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Antimalarial and anti-inflammatory activities of new chloroquine and primaquine hybrids: Targeting the blockade of malaria parasite transmission

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ABSTRACT

Malaria is a disease that requires new drugs not only to fight *Plasmodium* but also to reduce symptoms of infection such as fever and inflammation. A series of 21 hybrid compounds were designed from chloroquine (CQ) and primaquine (PQ) linked to the pharmacophoric group present in phenylacetic anti-inflammatory drugs. These compounds were designed to have dual activity: namely, to be capable of killing *Plasmodium* and still act on the inflammatory process caused by malaria infection. The compounds were assayed with nine different biological methods. The carbonylated CQ derivative **6** (n = 3; R_1 = Cl) was more potent than CQ *in vitro*, and **8** (n = 4; R_1 = H) reduced *P. berghei* parasitemia up to 37% on day 7. The carbonylated PQ derivative **17** (R = Br) was slightly less potent than PQ. The *gem*-difluoro PQ derivative **20** (R = Cl) exhibited high transmission blockade of the malaria sporogonic cycle in mosquitoes. Compounds **6** and **20** dose-dependently reduced nitric oxide (NO) production and inhibited TNFα production by LPS-stimulated J774A.1 macrophages. Our results indicate a viable and interesting approach in planning new chemical entities that act as transmission-blocking drugs for treating malaria caused by *P. falciparum* and *P. vivax* and the anti-inflammatory process related to this disease.

1. Introduction

Malaria is still the most important parasitic disease worldwide and a major public health problem. Data from the World Health Organization (WHO) have revealed a significant reduction in the number of cases of malaria across the globe. In 2018, a total of 228 million cases of malaria were reported by the WHO, compared with 251 million cases in 2010 and 231 million cases in 2017, with an estimated 405,000 deaths yearly[1] However, 405,000 malaria deaths were still recorded worldwide in 2018.[1]

Quinolines are among the most widely used drugs for malaria treatment. Quinine was first used to treat malaria followed by other quinoline synthetic derivatives, such as chloroquine (CQ) and primaquine (PQ) (Fig. 1).[2] For treating malaria, CQ is one of the safest, financially affordable and effective drugs against the different species of *Plasmodium* and has been the first treatment choice.

However, its abusive use has led to the emergence of chloroquine-resistant strains (CQR), making it ineffective in many parts of the world.[3–6] There is a widespread resistance of the parasite, which threatens the effectiveness of drug treatment for malaria.[7] This resistance is defined as a delay in the parasite elimination rate after the administration of artemisinin derivatives,[8–11] the which justifies the development of new antimalarials. The 8-aminoquinoline PQ and recently approved tafenoquine are both the only drug options for treating relapsing *P. vivax* malaria and act as transmission blocking drugs, despite their side effects.[12,13]

Killing the parasite that causes malaria is the main goal of any antimalarial drug candidate. However, the most serious manifestation of this disease is cerebral malaria (CM), which is associated with increased levels of inflammatory mediator cytokines and chemokines.[14] Severe

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cases of malaria are life threatening and demand effective treatment.[15] Thus, a new chemical entity with dual activities that can combat the parasitic *Plasmodium spp* and attenuate the hazardous inflammation caused by the parasitic infection could be considered as an interesting approach in innovative pharmaceutical research. One drug with this scope in the market for malaria chemotherapy could allow a revolution in the treatment of patients, ensuring an improvement in the quality of their lives. We previously used this approach in developing some hybrids from aminoquinolines and atorvastatin[16] since this statin drug reduces systemic and brain inflammation and is used as an adjuvant in CM treatment.[17]

Additionally, PQ is a drug that also prevents the transmission of the parasite, acting against gametocytes, which is the sexual form of the parasite responsible for its development in mosquito vectors.[18] Dual-activity antimalarial drugs able to kill the parasite in the human blood and block transmission, i.e., those that can target both asexual parasites and gametocytes, should be value and required.[19]

Molecular hybridization consists of linking two pharmacophoric groups of distinct compounds into a single molecule.[20] This new molecule, now called a hybrid, may exhibit equal or better activity than the precursor compound. It can act by the same mechanism of action as the precursor substance or in different ways, thus making it possible to avoid the mechanisms of resistance.[21] These characteristics are important in regard to obtaining drugs for the treatment of endemic diseases in neglected populations, as is the case among patients with malaria. We have exhaustively used the hybridization approach to obtain many different hybrids against *P. falciparum*.[22] CQ and sulfadoxine hybrids were designed, which gave rise to a prototype that was more active than both precursor drugs.[23] Among the nonquinoline derivatives, we obtained new compounds, namely, inhibitors of the *P. falciparum* dihydroorotate dehydrogenase (*Pf*DHODH) enzyme. These triazolopyrimidine and pyrazolopyrimidine derivatives are prototypes used to synthesize new compounds.[24,25]

To plan our hybrids (**1**–**21**, Fig. 1), compounds **(I** and **II)** were used as bioisosteres of anti-inflammatory drugs such as ibuprofen and diclofenac. They contain the 2-(2-acetamidophenyl)-2,2-*gem*-difluoroacetamide **(I)** or 2-(2-acetamidophenyl)-2-oxoacetamide **(II)** moieties, which have previously shown anti-inflammatory activity.[26] As they are easy to prepare and have relatively low cost, the molecular hybridization of these scaffolds was performed by connecting the quinolinic pharmacophoric group of CQ to linker groups containing 2–4 (CH_2) units to yield compounds **1**–**13**. The acetamidic skeletons were also directly bound to PQ to yield compounds **14**–**21**. This study aims to obtain compounds capable of killing *Plasmodium* that can still act on the inflammatory process caused by malaria infection. Novel compounds derived from PQ may represent a broad spectrum of action against all forms of *Plasmodium* involved in the biological cycle of malaria in the human host and be new alternatives for the treatment of *P. vivax* malaria. These PQ derivatives can also block malaria transmission.

2. Results and discussion

2.1. Chemistry

The synthetic route to prepare the 2-(2-acetamidophenyl)-*N*-(3-((7-chloroquinolin-4-yl)amino)alkyl)-2-oxoacetamides **(1**–**10)** and 2-(2-acetamidophenyl)-*N*-(3-((7-chloroquinolin-4-yl)amino)alkyl)-2,2-difluoroacetamides **(11**–**13)** is shown in Scheme 1.

Initially, the intermediates N^1-(7-chloroquinolin-4-yl)alkyldiamines **(22–24)** were synthesized with 85–90% yield by the nucleophilic substitution reaction between 4,7-dichloroquinoline **(25)** with the corresponding diamines: ethane-1,2-diamine, propane-1,3-diamine and butane-1,4-diamine without the use of a solvent under reflux for 4 hours.[23] The acetylation of isatins **(26a-d)** with acetic anhydride under reflux for 4 h provided the 1-acetylindoline-2,3-diones **(27a-d)** with 89–82% yield. The acetylated isatins **(27a-d)** were characterized by

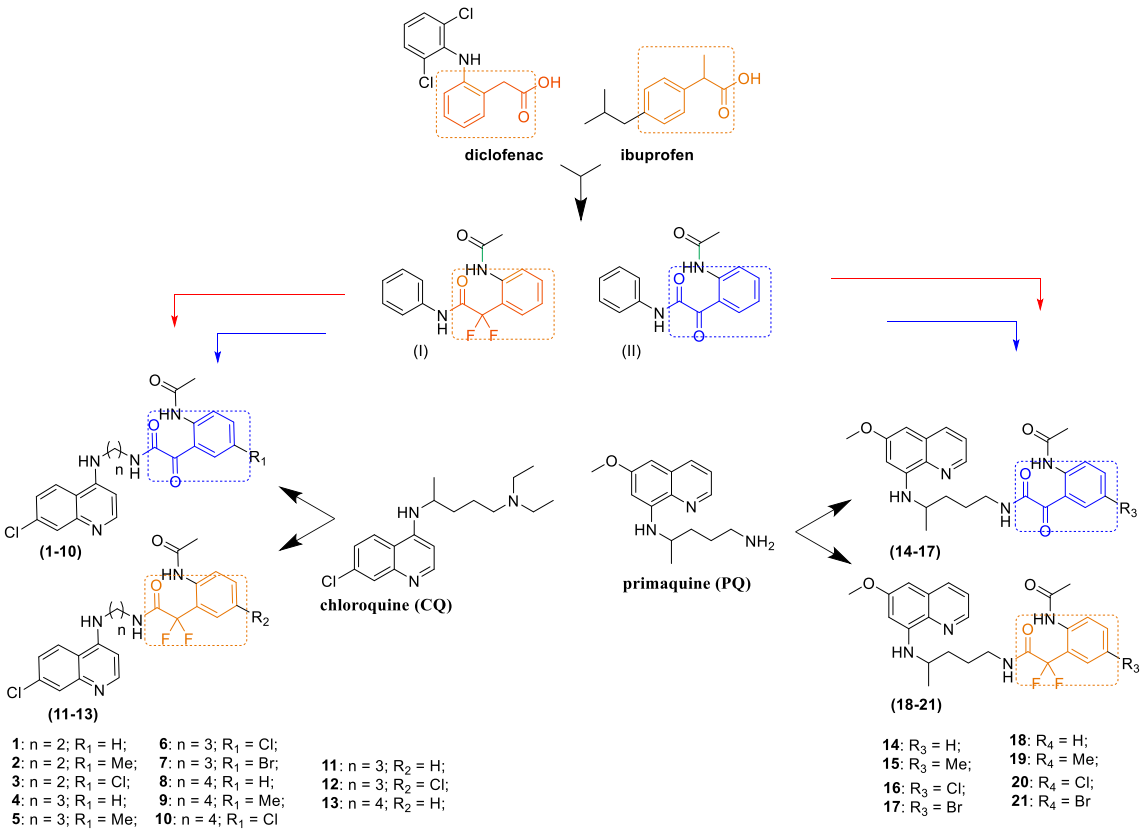
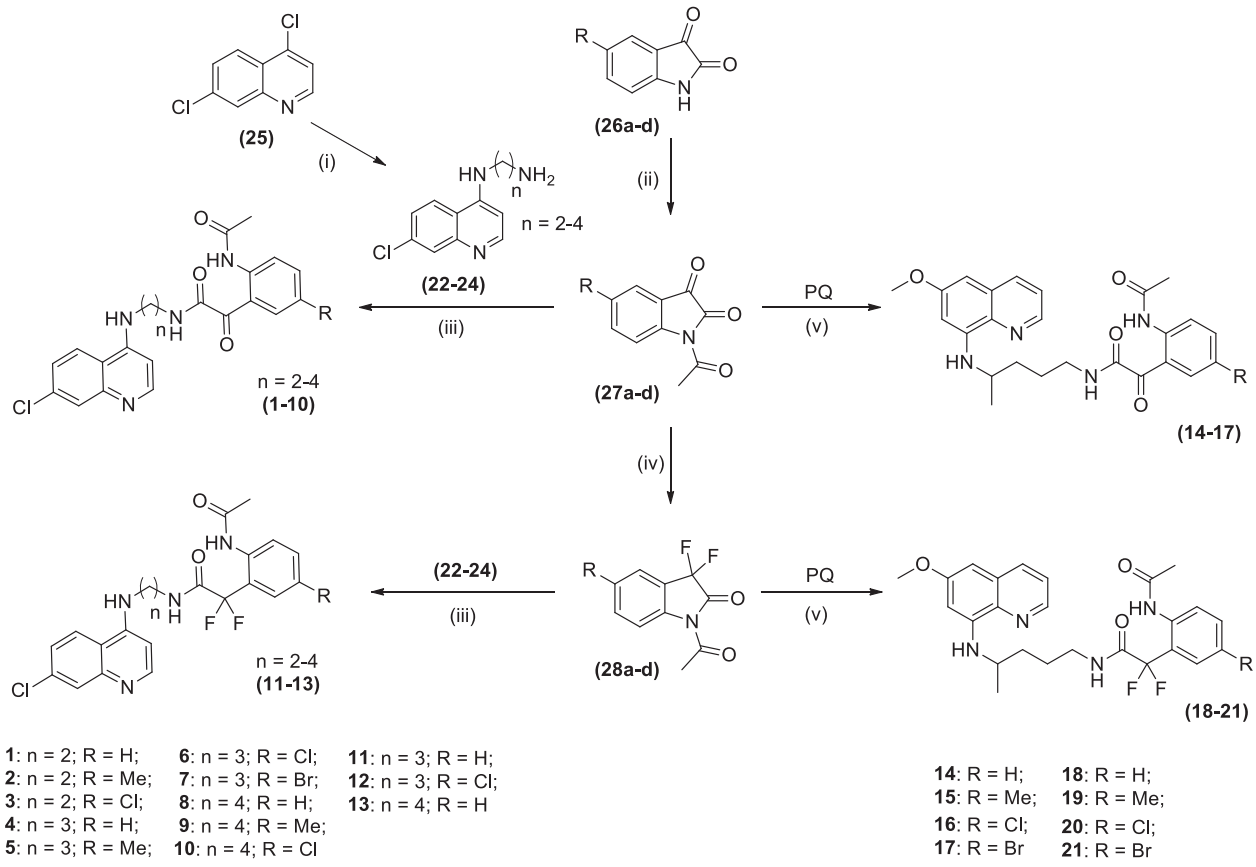


Figure 1. Rational approach to the design of compounds **1**–**21**.



