Beta-carboline-3-carboxamide derivatives as promising antileishmanial agents

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Leishmaniasis has an overwhelming impact on global public health especially in tropical and subtropical countries and the currently available antileishmanial drugs have serious side effects and low efficacy. Natural and synthetic compounds have been tested in the past few years against *Leishmania* and the beta-carboline class of compounds have shown great results in antiparasitic chemotherapy. In the present study, three 1-substituted beta-carboline-3-carboxamides (3–5) and 1-substituted beta-carboline-3-carboxylic acid (2) were synthesized and screened for *in vitro* activity against *L. amazonensis*. Compound 5 (*N*-benzyl 1-(4-methoxy)phenyl-9H-beta-carboline-3-carboxamide) had the best activity against promastigote and axenic amastigote forms with IC₅₀ of 2.6 and 1.0 µM, respectively. Its CC₅₀ on macrophages cell line was higher than 2457.0 µM with an SI ratio of 930.2. Against intracellular amastigote forms, it had a dose-dependent relationship with a 50% growth inhibitory concentration of 1.0 µM. Through morphological and ultrastructure analysis of promastigote forms treated with compound 5, alterations on cell shape and number of flagella and nuclear membrane damage were observed. For this, compound 5 supports the idea for more *in vitro* and *in vivo* studies.

INTRODUCTION

Leishmaniasis are a complex of diseases caused by different species of the genus *Leishmania* and has an overwhelming impact on the global public health especially in tropical and subtropical countries with a prevalence of estimated 12 million cases. The

parasite exists as the flagellated promastigote form in the female phlebotomine sandfly vector and the obligate intracellular amastigote form in the mammalian host. Infection by various strains of *Leishmania* causes a wide spectrum of diseases in humans, with many different clinical manifestations (cutaneous, mucocutaneous and visceral). Cutaneous leishmaniasis affect approximately 1–2 million new cases per year and the species *Leishmania amazonensis* is one of the causative agents of American Cutaneous Leishmaniasis. This illness frequently self-cures within 3–18 months, but it leaves disfiguring scars, followed by discrimination, stigma and sub-standard living

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conditions for those infected (Croft and Coombs, 2003; WHO, 2007; WHO, 2010).

The first-line antileishmanial compounds used are the pentavalent antimonials such as meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostan), and other drugs may be included as alternative compounds such as amphotericin B, pentamidine, miltefosine and paromomycin among others (WHO, 2010). Most of the drugs used require parenteral administration of high doses and a lengthy course of treatment, resulting in a marked increase in serious side effects and low efficacy (Croft and Coombs, 2003). Moreover, the pentavalent antimonials, as an example, are toxic and prone to stimulate drug resistance (Croft *et al.*, 2005). Searching alternatives for these effects, researches have been studying new substances (natural and synthetic) *in vitro* and *in vivo* against this disease reviewed by Tiuman and co-workers (2011) and new liposomal (Amphocil) and colloidal amphotericin B formulations that have shown great results on clinical trials when compared to amphotericin B (Romero and Boelaert, 2010).

A great number of natural and synthetic compounds have been tested in the past few years against leishmaniasis (Brenzan *et al.*, 2008; Santos *et al.*, 2008) and the importance of beta-carboline class compounds is well established in antiparasitic chemotherapy research (Chauhan and Srivastava, 2001; Croft and Coombs, 2003; Valdez *et al.*, 2009); moreover, natural and synthetic beta-carbolines alkaloids, also showed biological activities as antitumoral (Formagio *et al.*, 2008), anti-viral (HSV-1, HSV-2 and Poliovirus) (Formagio *et al.*, 2009) and parasiticidal, mainly against *T. cruzi* (Rivas *et al.*, 1999; Tonin *et al.*, 2009).

In a recent work, we demonstrated the *in vitro* activity of some *N*-alkyl-(1-phenylsubstituted-beta-carboline)-3-carboxamides against epimastigote form of *T. cruzi* and promastigote form of *L. amazonensis* (Tonin *et al.*, 2010). In the present study, the 1-(4-methoxy)phenyl-beta-carboline-3-carboxamides

FIG. 1. Reagents and conditions: (a) CH_3OH , NaOH aqueous, reflux, 12 hours; 85%. (b) SOCl_2 , reflux, 3 hours; 73%. (c) Isopropylamine, cyclohexylamine or benzylamine, Et_3N , rt, 12 hours; 70–75%.

