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Letter

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Novel aminoquinoline derivatives significantly reduce parasite load in *Leishmania infantum* infected mice

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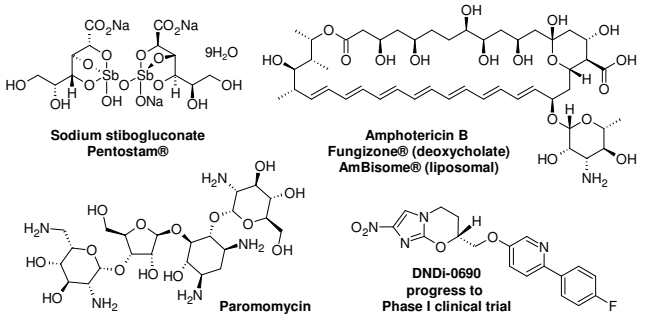
KEYWORDS: Leishmania infantum, promastigote, amastigote, mice model, aminoquinoline

ABSTRACT: In this paper a detailed analysis of thirty 4-aminoquinoline-based compounds with regard to their potential as antileishmanial drugs has been carried out. Ten compounds demonstrated IC_{50}<1 μM against promastigote stages of *L. infantum* and *L. tropica*, and five compounds showed IC_{50}<1 μM against intramacrophage *L. infantum* amastigotes. Two compounds showed dose-dependent enhancement of NO and ROS production by bone marrow-derived macrophages and remarkable reduction of parasite load *in vivo*, with advantage of being short-term and orally active. To the best of our knowledge, this is the first example of 4-amino-7-chloroquinoline derivatives active in *Leishmania infantum* infected mice.

Leishmaniasis is a neglected parasitic disease transmitted by more than 90 sand fly species. The disease may occur in humans and animals, including dogs and rodents. Human infection is caused by about 21 of the 30 species of *Leishmania* parasites that infect mammals.[1,2] Currently, 2 million people are infected every year and more than 350 million people are at risk mostly in tropical and subtropical areas.[3] The life cycle of *Leishmania* parasite includes sand fly and vertebrate host stages. After an infected sand fly deposits metacyclic promastigotes into the host's skin during blood feeding, they are phagocytized by macrophages and then transformed into aflagellated amastigotes. In macrophages, amastigotes multiply and after being released they infect new macrophages. The sand fly ingests infected macrophages during a blood meal and the life cycle continues within the sand fly gut.[2] Treatment of leishmaniasis varies and adapts to the severity of the disease and species of the parasite.[1] Antileishmanial drugs that are presently in use are pentavalent antimonials, amphotericin B, pentamidine, miltefosine and others, with AmBisome® and sodium stibogluconate – paromomycin combination therapy being the first choice for fighting leishmania infection (Chart 1).[3,4] All current therapies present serious side effects, including toxicity, and for some of them resistance development are emerging.[5] In addition, the vaccine for preventing human leishmaniasis, which could have an immense influence on suppression of the disease, is still not available.[6] Thus, the development of potent small molecule inhibitors of *Leishmania* parasites and clinical trials of new drugs are the priorities (**DNDI-0690**, Chart 1).[4,7]

Chart 1. Examples of current antileishmanial medicines and new potent drug candidate.

