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PII: S0223-5234(18)30249-6

DOI: 10.1016/j.ejmech.2018.03.014

Reference: EJMECH 10279

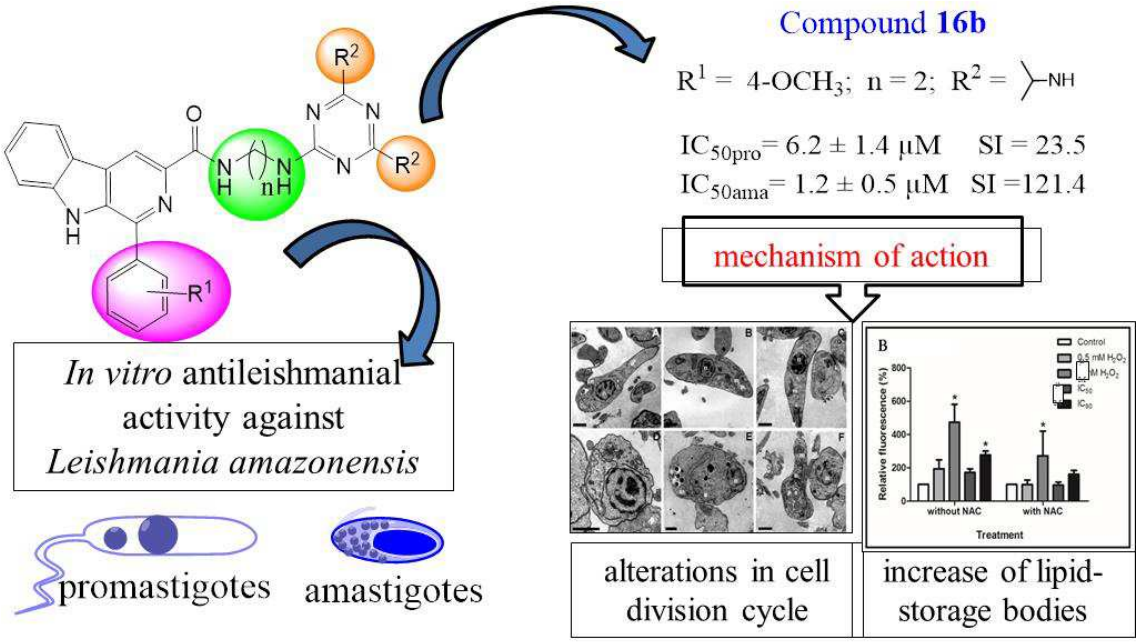
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 29 December 2017

Revised Date: 15 February 2018

Accepted Date: 2 March 2018

Please cite this article as: P. Baréa, Valé.Aquilino. Barbosa, D.L. Bidóia, Jé. Carreira de Paula, T.F. Stefanello, W. Ferreira da Costa, C.V. Nakamura, M.H. Sarragiotto, Synthesis, antileishmanial activity and mechanism of action studies of novel *β*-carboline-1,3,5-triazine hybrids, *European Journal of Medicinal Chemistry* (2018), doi: 10.1016/j.ejmech.2018.03.014.



Synthesis, antileishmanial activity and mechanism of action studies of novel *β*-carboline-1,3,5-triazine hybrids

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ABSTRACT

A series of novel hybrids *β*-carboline-1,3,5-triazine were synthesized and evaluated for their *in vitro* antileishmanial activity against promastigote and amastigote forms of *Leishmania amazonensis*. Among the compounds tested, the hybrids **9d**, **9e**, **16a** and **16b** showed potent activity against the promastigote forms with IC_{50} values less than 8 µM. Compounds **9e** and **16b** were also active against amastigote forms, displaying IC_{50} values of 1.0 ± 0.1 µM and 1.2 ± 0.5 µM, respectively. Besides that, the hybrid **16b** bearing the 4-methoxyphenyl group at C-1 of *β*-carboline and isopropylamino group at 1,3,5-triazine, showed low toxicity, being 23.5 and 121.4 times more toxic for promastigotes and axenic amastigotes, respectively, than for macrophage J774-A1 cell lines. Investigation of action mechanism in promastigotes showed that compound **16b** caused alterations in cell division cycle and an increase of lipid-storage bodies, leading the cells to death through various factors. The accumulation of lipid bodies may be associated with apoptotic cell death.

Keywords: *β*-carboline, 1,3,5-triazine, antileishmanial, *Leishmania amazonensis*, cell death mechanism

1. Introduction

Leishmaniasis is considered a neglected disease, and it is caused by protozoan parasites of the genus *Leishmania*. According to World Health Organization (WHO), leishmaniasis is endemic in 97 countries and territories, affecting mainly people in Africa, Asia and Latin American [1]. *Leishmania* infections are manifested in three clinical different forms: visceral (VL), cutaneous (CL) and mucocutaneous [1]. In South America, the species *L. amazonensis* is one of the etiological agents of cutaneous leishmaniasis, which may progress to diffuse cutaneous leishmaniasis in immunosuppressed individuals [2]. Current chemotherapies for leishmaniasis include treatment with pentavalent antimonials as first-line drugs, and amphotericin B, pentamidine, paromomycin, and miltefosine as second-line drugs [3]. However, the current drugs used clinically exhibit high toxicity, resistance, various side effects, and an increased incidence of treatment failure [2-5]. Beside this, no vaccines against *Leishmania* infections are available [6]. Therefore, there is a significant need to develop new more effective and less toxic antileishmanial agents.

