Title: Synthesis, biological evaluation and bioavailability prediction of novel furoxan derivatives as leishmanicidal compounds

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ABSTRACT

Four novel furoxan derivatives were synthesized, characterized and evaluated for *in vitro* activity against promastigote form of *Leishmania amazonensis* and *Leishmania infantum*, and amastigote form of *L. infantum*. Compound 7 presented low cytotoxicity, being 30 times less toxic than pentamidine. All compounds were more active against *L. infantum* than *L. amazonensis* promastigote form, with IC_{50} reaching values around 8 μM. Regarding amastigote form of *L. infantum*, the compounds (3b and 7) have exhibited IC_{50} values around 60 μM. The *in silico* prediction of ADME properties has shown that all compounds demonstrated drugability according to 'Lipinski rules' and presented intestinal absorption ranging from 67 to 70%. In conclusion, the compounds presented here have demonstrated to be interesting prototypes against the visceral form of leishmaniasis.

Key words: Leishmaniasis, furoxan, mucosal, visceral

INTRODUCTION:

Leishmaniasis is a neglected tropical disease caused by more than 20 species of the protozoan parasite *Leishmania* [1] and transmitted to humans by the bite of the female sanfly of the genera Phlebotomus, which is the only vector responsible for transmitting the disease [2].

There are three forms of leishmaniasis: cutaneous, visceral and mucocutaneous. Afghanistan, Algeria, Brazil and Colombia are among the countries with the majority of cutaneous leishmaniasis cases. Only in 2015, Brazil has reported more than one thousand new cases of visceral leishmanisis [3].

The increasing drug resistance in leishmaniasis treatment and the lack of recent cost-effective drugs are important issues to be considered in order to discover new drugs. Therefore, there is a high urgency for new compounds active against leishmaniasis. In the present study, four furoxan derivatives were evaluated against promastigote and amastigote forms of *L. amazonensis* and *L. infantum*, which are responsible for cutaneous and visceral leishmaniasis, respectively.

METHODS

Chemistry

^{1}H nuclear magnetic resonance (NMR) spectra were scanned on a Bruker Fourier 300 (300-MHz) NMR spectrometer using dimethyl sulfoxide (DMSO)-d_6 as the solvent. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane.

The compounds 1, 2a, 2b, 5, 6, 10 and 13 were synthesized according to a previously described methodology [4–9].

General procedure for the synthesis of compounds 3a and 3b. A mixture of 4-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy} benzaldehyde or 3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy} benzaldehyde (100mg; 0.3 mmol), a solution of aminoguanidine hydrochloride (45mg; 0,4mmol) and 3mL of ethanol was stirred at room temperature for 2 to 3h and monitored by TLC (100% ethyl acetate). Then, the precipitated was filtered and washed with cold distilled water, dried at room temperature to give compounds 3a and 3b as a white powder.

Preparation of compound 7. A mixture of 4-phenyl-1,2,5-oxadiazol-3-carbaldehyde 2-oxide (100mg, 0.5mmol) (compound 6), a solution of aminoguanidine hydrochloride

(65mg; 0.6mmol) and 3mL of ethanol was stirred at room temperature for 2 to 3h and monitored by TLC (100% ethyl acetate). Then, the precipitated was filtered and washed with cold distilled water, dried at room temperature to give compound 7 as yellow powder.

Preparation of compound 11. A mixture of 6-formyl-2,1,3-benzoxadiazol 1-oxide (100mg, 0.6mmol) (compound 10), a solution of aminoguanidine hydrochloride (78mg; 0.7mmol) and 3mL of ethanol was stirred at room temperature for 2 to 3h and monitored by TLC (100% ethyl acetate). Then, the precipitated was filtered and washed with cold distilled water, dried at room temperature to give compound 11 as yellow powder.

