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Research Article

7-Chloro-4-quinolinyl Hydrazones: A Promising and Potent Class of Antileishmanial Compounds

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In this work, we report the antileishmanial evaluation of twenty 7-chloro-4-quinolinyl hydrazone derivatives (1–20). Firstly, the compounds were tested against promastigotes of four different *Leishmania* species. After that, all derivatives were assayed against *L. braziliensis* amastigotes and murine macrophages. Furthermore, it was investigated whether the antiamastigote *L. braziliensis* effect of the compounds could be associated with nitric oxide production. Compounds 6 and 7 showed a strong leishmanicidal activity against intracellular parasite with IC_{50} in nanogram levels (30 and 20 ng/mL, respectively). Appreciable activity of three compounds tested can be considered an important finding for the rational design of new leads for antileishmanial compounds.

Key words: 7-chloro-4-quinolinyl hydrazones, amodiaquine, antileishmanial

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Leishmaniasis is a disease caused by a protozoan parasite of the genus *Leishmania*. The severity of the disease depends on the species of *Leishmania* responsible for the infection and the immune response to the infection. Therefore, clinical manifestations in humans can occur in three main forms: the most common one, cutaneous leishmaniasis; mucocutaneous leishmaniasis, which causes damages to nose and mouth tissues; and the most serious visceral leishmaniasis, which is fatal if untreated (1). This disease is endemic to the American, Asian, and African tropical countries, and there are 12 million people currently infected in about 88 countries. Among these, 72 are developing countries (a).

The first-line treatment against leishmaniasis is still based on pentavalent antimonial drugs, such as sodium stibogluconate and meglumine antimoniate. Other medications are used when the first-line regimen fails, such as pentamidine, amphotericin B, and paromycine, despite their high toxicity. In addition, antileishmanial drugs are expensive, and they are not orally active, requiring long-term parenteral administration, which might cause some problems regarding access to treatment, such as long distances to the treatment center, lack of transport and serious financial burden to the patients (2,3). Such problems favor the abandonment of the treatment and drug resistance may emerge. That being considering, there is an urgent need to develop new potent, nontoxic, and selective antileishmanial drugs to treat this neglected disease (4,5).

In this context, quinoline derivatives can be considered promising for the discovery of new antileishmanial agents, which can be exemplified by the activity of the antimalarial drug amodiaquine (AQ) (Figure 1), which is active against different species of *Leishmania* sp at μM concentration (6–8). Therefore, our research group decided to investigate the use of AQ as a prototype with the aim to develop new active compounds. As a result, we previously described the synthesis and antileishmanial activity of two series of AQ analogs: 7-chloro-4-aminoquinoline-aryl and 7-chloro-4-quinolinyl hydrazones derivatives (9). Among these, the compound **1** (Figure 1) displayed a significant activity against all promastigote forms of *Leishmania* species (IC_{50} values of 4.2, 4.9, 5.1, and 19.1 μg/mL for *Leishmania amazonensis*, *L. braziliensis*, *L. chagasi*, and *L. major*, respectively), being more effective than the AQ. Although, this compound has been cytotoxic in macrophages, we decided to continue exploring the antileishmanial potential of the 4-quinolinyl hydrazones series. As the presence of substituents at R^3 position, such as fluorine, chlorine, bromine, hydroxyl, methoxy, nitro, and cyano, was harmful to the biological activity of said series, we proposed in this work to evaluate the antileishmanial activity of other two series of 7-chloro-4-quinolinyl hydrazones (Figure 1):

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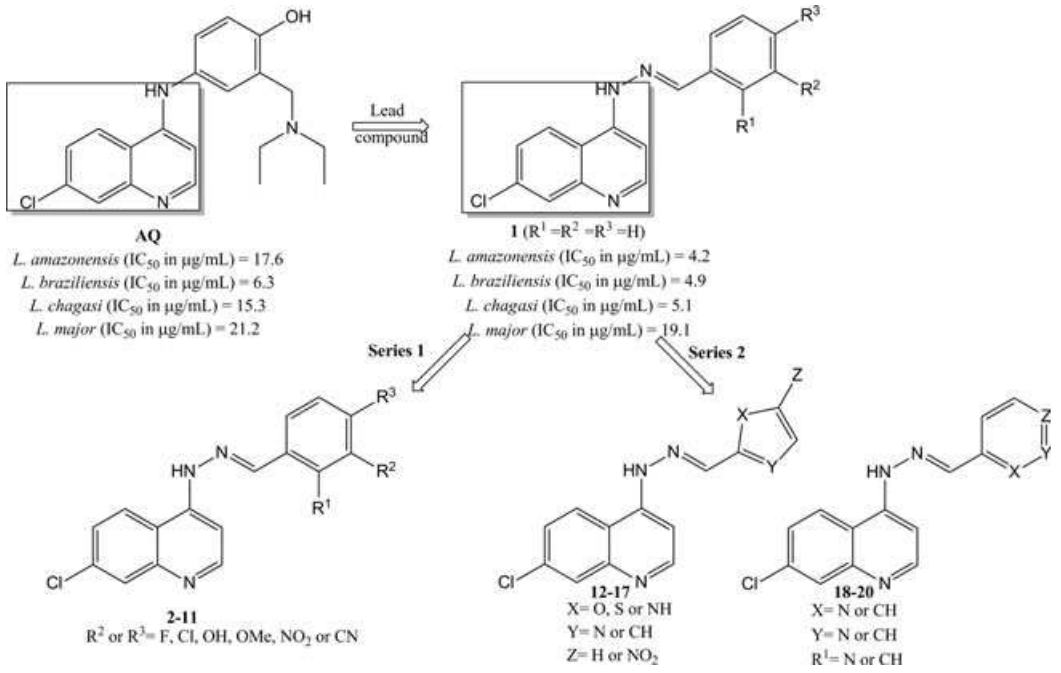

Figure 1: Amodiaquine analogs with potential antileishmanial activity synthesized by our research group.

(i) monosubstituted derivatives at R^1 or R^2 positions (**2**–**11**) and (ii) heteroaromatic derivatives (**12**–**20**). The choice of the substituents attached to the hydrazone moiety was made based on the different electronic and steric characteristics of these groups. In addition, the relevance of the present report also arises from the need for developing ongoing studies on the structure-activity relationship in this class of compounds.

Experimental

Synthesis

All 7-chloro-4-quinolinyl hydrazone derivatives described in this work (**1**–**20**) were previously synthesized by our research group. They were fully characterized by spectroscopic and spectrometric methods (10,11).

Antileishmanial activity

Four species of *Leishmania* were used: *L. chagasi* (MHOM/Br/74/PP75), *L. braziliensis* (MHOM/Br/75/M2903), *L. major* (MRHO/SU/59/P), and *L. amazonensis* (IFLA/Br/67/PH8). Promastigotes of *L. amazonensis* were cultured in Warren's medium [brain heart infusion (BHI)-plus hemin and folic acid], promastigotes of *L. major* and *L. braziliensis* were maintained in BHI medium, and promastigotes of *L. chagasi* were maintained in LIT medium, both supplemented with 10% fetal bovine serum at 24 °C. Fetal bovine serum was purchased from Cultilab (Campinas, São Paulo, Brazil); BHI and liver infusion tryptose from Himédia (Mumbai, India), and hemin and folic acid were purchased from Sigma Chemical Co (St. Louis, MO, USA). Amphotericin B was supplied by Cristália (São Paulo, Brazil), miltefosine was supplied by Cayman Chemical Company (Michigan, USA), and AQ was supplied by Ellipse Pharmaceuticals (Pessac, France).

Promastigotes assay

Antileishmanial activity was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), method based on tetrazolium salt reduction by mitochondrial dehydrogenase (12,13). The screening was performed in 96-well microtiter plates maintained at 24 °C. Briefly, promastigotes from a logarithmic phase culture were suspended to yield 2 millions of cells per mL (*L. amazonensis*) or 3 millions of cells per mL (*L. chagasi*, *L. braziliensis* and *L. major*) after Neubauer chamber counting. The analysis was performed in duplicate. The parasites were exposed to increasing concentrations of the compound (at minimum six serial dilutions) for 72 h at 24 °C. Controls containing 0.5% DMSO and medium alone were also included. The viability of promastigotes was assessed by MTT colorimetric method, and the absorbance was measured at 570 nm (Multiskan MS microplate reader; LabSystems Oy, Helsink, Finland). Amphotericin B and miltefosine were used as the reference drug**.**

Amastigotes assay

In vitro model of the amastigotes, inflammatory macrophages were obtained from BALB/c mice previously inoculated by the intraperitoneal route with 3% thioglycollate medium (Sigma). Briefly, peritoneal macrophages were pla-

ted at 2×10^6 cells/mL on coverslips (13 mm diameter) previously arranged in a 24-well plate in RPMI 1640 medium supplemented with 10% inactivated FBS and allowed to adhere for 24 h at 37 °C in 5% CO_2. Adherent macrophages were infected with *L. braziliensis* (MHOM/Br/75/M2903) promastigotes in the stationary growth phase using a ratio of 1:10 at 33 °C for 4 h. Non-internalized promastigotes were eliminated, and solutions of tested compounds were added in different concentrations and maintained at 33 °C in 5% CO_2 for 72 h. After 48 h, all culture supernatants were collected for nitric oxide (NO) measurement. Slides were fixed and stained with Giemsa for parasite counting (optical microscopy, 1000× magnification). The parasite burden was evaluated by counting intracellular parasite and the infected macrophages (100 cells infected). Amphotericin B and miltefosine were used as reference drug.

Cytotoxicity on macrophages

Mouse peritoneal macrophages were obtained and cultured as previously described. In brief, the macrophages were used for cytotoxicity assay in a concentration of 2×10^6 cells/mL in 96-well culture plates in RPMI 1640 medium supplemented with 10% inactivated FBS, at 37 °C and 5% CO_2 atmosphere. After 24 h, the adherent macrophages were incubated with the compounds in a serial dilution, in duplicate at each concentration for 72 h at 37 °C and 5% CO_2 atmosphere. The viability of the macrophages was determined with the MTT assay and was confirmed by comparing the control group morphology via light microscopy (12,13). Dose–response curves were plotted (values expressed as percentage of control optical density), and the values were expressed as CC_{50} values (50% cytotoxicity concentration).

Nitric oxide production

Nitric oxide production was determined in the supernatants of *L. braziliensis*-macrophages which were incubated for 48 h in the presence of the compounds. The assay was performed as described by Green *et al.* (14). In brief, 50 μL of Griess reagent 1% sulfanilamide in 2.5% of H_3PO_4 and 0.1% N-1-diidrocloreto de naftiletilenodiamina in 2.5% H_3PO_4 (v/v) was added to 50 μL of each sample in a 96-well microplate. Blank reference and standard curve were determined. The absorbance was measured at 540 nm using a microplate reader (Multiskan MS microplate reader; LabSystems Oy). Nitrite content was quantified by the extrapolation from sodium nitrite standard curve in each experiment. All the assays were performed in duplicate. The results were expressed as percentages in comparison with the non-treated macrophages infected. Lipopolysaccharides (LPS from *Escherichia coli* J5; Sigma-Aldrich, St. Louis, MO, USA) was used as positive control at 10 μg/mL.

Statistical analysis

For promastigote and amastigote forms of *Leishmania* and cytotoxicity against macrophages, the IC_{50} or CC_{50} values were carried out, and the 95% confidence intervals were included, calculated by Litchtfiet and Wilcoxon method using the Probit analysis, and the graphs were plotted by the program GRAPHPAD PRISM 4 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

All the 7-chloro-4-quinolinyl hydrazones derivatives **1**–**20** (Tables 1 and 2) were previously synthesized by our

Table 1: IC_{50} values (μg/mL) of Series 1 on promastigotes of *Leishmania* species

	Substituents			Antileishmanial activity (95% C.I.)[a]			
Compounds	R^1	R^2	R^3	*L. amazonensis*	*L. braziliensis*	*L. chagasi*	*L. major*
1	H	H	H	4.2 (3.3–5.4)	4.9 (3.6–6.6)	5.1 (4.0–6.6)	19.1 (15.6–23.3)
2	F	H	H	40.9 (34.0–49.2)	7.7 (6.4–9.3)	>100.0	30.1 (26.1–34.8)
3	H	F	H	8.1 (6.4–10.3)	2.1 (1.7–2.6)	32.5 (28.2–37.5)	2.0 (1.9–2.3)
4	H	Cl	H	5.8 (4.5–7.5)	2.1 (1.7–2.5)	17.8 (14.8–21.3)	1.6 (1.4–1.9)
5	OH	H	H	15.6 (13.3–18.3)	4.7 (3.4–6.7)	>100.0	>100.0
6	H	OH	H	>100.0	4.8 (3.4–6.9)	>100.0	>100.0
7	OMe	H	H	>100.0	1.0 (0.5–1.8)	>100.0	>100.0
8	H	OMe	H	4.0 (2.8–5.7)	1.6 (1.3–1.9)	10.3 (6.8–15.7)	2.4 (2.0–2.8)
9	NO_2	H	H	>100.0	1.0 (0.7–1.6)	>100.0	10.3 (7.0–15.1)
10	H	NO_2	H	>100.0	>100.0	>100.0	>100.0
11	H	CN	H	>100.0	3.9 (2.3–6.8)	>100.0	>100.0
AQ				17.6 (14.7–21.1)	6.3 (5.4–7.4)	15.3 (12.9–18.2)	21.2 (18.1–24.9)
AmB[b]				0.11 (0.09–0.12)	0.12 (0.09–0.14)	0.05 (0.05–0.06)	0.10 (0.09–0.11)
Miltefosine				14.1 (12.5–15.9)	10.4 (9.0–12.1)	8.3 (7.2–9.5)	8.8 (7.6–10.1)

AQ, amodiaquine.

[a]Data are IC_{50} values in μg/mL and 95% confidence intervals are in brackets. These data represent the average of three independent experiments.

[b]Amb (amphotericin B) and miltefosine were used as reference drugs.

Table 2: IC_{50} values (μg/mL) of Series 2 on promastigotes of *Leishmania* species

	Substituents			Antileishmanial activity[a] (95% C.I.)			
Compounds	X	Y	Z	*L. amazonensis*	*L. braziliensis*	*L. chagasi*	*L. major*
12	O	CH	H	>100.0	6.9 (5.8–8.2)	30.6 (22.6–41.4)	34.0 (25.2–45.9)
13	O	CH	NO_2	2.4 (2.0–2.9)	1.8 (1.5–2.2)	2.8 (2.0–3.9)	0.6 (0.5–0.8)
14	S	CH	H	3.0 (2.5–3.5)	0.5 (0.4–0.6)	2.6 (2.3–2.9)	0.5 (0.4–0.6)
15	S	CH	NO_2	9.3 (7.4–11.7)	3.4 (2.8–4.1)	6.6 (5.8–7.5)	2.3 (2.0–2.7)
16	NH	CH	H	2.9 (2.6–3.3)	0.8 (0.7–1.0)	0.5 (0.4–0.5)	0.8 (0.5–0.7)
17	NH	N	H	>100.0	>100.0	>100.0	>100.0
18	N	CH	CH	>100.0	>100.0	>100.0	>100.0
19	CH	N	CH	>100.0	>100.0	>100.0	>100.0
20	CH	CH	N	>100.0	>100.0	>100.0	>100.0
AQ				17.6 (14.7–21.1)	6.3 (5.4–7.4)	15.3 (12.9–18.2)	21.2 (18.1–24.9)
AmB[b]				0.11 (0.09–0.12)	0.12 (0.09–0.14)	0.05 (0.05–0.06)	0.10 (0.09–0.11)
Miltefosine[b]				14.1 (12.5–15.9)	10.4 (9.0–12.1)	8.3 (7.2–9.5)	8.8 (7.6–10.1)

AQ, amodiaquine.
[a]Data are IC_{50} values in μg/mL and 95% confidence intervals (C.I.) are in brackets. These data represent the average of three independent experiments.
[b]Amb (amphotericin B) and miltefosine were used as reference drugs.

research group (10,11). Antileishmanial activities of the compounds were determined *in vitro* against both the insect promastigote and the intramacrophage amastigote forms of *Leishmania*. Furthermore, the cytotoxicity of the compounds was assayed against mammalian cells.

For antipromastigote activity, these derivatives were assayed against four *Leishmania* species, consisting of three different species of *Leishmania* from the New World (*L. braziliensis*, *L. chagasi* and *L. amazonensis*) and one species from the Old World (*L. major*) (Tables 1 and 2). The numerous *Leishmania* species associated with human diseases have important implications for clinical treatment, and the sensitivity of each species should be considered in both experimental and clinical studies (15). With this in mind, it is important to point that the compounds with leishmanicidal activity shown to be effective against all *Leishmania* species tested.