In this context, several classes of compounds [7], including natural and synthetic *β*-carboline alkaloids [8-16], were described as potential antileishmanial agents.

In our previous work we have demonstrated that *β*-carbolines containing substituents at 1- and 3-positions of *β*-carboline nucleus have shown potent antileishmanial activity [17-22]. For instance, Tonin et al. [19] and Pedroso et al. [20] demonstrated the potentiality of *N*-alkyl-(1-phenylsubstituted-*β*-carboline)-3-carboxamides as antileishmanial agents. The compound *N*-butyl-1-(4-methoxy)-phenyl-*β*-carboline-3-carboxamide (**I, Fig. 1**), for example, showed IC_{50} of 0.25 μM against *L. amazonensis* promastigotes, displaying a selectivity index ratio (SI) 2,084 times higher for the parasite than for macrophages J774G8 cells. Further studies showed that this compound was also active against axenic and intracellular amastigote forms of *L. amazonensis*, exhibiting high selectivity for the parasite. Studies on mechanism of action demonstrated that compound **I** exerts its antileishmanial activity through a cytostatic effect, thus preventing cellular proliferation [21]. In the same away, the analog containing the *N*-benzyl instead *N*-butyl group was active against promastigote and axenic amastigote forms with IC_{50} of 2.6 and 1.0 μM, respectively, and killed *L. amazonensis* promastigotes through different cell death pathways, including apoptosis and autophagy [20, 22].

Additionally, compounds having a 1,3,5-triazine motif possess a broad range of biological activities, which makes this heterocycle an important scaffold in the search and development of new drugs [23]. The potentiality of 1,3,5-triazine derivatives as antileishmanial agents has been demonstrated in several studies [24-28]. Studies on structure-activity relationship have pointed that the presence of appropriated substituents at 2-, 4- and 6-positions of the 1,3,5-triazine nucleus can enhance the antileishmanial activity. For instance, the 1,2,4-triazino[5,6-b]indol-3-ylthio-1,3,5-triazines derivatives (**II**, **Fig. 1**) containing *N*-ethylpiperazine, isopropylamino and tertbutylamino substituents in 1,3,5-triazine nucleus showed more than 90% inhibition against promastigotes form of *L. donovani* while compounds with the *N*-methylpiperazine and morpholine substituents showed no inhibition or less than 50% of inhibition [27]. Also, the importance of the chain length between the triazine units of triazine mimetic **III** (**Fig. 1**) was demonstrated by Chauhan et al. [28]; the dimer with 5-aminopentan-1-ol linker was found to be inactive while the dimer with 4-aminobutan-1-ol linker exhibited potent antiamastigote activity for *L. donovani*. This compound also showed significant *in vivo* inhibition (74.41%) in *L. donovani/hambster* model, and prevent the progression of leishmania parasite. Besides this, it was demonstrated that the incorporation of 1,3,5-triazine ring, bearing different amino groups, into *β*-carboline nucleus led to compounds (**IV**, **Fig. 1**) with significant *in vivo* inhibition of *L. donovani* amastigotes [14].

The inefficacy and high toxicity of the drugs used to treat leishmaniasis infection, and the antileishmanial properties of both *β*-carboline and 1,3,5-triazine nucleus, motivated us to synthetize *β*-carboline-1,3,5-triazine hybrids (**V**, **Fig. 1**) in order to obtain new compounds with potent antileishmanial activity and low toxicity. The novel hybrids were evaluated against promastigote and amastigote forms of *L. amazonensis* and their cytotoxicity were determined. Also, studies on the antileishmanial mechanism of action were carried out for the most active derivative synthetized.

