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Novel squaramides with in vitro liver stage antiplasmodial activity

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ABSTRACT

A structure–activity relationship study was performed with ten 8-aminoquinoline-squaramides compounds active against liver stage malaria parasites, using human hepatoma cells (Huh7) infected by *Plasmodium berghei* parasites. In addition, their blood-schizontocidal activity was assessed against chloroquine-resistant W2 strain *Plasmodium falciparum*. Compound **3** was 7.3-fold more potent than the positive control primaquine against liver-stage parasites, illustrating the importance of the squarate moiety to activity.

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1. Introduction

Malaria is the most deadly human protozoan infection. It is caused by parasites belonging to the genus *Plasmodium*, with an estimated 1.1 billion people at high risk of being infected and developing disease. There are five *Plasmodium* species that infect humans, with *Plasmodium falciparum* and *Plasmodium vivax* contributing the most to the public health burden posed by malaria.[1,2]

The life cycle of *Plasmodium* parasites includes infection of mammalian hosts and an invertebrate vector. In their mammalian host, sporozoites injected by an infected mosquito migrate to the liver, where they undergo an asymptomatic, yet obligatory phase of development inside hepatocytes.[3] This results in the formation of merozoites that emerge from hepatocytes and invade red blood cells to cause the symptoms of malaria.

Most drugs employed in the treatment of malaria act mainly as potent erythrocytic stage antimalarials.[4,5] However, to achieve malaria eradication, targeting the *Plasmodium* liver stage will be helpful.[6,7] Given the asymptomatic, yet obligatory nature of the liver stage, effective prophylaxis can be achieved by targeting *Plasmodium* liver forms. In addition, anti-liver stage drugs are essential to control *P. vivax* and *Plasmodium ovale* infections, since in these species latent hypnozoites may persist in the liver after *Plasmodium* bloodstream clearance, resulting in relapse and, therefore, representing a potential reservoir of infection.[8]

The 8-aminoquinoline derivative primaquine (**1**, Fig. 1) is the only available drug that is active against all *Plasmodium* exoerythrocytic forms, including latent hypnozoites and gametocytes that are transmitted to the *Anopheles* vector. In fact, the World Health Organization recommends primaquine for radical treatment of *P. vivax* infections and as a single dose for treatment of *P. falciparum* as a gametocidal agent.[1] More recently, other 8-aminoquinoline derivatives were developed, displaying better efficacy and lower toxicity, such as bulaquine (**2**), whose use has already been approved in India.[9] This scaffold continues to represent one of the most studied families of liver stage-targeting drugs, especially employing a hybridization strategy approach.[10–19] Nevertheless, finding new scaffolds capable of targeting the liver stage of infection continues to be challenging, due to technical difficulties in studying liver stage forms, as well as significant problems in the identification of valuable *Plasmodium*-specific liver stage targets.[8,20]

Squaramides represent a unique moiety capable of establishing up to four H-bonds, by acting as H-bond acceptor and donor and, therefore, they are widely used in organocatalysis,[21–24] in chemical biology (bioconjugation to proteins and carbohydrates, and as ion receptors)[22,23] and in medicinal chemistry (antiplasmodial,[25] antichagasic,[26] anticancer,[27,28] and antibacterial[29] activity).

We previously reported the antiplasmodial activity of squaramide derivatives.[30–32] In particular, squaramides linked to 4-aminoquinolines and squaramides linked to primaquine (**3**, **4**) were tested against the chloroquine-resistant W2 strain of *P. falciparum*.[30] The similarity of compound **3** to bulaquine prompted us

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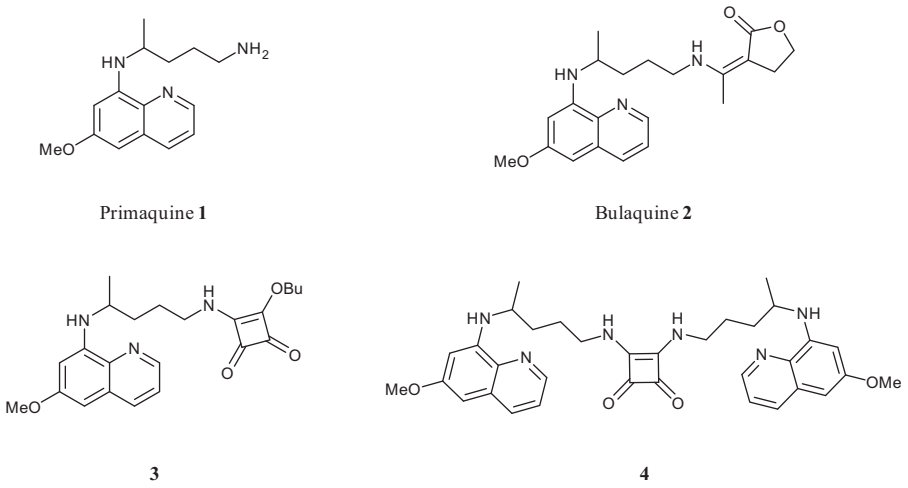


Figure 1. Chemical structure of 8-aminoquinoline derivatives with antimalarial activity.

to further explore the antiplasmodial potential of hybrid compounds containing an 8-aminoquinoline moiety linked to squaramide and to evaluate these novel compounds for their activity against *Plasmodium* liver stages. In order to perform a structure–activity relationship (SAR) study, particularly directed to understand the structural features for optimal liver stage activity, we synthesized compounds containing different linkers between the squaramide and the 8-aminoquinoline moieties, and different side chains attached to the squarate ring (Fig. 2).

2. Results and discussion

2.1. Chemistry

Compounds **5a**–**h** were synthesized starting from commercially available 6-methoxy-8-nitroquinoline (**6**), which was reduced by Pd/C catalyzed hydrogenation, forming 6-methoxy-8-aminoquinoline (**7**) in quantitative yield. Mono-squaramides **5a**–**d** were synthesized by reacting 8-aminoquinolines, with different linkers, with 3,4-dibutoxy-3-cyclobutene-1,2-dione (**8a**). To obtain the required starting materials, compounds **10a**–**d** were synthesized by reaction of compound **7** with different 2-bromoalkyl-isoindoline-1,3-diones (**9a**–**d**) in the presence of trimethylamine, as described previously[33] (Scheme 1). Starting materials **9a**–**b** were obtained commercially, while compounds **9c**–**d** were prepared by reacting the corresponding dibromoalkyl derivative with potassium isoindoline-2,3-dione in acetone.[34] Reflux of compounds **10** and hydrazine hydrate in ethanol led to compounds **11a**–**d** in 86–98% yields.[33] Mono-squaramides **5a**–**b** were synthesized by reacting compounds **11a**–**b** with **8a** in methanol, in low yields (21% and 39% yield, respectively), and compounds **5c**–**d** were synthesized by reacting **8a** with compounds **11c**–**d** in butanol, in the presence of triethylamine (45% and 35% yield, respectively). We also tried to synthesize the mono-squaramide **5** with R^1 = H, n = 3 (Scheme 1), but the corresponding starting material did not react with 3,4-dibutoxy-3-cyclobutene-1,2-dione (**8a**) (step d of Scheme 1).