Leishmanicidal activity

Animals

Adult male Swiss albino mice (20–35 g) were used in the experiments. They were housed in single-sex cages under a 12 h light:12 h dark cycle (lights on at 06:00) in a controlled-temperature room (22 ± 2 °C). The mice had free access to food and water. Groups of two animals were used in each test group. The experiments were performed after the protocol was approved by the local Institutional Ethics Committee (protocol number CEUA/FCF/Car n° 53/2012). All experiments were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals.

Parasite Culture

Promastigotes of *L. amazonensis* (MPRO/BR/1972/M1841-LV-79) and of *L. infantum* (MHOM/BR/72) recently isolated from golden hamsters were maintained at 28°C in Liver-Infusion Tryptose (LIT) and M199 medium, respectively, supplemented with 10% fetal bovine serum (FBS), penicillin (Sigma-Aldrich®) and streptomycin (Sigma-Aldrich®).

Promastigotes

Cultured promastigotes of *L. amazonensis* or *L. infantum* at the exponential growth phase were seeded at $1x10^7$ parasites/mL in 96 well flat-bottom plates (TPP®). Compounds and pentamidine (Sigma-Aldrich®) were dissolved in DMSO (the highest concentration was 1.4%, which was not hazardous to the parasites, as previously accessed), added to parasite suspension to final concentrations between 0.5 μM and 100.0 μM and incubated at

28 °C for 72 h. The assays were carried out in triplicate. Leishmanicidal effects were assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) method [10,11]. Absorbances were read at 490 nm. The drug concentration corresponding to 50% of parasite growth inhibition was expressed as the inhibitory concentration (IC_{50}) in μM [10].

Cytotoxicity using murine macrophages

To access the cytotoxicity thioglycolate-stimulated mice were used to collect peritoneal macrophages. Murine peritoneal macrophages were seeded in 96 well flat-bottom plates (TPP®) at a density of $3x10^5$ cells/well (100 μL/well) in RPMI-1640 medium supplemented with 10% heat inactivated FBS, 25 mM HEPES and 2 mM l-glutamine and incubated for 4 h at 37 °C in a 5% CO_2-air mixture. The medium was removed and then new medium was added to the cells which were treated with different concentrations of compounds and pentamidine (Sigma-Aldrich®). Cells without drugs were used as negative control. After that, plates were incubated for 24 h at 37 °C in a 5% CO_2-air mixture. Subsequently, the MTT colorimetric assay was carried out as described above. Absorbance was read in a 96-well plate reader (Robonik®) at 590 nm. The drug concentration corresponding to 50% of cell growth inhibition was expressed as the inhibitory concentration (CC_{50}) [11].

Amastigotes

Murine peritoneal macrophages were plated at $3x10^5$ cells/well 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 4 h at 37 °C in 5% CO_2. Adherent macrophages were infected with promastigotes in the end of the exponential growth phase using a ratio of 5:1 for *L. amazonensis* per cell at 37 °C in 5% CO_2 for 4 h. After that time, the non-internalized parasites were removed by washing, and infected cultures were incubated in RPMI-1640 medium for 24 h at 37 °C in 5% CO_2 to parasite multiplication. Then infected cells were treated with different concentrations of the compounds and pentamidine (Sigma-Aldrich®) for 24 h.

However, for *L. infantum* macrophages were infected with promastigotes in the end of the exponential growth phase using was used at a ratio of 10:1 per cell at 37 °C in 5% CO_2 for 18 h. After that time, infected cells were treated with different concentrations of the compounds and pentamidine (Sigma-Aldrich®) for 24 h.

The cells were then fixed in a methanol solution and stained with Giemsa. The number of amastigotes/100 cells and percent infected cells were determined. The concentration that caused a 50% decrease of growth inhibition compared to the control was determined by regression analysis and expressed as the inhibitory concentration (IC_{50}) in μM [11].

In silico prediction of ADME properties

In order to evaluate the human absorption of the compounds, the drugability through 'Lipinsk rule', Log P, water solubility and human absorption were predicted using the website pKCSM [12].

RESULTS AND DISCUSSION

Chemistry:

The synthetic routes for the preparation of compounds 3a, 3b, 7, 11 are summarized in Fig. 1 to 3.

