SYNTHESIS AND LEISHMANICIDAL ACTIVITY OF NEW BIS-ALKYLQUINOLINES

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ABSTRACT

Bis-alkylquinolines **1-6** were synthesized via Williamson reaction between 8-hydroxyquinoline with different 1,ω-dihaloalkanes. Structures of all the products were elucidated by spectroscopic analysis. Cytotoxic and antileishmanial activities of synthesized compounds were determined on U-937 cells and *L. (V) panamensis* amastigotes, respectively. Compound **5**, 1,9-bis[(2-methylquinolin-8-yl)oxy]nonane, was the most selective against axenic and intracellular amastigotes (EC₅₀ = 11.3 and 22.6, μg/mL), with selectivity indices greater than 17.7 and 8.8, respectively; which makes this compound promising for the developing of new leishmanicidal drugs.

Keywords: Leishmania, antiprotozoal, quinoline.

INTRODUCTION

Leishmaniasis is one of the world's most neglected diseases, affecting largely the poorest of the poor, mainly in developing countries; 350 million people are considered at risk of contracting leishmaniasis, and around 2 million new cases occur yearly¹ and WHO has classified leishmaniasis as a category 1 disease, i.e. emerging and uncontrolled².

Chemotherapy of leishmaniasis has been based on the pentavalent antimonials, sodium stibogluconate (Pentostam®) and meglumine antimonite (Glucantime®). These drugs contain multiple uncharacterized molecular structures with variable efficacies, toxicities, possess severe side effects^{3,4} prone to induce resistance⁵⁻⁷ and require lengthy treatments with parental administration under medical supervision⁸. Second-line drugs, such as Amphotericin B and its lipid formulations are either too toxic or expensive for routine use in developing countries. For these reasons, it is necessary to develop new, efficient, non-toxic and cheaper drugs for the treatment of this disease.

The quinolinic core is a structural feature of many bioactive compounds. Among the biological activities exhibited by compounds having this heteroaromatic ring are cytotoxic⁹, antimalarial¹⁰, antituberculosis¹¹, antimicrobial¹², anti-HIV¹³, antiinflammatory¹⁴ and antioxidant¹⁵ activities. Some of these compounds showed antiprotozoal activity^{16,17} including leishmanicidal activity¹⁸⁻²⁴.

Recently, four bis-quinolinoxyalkanes were examined against *Leishmania donovani*²⁵ the causative agent of Visceral Leishmaniasis. Based on these results, we investigated other compounds of this series in search of novel antileishmanial compounds against *Leishmania panamensis* that causes cutaneous leishmaniasis and, to establish a possible structure-activity relationship. Three new bis-alkylquinolines were synthesized via Williamson etherification using 1,ω-dihaloalkanes and 8-hydroxyquinoline and cytotoxic and leishmanicidal activities of these compounds as three of the reported compounds, were screened *in vitro* against U-937 cells and *L. (Viannia) panamensis*, respectively.

EXPERIMENTAL

Chemistry

IR spectra were recorded on a Perkin-Elmer Spectrum RX I FT-IR system in a KBr disk. ¹H NMR and ¹³C NMR spectra were recorded on Bruker 300 MHz spectrometer using CDCl₃ as solvent and TMS as an internal standard. The chemical shifts are expressed in δ ppm. HRMS were run on a Xevo Q-ToF Waters mass spectrometer. Silica gel 60 (Merck® 0.063-0.200 mesh) was used for column chromatography, and precoated silica gel plates (Merck® 60 F254 0.2 mm) were used for thin layer chromatography (TLC).

General method of synthesis of bis-alkylquinolines

To a solution of 2-methyl-8-hydroxyquinoline (1.60 g, 10 mmol) in dichloromethane (40 ml) was added aqueous NaOH solution (10%, 50 ml) followed by the addition of 1 mmol of tetrabutyl ammonium bromide and 10 mmol of 1,ω-dibromoalkane derivative. The mixture was stirred at room temperature for 24–48 h, then the organic layer was isolated using a separating funnel, washed with water to remove alkali, dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure. Column chromatography on silica gel (hexane-ethyl acetate) afford the expected bis-alkylquinoline in yields ranging between 15 and 22% and the corresponding monoethers in yields between 25 and 47% (figure 1).

Figure 1. Synthetic path to obtain the bisalkylquinolines.