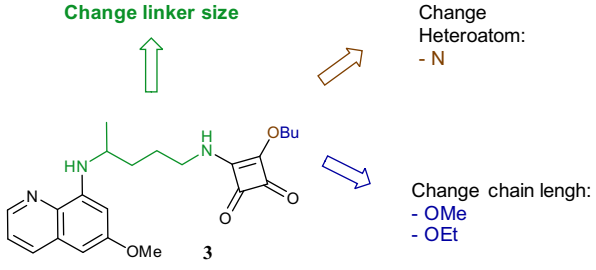


Figure 2. Synthetic strategy scope.

Compound **5e** was obtained by reaction of compound **7** with 3,4-dibutoxy-3-cyclobutene-1,2-dione (**8a**) in 50% yield (Scheme 1).

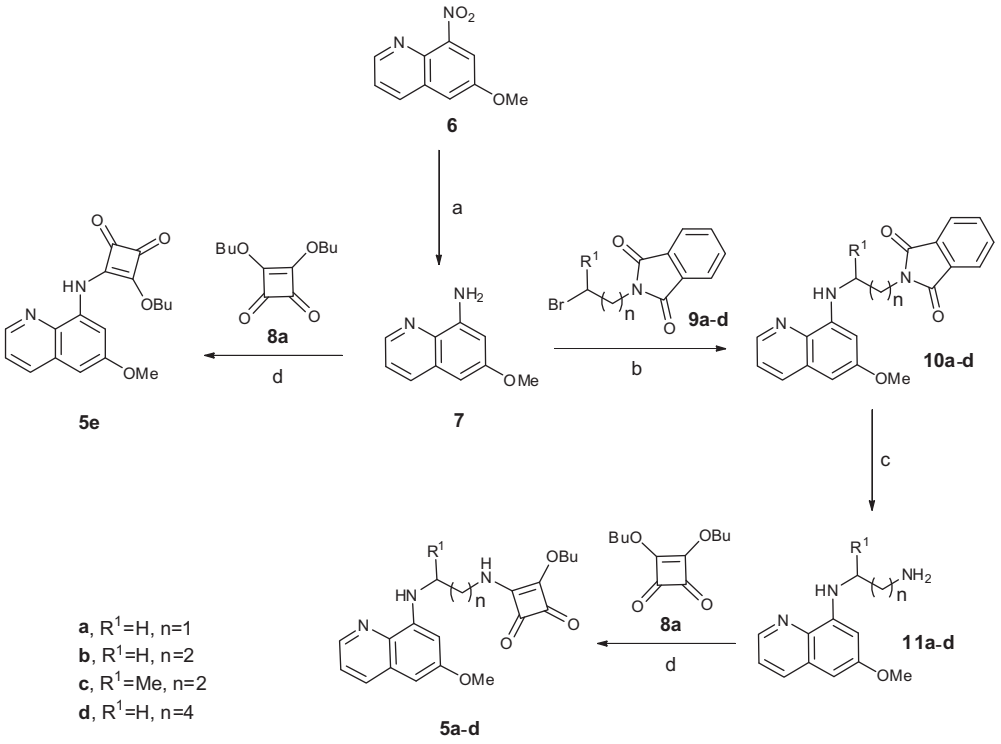
Mono-squaramides **5f**–**g**, with different alkoxy side chains, were also synthesized (Scheme 2). Compound **5f** was synthesized in 74% yield by reacting primaquine with 3,4-dimethoxy-3-cyclobutene-1,2-dione (**8b**) in methanol. The reaction of primaquine with 3,4-dibutoxy-3-cyclobutene-1,2-dione (**8a**) in ethanol gave rise to mono-squaramide **5g** as major product (61% yield) and as minor product, compound **3**.

Compound **5h**, with a *N*-butoxy side chain, was obtained by reaction of compound **5f** with *n*-butilamine, in the presence of triethylamine, with 86% yield (Scheme 2).

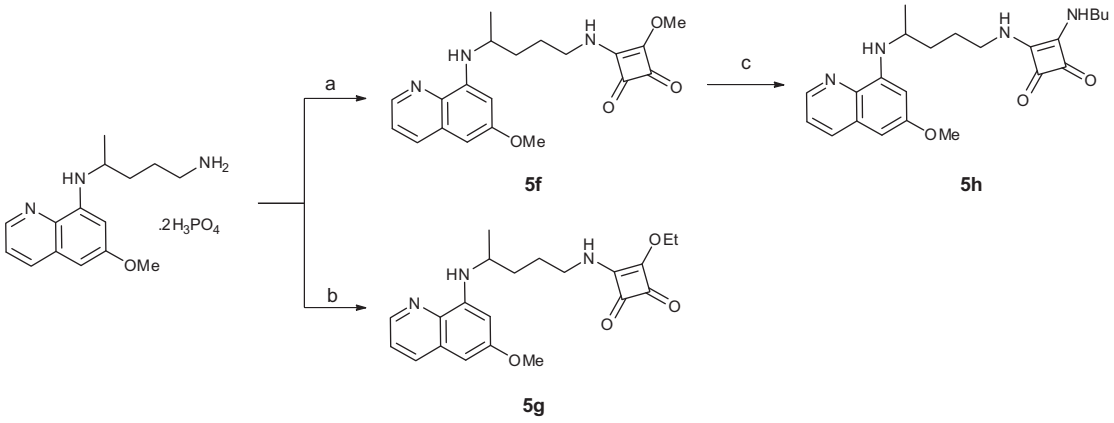
2.2. Biological activity and SAR study

The squaramide derivatives were evaluated for their ability to inhibit infection of the Huh7 human hepatoma cell line by *Plasmodium berghei* parasites. We started our work by testing the previously reported squaramides **3** and **4**. Both compounds had an IC_{50} value of ~1.3 μM, which represents a 7.3-fold increase in potency when compared to primaquine (Table 1). In addition, the IC_{50} of the starting compound **8a** was higher than 10 μM (Fig. 3), showing that linking primaquine and squaric moieties in the same molecule resulted in a more active compound. Furthermore, the initial observation that **3** and **4** are equipotent suggested that adding a second primaquine moiety to the squarate scaffold would not improve activity against the liver stage parasite. Therefore, our subsequent derivatizations focused only on the mono-squaramide derivative **3** (Fig. 2).