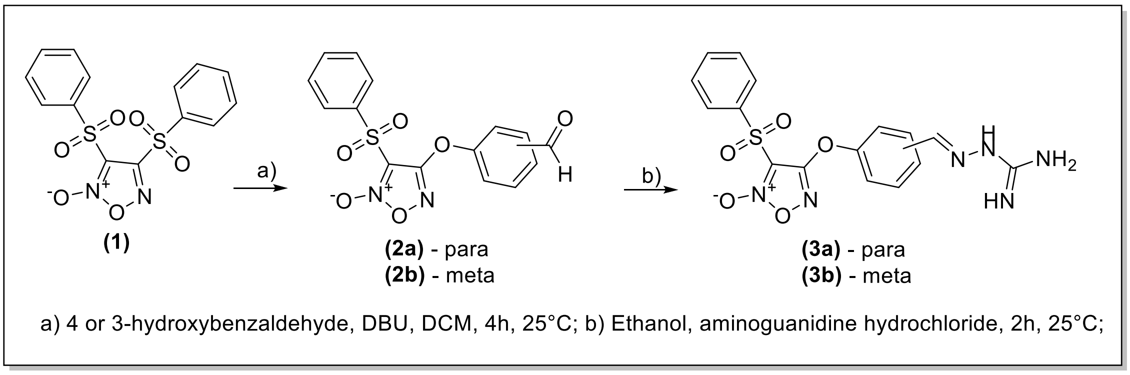


Figure 1. Reagents and conditions to obtain compounds 3a and 3b.

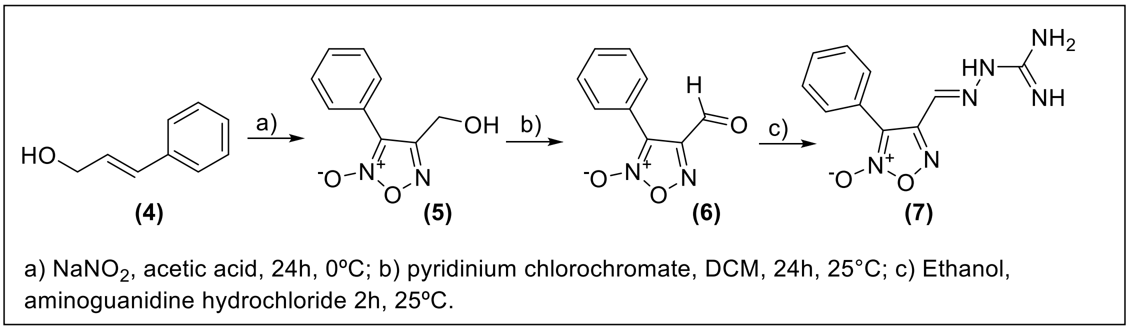
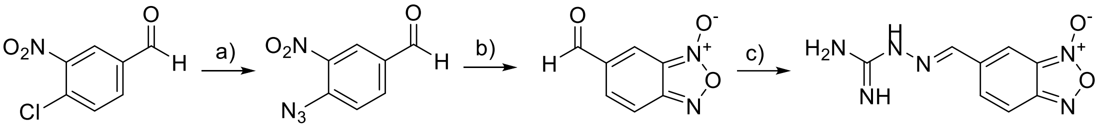


Figure 2. Reagents and conditions to obtain compound 7.



(8) (9) (10) (11)

a) NaN_3, DMSO, 1h, 75°C; b) toluene, 1.5h, 120°C; c) Ethanol, aminoguanidine hydrochloride, 2h, 25ºC.

Figure 3. Reagents and conditions to obtain compound 11.

Compounds 3a, 3b, 7, 11 were obtained reacting their respective aldehyde intermediate (2a, 2b, 6 and 10, respectively) with a solution of aminoguanidine hydrochloride in an ethanol and acetic acid medium with yields varying from 30 to 60%.