In general, for Series 1 (Table 1), it was observed that the presence of substituents attached to the benzene ring which are electron withdrawing groups (EWG) by polar and by resonance effects**,** such as nitro (**9** and **10**) and cyano (**11**), seem to be less important to the biological activity than the presence of substituents which are EWG by polar effect but are electron donating groups (EDG) through resonance, such as halogens (**2**–**4**), hydroxyl (**5** and **6**), and methoxyl groups (**7** and **8**). Regarding this type of substituents, it was observed that when they are at the R^2 position (**3**, **4** and **8**), the spectrum of antileishmanial activity is broader, except for the hydroxyl group (**6**).

In addition, the results presented in Table 2 indicate that the presence of two nitrogen atoms in a five-membered ring (**17**) or the presence of a six-membered ring containing one nitrogen atom (**18**–**20**) is detrimental to the biological activity of such series. Moreover, comparing the five-membered derivatives which were active, it can be observed that the presence of a sulfur atom leads to a compound (**14**) with better activity than the oxygenated derivative (**12**). However, the introduction of a nitro group at Z position in those substances (**13** and **15**) is advantageous only for the nitrofuran derivative (**13**), because the thiophene derivative (**14**) is still more active than the nitrothiophene compound (**15**). Furthermore, the introduction of a nitrogen atom in the five-membered ring (pyrrole) also produces a derivative (**16**) with expressive activity against all four *Leishmania* species tested with IC_{50} of 2.9, 0.8, 0.5, and 0.8 μg/mL (*L. amazonensis*, *L. braziliensis*, *L. chagasi*, and *L. major*, respectively). Additionally, the five-membered derivatives of the heteroaromatic series (**12**–**16**) are more active than compounds of Series 1, with IC_{50} values below 1.0 μg/mL.

Although tests in amastigote forms of *Leishmania* are more difficult to accomplish, it is a common agreement among many authors that such parasite forms should be those chosen for drug screening, and tests using promastigotes must be preliminarily considered (16,17). Furthermore, Vermeersch and coworkers emphasized that the intracellular amastigote model should be used as 'gold standard' for the evaluation of leishmanicidal activity *in vitro*, due to the fact that the amastigote stage of the parasite is found in mammalian hosts, and it is responsible for all clinical manifestations of leishmaniasis (16). Therefore, we decided to continue studying the leishmanicidal activity of these 7-chloro-4-quinolinyl hydrazones series against *L. braziliensis* amastigotes (Tables 3 and 4). That species was chosen because its promastigotes were the most sensitive to the tested compounds, because the majority of the active derivatives in both series have shown better activities against this parasite form.

Table 3: Effect of the compounds of Series 1 on macrophage murines and intracellular amastigotes of *Leishmania braziliensis*

Compounds	Macrophages CC_{50} (μg/mL) (95% C.I.)	Amastigotes IC_{50} (μg/mL) (95% C.I.)	Selectivity[a]	Specificity[b]
1	17.9 (16.1–20.0)	1.1 (0.8–1.5)	16.3	4.4
2	38.0 (28.8–50.0)	0.1 (0.07–0.2)	380.0	77.0
3	32.7 (25.4–42.2)	0.9 (0.7–1.3)	36.3	2.3
4	24.8 (15.5–39.6)	0.3 (0.2–0.4)	82.7	7.0
5	15.9 (10.8–23.1)	0.5 (0.4–0.6)	31.8	9.4
6	>150.0	0.03 (0.01–0.07)	>5000.0	160.0
7	>150.0	0.02 (0.009–0.07)	>7500.0	50.0
8	15.0 (9.9–22.8)	0.5 (0.4–0.7)	30.0	3.2
9	33.8 (27.2–42.0)	2.9 (2.2–3.9)	11.6	0.3
10	>150.0	45.2 (31.0–66.0)	>3.3	>2.2
11	37.0 (26.6–51.6)	0.5 (0.3–0.6)	74.0	7.8
AQ	20.9 (17.9–24.3)	1.3 (1.1–1.6)	16.1	4.8
AmB[c]	>150.0	0.01 (0.005–0.02)	>15 000.0	12.0
Miltefosine[c]	49.4 (43.8–55.7)	1.0 (0.9–1.2)	49.4	10.4

CI, Confidence interval; AQ, amodiaquine.
[a]Selectivity: CC_{50} of macrophages/IC_{50} of amastigotes of *L. braziliensis*.
[b]Specificity: is the ratio between promastigote IC_{50} and intracellular amastigote IC_{50}.
[c]AmB (amphotericin B) and miltefosine were used as reference drugs.

Table 4: Effect of the compounds of Series 2 on macrophage murines and intracellular amastigotes of *Leishmania braziliensis*

Compounds	Macrophages CC_{50} (μg/mL) (95% C.I.)	Amastigotes0 IC_{50} (μg/mL) (95% C.I.)	Selectivity[a]	Specificity[b]
12	>150.0	0.2 (0.1–0.3)	>750.0	34.5
13	73.4 (35.2–152.8)	0.1 (0.07–0.2)	734.0	18.0
14	3.4 (2.2–5.1)	0.4 (0.3–0.6)	8.5	1.2
15	9.4 (4.9–18.1)	0.5 (0.4–0.7)	18.8	6.8
16	10.0 (5.2–19.1)	0.5 (0.4–0.6)	20.0	1.6
17	>150.0	8.0 (6.7–9.6)	>18.7	>12.5
18	>150.0	25.5 (20.0–32.4)	>5.9	>3.9
19	>150.0	1.0 (0.7–2.4)	>150.0	>100.0
20	>150.0	0.8 (0.5–1.4)	>187.5	>125.0
AQ	20.9 (17.9–24.3)	1.3 (1.1–1.6)	16.1	4.8
AmB[c]	>150.0	0.01 (0.005–0.02)	>15 000.0	12.0
Miltefosine[c]	49.4 (43.8–55.7)	1.0 (0.9–1.2)	49.4	10.4

CI, confidence interval; AQ, amodiaquine.
[a]Selectivity: CC_{50} of macrophages/IC_{50} of amastigotes of *L. braziliensis*.
[b]Specificity: is the ratio between promastigote IC_{50} and intracellular amastigote IC_{50}.
[c]AmB (amphotericin B) and miltefosine were used as reference drugs.

These results showed that all the tested compounds were active against amastigotes of *L. braziliensis*. However, it can be observed that regarding Series 1 and 2, the activity profile against amastigotes was very different from that identified for promastigotes (Table 1 and 2). For example, in the Series 1, among the halogenated compounds (**2**–**4**), the presence of a fluorine atom at R^1 position led to the best biological activity against amastigotes, whereas substitutions of hydrogen by hydroxyl (**5**–**6**) or methoxyl groups (**7**–**8**) at R^2 or R^1 positions, respectively, have promoted improved activities. Interestingly, compounds **6** (R^2=OH) and **7** (R^1=OMe) were as active as the reference drug, amphotericin B, with IC_{50} values in the same range (IC_{50} = 0.03, 0.02 and 0.01 μg/mL, respectively). Moreover, among the 20 compounds tested, 13 showed to be more active against intracellular forms than miltefosine. Miltefosine is the first oral drug used for the treatment for visceral leishmaniasis and was considered a major breakthrough in antileishmanial chemotherapy (18). In general, comparing Series 1 and 2, the heteroaromatic compounds (Series 2) have been less active against amastigotes than the aromatic ones (Series 1), which is the opposite of what was found for promastigotes. Among heteroaromatic compounds, furan derivatives (**12** and **13**)

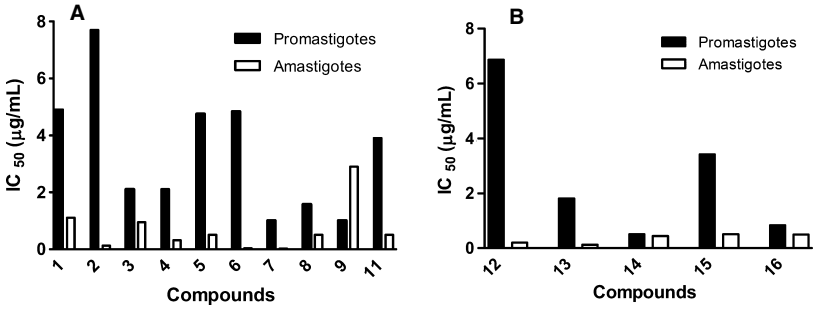

Figure 2: Comparative antipromastigotes and antiamastigotes activity of some compounds on *Leishmania braziliensis*.

have showed the best leishmanicidal activities, and in this case, the substitution with nitro group at Z position (**13**) promoted a slight improvement to the biological activity.

Despite the relevant activities, the majority of these substances are cytotoxic in macrophages with CC_{50} < 150.0 μg/mL (Tables 3 and 4). However, regarding the selectivity of those compounds, it is important mention that, when the compounds were assayed against amastigotes, they were much more destructive to that parasitic form than to the host cells. Such behavior can be noticed by the high values of selectivity presented in Tables 3 and 4. In fact, compounds **6** and **7** (Table 3) were 5000 and 7500 times more selective for the parasite than for macrophages, respectively.

Furthermore, Tables 3 and 4 also furnish information about the specificity of those compounds. Specificity value >2 was the cutoff point chosen to define a compound as being more active against the intracellular amastigote stage; while a specificity value <0.4 indicated a more active compound against promastigotes, compounds with specificity values between 0.4 and 2 were considered as being active against both stages (19). Therefore, it can be concluded that only compounds **14** and **16** were considered to be active against both amastigote and promastigote forms, while all the other compounds could be considered more active against amastigotes (Figure 2). These results reinforce the potential leishmanicidal activity of the 7-chloro-4-quinolinyl hydrazone compounds.

Table 5: Production of NO by *Leishmania braziliensis*-macrophages treated with the compounds

Compounds	Production of nitrite (%)[a]
1	N. T.
2	−1.1% (10 μg/mL)
3	−18.6% (10 μg/mL)
4	31.6% (10 μg/mL)
5	−9.3% (5 μg/mL)
6	31.6% (10 μg/mL)
7	−22.4% (10 μg/mL)
8	21.2% (10 μg/mL)
9	1.0% (10 μg/mL)
10	−45.0% (10 μg/mL)
11	−32.1% (10 μg/mL)
12	45.5% (10 μg/mL)
13	31.9% (10 μg/mL)
14	−28.5% (1 μg/mL)
15	−69.7% (5 μg/mL)
16	28.3% (10 μg/mL)
17	−78.2% (10 μg/mL)
18	−11.2% (10 μg/mL)
19	−29.0% (10 μg/mL)
20	−61.9% (10 μg/mL)
Stimulus[b]	320%

NO, nitric oxide.
[a]In parenthesis: maximum concentration tested for each compound.
[b]Stimulus used as positive control: 10 μg/mL of lipopolysaccharides. Tests were performed in duplicate.

Several investigations on the mechanisms of *Leishmania* killing by macrophages have clearly identified the generation of reactive nitrogen products as NO as the most likely cytotoxic molecules responsible for intracellular parasite destruction (20). So, we tried to investigate whether the antiamastigote *L. braziliensis* effect of the compounds could be associated with NO production. This assay was performed in parallel with the antiamastigote assay, collecting the supernatants of *L. braziliensis*-macrophages treated with several concentrations of compounds. Table 5 shows nitrite production by NO, only in the maximum concentration tested for each compound, when compared with non-treated, infected macrophages. For positive control, 10 μg/mL of LPS was used, and the results showed that this molecule was effective for stimulating NO production in high levels (320%). In general, a relation between leishmanicidal activity and nitrite production was not observed, and in fact, some of them even inhibited spontaneous NO production (negative values). However, some compounds were able to induce NO production in infected macrophages. For example, among the Series 1 (compounds **1**–**11**), only the compounds **4**, **6**, and **8** increased the production of nitrite at 31.6%, 31.6%, and 21.2%, respectively, when compared with the control. Among the Series 2 (compounds **12**–**20**), only compounds **12**, **13**, and **16** increased the production of nitrite at 45.5%, 31.9%, and 28.3%, respectively. It is interesting to note that among compounds **6** and **7**, which showed better

leishmanicidal activity against intracellular parasite (IC_{50} = 0.03 and 0.02 μg/mL), only compound **6** increased the NO production, but not compound **7**. These results showed that for some compounds, the leishmanicidal activity should be associated with the activation of NO production by macrophages, but it is not the only route, and the compounds can also act directly against the intracellular parasite. It is interesting to note that most of the compounds, including those which induced NO production, were more active against intracellular amastigotes than against free promastigotes, suggesting drug metabolization by macrophages, as observed with the pentavalent antimonials (21).

Conclusion

In summary, two series of 7-chloro-4-quinolinyl hydrazone derivatives have been evaluated against four different species of *Leishmania* promastigotes. Among these results, the majority of the active derivatives in both series have shown better activity against promastigotes of *L. braziliensis species.* Considering that, we decided to continue studying the leishmanicidal activity of such series against *L. braziliensis* amastigotes, and all the tested compounds were active, among which compounds **6** and **7** were as active as a reference drug used, amphotericin B, with IC_{50} values in the same range (IC_{50} = 0.03, 0.02 and 0.01 μg/mL, respectively). Furthermore, these derivatives were 5000 and 7500 times more selective for the parasite than for macrophages, respectively. Together with the structure-activity relationship study performed in this work, these results could be considered a significant contribution to the development of such class of compounds as potential leishmanicidal agents.

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Note

[a]WHO (World Health Organization). Leishmaniasis. Available at http://www.who.int/leishmaniasis/en/. Accessed on July 30 2012.

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Full length article

Aminoquinoline compounds: Effect of 7-chloro-4-quinolinylhydrazone derivatives against *Leishmania amazonensis*

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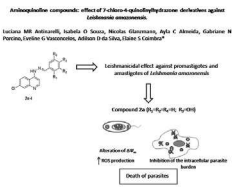
HIGHLIGHTS

- A series of quinoline derivatives was synthesized and showed leishmanicidal effect.
- Compound **2a** presented activity against promastigotes and amastigotes *L. amazonensis.*
- Compound **2a** induces formation of ROS and a reduction of mitochondrial membrane potential.
- Compound **2a** exhibited a selective and effective leishmanicidal effect.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, we have investigated the antileishmanial activity of ten 7-chloro-4-quinolinylhydrazone derivatives. Among the compounds tested, compounds **2a** and **2j** presented activity against promastigotes (IC_{50} values of 52.5 and 21.1 μM, respectively) and compounds **2a** and **2c** were active against intracellular amastigotes (IC_{50} of 8.1 and 15.6 μM, respectively) of *Leishmania amazonensis*. The majority of compounds did not show toxicity against murine macrophages. Compound **2a** exhibited low cytotoxicity to human erythrocytes and induced an oxidative imbalance in promastigote forms, reflected by an increase in the formation of reactive oxygen species (ROS) and a reduction of mitochondrial membrane potential. No alteration in the plasma membrane integrity of parasites was observed. Taken together, these results suggest that compound **2a** is a selective antileishmanial agent, and preliminary observations suggest that its effects appear to be mediated by mitochondrial dysfunction.

1. Introduction

Leishmaniasis remains a major public health problem in 98 countries and territories, resulting in 1.3 million new cases and 20,000 to 30,000 deaths worldwide (WHO, 2015). There is no available vaccine and the control of the disease relies on drug treatment of infected patients. Presently, chemotherapy has been

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hampered by significant toxicity, long-term therapy, high costs and drug resistance, making the development of new treatment strategies as urgent need (Croft and Olliaro, 2011; Tiuman et al., 2011).

Latin America presents several *Leishmania* species due to its large territorial dimension and its ecological diversity in tropical and subtropical areas. Among then, *L. amazonensis* is a very important *Leishmania* species and has been mainly associated with cutaneous form, such as a simple cutaneous or a diffuse cutaneous leishmaniasis (DCL) (Ameen, 2010; Convit et al., 2004). This last clinical manifestation (DCL) is considered severe and presents multiple nonulcerative lesions spread over the body, with features similar to lepromatous leprosy (Convit et al., 2004). DCL also exhibits an antigen-specific absence of manifestations of delayed hypersensitivity and protective immunity, consequently, responding very poorly to conventional chemotherapy (Convit et al., 2004). Fortunately, DCL is considered a much less common clinical manifestation. Besides cutaneous manifestation, *L. amazonensis* has been associated with the visceral form, considered as a fatal clinical manifestation if untreated (Ameen, 2010; Barral et al., 1986).