1: n = 2; R = H;
2: n = 2; R = Me;
3: n = 2; R = Cl;
4: n = 3; R = H;
5: n = 3; R = Me;
6: n = 3; R = Cl;
7: n = 3; R = Br;
8: n = 4; R = H;
9: n = 4; R = Me;
10: n = 4; R = Cl
11: n = 3; R = H;
12: n = 3; R = Cl;
13: n = 4; R = H

14: R = H;
15: R = Me;
16: R = Cl;
17: R = Br
18: R = H;
19: R = Me;
20: R = Cl;
21: R = Br

Reagents and conditions: (i) appropriate diamine, reflux, 4 h, 85-90%; (ii) acetic anhydride, reflux, 4 h, 89-82%; (iii) aminoquinoline **(22-24)**, CH_3CN, 25 °C for 2-3 h, 60-3%; (iv) DAST, CH_2Cl_2, 16 h, r.t., 96-75%; (v) primaquine, CH_3CN, 25 °C for 2-3 h, 45-3%.

Scheme 1. Synthetic route used to prepare compounds **1**–**21**.

melting point and mass spectrometry, and the data were consistent with the literature.[27] The deoxofluorination of **(27a-d)** was performed using sulfur diethylaminosulfur trifluoride (DAST) in anhydrous dichloromethane under an argon atmosphere for 16 h at 25 °C and furnished 1-acetyl-3,3-difluoroindolin-2-ones **(28a-d)** with 96–75% yield.[27] The reaction of aminoquinolines **(22**–**24)** with 1-acetylindoline-2,3-diones **(27a-d)** or 1-acetyl-3,3-difluoroindolin-2-ones **(28a-d)** in acetonitrile at 25 °C for 2–3 h furnished the compounds the 2-(2-acetamidophenyl)-*N*-(3-((7-chloroquinolin-4-yl)amino)alkyl)-2-oxoacetamides **(1**–**10)** or 2-(2-acetamidophenyl)-*N*-(3-((7-chloroquinolin-4-yl)amino)alkyl)-2,2-difluoroacetamides **(11**–**13)**, respectively, with 60–18% yield. PQ was obtained by the simple neutralization of PQ diphosphate with 10% aqueous NaOH solution at room temperature.[16]

The reaction of PQ with 1-acetylindoline-2,3-diones **(27a-d)** or 1-acetyl-3,3-difluoroindolin-2-ones **(28a-d)** using the same conditions described previously furnished the compounds the 2-(2-acetamidophenyl)-*N*-(4-((6-methoxyquinolin-8-yl)amino)pentyl)-2-oxoacetamides **(14**–**17)** or 2-(2-acetamidophenyl)-2,2-difluoro-*N*-(4-((6-methoxyquinolin-8-yl)amino)pentyl)acetamides **(18**–**21)**, respectively, with 45–3% yield.

2.2. Biology

Compounds were assayed with nine different biological methods such as continuous cultures of *P. falciparum, in vitro* assays with *P. falciparum*-infected erythrocytes, cell cultures and cytotoxicity tests, *P. berghei* and antimalarial tests in mice, *P. gallinaceum* sporogonic assays, resazurin cell viability assay for J774A.1 macrophages, nitric oxide production by J774A.1 macrophages, evaluation of TNFα production by J774A.1 macrophages and enzyme-linked immunosorbent assay (ELISA). The assay details are described below in experimental section.

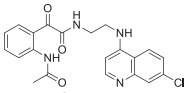
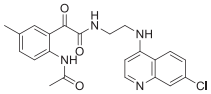
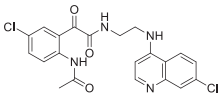
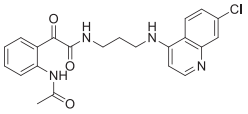
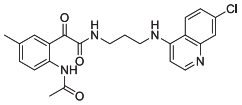
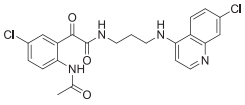
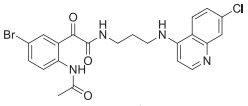
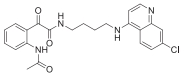
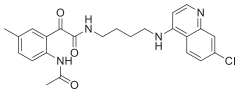
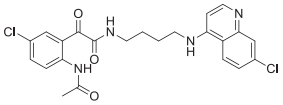
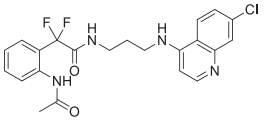
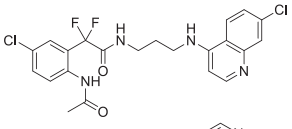
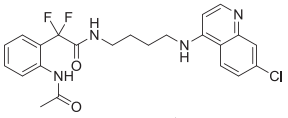
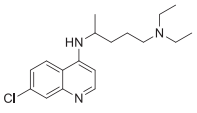
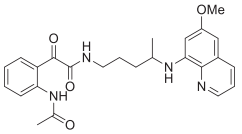
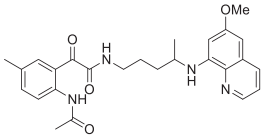
2.2.1. Continuous cultures of *P. falciparum*, in vitro assays with *P. falciparum*-infected erythrocyte cell cultures and cytotoxicity tests

Twenty-one new CQ and PQ synthesized derivatives **(1**–**21)** with different substituents in the 5- position of the acetamide moiety were tested *in vitro* against a *P. falciparum* CQR clone (W2), and the results are shown in Table 1. The carbonylated CQ derivatives **1**–**10** presented activity with IC_{50} values ranging from 0.12 to 3.18 μM with high selectivity index (SI) in the range of 270 - >700, excluding **10** (n = 4; R = Cl; SI = 29), **5** (n = 3; R = CH_3) and **9** (n = 4; R = CH_3), which were not determined. The most potent of the series was compound **6** (n = 3; R = Cl) with an IC_{50} = 0.12 μM and SI = 258. However, compound **8** (n = 4; R = H) was also potent (0.18 μM) and showed the best selectivity (>700).

The *gem*-difluorinated CQ derivatives (**11**–**13**) were also active, with IC_{50} values ranging from 0.40 to 2.70 μM. Compound **12** (n = 3; R = Cl) showed the largest SI (>1308) (Table 1). When comparing them by carbon numbers as linkers, it was not possible to say that one series was much better than the other. The *gem*-difluorinated CQ derivatives **11**–**13** did not showed significant differences when compared with the carbonylated derivatives.

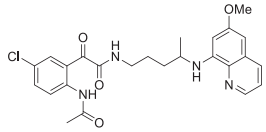
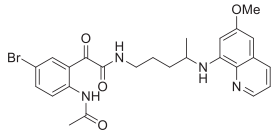
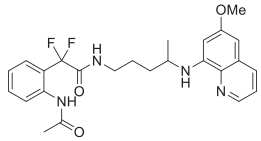
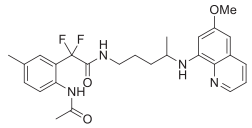
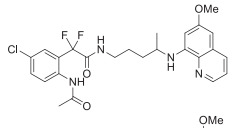
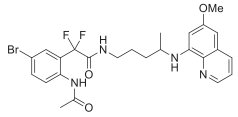
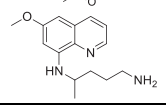
The carbonylated PQ derivatives **14**–**17** and *gem*-difluorinated derivatives **18**–**19** were the least potent CQ derivatives, with IC_{50} values between 2.50 and 26.8 μM and 3.50 to 25.5 μM, respectively. Compound **17** (R = Br) and its *gem*-difluorinated analogue **21** (R = Br) were the best of this series, with IC_{50} values of 2.50 and 3.50 μM, respectively. The SIs of this series were lower than those of the CQ series, suggesting the

Table 1
In vitro activity (IC_{50}) of compounds **1**–**21**, CQ and PQ, tested against a *P. falciparum* CQR (clone W2), cytotoxicity against BGM cell line (MDL_{50}), and the selectivity index (SI).

Compounds		Anti-*P. falciparum* activity IC_{50}* (μM)	Cytotoxicity to BGM cells MDL_{50} (μM)	SI** [MDL_{50} / IC_{50}]
	CQ derivatives			
1		0.71 ± 0.05	261 ± 61.0	>300
2		0.49 ± 0.12	>84	>170
3		0.52 ± 0.03	152.0 ± 9.0	292
4		0.20 ± 0.06	91.7 ± 20.8	>400
5		0.22	ND	ND
6		0.12 ± 0.02	30.9 ± 1.4	258
7		0.38 ± 0.01	102.6 ± 2.0	270
8		0.18 ± 0.08	123.8 ± 35.3	>700
9		0.84 ± 0.17	ND	ND
10		3.18 ± 1.0	91.1	29
***gem*-difluorinated CQ derivatives**				
11		0.40 ± 0.2	99.0 ± 84.0	>250
12		0.50 ± 0.3	654.0 ± 461.0	>1300
13		2.70 ± 3.3	140.0 ± 119.0	>50
CQ		0.20 ± 0.04	457 ± 22	2285
PQ derivatives				
14		26.8	ND	ND
15		25.3	> 2163.6	> 80

(*continued on next page*)

Table 1 (*continued*)

Compounds	Anti-*P. falciparum* activity IC_{50}* (μM)	Cytotoxicity to BGM cells MDL_{50} (μM)	SI** [MDL_{50} / IC_{50}]
16 	7.70 ± 2.1	164.0 ± 118.0	>20
17 	2.50 ± 1.4	309.0 ± 120.0	>100
***gem*-difluorinated PQ derivatives**			
18 	25.5 ± 1.8	113.4 ± 1.8	4
19 	20.7 ± 3.0	ND	ND
20 	8.40 ± 1.4	13.6 ± 5.0	1.6
21 	3.50 ± 0.07	> 1821.5	> 520
PQ 	1.90 ± 0.2	482.6 ± 11.0	255

ND = not determined. *Mean of 3–5 experiments; **Toxicity was considered at an SI < 10. MDL_{50} = minimum lethal dose for 50% of cells. IC_{50} = concentration inhibiting 50% of parasite growth.

associated toxicity of PQ.[28] An exception was compound **21**, which showed high selectivity (>520).

2.2.2. *P. Berghei and antimalarial tests in mice*

Compounds **4** (n = 3; R = H), **6** (n = 3; R = Cl), and **8** (n = 4; R = H), which showed activity equipotent to CQ (IC_{50} = 0.20 μM), were selected for *in vivo* assays in mice experimentally infected with malaria in parallel with the antimalarial CQ.[29] Compound **8** was partially active and reduced parasitemia up to 37% (Table 2). This result corroborated the *in vitro* findings, but it did not increase survival among mice, whereas CQ treatment resulted in 100% survival among mice until the last day of observation.

2.2.3. *In vivo assay in mosquitoes using a P. Gallinaceum model*

As PQ can also prevent the transmission of the parasite, the *gem*-difluorinate PQ derivative **20** (R = Cl) was selected for an *in vivo* assay in mosquitoes using a *P. gallinaceum* model aiming to find a PQ derivative that could be active against all developmental stages of the parasite.

