, 944w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): 5.73 (br s, H_2N–C(24)), 5.52 (br s, H_2N–C(24)), 5.09 (br s, H–C(12)), 4.91 (br s, H–C(7)), 2.13 (br s, CH_3COO–), 2.09 (br s, CH_3COO–), 0.94 (H_3C–C(10)), 0.82 (d, *J* = 6.0 Hz, H_3C–C(20)), 0.73 (H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 175.90, 170.62, 108.19, 107.88, 75.23, 70.61, 47.36, 44.99, 43.24, 37.55, 34.60, 32.63, 31.26, 28.37, 27.11, 25.66, 22.67, 22.03, 21.56, 21.40, 17.48, 12.17. (+)ESI LC–MS/MS (*m*/*z*): 579.9 (MH$^+$), 519.68 (MH−60)$^+$, 459.99 (MH−120)$^+$. Anal. Calcd for $C_{31}H_{49}NO_9$+0.5H_2O: C, 63.24; H, 8.56. Found: C, 63.32; H, 8.89.

4.2.2. *N*-Methyl-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (6)

Acid **4** (300 mg, 0.52 mmol) was transformed into amide **6** (281 mg, 92%) according to general procedure using a suspension of 6 equiv $MeNH_3Cl$ and 6 equiv Et_3N in dry CH_2Cl_2 (20 mL). Column chromatography: Lobar B, LichroPrep Si 60, eluent EtOAc. Colorless foam, softening at 117–118 °C. $[\alpha]_D^{20}$ +59.8 (*c* 0.132, $CHCl_3$). IR (KBr): 3430s, 2954m, 2875w, 1736s, 1651m, 1554w, 1445w, 1377w, 1245s, 1204m, 1164w, 1126w, 1076w, 1027m, 944w, 895w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): 5.60–5.45 (m, HN–C(24)), 5.09 (br s, H–C(12)), 4.95–4.85 (br s, H–C(7)), 2.80 (d, *J* = 4.8 Hz, H_3C–NH), 2.13 (br s, CH_3COO–), 2.08 (br s, CH_3COO–), 0.94 (H_3C–C(10)), 0.81 (d, *J* = 6.0 Hz, H_3C–C(20)), 0.73 (H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 173.91, 170.64, 108.22, 107.90, 75.27, 70.63, 47.43, 44.99, 43.24, 37.56, 34.69, 34.62, 33.34, 31.46, 28.41, 27.11, 26.24, 25.68, 22.71, 22.05, 21.58, 21.42, 17.50, 12.17. (+)ESI-MS (*m*/*z*): 658.29 (30), 657.29 (75), 616.29 ([M+Na]$^+$, 85), 594.29 ([M+H]$^+$, 100). Anal. Calcd for $C_{32}H_{51}NO_9$+0.5H_2O: C, 63.76; H, 8.70. Found: C, 63.49; H, 8.89.

4.2.3. *N*-(*n*-Propyl)-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (7)

Acid **4** (300 mg, 0.52 mmol) was transformed into amide **7** (284.6 mg, 86%) according to general procedure using a 6 equiv *n*-$PrNH_2$ (251 μL, 3.10 mmol) in dry CH_2Cl_2 (20 mL). Column chromatography: Lobar B, LichroPrep Si 60, eluent EtOAc/heptane = 95:5. Colorless foam, softening at 101–103 °C. $[\alpha]_D^{20}$ +36.05 (*c* 0.92, $CHCl_3$). IR (KBr): 3404m, 2958s, 2875m, 1737s, 1649s, 1547m, 1444m, 1377s, 1244s, 1204m, 1163w, 1126w, 1076w, 1027m, 964w, 944w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): 5.60–5.45 (m, HN–C(24)), 5.09 (br s, H–C(12)), 4.91 (br s, H–C(7)), 3.20–3.15 (m, $CH_3CH_2CH_2$–NH), 2.13 (br s, CH_3COO–), 2.08 (br s, CH_3COO–), 1.60–1.40 (m, $CH_3CH_2CH_2$–NH–), 1.00–0.85 (m, H_3C–C(10), and $CH_3CH_2CH_2$–NH–), 0.81 (d, *J* = 6.0 Hz, H_3C–C(20)), 0.73 (H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 173.17, 170.62, 108.19, 107.86, 75.25, 70.61, 47.41, 44.98, 43.23, 41.08, 37.55, 34.65, 34.60, 33.52, 31.52, 28.37, 27.10, 25.64, 22.80, 22.69, 22.03, 21.56, 21.40, 17.48, 12.15, 11.27. FAB-MS (*m*/*z*): 1243.8 ([2M]$^+$, 7), 757.5 (3), 622.4 ([M+H]$^+$, 100), 562.4 (6). Anal. Calcd for $C_{34}H_{55}NO_9$+0.5H_2O: C 64.74; H 8.95. Found: C, 64.68; H, 8.92.