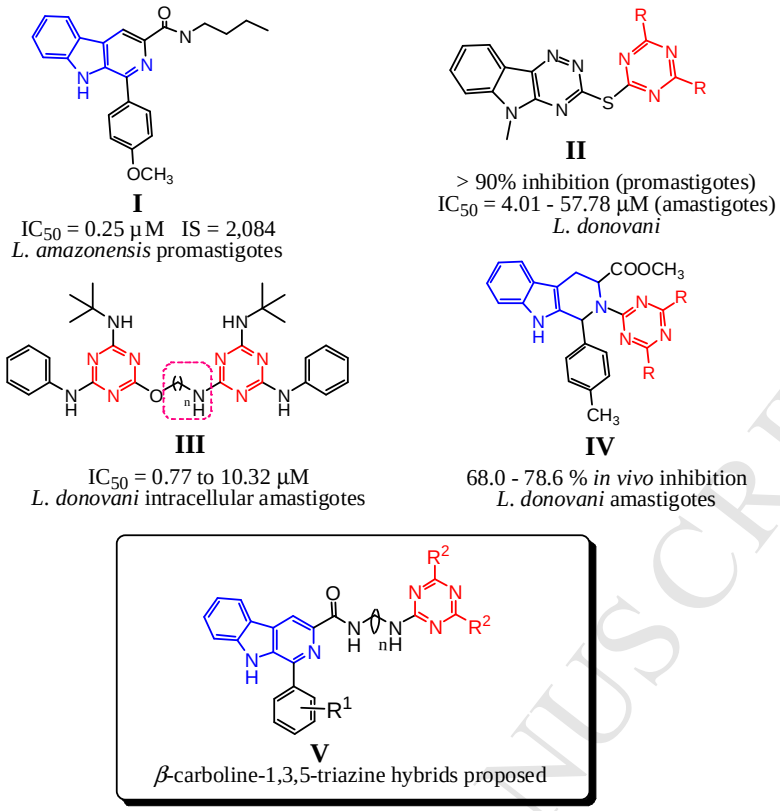


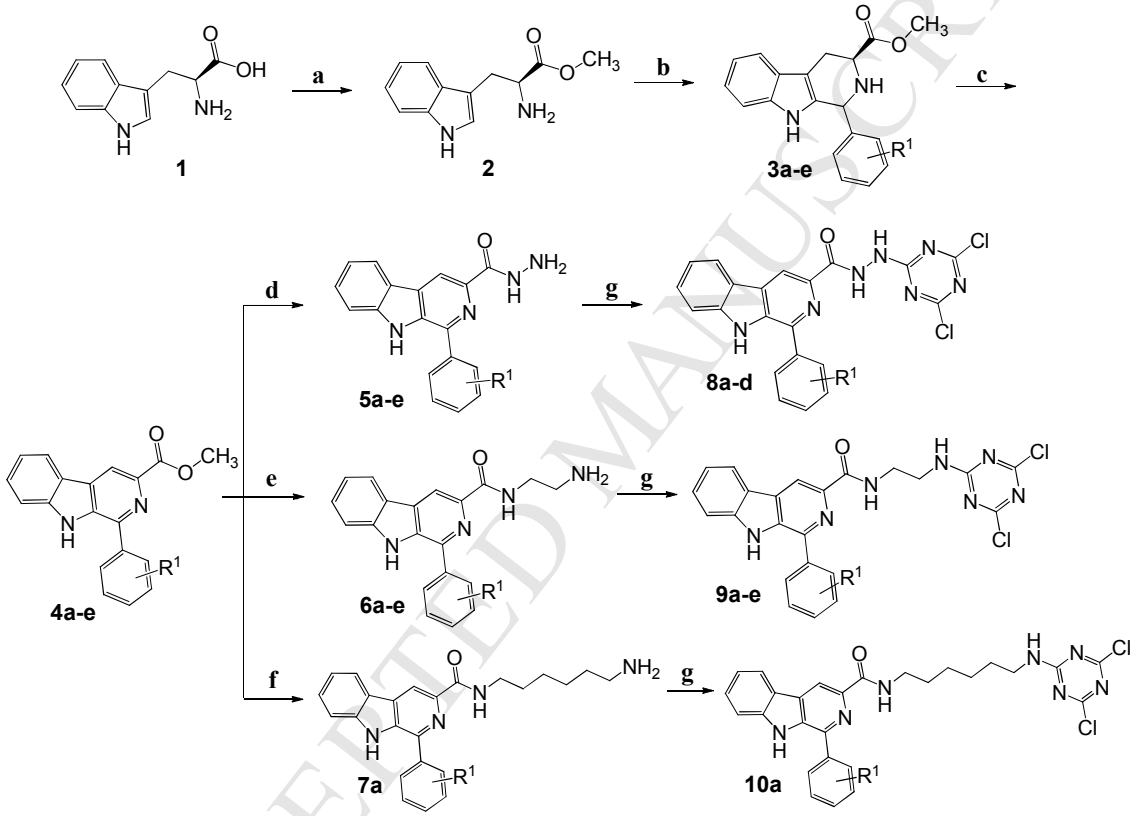
Figure 1: Structures of *β*-carboline and 1,3,5-triazine derivatives with antileishmanial activity and of *β*-carboline-1,3,5-triazine hybrids proposed.

2. Results and discussion

2.1. Chemistry

The novel designed *β*-carboline-1,3,5-triazine hybrids were synthesized by reaction of appropriate *β*-carboline intermediates with cyanuric chloride, a commercially useful reagent due the reactivity of its chlorine atoms toward nucleophiles [29], according the synthetic routes illustrated in **Schemes 1** and **2**. The hybrids **8a-d**, **9a-e** and **10a**, bearing a phenyl substituted group at 1-position of *β*-carboline nucleus and chlorine atoms at 6- and 4-positions of 1,3,5-triazine ring, were obtained from the intermediates **5a-d**, **6a-e** and **7a**, respectively (**Scheme 1**). These intermediates were prepared by a Pictet-Spengler condensation of the *L*-tryptophan methyl ester (**2**) with benzaldehyde (**a**), 4-methoxybenzaldeyde (**b**), 4-fluorobenzaldeyde (**c**), 2-chlorobenzaldeyde (**d**) and 3-nitrobenzaldehyde (**e**) followed by oxidation of the *cis*/*trans* mixture of **3a-e** in the presence of sulfur, in refluxing xylene, which afforded the derivatives **4a-e** [17-22]. Reaction of the methyl *β*-carboline-3-carboxylates (**4**) with hydrazine hydrate, ethylenediamine and hexamethylenediamine afforded the

corresponding *β*-carboline-3-carboxamides **5a-e**, **6a-e** and **7a**. Treatment of **5a-d, 6a-d** and **7a** with a suspension of cyanuric chloride in a mixture of water: acetonitrile 1:1, at 0 °C, followed by addition of sodium hydroxide in THF, gave the compounds **8a-d**, **9a-d** and **10a**, respectively, in good yields (45-98%). The hybrid **9e** was prepared from the reaction the intermediate **6e** with cyanuric chloride, at 0 ºC, using K_2CO_3 as basis and tetrahydrofuran as solvent. The intermediate **5e** was also submitted to reaction with cyanuric chloride, under the same conditions; however, in this case was observed the formation of a complex mixture of products.