The experimental model of *P. gallinaceum* avian malaria presently used for screening malaria transmission blockers was first developed by Gwadz.[30] After being modified using *Ae. fluviatilis*, a highly susceptible mosquito to *P. gallinaceum,*[31,32] the protocol has been successfully used in our routine to test compounds with such activity.[33]

Table 2
Antimalarial activity of synthetic compounds in mice infected with *P. berghei* treated with daily doses of 25 mg/kg body weight for three consecutive days.

Assay	Compounds	% Reduction (Mean Parasitemia ± SD)*		Survival (Mean ± SD)
		5th	7th	
1	**Non-treated**	4.6 ± 2	31 ± 6.4	13 ± 3
	CQ**	99% (0.1 ± 0)	99% (0.4 ± 0.4)	22 ± 6
	6	0% (4.9 ± 1.7)	11% (27.2 ± 6.8)	16 ± 3
	8	0% (5 ± 1.7)	2% (30 ± 16.2)	20 ± 7
2	**Non-treated**	0.5 ± 0.9	12.7 ± 3.8	23 ± 8.3
	CQ**	100% (0 ± 0)	100% (0 ± 0)	30 ± 0
	4	0% (1.9 ± 0.1)	15% (10.7 ± 4.2)	24 ± 7.3
	6	0% (1.7 ± 1.3)	0% (19.2 ± 2.2)	16 ± 6.1
	8	33% (0.4 ± 0.3)	37% (9.4 ± 2.5)	25 ± 1.2

* Reduction in parasitemia in relation to untreated controls; when < 30%, the compound was considered inactive, 30–40% was considered partially active, and > 40% was considered active; **20 mg/kg body weight.

At 50 mg/kg, compound **20** significantly inhibited ($p < 0.005$) the sporogonic cycle of *P. gallinaceum* up to 82%. In contrast to its strong activity, this compound was not active at a dose of 25 mg/kg (Table 3). No inhibition of mosquito infection was observed at any evaluated doses, but compound **20** can be considered a transmission blocker (at the 50 mg/kg dose), and structural modifications can be made to improve this characteristic.

The demonstration that compound **20** exhibited high transmission blocking of the malaria sporogonic cycle suggests that new PQ-derived compounds synthesized from structural modifications of the compounds evaluated here should be tested in this model and that they are less toxic than PQ. Compound **20** only had a blocking effect on the malaria sporogonic cycle in mosquitoes when used at a high dose (50 mg/kg); in contrast, PQ, at a dose of 15 mg/kg totally inhibited this cycle.

PQ is used to block malaria transmission in endemic areas aiming at malaria elimination. It is expected that transmission-blocking drugs will slow down the spread of resistant parasites.[34] Although a single 0.75 mg/kg dose of PQ is effective as a gametocytocidal agent,[35] PQ has restrictions because it is poorly tolerated and its metabolites cause hemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency.[28] showed high selectivity.

2.2.4. Cell viability, nitric oxide and TNFα production in J774.A1 macrophages

As the CQ and PQ derivatives were designed to have dual activity, the ability to act as anti-inflammatory agents was also investigated. The original concept was to achieve an optimal antiplasmodial compound with additional anti-inflammatory activity, considering the inflammatory comorbidities in severe malaria cases. Compounds **4**, **6**, **8**, **11**, **12**, **13**, **16**, **17** and **20** were selected according to the antiplasmodial activity screening against *P. falciparum*, *P. berghei*, and *P. gallinaceum* as described above. Taking this into account, the anti-inflammatory action of active antiplasmodial compounds was evaluated by nitric oxide (NO) and TNFα produced by macrophages stimulated by LPS.

Prior to the assays, the compounds were tested for cytotoxicity to establish a suitable noncytotoxic concentration. All compounds displayed cytotoxicity in concentrations above 25 μM (Table 4) in a resazurin cell viability assay with the J774A.1 macrophage cell line. Thus, to evaluate the action of these compounds on NO and TNFα production, the 25 μM concentration was selected.

Among the nine compounds tested, only compounds **6**, **12** and **20** dose-dependently reduced NO production (Fig. 2) and inhibited TNFα production by LPS-stimulated J774A.1 macrophages (Fig. 3). It is interesting to highlight that chlorinated compounds **6** and **12** (except **16**) also showed high activity against *P. falciparum,* although no decrease in parasitemia was detected in animal models. Compound **8** (n = 4; R = H) was the only one with moderate activity against *P. berghei* in mice (Table 2). However, it was not able to reduce the *in vitro* production of the inflammatory mediator TNFα (data not shown), which is closely related to the severe cases of malaria as well as cerebral malaria, as extensively reported.[15,36,37]

3. Conclusions

Twenty-one new CQ and PQ derivatives **(1–21)** were synthesized and tested *in vitro* against a *P. falciparum* CQR clone (W2). The carbonylated CQ derivatives **1**–**10** presented activity with IC_{50} values ranging from 0.12 to 3.18 μM. Compound **6** (n = 3; R = Cl) was the most potent of this series. However, compound **8** (n = 4; R = H) was also potent and showed the best selectivity. The *gem*-difluorinated CQ derivatives (**10**–**13**) did not show significant differences compared with carbonylated analogs. When comparing them by carbon numbers as linkers, it was not possible to say that one series was much better than the other. The PQ derivatives carbonylated **14**–**17** and *gem*-difluorinated **18**–**19** were the least potent CQ derivatives. Compound **17** (R = Br) and its *gem*-difluorinated analogue **21** were the best of this series, showing IC_{50} values of 2.50 and 3.50 μM, respectively. Compound **20** (R = Cl) exhibited high transmission blockade of the malaria sporogonic cycle in mosquitoes when used at a high dose (50 mg/kg) compared with PQ, which when used at a dose of 15 mg/kg totally inhibited this cycle. Compound **8** (n = 4; R = H) was tested in mice infected with *P. berghei* and reduced parasitemia up to 37% (25 mg/kg) on day 7 after inoculation. Preliminary studies showed that the hybridization of the 2-(2-acetamidophenyl)-2-oxoacetamidic and 2-(2-acetamidophenyl)-2,2-*gem*-difluoroacetamidic skeletons to CQ and PQ was promising in maintaining the anti-inflammatory activity. Compound **6** (n = 3; R = Cl) and the *gem*-difluorinated compounds **12** (n = 3; R = Cl), **20** (R = Cl) dose-dependently reduced NO production and inhibited TNFα production by LPS-stimulated J774A.1 macrophages.

The results of this work indicate a viable and interesting approach in planning new chemical entities for the treatment of malaria caused by *P. falciparum* and *P. vivax* and the anti-inflammatory process related to this disease.

4. Experimental section

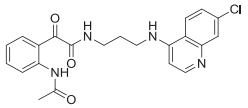
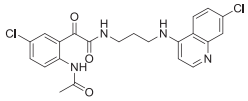
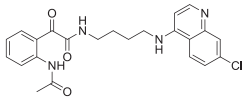
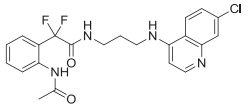
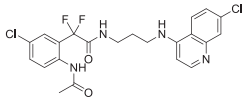
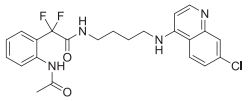
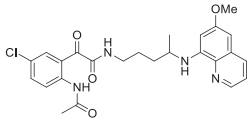
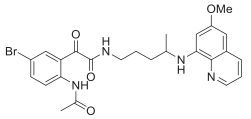
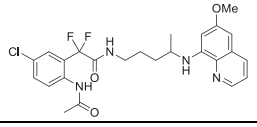
4.1. Chemistry

All reagents and solvents used were analytical grade. The ^{1}H, ^{13}C and ^{19}F nuclear magnetic resonance (NMR) spectra were obtained at 400.00, 100.00 and 376.00 MHz, respectively, using a BRUKER Avance instrument equipped with a 5-mm probe. Tetramethylsilane was used as an internal standard. The chemical shifts (δ) are reported in ppm, and the coupling constants (*J*) are reported in Hertz. Electron-ionization mass spectra (EI-MS, scan ES + capillary (3.0 kV)/cone (30 V)/extractor (1 V)/RF lens (1.0 V)/source temperature (150 °C)/desolvation temperature (300 °C) were recorded using a Micromass/Waters Spectrometer (model: ZQ-4000). High-resolution mass spectrometry (HRMS) data were obtained using an LC-MS Bruker Daltonics MicroTOF (time of flight analyzer). Fourier transform infrared (FT-IR) absorption spectra were recorded on a Shimadzu mode IR Prestige-21 spectrophotometer. The melting points (m.p.) were determined using a Büchi model B-545 apparatus. TLC (thin layer chromatography) was performed using a silica gel F-254 glass plate (20 × 20 cm). Column chromatography was performed using Merck Silica gel 60 (0.040–0.063 mm). All compounds were purified using the same gradient. Initially 100% $CHCl_3$ and then

Table 3
Oocyst number of *P. gallinaceum* in *Ae. fluviatilis*. Mosquitoes were allowed to blood feed on chickens before and after treatment with compound **20** and PQ.

Assay	% Parasitemia (Gametocythemia)	T = 0 h		Compounds (Dose)	T = 4 h		
		Oocyst Number (Mean ± SD)	% Mosquitoes Infection		Oocyst Number (Mean ± SD)	% Inhibition Mosquitoes Infection	% Oocyst Reduction
1	10.3 (42%)	256 ± 180	95	**20** (50 mg/kg)	64 ± 64	15	81.3
	7.3 (49%)	137 ± 100	95	**20** (25 mg/kg)	142 ± 118	5	0.0
	7.0% (45%)	117 ± 72	90	**PQ** (15 mg/kg)	0 ± 0	100	100
2	10.6 (42%)	166 ± 101	100	**20** (50 mg/kg)	30 ± 39	21	81.9
	4.7 (41%)	56 ± 57	89	**20** (25 mg/kg)	67 ± 52	7	0,0
	17.0 (45%)	41 ± 17	85	**PQ** (15 mg/kg)	0 ± 0	100	100

Table 4
Resazurin cell viability assay with the J774A.1 macrophage cell line and test compounds. Values represent the cell viability percentage (%) in different concentrations compared to vehicle (0.2% DMSO).

Compounds		% Viability Concentration				
		0	12,5 μM	25 μM	50 μM	100 μM
4		100%	100%	100%	89,1%	67,6%
6		100%	100%	100%	82,7%	10,7%
8		100%	98,4%	100%	83,4%	10,1%
11		100%	100%	100%	91,55	33,3%
12		100%	100%	95,5%	87,55%	10,4%
13		100%	100%	100%	83,7%	10,4%
16		100%	100%	100%	77,6%	54,6%
17		100%	100%	100%	75,5%	75,8%
20		100%	100%	96,8%	56,2%	33,9%

$CHCl_3$ / MeOH (99: 1 up until 95:5). The analysis by high-performance liquid chromatography (HPLC) was performed on Shimadzu liquid chromatography LC-10AD using Hypersil BDS C18 column (5 μm 250 × 4.6 mm).

4.1.1. General procedure for preparing 1-acetylindoline-2,3-dione (27a-d)

The 1-acetylindoline-2,3-diones **(27a-d)** were prepared according to the methodology described in the literature.[27] A total of 13 mmol of the proper indoline-2,3-dione **(26a-d)** and 15 equivalents of freshly distilled acetic anhydride were added to a round-bottom flask connected to a reflux condenser. The reaction medium was heated to reflux with magnetic stirring for 4 h. Then, the flask was cooled to room temperature and taken to the freezer (-20 °C) for 12 h. The formed solid was filtered and washed with water and hexane and allowed to air dry. The product was recrystallized in ethyl acetate: hexane (1:1) with activated charcoal. The solid formed was filtered and washed with cold hexane.

4.1.1.1. 1-acetylindoline-2,3-dione (27a). Yield: 82%. mp: 141 – 143 °C. MS (EI): *m/z* 189 (11%), *m/z* 146 (100%), *m/z* 147 (22%), *m/z* 90 (34%), *m/z* 43 (45%).

4.1.1.2. 1-acetyl-5-methylindoline-2,3-dione (27b). Yield: 89%. Yellow solid, mp: 172 – 173 °C. MS (EI): *m/z* 203 (17%), *m/z* 161 (52%), *m/z* 160 (100%), *m/z* 133 (21%), *m/z* 104 (30%), *m/z* 43 (27%).

4.1.1.3. 1-acetyl-5-chloroindoline-2,3-dione (27c). Yield: 87%. mp: 208 – 210 °C. MS (EI): *m/z* 223 (9%), *m/z* 181 (54%), *m/z* 180 (100%), *m/z* 153 (20%), *m/z* 124 (36%).