Aminoquinolines scaffolds belong to the N-containing heterocyclic compounds that have a wide range of reported biological effects, including anticancer, antimycobacterial and antioxidant activities (Srivastava and Lee, 2015). Antiprotozoal activity of quinolines is very relevant especially as antimalarial drugs, like chloroquine, tafenoquine and amodiaquine compounds (Visser et al., 2014). Quinolines also exhibit good potential as antileishmanials, which has been reported for sitamaquine, an 8-aminoquinoline derivative which is in phase IIb/III for the oral treatment of Visceral Leishmaniasis (Reynolds et al., 2013).

In previous work we showed that 4-aminoquinoline derivatives presented promising leishmanicidal results (Antinarelli et al., 2012; Carmo et al., 2011; Coimbra et al., 2010, 2011, 2013). Additionally, we verified that leishmanicidal activity of 7-chloro-4-quinolinylhydrazone derivatives can be associated with some parasite effects as depolarization of the mitochondrial membrane and increase of the reactive oxygen species (ROS) production, indicating that this compound induced mitochondrial dysfunction (Antinarelli et al., 2015). The aim of the present study is to identify new molecules of this class acting as leishmanicidal compounds and to investigate the mechanisms of action of these compounds in order to clarify the mechanism of the cell death against *L. amazonensis.*

2. Materials and methods

2.1. Chemistry

All the 7-chloro-4-quinolinylhydrazone derivatives **2a-i** were synthesized by our research group (Fig. 1). Firstly, 7-Chloro-4-hydrazinoquinoline **2** was prepared from 4,7-dichloroquinoline **1** using hydrazine hydrate (80%) in ethanol under reflux. After that, the compounds **3a-u** were obtained through reaction between the compound **2** and appropriated benzaldehydes as described in our previous work (Coimbra et al., 2011, 2013) (Table 1). In general, the ^{1}H NMR spectra showed the characteristic signal for the N=CH proton at 8.37–8.81 ppm. Furthermore, the IR spectra showed N–H and N=C stretching vibrations at 3197–3247 and 1570-1585 cm^{-1}, respectively.

2.2. Biological assays

2.2.1. Reagents

Miltefosine was supplied by Cayman Chemical Company (Michigan, USA). Geneticin (G418), Propidium Iodide (PI), Hank's Balanced Sal Solution (HBSS), dimethylsulfoxide (DMSO), hemin, saponin, folic acid, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Invitrogen Molecular Probes (Eugene, OR, USA). Mitotracker was supplied by Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS), RPMI 1640 medium, penicillin G and streptomycin were purchased from Cultilab (Campinas, São Paulo, Brazil); brain heart infusion (BHI) was purchased from Himedia (Mumbai, India).

2.2.2. Parasites

Promastigotes of *Leishmania amazonensis* (IFLA/Br/67/PH8) wild-type (WT) and *L. amazonensis* (WHOM/BR/75/Josefa) transfected with the gene of green fluorescent protein (GFP) were used. *Leishmania amazonensis*-GFP was used in promastigote and amastigote assays and *L. amazonensis*-WT was used in the study of action mechanism. Parasites were cultured in Warren's medium (brain heart infusion- BHI- plus hemin and folic acid), supplemented with 10% inactivated fetal bovine serum (FBS), 0.1% of the antibiotic 100 UI/mL penicillin G and 0.1 mg/mL of streptomycin at 25 °C in a BOD incubator. Promastigotes of L. *amazonensis* (WHOM/BR/75/Josefa) were periodically selected for green fluorescence by culturing in 1 mg/mL of geneticin antibiotic for 72 h (Antinarelli et al., 2015). Parasites were routinely isolated from cutaneous lesions of infected BALB/c mice and maintained as promastigote forms at 25 °C in Warren's medium supplemented with 10% inactivated FBS. All protocols were approved by the Ethical Committee for Animal Research of Federal University of Juiz de Fora (#055/2013 -CEUA).

2.2.3. Antipromastigote assay

The antipromastigote assay was accomplished by using the colorimetric assay MTT which is based on tetrazolium salt reduction by mitochondrial dehydrogenases (Mossman, 1983). Briefly, promastigotes of *L. amazonensis*-GFP (WHOM/BR/75/Josefa) from a logarithmic phase culture were suspended to yield 2×10^6 cells/mL after Neubauer chamber counting. Parasites were exposed to an increasing concentration of the compounds (6.2–100.0 μM) for 72 h at 25 °C in a BOD incubator. Final concentration of DMSO was 0.1% (v/v), which is not toxic to the parasites. Viability of the promastigotes was determined by measuring the absorbance at 570 nm (Multiskan MS microplate reader, LabSystems Oy, Helsink, Finland). Promastigotes incubated without the compounds were used as negative control (100% viable cells). Results were expressed as the concentration inhibiting parasite growth by 50% (IC_{50}) after 72 h incubation. Miltefosine (6.2–100.0 μM) was used as the reference drug.

2.2.4. Antiamastigote assay

Macrophages were obtained after peritoneal injection of 10 mL of Hank's solution in BALB/c mice previously inoculated with 3% thioglycollate medium (Sigma Chemical Co; St. Louis, MO, USA). Briefly, peritoneal macrophages were added at 2×10^6 cells/mL in a 24-well plate in RPMI 1640 medium supplemented with 10% inactivated FBS, and allowed to adhere for 16 h at 33 °C in 5% CO_2 atmosphere. Plates were washed with sterile Phosphate Buffered Saline (PBS) to remove nonadherent cell and the adherent macrophages were infected with *L. amazonensis*-GFP promastigotes in the stationary growth phase at a parasite cell ratio of 20:1 for 4 h at 33 °C in 5% CO_2 atmosphere. After 72 h of incubation with different concentrations of compounds (6.2–100.0 μM) dissolved in DMSO (final concentration 0.1% v/v), macrophages were lysed with 200 μL of distilled water to release amastigotes and transferred to black 96-well microplates (Antinarelli et al., 2015). Uninfected macrophages were used as blank. Fluorescence intensity of the cultures

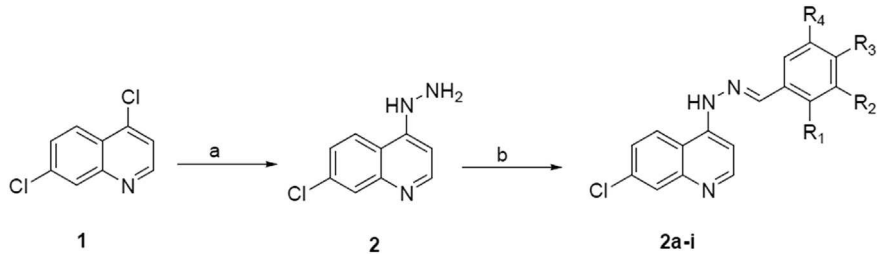

Fig. 1. Reagents and conditions: (a) $N_2H_4 \cdot H_2O$ (80%), EtOH, 80 °C, 2 h, 80%; (b) corresponding benzaldehyde, EtOH, r.t., 4–24 h, 64–91%.

Table 1
Yields and Melting Points of 7-chloro-4-quinolinylhydrazone derivatives **2a-j**.

Entry	Substituents	Yield (%)	mp (°C)
2a	$R_1 = R_2 = R_4 = H$; $R_3 = OH$;	82	233–235
2b	$R_2 = R_3 = R_4 = H$; $R_1 = OH$;	80	219–220
2c	$R_1 = R_2 = R_4$ H; $R_3 = HCO$;	84	282–285
2d	$R_1 = R_2 = R_4 = H$; $R_3 = COOH$;	79	>360
2e	$R_1 = R_2 = R_4 = H$; $R_3 = O{-}CH_3$;	85	144–145
2f	$R_1 = R_4 = H$; $R_2 = OH$; $R_3 = O{-}CH_3$;	81	272–275
2g	$R_1 = OH$; $R_2 = R_4 = H$; $R_3 = OH$;	75	309–312
2h	$R_1 = R_3 = H$; $R_2 = OH$; $R_4 = OH$;	80	340–343
2i	$R_1 = R_4 = H$; $R_3 = OH$; $R_2 = O{-}CH_3$;	82	272–275
2j	$R_1 = H$; $R_2 = R_3 = R_4 = O{-}CH_3$;	77	327–330

was measured using a plate-reader fluorometer (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) set at 485 nm excitation/528 nm emission. Results were expressed as percent inhibition in relation to control *L. amazonensis*-infected macrophages. Miltefosine (1.6–25.0 μM) was used as the reference drug. All protocols were approved by the Ethical Committee for Animal Research of Federal University of Juiz de Fora (#0056/2013-CEUA).

2.2.5. Cytotoxicity on macrophages

Murine macrophages were obtained as described before. Briefly, the macrophages were used for cytotoxicity assay in a concentration of 2×10^6 cells/mL in 96-well culture plates in RPMI 1640 medium supplemented with 10% inactivated FBS, at 33 °C in 5% CO_2 atmosphere. After 24 h, the adherent macrophages were incubated with the compounds in a serial dilution (9.4–150.0 μM) for 72 h at 33 °C in 5% CO_2 atmosphere. Macrophages incubated in RPMI 1640 medium supplemented with 10% inactivated FBS without the compounds were used as negative control (100% viable macrophages). Viability of the macrophages was determined with the MTT assay, as described above. All procedures were performed in agreement with the Ethical Principles in Animal Research and according to protocols approved by the Ethical Committee for Animal Research (#054/2013-CEUA). The selectivity index (SI) was calculated using the following equation: SI= CC_{50} (macrophages)/IC_{50} (*Leishmania* amastigotes).

2.2.6. Erythrocytes lysis

The hemolytic activity was determined by incubation of 180 μL of a 1% suspension of fresh human red blood cells in PBS with 20 μL of different concentrations (9.4–150.0 μM) of the compound **2a** and miltefosine for 16 h at 37 °C. After centrifugation, 100 μL of supernatant was transferred to a 96-well plate and hemoglobin content was quantified by spectrophotometrical measurement of absorbance at 540 nm (Multiskan MS microplate reader, LabSystems Oy, Helsink, Finland). Solution of 1% saponin was used as positive control, and the cell suspension was used as negative control. Percentage of erythrocytes lysis was determined by the formula: hemolysis (%) = (At-Ac)/ApX100, in which At (absorbance of the test compounds), Ac (absorbance of the negative control) and Ap (absorbance of the 1% saponin) (Machado et al., 2015). Results were obtained from three independent experiments performed in triplicate.

2.2.7. Determination of ROS production

Leishmania amazonensis promastigotes (10×10^6 cells/mL) were untreated or treated with 52.0 or 104.0 μM compound **2a** for 72 h at 25 °C in a BOD incubator in the dark. Promastigotes were then harvested, resuspended in PBS and parasite concentration was adjusted to 2×10^7 promastigotes in 200 μL of PBS. After that, 4 μL of H_2DCFDA at 1 mM was added and the samples were incubated for 30 min in the dark at room temperature (Antinarelli et al., 2015). Reactive oxygen species (ROS) were measured as an increase in fluorescence caused by the conversion of nonfluorescent dye to highly fluorescent 2′,7′-dichlorofluorescein using an excitation and emission wavelengths of 485 and 528 nm, respectively, in a fluorometer microplate reader (FLx800, BioTek Instruments, Inc., Winooski, VT, USA). Promastigotes incubated with miltefosine at 22.0 μM were used as positive control. Results were obtained from three independent experiments performed in triplicate.

2.2.8. Determination of the mitochondrial membrane potential ($\Delta\Psi m$)

Cationic probe JC-1 was used to determine the ΔΨm as previously described (Antinarelli et al., 2015). *Leishmania amazonensis* promastigotes (10×10^6 cells/mL) were cultured for 72 h in the absence or presence of 52.0 or 104.0 μM compound **2a** at 25 °C in a BOD incubator. Cells were harvested, resuspended in Hank's Balanced Sal Solution (HBSS) and the cell number was counted in Neubauer chamber. Promastigotes (5×10^6 cells/mL) were incubated with JC-1 (10 μg/mL) for 20 min in the dark at 37 °C. After washing twice with HBSS, fluorescence was spectrofluorometrically measured (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) at both 528 nm and 600 nm using excitation wavelength of 485 nm. Relative Δψm value was quantified using the ratio between the reading at 600 nm and the reading at 528 nm. Additionally, the ΔΨm estimations were also determined using the fluorescent dye Mitotracker, which passively diffuse through the plasma membrane and accumulate in active metabolically mitochondria (Singh et al., 2009). Promastigote forms of *L. amazonensis* were untreated or treated with 52.0 and 104.0 μM compound **2a** at 25 °C in a BOD incubator for 24 h. Cells were washed with PBS and incubated with 50 nM of Mitotracker for 40 min at 25 °C in a BOD incubator in the dark. After washing twice with PBS, data acquisition was performed using a spectrofluorometer (FLx800, BioTek Instruments, Inc., Winooski, VT, USA), at excitation and emission wavelengths of 540 and 600 nm, respectively (Mesquita et al., 2014). Promastigotes treated with miltefosine (22.0 μM) were utilized as positive control. Results were obtained from three independent experiments performed in triplicate.

2.2.9. Evaluation of cellular membrane integrity

Leishmania amazonensis promastigotes (10×10^6 cells/mL) were untreated or treated with 52.0 or 104.0 μM compound **2a** for 72 h at 25 °C in BOD incubator. After that, the parasites were harvested, washed with PBS, resuspended in PBS and incubated with 5 μL PI at 1 mg/mL for 15 min in the dark at room temperature. Fluorescence was spectrofluorometrically measured (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) using an excitation wavelength of 540 nm and an emission wavelength of 600 nm (Antinarelli et al., 2015). As positive control, cells heated at 65 °C for 10 min were used to obtain a maximum value for the fluorescence intensity. Alterations in the PI fluorescence were quantified as the percentage of increase in the fluorescence compared with the untreated promastigotes. Results were obtained from three independent experiments performed in triplicate.

2.2.10. Statistical analysis

The compound concentration that inhibited 50% of the survival of the *Leishmania* (IC_{50} values) and the viability of the murine macrophages (CC_{50} values) were determined and the 95% confidence intervals were included, calculated by Litchtfiet and Wilcoxon method using Probit regression model considering the means from three independent experiments. Data were statistically analysed using One-way-ANOVA followed by Dunnett post-test to compare all the groups to the negative control using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Differences were regarded as significant when $p < 0.0001$ (***), $p < 0.001$ (**) and $p < 0.01$ (*).

3. Results and discussion

In this work, we evaluated the *in vitro* activity of ten aminoquinoline derivatives, with emphasis in the 7-chloro-4-quinolinylhydrazones, against promastigote and amastigote forms of *L. amazonensis*.

Table 2 displays the effect of 7-chloro-4-quinolinylhydrazone derivatives on *L. amazonensis* promastigotes, and intramacrophagic amastigotes and murine macrophages. In the antipromastigote assay, among the compounds tested, only compounds **2a** and **2j** presented antipromastigote activity with IC_{50} values of 52.5 and 21.1 μM, respectively. Other compounds showed no promising antipromastigote activity until maximum concentration used (100 μM). Notwithstanding, we carried out antiamastigote assays with all compounds to evaluate more precisely their potential as leishmanicidal agents. As can be seen in Table 2, only compounds **2a** and **2c** were active against intracellular amastigote of *L. amazonensis* with IC_{50} of 8.1 and 15.6 μM, respectively. For miltefosine, a reference drug, the IC_{50} values obtained were 13.4 and 6.5 μM for promastigotes and intracellular amastigotes, respectively. Table 2 shows some differences in the effect of the tested compounds on promastigote and amastigote forms of *L. amazonensis*. The compound **2c** showed a strong effect on amastigote forms (CI_{50} = 15.6 μM) and no promising antipromastigote activity until maximum concentration used (100 μM). In contrast, the compound **2j** showed effect on promastigote forms (CI_{50} = 21.1 μM), but it was ineffective against intracellular parasite ($CI_{50} > 100.0$ μM). The stage-specific activity has been reported for several antileishmanial drugs, including classical drugs for the treatment of leishmaniasis (Carrio' et al., 2000; Escobar et al., 2002; Cortes et al., 2015). This variation in sensitivity could be due to several differences between the both stages of parasite, including the rate of division, biochemical targets, drug metabolism and metabolic enzymes (Escobar et al., 2002; Abdel-Sattar et al., 2010; Brotherton et al., 2010). Furthermore, the inefficacy of some compounds against intracellular amastigotes might have been due to poor drug uptake by host cell, inactivation of the compounds inside macrophages, or metabolic differences of amastigotes (Brotherton et al., 2010; de Muylder et al., 2011).