To investigate the importance of the linker between the 8-aminoquinoline and squaramide moieties, we synthesized and evaluated compounds **5a**–**e** (Fig. 3, Table 1). Derivative **5e**, without linker, was inactive up to 10 μM concentration. All derivatives with a linker were more active than **5e**, with an IC_{50} value lower than 5 μM. Varying the linker length from two to five carbon atoms revealed that the most potent derivatives were compounds **5a** (R^1 = H, n = 2) and **5d** (R^1 = H, n = 4), with an IC_{50} of 1.8 and 2.2 μM, respectively. Next, we evaluated the importance of the



Scheme 1. Synthesis of mono-squaramides. Reagents and conditions: **5a**–**e**: (a) 15% Pd/C, MeOH, H_2, rt, 24 h, 99%; (b) **9**, NEt_3, 120 °C, 24 h, 34–92%; (c) $NH_2NH_2{\cdot}H_2O$, EtOH, reflux, 15 h, 86–98%; (d) for compounds **5a**–**b**: MeOH, reflux, 16–24 h, 21–39%; for compounds **5c**–**d**: BuOH, reflux, 48 h, 35–45%; for compound **5e**: MeOH, reflux, 16 h, 50% yield.



Scheme 2. Synthesis of mono-squaramides. Reagent and conditons: **5f**–**h**: (a) NEt_3, **8b**, MeOH, reflux, 12 h, 79% yield; (b) NEt_3, **8a**, EtOH, reflux, 48 h, 61% yield; (c) *n*-butilamine, MeOH, reflux, 24 h, 86% yield.

length of the *O*-chain to activity. Since compound **3** was the most potent derivative, we synthesized two additional mono-squaramides with different alkoxy side chains: **5f** (OMe) and **5g** (OEt). Interestingly, the potency increased with an increase in the length of the carbon aliphatic chain.

Since squaric moieties may function as electrophilic warheads and, therefore, could potentially be involved in malarial protease inhibition, we decided to test the effect of replacing the OBu (**3**) side chain with NHBu (**5h**). In general, bis-squaramides appear to be quite stable to nucleophilic attack,[23] especially when compared to squarate esters, as expected since alkoxys are far better leaving groups. Furthermore, squaramides may also be less reactive due to stabilization by a gain in aromaticity when compared to the corresponding squarate esters.[22]

Compound **5h** was at least 5-fold less active than its OBu derivative counterpart **3**, displaying an activity similar to primaquine (Table 1). This observation, and the increase in potency noticed in derivatives with better leaving alkoxy groups (OBu > OEt > OMe) suggested that the mechanism of action may involve nucleophilic substitution. In addition, Huh7 cell proliferation was not significantly affected by squaramides **5a**–**h** at the concentrations tested, indicating selectivity towards liver stage *Plasmodium* forms (Fig. 3).

All derivatives were further evaluated for their blood schizonticidal activity against W2 strain chloroquine-resistant *P. falciparum*. Interestingly, for the mono-squaramide series, a three carbon linker (**5a**–**b**) was preferred over the primaquine-squaramide derivative **3**, with a 3.6-fold increase in potency (5.1 vs 18.6 μM,

Table 1
Squaramides antiplasmodial activities and cytotoxicity against Hek 293T cells

Compounds	Liver stage IC_{50}[a] (μM)	*Pf*W2 IC_{50}[b] (μM)	HEK 293T IC_{50}[c] (μM)	Log *P*[d]
3	1.3 ± 0.1	18.6[30]	ND	3.94
4	1.3 ± 0.1	2.2[30]	ND	5.63
5a	1.8 ± 0.6	5.1 ± 0.5	>100	3.36
5b	ND	5.1 ± 0.2	>100	3.79
5c	ND	8.5 ± 0.8	>100	3.08
5d	2.2 ± 0.3	>10	>100	4.12
5e	ND	>10	ND	3.38
5f	ND	>10	ND	3.19
5g	ND	7.3 ± 0.5	>100	3.42
5h	ND	>10	ND	3.79
Primaquine	9.5 ± 2.3	3.3[35]	ND	2.76
Bulaquine	10–20[36]	1.6 ± 0.3[36]	ND	3.45

[a] Data represents the mean ± SD of $n = 3$ independent experiments performed in triplicate obtained for inhibition of the infection of human hepatoma cells (Huh7) by *P berghei*; ND: not determined.
[b] Data represents the mean ± SD of $n = 2$ independent experiments performed in quadruplicate. *Pf*W2: *Plasmodium falciparum* W2 strain.
[c] Data represents the mean ± SD of $n = 2$ independent experiments performed in quadruplicate.
[d] Estimated by the ALOGPS 2.1 algorithm.[37]

respectively). Additionally, an ethoxy side chain was also favored, representing a 2.5-fold increase in activity (**5g** vs **3**). As dual stage antiplasmodial agents, the most promising compounds were mono-squaramide **5a** and bis-squaramide **4**. They showed similarly modest activity against blood stage parasites to that of primaquine, but at least a 5.3-fold increase in potency against liver stage forms.

Additionally, the in vitro cytotoxicity of compounds **5a–d** and **5g** was evaluated using HEK 293T cell lines. The 5 squaric derivatives tested had IC_{50} values higher than 100 μM which indicates they are non-cytotoxic at concentrations up to 100 μM (Table 1).