4.1.1.4. 1-acetyl-5-bromoindoline-2,3-dione (27d). Yield: 85%. mp: 169 – 170 °C. MS (EI): *m/z* 170 (33%); *m/z* 224 (100%); *m/z* 226 (100%); *m/z* 267 (14%); *m/z* 269 (14%).

4.1.2. General procedure for preparing 1-acetyl-3,3-difluoroindolin-2-ones (28a-d)

The 1-acetyl-3,3-difluoroindolin-2-ones **(28a-d)** were prepared according to methodology described in the literature.[27] The corresponding 1-acetyl-indoline-2,3-dione **(27a-d)** (6 mmol) was added to a round-bottom flask and then sealed with a septum. The atmosphere of the system was replaced by argon, and 37 equivalents of freshly distilled anhydrous dichloromethane were added. The mixture was stirred for 5 min. Then, 2 equivalents of diethylaminosulfur trifluoride (DAST) were added through a syringe. The reaction medium was stirred for 16 h at room temperature under argon atmosphere. After this period, the reaction mixture was poured into crushed ice. The organic phase was separated, washed 2 times with distilled water, dried with anhydrous

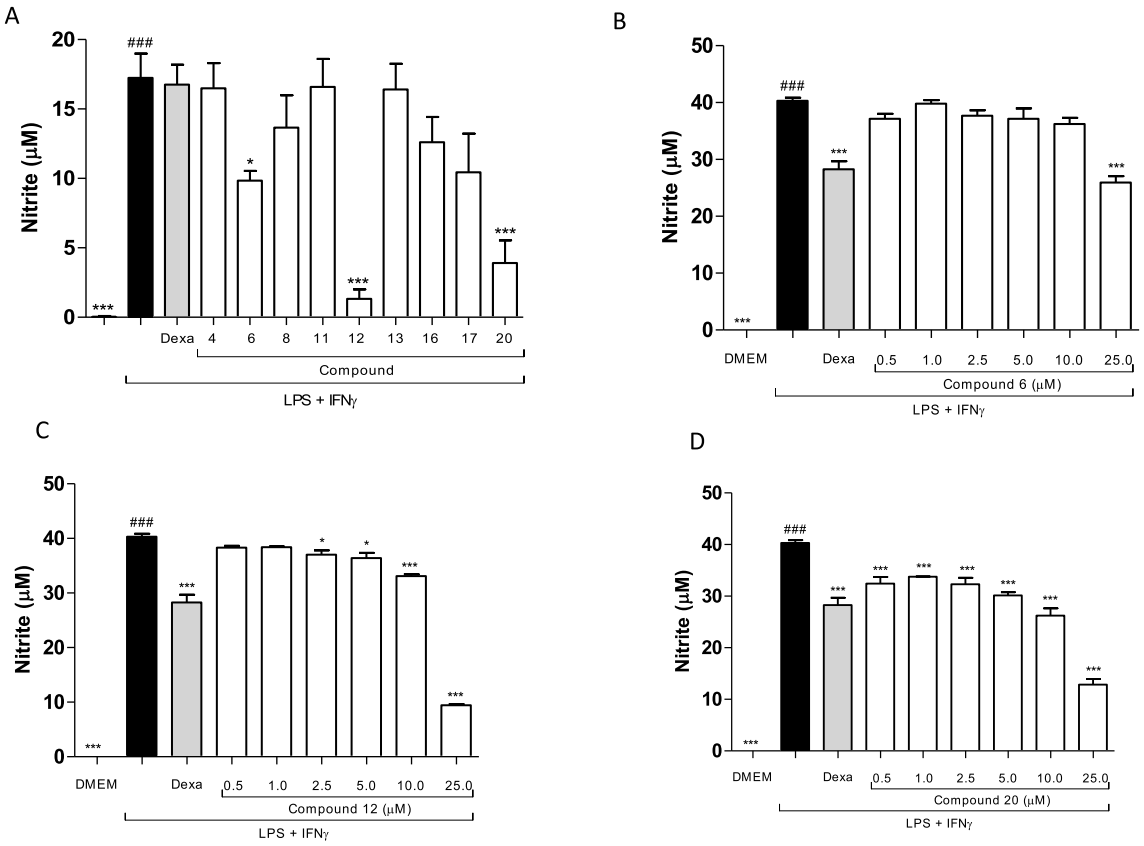


Figure 2. Initial screening for anti-inflammatory activity of selected compounds on nitric oxide (NO) production by LPS (37.5 ng/ml) + IFNγ (25 U/ml)-stimulated J774A.1 macrophages (A). Concentration-response curves for compounds **6** (B), **12** (C) and **20** (D) on NO production by J774A.1 macrophages. The results are represented by the mean ± SEM002C and significant differences from the positive control were determined by ANOVA followed by Dunnet's post hoc test. ### $P < 0.0001$ compared to vehicle (0.2% DMSO) and *** $P < 0.0001$ compared to the positive control.

Na_2SO_4 and concentrated under a vacuum. The product was purified by vacuum sublimation and stored in amber vials in an argon atmosphere.

4.1.2.1. 1-acetyl-3,3-difluoroindolin-2-ones (28a). Yield: 96%. mp: 110 – 112 °C. MS (EI): *m/z* 211 (13%), *m/z* 169 (100%), *m/z* 141 (66%), *m/z* 114 (12%), *m/z* 43 (61%).

4.1.2.2. 1-acetyl-3,3-difluoro-5-methylindolin-2-one (28b). Yield: 88%. mp: 71 – 73 °C. MS (EI): *m/z* 225 (12%), *m/z* 183 (100%), *m/z* 184 (10%), *m/z* 155 (74%), m/ z 43 (35%).

4.1.2.3. 1-acetyl-5-chloro-3,3-difluoroindolin-2-one (28c). Yield: 94%. mp: 131 – 133 °C. MS (EI): *m/z* 245 (14%), *m/z* 205 (32%), *m/z* 203 (100%), *m/z* 175 (56%), *m/z* 43 (91%).

4.1.2.3.1. 1-acetyl-5-bromo-3,3-difluoroindolin-2-one (28d). Yield: 75%. mp: 142 – 144 °C. MS (EI): *m/z* 219 (36%); *m/z* 221 (34%); *m/z* 249 (100%); *m/z* 249 (100%); *m/z* 289 (14%); *m/z* 290 (14%).

4.1.3. General procedure for preparing N^1-(7-chloroquinolin-4-yl) alkyldiamines (22–24)

The N^1-(7-chloroquinolin-4-yl)alkyldiamines **(22–24)** were prepared according to the methodology described.[23] The 4,7-dichloroquinoline **(25)** (1 mmol) and the appropriate diamine (1 equivalent) was added to a bound-bottom flask connected to a reflux condenser and stirred at reflux for 4 h. The reaction mixture was concentrated and poured into 50 mL of ice-cold water. The precipitate was collected by filtration and washed with water to give compounds **22–24** with yields of 85–90%.

4.1.3.1. N^1-(7-chloroquinolin-4-yl)ethane-1,2-diamine (22). Yield: 85%. m.p.: 168 – 170 °C. IR (KBr, cm^{-1}): 3233; 2922; 1614; 1584. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 2.49–2.50 (m, 2H, CH_2); 3.27–3.29 (m, 2H, CH_2); 6.49 (d, 1H, $J = 5.4$ Hz, H3); 7.41 (dd, $J = 2.0$; 9.0 Hz, H6); 7.77 (d, 1H, $J = 2.0$ Hz, H8); 8.26 (d, 1H, $J = 9.0$ Hz, H5); 8.39 (d, 1H, $J = 5.4$ Hz, H2). ^{13}C NMR (100 MHz, DMSO-d_6, δ in ppm): 40.14; 45.69; 98.68; 117.46; 123.95; 124.11; 127.44; 133.31; 149.06; 150.24; 151.89.

4.1.3.2. N^1-(7-chloroquinolin-4-yl)propane-1,3-diamine (23). Yield: 90%. m.p.: 161.5 – 163.5 °C. IR (KBr, cm^{-1}): 3253; 2935; 1610; 1581. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.44–1.47 (m, 2H, CH_2); 1.70–1.77 (m, 2H, CH_2); 2.61–2.70 (m, 2H, CH_2); 6,47 (d, 1H, $J = 5.4$ Hz, H3); 7.42 (dd, $J = 2.0$; 8.9 Hz, H6); 7.76 (d, 1H, $J = 2.0$ Hz, H8); 8.23 (d, 1H, $J = 8.9$ Hz, H5); 8.37 (d, 1H, $J = 5.4$ Hz, H2). ^{13}C NMR (100 MHz, DMSO-d_6, δ in ppm): 30.96; 39.93; 40.58; 98.59; 117.47; 124.00; 127.45; 133.34; 149.05; 150.16; 151.92.

4.1.3.3. N^1-(7-chloroquinolin-4-yl)butane-1,4-diamine (24). Yield: 86%. m.p.: 121 – 123 °C. IR (KBr, cm^{-1}): 3062; 2931; 1610; 1575. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.43–1.50 (m, 2H, CH_2); 1.65–1.72 (m, 2H, CH_2); 2.60 (m, 2H, CH_2); 3.23–3.26 (m, 2H, CH_2); 6.44 (d, 1H, $J = 5.4$ Hz, H3); 7.40 (dd, $J = 2.0$; 9.0 Hz, H6); 7.77 (d, 1H, $J = 2.0$ Hz, H8); 8.26 (d, 1H, $J = 9.0$ Hz, H5); 8.38 (d, 1H, $J = 5.4$ Hz, H2). ^{13}C NMR (100 MHz, DMSO-d_6, δ in ppm): 25.27; 30.57; 41.17; 42.33; 98.50; 117.41; 123.78; 123.94; 127.32; 133.22; 149.01; 150.08; 151,75.

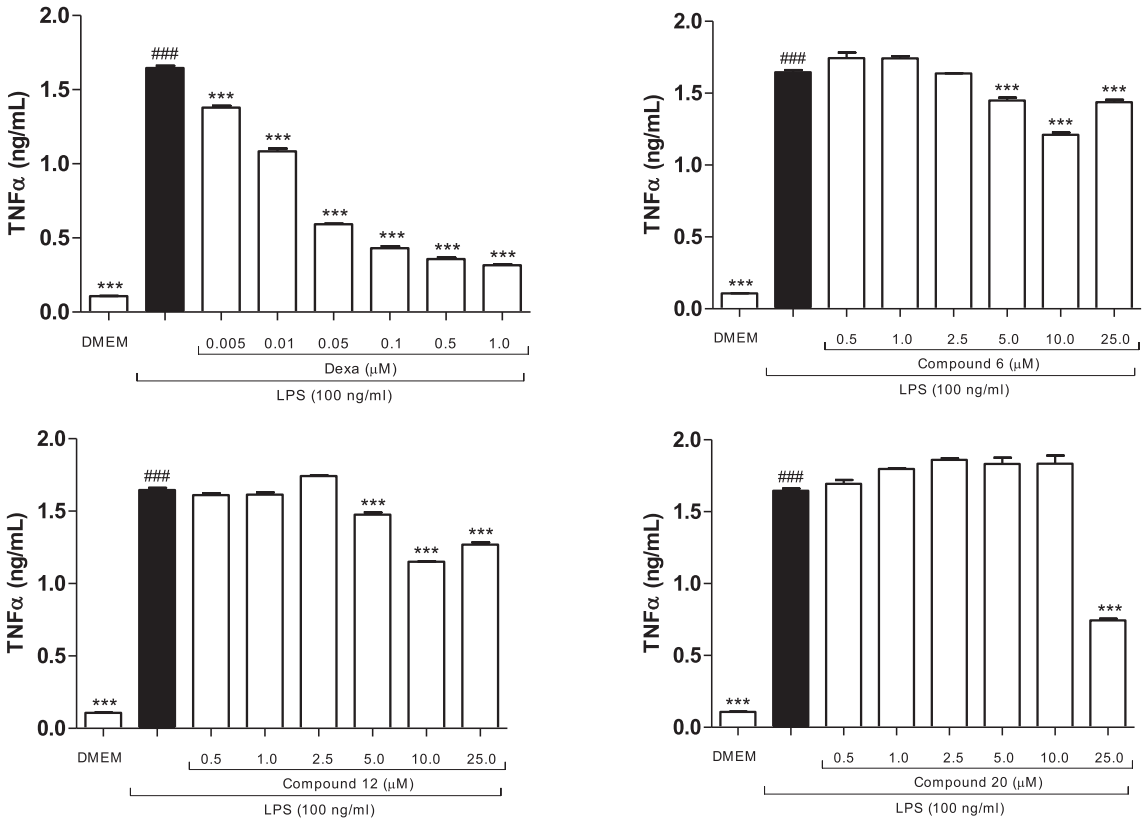


Figure 3. Effect of dexamethasone and compounds **6**, **12** and **20** on LPS-induced TNFα production by J774A.1 macrophages. The results are represented by the mean ± SEM, and significant differences from the positive control were determined by ANOVA followed by Dunnet's post hoc test. ### $P < 0.0001$ compared to vehicle (DMSO) and *** $P < 0.0001$ compared to the positive control.