3–5 were synthesized and screened for leishmanicidal activity against promastigote and axenic amastigote forms of *L. amazonensis* and for cytotoxicity. The activity of *N*-benzyl 1-(4-methoxy)phenyl-9H-beta-carboline-3-carboxamide (**5**) was evaluated against intracellular amastigote forms and compounds 1-(4-methoxy)phenyl-9H-beta-carboline-3-carboxylic acid (**2**) and *N*-benzyl 1-(4-methoxy)phenyl-9H-beta-carboline-3-carboxamide (**5**) for its morphological and ultrastructural analysis.

MATERIAL AND METHODS

General Procedure for the Synthesis of beta-carbolines 3-carboxamides

The synthetic route for beta-carboline-3-carboxylic acid **2** and *N*-alkyl-beta-carboline-3-carboxamides **3–5** is outlined in Fig. 1. The methyl beta-carboline-3-carboxylate (**1**) was synthesized through a Pictet-Spengler condensation of the L-tryptophan methyl ester with 4-methoxybenzaldehyde, in acid media, followed by oxidation with sulphur in refluxing xylene, in accordance with procedures previously reported (Formagio *et al.*, 2008). The methyl beta-carboline-3-carboxylate **1** was treated with water/methanol in basic media, to afford the beta-carboline-3-carboxylic acid **2**. The compound **2** was treated with thionyl chloride and then with the amines as isopropylamine, cyclohexylamine and benzylamine, in

THF and triethylamine, to afford compounds **3–5**, respectively.

Parasites

Promastigote forms of *L. amazonensis* (MHOM/BR/75/Josefa-isolated from a patient with diffuse cutaneous leishmaniasis by C.A. Cuba-Cuba of Universidade de Brasília, Brazil) were maintained by weekly transfers in Warren's medium pH 7.2 (brain heart infusion broth enriched with haemin and folic acid) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 25°C. The infectivity of the parasites was maintained by periodic inoculation into BALB/c mice footpads. Axenic amastigote forms were obtained by *in vitro* incubation of infective promastigotes (Ueda-Nakamura *et al.*, 2001) and maintained by weekly transfers in Schneider's medium pH 4.2 supplemented with 20% FBS at 32°C.

Cells

J774G8 murine macrophages were maintained at 37°C with 5% CO₂-air mixture in RPMI 1640 medium (Gibco Invitrogen Corporation, Grand Island, NY, USA) with sodium bicarbonate and L-glutamine and supplemented with 10% FBS.

Antileishmanial activity

Promastigote forms, in log growth phase, 48 hours culture (1×10^6 parasites) were grown in a 24-well plate in Warren's medium supplemented with 10% inactivated FBS and different concentrations of compounds **2**, **3**, **4** or **5**. Axenic amastigotes also in log growth phase, 72 hours culture (1×10^6 parasites) were grown in a 12-well plate in Schneider's medium, pH 4.2, supplemented with 20% FBS and different concentrations of the same compounds. The cell density, for each treatment, after 72 hours of incubation, was obtained with a haemocytometer (Improved Double Neubauer) and the 50% (IC₅₀) and 90% (IC₉₀) inhibitory growth concentrations were determined.

Cytotoxicity assay

Adherent J774G8 macrophage cells were suspended to yield 5×10^5 cells/ml in RPMI 1640 medium supplemented with 10% FBS and added to each well in 96-well microtitre plates. The plates were incubated in a 5% CO₂-air mixture at 37°C to obtain confluent cell growth. After 24 hours, the cells were treated with different concentrations of compounds **2**, **3**, **4** or **5**. The plates were incubated in a 5% CO₂-air mixture at 37°C for 48 hours. The cultures were then fixed with 10% trichloroacetic acid for 1 hour at 4°C, stained for 30 minutes with 0.4% sulforhodamine B in 1% acetic acid and subsequently washed four times with deionized water. Bound sulforhodamine B was solubilized with 150 µl of 10 mM unbuffered Tris-base solution. Absorbance was read in a 96-well plate reader (Power Wave XS; Bio-Tek, Winooski, VT, USA) at 530 nm. Dose-response curves were plotted (values expressed as percentage of control optical density) and CC₅₀ values (50% cytotoxicity concentration) were estimated by regression analysis.