3. Summary and conclusions

Liver and blood stage antiplasmodial activities were assessed in vitro for novel 8-aminoquinoline-squaramides compounds. Compounds **3**, **4**, **5a** and **5d** had 4.3- to 7.3-fold more potency against the liver stage of *P. berghei* infection than primaquine or bulaquine, corroborating the importance of the squaramide moiety for anti-plasmodial activity. In the future, the synthesis of squaramide-primaquine derivatives may help to shed some light on the still unclear primaquine mechanism of action, by means of localization studies with already established squaramide-based fluorescent probes.[38]

4. Experimental section

All reagents and solvents were obtained from commercial suppliers and were used without further purification, with exception of NEt_3 and reaction solvents, which were dried prior to their use. Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected. The infrared spectra were collected on a Shimadzu FTIR Affinity-1 spectrophotometer. Merck Silica Gel 60 F254 plates were used for analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh) and Combi-Flash Rf from Teledyne ISCO (columns RediSep Rf, silica). Preparative TLC was performed on Merck Silica Gel 60 GF_{254} over glass plates with 1 mm thickness. ^1H and ^{13}C NMR spectra were recorded on a Bruker 300 Avance at 300 MHz (^1H NMR) and 75 MHz (^{13}C NMR). Data are reported as follows: chemical shift (δ), multiplicity (s: singlet, d: doublet, dd: doublet of doublet; t: triplet, m: multiplet, br: broad), coupling constants (J) in Hertz and integration. ^1H and ^{13}C chemical shifts are expressed in ppm using the solvent as internal reference. All new compounds showed purity ⩾90% by LC–MS, performed in a Waters Alliance 2695 HPLC with a LiCrospher® 100 RP-18 column (250 × 4 mm; 5 μm) at 35 °C, using as mobile phase a gradient from 95% solution A (Milli-Q water containing 0.5% formic acid (v/v)) to 95% solution B (acetonitrile), and employing a photodiode array detector to scan wavelength absorption from 210 to 600 nm; MS experiments were performed on Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) with an electrospray in positive ion mode (ESI +), ion source at 120 °C, capillary voltage of 3.0 kV and source voltage of 30 V, at the Liquid Chromatography and Mass Spectrometry Laboratory, Faculty of Pharmacy, University of Lisbon.

4.1. Synthesis of 8-amino-6-methoxyquinoline (7)

To a stirred solution of 6-methoxy-8-nitroquinoline (**6**, 500.0 mg, 2.449 mmol) in 10 mL of dry methanol, Pd/C (15% m/m, 75 mg) was slowly added. After purging the system with nitrogen, the reaction was stirred for 24 h under hydrogen atmosphere at room temperature. The catalyst was removed by vacuum filtra-

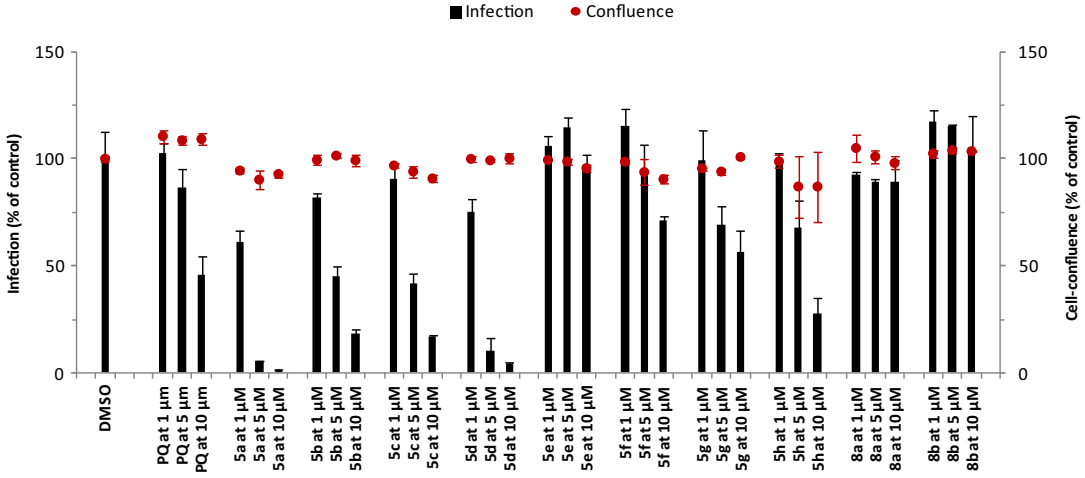


Figure 3. Luminescence-based measurements of dose-dependent effects of synthesized squaramides on luciferase-expressing *P. berghei*-infected Huh7 cells. Bars represent infection loads, as measured by bioluminescence of infected cell lysates, and dots represent cell confluence, as an estimate of the number of adherent cells in the culture dish, measured by Alamar Blue fluorescence. Results are expressed as mean ± SD (triplicate wells). PQ: primaquine, used as positive control. DMSO: solvent-treated control.

tion through Celite and the filtrate evaporated to afford compound **7** (lit. 39) as a dark colored oil; 421.5 mg (99% yield); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.60 (dd, J = 4.2, 1.6 Hz, 1H), 7.94 (dd, J = 8.3, 1.6 Hz, 1H), 7.31 (dd, J = 8.3, 4.2 Hz, 1H), 6.58 (d, J = 2.6 Hz, 1H), 6.47 (d, J = 2.6 Hz, 1H), 4.99 (br s, 2H), 3.87 (s, 3H).

4.2. General procedure for compounds 5a–b, 5e

A reaction mixture of 8-amino-6-methoxyquinoline derivative **7** or **11** (1 equiv), 3,4-dibutoxy-3-cyclobutene-1,2-dione (**8a**, 1.2 equiv) in dry methanol (1 ml/mmol of compound **7** or **11**) was heated at reflux for 16–24 h, under nitrogen atmosphere. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography on silica gel using as eluent a gradient from *n*-hexane (100%) to *n*-hexane/EtOAc (1:2), followed by preparative TLC (*n*-Hexane/EtOAC 2:1).