Leishmanicidal activity:

The first step of the biological evaluation was the IC_{50} determination of the compounds against promastigote forms of *Leishmania amazonensis* and *Leishmania infantum.* The intermediates 2a, 2b, 6 and 10 were previously evaluated against the promastigote form of *L. amazonensis* with an IC_{50} range from 0.79 to 4.29 µM and selective index range from 0.38 to 3.61, suggesting a relative cytotoxicity of these molecules [8].

Regarding *L. amazonensis* promastigote form, the compounds presented IC_{50} values of 2.7 to 19 times higher than pentamidine; however, the CC_{50} has demonstrated that the cytotoxicity of the new molecules is expressive lower than pentamidine, also demonstrated by the SI. Compound 7 is the less toxic among the molecules, with an outstanding CC_{50} more than 30 times higher than pentamidine. During the evaluation against promastigote form of *L. infantum*, all compounds presented an IC_{50} values lower than pentamidine, reaching values around 8 µM. Compound 3b and 7 have exhibited selective index superior to 15, therefore, these compounds were selected to further studies involving amastigote form of *L. infantum*. Interestingly, both compounds (3b and 7) have exhibited related IC_{50} values around 60 µM.

Table 1. Biological activity of compounds and pentamidine against promastigotes and amastigotes forms of *L. amazonensis* and *L. infantum* (IC_{50}), macrophages (CC_{50}) and selectivity index (SI).

Compounds	**CC_{50} (µM) macrophages**	**IC_{50} (µM) promastigotes**				**IC_{50} (µM) amastigotes**	
		L. amazonensis	**SI**	***L. infantum***	**SI**	***L. infantum***	**SI**
3a	52,41 ± 4,17	35,83 ± 0,08	1,46	8,87 ± 0,19	5,91	-	-
3b	315,73 ± 4,72	27,73 ± 0,03	11,38	12,37 ± 0,32	25,51	60,0 ± 0,01	5,26
7	1095,52 ± 13,91	197,37 ± 1,13	5,55	32,28 ± 0,92	33,93	59,6 ± 0,56	18,38
11	268,08 ± 2,11	42,69 ± 0,90	6,28	52,90 ± 0,78	5,07	-	-
Pentamidine	35,69 ± 2,33	10,19 ± 0,29	3,50	67,71 ± 2,76	0,53	19,77 ± 0,5	1,8

In silico prediction of ADME properties

Lipinski rule, LogP, water solubility and intestinal absorption were parameters predicted of the compounds and presented in Table 2. For the Lipinski rule evaluation, the molecular weight, hydrogen bond donors and acceptors and LogP were analyzed, and the compounds were classified in "yes", as the compounds which follow the rules, and "no" as the compounds that do not follow the rules. Based on these parameters, all compounds exhibited drugability through Lipinski rules.

Intestinal absorption is a parameter that results from LogP and water solubility. The results presented here demonstrates that the predicted absorption of the compounds is close, varying from 67 to 70%. The compounds with higher absorption presented a higher water solubility, as observed in compounds 7 and 11.

Table 2. Results of *in silico* evaluation of ADME properties.

Compounds	Lipinski rule	LogP	Water solubility (log.mol/L)	Intestinal absorption (%)
3a	Yes	0.75	-3.115	67.169
3b	Yes	0.75	-3.083	67.224
7	Yes	-0.21	-2.286	70.774
11	Yes	-0.72	-2.419	70.216

CONCLUSION

The new series of furoxan derivatives (compounds 3a, 3b, 7 and 11) were synthesized and characterized using analytical methods. The compounds presented CC_{50} values superior to that of pentamidine. The CC_{50} of compound 7 is 30 times higher than the control drug. All compounds have demonstrated to be more active against *L. infantum* than *L. amazonensis*, which leaded to an evaluation against amastigote form of *L. infantum* and resulted in IC_{50} three times higher than pentamidine. The *in silico* study has demonstrated that the compounds exhibits drug-like properties. The results presented here demonstrate that the compounds showed low toxicity and interesting activity against *L. infantum*, therefore they are interesting prototypes against the visceral form of leishmaniasis.

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