In this work, the compound **2a** exhibited a strong activity against amastigote forms, the obligate intracellular form responsible for human leishmaniasis. The leishmanicidal effect of the compound **2a** is not surprising since we showed that this compound displayed a good activity against promastigotes of several *Leishmania* species, including *L. amazonensis* in previous work (Coimbra et al., 2011). Recently, antitubercular and antifungic activity of the compound **2a** also has also been described (Candéa et al., 2009; Duval et al., 2011) which confirms the biological potential of this molecule. Besides the antileishmanial activity, we also assessed the cytotoxic activity of all compounds on host cells macrophages, not observing toxic effect for the majority of them at the maximum tested concentration (150.0 μM) (Table 2). In this sense, when the compound **2a** was assayed against amastigotes, it was much more destructive to this parasitic form than to the macrophages and the selectivity index (SI = CC_{50} of macrophages/IC_{50} of amastigotes) was close to 20 (SI > 18.5).

Since compound **2a** exhibited a remarkable effect on promastigotes and amastigotes of *L. amazonensis*, without toxic effect on mammalian cells, we chose this compound to better explore its leishmanicidal effect and mechanism of action. First, as can be seen in Fig. 2, the incubation with compound **2a** led to a dose-dependent effect on intracellular parasites. Furthermore, compound **2a** was able to act against amastigotes in all concentrations used, decreasing the parasite burden in 86.9; 80.0; 61.5; 53.5 and 42.1% at 100.0; 50.0; 25.0, 12.5 and 6.2 μM of the compound, respectively.

Aiming to determine the safety of compound on the mammalian cells, we also evaluated the toxic effect of the compound **2a** in human erythrocytes (Fig. 3). This compound did not cause significant alterations to cells in all concentrations available showing hemolysis levels of less than 10%, indicating a good safety profile of cytotoxicity. Miltefosine caused significant damage to erythrocytes

Table 2
Effect of 7-chloro-4-quinolinylhydrazone derivatives on *L. amazonensis* promastigotes, intracellular amastigotes and murine macrophages.

Compounds	Promastigote IC_{50} (μM) (95% C.I.)[a]	Amastigote IC_{50} (μM) (95% C.I.)[a]	Murine Macrophages CC_{50} (μM) (95% C.I.)[a]
2a	52.5 (41.1–69.6)	8.1 (6.7–9.9)	>150.0
2b	> 100.0	> 100.0	15.1 (10.6–21.7)
2c	> 100.0	15.6 (11.7–20.8)	>150.0
2d	> 100.0	> 100.0	>150.0
2e	> 100.0	> 100.0	>150.0
2f	> 100.0	> 100.0	57.4 (47.0–70.1)
2g	> 100.0	> 100.0	>150.0
2h	> 100.0	> 100.0	>150.0
2i	> 100.0	> 100.0	>150.0
2j	21.1 (16.6–26.7)	> 100.0	76.5 (63.3–92.5)
Miltefosine	13.4 (10.3–17.4)	6.5 (5.2–8.2)	99.8 (85.6–116.3)

[a] IC_{50} values in μM and 95% confidence intervals are in brackets. These data represent the average of three independent experiments. Miltefosine was used as reference drug.

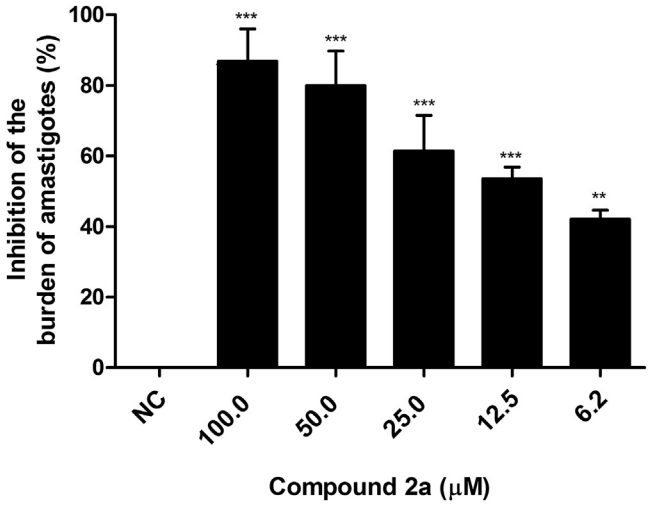

Fig. 2. Effect of the compound **2a** on intracellular amastigotes of *L. amazonensis*. Murine macrophages were infected with *L. amazonensis*-GFP promastigotes in stationary growth (20:1) for 4 h at 33 °C in 5% CO_2 atmosphere. After 72 h of incubation with different concentrations of compounds the fluorescence intensity of the cultures was measured using a plate-reader fluorometer set at 485 nm excitation/528 nm emission. Uninfected macrophages were used as blank. Results were expressed as percent inhibition of intracellular amastigotes in relation to control *L. amazonensis*-infected macrophages. Differences were regarded as significant when $p < 0.001$ (***) and $p < 0.01$ (**) compared with the NC (negative control). Data were expressed as the mean of three independent experiments performed in duplicate.

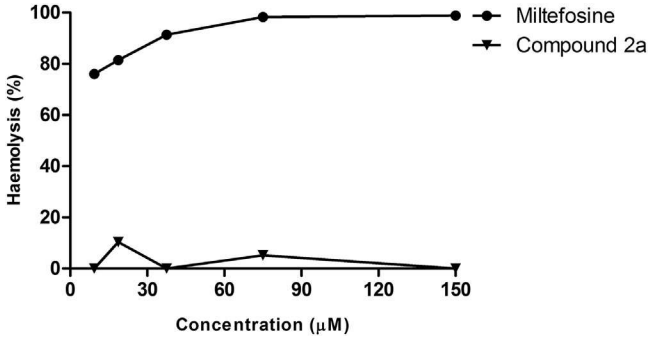

Fig. 3. Cytotoxicity of the compound **2a** against human erythrocytes. Cells were exposed to the compound **2a** and miltefosine in concentrations ranging 9.4–150.0 μM for 16 h and hemolysis levels were estimated by measuring the absorbance of the cells supernatants at 540 nm. Data were expressed as the means of three independent experiments performed in triplicate.

over 70% in all concentrations tested (Fig. 3). An important limitation of sitamaquine, an 8-aminoquinoline derivative, is related to their hematological toxicity, such as hemolysis and methemoglobinemia (Loiseau et al., 2011). Recently, it was evidenced that miltefosine exhibits high *in vitro* erythrocytes toxicity (Munoz et al., 2013).

Considering the ability of the compound **2a** to inhibit both *L. amazonensis* promastigotes and intramacrophage amastigotes growth with high selectivity index for the parasite, *in vitro* assays were performed in order to elucidate its mechanism of action related to the cell death of this protozoan. In trypanossomatidae parasites, oxidative stress can be induced by the increase of reactive oxygen species (ROS) production by effect of various drugs and associated with an apoptotic event (Menna-Barreto and de Castro, 2014). In this sense, we evaluated the effects of ROS production in *L. amazonensis* promastigotes treated with compound **2a** using a fluorescent probe, H_2DCFDA. Our results showed that after 72 h incubation with this compound at 52.0 and 104.0 μM (one time and two times the IC_{50} values of promastigotes, respectively), the ROS production was increased by 45.0% and 76.0%, respectively, when compared with the negative control ($p < 0.001$) (Fig. 4). Miltefosine (22.0 μM) used as the positive control, induced an increase of 114.0% in the ROS level.

In trypanossomatids, collapse of the mitochondrial membrane potential ($\Delta\psi m$) is known as one characteristic metabolic process of cell death by apoptosis-like (Fidalgo and Gille, 2011; Smirlis et al., 2010). To determine the effects of the compound **2a** on the mitochondrial function of *L. amazonensis* promastigotes, we measured the $\Delta\psi m$ using the JC-1 and Mitotracker fluorescent probes. Spectrofluorometric data using the fluorescent dye JC-1 show a significant decrease in the $\Delta\psi m$, corresponding to 14.8 and 14.9% with 52.0 μM and 104.0 μM compound **2a**, respectively, after treatment for 72 h. Furthermore, using the fluorescent probe Mitotracker, the results also confirmed that treatment with 52.0 and 104.0 μM compound **2a** for 72 h reduced by 16.9% and 14.9% the $\Delta\psi m$ compared to the untreated control promastigotes (Fig. 5). Miltefosine (22.0 μM) was used as positive control and reduced the $\Delta\psi m$ by 17.0% and 15.3% (JC-1 and Mitotracker assays, respectively). In fact, in recent years more studies have associated quinoline derivatives with target *Leishmania* mitochondria (Bompart et al., 2013; Carvalho et al., 2010, 2011).

To evaluate whether the mechanism of cell death triggered by compound **2a** also involves the necrotic death, we evaluated the plasma membrane integrity in promastigotes compound **2a** treated and stained with propidium iodide (PI). PI is a nucleic acid stain that selectively passes across plasma membrane and binds to DNA and RNA in cells which membrane plasma integrity was disrupted (da Silva et al., 2015). As shown in Fig. 6, promastigotes treated with 52.0 and 104.0 μM compound **2a** for 72 h did not show significant permeabilization of the plasma membrane compared with the negative control (untreated cells), demonstrating that the parasites probably did not undergo a necrotic cell death. Positive control (cells heated at 65 °C for 10 min) showed a significant increase in

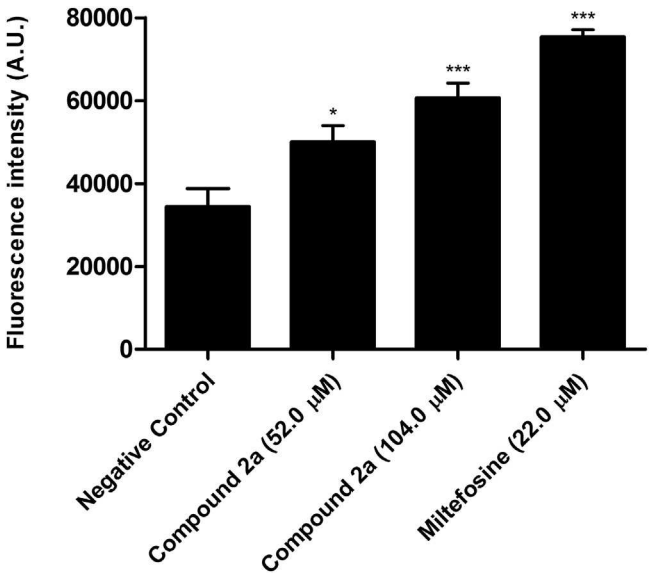

Fig. 4. ROS production in *L. amazonensis* promastigote forms treated with the compound **2a**. Parasites were untreated (negative control) or treated with compound **2a** at 52.0 or 104.0 μM and after 72 h were probed with H_2DCFDA. Miltefosine (22.0 μM) was used as positive control. Fluorescence was measured fluorometrically and the data were expressed as the means of fluorescence intensity in arbitrary units (A.U.) of three independent experiments performed in triplicate. $P < 0.001$ (***) and $p < 0.1$ (*): significant difference compared with the negative control.

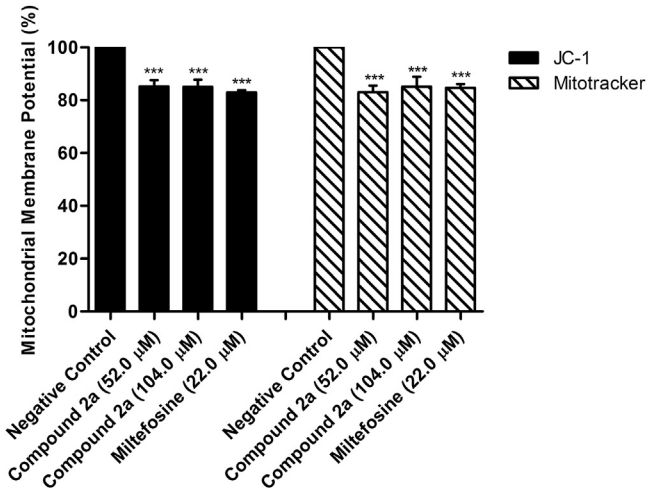

Fig. 5. Changes on the mitochondrial membrane potential of *L. amazonensis* promastigotes. Parasites were untreated (negative control) or treated with compound **2a** at 52.0 or 104.0 μM and after 72 h were probed with JC-1 or Mitotracker. Fluorescence was measured fluorometrically. Negative control was regarded as 100% and the results expressed as a percentage in relation to this control. Miltefosine (22.0 μM) was used as positive control. $P < 0.001$ (***), significant difference compared with the negative control. Data were expressed as the means of three different experiments performed in triplicate.

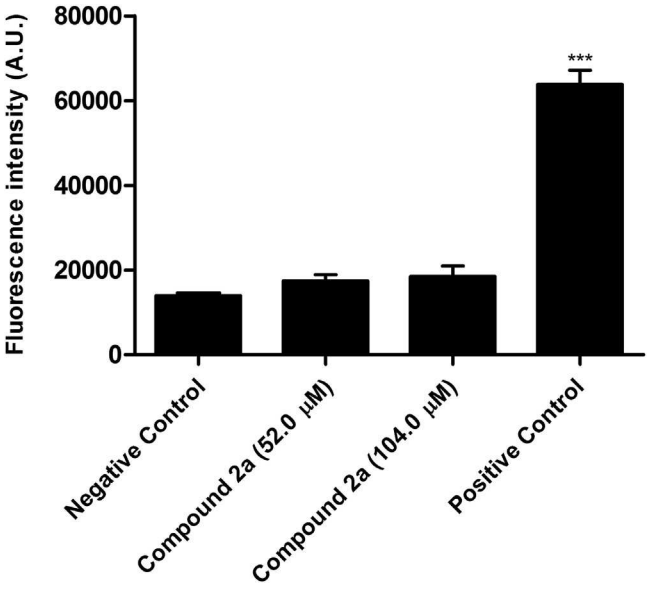

Fig. 6. Cell membrane integrity assay in *L. amazonensis* promastigote forms. Parasites were untreated (negative control) or treated with compound **2a** at 52.0 or 104.0 μM for 72 h and stained with PI. Fluorescence was measured fluorometrically and the data were expressed as the means of fluorescence intensity in arbitrary units (A.U.) of three independent experiments performed in triplicate. $P < 0.001$ (***), significant difference compared with the negative control. Positive control: cells heated at 65 °C for 10 min.

the percentage of PI-stained promastigotes.

Finally, our preliminary hypothesis about the mechanism of action of compound **2a** on *L. amazonensis* involves an increase in reactive oxygen species (ROS) through the electron transport chain that acts in the mitochondrial membrane of the parasite, leading to membrane permeabilization followed by mitochondrial dysfunction (de Souza et al., 2009; Menna-Barreto and de Castro, 2014). In addition, no alteration in the plasma membrane integrity of parasites was observed suggesting that necrosis is not the mechanism of parasite death.

4. Conclusion

In summary, the potential biological activity of 7-chloro-4-quinolinylhydrazone derivatives as leishmanicidal agents was confirmed. Furthermore, compound **2a** showed an effective and selective activity against *L. amazonensis* and its activity suggests the involvement of ROS production associated with the mitochondrial depolarization without rupture of *L. amazonensis* promastigotes plasma membrane. Further studies with this class of compounds could contribute to the development of new antileishmanial drugs.

Conflict of interest

The authors have not supplied their declaration of conflict of interest.

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SELECTIVE ACTION OF FLUOROQUINOLONES AGAINST INTRACELLULAR AMASTIGOTES OF *LEISHMANIA* (*VIANNIA*) *PANAMENSIS* IN VITRO

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SELECTIVE ACTION OF FLUOROQUINOLONES AGAINST INTRACELLULAR AMASTIGOTES OF *LEISHMANIA* (*VIANNIA*) *PANAMENSIS* IN VITRO

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ABSTRACT: We have demonstrated that fluoroquinolones, a class of antibacterial agents that act through inhibition of type II DNA topoisomerases, exert selective action against intracellular amastigotes of *Leishmania* (*Viannia*) *panamensis* at concentrations that are achievable in vivo. Drug cytotoxicity assays employing the luciferase reporter gene revealed that intracellular amastigotes were 6.6- to 25.9-fold more sensitive than human macrophages ($P < 0.05$) to second-generation fluoroquinolones in vitro. The most selective agents (enoxacin and ciprofloxacin) exhibited 2 orders of magnitude greater potency against parasites (50% effective dose [ED_{50}] = 54.9–83.4 μM) than host cells (ED_{50} = 1,425–1,740 μM). Linear regression analysis of ED_{50} data confirmed a complete lack of correlation ($r = 0.001$) between the relative drug sensitivities of parasites and host cells. A potential relationship between the structures of fluoroquinolones and their relative leishmanicidal activities was observed. The key substituents of the basic pyridone β-carboxylic acid nucleus accounting for enhanced antiparasite potency and selectivity appear to be a nitrogen at position 8 of the bicyclic nucleus (enoxacin), a cyclopropyl substituent at the R_1 site (ciprofloxacin), and linkage of the R_1 and X_8 groups by a CH_3CHO bridge to form a tricyclic compound (ofloxacin). These findings support the potential of fluoroquinolones and derivatives as novel antileishmanials and encourage their clinical evaluation.