4.2.1. 3-Butoxy-4-({3-[(6-methoxyquinolin-8-yl)amino]propyl}amino)cyclobut-3-ene-1,2-dione (5a)

Following the general procedure, starting with **11a** (50.0 mg, 0.216 mmol), **8a** (56 µL, 0,259 mmol) in 2.2 ml of dry methanol (reaction time: 24 h), compound **5a** was obtained as brown oil (32.3 mg, 0.084 mmol, 39% yield). IR (KBr, selected peaks): 2928, 1802, 1701, 1597, 1522 cm^{-1}; ^{1}H NMR (300 MHz, Acetone-d_6) δ (ppm): 8.52 (dd, J = 4.2, 1.5 Hz, 1H, H_{Ar}), 8.04 (d, J = 8.3 Hz, 1H, H_{Ar}), 7.88–7.58 (m, 1H, NH), 7.38 (dd, J = 8.3, 4.2 Hz, 1H, H_{Ar}), 6.48 (d, J = 2.4 Hz, 1H, H_{Ar}), 6.45 (br s, 1H, NH), 6.29 (d, J = 2.4 Hz, 1H, H_{Ar}), 4.69–4,54 (m, 2H, OCH_2CH_2), 3.86 (s, 3H, OCH_3), 3.86–3.77 (m, 1H, NH_2, partially obscured by OCH_3 signal), 3.72–3.60 (m, 1H, NCH_2), 3.47 (q, J = 6.4 Hz, 1H, NCH_2), 2.16–2.07 (m, 2H, CH_2), 1.79–1.59 (m, 2H, OCH_2CH_2), 1.48–1.30 (m, 2H, CH_2CH_3), 0.87 (t, J = 7.2 Hz, CH_2CH_3); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 189.90 (C=O), 183.92 (C=O), 177.82 (Cq), 173.98 (Cq), 160.49 (Cq_{Ar}), 146.63 (Cq_{Ar}), 145.11 (CH_{Ar}), 136.18 (Cq_{Ar}), 135.48 (CH_{Ar}), 130.81 (Cq_{Ar}), 122.79 (CH_{Ar}), 97.06 (CH_{Ar}), 92.69 (CH_{Ar}), 73.38 ($CH_{2\text{-butoxi}}$), 55.45 (OCH_3), 43.09 (NCH_2), 40.58 (NCH_2), 32.69 ($CH_{2\text{-butoxi}}$), 30.55 (CH_2), 19.21 ($CH_{2\text{-butoxi}}$), 13.90 (CH_3). MS (ESI) m/z calcd for $C_{21}H_{25}N_3O_4$ 383, found 384 $[M+H]^+$.

4.2.2. 3-Butoxy-4-({3-[(6-methoxyquinolin-8-yl)amino]butyl}amino)cyclobut-3-ene-1,2-dione (5b)

Following the general procedure, starting with **11b** (44.1 mg, 0.180 mmol), **8a** (47 µL, 0,216 mmol) in 1.8 ml of dry methanol (reaction time: 24 h), compound **5b** was obtained as brown oil (15.0 mg, 21% yield). IR (KBr, selected peaks): 2926, 1802, 1701, 1605, 1522 cm^{-1}; ^{1}H NMR (300 MHz, Acetone-d_6) δ (ppm): 8.52 (dd, J = 4.2, 1.5 Hz, 1H, H_{Ar}), 8.04 (dd, J = 8.3, 1.3 Hz, 1H, H_{Ar}), 7.67 (br s, 1H, NH), 7.38 (dd, J = 8.3, 4.2 Hz, 1H, H_{Ar}), 6.48 (d, J = 2.5 Hz, 1H, H_{Ar}), 6.29 (d, J = 2.5 Hz, 1H, H_{Ar}), 6.17 (d, J = 8.9 Hz, 1H, NH), 4.66–4.32 (m, 2H, OCH_2CH_2), 3.94–3.75 (m, 5H, OCH_3, CH, NCH_2), 3.73–3.56 (m, 1H, NCH_2), 2.15–1.95 (m, $CHCH_2$, partially obscured by acetone) 1.78–1.49 (m, 2H, OCH_2CH_2), 1.40–1.24 (m, 5H, CH_2CH_3, $CHCH_3$), 0.96–0.78 (m, 3H, CH_2CH_3). ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 189.89 (C=O), 183.84 (C=O), 177.74 (Cq), 173.84 (Cq), 160.48 (Cq_{Ar}), 145.80 (Cq_{Ar}), 145.07 (CH_{Ar}), 136.18 (Cq_{Ar}), 135.53 (CH_{Ar}), 130.92 (Cq_{Ar}), 122.80 (CH_{Ar}), 97.42 (CH_{Ar}), 92.61 (CH_{Ar}), 73.27 ($CH_{2\text{-butoxi}}$), 55.46 (OCH_3), 46.15 (CH), 42.58 (NCH_2), 38.17 (CH_2), 32.56 ($CH_{2\text{-butoxi}}$), 20.89 ($CHCH_3$), 19.18 ($CH_{2\text{-butoxi}}$), 13.88 ($CH_{3\text{-butoxi}}$). MS (ESI) m/z calcd for $C_{22}H_{27}N_3O_4$: 397, found 398 $[M+H]^+$.

4.2.3. 3-Butoxy-4-[(6-methoxyquinolin-8-yl)amino]cyclobut-3-ene-1,2-dione (5e)

Following the general procedure, starting with **7** (52,7 mg, 0.303 mmol), **8a** (79 µL, 0,364 mmol) in 3.0 ml of dry methanol (reaction time: 16 h), compound **5e** was obtained as a yellow solid (49.0 mg, 0.150 mmol, 50% yield). Mp 138–139 °C; IR (KBr, selected peaks): 3726, 2962, 1813, 1723, 1589, 1526, 1345 cm^{-1}; ^{1}H NMR (300 MHz, Acetone-d_6) δ (ppm): 9.60 (br s, 1H, NH), 8.71 (dd, J = 4.2, 1.6 Hz, 1H, H_{Ar}), 8.24 (dd, J = 8.4, 1.6 Hz, 1H, H_{Ar}), 7.91 (d, J = 2.4 Hz, 1H, H_{Ar}), 7.55 (dd, J = 8.4, 4.2 Hz, 1H, H_{Ar}), 7.05 (d, J = 2.4 Hz, 1H, H_{Ar}), 4.87 (t, J = 6.5 Hz, 2H, OCH_2CH_2), 3.94 (s, 3H, OCH_3), 1.95–1.83 (m, 2H, OCH_2CH_2), 1.63–1.48 (m, 2H, CH_2CH_3), 1.01 (t, J = 7.4 Hz, 3H, CH_2CH_3).[13] ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 188.19 (C=O), 185.54 (C=O), 180.47 (Cq), 169.39 (Cq), 159.18 (Cq_{Ar}), 147.32 (CH_{Ar}), 136.16 (CH_{Ar}), 135.55 (Cq_{Ar}), 135.32 (Cq_{Ar}), 130.52 (Cq_{Ar}), 123.69 (CH_{Ar}), 108.19 (CH_{Ar}), 101.32 (CH_{Ar}), 74.78 (OCH_2), 56.12 (OCH_3), 32.73 (CH_2), 19.30 (CH_2), 13.93 (CH_3). MS (ESI) m/z calcd for $C_{18}H_{18}N_2O_4$: 326, found 327 $[M+H]^+$.