4.2.4. *N*-(2-Dimethylamino)ethyl-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (8)

Acid **4** (312.1 mg, 0.54 mmol) was transformed into amide **8** (300.4 mg, 86%) according to general procedure using a 6 equiv *N*,*N*-dimethylethane-1,2-diamine (343 μL, 3.23 mmol) in dry CH_2Cl_2 (20 mL). Column chromatography: Lobar B, LichroPrep Si 60, eluent $CHCl_3$/MeOH/NH_3 = 9:1:1. Colorless foam, softening at 86–88 °C. $[\alpha]_D^{20}$ 48.5 (*c* 0.194, $CHCl_3$). IR(KBr): 3443m, 2951s, 2873m, 1734s, 1653w, 1544m, 1449m, 1376m, 1247s, 1203w, 1125w, 1076w, 1028m, 957w cm^{-1}. ^{1}H NMR (200 MHz, $CDCl_3$): 6.20–6.10 (m, HN–C(24)), 5.09 (br s, H–C(12)), 4.91 (br s, H–C(7)), 3.40–3.25 (m, –NH–$CH_2CH_2N(CH_3)_2$), 2.50–2.42 (m, –NH–$CH_2CH_2N(CH_3)_2$), 2.27 (s, –$N(CH_3)_2$), 2.13 (br s, CH_3COO–), 2.09 (br s, CH_3COO–), 0.94 (s, H_3C–C(10)), 0.82 (d, *J* = 6.0 Hz, H_3C–C(20)), 0.73 (H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 173.44, 170.62, 108.21, 107.88, 75.27, 70.63, 57.83, 47.41, 44.99, 44.96, 43.24, 37.60, 36.42, 34.62, 33.27, 31.37, 30.54, 28.41, 27.10, 25.66, 22.71, 22.05, 21.58, 21.40, 17.50, 12.17. (+)ESI-MS (*m*/*z*): 725.42 (10), 699.49 (5), 652.56 (35), 651.45 ([M+H]$^+$, 100). FAB-MS (*m*/*z*): 651.3 ([M+H]$^+$, 100), 649.3 (11), 561.3 (7), 307.0 (12), 289.0 (7), 154.0 (64), 59.2 (60), 44.4 (9). HRMS-ESI: *m*/*z* 651.4236 corresponds to $C_{35}H_{59}O_9N_2$ (error in ppm: 2.3).

4.2.5. *N*-Phenyl-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (9)

Acid **4** (300.0 mg, 0.52 mmol) was transformed into amide **9** (277.3 mg, 82%) according to general procedure using a 6 equiv $PhNH_2$ (288 μL, 3.12 mmol) in dry CH_2Cl_2 (20 mL). Column chromatography: Lobar B, LichroPrep Si 60, eluent EtOAc/heptane = 3:7.