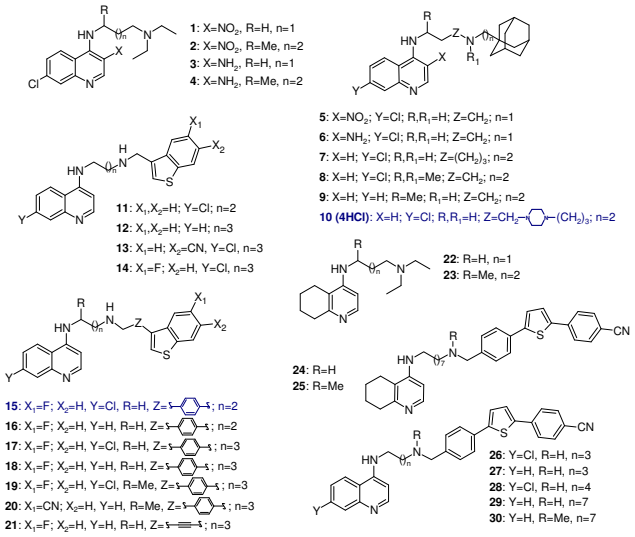


Beside their antimalarial activity, derivatives of 4-aminoquinoline also demonstrated antibacterial, antifungal, antitumor and antileishmanial activity.[8] 4-Amino-7-chloroquinoline analogues and their Pt(II) complexes were shown to be active against promastigotes of different *Leishmania* species.[8] Another study investigated the *in vitro* activity of a series of 4-amino-7-chloroquinolines conjugated to sulfonamide, hydrazide and hydrazine against *L. amazonensis* promastigotes and amastigotes, revealing the ability of these

compounds to induce depolarization of the mitochondrial membrane potential in promastigotes and infected macrophages *in vitro*.[9,10] Steroid linked aminoquinolines also proved to have significant activity against both promastigote and amastigote forms of *L. major in vitro*.[11]

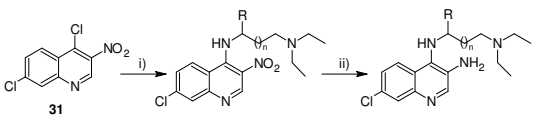
Here we report on the synthesis and antileishmanial potential of novel 4-aminoquinoline derivatives and some aminoquinoline-based drugs previously published by our group (Chart 2).[12-16]

Chart 2. Structures of examined compounds



The introduction of different substituents at C(3) of quinoline moiety was of interest since it is expected to influence the electronic density to substantial extent. Sontochin-like compounds, as well as our previously published aminoquinoline derivatives with fluorine atom at the same position proved to be of significant antiplasmodial potential.[12] Here, we explore the effect of nitro and amino substituents on antileishmanial activity (Scheme 1). Nitro substituent in **31**[12] enabled nucleophilic substitution under mild conditions,[17] resulting in compounds **1** and **2** in reasonably good yield. Reduction of nitro group to amino using tin(II)-chloride[12] afforded compounds **3** and **4**.

Scheme 1. Synthesis of substituted chloroquine-like compounds **1**-**4**

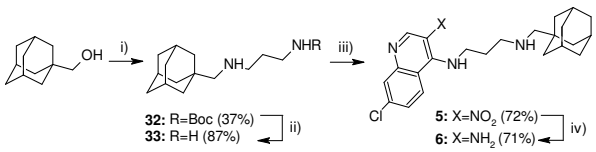


i) amine, Et_3N, CH_2Cl_2, 0 °C to reflux; ii) $SnCl_2$, EtOH

Aminoquinolines with adamantane carrier were synthesized as presented in Scheme 2. Commercially available 1-adamantanemethanol was transformed into mono-Boc protected amine **32** in moderate yield using procedure we established earlier.[12] Removal of protecting group under standard conditions gave amine **33** in good yield. Dichloride **31** was submitted to nucleophilic substitution with amine **33** affording aminoquinoline **5** in good yield. Nitro group in **5** was further reduced to amine **6** using $SnCl_2$.

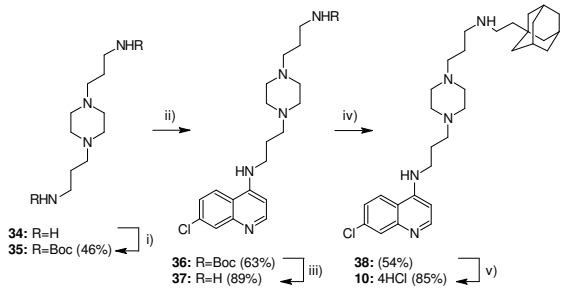
Compound **10** with piperazine moiety in linker was synthesized in several reaction steps, starting from commercially available 3,3'-piperazine-1,4-diyldipropan-1-amine **34** (Scheme 3). After protection of amino groups and nucleophilic substitution with 4,7-dichloroquinoline, compound **36** was obtained in moderate yield. Removal of protecting group under standard conditions gave amine **37** in high yield. Reductive amination of the obtained amine with adamantane-1-acetaldehyde furnished the compound **38**. Compound **10** was obtained as HCl salt of amine **38** (confirmed by elemental analysis).