Activity Against Intracellular Amastigote Forms

Peritoneal macrophages from male BALB/c mice (6–8 weeks of age) were collected with 0.01M phosphate-buffered saline, harvested in RPMI 1640 medium pH 7.6 supplemented with 10% FBS (5×10^5 cells/ml) and plated on 13 mm coverslips in 24-well culture plates for adherence at 37°C in a 5% CO₂ atmosphere. Non-adherent cells were then removed and adhered macrophages cultured for 12–16 hours in RPMI 1640 medium supplemented with 10% FBS. Next, macrophages were infected in multiples of 10 promastigotes per host cell and incubated at 37°C in 5% CO₂ atmosphere. After 6 hours, infected macrophages were treated with different concentrations of compound **5**. After 24 hours, the monolayers were fixed with methanol, and stained with Giemsa. The number of amastigotes was determined by counting at least 200

macrophages, in duplicate cultures, and results were expressed as the inhibition index (the percentage of infected macrophages by the mean number of internalized parasites per cell).

Scanning Electron Microscopy

After treatment with IC₅₀ of compounds **2** or **5** for 72 hours, promastigote forms were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 2 hours. The parasites were washed three times in 0.1M sodium cacodylate buffer, placed on the poly-L-lysine-coated coverslip, dehydrated in different concentrations of ethanol, critical point-dried in CO₂, sputter-coated with gold and observed in an SS-550 scanning electron microscope (Shimadzu, Tokyo, Japan).

Transmission Electron Microscopy

After treatment with IC₅₀ of compounds **2** or **5** for 72 hours, promastigotes were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at 4°C, post-fixed in a solution containing 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1M cacodylate buffer, dehydrated in different concentrations of acetone and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined in a Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR, USA).

Statistical Analysis

The experiments were performed in triplicate, in three independent experimental sets. The data were analysed statistically by non-parametric Kruskal-Wallis test to evaluate significant differences. Alternatively, the analysis of variance between groups was made by means of ANOVA test using Statistics 8.0®. Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Natural and synthetic beta-carboline alkaloids have been studied for their biological

activities. These compounds can be found in biological tissues and fluids (Harraiz and Cuq, 2000), in food products as juices and fruits (Harraiz and Galisteo, 2003), and in plants (Dusman *et al.*, 2004). Beta-carboline compounds have shown great activity against *Trypanosoma cruzi* and *Leishmania* spp. (Rivas *et al.*, 1999; Boursereau and Coldham, 2004; Giorgio *et al.*, 2004; Tanaka *et al.*, 2007; Barbaras *et al.*, 2008; Tonin *et al.*, 2009). In a recent work, we demonstrated the *in vitro* activity of some *N*-alkyl-(1-phenylsubstituted-beta-carboline)-3-carboxamides (Tonin *et al.*, 2010) against epimastigote form of *T. cruzi* and promastigote form of *L. amazonensis*. In this work, we evaluated the biological activity of three *N*-alkyl-1-(4-methoxy)phenyl-beta-carboline-3-carboxamides and a 1-substituted beta-carboline-3-carboxylic acid against the protozoa *L. amazonensis*.

The treatment of promastigote and axenic amastigote forms with these synthetic beta-carbolines demonstrated that compound **5** had a great activity with 50 and 90% growth inhibitory concentration values (IC₅₀ and IC₉₀) of 2.6 ± 0.0 and 11.2 ± 0.5 μ M for promastigote and 1.0 ± 0.2 and 41.0 ± 2.9 μ M for axenic amastigote forms, respectively. The compounds **3** and **4** also had their inhibitory activity enhanced to both forms of protozoan when compared to compound **2** (Table). Valdez and co-workers (2009) observed that some tetrahydro-beta-carboline derivatives had their trypanocidal activity enhanced when modifications of the groups attached to C-3 of beta-carboline nucleus were done.