4.3. General procedure for compounds 5c–d

A reaction mixture of 8-amino-6-methoxyquinoline derivative **11** (1.0 equiv), 3,4-dibutoxy-3-cyclobutene-1,2-dione (**8a**, 1.2 equiv), and NEt_3 (1.0 equiv) in dry butanol (1 ml/mmol of compound **11**) was heated at reflux for 48 h, under nitrogen atmosphere. The solvent was removed under reduced pressure and the residue obtained dissolved in EtOAc. The mixture was then washed with water (2×), dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure. The crude product was purified by flash chromatography on silica gel using as eluent a gradient from *n*-hexane (100%) to *n*-hexane/EtOAc (1:2), followed by preparative TLC (*n*-Hexane/EtOAC 2:1).

4.3.1. 3-Butoxy-4-({2-[(6-methoxyquinolin-8-yl)amino]ethyl}amino)cyclobut-3-ene-1,2-dione (5c)

Following the general procedure, starting with **11c** (51.4 mg, 0.237 mmol), **8a** (61 µL, 0.284 mmol), and NEt_3 (33 µL, 0.237 mmol) in 2.4 mL of dry butanol, compound **5c** was obtained as brown oil (39.1 mg, 0.106 mmol, 45% yield). IR (KBr, selected peaks): 3254, 2930, 1802, 1701, 1597, 1522 cm^{-1}; ^{1}H NMR (300 MHz, Acetone) δ (ppm): 8.50 (dd, J = 4.2, 1.6 Hz, 1H, H_{Ar}), 8.05 (d, J = 8.3, 1H, H_{Ar}), 7.87–7.70 (m, 1H, NH), 7.38 (dd, J = 8.3, 4.2 Hz, H_{Ar}), 6.66 (br s, 1H, NH), 6.51 (s, 1H, H_{Ar}), 6.41 (s, 1H, H_{Ar}), 4.65–4.46 (m, 2H, OCH_2CH_2), 4.02–3.91 (m, 1H, NCH_2), 3.87 (s, 3H, OCH_3), 3.84–3.73 (m, 1H, NCH_2), 3.72–3.61(m, 2H, NCH_2), 1.77–1.58 (m, 2H, OCH_2CH_2), 1.47–1.31 (m, 2H, CH_2CH_3), 0.90 (dd, J = 7.4 Hz, CH_2CH_3); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 189.82 (C=O), 183.87 (C=O), 177.91 (Cq), 174.28 (Cq), 160.47 (Cq_{Ar}), 146.34 (Cq_{Ar}), 145.19 (CH_{Ar}), 136.20 (Cq_{Ar}), 135.49 (CH_{Ar}), 130.84 (Cq_{Ar}), 122.84 (CH_{Ar}), 97.36 (CH_{Ar}), 93.06 (CH_{Ar}), 73.39 ($CH_{2\text{-butoxi}}$), 55.49 (OCH_3), 44.12 (NCH_2), 44.01 (NCH_2), 32.59 ($CH_{2\text{-butoxi}}$), 19.21 ($CH_{2\text{-butoxi}}$), 13.93 (CH_3). MS (ESI) m/z calcd for $C_{20}H_{23}N_3O_4$: 369, found 370 $[M+H]^+$.

4.3.2. 3-Butoxy-4-({5-[(6-methoxyquinolin-8-yl)amino]pentyl}amino)cyclobut-3-ene-1,2-dione (5d)

Following the general procedure, starting with **11d** (153.4 mg, 0.591 mmol), **8a** (153 µL, 0.284 mmol), and NEt_3 (82 µL, 0.591 mmol) in 5.9 mL of dry butanol, compound **5d** was obtained as light brown semi-solid (84.9 mg, 0.206 mmol, 35% yield). IR (KBr, selected peaks): 3257, 2933, 1807, 1703, 1613, 1519, 1349 cm^{-1}; ^{1}H NMR (300 MHz, Acetone-d_6) δ (ppm): 8.51 (dd, J = 4.2, 1.6 Hz, 1H, H_{Ar}), 8.04 (dd, J = 8.3, 1.6 Hz, 1H, H_{Ar}), 7.74–7.48 (m, 1H, NH), 7.37 (dd, J = 8.3, 4.2 Hz, 1H, H_{Ar}), 6.47 (d, J = 2.5 Hz, 1H, H_{Ar}), 6.30 (br s, 1H, NH), 6.27 (d, J = 2.5 Hz, 1H, H_{Ar}), 4.72–4,59 (m, 2H, OCH_2CH_2), 3.86 (s, 3H, OCH_3), 3.75–3.63 (m, 1H, NCH_2), 3.58–3.44 (m, 1H, NCH_2), 3.38–3.27 (m, 2H, NCH_2), 1.87–1.67 (m, 6H, $2NCH_2CH_2$, OCH_2CH_2), 1.65–1.50 (m, 2H, CH_2), 1.50–1.35 (m, 2H, CH_2CH_3), 0.92 (t, J = 7.4 Hz, 3H, CH_2-CH_3). ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 189.99 (C=O), 183.85 (C=O), 177.77 (Cq), 173.94 (Cq), 160.56 (Cq_{Ar}), 146.85

(Cq_{Ar}), 145.10 (CH_{Ar}), 136.15 (Cq_{Ar}), 135.47 (CH_{Ar}), 130.80 (Cq_{Ar}), 122.78 (CH_{Ar}), 96.95 (CH_{Ar}), 92.54 (CH_{Ar}), 73.35 ($CH_{2\text{-butoxi}}$), 55.44 (OCH_3), 45.07 (NCH_2), 43.53 (NCH_2), 32.75 ($CH_{2\text{-butoxi}}$), 31.17 (CH_2), 29.25 (CH_2), 24.70 (CH_2), 19.28 ($CH_{2\text{-butoxi}}$), 13.90 (CH_3). MS (ESI) *m*/*z* calcd for $C_{23}H_{29}N_3O_4$ $[M+H]^+$ 412, found 412.