Scheme 2. Synthesis of adamantane derivatives **5** and **6**



i) 1) PCC, DCM, 2) *tert*-butyl (3-aminopropyl)carbamate, MeOH/DCM, AcOHglac, 3) $NaBH_4$; ii) TFA, DCM; iii) **31**, DCM, 0 °C to reflux; iv) $SnCl_2$, EtOH

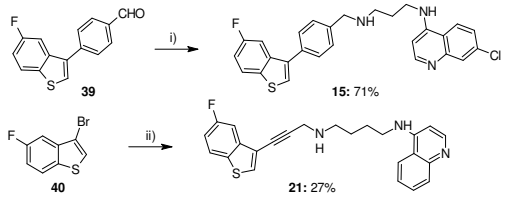
Scheme 3. Synthesis of compound **10**



i) Boc_2O, DCM, r.t.; ii) 4,7-dichloroquinoline, 80 °C, 1 h; 125 °C, 6-8 h; iii) TFA/DCM (v:v 1:10), r.t., 24 h; 2,5 M NaOH; iv) aldehyde, $NaBH(OAc)_3$, DCM, r.t., 24 h; v) MeOH/HCl

Benzothiophene derivative **15** was obtained by reductive amination starting from aldehyde **39**[13] in good yield. Compound **21** was obtained in rather low yield by Sonogashira coupling reaction of **40**[13] and *N*-(prop-2-yn-1-yl)-*N*'-(quinolin-4-yl)butane-1,4-diamine **41**. (Scheme 4).

Scheme 4. Synthesis of novel benzothiophene derivatives **15** and **21**

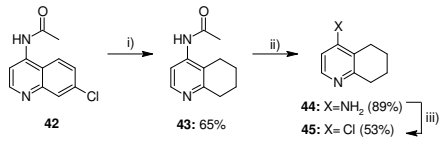


i) 1) **AQ3**, AcOHglac, MeOH/DCM, 2) $NaBH_4$; ii) **41**, $PdCl_2(PPh_3)_2$, PPh_3, CuI, Et_2NH, DMF, MW, 120 °C, 25 min

Reduction of quinoline core was designed in order to explore the effect of resulting deviation from planarity on inhibition of *Leishmania* proliferation (Scheme 5). Compound **42**, synthesized according to the procedure described in literature,[18] was transformed into **43** in acceptable yield. Selective reduction of benzene core of quinoline and hydrogenolysis of chlorine were performed by hydrogenation method using platinum(IV) oxide in acetic acid as solvent in the presence of

perchloric acid. Deacetylation of compound **43** in 2M HCl gave compound **44** in high yield. Using Sandmeyer reaction conditions ($NaNO_2$, AcOH, HCl; CuCl), compound **44** was transformed into **45**.

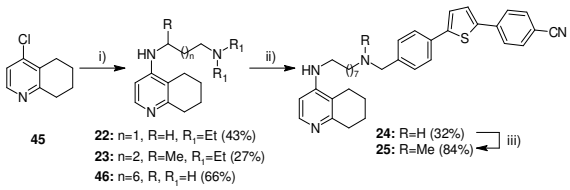
Scheme 5. Synthesis of tetrahydroquinoline core



i) PtO_2, H_2, AcOH, $HClO_4$, ii) 2M HCl, 70 °C; iii) 1) AcOHglac, 28% HCl, $NaNO_2$aq, 0 °C, 2) CuCl, 28% HCl, 0 °C to r.t.

Syntheses of thiophene-based tetrahydroquinoline compounds are presented on Scheme 6. Compound **45** was submitted to Buchwald-Hartwig amination affording the amines **22**, **23** and **46** in low to moderate yield. Compound **46** with eight methylene groups in linker was subjected to reductive amination with 4-[5-(4-formylphenyl)thiophen-2-yl]benzonitrile[13] to obtain **24**. Methylation of secondary nitrogen using 37% formaldehyde gave compound **25** in high yield.