Colorless foam, softening at 126–128 °C. $[\alpha]_D^{20}$ +46.3 (*c* 0.082, $CHCl_3$). IR (KBr): 3453m, 2949m, 2876w, 1736s, 1669w, 1602m, 1541m, 1498m, 1442m, 1377m, 1245s, 1204w, 1126w, 1077w, 1027m, 965w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 7.60–7.00 (m, *Ph*–NHC(24)), 5.10 (br s, H–C(12)), 4.92 (br s, H–C(7)), 2.13 (br s, CH_3COO–), 2.08 (br s, CH_3COO–), 0.94 (H_3C–C(10)), 0.85 (d, *J* = 5.6 Hz, H_3C–C(20)), 0.73 (H_3C–C(13)). ^{13}C NMR (50 Hz, $CDCl_3$): 171.51, 170.67, 137.94, 128.96, 124.16, 119.71, 108.21, 107.90, 75.31, 70.65, 47.52, 45.05, 43.26, 37.58, 34.69, 34.62, 31.28, 28.41, 27.15, 25.68, 22.73, 22.05, 21.58, 21.43, 17.59, 12.20. (+)ESI-MS (*m*/*z*): 720.55 (30), 719.41 (85), 694.38 ($[M+K]^+$, 30), 678.45 ($[M+Na]^+$, 90), 657.35 (30), 656.31 ($[M+H]^+$, 100). Anal. Calcd for $C_{37}H_{53}NO_9$: C, 67.76; H, 8.15. Found: C, 67.34; H, 8.37.

4.2.6. *N*-(Methoxycarbonylethyl)-7α,12α-diacetoxy-5β-cholan-24-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (10)

Acid **4** (300.0 mg, 0.52 mmol) was transformed into amide **10** (276.1 mg, 82%) according to general procedure using a suspension of 6 equiv methyl glycinate hydrochloride and 6 equiv Et_3N in dry CH_2Cl_2 (20 mL). Column chromatography: Lobar B, LichroPrep Si 60, eluent EtOAc/heptane = 95:5. Colorless foam, softening at 98–100 °C. $[\alpha]_D^{20}$ +36.09 (*c* 0.46, $CHCl_3$). IR(KBr): 3350m, 2951s, 2876w, 1736s, 1661m, 1539m, 1442m, 1377s, 1244s, 1205s, 1127w, 1076m, 1027m, 965w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 6.05–5.95 (m, HN–C(24)), 5.09 (br s, H–C(12)), 4.95–4.85 (m, H–C(7)), 4.04 (d, *J* = 5.2 Hz, H_2C–N), 3.77 (s, CH_3O_2C(26)), 2.14 (br s, CH_3COO–), 2.09 (br s, CH_3COO–), 0.95 (s, H_3C–C(10)), 0.82 (d, *J* = 6.0 Hz, H_3C–C(20)), 0.73 (H_3C–C(13)). ^{13}C NMR (50 Hz, $CDCl_3$): 173.41, 170.62, 108.22, 107.90, 75.27, 70.65, 52.35, 47.40, 45.01, 43.26, 41.11, 37.60, 34.63, 33.01, 31.24, 30.55, 28.40, 27.11, 25.67, 22.71, 22.07, 21.58, 21.0, 17.50, 12.16. (+)ESI LC–MS/MS (*m*/*z*): 652.2 (MH^+), 270.20 $(MH-38)^+$, 261.10 $(MH-391)^+$, 235.30 $(MH-417)^+$. Anal. Calcd for $C_{34}H_{53}NO_{11}$: C, 62.65; H, 8.20. Found: C, 62.31; H, 8.37.