4.4. General procedure for derivatives 5f–g

A reaction mixture of primaquine diphosphate (1.2 equiv), and NEt_3 (2.4 equiv) in dry solvent (1 mL/3-cyclobutene-1,2-dione derivative **8**) was stirred for 30 min at room temperature under nitrogen atmosphere, before addition of 3-cyclobutene-1,2-dione derivative **8** (1.0 equiv). After heating at reflux for 12–48 h, the solvent was removed under reduced pressure and the residue obtained dissolved in EtOAc. The mixture was then washed with water (2×) and dried over anhydrous Na_2SO_4. The crude product was purified by flash chromatography on silica gel using as eluent a gradient from *n*-hexane (100%) to *n*-hexane/EtOAc (1:2), followed by preparative TLC (*n*-Hexane/EtOAC 2:1).

4.4.1. 3-Methoxy-4-({4-[(6-methoxyquinolin-8-yl)amino] pentyl}amino)cyclobut-3-ene-1,2-dione (5f)

Following the general procedure, starting with primaquine diphosphate (192.2 mg, 0.422, 1.2 equiv), NEt_3 (118 μL, 0.845 mmol, 2.4 equiv) in 3.5 mL of dry methanol, and then 3,4-dimethoxy-3-cyclobutene-1,2-dione (**8b**, 50 mg, 0,352 mmol, 1.0 equiv) (reaction time: 12 h), compound **5f** was obtained as a light yellow solid (102.7 mg, 0.278 mmol, 79% yield). Mp: 139–141 °C; IR (KBr, selected peaks): 3272, 2971, 1802, 1696, 1614, 1521, 1382 cm^{-1}, ^1H NMR (300 MHz, $CDCl_3$) δ (ppm): 8.52 (dd, *J* = 4.2, 1.6 Hz, 1H, H_{Ar}), 7.92 (dd, *J* = 8.3, 1.6 Hz, 1H, H_{Ar}), 7.31 (dd, *J* = 8.3, 4.2 Hz, 1H, H_{Ar}), 6.71 (br s, 1H, NH), 6.34 (d, *J* = 2.4 Hz, 1H, H_{Ar}), 6.26 (d, *J* = 2.4 Hz, 1H, H_{Ar}), 5.96 (d, *J* = 8.3 Hz, 1H, NH), 4.30 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 3.73–3.56 (m, 1H, CH), 3.49–3.35 (m, 1H, NCH_2), 1.84–1.62 (m, 4H, $2CH_2$), 1.30 (d, *J* = 6.4 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 189.60 (C═O), 183.01 (C═O), 177.69 (Cq), 172.26 (Cq), 159.48 (Cq_{Ar}), 144.88 (Cq_{Ar}), 144.53 (CH_{Ar}), 135.39 (Cq_{Ar}), 135.03 (CH_{Ar}), 130.04 (Cq_{Ar}), 122.07 (CH_{Ar}), 97.03 (CH_{Ar}), 91.96 (CH_{Ar}), 60.52 (OCH_3), 55.36 (OCH_3), 47.71 (CH), 44.87 (CH_2), 33.27 (CH_2), 27.27 (CH_2), 20.82 (CH_3). MS (ESI) *m*/*z* calcd for $C_{20}H_{23}N_3O_4$: 369, found 370 $[M+H]^+$.

4.4.2. 3-Ethoxy-4-({4-[(6-methoxyquinolin-8-yl)amino]pentyl} amino)cyclobut-3-ene-1,2-dione (5g)

Following the general procedure, starting with primaquine diphosphate (253.0 mg, 0.556 mmol), NEt_3 (155 μL, 1.111 mmol) in 4.6 mL of dry ethanol, and then 3,4-dibutoxy-3-cyclobutene-1,2-dione (**8a**, 100 μL, 0.463 mmol) (reaction time: 48 h), compound **5g** was obtained as a yellow solid (108.5 mg, 0.283 mmol, 61% yield). Mp: 134–136 °C; IR (KBr, selected peaks): 3294, 2970, 1800, 1702, 1611, 1520, 1385, 1344 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 8.38 (dd, *J* = 4.2, 1.6 Hz, 1H, H_{Ar}), 7.90 (dd, *J* = 8.3, 1.6 Hz, 1H, H_{Ar}), 7.62–7.34 (m, 1H, NH), 7.24 (dd, *J* = 8.3, 4.2 Hz, 1H, H_{Ar}), 6.33 (d, *J* = 2.4 Hz, 1H, H_{Ar}), 6.16 (d, *J* = 2.4 Hz, 1H, H_{Ar}), 6.01 (d, *J* = 8.6 Hz, 1H, NH), 4.52 (q, *J* = 7.0 Hz, 2H, OCH_2), 3.65–3.50 (m, 2H, CH, NCH_2), 3.43–3.32 (m, 1H, NCH_2), 1.77–1.48 (m, 4H, $2CH_2$), 1.23 (t, *J* = 7.0 Hz, 3H, $CHCH_3$), 1.17 (d, *J* = 6.3 Hz, 3H, OCH_2CH_3); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 189.91 (C═O), 183.79 (C═O), 177.64 (Cq), 174.01 (Cq), 160.52 (Cq_{Ar}), 145.93 (CH_{Ar}), 145.03 (Cq_{Ar}), 136.15 (Cq_{Ar}), 135.53 CH_{Ar}), 130.91 (Cq_{Ar}), 122.80 (CH_{Ar}), 97.34 (CH_{Ar}), 92.44 (CH_{Ar}), 69.61 ($CH_{2\text{-ethoxy}}$), 55.43 (OCH_3), 48.25 (CH), 45.10 (NCH_2), 34.02 (CH_2), 28.07 (CH_2), 20.81 ($CHCH_3$), 16.04 ($CH_{3\text{-ethoxy}}$). MS (ESI) *m*/*z* calcd for $C_{21}H_{25}N_3O_4$ $[M+H]^+$ 384, found 384.