Scheme 6. Synthesis of compounds **22-25**



i) amine, $Pd(OAc)_2$, SPhos, K_3PO_4, dioxane, 85 °C, 24h; ii) for compound **46**: 1) 4-[5-(4-formylphenyl)thiophen-2-yl]benzonitrile, AcOHglac, MeOH/DCM, 2) $NaBH_4$; iii) 37% HCHO, $ZnCl_2$, $NaBH_3CN$, MeOH

The syntheses of other compounds are presented in our previous papers.[12-16] Full details of synthetic procedures, NMR spectra and HPLC purities are given in the Supporting Information.

Thirty compounds presented on Chart 2 were first examined for their activity against *L. infantum* and *L. tropica* promastigote stage using standard MTT assay (Table 1, Table S1). Ten compounds showed IC_{50} values of the same order of magnitude as amphotericin B (IC_{50} < 1 μM), which was used as a positive control. C(3)-substituted chloroquine-like compounds (**1-4**) displayed poor antileishmanial activity against both promastigote species. However, hybrids of such compounds with adamantane carrier resulted in more active derivatives **5** and **6**. Among adamantane derivatives without substituent at C(3), compound **7** showed clear improvement of potency. The most potent compound was **10**, with piperazine moiety in linker.

Benzothiophene compounds **15** and **17** with chlorine atom at C(7) position of quinoline moiety were more potent than their des-chloro analogues **16** and **18**, suggesting that chlorine atom would be important for the activity. Replacing phenyl group with C≡C bond did not produce any significant effect on the activity (**18** vs **21**).

While chloroquine-like compounds, tetrahydroquinolines **22** and **23** were completely inactive, hybrids with thiophene carrier **24** and **25** showed >100-fold increase in activity. Among other thiophenes, compound **29** with eight methylene groups between two nitrogens, demonstrated the highest potency against both *Leishmania* promastigote species. All compounds were also checked for cytotoxicity against differentiated THP-1 cells. For both species moderate selectivity indices were obtained ($SI_{THP\text{-}1/L.i.}$=1.3-9.9; $SI_{THP\text{-}1/L.t.}$=1.1-10.9, Table 1).

Table 1. *In vitro* activities against *L. infantum* and *L. tropica* promastigotes and cytotoxicity against THP-1 human cells[a]

Comp.	L. *infantum* IC_{50} (μM)[b]	L. *tropica* IC_{50} (μM)[b]	THP-1 IC_{50} (μM)[c]	SI (THP/ *L.i.*)[d]	SI (THP/ *L.t.*)[d]
1	8.67	2.77	23.66	2.7	8.5
2	6.49	2.96	>109.6	>16.9	>37.0
3	16.60	9.35	>65.2	>3.9	>7.0
4	16.60	6.63	>59.7	>3.6	>9.0
5	1.91	2.24	12.59	6.6	5.6
6	1.77	1.30	6.39	3.6	4.9
7	0.73	0.66	1.81	2.5	2.7
8	2.46	1.84	3.29	1.3	1.8
9	2.40	2.35	4.90	2.0	2.1
10	0.52	0.51	1.00	1.9	2.0
11	1.14	1.31	2.96	2.6	2.3
12	0.64	0.68	3.76	5.8	5.5
13	1.23	1.24	4.25	3.4	3.4
14	0.51	0.50	1.91	3.8	3.8
15	0.48	0.43	4.73	9.9	10.9
16	1.03	0.81	2.31	2.2	2.8
17	1.02	0.85	4.28	4.2	5.0
18	1.24	1.02	2.35	1.9	2.3
19	0.98	0.91	2.44	2.5	2.7
20	1.55	1.22	2.79	1.8	2.3
21	1.02	1.37	7.11	7.0	5.2
22	>76.5	>76.5	>76.5	>1	>1
23	>69.1	>69.1	>69.1	>1	>1
24	0.72	0.75	2.31	3.2	3.1
25	0.83	0.80	3.68	4.4	4.6
26	2.30	1.94	5.01	2.2	2.6
27	1.22	1.54	2.80	2.3	1.8
28	5.42	7.11	8.10	1.5	1.1
29	0.35	0.30	1.38	4.0	4.6
30	0.80	1.06	3.85	4.8	3.6
Control[e]	0.13	0.14	>10.8	>83.1	>77.1

aAntileishmanial IC50 values against promastigote stages (μM), MTT assay; bAll *in vitro* experiments were performed in duplicate, mean values are given; cCytotoxicity against differentiated THP-1, human monocytic cell line derived from an acute monocytic leukemia patient. dSelectivity index; eControl drug: amphotericin B