J774G8 murine macrophages were treated with beta-carboline compounds to test the non-toxicity of these substances for mammalian cells. When macrophages were treated with compound **5**, the CC₅₀ was higher than 2457.0 ± 0.0 μ M while compound **2** had a CC₅₀ of 69.0 ± 1.2 μ M (Table). When compared to the growth inhibitory activity against promastigotes, by using the selective index (SI) (ratio: CC₅₀ J774G8 cells/IC₅₀ protozoa), compound **2**

TABLE. Antileishmanial activity against promastigote and axenic amastigote forms of *L. amazonensis*, cytotoxic effects and selectivity index of the beta-carboline derivatives

Compound	Molecular weight	Promastigote		Amastigote		Macrophages J774G8*	
		IC ₅₀ (μM)	IC ₉₀ (μM)	IC ₅₀ (μM)	IC ₉₀ (μM)	CC ₅₀ (μM)*	SI (CC ₅₀ /IC ₅₀)
(2)	318.0	71.2 ± 2.8	131.7 ± 2.0	15.0 ± 3.7	110.7 ± 6.7	69.0 ± 1.2	0.96
(3)	359.0	5.3 ± 0.3	12.8 ± 0.4	0.5 ± 0.0	31.6 ± 4.0	719.1 ± 12.5	135.6
(4)	399.0	3.6 ± 0.5	11.1 ± 0.0	1.6 ± 0.2	72.9 ± 1.3	1942.3 ± 44.3	539.4
(5)	407.0	2.6 ± 0.0	11.2 ± 0.5	1.0 ± 0.2	41.0 ± 2.9	>2457.0 ± 0.0	>930.2

Values represent the mean ± SD of at least three experiments performed in triplicate. SI=CC₅₀ J774G8/IC₅₀ promastigote forms.

*On macrophage J774G8 at 48 hours of culture.

FIG. 2. Effect of compound 5 on *L. amazonensis*–macrophage interaction. Peritoneal macrophages were infected with promastigotes and then treated with different concentrations of the compound. After 72 hours, the inhibition percentage was calculated by the equation $(P2/P1) \times 100$, where P1 is the inhibition index for the control and P2 is the inhibition index for the treated cells. Inhibition index was calculated by multiplying the percentage of macrophages with internalized parasites by the mean number of internalized parasites per macrophage. Each bar represents $\pm$ the standard deviation. Significant differences of each group from untreated cell (control) were done using ANOVA test with $P \leq 0.05$.

showed an SI of 0.96. On the other hand, compounds 3, 4 and 5 had higher SI indexes with values of 135.6, 539.4 and 930.2, respectively. SI values lower than 1.0 are considered more toxic to the host cell than to the parasite, inferring that compounds 3, 4 and 5 are much more selective to the parasite than to the mammalian host cell, indicating them as promising antileishmanial agents and prone to *in vivo* assays. Ferreira *et al.* (2002) tested some beta-carboline alkaloids in *L. amazonensis* with interesting antileishmanial effect and low toxicity results, indicating that those alkaloids could be used for the treatment of cutaneous leishmaniasis. In the present work, it was observed that the introduction of an *N*-benzylcarboxamide group on the beta-carboline nucleus improved the cytotoxicity index of compound 5 when compared to its carboxylic acid precursor (2). All results were significant at $P \leq 0.05$ by non-parametric Kruskal–Wallis test.

Against the clinically important form, the intracellular amastigote, compound 5 showed a dose-dependent relationship (Fig. 2)

FIG. 3. Scanning electron microscopy of *L. amazonensis* treated with beta-carboline 2 or 5 for 72 hours at 25°C. Control parasite (A) shows the typical elongated body. Protozoa treated with IC_{50} of compound 2 (B) shows a rounded and swelled body. Protozoa treated with IC_{50} of compound 5 (C and D) shows alterations on the cell shape and on the number of flagella. Protozoa treated with IC_{90} of compound 5 (E and F) shows alterations on cytokineses and multiple flagella. Bar = 1 µm.

with a reduction in the number of infected cells as well as in the number of intracellular amastigotes. At 40.7 µM, an inhibition of 87.7% of intracellular amastigote forms was observed, at 4.07 µM, 75.5%, at 0.40 µM, 48.5% and at 0.04 µM, 45.8%. For this, 50% growth inhibitory concentration of intracellular amastigote was calculated as 1 µM.