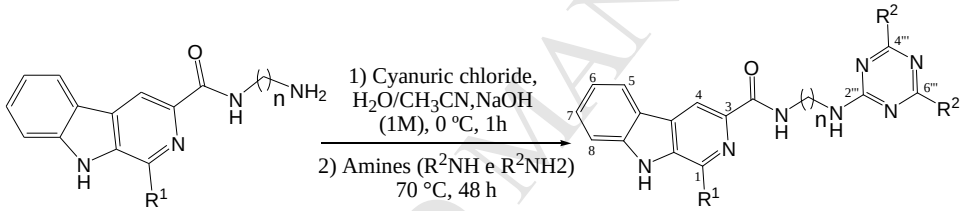
R^1 = **a)** H; **b)** 4-OCH_3; **c)** 4-F; **d)** 2-Cl; **e)** 3-NO_2;

Scheme 1: Synthesis of compounds **8a-d**, **9a-e** and **10a**. Reagents and conditions: (a) MeOH, H_2SO_4 (*cat.*), reflux, 48 h; (b) R^1Ph-CHO, CH_2Cl_2, TFA, rt, 48 h; (c) Sulfur, xylene, reflux, 48 h; (d) Hydrazine hydrate, EtOH, reflux, 48 h; (e) Ethylenediamine, rt, 24 h; (f) Hexamethylenediamine, MeOH, $CHCl_3$, reflux, 36 h; (g) i) Cyanuric chloride/H_2O: CH_3CN 1:1, NaOH (1 M)/THF, 0 ºC, 1 h for **8a-d** and **9a-d**; ii) Cyanuric chloride, K_2CO_3, THF, 0 ºC, 1 h for **9e**.

In order to evaluate the effect of other substituents than chlorine into 1,3,5-triazine portion on antileishmanial activity, we synthetized the *β*-carboline-1,3,5-triazine hybrids **11a-16a**, bearing different amino substituents in the 4- and 6-positions of 1,3,5-

triazine ring (**Scheme 2**). For this, the intermediate **6a** (n = 2) was subjected to reaction with cyanuric chloride, at 0 °C for 1 hour, using NaOH as basis and water-acetonitrile as solvent, followed by addition to the reaction mixture, without previous treatment, of 10 equivalents of an appropriate amine. Heating of the reaction mixture at 70 °C for 48 hours afforded the compounds **11a-16a** (**Scheme 2**).

The antileishmanial activity results for **11a-16a** (**Table 1**) showed that the hybrid **16a**, bearing the isopropylamino group at 1,3,5-triazine ring, was the only one active compound of this series. Therefore, compounds **16b-d** (n = 2) and **17a-d** (n = 0), containing the isopropylamino group at 1,3,5-triazine unity and the phenyl substituted group at position-1 of the *β*-carboline nucleus, were synthetized from the reaction of the respective intermediates **6b-d** and **5a-d** with cyanuric chloride in basic medium, followed by addition of 10 equivalents of isopropylamine (**Scheme 2**). The reaction of **6e** with cyanuric chloride in the presence of K_2CO_3 and excess isopropylamine afforded the compound **16e** in 40% yield.



5a-d (n = 0)
6a-e (n = 2)

11a-16a (n = 2); **16b-e** (n = 2)
17a-d (n = 0)

Comp.	R^1	R^2
11a	Ph	H_2NNH-
12a	Ph	
13a	Ph	
14a	Ph	
15a	Ph	

Comp.	R^1	R^2
16a; 17a	Ph	
16b; 17b	4-OCH_3-Ph	
16c; 17c	4-F-Ph	
16d; 17d	2-Cl-Ph	
16e[a]	3-NO_2-Ph	

[a]For **16e** was used K_2CO_3 as basis and THF as solvent

Scheme 2: Synthesis of compounds **11a-16a**, **16b-e** and **17a-d**.

All hybrids were characterized by their spectral data (HRMS, ^{1}H NMR and ^{13}C). The characterization of the *β*-carboline-1,3,5-triazine hybrids **8a-d**, **9a-e** and **10a** bearing chlorine at 6- and 4-positions of 1,3,5-triazine ring was mainly supported by the signals at δ_C 167.1 – 170.3 (C_0), characteristic of C-Cl bonds, and at δ_C 163.9 – 165.3 (C_0) related to C-2 of the1,3,5-triazine ring, in ^{13}C NMR spectra. The hybrids **11a-16a**, **16b-e** and **17a-d** were characterized by the signal of aminoalkyl substituents linked to

6- and 4-positions of 1,3,5-triazine. For example, the compounds containing the isopropylamino substituent (**16a-e** and **17a-d**) showed the presence of signals at δ_H 0.86 – 1.12 (12H, CH_3), δ_H 4.01 – 4.09 (2H, CH), in ^{1}H NMR spectra, and δ_C 22.5 – 22.7 (CH_3) and δ_C 40.1 – 41.6 (CH), in ^{13}C NMR spectra, related to methylene hydrogens and carbons, respectively.

2.2. Antileishmanial Activity

In this work, novel *β*-carboline-1,3,5-triazine hybrids synthesized (**8a-d**, **9a-e**, **10a-15a**, **16a-e** and **17a-d**) were evaluated *in vitro* against the promastigote and amastigote forms of *L. amazonensis*. The antileishmanial assay results are shown in **Table 1**. The compounds that showed IC_{50} (50% Inhibitory Concentration) values greater than 100 µM were considered inactive. The toxic effects on the host cells were determined by the selectivity index (SI). The SI for each active compound was calculated as the ratio between the cytotoxicity (CC_{50}) for the macrophage J774-A1 cell lines and IC_{50} against the promastigote and amastigote forms of *L. amazonensis*.