All compounds were tested against intramacrophage amastigotes of *L. infantum* at 0.5 μM, non-toxic concentration on human cells (Table S2). Compounds that showed more than 25% inhibition were tested in dose-response experiments and IC_{50} were calculated. Five compounds showed IC_{50} less than 1 μM. Among them, compounds **10**, **15** and **18** were the most active, while compound **15** was the least toxic with good selectivity index (Table 2).

Three compounds with good antileishmanial potential (**10**, **15** and **18**) were subjected to *in vivo* tolerability evaluation in a mice model. All compounds were tested orally at 300 mg/kg (single dose) as suspension in 0.1%Tween/0.5% HEC in wa-

ter. Compound **15** was also tested at lower 50 mg/kg dose by subcutaneous route of administration (in sunflower oil). Compound **18** showed toxic effects when given orally with 3/5 mice alive at the end of experiment, while compounds **10** and **15** given either p.o. or s.c. proved to be tolerable, since all mice survived 30 days after administration and showed normal appearance and behavior.

Table 2. *In vitro* activities against intramacrophage *L. infantum* amastigotes

Comp.	In Vitro Antiamastigote Activity at 0.5 μM[a]	In Vitro Antiamastigote Activity IC_{50} (μM)[b]	THP-1[c] IC_{50} (μM)	SI (THP/IPT)[d]
8	29.6	1.91	3.29	1.72
10	72.2	0.31	1.00	3.22
11	26.4	1.85	2.96	1.60
13	38.9	1.29	4.25	3.29
14	26.4	>1	1.91	<1.91
15	47.6	0.58	4.73	8.15
18	42.7	0.65	2.35	3.61
19	36.9	0.73	2.44	3.34
20	42.2	0.79	2.79	3.51
24	29.6	>1	2.31	<2.31
Control[e]	95.5	0.21	>10.8	>51.4

aMean value of two or three experiments. bMean value of two experiments. cCytotoxicity against differentiated THP-1, human monocytic cell line derived from an acute monocytic leukemia patient. dSelectivity Index (IC50 against THP-1/IC50 against intracellular amastigotes); eControl drug: amphotericin B.

Compounds **10** and **15** were further evaluated for reduction of liver parasite load in a mouse model of visceral leishmaniasis (Balb/c mice infected intravenously with *L. infantum* amastigotes). Results presented in Figure 1 are given as % of reduction relative to control (untreated infected mice, Table S3, Table S4). Compound **15** was tested per os at two different doses 50 mg/kg × 4 days and 100 mg/kg × 4 days and showed significant reduction of parasites in the liver, 95% and 99%, respectively. Compound **10** was also tested per os at 60 mg/kg × 4 days and 100 mg/kg × 4 days. At lower applied dose it reduced parasite load 96% compared to control. Although at higher dose complete clearance was achieved, it showed signs of toxicity, since 1 mouse died on day 10 and 1 mouse died on day 12. Both compounds were also subjected to s.c. administration at lower doses (Figure 1). Compound **10** administered at dose 10 mg/kg × 4 days reduced parasite load by 81%. At 5 mg/kg × 5 days and lower both compounds exhibited poorer activities (57% and 18-48% for **10** and **15**, respectively). However, these results are extremely important, giving us the essential information about dose-dependent behavior of selected 4-aminoquinolines.