1,8-Bis[(2-methylquinolin-8-yl)oxy]octane, 4, Amorphous brown solid, m.p. = 91–93°C, 20% yield. IR cm⁻¹: 2993, 1602, 1561, 1104, 761. ¹H NMR (300MHz, CDCl₃): δ 2.73 (s, 6H, 2 CH₃), 7.24 (d, 2H, J = 8.4 Hz, 2 H-3), 7.95 (d, 2H, J = 8.4 Hz, 2 H-4), 7.30 (d, 2H, J = 7.4 Hz, 2 H-5), 7.33 (t, 2H, J = 7.4 Hz, 2 H-6), 7.0 (dd, 2H, J = 7.0, 1.8 Hz, 2 H-7), 4.18 (t, 4H, J = 7.2 Hz, H-1', H-8'), 2.01 (q, 4H, J = 7.1 Hz, H-2', H-7'), 1.51 (m, 4H, H-3', H-6'), 1.48 (m, H-4', H-5'). ¹³C NMR (75MHz, CDCl₃): δ 25.6 (2 CH₃), 154.2 (2 C-2), 122.3 (2 C-3), 135.9 (2 C-4), 127.6 (2 C-4a), 119.1 (2 C-5), 125.5 (2 C-6), 108.9 (2 C-7), 157.9 (2 C-8), 68.9 (C-1'), 28.7 (C-2'), 25.8 (C-3'), 29.2 (C-4'), 29.2 (C-5'), 25.8 (C-6'), 28.7 (C-7'), 68.9 (C-8'). APCI MS m/z 429.2562 [M+ H]⁺.

1,9-Bis[(2-methylquinolin-8-yl)oxy]nonane, 5, Amorphous yellow solid, m.p. = 110–112°C, 22% yield. IR cm⁻¹: 1654, 1605, 1560, 1431, 1107, 760. ¹H NMR (300MHz, CDCl₃): δ 2.72 (s, 6H, 2 CH₃), 7.27 (d, 2H, J = 8.4 Hz, 2 H-3), 7.98 (d, 2H, J = 8.4 Hz, 2 H-4), 7.35 (d, 2H, J = 7.0 Hz, 2 H-5), 7.36 (t, 2H, J = 7.4 Hz, 2 H-6), 7.03 (dd, 2H, J = 7.0, 1.8 Hz, 2 H-7), 4.16 (t, 4H, J = 7.2 Hz, H-1', H-9'), 1.97 (q, 4H, J = 7.2 Hz, H-2', H-8'), 1.47 (m, 8H, H-3', H-4', H-6', H-7'), 1.35 (m, 2H, H-5'). ¹³C NMR (75MHz, CDCl₃): δ 25.6 (2 CH₃), 157.8 (2 C-2), 122.2 (2 C-3), 135.9 (2 C-4), 127.6 (2 C-4a), 119.1 (2 C-5), 125.5 (2 C-6), 108.9 (2 C-7), 154.2 (2 C-8), 68.9 (C-1'), 29.3 (C-2'), 25.9 (C-3'), 28.7 (C-4'), 29.4 (C-5'), 28.7 (C-6'), 25.9 (C-7'), 29.3 (C-8'), 68.9 (C-9'). APCI MS m/z 443.2710 [M+ H]⁺.

1,12-Bis[(2-methylquinolin-8-yl)oxy]dodecane, 6, Amorphous brown solid, m.p. = 153–155°C, 15% yield. IR cm⁻¹: 1603, 1562, 1430, 1260, 1106, 745. ¹H NMR (300MHz, CDCl₃): δ 2.72 (s, 6H, 2 CH₃), 7.28 (d, 2H, J = 8.4 Hz, 2 H-3), 7.99 (d, 2H, J = 8.4 Hz, 2 H-4), 7.23 (d, 2H, J = 7.2 Hz, 2 H-5), 7.37 (t, 2H, J = 7.2 Hz, 2 H-6), 7.03 (dd, 2H, J = 7.2, 1.8 Hz, 2 H-7), 4.16 (t, 4H, J = 7.3 Hz, H-1', H-12'), 1.97 (q, 4H, J = 7.3 Hz, H-2', H-11'), 1.45 (m, 4H, H-3', H-10'), 1.30 (m, 12H, H-4', H-5', H-6', H-7', H-8', H-9'). ¹³C NMR (75MHz,

CDCl₃): δ 25.7 (2 CH₃), 158.0 (2 C-2), 122.4 (2 C-3), 136.0 (2 C-4), 127.6 (2 C-4a), 119.2 (2 C-5), 125.3 (2 C-6), 108.9 (2 C-7), 154.3 (2 C-8), 154.3 (2 C-8a), 69.1 (C-1'), 29.4 (C-2'), 25.9 (C-3'), 28.8 (C-4'), 29.5 (C-5'), 29.5 (C-6'), 29.5 (C-7'), 29.5 (C-8'), 28.8 (C-9'), 25.9 (C-10'), 29.4 (C-11'), 69.1 (C-12'). APCI MS m/z 485.3193 [M + H]⁺.