In order to evaluate the influence of the linkers between the *β*-carboline and 1,3,5-triazine units, firstly, the *β*-carboline-1,3,5-triazine hybrids **8a** (n=0), **9a** (n=2) and **10a** (n=6), bearing the phenyl group at 1-position of *β*-carboline nucleus and chlorine atoms at 4- and 6-positions of triazine ring, were assayed for their antileishmanial activity. As showed in the **Table 1**, compounds **8a** (n = 0) and **9a** (n = 2) were moderately active for *L. amazonensis* promastigotes, with C_{50} values of 43.3 µM and 30.9 µM, respectively, while the compound **10a** (n = 6) was inactive. The hybrids **8a** and **9a** showed also potent activity and high selectivity against amastigote forms of *L. amazonensis*, with IC_{50} values minor than 2 µM and SI of 85.9 and 70.8, respectively.

Aiming to increase the activity of compounds **8a** and **9a** against *L. amazonensis* promastigotes we introduced electron-donating and electron-withdrawing groups at the phenyl group in the 1-position of the *β*-carboline nucleus. The 4-methoxy, 2-chloro and 3-nitro substituents were chosen based on our previous studies, which demonstrated that these groups at 1-phenyl provided *β*-carboline derivatives with promising antileishmanial activities [19-21]. Besides that, a new withdrawing substituent (4-fluoro) was chosen in order to compare its effects on the antileishmanial activity with those of the other groups.

In this work, the introduction of 4-methoxy (**8b** and **9b**) and 4-fluoro (**8c** and **9c**) at 1-phenyl group led to a decrease or loss of activity against promastigote forms in

comparison to **8a** and **9a**. On the other hand, the presence of 2-chloro and 3-nitro substituents at 1-phenyl group in **9d** and **9e** resulted in a 4- and 6-fold increase, respectively, in the activity towards promastigotes. The hybrids **9b** and **9e**, as well as **9a** presented potent activity and high selectivity index for the amastigote forms.

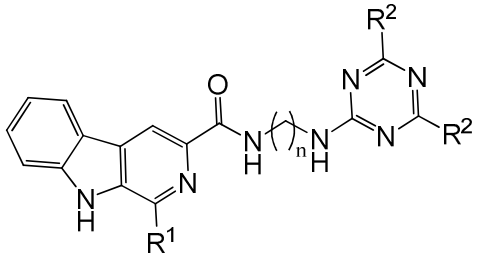
From the IC_{50} data for the *β*-carboline-1,3,5-triazine derivatives **11a-16a** we verified that only the compound **16a,** with the isopropylamino group in 1,3,5-triazine ring, was active, showing IC_{50} values of 7.5 ± 2.5 µM and 35.0 ± 0.5 µM, respectively, against promastigotes and amastigotes forms of *L. amazonensis*. Comparison of these results with those obtained for **9a** show that replacement of the chlorine atoms at C-4 and C-6 of 1,3,5-triazine ring by isopropylamino group increases about 4-fold the activity to the promastigotes form. On the other hand, the insertion of isopropylamino group in **16a** resulted in a decrease of activity for the amastigote forms by approximately 18 times in comparison with those of **9a**. The introduction of the others amino substituents in 1,3,5-triazine ring (**11a-15a**) also led to loss of activity for promastigote forms.

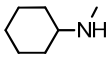
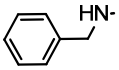
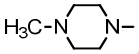
Finally, we evaluated the antileishmanial activity for the hybrids bearing the isopropylamino group at C-4 and C-6 of 1,3,5-triazine and the phenyl substituted groups at the *β*-carboline nucleus **16b-e** (n = 2) and **17a-d** (n=0).

Analysis of IC_{50} data for the series where n=2 showed that the insertion of the isopropylamino group led to a loss and decrease of the activity towards the promastigotes for compounds **16d** (IC_{50} > 100 µM) and **16e** (IC_{50}=44.6 ± 1.2 µM) compared to **9d** (IC_{50} = 7.6 ± 2.02 µM) and **9e** (IC_{50} = 5.1 ± 0.1 µM), respectively. On the other hand, the replacement of chlorine atoms of **9b** (IC_{50} = 67.5 ± 0.0 µM) by isopropylamino group in compound **16b** (IC_{50} = 6.2 ± 1.4 µM) increased the antileishmanial activity against to the promastigote forms by about 10 times. Compound **16b** was also active for amastigotes (IC_{50} = 1.2 ± 0.5 µM), with increase of the activity by about 30-fold when compared with the compound **16a** (IC_{50} = 35.0 ± 0.5 µM). Besides that, the hybrid **16b** showed a decreasing toxicity, being 23.5 and 121.4 times more toxic for promastigotes and axenic amastigotes of the parasites, respectively, than for the host cells (**Table 1**). The positive control miltefosine, a drug used for treatment of cutaneous leishmaniasis [30], showed IC_{50} of 18.5 and 2.4 µM, and SI of 2.2 and 16.9, for promastigotes and amastigotes, respectively (**Table 1**). Thus, **16b** becomes promising for other studies.