4.1.4. General procedure for preparing 2-(2-acetamidophenyl)-N-(3-((7-chloroquinolin-4-yl)amino)alkyl)-2-oxoacetamides (1–10) and 2-(2-acetamidophenyl)-N-(3-((7-chloroquinolin-4-yl)amino)alkyl)-2,2-difluoroacetamides (11–13)

A total of 1.0 mmol of the corresponding 1-acetylindoline-2,3-diones **(27a-d)** or 1-acetyl-3,3-difluoroindolin-2-ones **(28a-d)** and 50 mL of CH_3CN were added to a round-bottom flask coupled to a condenser. The mixture was stirred at 25 °C for 5–10 min. Then, 1 equivalent of proper aminoquinoline **(22–24)** or primaquine was added. The reaction was kept under stirring at 25 °C for 2–3 h. At the end of this period, the formed precipitate was vacuum filtered and washed with cold CH_3CN. The residual crude product was purified via silica gel column chromatography using a gradient mixture of $CHCl_3$/MeOH. Compounds **1**–**13** were obtained as white solids with 60–3% yield.[27]

Primaquine was obtained from the treatment of primaquine diphosphate with NaOH (10% solution aq.) for 2 h. The obtained brown oil was extracted from the medium with CH_2Cl_2 (30 mL) washed with water (3 × 10 mL), dried (magnesium sulfate anhydrous), concentrated under vacuum and stored under argon atmosphere with protection from light.[16]

4.1.4.1. 2-(2-acetamidophenyl)-N-(2-((7-chloroquinolin-4-yl)amino) ethyl)-2-oxoacetamide (1). Yield: 36%. MP: 120–122 °C. IR (cm^{-1}): 3300; 3260; 3125–2875; 1674; 1611; 1581; 1529; 1484; 1451; 1371; 1300; 1208; 1100; 798; 748. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 2.02 (s, 3H, CH_3); 3.53 (s, 4H, CH_2); 6.72 (d, 1H, $J = 5.8$ Hz, H3); 7.16 (t, 1H, $J = 8.0$ Hz, H4′); 7.54–7.61 (m, 3H, H5′, H6′; H6); 7.72 (d, 1H, $J = 8.0$ Hz, H3′); 7.85 (d, 1H, $J = 2.0$ Hz, H8); 7.93 (s, 1H, NH); 8.30 (d, 1H, $J = 9.0$ Hz, H5); 8.47 (d, 1H, $J = 5.8$ Hz, H2); 8.95 (s, 1H, NH); 10.54 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 23.79; 37.18; 41.83; 98.75; 116.97; 121.55; 123.42; 124.34; 124.72; 124.87; 125.49; 131.18; 133.65; 134.59; 137.85; 146.63; 149.78; 151.42; 163.62; 168.74; 190.47. HRMS (ESI) calc. For $C_{21}H_{19}ClN_4O_3$ 410.11146, found $[M + 1]^+$ 411.1237. HPLC: 86.8%.

4.1.4.2. 2-(2-acetamido-5-methylphenyl)-N-(2-((7-chloroquinolin-4-yl) amino)ethyl)-2-oxoacetamide (2). Yield: 29%. MP: 118–119 °C. IR (cm^{-1}): 3292; 3231; 3062; 1682; 1639; 1577; 1531; 1457; 1371; 1300; 1243; 1182; 1143; 1079; 859; 793. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.99 (s, 3H, CH_3); 2.23 (s, 3H, CH_3); 3.42–3.47 (m, 2H, CH_2); 3.50–3.55 (s, 2H, CH_2); 6.63 (d, 1H, $J = 5.5$ Hz, H3); 7.36–7.44 (m, 3H, H4′, H6′; NH); 7.48 (dd, 1H, $J = 2.0$; 9.0 Hz, H6); 7.57 (d, 1H, $J = 8.0$ Hz, H3′); 7.81 (d, 1H, $J = 2.0$ Hz, H8); 8.22 (d, 1H, $J = 9.0$ Hz, H5); 8.43 (d, 1H, $J = 5.5$ Hz, H2); 8.89 (s, 1H, NH); 10.40 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 21.10; 23.69; 37.26; 41.78; 98.75; 117.41; 121.79; 123.94; 124.27; 125.08; 127.40; 130.98; 132.77; 133.55; 134.13; 135.42; 148.85; 150.13; 151.80; 163.67; 168.61; 190.58. HRMS (ESI) calc. For $C_{22}H_{21}ClN_4O_3$ 424.1302, found $[M + 1]^+$ 425.1384. HPLC: 99.4%.

4.1.4.3. 2-(2-acetamido-5-chlorophenyl)-N-(2-((7-chloroquinolin-4-yl) amino)ethyl)-2-oxoacetamide (3). Yield: 36%. MP: 147–149 °C. IR (cm^{-1}): 3298; 2890; 1646; 1609; 1577; 1506; 1450; 1396; 1367; 1286; 1194; 1140; 1109; 804; 749. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.97 (s, 3H, CH_3); 3.49 (m, 4H, CH_2); 6.71 (d, 1H, $J = 5.8$ Hz, H3); 7.47 (d, 1H, $J = 8.7$ Hz, H3′); 7.52 (d, 1H, $J = 2.5$ Hz, H6′); 7.56 (dd, 1H, $J = 2.2$; 9.0 Hz, H6); 7.62 (dd, 1H, $J = 2.5$; 8.7 Hz, H4′); 7.80 (s, 1H, NH); 7.83 (d, 1H, $J = 2.2$ Hz, H8); 8.27 (d, 1H, $J = 9.0$ Hz); 8.46 (d, 1H, $J = 5.8$ Hz, H2); 8.90 (s, 1H, NH); 10.46 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 23.27; 37.18; 41.71; 98.63; 116.91; 123.63; 124.14; 124.75; 125.99; 127.36; 128.04; 129.39; 132.25; 134.38; 135.30; 146.58; 149.88; 151.19; 162.02; 168.54; 187.20. HRMS (ESI) calc. For $C_{21}H_{18}Cl_2N_4O_3$ 444.0756, found $[M + 1]^+$ 445.0872. HPLC:

93.8%.

4.1.4.4. 2-(2-acetamidophenyl)-N-(3-((7-chloroquinolin-4-yl)amino)propyl)-2-oxoacetamide (4). Yield: 40%. MP: 166–168 °C. IR (cm^{-1}): 3286; 3217; 2179; 1641; 1576; 1525; 1447; 1210. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.93–1.86 (m, 2H, CH_2); 2.04 (s, 3H, CH_3); 3.38–3.32 (m, 4H,CH_2); 6.52 (d, 1H, J = 5.5 Hz, H3); 7.21 (td, 1H, J = 1.0; 7.5 Hz, H4′); 7.40 (t, 1H, J = 5.2 Hz, NH); 7.47 (dd, 1H, J = 2.2 ; 7.4 Hz, H6); 7.62–7.58 (m, 2H, H5′; H6′); 7.80–7.78 (m, 2H, H3′; H8); 8.26 (d, 1H, J = 9.0 Hz, H5); 8.41 (d, 1H, J = 5.5 Hz, H2); 8.82 (t, 1H, J = 5.8 Hz, NH); 10.56 (s,1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm):23.9; 27.3; 36.4; 79.1; 98.6; 117.4; 121.4; 123.4; 124.0; 124.1; 124.2; 127.1; 131.3; 133.5; 133.8; 138.1; 148.6; 150.1; 151.5; 163.5; 168.7; 191.1. HRMS (ESI) calc. For $C_{22}H_{21}ClN_4O_3$ 424.1302, found $[M + 1]^+$ 425.1366. HPLC: 99.0%.

4.1.4.5. 2-(2-acetamido-5-methylphenyl)-N-(3-((7-chloroquinolin-4-yl) amino)propyl)-2-oxoacetamide (5). Yield: 38%. MP: 158–159 °C. IR (cm^{-1}): 3365; 2927; 2864; 1702; 1643; 1581; 1514; 1438; 1371; 1296; 1237; 1181; 1080; 905; 805; 779. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.87–1.93 (m, 2H, CH_2); 2.02 (s, 3H, CH_3); 2.28 (s, 3H, CH_3); 3.35 (m, 4H, CH_2); 6.51 (d, 1H, J = 5.5 Hz, H3); 7.36 (t, 1H, J = 5.4 Hz, NH); 7.40–7.43 (m, 2H, H4′; H6′); 7,46 (dd, 1H, J = 2.0; 9.0 Hz, H6); 7.65 (d, 1H, J = 8.0 Hz, H3′); 7.79 (d, 1H, J = 2.0 Hz, H8); 8.26 (d, 1H, J = 9.0 Hz, H5); 8.40 (d, 1H, J = 5.5 Hz, H2); 8.79 (t, 1H, J = 5.8 Hz, NH); 10.44 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.68; 25.40; 26.01; 34.02; 39.62; 47.82; 55.19; 91.85; 96.95; 118.70; 120.65; 121.90; 129.90; 132.15; 134.24; 134.83; 135.33; 137.28; 139.69; 144.37; 144.81; 159.37; 163.06; 169.08; 192.09. HRMS (ESI) calc. For $C_{23}H_{23}ClN_4O_3$ 438.1459, found $[M + 1]^+$ 439.155. HPLC: 99.8%.

4.1.4.6. 2-(2-acetamido-5-chlorophenyl)-N-(3-((7-chloroquinolin-4-yl) amino)propyl)-2-oxoacetamide (6). Yield: 35%. MP: 202–204 °C. IR (cm^{-1}): 3253; 3061; 2369; 1691; 1638; 1571; 1514; 1448; 1194. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.92–1.85 (m, 2H, CH_2); 1.99 (s, 3H, CH_3); 3.37–3.29 (m, 4H,CH_2); 6.51 (d, 1H, J = 5.5 Hz, H3); 7.34 (t, 1H, J = 5.3 Hz, NH); 7.46 (dd, 1H, J = 2.2; 9.0 Hz, H6); 7.52 (d, 1H, J = 8.7 Hz, H4′); 7.56 (d, 1H, J = 2.5 Hz, H6′); 7.63 (dd, 1H, J = 2.5; 8.7 Hz, H3′); 7.78 (d, 1H, J = 2.2 Hz, H8); 8.25 (d, 1H, J = 9.0 Hz, H5); 8.40 (d, 1H, J = 5.5 Hz, H2); 8.79 (t, 1H, J = 5.9 Hz, NH); 10.47 (s,1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 23.3; 27.2; 36.4; 39.7; 98.5; 117.3; 123.5; 123.9; 124.0; 127.3; 127.4; 127.8; 129.5; 132.3; 133.3; 135.5; 148.8; 149.9; 151.6; 161.9; 168.5; 187.7. HRMS (ESI) calc. for $C_{22}H_{20}Cl_2N_4O_3$ 458.0912, found $[M + 1]^+$ 459.0983. HPLC: 100.0%

4.1.4.7. 2-(2-acetamido-5-bromophenyl)-N-(3-((7-chloroquinolin-4-yl) amino)propyl)-2-oxoacetamide (7). Yield: 18%. MP: 146–147 °C. IR (cm^{-1}): 3285; 2944; 1694; 1667; 1615; 1581; 1529; 1480; 1371; 1289; 1193; 1137; 1085; 814; 752; 691. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.87–1.91 (m, 2H, CH_2); 1.99 (s, 3H, CH_3); 3.35 (m, 4H, CH_2); 6.52 (d, 1H, J = 5.5 Hz, H3); 7.39 (t, 1H, J = 5.2 Hz, NH); 7.45–7.48 (m, 2H, H6; H3′); 7.68 (d, 1H, J = 2.4 Hz, H6′); 7.76 (dd, 1H, J = 2.4; 9.0 Hz, H4′); 7.79 (d, 1H, J = 2.2 Hz, H8); 8.26 (d, 1H, J = 9.0 Hz, H5); 8.40 (d, 1H, J = 5.5 Hz, H2); 8.79 (t, 1H, J = 5.9 Hz, NH); 10.47 (s, 1H, H). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 23.39; 27.20; 36.48; 98.60; 115.17; 117.29; 123.80; 123.94; 124.07; 127.09; 128.08; 132.41; 133.44; 135.27; 135.97; 148.57; 150.06; 151.47; 161.95; 168.58; 187.67. HRMS (ESI) calc. For $C_{22}H_{20}BrClN_4O_3$ 502.0407, found $[M + 1]^+$ 505.0497. HPLC: 96.6%.