4.2.7. *N*,*N*-Dimethyl-7α,12α-diacetoxy-5β-cholan-21-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (11)

Acid **4** (300.0 mg, 0.52 mmol) was transformed into amide **11** (276.1 mg, 86%) according to general procedure using a solution of 6 equiv $NH(CH_3)_2$ (156 μL, 3.10 mmol) in dry CH_2Cl_2 (20 mL). Column chromatography: Lobar B, LichroPrep Si 60, eluent EtOAc/heptane = 95:5. Colorless foam, softening at 93–94 °C. $[\alpha]_D^{20}$ +60.5 (*c* 0.076, $CHCl_3$). IR(KBr): 3475m, 2954s, 2875m, 1735s, 1649s, 1446w, 1377m, 1244s, 1204m, 1163w, 1127w, 1076w, 1027m, 943w, 891w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 5.10 (br s, H–C(12)), 4.91 (br s, H–C(7)), 3.00 (s, H_3C–N), 2.94 (s, H_3C–N), 2.13 (br s, CH_3COO–), 2.08 (br s, CH_3COO–), 0.95 (H_3C–C(10)), 0.83 (d, *J* = 6.2 Hz, H_3C–C(20)), 0.74 (H_3C–C(13)). ^{13}C NMR (50 Hz, $CDCl_3$): 173.33, 170.66, 108.21, 107.86, 75.25, 70.61, 47.56, 44.99, 43.23, 37.55, 37.22, 35.33, 34.82, 34.60, 30.87, 30.28, 28.37, 27.10, 25.66, 24.06, 22.73, 22.40, 22.05, 21.58, 21.40, 20.61, 17.63, 12.17. (+)ESI-MS (*m*/*z*): 672.48 (10), 671.38 (30), 631.43 (10), 630.35 ($[M+Na]^+$, 30), 608.38 ($[M+H]^+$, 100). Anal. Calcd for $C_{33}H_{53}NO_9$: C, 65.21; H, 8.79. Found: C, 64.80; H, 9.04.

4.2.8. 7α,12α-Diacetoxy-5β-cholan-24-hydrazide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (12)

Acid **4** (300.0 mg, 0.52 mmol) was transformed into amide **11** (182,6 mg, 59%) according to general procedure using a solution of 6 equiv $NH_2NH_2 \times 2HCl$ and 12 equiv Et_3N in dry CH_2Cl_2 (20 mL). Colorless solid, m.p. 174–177 °C. $[\alpha]_D^{20}$ +58.6 (*c* 0.07, $CHCl_3$). IR (KBr):3528m, 3496m, 3386m, 2950s, 2876w, 1737s, 1638m, 1446m, 1377s, 1244s, 1204m, 1165w, 1126w, 1076w, 1027m, 965w, 944w, 892w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 8.62 (br s, H_2N–N*H*–C(24)), 7.28 (s, *H*$_2$N–NH– C(24)), 5.08 (br s, H–C(12)), 4.92 (br s, H–C(7)), 2.13 (br s, CH_3COO–), 2.08 (br s, CH_3COO–), 0.94 (H_3C–C(10)), 0.81 (d, *J* = 5.6 Hz, H_3C–C(20)), 0.73 (H_3C–C(13)). ^{13}C NMR (50 Hz, $CDCl_3$): 170.62, 170.20, 108.21, 107.90, 75.20, 70.61, 47.32, 45.01, 43.26, 37.56, 34.62, 31.05, 30.88, 28.39, 27.13, 25.68, 22.69, 22.38, 22.07, 21.58, 21.43, 20.63, 17.48, 12.18. Anal. Calcd for $C_{31}H_{50}N_2O_9$: C, 62.61; H, 8.47. Found: C, 62.70; H, 8.35.

4.2.9. 7α,12α-Dihydroxy-5β-cholan-24-amide-3-spiro-6′-(3′,3′-dimethyl-1′,2′,4′,5′-tetraoxacyclohexane) (13)