The intracellular protozoan parasite *Leishmania* spp. is the causative agent of a clinically diverse group of diseases, known collectively as leishmaniasis, which affect an estimated 12 million people with 2 million new cases annually (Herwaldt, 1999; Desjeux, 2001). Control of leishmaniasis relies entirely on chemotherapy, but the available armory of effective drugs is limited. Pentavalent antimonials, for many years the first line of antileishmanial defense, have been rendered obsolete in several Old World regions by the emergence of antimony resistance (Faraut-Gambarelli et al., 1997; Sundar, 2001), and treatment failure has also been reported in the New World (Martinez et al., 1997; Velez et al., 1997; Palacios et al., 2001). Although the alkyl phospholipid compound miltefosine provides an effective new oral therapy for Old World visceral leishmaniasis (Jha et al., 1999), variable results were obtained in trials against parasites of the *L. Viannia* subgenus (Soto et al., 2004). Treatment options for American (muco)cutaneous leishmaniases thus remain restricted by a lack of novel agents combined with the limitations of existing second-line drugs, such as pentamidine and amphotericin B (Herwaldt, 1999). The need to define biochemical targets for the development of new treatment strategies has therefore become an urgent priority.

In this context, the DNA topoisomerases (TOPs) of trypanosomatids (*Leishmania* spp. and *Trypanosoma* spp.) have emerged as attractive potential targets due to variations in structure, biochemical properties, and inhibitor sensitivities from the counterpart human enzymes, as well as their roles in organizing the kinetoplast DNA (kDNA) network unique to these parasites (Burri et al., 1996; Cheesman, 2000; Das et al., 2001; Marquis et al., 2003; Villa et al., 2003; Walker and Saravia, 2004). TOPs play essential roles in modulating DNA topology during nucleic acid biosynthesis and cell division and have been exploited as targets for antibacterial and antitumor agents (Hooper, 1998; Liu, 1989). Bacterial type II TOPs (DNA gyrase and TOPIV) are the sites of action for the fluoroquinolones (FQs), a family of potent antimicrobial agents with a wide range of clinical applications (Hooper, 1998). FQs act as poisons, interrupting the catalytic cycle of type II TOPs by promoting the formation of covalent complexes between enzyme and DNA substrate, resulting in irreparable cleavage of both DNA strands and cell death (Drlica and Zhao, 1997; Takei et al., 1998; Morrissey and George, 1999). While the reported potencies of FQs against trypanosomes in vitro vary widely according to both species and life stage, bloodstream forms of *T. brucei brucei* and *T. b. rhodesiense* appear particularly sensitive to these compounds, and novel experimental derivatives with improved antitrypanosomal selectivities were identified recently (Betbeder et al., 1988; Croft and Hogg, 1988; Nenortas, Burri and Shapiro, 1999; Keiser and Burri, 2001; Nenortas et al., 2003). Studies on antileishmanial effects of FQs lag well behind research on African trypanosomes, but the limited available data indicate significant interspecies variation in the in vitro sensitivities of *Leishmania* spp. amastigotes (Croft and Hogg, 1988; Savoia et al., 1993; Bianciardi et al., 2004). Nevertheless, certain FQs have shown promising in vivo efficacies against *L. donovani*, *L. infantum* and *L. major* in animal models (Raether et al., 1989; Savoia et al., 1993; Zucca et al., 1996) and have been employed in combination therapy of canine leishmaniasis (Bianchini and DAmico, 2004; Bianciardi et al., 2004).

Here, we report that second-generation FQs exhibit significant selective toxicities in vitro against intracellular amastigotes of *Leishmania* (*Viannia*) *panamensis* compared with uninfected human macrophages.

MATERIALS AND METHODS

Chemicals

With the exception of ciprofloxacin (purchased from ICN Biomedicals, Aurora, Ohio), all generic quinolones were obtained from Sigma Chemical Co. (St. Louis, Missouri). D-luciferin was obtained from Molecular Probes Inc. (Eugene, Oregon).

Parasites

Promastigotes of *L.* (*Viannia*) *panamensis* (strain MHCOM/CO/86/1166) were cultured by standard procedures (Bosque et al., 1998) in Schneiders' *Drosophila* medium (Sigma) containing 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, New York), 1% glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 7.7 μM hemin hydrochloride (ICN), and 10 μM biopterin (Sigma).

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TABLE I. Cytotoxicity of quinolones against *L. panamensis* intracellular amastigotes and human macrophages.

Quinolone	ED_{50} (μM)*		Specificity of inhibition (A/B ratio)
	Macrophages (A)	*L. panamensis* amastigotes (B)	
Enoxacin	1,425 ± 107 (4)†	54.9 ± 10 (4)†	25.9
Ciprofloxacin	1,740 ± 280 (4)†	83.4 ± 19.9 (4)†	20.8
Lomefloxacin	870 ± 378 (4)†	115.6 ± 26.5 (4)†	7.5
Ofloxacin	2,195 ± 287 (5)†	151.6 ± 23.5 (4)†	14.5
Norfloxacin	991 ± 317 (3)†	150.2 ± 23.8 (3)†	6.6
Cinoxacin	12% inhibition at 2,000 μM (1)	715 (1)	>2.8
Nalidixic acid	19% inhibition at 2,000 μM (1)	2 ± 0.9% inhibition at 500 μM (2)	—

* The ED_{50} (= 50% effective dose) is the concentration of drug (in μM) required to cause 50% inhibition of growth. Results are presented as mean ED_{50} + SE, with number of replicate assays in parentheses.
† Data pairs exhibiting statistically significant differences ($P < 0.05$) as determined by Mann–Whitney analyses.

Transfection of parasites with the luciferase reporter gene

Promastigotes were transfected with the firefly luciferase (LUC) reporter gene using the pGL2-α-NEOαLUC expression vector essentially as described (Roy et al., 2000). Cultures of late log-phase promastigotes containing a total of 3×10^8 parasites were harvested after 4 days of growth by centrifugation (2,500 *g*, 5 min), and washed with phosphate-buffered saline (PBS, pH 7.2; 1% [w/v] NaCl, 0.5% [w/v] Na_2HPO_4, 0.5% [w/v] KH_2PO_4). Parasites were resuspended in 0.4 ml of PBS and prepared for transfection by mixing with 20 μg of purified pGL2-α-NEOαLUC expression vector and incubated on ice for 10 min. Electroporation was performed by subjecting parasites to a single electrical pulse at a voltage of 450 V and capacitance of 500 micro-Farads using the Gene-Pulser XL Electroporator (Bio-Rad, Hercules, California). Parasites were immediately mixed with 1 ml of complete Schneider's, incubated on ice for 10 min, then transferred to 10 ml complete medium and grown for 24 hr at 25 C. Antibiotic selection of stable LUC-transfectants by incremental exposure to geneticin (G418; Sigma) was initiated at 20 μg/ml on day 2 and continued up to final concentrations 80–100 μg/ml by day 21; thereafter, parasites were permanently maintained in the presence of appropriate G418 concentrations.

Human U-937 macrophages

The human promonocytic cell line U-937 (ATCC CRL-159302) was cultured at 37 C in a 5% CO_2 atmosphere, using RPMI 1640 medium containing 1% glutamine, 10% fetal calf serum, 100 IU penicillin, and 100 μg/ml streptomycin (Bosque et al., 1998). Monocytes were harvested during the logarithmic stage of growth (48 hr after subculture), quantified by microscopy, and transferred to standard medium containing 100 ng/ml phorbol myristate acetate (PMA; Sigma) for the induction of adherence and differentiation into macrophages; samples containing 1×10^5 cells (for infection with parasites and drug testing) were seeded into 96-well microtiter plates and incubated for 96 hr at 37 C, 5% CO_2 (Bosque et al., 1998).

Macrophage infections

Stationary-phase LUC-transfected parasites were harvested at 6 days after subculture, opsonized by treatment with RPMI 1640 containing 10% human AB+ serum for 1 hr as described (Bosque et al., 1998). Parasites were added to each sample of 1×10^5 differentiated macrophages in microplate wells using experimentally determined optimum parasite-to-cell ratio of 20 : 1, and infection was allowed to proceed for 2 hr (34 C, 5% CO_2). Free parasites were removed by washing 2–3 times with serum-free Hanks' medium (Sigma). Following a further 24-hr incubation (34 C, 5% CO_2) to allow the complete development of amastigotes, infected macrophages were treated with serial dilutions of test drug.

Viability assays (ED_{50} tests)

Drug cytoxicities: Drug cytoxicities were determined as 50% effective dose (ED_{50}) values using acid phosphatase (Bodley et al., 1995) and LUC activities (Roy et al., 2000) as indicators of cellular viability for uninfected macrophages and intracellular amastigotes, respectively. In all experiments, ED_{50} values were calculated by probit analyses (Atchinson and Silvey, 1957). Stock solutions of quinolones were prepared in 0.1 M NaOH, sterilized by filtration, then serially diluted in the appropriate culture medium for immediate use in ED_{50} assays. Preliminary standardization of the LUC/phosphatase assays revealed excellent agreement with ED_{50} values obtained via microscopical analysis. Untreated control cells and reagent blanks were included in all experiments, and additional controls revealed that the FQ solvent (0.1 M NaOH) did not adversely affect either parasite or macrophage growth. Direct inhibition assays confirmed that FQs had no inhibitory effects on either the acid phosphatase or LUC marker enzyme activities. Calibration-curve data confirmed linear correlations between cell numbers and LUC/phosphatase activities.

LUC-reporter gene assay: Infected macrophages were treated with serial dilutions of test quinolone for 72 hr (at 34 C, 5% CO_2), with 1 change of drug-containing medium at 48 hr. Surviving LUC-transfected amastigotes were determined by the luminometric luciferase assay (Roy et al., 2000). Following aspiration of drug-containing medium, infected macrophages were lysed in microplate wells by the direct addition of 50 μl of lysis buffer (125 mM Tris-HCl, pH 7.8, 50% glycerol, 10 mM DTT, 5% Triton X-100) and incubated for 30 min at room temperature. Lysates were then transferred to a Lucy 1 Microplate luminometer (Anthos Labtech GmBH, Salzburg, Austria). Bioluminescence due to LUC activity was determined at 37 C as anthos relative light units (ALU) via the automated addition of 100 μl of assay buffer (25 mM Tris-HCl, pH 7.8, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 4.7 μM D-luciferin, 0.53 mM ATP, and 33.3 mM DTT) to each well. Light output was measured for 20 sec per well after a 1-sec delay.

Acid phosphatase assay: Following incubation of uninfected macrophages (1×10^5 cells) in microplate wells with 200 μl of medium containing serial dilutions of test drug for 48 hr at 37 C, the acid phosphatase activity of surviving cells was determined by direct addition of 20 μl of lysis buffer (1 M sodium acetate, pH 5.5, and 1% Triton X-100) containing 10 mg/ml *p*-nitrophenyl phosphate. Incubation was continued for a further 6 hr at 37 C, whereupon the production of *p*-nitrophenol was determined by optical-density measurements at 405 nm using an ELISA plate reader (Revelation MRX; Dynex Laboratories, Chantilly, Virginia).

Statistical analysis

The ED_{50} data (mean ± SE) for quinolones against amastigotes and host cells were evaluated by the Mann–Whitney test and differences of $P < 0.05$ were significant.

RESULTS

All of the quinolones tested exhibited greater inhibitory effects on intracellular amastigotes of *L. panamensis* than against uninfected host macrophages (Table I). Although the first-generation (nonfluorinated) quinolones nalidixic acid and cinoxacin were weak inhibitors and showed only modest antiparasite selectivity, second-generation (fluorinated) derivatives exhibited

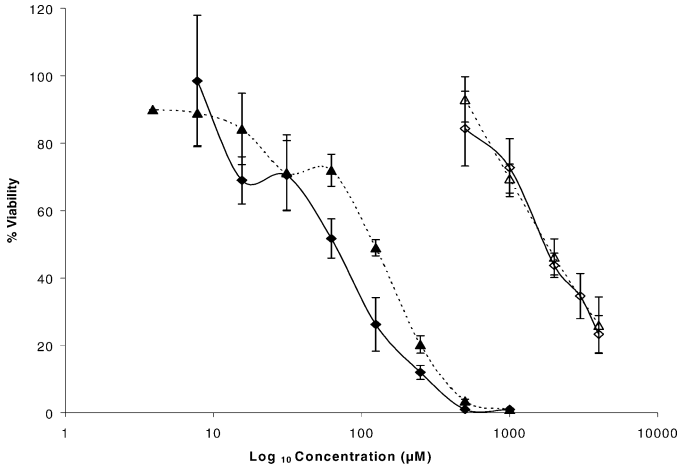

FIGURE 1. Selective inhibitory effects of enoxacin and ciprofloxacin upon the viability of *L. panamensis* amastigotes compared with human macrophages. Plots of percentage viability against both enoxacin and ciprofloxacin concentrations are presented for parasite and host cells, respectively. Each point is the mean of at least 3 replicate ED_{50} determinations and the bars represent ± SE. Key to symbols: ◊, enoxacin/macrophages; ♦, enoxacin/amastigotes; △, ciprofloxacin/macrophages; ▲, ciprofloxacin/amastigotes.

significantly higher potency (6.6- to 25.9-fold) against amastigotes than host cells ($P < 0.05$) (Table I). Of the 3 most potent and selective inhibitors (enoxacin, ciprofloxacin, and ofloxacin), the former 2 FQs were notable in exhibiting ED_{50} values 2 orders of magnitude lower for amastigotes than against host macrophages (Table I, Fig. 1). These levels of selectivity closely resemble the 2 orders of magnitude difference observed in our laboratory between the relative potencies of the pentavalent antimony (Sb^V)-containing antileishmanial sodium stibogluconate (SSG) against amastigotes of *L. panamensis* 1166 (27.9 ± 4.9 μM Sb^V) and U-937 macrophages (4,718 ± 872 μM Sb^V) ($P < 0.05$) (R. Rojas and N. G. Saravia, pers. comm.). Linear regression analysis of a plot of the ED_{50} values of each FQ for both cell types demonstrated the complete lack of correlation between the relative drug sensitivities of amastigotes and macrophages ($r = 0.001$), and Mann–Whitney analysis confirmed significant variation between the overall mean ED_{50} values (of all 5 FQs combined) for parasites and host cells ($P = 0.005$) (data not shown). Comparison of the structures and activities of the 5 second-generation FQs relative to the base compound norfloxacin revealed that the superior antiparasite potencies and selectivities of ciprofloxacin, enoxacin, and ofloxacin are potentially attributable to the presence of a nitrogen at ring-position 8 of the pyridone β-carboxylic acid nucleus (enoxacin), a cyclopropyl substituent at the R_1 site (ciprofloxacin), or the bridging of the R_1 and X_8 sites by a CH_3CHO group (ofloxacin) (Table I, Fig. 2). Antiparasite potency was significantly increased (relative to norfloxacin) by the presence of the additional fluorine substituent at X_8 in lomefloxacin, although this modification also enhanced activity against host cells, thus reducing selectivity. The very minor structural differences between the R_7 substituents of the test compounds (identical in ciprofloxacin, enoxacin, and norfloxacin) indicate that this position plays a minimal role in the observed differences in antiparasite activities (Table I, Fig. 2).

DISCUSSION

Second-generation FQs have significant activity against *L. panamensis* amastigotes in vitro. The most selective agents (enoxacin and ciprofloxacin) exhibited antiparasite potencies 2 orders of magnitude higher than against human macrophages, but 1 to 3 orders of magnitude lower than the corresponding MIC values reported for *Escherichia coli* and the Gram-positive pathogens *Streptococcus pneumoniae* and *Staphylococcus aureus* (Takei et al., 1998; Morrissey and George, 1999). The intermediate FQ sensitivity of *Leishmania* as compared with prokaryotic and mammalian cells is probably influenced by a combination of differences in molecular characteristics and relative accessibility of the drug target and in drug uptake mechanisms.