Biological activity assays

The compounds were subjected to *in vitro* leishmanicidal activity on axenic and intracellular amastigotes of *L. (V) panamensis* and cytotoxic activity on mammalian cells.

In vitro cytotoxic activity in mammalian cells

The cytotoxic activity of compounds was assessed based on the viability of the human promonocytic cell line U937 (ATCC CRL-1593.2^{TM}) evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method²⁶. Briefly, cells were grown in 96 wells-cell culture dishes at a concentration of 100,000 cells/ml in RPMI-1640 supplemented with 10% FBS and the corresponding concentration of the compounds, starting at 200 mg/ml in duplicate. The cells were incubated at 37°C with 5% CO₂ for 72 hours in the presence of the compounds and then the effect was determined using MTT assay, incubating at 37°C for 3 hours. After 72 hours of incubation the effect of compounds was determined by measuring the activity of the mitochondrial dehydrogenase by adding 10 ml/well of MTT solution (0.5 mg/ml) and incubating at 37 °C for 3 hours. The reaction was stopped by adding a 50% isopropanol solution with 10% sodium dodecyl sulfate for 30 min. Cell viability was determined based on the quantity of formazan produced, which was measured with a Bio-Rad ELISA reader set at 570 nm. As a viability test was used cultured cells in the absence of extracts, as cytotoxicity control was used Amphotericin B and meglumine antimoniate. The results are expressed as the lethal concentration 50 (LC₅₀) calculated by the Probit method²⁷.

In vitro leishmanicidal activity on axenic and intracellular amastigotes

Axenic and intracellular amastigotes of GFP-transfected *L. (V) panamensis* strain (MHOM/CO/87/UA140epir GFP) were used for the *in vitro* testing of leishmanicidal activity of the bis-alkylquinolines.

Activity against axenic amastigotes

The ability of the bis-alkylquinolines to kill axenic amastigotes of *L. (V) panamensis* was determined based on the viability of the parasites evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method²⁸. In brief, parasites were cultivated in Schneider's medium pH 5.4 supplemented with 20% heat inactivated FBS for 3 days at 32°C. Afterwards they were harvested, washed and resuspended at 2 x 10⁶ axenic amastigotes/ml in fresh medium. Each well of a 96-well plate was seeded with 100 μ l of each parasite suspension (in duplicate) and 100 μ l of each concentration of the test compound, starting at 100 mg/ml, was added. Plates were incubated at 32°C. After 72 hours of incubation the effect of drugs was determined by adding 10 ml/well of MTT and incubating at 32°C for 3 hours. The reaction was stopped

and the quantity of formazan produced was measured with a Bio-Rad ELISA reader set at 570 nm. Parasites cultivated in the absence of the compound but maintained under the same conditions were used as controls for growth and viability. Parasites cultivated in the presence of amphotericin B and meglumine antimoniate were used as controls for leishmanicidal activity.

Activity against intracellular amastigotes

The effect of the bis-alkylquinolines against intracellular amastigotes of *L. (V) panamensis* was evaluated by flow cytometry using the methodology described by Varela et al. (2009)²⁹. Briefly, U937 cells were dispensed in 24 well plates at a concentration of 300,000 cells/well, which were treated with 1 μ M of Phorbol Myristate Acetate (PMA) for 48h at 37°C, after which they were infected with promastigotes of *L. (V) panamensis* in stationary growth phase (day 5) in modified NNN medium, a proportion of 1:25 cell/parasite, after 3 hours of incubation at 34°C in 5% CO₂, non-internalized parasites were washed and incubated again at 34°C and 5% CO₂ to allow differentiation to amastigotes form. After 24 hours of incubation, the compounds with the appropriate dilution, not exceeding the LC₅₀, were added. Infected and treated cells were maintained at 34°C and 5% CO₂ for 72 hours. The leishmanicidal effect was measured in a flow cytometer at 488 nm of excitation and 525 nm of emission and determined as described by Valera et al. (2009). The results are expressed as the Effective Concentration 50 (EC₅₀) calculated by the Probit statistical method. The data are the average of three independent experiments conducted in duplicate. Infected but untreated cells were used as control of viability. Additionally, infected cells exposed to amphotericin B and meglumine antimoniate were used as control for leishmanicidal activity. The Selectivity Index (SI) was calculated by dividing the cytotoxic activity between the leishmanicidal activity (SI = LC₅₀/EC₅₀).