Compounds **17a-d** (n=0) were moderately active or inactive against *L. amazonensis* promastigotes, showing that the best linker between the *β*-carboline and 1,3,5-triazine moieties is those having the ethylene unity (n=2).

Table 1: Antileishmanial activity data for compounds **8a-d**, **9a-e**, **11a-15a**, **16a-e** and **17a-d** against *L. amazonensis*.



Comp.	n	R^1	R^2	Promastigotes IC_{50} (µM)	Amastigotes IC_{50} (µM)	J774A1 CC_{50} (µM)	IS PRO	IS AMA
8a	0	Ph	Cl	43.3 ± 10.3	**1.1 ± 0.1**	94.5 ± 7.8	2.2	85.9
8b	0	4-OCH_3-Ph	Cl	>100	n.t	n.t	n.d	n.d
8c	0	4-F-Ph	Cl	>100	n.t	n.t	n.d	n.d
8d	0	2-Cl-Ph	Cl	34.1 ± 10.6	n.t	22.0 ± 4.2	0.6	n.d
9a	2	Ph	Cl	30.9 ± 0.9	**1.9 ± 0.4**	134.6 ± 11.8	4.4	70.8
9b	2	4-OCH_3-Ph	Cl	67.5 ± 0.1	**1.0 ± 0.1**	201.3 ± 3.9	3.0	201.3
9c	2	4-F-Ph	Cl	>100	n.t	n.t	n.d	n.d
9d	2	2-Cl-Ph	Cl	**7.6 ± 2.02**	n.t	28.5 ± 3.6	3.8	n.d
9e	2	3-NO_2-Ph	Cl	**5.1 ± 0.1**	**1.1 ± 0.2**	83.1 ± 7.8	**16.3**	**75.5**
10a	6	Ph	Cl	>100	n.t	n.t	n.d	n.d
11a	2	Ph	-$NHNH_2$	>100	n.t	n.t	n.d	n.d
12a	2	Ph		>100	n.t	n.t	n.d	n.d
13a	2	Ph		>100	n.t	n.t	n.d	n.d
14a	2	Ph		>100	n.t	n.t	n.d	n.d
15a	2	Ph		>100	n.t	n.t	n.d	n.d
16a	2	Ph		**7.5 ± 2.5**	35.0 ± 0.5	98.1 ± 6.5	13.1	2.80
16b	2	4-OCH_3-Ph		**6.2 ± 1.4**	**1.2 ± 0.5**	145.7 ± 10.1	**23.5**	**121.4**
16c	2	4-F-Ph		>100	n.t	n.t	n.d	n.d
16d	2	2-Cl-Ph		>100	n.t	n.t	n.d	n.d
16e	2	3-NO_2-Ph		44.6 ± 1.2	n.t	86.3 ± 11.8	1.9	n.d
17a	0	Ph		39.7 ± 2.9	32.5 ± 3.4	102.7 ± 9.9	2.6	3.16
17b	0	4-OCH_3-Ph		>100	n.t	n.t	n.d	n.d
17c	0	4-F-Ph		43.4 ± 1.2	n .t	50.0 ± 14.1	1.2	n.d
17d	0	2-Cl-Ph		>100	n.t	n.t	n.d	n.d
Miltefosine[a]	-	-	-	18.5 ± 1.1	2.4 ± 0.1	40.5 ± 1.7	2.2	16.9

[a] positive control; n.t: not tested; n.d: not determined.

2.3.Transmission Electron Microscopy

As the compound **16b** showed potent activity against promastigotes and axenic amastigotes of *L. amazonensis*, and higher selectivity index than the other derivatives synthetized for both forms, it was submitted to studies for investigate their mechanism of protozoan cell death.

The treatment of *L. amazonensis* promastigotes with compound **16b** during 24 h caused ultrastructural alterations, mainly in mitochondria and plasma membrane. After 48 h of treatment, it was observed storage-lipic bodies, mitochondrial swelling and plasma membrane alterations. The treatment with 72 h showed alterations in flagellar pocket with vacuoles and mitochondrial swelling. These results initially demonstrated that in promastigotes the compound **16b** caused alterations in cell division cycle and, due to stress, it started a process of energy storage (storage-lipid bodies) [31], leading the cells to death thought various factors (**Figure 2**). For scanning electron microscopy (SEM) the treatment after 24, 48 and 72 h caused cell rounding and cell volume reduction (**Figure 3**).