Our results for *L. panamensis* complement the reported efficacies of FQs against *L. major* and *L. infantum* amastigotes in vitro (Savoia et al., 1993). In marked contrast, other studies have indicated insensitivity of *Leishmania* parasites to FQs in vitro; *L. donovani* amastigotes were unaffected by ciprofloxacin, ofloxacin, and norfloxacin at concentrations of up to 250 μM (Croft and Hogg, 1988), and the fourth-generation compound enrofloxacin was ineffective against *L. infantum* amastigotes at 140 μM (Bianciardi et al., 2004). A similar dichotomy exists with trypanosomes. Ofloxacin and norfloxacin were inactive and ciprofloxacin of very limited efficacy against *T. cruzi* amastigotes (Croft and Hogg, 1988), yet all 3 compounds (plus enoxacin) were potent inhibitors of *T. b. brucei* bloodstream forms with ED_{50} values similar to our present data for *L. panamensis* (Nenortas, Burri, and Shapiro, 1999; Nenortas et al., 2003). The selectivity of these FQs for *L. panamensis* compared with macrophages (14.5- to 26-fold) is higher than reported previously for *T. b. brucei* in comparison with mouse leukemia cell line L1210 (1- to 1.4-fold) because the latter overexpresses TOPs and is, therefore, abnormally FQ sensitive (Nenortas, Burri, and Shapiro, 1999; Nenortas et al., 2003). Collectively, the significant variations in sensitivities to FQs between trypanosomatid species and developmental stages may reflect differences in drug uptake or in expression levels of the presumed TOP target. The effects of FQs may also be more subtle in certain species, as exemplified by the ofloxacin-mediated inhibition of transformation in *T. cruzi* (Gonzales-Perdomo et al., 1990).

The differential sensitivities of *L. panamensis* amastigotes and human macrophages to FQs in vitro correlate very well with our ongoing TOP inhibition analyses, which have so far revealed that second-generation FQs exhibit up to 3 orders of magnitude greater inhibitory potency against *Leishmania* spp. TOPII than against monocyte/macrophage TOPII, and are ineffective inhibitors of both *Leishmania* spp. and human TOPI (T. Cortázar, V. Jean-Moreno, and J. Walker, pers. comm.). These data strongly implicate *Leishmania* spp. TOPII as the likely target for FQs, and it is notable that the sequences of *Leishmania* spp. and human TOPII subunits differ sufficiently (30–40% amino acid identities) to influence structural and functional variations that could provide the molecular basis for the parasite-selective action of FQs. Indeed, substantial differences exist between the catalytic domains of prokaryotic DNA gyrase and lower eukaryotic (yeast) TOP II (Morais-Cabral et al.,

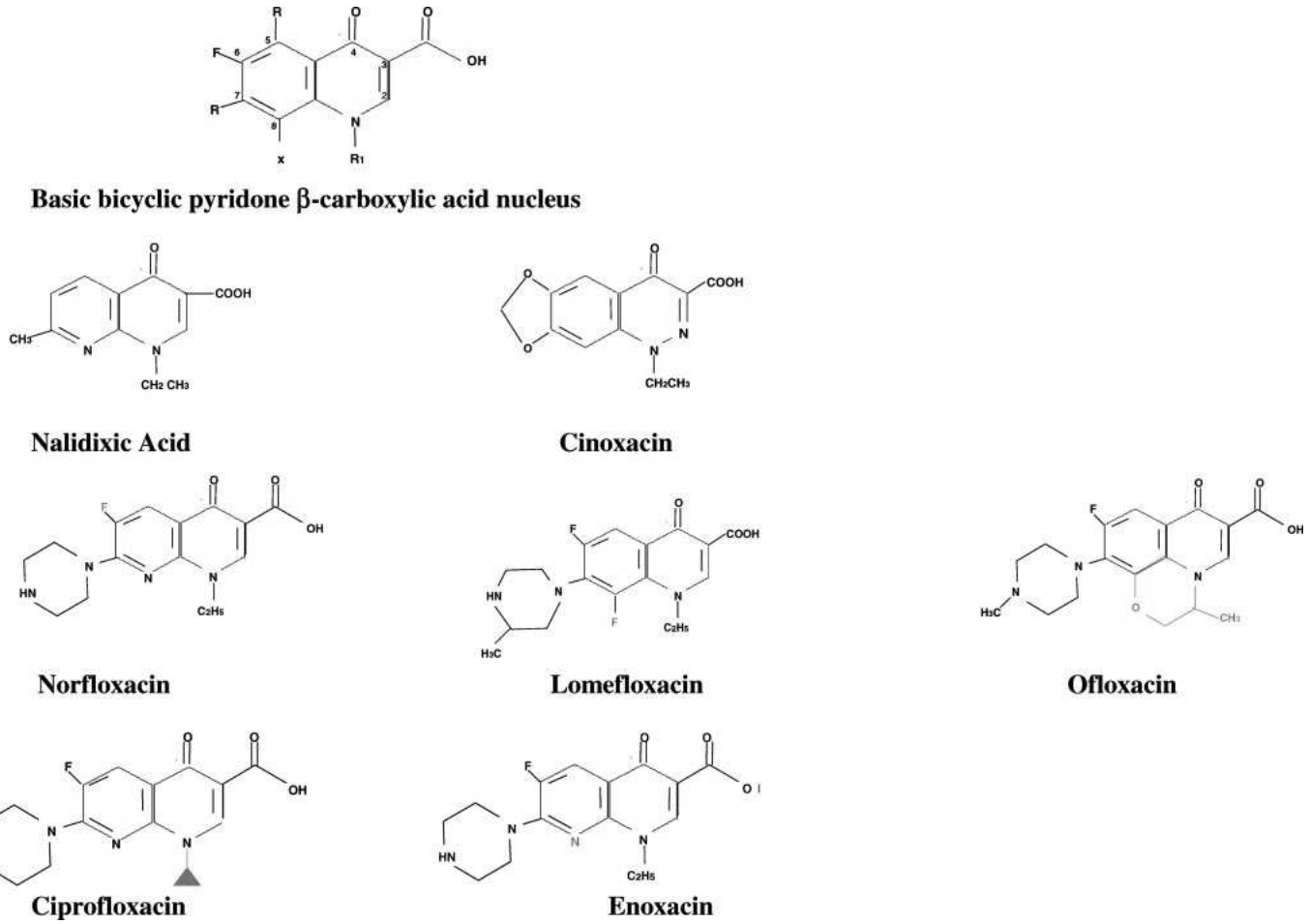

FIGURE 2. Structures of nonfluorinated quinolones and second-generation fluoroquinolones. The molecular structures of the quinolones nalidixic acid and cinoxacin together with the second-generation FQs norfloxacin, lomefloxacin, enoxacin, ciprofloxacin, and ofloxacin are presented alongside a systematically annotated diagram of the basic bicyclic pyridone β-carboxylic acid nucleus. Key substituents differing between individual compounds are highlighted.

1997), and similar structural variability may be expected between *Leishmania* spp. and human TOPII (Das et al., 2001).

The implication of R_1 and X_8 as key positions influencing the antileishmanial activities of second-generation FQs is highly significant because R_1 substituents, such as the cyclopropyl moiety and fluorinated benzene rings, plus X_8 substitutions are frequent features of third and fourth generation compounds, which exhibit enhanced antibacterial activities in vitro and in vivo (Hooper, 1998; Nenortas, Burri and Shapiro, 1999; Keiser and Burri, 2001; Nenortas et al., 2003). Although the negligible differences between the R_7 substituents of second-generation FQs did not permit evaluation of the potential contribution of this position to antileishmanial activity, recent studies demonstrated that replacement of the R_7-piperazinyl groups of existing FQs by pyrrolidine substituents, plus the creation of novel tri- and tetracyclic derivatives, enhances antitrypanosomal potency and selectivity (Nenortas, Burri, and Shapiro, 1999; Keiser and Burri, 2001; Nenortas et al., 2003).

Oral formulations of FQs have excellent pharmacokinetic profiles, reaching reported serum concentrations of up to 31 μM before being concentrated still further inside macrophages (Carlier et al., 1990; Physicians' Desk Reference, 2004), thus providing a feasible mechanism for attaining intracellular concentrations lethal to *L. panamensis* amastigotes. The limited in vivo studies performed to date have indicated significant efficacies of second-generation FQs against *Leishmania* infections in animal models. For example, reductions in liver parasitemia of between 33.5–80.3% were achieved in hamsters infected with *L. donovani* following treatment with pefloxacin, enoxacin, ciprofloxacin, norfloxacin, and ofloxacin at a remarkably low dosage of 10 mg/kg/day, which represents only 1.3–4% of the standard regime (250–750 mg/kg/day) used clinically for the treatment of bacterial infections in humans (Raether et al., 1989; Physicians' Desk Reference, 2004). Similarly, treatment of *L. major* infections in mice with pefloxacin, either alone (at 180 μg/mouse/day) or in combination with IFN-γ, significantly reduced lesion sizes (Zucca et al., 1996). Moreover, the successful treatment of canine leishmaniasis has recently been reported using enrofloxacin (Baytril®, Bayer) at 20 mg/kg/day in combination with metronidazole (Bianchini and D'Amico, 2004; Bianciardi et al., 2004). However, despite the significant clinical improvements attributable to coadministration of enrofloxacin, the drug appears to act through activation of host macrophages

rather than direct targeting of the parasite itself (Bianciardi et al., 2004).

Although focused on a single New World species, this study provides evidence that FQs have potential as a basis for alternative antileishmanial therapies. These findings underpin our current objectives to identify, via in vitro and in vivo screening (in animal models), highly selective third/fourth-generation compounds or novel derivatives for clinical trials in CL patients or for use as templates in rational drug design, respectively. FQs could assume particular importance in view of the urgent need to identify novel oral drugs that can be combined with existing agents (pentavalent antimonials, Miltefosine) to offset the development of resistance, especially in zones of proven anthroponotic transmission.

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ORIGINAL ARTICLE

Synthesis, characterization and antimalarial activity of hybrid 4-aminoquinoline-1,3,5-triazine derivatives

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Abstract A novel series of hybrid 4-aminoquinolines-1,3,5-triazine were synthesized by means of aromatic nucleophilic displacement of chlorine atoms of 2,4,6-trichloro-1,3,5-triazine. Afforded title analogs were subsequently characterised by elemental analysis, FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy and subjected to screening against chloroquine sensitive *RKL2* strain of *Plasmodium falciparum* in 96 well-microtitre plates. However, synthesized derivatives exhibit mild to moderate antimalarial activity and acute toxicity studies of the most active (**6a** and **6g**) compounds were shown to have no significant change in body insight and toxic sign.

1. Introduction

Multidrug resistant *Plasmodium* parasites are the biggest therapeutic challenge to health care in most malaria-endemic areas specifically tropical and sub-tropical areas (Kremsner and Krishna, 2004). Resistance to former first-line treatment, chloroquine and sulfadoxine-pyrimethamine is becoming most apparent in *Plasmodium falciparum* species (Marfurt et al., 2010). Moreover, it has rendered monotherapy for malaria useless in most parts of the world (Guidelines for the treatment of malaria, 2010). To improve efficacy and delayed onset of resistance, the World Health Organization began recommending the use of Artemisinin Combination Therapies (ACTs) since 2005 (Rogerson and Menendez, 2006). Currently, ACTs demonstrate excellent clinical efficacy, paradoxically the history of antimalarial chemotherapy predicts that it is a matter of time before parasitic resistance re-emerges (Ekland and Fidock, 2008). Nevertheless, safe and cost effective new antimalarial agents are urgently needed to treat malaria (Guerin et al., 2002).

One important pipeline approach is the new generation of hybrid molecules against both chloroquine sensitive and resistant strains of *P. falciparum* by diverse functionalization of

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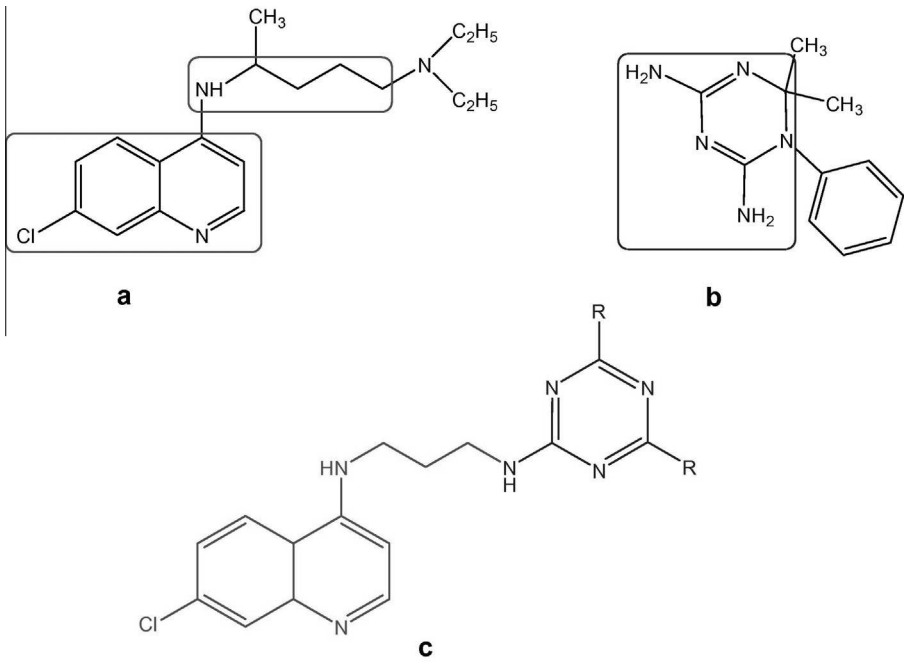


Figure 1 Structure of (a) chloroquine, (b) cycloguanil and (c) hybrid 4-aminoquinoline-1,3,5-triazine derivatives.

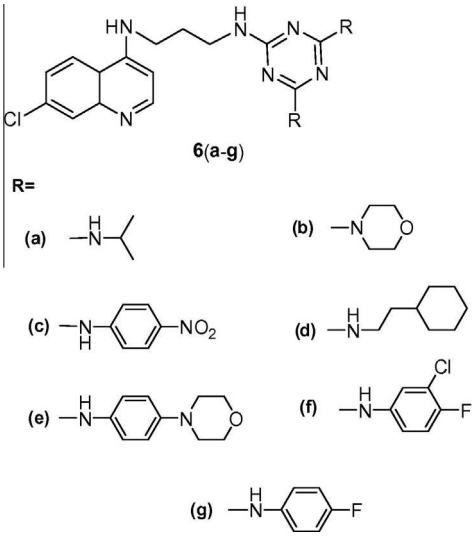


Figure 2 Hybrid 4-aminoquinoline-1,3,5-triazine derivatives (**6a**–**g**).

the lateral side chain of 4-aminoquinoline, such as isatin derivatives (Chiyanzu et al., 2005), β-carbolines (Gupta et al., 2008), the peroxide based trioxaquine derivatives (Singh et al., 2004) etc. The Structure activity relationships (SARs) of 4-aminoquinolines with propyl side chain [$-HN(CH_2)_3NH-$] exhibit most potent activity against chloroquine-susceptible *P. falciparum* (Kgokong et al., 2008). Encouraged by these observations and in continuation of our investigation in search of new and effective pharmacophores from 1,3,5-triazine (Singh et al., in press; Gahtori et al., 2009), we herein report a new series of hybrid 4-aminoquinoline-1,3,5-triazine (Fig. 1c), as a core bioactive lead fragment derived from chloroquine (Fig. 1a) and cycloguanil (Fig. 1b) to obtain seven novel hybrid 4-aminoquinoline-1,3,5-triazine derivatives (**6a–6g**) (Fig. 2).

2. Results

A series of hybrid 7-chloro-4-aminoquinoline substituted 1,3,5-triazines derivatives **6a–g** were synthesized, characterized and found in agreement with spectroscopic analysis. IR spectra of the all products **6a**–**g** nearer at 3350 cm^{-1} is due to the primary amino groups, where as the secondary –NH linker between 4-aminoquinoline and 1,3,5-triazine appears in the region 3214–3140 cm^{-1}. The strong absorption bands at 850–670 cm^{-1} confirm the existence of aromatic skeleton. The ^1H NMR spectrums report a signal corresponding to the quinolyl proton at 6.46–10.01 ppm. ^{13}C NMR of the carbon atom of 1,3,5-triazine was detected at 152.30–168.61 ppm. The tested compounds, **6e**, **6f** and **6g** have shown good in vitro antimalarial efficacy under similar experimental conditions with reference to the standard drug chloroquine.