4.5. Synthesis of 3-(butylamino)-4-({4-[(6-methoxyquinolin-8-yl)amino]pentyl}amino)cyclobut-3-ene-1,2-dione (5h)

A reaction mixture of compound **5f** (150.0 mg, 0.406 mmol, 1.0 equiv), *n*-butylamine (48 μL, 0.487 mmol, 1.2 equiv), and NEt_3 (68 μL, 0.487 mmol, 1.2 equiv) in 4.1 mL of dry methanol was heated at reflux for 24 h, under nitrogen atmosphere. The solvent was removed under reduced pressure and the residue obtained dissolved in EtOAc. The mixture was then washed with water (2×) and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography on silica gel using as eluent a gradient from *n*-hexane (100%) to *n*-hexane/EtOAc (1:2), followed by preparative TLC (*n*-Hexane/EtOAC 2:1). Compound **5h** was obtained as a yellow solid (144.0 mg, 0.351 mmol, 86% yield). Mp: 109–111 °C; IR (KBr, selected peaks): 3174, 2958, 1801, 1649, 1576, 1387, 1159 cm^{-1}; ^1H NMR (300 MHz, Acetone-d_6) δ (ppm): 8.50 (dd, *J* = 4.2, 1.6 Hz, 1H, H_{Ar}), 8.02 (dd, *J* = 8.3, 1.6 Hz, 1H, H_{Ar}), 7.36 (dd, *J* = 8.3, 4.2 Hz, 1H, H_{Ar}), 7.01 (t, *J* = 5.6 Hz, 1H, NH), 6.94 (t, *J* = 5.6 Hz, 1H, NH), 6.46 (d, *J* = 2.4 Hz, 1H, H_{Ar}), 6.30 (d, *J* = 2.4 Hz, 1H, H_{Ar}), 6.13 (d, *J* = 8.7 Hz, 1H, NH), 3.85 (s, 3H, OCH_3), 3.76–3.65 (m, 3H, CH, NCH_2), 3.64–3.54 (m, 2H, NCH_2), 1.86–1.66 (m, 4H, $2CH_2$), 1.62–1.50 (m, 2H, CH_2), 1.42–1.31 (m, 2H, CH_2CH_3), 1.28 (d, *J* = 6.3 Hz, 3H, $CHCH_3$), 0.89 (t, *J* = 7.3 Hz, 3H, CH_2CH_3); ^{13}C NMR (75 MHz, Acetone-d_6) δ (ppm): 183.86 (2C═O), 169.05 (Cq), 168.91 (Cq), 160.53 (Cq_{Ar}), 145.94 (CH_{Ar}), 145.01 (Cq_{Ar}), 136.14 (Cq_{Ar}), 135.50 (CH_{Ar}), 130.90 (Cq_{Ar}), 122.78 (CH_{Ar}), 97.33 (CH_{Ar}), 92.47 (CH_{Ar}), 55.44 (OCH_3), 48.30 (CH), 44.70 (NCH_2), 44.42 (NCH_2), 34.22 (CH_2), 34.17 (CH_2), 28.86 (CH_2), 20.81 ($CHCH_3$), 20.16 (CH_2), 13.96 (CH_3). MS (ESI) *m*/*z* calcd for $C_{23}H_{30}N_4O_3$: 410, found 411 $[M+H]^+$.

4.6. In vitro activity against liver-stage *P. berghei*

Huh-7 cells, a human hepatoma cell line, were cultured in 1640 RPMI medium supplemented with 10% v/v fetal calf serum (FCS), 1% v/v nonessential amino acids, 1% v/v penicillin/streptomycin, 1% v/v glutamine, and 10 mM HEPES, pH 7, and maintained at 37 °C with 5% CO_2. Inhibition of *P. berghei* liver-stage infection was determined by measuring the luminescence of Huh-7 cell lysates 48 h after infection with a firefly luciferase-expressing *P. berghei* line, PbGFP-Luccon, as previously described.[40,41] Briefly, cells (12×10^3 cells per well) were seeded in 96-well plates the day before drug treatment and infection. Test compounds were prepared as 10 mM stock solutions in MeOH and diluted with medium to desired concentrations. Medium was replaced by fresh medium containing the appropriate concentration of each compound 1 h prior to infection. Sporozoites (10,000 spz per well), freshly obtained through disruption of salivary glands of infected female *Anopheles stephensi* mosquitoes, were added to the wells 1 h after compound addition, followed by centrifugation at 1700 g for 5 min and incubation at 37 °C, with 5% CO_2. At 24 h post-infection, medium was again replaced by fresh medium containing the appropriate concentration of each compound. Parasite load was determined 48 h after infection by luminescence measurement by using the Biotium Firefly Luciferase Assay Kit. The effect of the compounds on the viability of Huh-7 cells was assessed by the Alamar Blue assay (Invitrogen, UK) following the manufacturer's protocol. Nonlinear regression analysis was employed to fit the normalized results of the dose–response curves, and IC_{50} values were determined with SigmaPlot software.

4.7. Activity against erythrocyte-stage *P. falciparum*

Human erythrocytes infected with 1% ring-stage W2-strain *P. falciparum* synchronized with 5% sorbitol were incubated with test

compounds in 96-well plates at 37 °C for 48 h in RPMI-1640 medium, supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 10% heat inactivated human serum (or 0.5% Albumax, 2% human serum), and 100 μM hypoxanthine under an atmosphere of 3% O_2, 5% CO_2, and 91% N_2. After 48 h, the cells were fixed in 2% formaldehyde in phosphate-buffered saline (PBS) and transferred into PBS with 100 mM NH_4Cl, 0.1% Triton X-100, 1 nM YOYO-1, and infected erythrocytes were counted in a flow cytometer (FACSort, Beckton Dickinson; EX 488 nm, EM 520 nm). IC_{50}s based on comparisons with untreated control cultures were calculated with GraphPad PRISM software. Two independent experiments were performed, each with four replicates for each of the experimental conditions.

4.8. In vitro cytotoxicity

Cytotoxicity was assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT). The day before experiments HEK 293T human embryonic kidney epithelial cells (ATCC CRL-11268) were seeded in 96 well tissue culture plates, in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units of penicillin G (sodium salt), 100 μg of streptomycin sulfate and 2 mM L-glutamine, at 2×10^4 cells per well. Test compounds were diluted in DMSO, serially diluted in the culture medium and added to the cells. Each compound concentration was tested in quadruplicate in a single experiment which was repeated at least 2 times; controls contained equivalent concentrations of DMSO. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 48 h, cell medium was removed and replaced with fresh medium, MTT was added to each well (5 mg/mL in 10 mM phosphate buffer at pH 7.4), and after 3 h the medium was removed and intracellular formazan crystals were solubilized and extracted with DMSO. After 15 min at room temperature absorbance was measured at 570 nm in a microplate reader (Infinite M200, Tecan, Austria), and the percentage of viable cells was determined as described previously.[30] IC_{50}s were determined by non-linear regression using GraphPad PRISM software.

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