4.1.4.8. 2-(2-acetamidophenyl)-N-(4-((7-chloroquinolin-4-yl)amino) butyl)-2-oxoacetamide (8). Yield: 60%. MP: 169–171 °C. IR (cm^{-1}): 3363; 3268; 2855; 2024; 1664; 1573; 1525; 1444; 1205. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.71–1.61 (m, 4H, 2 CH_2); 2.04 (s, 3H, CH_3); 3.35–3.24 (m, 4H, 2 CH_2); 6.52 (d, 1H, J = 5.5 Hz, H3); 7.20 (td, 1H, J = 1.0; 7.6 Hz, H4′ or H5′); 7.50–7.45 (m, 2H, H4′ or H5′; NH); 7.63–7.58 (m, 2H, H3′; H6); 7.79 (d, 1H, J = 2.2 Hz, H8); 7.87 (d, 1H, J = 7.8 Hz, H3′); 8.30 (d, 1H, J = 9.0 Hz, H5); 8.39 (d, 1H, J = 5.5 Hz, H2); 8.76 (t, 1H, J = 5.8 Hz, NH); 10.58 (s,1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 24.0; 25.1; 26.4; 38.2; 42.0; 98.6; 117.3; 121.3; 123.3; 124.1; 124.2; 126.8; 131.6; 133.6; 134.0; 138.5; 148.3; 150.4; 151.2; 163.7; 168.7; 191.6. HRMS (ESI) calc. for $C_{23}H_{23}ClN_4O_3$ 438.9067, found $[M + 1]^+$ 439.1545. HPLC: 99.9%.

4.1.4.9. 2-(2-acetamido-5-methylphenyl)-N-(4-((7-chloroquinolin-4-yl) amino)butyl)-2-oxoacetamide (9). Yield: 25%. MP: 142–144 °C. IR (cm^{-1}): 3386; 3223; 3099; 3024; 2925; 2869; 1695; 1631; 1610; 1589; 1525; 1452; 1363; 1304; 1237; 1181; 1135; 1090; 817; 766. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.60–1.75 (m, 4H, CH_2); 2.02 (s, 3H, CH_3); 2.27 (s, 3H, CH_3); 3.24–3.29 (m, 2H, CH_2); 3.50–3.55 (m, 2H, CH_2); 6.82 (d, 1H, J = 7.0 Hz, H3); 7.38–7.42 (m, 2H, H4′; H6′); 7.67–7.72 (m, 2H, H6; H3′); 8.08 (d, 1H, J = 2.0 Hz, H8); 8.48 (d, 1H, J = 7.0 Hz, H2); 8.71 (d, 1H, J = 9.0 Hz, H5); 8.77 (t, 1H, J = 6.0 Hz, NH); 9.38 (s, 1H, NH); 10.58 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.18; 23.89; 25.04; 26.24; 38.15; 45.55; 98.58; 115.88; 120.60; 121.67; 124.24; 125.76; 126.16; 131.26; 132.61; 134.43; 135.99; 136.98; 140.60; 144.34; 154.33; 163.79; 168.63; 191.53. HRMS (ESI) calc. For $C_{24}H_{25}ClN_4O_3$ 452.1615, found $[M + 1]^+$ 453.1693. HPLC: 97.1%.

4.1.4.10. 2-(2-acetamido-5-chlorophenyl)-N-(4-((7-chloroquinolin-4-yl) amino)butyl)-2-oxoacetamide (10). Yield: 55%. MP: 168–169 °C (dec.). IR (cm^{-1}): 3239; 3097; 3011; 2877; 1701; 1660; 1582; 1518; 1476; 1366; 1336; 1261; 1133; 1079; 851; 707. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.60–1.72 (m, 4H, CH_2); 1.99 (s, 3H, CH_3); 3.22–3.31 (m, 2H, CH_2); 6.49 (d, 1H, J = 5.5 Hz, H3); 7.33 (t, 2H, J = 5.3 Hz, CH_2) 7.43 (dd, 1H, J = 2.0; 9.0 Hz, H4′); 7.56–7.59 (m, 2H, H3′; H6′); 7.64 (dd, 1H, J = 2.0; 9.0 Hz, H6); 7.77 (d, 1H, J = 2.0 Hz, H8); 8.27 (d, 1H, J = 9.0 Hz, H5); 8.38 (d, 1H, J = 5.5 Hz, H2); 8.73 (t, 1H, J = 6.0 Hz, NH); 10.46 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 23.43; 25.04; 26.32; 38.31; 41.91; 98.58; 117.34; 123.58; 123.87; 124.00; 127.31; 127.32; 127.42; 129.71; 132.58; 133.25; 135.90; 148.94; 149.99; 151.76; 162.08; 168.61; 188.22. HRMS (ESI) calc. For $C_{23}H_{22}Cl_2N_4O_3$ 472.1069, found $[M + 1]^+$ 473.1136. HPLC: 96.5%.

4.1.4.11. 2-(2-acetamidophenyl)-N-(3-((7-chloroquinolin-4-yl)amino) propyl)-2,2-difluoroacetamide (11). Yield: 44%. MP: 207–209 °C. IR (cm^{-1}): 3337; 3260; 1670; 1573; 1514; 1448; 1217. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm):): 1.89–1.82 (m, 2H, $CH_2CH_2CH_2$); 2.03 (s, 3H, CH_3); 3.36–3.22 (m, 4H, $CH_2CH_2CH_2$ inside H_2O of DMSO); 6.38 (d, 1H, J = 5.4 Hz, H3); 7.32–7.29 (m, 2H, H6 and NH); 7.45 (dd, 1H, J = 2,2 and 9.0 Hz, H4′ or H5′); 7.58–7.51 (m, 2H, H4′ or H5′ and H8); 7.78–7.74 (m, 2H, H3′ and H6′); 8.23 (d, 1H, J = 9.0 Hz, H5); 8.36 (d, 1H, J = 5.4 Hz, H2); 9.15 (s, 1H, NH); 9.58 (s,1H, $NHCOCH_3$). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 23.5; 27.0; 37.1; 98.4; 114.3 (t, J = 253 Hz, CF_2); 117.3; 123.9; 124.0; 124.8; 125.3 (t, J = 24 Hz, C1′); 125.8 (t, J = 8 Hz, C6′); 126.1; 127.2; 131.4; 133.3; 135.8; 148.7; 149.9; 151.5; 163.9 (t, J = 30 Hz, $COCF_2$); 168.2. ^{19}F NMR (376 MHz, DMSO-d_6, TMS, δ in ppm): −100.7. HRMS (ESI) calc. for $C_{22}H_{21}ClF_2N_4O_2$ 446.1321, found $[M + 1]^+$ 447.1396. HPLC: 98.4%.

4.1.4.12. 2-(2-acetamido-5-chlorophenyl)-N-(3-((7-chloroquinolin-4-yl) amino)propyl)-2,2-difluoroacetamide (12). Yield: 37%. MP: 221–223 °C. IR (cm^{-1}): 3356; 2935; 1678; 1582; 1520; 1365; 1298; 1215; 1115. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.89–1.82 (m, 2H, $CH_2CH_2CH_2$); 2.01 (s, 3H, CH_3); 3.33–3.22 (m, 4H, $CH_2CH_2CH_2$); 6.40 (d, 1H, J = 5.5 Hz, H3); 7.35 (t, 1H, J = 5.3 Hz, NH); 7.46 (dd, 1H, J = 2.2 and 9.0 Hz, H6); 7.58 (d, 1H, J = 2.4 Hz, H6′); 7.62 (dd, 1H, J = 2.4 and 8.6 Hz, H3′); 7.78–7.75 (m, 2H, H4′ and H8); 8.24 (d, 1H, J = 9.0

Hz, H5); 8.37 (d, 1H, $J = 5.5$ Hz, H2); 9.14 (t, 1H, $J = 5.5$ Hz, NH); 9.54 (s,1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm):): 23.4; 26.9; 37.1; 79.0; 98.4; 113.3 (t, $J = 254$ Hz, CF_2); 117.2; 123.9; 124.0; 125.6 (t, $J = 9$ Hz, C6′);127.0; 128.1; 128.0; 131.3; 133.4; 134.7; 148.4; 148.5; 150.0; 151.3; 151.4; 163.2 (t, $J = 30$ Hz, $COCF_2$); 168.4. ^{19}F NMR (376 MHz, DMSO-d_6, TMS, δ in ppm): −101.9. HRMS (ESI) calc. for $C_{22}H_{20}Cl_2F_2N_4O_2$ 480.0931, found $[M + 1]^+$ 481.1007. HPLC: 99.2%.

4.1.4.13. 2-(2-acetamidophenyl)-N-(4-((7-chloroquinolin-4-yl)amino) butyl)-2,2-difluoroacetamide (13). Yield: 40% MP: 172–174 °C. IR (cm^{-1}): 3359; 2936; 2015; 1680; 1579; 1535; 1448; 1309; 1221. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.60–1.59 (m, 4H, CH_2-CH_2); 2.03 (s, 3H, CH_3); 3.29–3.19 (m, 4H, 2 CH_2); 6.46 (d, 1H, $J = 5.5$ Hz, H3); 7.29 (t, 1H, $J = 7.5$ Hz, H6); 7.37 (t, 1H, $J = 5.2$ Hz, NH), 7.45 (dd, 1H, $J = 2$,2 and 9.0 Hz, H4′ or H5′); 7.56–7.50 (m, 2H, H4′ or H5′ and H8); 7.78–7.75 (m, 2H, H3′ and H6′); 8.26 (d, 1H, $J = 9.0$ Hz, H5); 8.38 (d, 1H, $J = 5.5$ Hz, H2); 9.11 (t, 1H, $J = 5.0$ Hz, NH); 9.59 (s,1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 23.6; 24.9; 26.0; 38.8; 41.8; 98.5; 114.3 (t, $J = 253$ Hz, CF_2); 117.2; 123.92; 123.99; 124.7; 125.2 (t, $J = 24$ Hz, C1′); 125.7 (t, $J = 8$ Hz, C6′); 126.0; 127.2; 131.3; 133.3; 135.8; 148.8; 150.0; 151.6; 163.8 (t, $J = 30$ Hz, $COCF_2$); 168.2. ^{19}F NMR (376 MHz, DMSO-d_6, TMS, δ in ppm): −100.7. HRMS (ESI) calc. for $C_{23}H_{23}ClF_2N_4O_2$ 460.1478, found $[M + 1]^+$ 461.1562. HPLC: 98.9%.