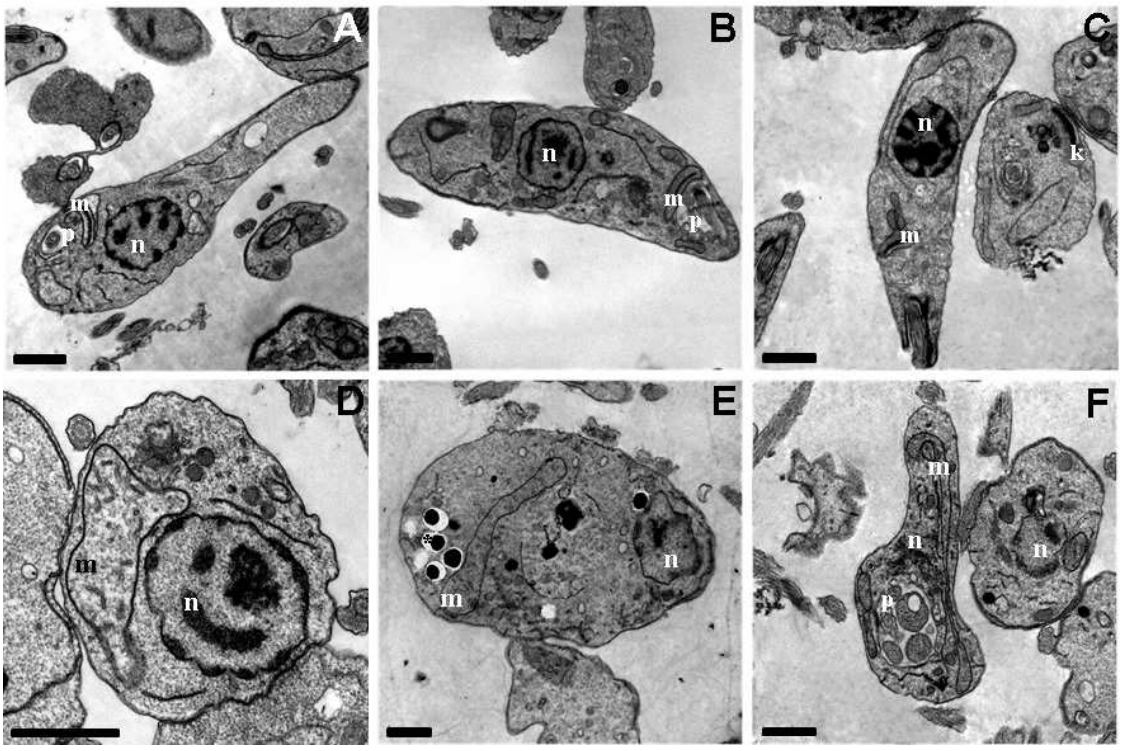


Figure 2. Ultrastructural alterations in promastigotes of *Leishmania amazonensis* treated with **16b**, visualized by transmission electron microscopy. Control cells (A-C) and parasites treated with IC_{50} (6.2 µM) (D-F) for 24 h (A; D), 48 h (B; E) and 72 h (C; F). (n) nucleus; (m) mitochondrion; paraflagellar pocket; (*) lipid-storage bodies. Bars = 1 µm.

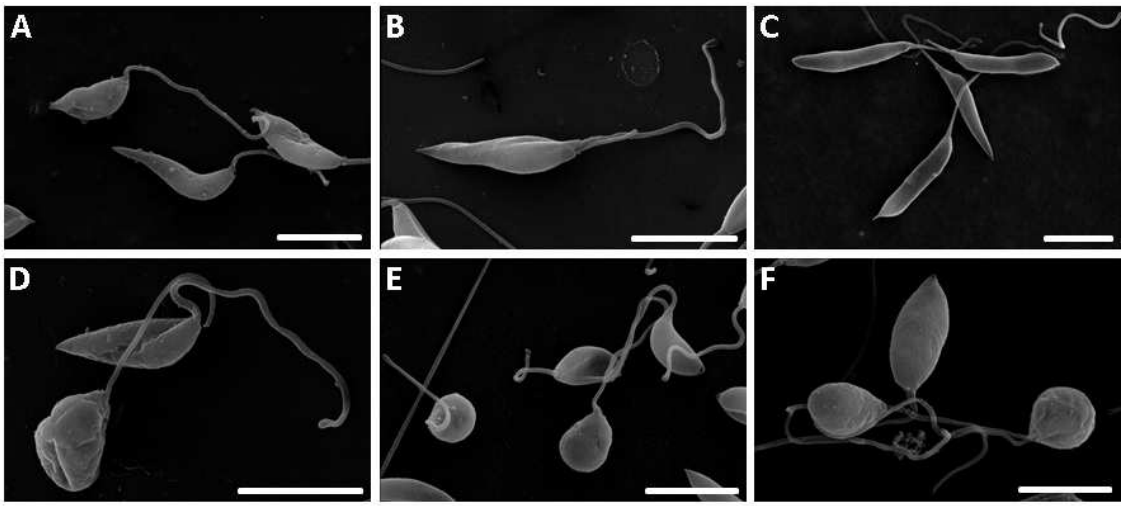


Figure 3. Morphological alterations in promastigotes of *Leishmania amazonensis* treated with **16b**, visualized by scanning electron microscopy. Control cells (A-C) and parasites treated with IC_{50} (6.2 µM) (D-F) for 24 h (A;D), 48 h (B; E) and 72 h (C;F). Bars = 5 µm.

2.4. Biochemical assays

Leishmania amazonensis promastigotes were treated with compound **16b** at the concentrations corresponding to IC_{50} and IC_{90}, for 24 h, and loaded with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), a probe that is permeable to the plasma membrane and chemically reduced in 2',7'-dichlorofluorescein (DCF) when in contact with reactive oxygen species (ROS), producing fluorescence. The parasites treated with positive control (0.5 and 4 µM of H_2O_2) showed a fluorescence increase of 45.9 and 230.8%, while treatment with **16b** with concentrations corresponding to IC_{50} and IC_{90} showed increase 81.9 and 184.8%. The parasites pre-incubated with *N*-acetyl-cysteine (NAC) and treatment with H_2O_2 (0.5 and 4 µM) showed reversible effects, but the treatment with **16b** not showed such effects (**Fig. 4A**).