2.1. Acute toxicity

The three active hybrid 4-aminoquinoline-1,3,5-triazine derivatives **6e**, **6f** and **6g**, were further tested for acute toxicity testing-Up and Down procedure (UDP) as recommended by the Organization for Economic Co-operation and Development (OECD) (Guidelines for the Testing of Chemicals, 2006). These test compounds at a test dose of 2000 mg/kg for 48 h intervals and serially for a total of 14 days have exhibited no significant changes in body weight and toxic signs.

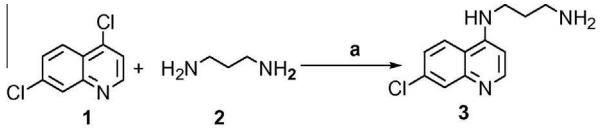
3. Discussion

The antimalarial screening result reflects that the compounds **6e**, **6f** and **6g** possessing aromatic group along with chloro,

fluoro and morpholino substitution have shown comparatively good in vitro antimalarial activity ranges from 47.5 to 56 in comparison to chloroquine under similar test conditions. Out of seven evaluated compounds, N^2,N^4-bis (3-chloro-4-fluorophenyl)-N^6-(3-(7chloroquinolin-4-ylamino) propyl)-1,3,5-triazine-2,4,6-triamine (**6f**) was found to be the most active against chloroquine sensitive strain and the cut off value (LD_{50} > 2000 mg/kg) for **6e**, **6f** and **6g** was recorded via oral administration proves its efficacy. These new hybrid series of 4-aminoquinoline-1,3,5-triazine were found to be less effective than chloroquine, however their in vitro results prove these new hybrids as a promising model for further optimization work in malarial chemotherapy.

4. Experimental

All commercially available solvents and reagents were of AR grade and used without further purification. Melting points were determined on a Veego, MPI melting point apparatus and are uncorrected. UV_{max} (DMSO) were recorded on Shimadzu UV-1700 and FT-IR (2.0 cm^{-1}, flat, smooth, abex) were taken on Perkin Elmer RX-I Spectrophotometer. ^1H NMR spectra were recorded on Bruker Avance II 400 NMR and ^{13}C NMR spectra on Bruker Avance II 100 NMR spectrometer in DMSO-d_6 using TMS as the internal standard. Mass spectra were obtained on VG-AUTOSPEC spectrometer equipped with electrospray ionization (ESI) sources. Elemental analysis was carried out on Vario EL-III CHNOS element or analyzer.



Scheme 1 Reagents and conditions: (a) Reflux/80 °C/1 h followed by reflux at 6–8 h at 120–130 °C.

4.1. Preparation of parasites

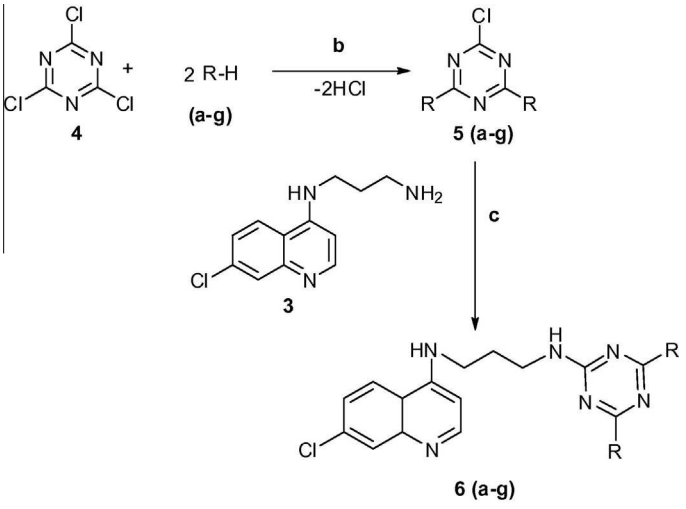
The chloroquine sensitive *RKL-2* strain (Raurkela, Orissa, India) of *P. falciparum* were routinely maintained in stock cultures in medium RPMI-1640 supplemented with 25 mmol HEPES, 1% D-glucose, 0.23% sodium bicarbonate and 10% heat inactivated human serum. The asynchronous parasites of *P. falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage parasitized cells. For carrying out the assay, the initial ring stage parasitaemia of 0.8–1.5% at 3% hematocrit in a total volume of 200 μL of medium RPMI-1640 was uniformly maintained.

4.2. In vitro antimalarial efficacy test

The in vitro antimalarial assay was carried out according to microassay of Rieckmann et al. (1978) in 96 well-microtitre plates, with minor modifications. A stock solution of 5 mg/mL of each of the test samples was prepared in DMSO and subsequent dilutions were prepared with the culture medium. The test compounds in 20 μL volume concentration at 50 μg/mL in a duplicate well were incubated with parasitized cell preparation at 37 °C in a candle jar. After 36–40 h of incubation, the blood smears were prepared from each well and stained with Giemsa stain. The level of parasitemia in terms of % dead rings along with Schizonts was determined by counting a total of 100 asexual parasites (both live and alive) microscopically using chloroquine as the reference drug.

4.3. Synthesis and structural investigation

The desired compounds **3** and **6a**–**g** were synthesised by the synthetic protocols as outlined in Schemes 1 and 2, respectively. Synthesis of compound (**3**) was achieved by the nucleophilic substitution of 1,3-diaminopropane (**2**) at the 4-Cl atom of 4,7-dichloroquinoline (**1**). The synthesis of di-substituted 1,3,5-triazines **5**(**a**–**g**) were accomplished by the nucleophilic substitution of the Cl atom of the 1,3,5-triazines (**4**) with different primary amines (**a**–**g**) as shown in Fig. 2. Finally title analogs **6**(**a**–**g**) were synthesized by incorporating di-substituted



Scheme 2 Reagents and conditions: (a) R–H (a–g) distinguished amines, (b) 1,4 dioxane 0–5 °C 1 h, 40–45 °C 3 h, $KHCO_3$, (c) 1,4 dioxane 120–130 °C 5–6 h.

1,3,5-triazine moiety **5(a–g)** with the side chain attached to 4-aminoquinoline pharmacophore (**3**).

*4.4. Synthesis of N^1-(7-chloro-quinolin-4-yl)-propane-1,3-diamine (**3**)*

The compound **3** is prepared by the published procedure (De et al., 1997) to yield an off-white solid, characterized by the following physicochemical properties % yield: 83.2, mp: 77–78 °C; FTIR (cm^{-1}): 3346, 3333 ($_{sym.\ \&\ asym.}$ N–$H_{stretch}$, –NH_2), 3253 (N–$H_{stretch}$ > NH), 2987, 2893 (C–$H_{stretch}$, > CH_2), 2029, 1950, 1636, 1603, 1571 (N–H_{bend}), 1464, 1360, 1320, 1240, 1190 (C–$H_{stretch}$), 1090, 910, 850, 800, 760, 610; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.29–8.14 (m, 1H, quinolyl), δ 8.04–8.02 (d, 1H, J = 9.20 Hz, quinolyl), δ 7.90–7.89 (d, 1H, J = 6.00 Hz, quinolyl), δ 7.77–7.56 (dd, 1H, J = 18.00, 18.00 Hz, quinolyl), δ 7.51 (s, 2H, NH), δ 7.35–7.33 (d, 1H, J = 7.60 Hz, quinolyl), δ 7.25 (br s, 1H, NH), δ 6.55–6.08 (m, 2H, CH), δ 3.32–3.27 (t, 2H, J = 9.6 Hz, CH_2), δ 2.81–2.71 (t, 2H, J = 19.2 Hz, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.94, 152.53, 150.75, 146.89, 136.79, 134.26, 127.59, 125.07, 117.72, 109.54, 103.24, 99.14, 44.67, 40.46, 37.95, 27.65, 25.07; Mass: 236.3 $[M+H]^+$.

*4.5. General procedure for the synthesis of di-substituted 1,3,5-triazine derivative **5a–g***

These derivatives were synthesized according to the published procedures (Thruston et al., 1951; Richard et al., 2007). Finally obtained reaction mixture was poured into crushed ice. The solid separated out was filtered, washed with water and recrystallized from different solvents to yield **5a–g**.

*4.5.1. 6-Chloro-N^2,N^4-di-isopropyl-1,3,5-triazine-2,4-diamine (**5a**)*

%Yield: 84.11, mp: 86–88 °C; FTIR (cm^{-1}): 2969.66 (C–$H_{stretch}$), 1582.26 (N–H_{bend}), 1324.20 (N–$H_{stretch}$, > NH), 1019.08 (C–$H_{bend\ out\ of\ plane}$); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.35 (d, J = 6.9 Hz, 12H, 4× CH_3), 2.04 (m, 2H, 2× CH), 3.42 (t, 2H, 2× NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 20.53, 47.35, 163.56, 168.96; Mass: 229.11 $(M+H)^+$; Anal. Calcd. for $C_9H_{16}ClN_5$: C, 47.06; H, 7.02; N, 30.49. Found: C, 47.04; H, 6.99; N, 30.50.

*4.5.2. 6-Chloro-2,4-dimorpholino-1,3,5-triazine (**5b**)*

%Yield: 73.32, mp: 132–135 °C; FTIR (KBr) cm^{-1} 2966.61, 1574.98–1451.24, 1362.21, 1116.51; H^1 NMR (400 MHz, $CDCl_3$) δ 3.70 (t, J = 4.9 Hz, 8H, 4× CH_2–N), 3.78 (t, 8H, 4× CH_2–O); C^{13} NMR (100 MHz, $CDCl_3$) 43.86, 66.56, 164.48, 169.69; Mass: 286.10 $(M+H)^+$; Anal. Calcd. for $C_{11}H_{16}ClN_5O_2$: C, 46.24; H, 5.64; N, 24.51. Found: C, 46.28; H, 5.58; N, 24.56.

*4.5.3. 6-Chloro-N^2,N^4-bis(4-nitrophenyl)-1,3,5-triazine-2,4-diamine (**5c**)*

%Yield: 84.16; mp: 86–88 °C; FTIR (cm^{-1}) 3055.70, 1548.28–1446.06, 1342.89, 1079.19; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (t, 4H, 4× ═CH–), 7.32 (t, 4H, 4× ═CH–), 3.62 (d, 2H, 2× –NH); ^{13}C NMR (100 MHz, $CDCl_3$) 126.23, 131.36 (Ar–C), 143.16 (Ar–C–NO_2), 148.26 (Ar–C–NH), 168.85 (Ar–C–Cl), 173.56 (Ar–C–N, s-triazine); Mass: 388.10 $(M+H)^+$; Anal. Calcd. for $C_{15}H_{10}ClN_7O_4$: C, 46.46; H, 2.60; N, 25.29. Found: C, 46.42; H, 2.62; N, 25.28.

*4.5.4. 6-Chloro-N^2,N^4-bis(2-cyclohexylethyl)-1,3,5-triazine-2,4-diamine (**5d**)*

%Yield: 95.12, mp: 145–147 °C; FTIR (cm^{-1}) 3058.50, 2964.12, 1549.12, 1446.06, 1342.89, 1076.11; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.62 (q, 4H, 2× –CH_2 ethyl), 2.62 (m, 2H, 2× –CH cyclohexyl), 1.48(m, 8H, –CH_2 cyclohexyl), 1.42 (m, 8H, –CH_2 cyclohexyl), 1.24 (t, 6H, 2×CH_3 ethyl); ^{13}C NMR (100 MHz, $CDCl_3$) 29.23, 25.26, 33.18 (cyclohexyl), 18.18, 52.12 (ethyl), 158.26 (Ar–C–NH), 169.65 (Ar–C–Cl), 172.16 (Ar–C–N, s-triazine); Mass: 367.25 $(M+H)^+$; Anal. Calcd. for $C_{19}H_{32}ClN_5$: C, 62.36; H, 8.81; N, 19.14. Found: C, 62.34; H, 8.90; N, 19.16.

*4.5.5. 6-Chloro-N^2,N^4-bis(4-morpholinylphenyl)-1,3,5-triazine-2,4-diamine (**5e**)*

%Yield: 85.22; mp: 108–110 °C; FTIR (cm^{-1}) 3057.70, 2965.12, 1549.18,1447.16, 1342.91, 1076.12, 974.13; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (t, 4H, 2× –CH), 6.75 (t, 2H, –CH), 6.65 (t, 4H, 2× –CH), 3.84 (m, 8H, –CH_2), 3.31(m, 8H, –CH_2); ^{13}C NMR (100 MHz, $CDCl_3$) 71.23, 78.25 (C-morpholine), 113.46, 118.36, 128.26 (Ar–C), 168.85 (Ar–C–Cl), 172.24 (Ar–C–N, s-triazine); Mass: 468.18 $(M+H)^+$; Anal. Calcd. for $C_{23}H_{26}ClN_7O_2$: C, 59.03; H, 5.60; N, 20.95. Found: C, 59.05; H, 5.61; N, 20.95.

*4.5.6. 6-Chloro-N^2,N^4-bis(3-chloro-4-flurophenyl)-1,3,5-triazine-2,4-diamine (**5f**)*

%Yield: 91.86; mp: 152–155 °C; FTIR (cm^{-1}) 3052.32, 1549.58, 1453.25, 1345.91, 978.21; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 (d, 2H, 2× –CH), 6.54 (d, 2H, 2× –CH), 6.84 (d, 2H, 2× –CH), 4.66 (d, 2H, –NH); ^{13}C NMR (100 MHz, $CDCl_3$) 118.11, 122.13, 152.13 (Ar–C), 171.19 (Ar–C–Cl), 179.54 (Ar–C–N, s-triazine); Mass: 401.03 $(M+H)^+$; Anal. Calcd. for $C_{15}H_8Cl_3F_2N_5$: C, 44.75; H, 2.00; N, 17.39. Found: C, 44.74; H, 2.01; N, 17.40.

*4.5.7. 6-Chloro-N^2,N^4-bis(4-flurophenyl)-1,3,5-triazine-2,4-diamine (**5g**)*

%Yield: 88.86; mp: 143–145 °C; FTIR (cm^{-1}) 3054.72, 1548.58, 1448.25, 1342.91, 978.16; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, 4H, 2× –CH), 7.14 (d, 4H, 2× –CH), 4.84 (t, 2H, –NH); ^{13}C NMR (100 MHz, $CDCl_3$) 117.46, 118.11 (Ar–C), 153.43 (Ar–C–F), 172.89 (Ar–C–Cl), 181.24 (Ar–C–N, *s*-triazine); Mass: 334.10 $(M+H)^+$; Anal. Calcd. for $C_{15}H_{10}ClF_2N_5$: C, 53.99; H, 3.02; Cl, 10.62; F, 11.39; N, 20.99. Found: C, 54.00; H, 3.01; Cl, 10.64; F, 11.35; N, 20.98.

*4.6. General procedure for the synthesis of compounds (**6a–g**)*

A mixture of **3** (0.01 M) and **5a–g** (0.01 M) were dissolved in acetone (50 mL) and the reaction mixture was refluxed for 6–8 h. At a regular interval 10% sodium carbonate solution was added to neutralize hydrochloric acid evolved during the reaction. Then the reaction mixture was poured into crushed ice. The product separated was filtered, washed with water and recrystallized from ethanol (see Table 1).

Table 1 In vitro antimalarial activity of the synthesized compounds **6a**–**g**.

Compounds[a]	Antimalarial activity (% dead rings + schizonts[b])
6a	26.5
6b	17.0
6c	10.0
6d	8.5
6e	47.5
6f	56.0
6g	51.5
Chloroquine	50.5

[a] Dose for synthesized compounds 50 μg/ml whereas for chloroquine 0.4 μg/mL.
[b] Mean of two replicates counted against 100 asexual parasites per replicate.