RESULTS AND DISCUSSION

Chemistry

Compounds described in this report were prepared as outlined in Figure 1. Reaction of 8-hydroxy quinoline and 1,w-dibromoalkanes in aqueous solution of NaOH and dichloromethane to synthesize (1,w-bis(2-methylquinolin-8-yloxy)alkane), was carried out under phase transfer catalysts condition. Two tandem substitutions of bromide from bromoalkane by quinoline occurred in 15 to 22% yield and allowed the preparation of compounds **4-6** for the first time. Compounds **1-3** have been synthesized and their leishmanicidal activity against *L. (L) donovani* reported²⁵. The novel compounds **4-6** were characterized by their spectral data (IR, EIMS, ¹H and ¹³C NMR, see materials and methods).

Leishmanicidal and cytotoxicity activity

The leishmanicidal and cytotoxicity activity of synthetic compounds as well as glucantime and amphotericin B, which were used as control drugs, was evaluated following the method reported in the literature²⁶⁻³⁰. The results were expressed as EC₅₀ and LC₅₀ values of compounds and are shown in the tables 1 and 2.

Table 1: *In vitro* leishmanicidal activity against axenic amastigotes of *L. (V) panamensis* and toxicity of bis-alkylquinolines.

Compound	Cytotoxicity U937 cells LC ₅₀ (μg/ml) ^a	Leishmanicidal Activity EC ₅₀ (μg/ml) ^b	SI ^c	Comment
1	9.2 ± 0.2	8.5 ± 1.4	1.1	Cytotoxic and active ^d
2	3.3 ± 0.9	> 100.0	< 0.03	Cytotoxic and no active
3	4.2 ± 0.2	6.2 ± 0.4	0.7	Cytotoxic and active
4	>200.0	>100.0	>2.0	No cytotoxic and no active
5	>200.0	11.3 ± 1.9	>17.7	No cytotoxic and active
6	>200.0	>100.0	>2.0	No cytotoxic and no active
8-hydroxy quinoline	7.6 ± 0.2	2.6 ± 0.3	2.9	Cytotoxic and active
Amphotericin B	38.8 ± 2.2	0.05 ± 0.01	776	Cytotoxic and active
Meglumine antimoniate	>1000.0	>1000.0	>1.0	No cytotoxic and no active

^aLC₅₀: Lethal Concentration 50; ^bEC₅₀: Effective Concentration 50; ^cSI: selectivity index: LC₅₀/EC₅₀; ^dCytotoxicity: LC₅₀ <100 mg/ml; No Cytotoxicity: LC₅₀ >200 mg/ml; Active: EC₅₀ <20 mg/ml; Moderately Active: EC₅₀ <100 mg/ml; No Active: EC₅₀ >100 mg/ml

The results show that compounds **1**, **3**, **5** (bisquinolines), are very active against axenic amastigotes of *L. panamensis* exhibiting an $EC_{50} \leq 12.0$ mg/ml (Table 1). No leishmanicidal activity was observed for the compounds **2**, **4** and **6** with $EC_{50} > 100$ µg/ml (Table 1). Regarding to the toxic activity against macrophages, the results showed a high toxicity level for the compounds **1**, **2**, **3** and the 8-hydroxy quinoline with a $LC_{50} < 10$ µg/ml (Table 1). No apparent toxicity was observed for the compounds **4**, **5** and **6**. The LC_{50} for these compounds was > 200 µg/ml (Table 1). The best Selectivity Index was observed for the compound **5** with value of > 17.7 .

The leishmanicidal activity against the intracellular forms of *Leishmania* parasites was also determined only for those compounds that showed low toxicity levels. (Table 2). Among these compounds, only the compound **5** showed leishmanicidal activity against the intracellular form with an EC_{50} of 22.6 mg/ml. The compounds **4** and **6** showed no activity against intracellular amastigotes of *L. (V) panamensis* ($EC_{50} > 100$ mg/ml) (Table 2).

Table 2: *In vitro* activity of bis-alkylquinolines against intracellular amastigotes of *L. panamensis*.