Amide **5** (50 mg, 0.086 mmol) was hydrolyzed at 70 °C using NaOH (20.7 mg, 0.52 mmol) in MeOH/H_2O mixture (2 mL, 3:1, v/v). After 6 h, reaction was cooled and diluted with 20 mL H_2O and 50 mL CH_2Cl_2. Water layer was acidified with diluted HCl to pH 2, and layers were separated. Water layer was further extracted with CH_2Cl_2 (3× 30 mL), combined organic layers were washed with water and brine, dried over anhyd Na_2SO_4 and evaporated to dryness. Column chromatography. Lobar A, LichroPrep, eluent EtOAc/heptane = 9:1. Yield 42.7 mg (96%). Colorless foam, softening at 117–119 °C. $[\alpha]_D^{20}$ +25.0 (*c* 0.112, $CHCl_3$). IR(KBr): 3418m, 2940s, 2873m, 1666s, 1615w, 1460w, 1407w, 1377m, 1256w, 1204m, 1164w, 1123w, 1080m, 1042m, 980w, 946w, 922w cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): 6.24, 6.03 (both br s, H_2N–C(24)), 3.98 (br s, H–C(12)), 3.84 (br s, H–C(7)), 0.99 (d, *J* = 5.6, H_3C–C(10)), 0.92 (s, H_3C–C(20)), 0.68 (H_3C–C(13)). ^{13}C NMR (50 MHz, $CDCl_3$): 177.46, 108.73, 107.64, 73.05, 68.21, 46.40, 41.77, 39.17, 35.34, 35.05, 32.08, 31.39, 28.17, 27.57, 25.86, 23.16, 21.91, 17.30, 12.33. (+)ESI-MS (*m*/*z*): 1014.21 (40), 1013.39 ($[2M+Na]^+$, 100), 991.32 ($[2M]^+$, 30), 559.39 (70), 543.31 ($[M+K]^+$, 50), 496.36 ($[M+H]^+$, 5). Anal. Calcd for $C_{27}H_{45}NO_7$: C, 65.43; H, 9.15. Found: C, 65.13; H, 9.39.

4.3. In vitro antimalarial activity

The in vitro antimalarial drug susceptibility screen is a modification of the procedures first published by Desjardins et al.,[13] with modifications developed by Milhous et al.,[14] and the details are given in Ref. 4b. All 11 synthesized mixed tetraoxanes were screened in vitro against *P. falciparum* strains: CQ- and MFQ-susceptible strain D6 (clone of Sierra I/UNC isolate), CQ-resistant but MFQ-susceptible strain W2 (clone of Indochina I isolate), and CQ- and MFQ-resistant strain TM91C235 (clone of South-East Asian isolate). Compounds **9**, **12**, and **13** were additionally screened against CQ-resistant strain RCS (clone of South America isolate).

4.4. In vivo antimalarial activity

The *P. berghei* mouse efficacy tests were conducted using a modified version of the Thompson test. Basically, groups of five mice were inoculated intraperitoneally with erythrocytes infected with a drug-sensitive strain of *P. berghei* on day 0. For po administration, drugs were suspended in 0.5% hydroxyethylcellulose–0.1% Tween 80 and administered beginning on day 3 postinfection once a day. For sc administration, drugs were suspended in sesame oil, and administered beginning on day 3 postinfection two times a day. Dosings are given in Table 3. Cure was defined as survival until day 31 posttreatment. Untreated control mice usually die on day 6–8 postinfection.

4.5. In vitro metabolism studies

The metabolic stability assay sample preparation was performed in a 96-well plate on a TECAN Genesis robotic sample processor. All incubations were carried out in 0.1 M sodium phosphate

buffer (pH 7.4) in the presence of an NADPH-regenerating system ($NADP^+$ sodium salt, $MgCl_2 \cdot 6H_2O$, and glucose-6-phosphate). Test drug (10 μM), microsomes (1 mg/mL total protein), buffer, and NADPH-regenerating system were warmed to 37 °C, and the reaction was initiated by the addition of glucose-6-phosphate dehydrogenase (G6PD). Samples were quenched using an equal volume of cold methanol. Samples were centrifuged to pellet the proteins, and the supernatant was analyzed by LC–MS/MS using fast LC gradient or isocratic methods. Percentages of parent drug remaining at each time point were calculated using a ratio of the peak areas at each time point to the area of the time zero point. To calculate the half-life, a first-order rate of decay was assumed. A plot of the natural log (LN) of the drug concentration versus time was generated, where the slope of that line was $-k$. Half-life was calculated as $0.693/k$.

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