4.1.4.14. 2-(2-acetamidophenyl)-N-(4-((6-methoxyquinolin-8-yl)amino) pentyl)-2-oxoacetamide (14). Yield: 43%. MP: 48–52 °C. IR (cm^{-1}): 3292; 3075; 2933; 2861; 1673; 1644; 1577; 1515; 1448; 1385; 1299; 1203; 1160; 1055; 1028; 820; 790; 753. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.32 (d, 3H, $J = 6.0$ Hz, CH_3); 1.76–1.80 (m, 4H, CH_2); 2.19 (s, 3H, CH_3); 3.41–3.47 (m, 2H, CH_2); 3.65–3.68 (m, 1H, CH); 3.86 (s, 3H, OCH_3); 6.0 (d, 1H, $J = 8.0$ Hz, NH); 6.28 (d, 1H, $J = 2.5$ Hz, H5 or H7); 6.32 (d, 1H, $J = 2.5$ Hz, H7 or H5); 6.99 (t, 1H, $J = 5.5$ Hz, NH); 7.08 (dt, 1H, $J = 1.0$; 8.0 Hz, H5′); 7.29 (q, 1H, $J = 4.2$; 8.2 Hz, H3); 7.57 (dt, 1H, $J = 1.0$; 8.0 Hz, H4′); 7.91 (dd, 1H, $J = 1.6$; 8.2 Hz, H4); 8.31 (dd, 1H, $J = 1.6$; 8.2 Hz, H6′); 8.50 (dd, 1H, $J = 1.6$; 4.2 Hz, H2); 8.63 (dd, 1H, $J = 1.0$; 8.0 Hz, H3′); 10.92 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.67; 25.46; 25.98; 34.00; 39.62; 47.80; 55.20; 91.85; 96.95; 118.61; 120.60; 121.90; 122.53; 129.90; 134.39; 134.89; 135.32; 136.45; 142.05; 144.37; 144.81; 159.36; 162.95; 169.25; 192.08. HRMS (ESI) calc. For $C_{25}H_{28}N_4O_4$ 448.2111, found $[M + 1]^+$ 449.2197. HPLC: 99.5%.

4.1.4.15. 2-(2-acetamido-5-methylphenyl)-N-(4-((6-methoxyquinolin-8-yl)amino)pentyl)-2-oxoacetamide (15). Yield: 36%. MP: 58–60 °C. IR (cm^{-1}): 3300; 2931; 2856; 1644; 1620; 1592; 1514; 1454; 1386; 1296; 1224; 1171; 1055; 1028; 820; 790. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.32 (d, 3H, $J = 6.0$ Hz, CH_3); 1.75–1.80 (m, 4H, CH_2); 2,18 (s, 3H, CH_3); 2.31 (s, 3H, CH_3); 3.42–3.48 (2H, m, CH_2); 3.67 (m, 1H, CH); 3.86 (s, 3H, OCH_3); 6.00 (d, 1H, $J = 8.0$ Hz, NH); 6.28 (d, 1H, $J = 2$,4 Hz, H5 or H7); 6.32 (d, 1H, $J = 2.4$ Hz, H7 or H5); 6.95 (t, 1H, NH); 7.29 (q, 1H, $J = 4.2$; 8.2 Hz, H3); 7.36 (d, 1H, $J = 9.0$ Hz, H4′); 7.91 (dd, 1H, $J = 1.6$; 8.2 Hz, H4); 8.08 (s, 1H, H6′); 8.50–8.52 (m, 2H, H3′; H2); 10.80 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.68; 25.40; 26.01; 34.02; 39.62; 47.82; 55.19; 91.85; 96.95; 118.70; 120.65; 121.90; 129.90; 132.15; 134.24; 134.83; 135.33; 137.28; 139.69; 144.37; 144.81; 159.37; 163.06; 169.08; 192.09. HRMS (ESI) calc. For $C_{26}H_{30}N_4O_4$ 462.2267, found $[M + 1]^+$ 463.2344. HPLC: 98.8%.

4.1.4.16. 2-(2-acetamido-5-chlorophenyl)-N-(4-((6-methoxyquinolin-8-yl)amino)pentyl)-2-oxoacetamide (16). Yield: 32%. MP: 136–138 °C. IR (cm^{-1}): 3297; 2931; 2870; 2332; 1973; 1643; 1511; 1385;1196;1159. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.22 (d, 3H, $J = 6.2$ Hz, CH_3); 1.66–1.58 (m, 4H, CH_2); 1.97 (s, 3H, CH_3); 3.23–3.19 (m, 2H, CH_2); 3.68–3.63 (m, 1H, CH); 3.81 (s, 3H, OCH_3); 6.14 (d, 1H, $J = 8.8$ Hz, H3′); 6.27 (d, 1H, $J = 2.4$ Hz, H5 or H7); 6.47 (d, 1H, $J = 2.4$ Hz, H5 or H7); 7.42 (q, 1H, $J = 4.2$ Hz, H3); 7.65–7.55 (m, 3H, H4′, H6′ and NH); 8.07 (dd, 1H, $J = 1.6$; 8.3 Hz, H4); 8.53 (dd, 1H, $J = 1.6$; 4.2 Hz, H2); 8.73 (t, 1H, $J = 5.9$ Hz, NH); 10.4 (s,1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.1; 23.4; 25.5; 33.3; 38.6; 46.8; 54.8; 91.5; 96.0; 122.0; 123.5; 127.2; 127.3; 129.4; 129.7; 132.5; 134.4; 134.6; 135.9; 144.1; 144.5; 158.9; 162.0; 168.6; 188.3. HRMS (ESI) calc. for $C_{25}H_{27}ClN_4O_4$ 482.1721, found $[M + 1]^+$ 483.1802. HPLC: 91.4%

4.1.4.17. 2-(2-acetamido-5-bromophenyl)-N-(4-((6-methoxyquinolin-8-yl)amino)pentyl)-2-oxoacetamide (17). Yield: 45%. MP: 146–148 °C. IR (cm^{-1}): 3306; 1695; 1642; 1510; 1386; 1195; 1161. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.22 (d, 3H, $J = 6.2$ Hz, CH_3); 1.73–1.55 (m, 4H, CH_2); 1.97 (s, 3H, CH_3); 3.23–3.17 (m, 2H, CH_2); 3.67–3.65 (m, 1H, CH); 3.81 (s, 3H, OCH_3); 6.14 (s, 1H, NH); 6.27 (d, 1H, $J = 2.4$ Hz, H5 or H7); 6.47 (d, 1H, $J = 2.4$ Hz, H5 or H7); 7.42 (q, 1H, $J = 4.2$ Hz, H3); 7.54 (d, 1H, $J = 8.7$ Hz, H3′); 7.67 (d, 1H, $J = 2.4$ Hz, H6′); 7.75 (dd, 1H, $J = 2.4$; 8.7 Hz, H4′); 8.08 (dd, 1H, $J = 1.6$; 8.3 Hz, H4); 8.53 (dd, 1H, $J = 1.6$; 4.2 Hz, H2); 8.73 (t, 1H, $J = 5.8$ Hz, NH); 10.4 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.2; 23.5; 25.6; 33.4; 38.7; 47.0; 54.9; 91.6; 96.1; 115.1; 122.1; 123.8; 127.6; 129.6; 132.7; 134.4; 134.8; 135.5; 136.5; 144.2; 144.5; 159.0; 162.1; 168.7; 188.3. HRMS (ESI) calc. for $C_{25}H_{27}BrN_4O_4$ 526.1216, found $[M + 1]^+$ 527.1288. HPLC: 99.3%.

4.1.4.18. 2-(2-acetamidophenyl)-2,2-difluoro-N-(4-((6-methoxyquinolin-8-yl)amino)pentyl)acetamide (18). Yield: 7%. MP: 86–89 °C. IR (cm^{-1}): 3273; 3051; 2935; 1682; 1614; 1590; 1516; 1449; 1386; 1302; 1265; 1238; 1219; 1202; 1142; 1087; 1050; 1030; 820; 755. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.25 (d, 3H, $J = 6.0$ Hz, CH_3); 1.62–1.72 (m, 4H, CH_2); 2.14 (3H, s, CH_3); 3.30–3.34 (m, 2H, CH_2); 3.59–3.66 (m, 1H, CH); 3.88 (s, 3H, OCH_3); 5.99 (d, 1H, $J = 5.0$ Hz, NH); 6.28 (d, 1H, $J = 2.4$ Hz, H5 or H7); 6.38 (d, 1H, $J = 2.4$ Hz, H7 or H5); 6.78 (s, 1H, NH); 7.12 (1H, t, $J = 8.0$ Hz, H5′ or H4′); 7.33 (q, 1H, $J = 4.2$; 8.0 Hz, H3); 7.42–7.46 (m, 2H, H4; H6′); 7.96 (d, 1H, $J = 8.0$ Hz, H3′); 8.14 (d, 1H, $J = 8.0$ Hz, H4′ or H5′); 8.50 (dd, 1H, $J = 1.6$; 4.2 Hz, H2); 9.76 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.87; 24.96; 26.25; 34.24; 40.50; 48.29; 55.73; 92.39; 97.45; 115.10 (t, $J = 253.0$ Hz); 122.48; 123.29 (t, $J = 24.0$ Hz); 124.29; 124.80; 125.83 (t, $J = 8.0$ Hz); 130.50; 132.21; 135.31; 137.42 (t, $J = 3.6$ Hz); 144.86; 145.39; 160.07; 165.96 (t, $J = 31.0$ Hz); 169,02. ^{19}F NMR (376 MHz, DMSO-d_6, TMS, δ in ppm): −104.4. HRMS (ESI) calc. For $C_{25}H_{28}F_2N_4O_3$ 470.2129, found $[M + 1]^+$ 471.22230. HPLC: 99.0%.

4.1.4.19. 2-(2-acetamido-5-methylphenyl)-2,2-difluoro-N-(4-((6-methoxyquinolin-8-yl)amino)pentyl)acetamide (19). Yield: 3%. MP: 56–58 °C. IR (cm^{-1}): 3272; 2929; 1682; 1614; 1515; 1454; 1386; 1300; 1202; 1184; 1156; 1121; 1097; 1050; 1030; 818; 790. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.25 (d, 3H, $J = 6.0$ Hz, CH_3); 1.61–1.71 (m, 4H, CH_2); 2.12 (s, 3H, CH_3); 2.31 (s, 3H, CH_3); 3.29–3.35 (m, 2H, CH_2); 3.62 (m, 1H, CH); 3.87 (s, 3H, OCH_3); 6.01 (s, 1H, NH); 6.28 (d, 1H, $J = 2.4$ Hz, H5; H7); 6.38 (d, 1H, $J = 2.4$ Hz, H7; H5); 6.76 (s, 1H, NH); 7.24–7.26 (m, 2H, H4′or H4; H6′); 7.33 (q, 1H, $J = 4.2$; 8.0 Hz, H3); 7.97 (m, 2H, H3′; H4 or H4′); 8.51 (dd,1H, $J = 1.6$; 4.0 Hz, H4′ or H5′); 9.57 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 21.10; 20.87; 24.86; 26.28; 34.26; 40.48; 48.30; 55.72; 92.36; 97.40; 115.13 (t, $J = 253.0$ Hz); 122.47; 123.34 (t, $J = 24.0$ Hz); 125.06; 126.14 (t, $J = 9.0$ Hz); 130.51; 132.73; 134.39; 134.72 (t, $J = 3.7$ Hz); 135.32; 135.82; 144.84; 145.39; 160.07; 165.97 (t, $J = 31.0$ Hz); 168.95. ^{19}F NMR (376 MHz, DMSO-d_6, TMS, δ in ppm): −104.4. HRMS (ESI) calc. For $C_{26}H_{30}F_2N_4O_3$ 484.2286, found $[M + 1]^+$ 485.2376. HPLC: 90.0%.

4.1.4.20. 2-(2-acetamido-5-chlorophenyl)-2,2-difluoro-N-(4-((6-methoxyquinolin-8-yl)amino)pentyl)acetamide (20). Yield: 32%. MP: 122–124 °C. IR (cm^{-1}): 3308; 3052; 2950; 2364; 2155; 2016; 1683;

1514; 1453; 1209. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.17 (d, 3H, CH_3); 1.62–1.48 (m, 4H, CH_2); 2.02 (s, 3H, CH_3); 3.17–3.14 (m, 2H, CH_2); 3.64–3.59 (m, 1H, CH); 3.82 (s, 3H, OCH_3); 6.10 (d, 1H, J = 8.7 Hz, H3′); 6.25 (d, 1H, J = 2.4 Hz, H5); 6.47 (d, 1H, J = 2.4 Hz, H7); 7.42 (q, 1H, J = 4.2 Hz, H3); 7.55 (d, 1H, J = 2.4 Hz, H6′); 7.60 (dd, 1H, J = 2.4; 8.7 Hz, H4′); 7.78 (d, 1H, NH); 8.07 (dd, 1H, J = 1.6; 8.2 Hz, H4); 8.52 (dd, 1H, J = 1.6; 4.2 Hz, H2); 9.10 (t, 1H, NH); 9.53 (s,1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.1; 23.5; 25.2; 33.1; 46.8; 54.9; 91.6; 96.1; 113.4 (t, J = 254 Hz, CF_2); 122.0; 125.7 (t, J = 8.4 Hz); 127.2 (t, J = 25.0 Hz); 128.0; 128.9; 129.5; 131.3; 134.5; 134.7; 134.9 (t, J = 9.0); 144.2; 144.5; 158.9; 163.1 (t, J = 30 Hz); 168.4. ^{19}F NMR (376 MHz, DMSO-d_6, TMS, δ in ppm): −101.2. HRMS (ESI) calc. for $C_{25}H_{27}ClF_2N_4O_3$, 504.1740, found $[M + 1]^+$ 505.1827. HPLC: 98.6%.