To obtain more information about the possible mechanism of action of compounds 2 and 5 on *L. amazonensis*, the cells were analysed by scanning and transmission electron microscopy. Morphological alterations, visualized by scanning electron microscopy (Fig. 3), showed control cells with its elongated body and terminal flagellum (A) and cells treated with IC_{50} of compound 2 with a rounding and swelling of the cell body (B). Cells treated with IC_{50} of compound 5 had alterations on cell shape and on the number of flagellum (C and D), and cells treated with IC_{90} had their cell division altered, not splicing completely and with a higher number of flagella (E and F).

Ultrastructural alterations (Fig. 4) showed control cells with some characteristic organelles such as kinetoplast, mitochondria and nucleus (A). Cells treated with IC_{50} of compound 2 demonstrated remarkable alterations on the nuclear membrane and multiple flagella (B and C). Cells treated with IC_{50} of compound 5 also demonstrated alterations at the nuclear membrane, multiple flagella (D) and alteration on cell division (F), confirming what was seen by scanning electron microscopy. The presence of chromocenters can also be observed in these cells (E).

Similar results as nuclear chromatin condensation with alteration on nucleus morphology, and appearance of large multi-vesicular bodies in promastigote forms of *L. amazonensis* treated with BPQ-OH, a specific inhibitor of squalene synthase, were observed by Rodrigues and co-workers (2005). de Souza *et al.* (2004) observed severe alterations on nuclei of *T. cruzi* and *L. amazonensis* treated with a phenyl-substituted furamidine. Another ultrastructural change in treated promastigotes with compound 5 was the intense exocytic activity in the region of the flagellar pocket. Some studies have reported that this exocytic activity might occur as a result of the secretion into this region of abnormal lipids, which accumulate as a consequence of drug action, or might indicate a process of exacerbated protein production by the cells as they attempt to survive (Tiuman *et al.*, 2005).

Chromocenter is a fuelgen-positive region frequently observed in higher organisms at interphasic nuclei. These chromocenters are extremely rare among protozoa where they are observed as a bud-like projection attached to nucleolus (Bhamrah and Juneja, 2001). Smith and Virkki (1978) and Drets *et al.* (1983) reported that the heterochromatic segments in nuclei of insects that presented different degrees of ectopic pairing led to the formation of chromocenters and seemed to play an

FIG. 4. Transmission electron microscopy of *L. amazonensis* treated with beta-carbolines 2 or 5 for 72 hours at 25°C. (A) Control parasite shows characteristic organelles. (B and C) Cells treated with IC_{50} of compound 2 show remarkable alterations on the nuclear membrane and multiple flagella. (D–F) Cells treated with IC_{50} of compound 5 demonstrate nuclear membrane alterations, cytoskeleton alterations and multiple flagella. Arrows indicate nuclear membrane alterations and small arrow indicate chromocenters. F: flagellum; Fp: flagellar pocket; K: kinetoplast; M: mitochondria; N: nucleus; V: vacuoles. Bar = 1 μ m.

important role in nuclear organization and in segregation of meiotic chromosomes.

The mechanism of leishmanicidal action of alkaloid beta-carbolines is not well established. Some authors suggested that these compounds associate by intermolecular interaction with prosthetic groups of flavoenzymes, dinucleotids of adenine-flavine and riboflavin and that they interfere in the synthesis of deoxyribonucleic acid in cancer cells. They also suggest that the association with flavoenzymes and alteration in the synthesis of DNA of the parasite could be the reason of the activity for the compounds tested (Rivas *et al.*, 1999; Boursereau and Coldham, 2004).

The present data showed the effect of a series of beta-carboline-carboxamides derivatives against promastigote, axenic amastigote and intracellular amastigote forms of *L. amazonensis* and also morphological and ultrastructural alterations of the parasite. The compound *N*-benzyl-1-(4-methoxy)-phenyl-9H-beta-carboline-3-carboxamide (5) had a great activity on this protozoan with low toxicity and a high selective index that supports further *in vitro* studies of this compound to pursue a mechanism of action and *in vivo* studies to evaluate its activity in rodent models.

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