Compound	Leishmanicidal Activity EC_{50} (µg/ml)	SI	Comment
4	> 100.0	> 2.0	No Active
5	22.6 ± 1.0	> 8.9	Active
6	> 100.0	> 2.0	No Active
Amphotericin B	0.04 ± 0.01	970.0	Active
Meglumine antimoniate	6.8 ± 0.5	> 147.05	Active

All results together show that the compound **5** is apparently the most promising compound because it showed low toxic activity on mammalian cells with $LC_{50} > 200$ mg/ml but high leishmanicidal activity against both axenic and intracellular amastigotes with EC_{50} values of 11.3 and 22.6 mg/ml, respectively (Table 1 and Table 2). On the other hand, compounds **1** and **3** showed high leishmanicidal activity against axenic amastigotes. Nevertheless, these compounds also showed high toxicity against mammalian cells (CE_{50} and CL_{50} values < 10 mg/ml). The compound **2**, that was highly toxic, showed no leishmanicidal activity on axenic amastigotes ($CE_{50} > 100$ mg/ml and $CL_{50} = 3.3$ mg/ml). The leishmanicidal activity observed with these compounds is similar to that found by Palit et al (2009) on promastigotes and intracellular amastigotes of *L. (L.) infantum*, where compounds **1**, **2** and **3** showed EC_{50} values of 13.5 mg/ml, 3.2 mg/ml and 2.8 mg/ml for promastigotes and EC_{50} values of 10 mg/ml, 2.7 mg/ml and 2.4 mg/ml for intracellular amastigotes, respectively. In this paper, these same compounds showed a lower toxicity on marine peritoneal macrophages than U937 cells. This, the CL_{50} values on peritoneal macrophages were > 200 mg/ml, > 90 mg/ml and > 85 mg/ml, for compounds **1**, **2**, and **3**, respectively, in comparison to the toxicity observed on U937 cells where the CL_{50} values were < 10 mg/ml. The differential sensitivity exhibited by cell lines and primary culture cells has been demonstrated³⁰.

8-hydroxy quinoline showed activity against U937 cells and axenic amastigotes of *L. (V) panamensis*. Because of their high toxicity level, the activity of this compound against intracellular amastigotes was not determined. The dimer **5** was the least toxic compound showing high leishmanicidal activity for both axenic and intracellular amastigotes of *L. (V) panamensis*. In addition, some bisquinolines showed good activity and a low selectivity index.

Many quinolones have shown to be highly potent against protozoan parasites such as *Plasmodium* and *Toxoplasma* but also *Leishmania*³¹. Specifically fluoroquinolones, indolylquinolones and alkylquinolones have shown *in vitro* and/or *in vivo* leishmanicidal activity³²⁻³⁴. In these types of compounds the substituents may change the electronic structure of the molecule and in the case of alkyl group its hydrophilicity.

The analysis of the relationship between the leishmanicidal activity and structural factors suggest that the alkyl group gives a higher hydrophobicity to the molecules making them less soluble in aqueous systems but facilitating the cell membrane penetration. The biological activity in these alkyl quinolones is presented only when the ether bridge has an odd number of carbon atoms.

Although the mechanism of action of quinolone type compounds is unknown

or not completely understood, several quinolones (e.g fluoroquinolones), can inhibit DNA replication by interacting with DNA gyrase and topoisomerase IV³⁵, two essential bacterial type II topoisomerases present in *Leishmania*³⁶ and other protozoal parasites; the stabilization of cleavable complexes by these inhibitors can lead to cell death through different pathways including activated kinases and death receptor pathways and caspase mediated cleavages^{37,38}. Other quinolones such as sitamaquine (8-amino-quinoline) may induce structural alterations in intracellular *Leishmania* parasites³⁹, leads to collapse of the mitochondrial inner membrane potential of *Leishmania* promastigotes⁴⁰ or induce the alkalization of the acidocalcisomes in *Leishmania* parasites⁴¹.

CONCLUSION

The results obtained are very promising since one of these compounds has potential as a leishmanicidal drug. Due to the high leishmanicidal activity and low cytotoxicity we consider that compound **5** is a good candidate and therefore preclinical studies on the animal model for cutaneous and visceral leishmaniasis disease should be done to confirm the results observed *in vitro*. On the other hand, compounds **1**, **3** and 8-hydroxyquinoline that were active against *Leishmania* parasite but toxic for mammalian cells. Although more studies on toxicity using other cell lines are needed in order to discriminate whether the toxicity showed by these compounds is selective against tumoral or non-tumoral cells. These molecules may be promising candidates to develop antileishmanial drugs by modifying their toxicity with bioguided studies. The mechanism of action of this promising compounds also need to be addressed.

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