4.1.4.21. 2-(2-acetamido-5-bromophenyl)-2,2-difluoro-N-(4-((6-methoxyquinolin-8-yl)amino)pentyl)acetamide (21). Yield: 6%. MP: 84–87 °C. IR (cm^{-1}): 3272; 3072; 2926; 1685; 1613; 1516; 1455; 1386; 1294; 1208; 1154; 1108; 1051; 1030; 819; 790. ^{1}H NMR (400 MHz, DMSO-d_6, TMS, δ in ppm): 1.29 (d, 3H, J = 6.0 Hz, CH_3); 1.67–1.77 (m, 4H, CH_2); 2.20 (s, 3H, CH_3); 3.29–3.37 (2H, m, CH_2); 3.64 (m, 1H, CH); 3.90 (s, 3H, OCH_3); 6.00 (s, 1H, NH); 6.29 (d, 1H, J = 2.4 Hz, H5); 6.37 (d, 1H, J = 2.4 Hz, H7); 6.80 (s, 1H, NH); 7.32 (q, 1H, J = 4.0; 8.0 Hz, H3); 7.36–7.41 (m, 2H, H4′; H6′); 7.93; 7.95 (dd, 1H, J = 1.6; 8.0 Hz, H4); 8.19 (d, 1H, J = 9.0 Hz, H3′); 8.54 (dd, 1H, J = 1.6; 4.0 Hz, H2); 9.89 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6, TMS, δ in ppm): 20.90; 24.99; 26.22; 34.24; 40.62; 48.28; 55.73; 92.40; 97.43; 114.17 (t, J = 255.0 Hz); 116.65; 122.50; 124.82 (t, J = 25.0 Hz); 126.16; 128.80 (t, J = 10.0 Hz); 130.49; 135.12; 135.32; 135.82; 136.73 (t, J = 3.6 Hz); 144.86; 145.38; 160,04; 165.47 (t, J = 31.0 Hz); 169.06. ^{19}F NMR (376 MHz, DMSO-d_6, TMS, δ in ppm): −104.7. HRMS (ESI) calc. For $C_{25}H_{27}BrF_2N_4O_4$ 548.1235, found $[M + 1]^+$ 549.1340. HPLC: 97.8%.

4.2. Biological evaluation

4.2.1. Continuous cultures of P. Falciparum

P. falciparum CQR (W2 clone) parasites isolated by Oduola and coworkers[38] were cultured in human red blood cells as described by Trager and Jensen[39] with minor modifications.[40] The parasites were kept at 37 °C in human erythrocytes (A^+) in complete medium (RPMI 1640 supplemented with ALBUMAX II, 2% glutamine, and 7.5% $NaHCO_3$) in Petri dishes using the candle jar method.

4.2.2. In vitro assays with P. falciparum-infected erythrocytes

Before testing, the ring-stage parasites were synchronized using sorbitol solution.[41] Then, the infected red blood cells were distributed in a 96-well microtiter plate (Corning, Santa Clara, CA, USA) at 180 μL/well already containing 20 μL of different concentrations of test compounds and the antimalarials controls.

The effects of compounds against the *P. falciparum* blood cultures were evaluated through SYBR test as described.[42] Briefly, the test compounds, in serial dilutions, were incubated with the parasite suspensions (0.5% parasitemia, 2% hematocrit) in 96-well plates ('U' bottom). After 48 h at 37 °C, the culture supernatant was removed and replaced by 100 μL of lysis buffer solution [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; vol/vol)] followed by the addition of 0.2 μL/mL SYBR Safe (Sigma-Aldrich, Carlsbad, CA, USA). The plate content was transferred to a flat bottom plate and incubated in the dark for 30 min. Then, the plate was read in a fluorometer (Synergy H4 Hibrid Reader, BioteK) with excitation at 485 nm and an emission of 535 mm.

The antiplasmodial activity was calculated by comparing the inhibition of parasite growth in the drug-exposed cultures to those in the drug-free control culture. The tests, performed using serial drug dilutions, generated sigmoid dose–response curves with curve-fitting software (Microcal Origin Software 5.0, Inc.), which enabled the determination of the 50% inhibitory concentration (IC_{50}).

4.2.3. Cell cultures and cytotoxicity tests

For the cytotoxicity tests, a monkey kidney cell line (BGM), originally acquired from ATCC (American Type Culture Collection, Manassas, VA, USA) by the University of Minas Gerais, Brazil, was cultured in 75 cm^2 sterile flasks containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal serum and 40 mg/L of gentamicin in a 5% CO_2 atmosphere at 37 °C.[43] When the cell monolayer was confluent, it was trypsinized, washed with culture medium distributed in a flat-bottomed 96-well plate (2.5×10^5 cells/mL) and incubated for 24 h at 37 °C to ensure cell adherence.

Cytotoxicity assays were performed with cells properly diluted and incubated with 20 μL of the compounds at different concentrations (1000–1 μg/mL) for 24 h in a 5% CO_2 air atmosphere at 37 °C. The neutral red (NR) assay was used to evaluate cell viability with minor modifications.[44,45] Briefly, the cells were incubated for 3 h with 100 μL of an NR solution (4 mg/mL), the supernatants were discarded, and 200 μL of formaldehyde (0.5% v/v) and $CaCl_2$ (1%) solution were added followed by another 5 min incubation period. Then, the supernatant was removed, and 100 μL of an alcohol-acetic acid (50–1%) solution was added to the sediment. The absorbance was read at 540 nm on an ELISA reader (SpectraMax340PC384, Molecular Devices).

Cell viability was expressed as the percentage of control absorbance obtained in untreated cells after subtracting the absorbance from the appropriate background. Last, the minimum lethal dose for 50% of the cells (MLD_{50}) was determined as previously described in the literature.[46] The ratio between the MLD_{50} and drug activity (IC_{50}), allowed the determination of the drug specificity or selectivity index (SI) as described.[47]

4.2.4. P. Berghei and antimalarial tests in mice

The suppressive test was performed as described.[29] *P. berghei* NK65 parasites were maintained through weekly blood passages in mice. For the experiments, groups of up to 30 mice were inoculated i.p. with 1×10^6 infected erythrocytes. The mice were kept together for approximately 24 h and then randomly distributed into groups of five per cage. They were orally treated daily for three consecutive days with compounds (25 mg/kg) freshly diluted in 3% DMSO (Sigma-Aldrich) in RPMI medium. The control groups received either the drug vehicle or the antimalarial CQ administered at 20 mg/kg. On days 5 and 7 after the parasite inoculation, thin blood smears were taken, methanol-fixed, Giemsa stained, and examined microscopically (1000x) to determine parasitemia.

The inhibition of parasite growth was determined in relation to parasitemia in the untreated mice, which was considered to be 100% parasite growth. Compounds reducing the parasitemia by $\geq$ 40% were considered active; between 20% and 39%, partially active; and <20%, inactive.[48]

4.2.5. P. Gallinaceum sporogonic assays

The *P. gallinaceum* strain, received from the Oswaldo Cruz Institute, Rio de Janeiro, was maintained by weekly blood passages in domestic chickens (*Gallus gallus domesticus*) alternating with every 2–3 months by passage through *Aedes fluviatilis* mosquitoes.[28] *Ae. fluviatilis* mosquitoes were raised and kept at 28 °C ± 2 °C, 80% relative air humidity, with 10% glucose solution, and starved 12 h prior to an infectious blood meal.

For the testes, one-day old chickens were inoculated by the intramuscular route with *P. gallinaceum*-infected erythrocytes. Blood smears were taken daily, Giemsa stained and microscopically examined to determine parasitemia and gametocytemia. Chickens with increasing parasitemia below 10% were offered for the blood meal for *Ae. fluviatilis* female mosquitoes, i.e., at time 0 h (control) and 4 h after drug treatment (test group), as previously described.[27,29] Test compound 20 was used at 25–50 mg/kg; and PQ was used at 15 mg/kg (a nontoxic dose).

PQ was diluted in water, and compound 20 was suspended in RPMI with Arabic gum. The unfed female mosquitoes were discarded from the cages. Seven days later, 20 mosquitoes from each group were dissected, their midguts removed, stained with 0.2% mercurochrome and microscopically examined (400x) to evaluate the numbers of infected mosquitoes and oocysts as criteria to evaluate the drug's ability to block parasite sporogony.

A compound was considered active when the numbers of oocysts and mosquitoes infected were reduced by at least 50% in relation to the nontreated groups.

4.2.6. Resazurin cell viability assay for J774A.1 macrophages

Prior to the assessment of the anti-inflammatory activity, each compound was assayed for cytotoxicity on the J774A.1 macrophage cell line in order to determine a suitable noncytotoxic concentration. J774A.1 macrophages were cultured in DMEM supplemented with 10% FBS and 0.1% gentamycin. The cells were seeded in a flat-bottomed 96-well microplate at a density of 1×10^5 cells/well. After 24 h of incubation with the test compounds (0, 12.5, 25, 50, and 100 μM), the cells were incubated with Resazurin (10 μg/ml) for 3 h, and the fluorescence was read at $\lambda_{exc} = 555$ nm and $\lambda_{em} = 555$ nm. Each compound was tested in triplicate, and cell viability was expressed as a percentage of viable cells compared to 0.2% DMSO-treated cells.

4.2.7. Nitric oxide production by J774A.1 macrophages

Preliminary anti-inflammatory activity was assessed in J774A.1 macrophages. The test compounds were dissolved in DMSO at 10 mM and serially diluted in supplemented DMEM culture medium for the *in vitro* anti-inflammatory screening. Each compound was tested at the noncytotoxic concentration of 25 μM in triplicate. Macrophages were pre-incubated with dexamethasone (1 μM), 0.2% DMSO or 25 μM of each test compound in triplicate prior to LPS (37.5 ng/ml) + IFNγ (25 U/ml) stimulation for NO production after 24 h of incubation at 37 °C and 5% CO_2. Culture supernatants were collected and subjected to Griess reaction for colorimetric determination of nitrites at 540 nm. Dose-response curves (0.5–25 μM) for NO production were performed for compounds 6, 12 and 20 after the initial screening. The results are expressed as the mean ± SEM of nitrite concentration (μM) in the supernatants from each treatment.

4.2.8. Evaluation of TNFα production by J774A.1 macrophages

TNFα production was assessed in J774A.1 macrophages after LPS (100 ng/ml) stimulation for 24 h in 24-well culture plates. Cells were seeded in the density of 2×10^6 cells/well and pretreated with vehicle, 1 μM of dexamethasone or test compound 6, 12 or 20 (0.5–25 μM). Culture supernatants were collected and stored at −20 °C before TNFα determination by enzyme-linked immunosorbent assay (ELISA). Experiments were performed in triplicate, and the results are expressed as the mean ± SEM of TNFα concentration (pg/mL) in the supernatants from each treatment.

4.2.9. Enzyme-Linked immunosorbent assay (ELISA)

The levels of TNF-α were determined 24 h after stimulation with LPS (100 ng/ml). Sandwich enzyme-linked immunosorbent assay (ELISA) was performed according to standard procedures supplied by the manufacturers (R&D Systems, Minneapolis, MN, USA). The results are expressed as picograms per milliliter (pg/mL).

4.2.10. Animal ethical approval

The conditions to use laboratory animals were approved by the Ethics Committee for Animal Use from the Oswaldo Cruz Foundation - Fiocruz (CEUA LW-6/18).

4.2.11. Statistics

Statistical differences between means were determined by analysis of variance (ANOVA) followed by Dunnet's *post hoc* test and were considered significant for $p \leq 0.05$.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2020.115832.

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