*4.6.1. N^2-(3-(7-chloroquinolin-4-ylamino)propyl)-N^4,N^6-diisopropyl-1,3,5. triazine-2,4,6-triamine (**6a**)*
%Yield 64.86; mp: 184–186 °C; UV λ_{max} (DMSO): 334.0 nm; FTIR (cm^{-1}): 3251 (N–$H_{stretch}$, > NH), 2969 (C–$H_{stretch}$), 2872 (C–$H_{stretch}$, > CH2), 1554 (N–H_{bend}), 1461 (> C═C < stretch), 1383 ($_{Isopropyl\ group}$), 1364,1338, 1313 (C–$H_{stretch}$), 1243 (C–$H_{bending}$), 1167 (C–$H_{stretch}$), 1129, 1080 (C–$N_{stretch}$), 1018, 967, 891 (C–$H_{bend\ out\ of\ plane}$), 851, 805 (C–$H_{stretch\ out\ of\ plane}$), 761, 692 (C–$H_{bend\ out\ of\ plane}$), 557, 497; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.28–8.26 (d, 1H, *J* = 9.60 Hz, quinolyl), δ 7.75 (s, 1H,NH), δ 7.68–7.66 (d, 1H, *J* = 7.60 Hz, quinolyl), δ 7.57 (s, 1H, NH), δ 7.40–7.30 (d, 1H, *J* = 40.80 Hz, quinolyl), δ 6.47–6.39 (d, 1H, *J* = 32.00 Hz, Ar–H), δ 6.25–6.00 (d, 1H, *J* = 32.00 Hz, Ar–H), δ 4.38–4.14 (d, 1H, *J* = 96.80 Hz, CH), δ 3.98–3.96 (d, 2H, CH_2), δ 3.74 (s, 2H, CH_2), δ 3.39–3.27 (m, 2H, CH_2), δ 2.03–1.83 (d, 1H, *J* = 80.00 Hz, Isopropyl, CH_3), δ 1.69 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.61, 166.26, 165.40, 165.18, 164.93, 164.56, 152.29, 150.55, 149.51, 133.83, 127.90, 124.50, 124.44, 117.95, 99.17, 99.02, 42.49, 42.28, 42.06, 41.29, 40.56, 40.35, 40.14, 39.93, 39.51, 39.31, 38.14, 28.48, 26.90, 23.15, 22.74, 22.62, 22.38; Mass: 429.28 $(M+H)^+$; Anal. Calcd. for $C_{21}H_{29}ClN_8$: C, 58.80; H, 6.81; N, 26.12 Found: C, 58.69; H, 6.63; N, 26.34.

*4.6.2. N^2-(3-(7-chloroquinolin-4-ylamino)propyl)-4,6-dimorpholino-1,3,5. triazine-2-amine (**6b**)*
%Yield: 73.0; mp: 207–209 °C; UV λ_{max} (DMSO): 337.0 nm; FTIR (cm^{-1}): 3354 (N–$H_{stretch}$, > NH), 2959 (C–$H_{stretch}$, > CH_2), 2853 (C–$H_{stretch}$), 1583 (N–H_{bend}), 1541 (C═$O_{stretch}$), 1481 (> C═C < ring stretch), 1441, 1361, 1330, 1303, 1286, 1255, 1213 (C–$H_{in\ plane\ bend}$), 1136 (C–$H_{stretch}$), 1110, 1067 (C–$N_{stretch}$), 1005 (C–$O_{stretch}$), 906, 855 (C–$H_{stretch\ out\ of\ plane}$), 805, 765 (C–$H_{bend\ out\ of\ plane}$),737, 639 (C–$H_{bend}$), 544; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.33–8.32 (d, 1H, *J* = 4.80 Hz, quinolyl), δ 8.29–8.27 (d, 1H, *J* = 8.80 Hz, quinolyl), δ 8.23–8.21 (d, 1H, *J* = 8.80 Hz, quinolyl, δ 7.75 (br s, 1H, NH), δ 741–7.39 (d, 1H, *J* = 9.60 Hz, quinolyl), δ 7.33 (br s, 1H, NH), δ 6.90–6.88 (d, 1H, *J* = 7.60 Hz, Ar–H), δ 6.46–6.44 (d, 1H, *J* = 8.80 Hz, quinolyl), δ 6.40–6.39 (d, 1H, *J* = 6.40 Hz, morpholine CH), δ 6.31 (br s, 1H, morpholine NH), δ 3.55 (s, 1H, CH), δ 3.39–3.32 (d, 2H, CH_2), δ 2.47 (s, 2H, CH_2), δ 2.036–1.828 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.17, 165.17, 152.34, 150.54, 150.48, 149.55, 133.81, 127.93, 124.65, 124.44, 117.94, 99.19, 99.06, 66.51, 66.35, 43.70, 43.64, 43.48, 40.36, 40.16, 39.95, 39.74, 39.53, 39.32, 28.34, 26.88; Mass: 485.2 $(M+H)^+$; Anal. Calcd. for $C_{23}H_{29}ClN_8O_2$, C, 56.96; H, 6.03; N, 23.10, Found: C, 57.00; H, 6.01; N, 22.98.

*4.6.3. N^2-(3-(7-Chloroquinolin-4ylamino)propyl)-N^4,N^6-bis(4-nitrophenyl)-1,3,5-triazine -2,4,6-triamine (**6c**)*
%Yield: 66.67; mp: 230–232 °C; UV λ_{max} (DMSO): 373.0 nm; FTIR (cm^{-1}): 3214 (N–$H_{stretch}$, > NH), 2923 (C–$H_{stretch}$, > CH_2), 1580 (N–H_{bend}), 1530, 1494 (> C═C < ringstretch), 1418 (C–O–H_{bend}), 1369, 1301 (C═$O_{stretch}$), 1245, 1177, 1135 (C–$H_{stretch}$), 1108, 899, 847 (C–$H_{stretch\ out\ of\ plane}$), 804, 750 (C–$H_{bend\ out\ of\ plane}$), 691 (C–$H_{bend}$), 628,492; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.01–9.93 (d, 2H, *J* = 33.20 Hz, quinolyl –H), δ 9.68–9.60 (d, 2H, *J* = 30.00 Hz, quinolyl –H), δ 8.53 (s,1H, NH), δ 8.33–8.26 (d, 1H, *J* = 26.80 Hz, quinolyl), δ 8.11–8.05 (d, 1H, *J* = 21.20 Hz, quinolyl), δ 7.92–7.74 (d, 1H, *J* = 69.20 Hz, quinolyl), δ 7.42–7.37 (d, 1H, *J* = 21.20 Hz, quinolyl), δ 7.12–7.07 (d, 1H, *J* = 20.80 Hz, quinolyl), δ 6.71 (s, 1H, NH), δ 6.58–6.47 (d, 1H, *J* = 45.20 Hz, Ar–H), δ 5.84–5.83 (d, 2H, *J* = 56.40 Hz, CH_2), δ 3.52–3.38 (d, 2H, CH_2), δ 2.03–1.92 (d, 2H, *J* = 41.20 Hz,; ^{13}C NMR (100 MHz, DMSO-d_6): δ 152.31, 151.03, 149.45, 134.02, 127.91, 124.55, 117.92, 99.22, 40.56, 40.37, 40.16, 39.95, 39.75, 39.54, 39.33; Mass: 587.2 (4.45) $(M+H)^+$, 324.1 (100), 397.1 (72.64) 399.1 (49.09). Anal. Calcd. for $C_{27}H_{23}ClN_{10}O_4$: C, 55.25; H, 3.95; N, 23.86, Found: C, 55.23; H, 3.91; N, 23.89.

*4.6.4. N^2-(3-(7-Chloroquinolin-4-ylamino)propyl)-N^4,N^6-bis(2-cyclohexylethyl)-1,3,5-triazine-2,4,6-triamine (**6d**)*
%Yield: 65.53; mp: 252–254 °C; UV λ_{max} (DMSO): 269.0 nm; FTIR (cm^{-1}): 3242 (N–$H_{stretch}$, > NH), 2924 (C–$H_{stretch}$, > CH_2), 2852 (C–$H_{stretch}$), 2872, 2364, 1579 (N–H_{bend}), 1463 ($_{n\text{-}hexene}$), 1364, 1201, 1135 (C–$H_{stretch}$), 899, 851 (C–$H_{stretch\ out\ of\ plane}$), 808, 598; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.32–8.24 (d, 1H, *J* = 30.40 Hz, quinolyl), δ 8.03 (d, 1H, *J* = 2.08 Hz, quinolyl), δ 7.75–7.55 (d, 1H, *J* = 80.00 Hz, quinolyl), δ 7.39–7.23 (d, 1H, *J* = 64.00 Hz, cyclohexyl), δ 6.46–6.40 (d, 1H, *J* = 26.00 Hz, Ar–H), δ 3.58 (s, 2H, CH_2), δ 2.50–2.46 (d, 1H, CH), δ 1.63 (s, 2H, CH_2), δ 1.43 (s, 1H, NH), δ 1.43–1.13 (d, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6): δ 152.30, 150.60, 133.94, 127.79, 124.58, 117.90, 99.22, 40.70, 40.43, 40.23, 40.02, 39.81, 39.60, 39.39, 39.18, 36.28; Mass: 551.4 (5.25) $(M+H)^+$, 381 (100%), 397.1 (78.04), 399 (53.07); Anal. Calcd. for $C_{31}H_{45}ClN_8$: C, 65.88; H, 8.03; N, 19.83. Found: C, 65.70; H, 8.09; N, 19.98.

*4.6.5. N^2-(3-(7-Chloroquinolin-4-ylamino)propyl)-N^4,N^6-bis(4-morpholinophenyl)-1,3,5-triazine-2,4,6-triamine (**6e**)*
%Yield: 58.33; mp: 258–260 °C; UV λ_{max} (DMSO): 260.5 nm; FTIR (cm^{-1}): 3294 (N–$H_{stretch}$, > NH), 2922 (C–$H_{stretch}$, > CH_2), 2853 (C–$H_{stretch}$), 1579 (N–H_{bend}), 1451 (> C═C < stretch), 1367, 1207, 1136 (C–$H_{stretch}$), 852 (C–$H_{stretch\ out\ of\ plane}$), 807, 645 (C–$H_{bend}$); ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.37 (d, 1H, quinolyl), δ 8.28–8.25 (d, 1H, *J* = 12.00 Hz, quinolyl), δ 7.74 (d, 1H, quinolyl), δ 7.45 (d, 1H, quinolyl), δ 6.83 (s, 1H, morpholine), δ 6.48 (d, 1H, Ar–H), δ 3.71 (s, 2H, CH_2), δ 3.43 (s, 2H, CH_2), δ 2.99 (s, 1H, NH), δ 2.48 (s, 1H, NH), δ 2.07–2.03 (d, 2H, phenyl); ^{13}C NMR (100 MHz, DMSO-d_6): δ152.31, 127.91, 124.55, 40.58,

40.37, 40.16, 39.95, 39.75, 39.54, 39.33; Mass: 667.2 (3.06) $(M+H)^+$, 353.3 (100), 381.3 (61.98), 711.6 (4.26); Anal. Calcd. for $C_{35}H_{39}ClN_{10}O_2$: C, 63.01; H, 5.89; N, 20.99. Found: C, 63.10; H, 6.01; N, 20.74.

*4.6.6. N^2-(3-(7chloroquinolin-4-ylamino)propyl)-N^4,N^6-bis(3-chloro-4-fluorophenyl)-1,3,5-triazine-2,4,6-triamine (**6f**)*

%Yield: 46.78; mp: 202–204 °C; UV λ_{max} (DMSO): 262.0 nm; FTIR (cm^{-1}): 3413, 3271 ($N–H_{stretch}$ > NH), 3106 ($C–H_{stretch}$), 2929 ($C–H_{stretch}$, > CH_2), 1581 ($N–H_{bend}$), 1493 (> C=C <$_{stretch}$), 1415, 1259, 1210, 1135 ($C–H_{stretch}$), 1054 ($C–N_{stretch}$), 870 ($C–H_{stretch\ out\ of\ plane}$), 807, 699 ($C–H_{bend\ out\ of\ plane}$), 653 ($C–H_{bend}$), 576, 554, 513; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.53–8.34 (d, 1H, J = 78.00 Hz, quinolyl), δ 8.30–8.28 (d, 1H, J = 9.20 Hz, quinolyl), δ 8.09 (s, 1H, quinolyl), δ 8.02 (s, 1H, quinolyl), δ 7.75–7.73 (d, 1H, J = 8.80 Hz, quinolyl), δ 7.71–7.68 (d, 1H, J = 13.60 Hz, quinolyl), δ 7.61 (s, 2H, NH), δ 7.42–7.39 (d, 1H, J = 10.40 Hz, quinolyl), δ 7.34–7.33 (d, 1H, J = 4.40 Hz, quinolyl), δ 7.28–7.20 (m, 1H, quinolyl), δ 6.46 (s, 2H, phenyl), δ 3.41–3.36 (d, 2H, J = 19.20 Hz, CH_2), δ 2.47 (s, 1H, NH), δ 1.16–0.787 (d, 2H, J = 151.20 Hz, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6): δ166.03, 164.35, 164.19, 153.92, 151.52, 151.17, 151.10, 150.06, 148.15, 137.99, 136.58, 134.42, 126.83, 124.80, 124.36, 121.47, 120.38, 119.39, 119.21, 117.68, 116.93, 116.72, 99.04, 40.74, 40.36, 40.15, 39.94, 39.73, 39.52, 39.32, 38.51, 28.17; Mass: 601.1 $(M+H)^+$; Anal. Calcd. for $C_{27}H_{21}Cl_3F_2N_8$: C, 53.88; H, 3.52; N, 18.62. Found: C, 54.00; H, 3.11; N, 18.48.

*4.6.7. N^2-(3-(7-chloroquinolin-4-ylamino)propyl)-N^4,N^6-bis(4-fluorophenyl)-1,3,5-triazine-2,4,6-triamine (**6g**)*

%Yield: 55.56; mp: 183–185 °C; UV λ_{max} (DMSO): 261.0 nm; FTIR (cm^{-1}): 3265 ($N–H_{stretch}$, > NH), 3108, 2930 ($C–H_{stretch}$, > CH_2), 1617, 1582 ($N–H_{bend}$), 1502, 1415, 1209, 1154 ($C–H_{stretch}$), 984, 832 ($C–H_{stretch\ out\ of\ plane}$), 787 ($C–H_{bend\ out\ of\ plane}$), 542, 510; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.81–8.72 (d, 1H, J = 35.60 Hz, quinolyl), δ 8.55–8.52 (d, 1H, J = 14.00 Hz, quinolyl), δ 8.40–8.36 (d, 1H, J = 12.40 quinolyl), δ 7.88 (s, 1H, quinolyl), δ 7.73–7.66 (d, 1H, J = 30.80 Hz, quinolyl), δ 7.52–7.50 (d, 1H, J = 6.80 Hz, quinolyl), δ 7.20–7.17 (d, 1H, J = 13.20 Hz, quinolyl), δ 7.14–7.11 (d, 1H, J = 10.80 Hz, quinolyl), δ 7.07 (s, 2H, NH), δ 7.04–7.00 (m, 1H, quinolyl), δ 6.30 (s, 2H, phenyl), δ 6.62–6.60 (d, 1H, J = 5.60 Hz, phenyl), δ 3.72 (s, 1H, NH), δ 3.47–3.46 (d, 2H, J = 2.40 Hz, CH_2), δ 3.43–3.40 (dd, 2H, J = 4.00,5.60 Hz, CH_2), δ 2.47 (s, 1H, NH), δ 2.04–1.97 (d, 2H, J = 27.20 Hz, CH_2), δ 1.15 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.12, 164.46, 164.35, 156.54, 153.44, 147.03, 143.55, 137.09, 136.35, 125.91, 125.71, 124.16, 123.58, 123.10, 121.99, 116.83, 115.76, 115.53, 115.29, 115.08, 98.95, 95.87, 41.01, 40.58, 40.37, 40.16, 39.95, 39.53, 39.32, 38.20, 28.05; Mass: 533.2 (33.19) $(M+H)^+$, 377.2 (100), 535.2 (13.62), 536.2 (3.97); Anal. Calcd. for $C_{27}H_{23}ClF_2N_8$: C, 60.85; H, 4.35; N, 21.02. Found: C, 60.60; H, 4.51; N, 20.98.

5. Conclusion

On close perlustration and analysis, all the target compounds exhibit mild to moderate degrees of parasite inhibition. But, none of the compounds proved to be as effective as lead, although it contains 1,3,5-traizine and 4-aminoquinoline which already proved be an effective pharmacophore present in clinically used drugs such as cycloguanil and chloroquine, respectively. The present study illustrates that the existence of these two active pharmacophoric groups could not translate hybrid molecules into potent antimalarials. In the light of the above, our further studies are in progress to explain the plausible mechanism lying behind this untoward activity.

However, it can be concluded that this class of compounds will certainly hold a great promise by effective pharmacomodulation toward pursuit to discover a novel class of antimalarial agents to substitute expensive ACTs in the near future.

Conflict of interest

Authors declare no conflict of interest.

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