In order to investigate the putative mechanism of action (MOA),[9] we examined the production of nitric oxide and ROS by IFNγ primed murine bone marrow-derived macrophages (BMDM) treated with **10** or **15**. Experiments were performed at several concentrations not toxic on BMDM. Not toxic concentrations were determined by MTT assay (data not shown). The concentration of nitrite and ROS was determined using Griess reagent and H_2DCFDA, respectively. Results revealed that compounds **10** and **15** increased the production of nitric oxide by IFNγ-primed macrophages only at the highest dose used (Figure S1). Both compounds induced persistent increase of ROS at all the doses tested (Figure S2).

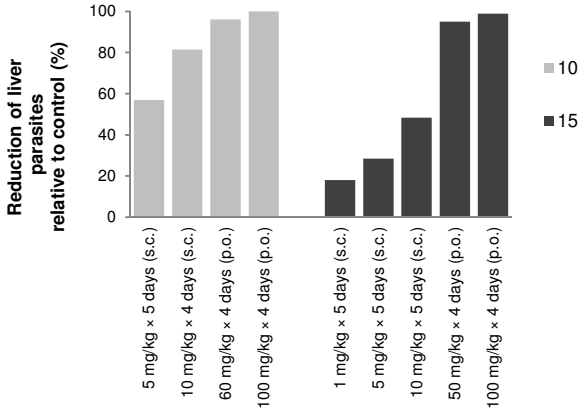


Figure 1. Reduction of parasite load in a mouse model by compounds **10** and **15**

Currently, several noteworthy *in vivo* studies have been published,[19,20,21] Nitroimidazo-oxazole compound **DNDI-VL-2098** previously identified as a favorable candidate did not proceed to the clinical study.[20,22] However, its oxazine derivative **DNDI-0690** was very recently recognized as a new promising candidate for Phase I trial for VL.[4,7] In the last ten years, only a few 4-amino- and 2-alkenylquinolines with modest activity against *Leishmania* parasites *in vivo* were also identified.[23-25] 8-Aminoquinoline derivative sitamaquine appeared to be orally active against visceral leishmaniasis and is presently under clinical investigation.[26]

Here, we identified two 4-amino-7-chloroquinoline compounds bearing benzothiophene or adamantane moieties as potent antileishmanial candidates with significant *in vivo* efficacy. The importance of this work lies in discovery of highly active short-term compounds, tolerable in mice, being the first example of a 4-amino-7-chloroquinoline active in *L. infantum* mice model of visceral leishmaniasis. Although a certain dose-dependent enhancement of NO and ROS production (which can contribute to the amastigotes killing) was observed in the presence of **10** and **15**, MOA still remains unclear. Further studies will be focused on discovering specific target in order to elucidate the mechanism of action. Based on compounds' tolerability in mice model and noteworthy *in vivo* activity, future efforts will be focused on improvement of pharmacokinetic profile and enhancement of the antileishmanial activity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Full details of synthetic procedures, biological assays, procedures for the determination of the HPLC purity, *in vitro* activities on promastigote stages (Table S1), *in vitro* activities against intramacrophages *L. infantum* amastigotes (Table S2), *in vivo* antileishmanial activity (Table S3, Table S4), nitric oxide and ROS production (Figure S1, Figure S2) (**Supporting information – I**, PDF).

NMR spectra and HPLC purity spectra of all tested compounds (**Supporting information – II**, PDF).

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Author Contributions

B.Š. and N.B. designed the research. The manuscript was written by J.K. with contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

Ethical approval: The study followed the International Guiding Principles for biomedical research involving animals (European Directive 2010/63/UE), and it was reviewed by a local Ethics Committees. The study was approved by the Veterinary Directorate at the Ministry of Agriculture and Environmental Protection of Serbia (decision no. 323-07-02444/2014-05/1) and by the Directorate of Animal Health and Veterinary Drugs at the Ministry of Health of Italy (authorization no. 120/2015-PR).

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ABBREVIATIONS

BMDM, murine bone marrow-derived macrophages; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; Tween 80, Polysorbate 80; HEC, hydroxyethyl cellulose; ROS, reactive oxygen species; MOA, mechanism of action; **AQ3**, *N*-(7-chloroquinolin-4-yl)propane-1,3-diamine.

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