Additionally, the evaluation of lipid bodies was performed by using the probe Nile red. The positive control (H_2O_2) at concentrations of 0.5 and 4 µM showed an increase in fluorescence of 19% and 241.2%, respectively. The treatment with **16b** at concentrations corresponding to IC_{50} and IC_{90} showed an increase in fluorescence of 9% and 428.6%, respectively, confirming the increase of lipid-storage bodies. However, this accumulates of lipid-storage bodies is not related with the ROS generation, initially observed. The accumulation of lipid bodies is caused for cellular stress, mitochondrial dysfunction and may be associated with apoptotic cell death [21, 32] (**Fig. 4B**).

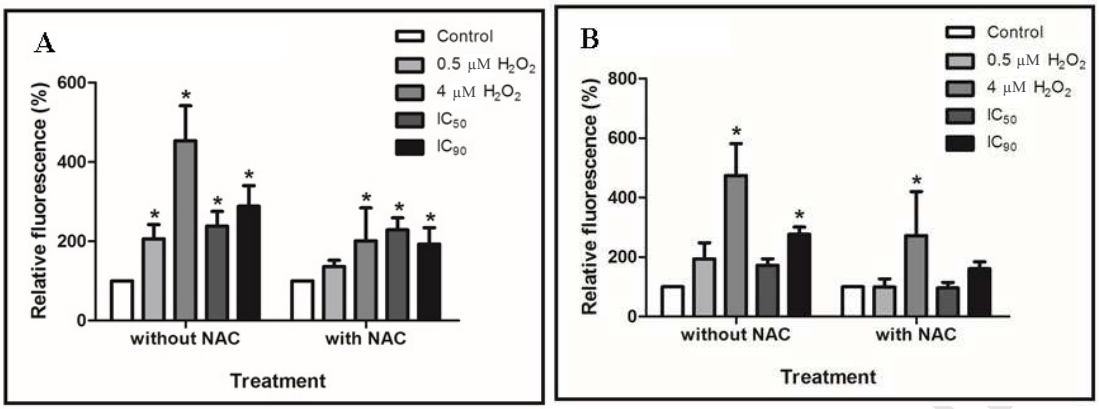


Figure 4. Total ROS production using the fluorescent probe H_2DCFDA (A) and Nile Red accumulation (B) in promastigotes of *Leishmania amazonensis* treated with **16b** for 24 h. IC_{50} (6.2 µM); IC_{90} (9.9 µM). The data are expressed as relative fluorescence of at least three independent experiments. H_2O_2 was used as a positive control. Two-way ANOVA followed by Bonferroni post hoc test. $^*p \leq 0.05$ compared to untreated parasites (control).

3. Conclusions

In this work, novel *β*-carboline-1,3,5-triazine hybrids containing different spacers between the *β*-carboline and 1,3,5-triazine units, chlorine and amino groups at 6- and 4-position of 1,3,5-triazine ring, and electron-donating and electron-withdrawing substituents at the 1-phenyl group of *β*-carboline nucleus have been synthesized and evaluated in promastigote and amastigote forms of *Leishmania amazonenis*. The assay results showed that the nature of substituents attached at *β*-carboline and 1,3,5-triazine moieties influenced the activity. The hybrid **9e**, containing the 3-nitrophenyl at C-1 of *β*-carboline was the most active among the compounds bearing chlorine atoms at 1,3,5-triazine ring, displaying potent activity for both promastigote and amastigote forms.

The presence of amino groups at 1,3,5-triazine nucleus led to loss of activity for almost all compounds, except for those bearing the isopropylamino group (**16a, 16b, 16e**, **17a** and **17c**). Compound 1**6b** containing the 4-methoxyphenyl at C-1 of *β*-carboline nucleus showed potent activity and low toxicity to amastigote and promastigote forms of *L. amazonenis*, being a promising candidate for antileishmanial agent.

Studies on mechanism of action showed that in promastigotes the compound **16b** caused alterations in cell division cycle and an increase of lipid-storage bodies, leading the cells to death thought various factors. This accumulation of lipid bodies is caused of cellular stress, mitochondrial dysfunction and may be associated with apoptotic cell death.

In summary, from our results it was possible to obtain new hybrids *β*-carboline-1,3,5-triazine with remarkable antileishmanial activity, which can be considered as potential compounds for future studies in view to development of new antileishmanial agents.

4. Experimental section

4.1.General methods

All reagents were purchased from commercial suppliers. The reactions were monitored by thin layer chromatography conducted on Whatman TLC plates (Silica Gel 60 F_{254}). NMR spectra were recorded in a in Varian spectrometer model Mercury plus BB at 300 MHz (for ^{1}H) and 75 MHz (for ^{13}C), and in a Bruker spectrometer model Avance III HD at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C), with TMS and deuterated solvents, dimethyl sulfoxide (DMSO-d_6) and chloroform ($CDCl_3$), as internal standard. Mass spectra (ESI/MS) were recorded on Thermoelectron Corporation Focus-DSQ II spectrometer. Melting points were determined in Microquímica apparatus model MQAPF-301 and are uncorrected.

The following chemicals were used in biological assay: Warren medium; fetal bovine serum (FBS); carbonylcyanide *m*-chlorophenylhydrazone (CCCP), dimethylsulfoxide (DMSO), Roswell Park Memorial Institute Medium (RPMI 1640; Gibco 99 Invitrogen, Grand Island, NY, USA), 3-[4,5-dimethylthiazol-2-yl]-2,5-100 diphenyltetrazolium bromide (MTT), potassium sodium buffer; Nile red (Sigma); 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA-ThermoFisher); sodium cacodilate (Electron Microscopy Scanning - EMS); glutaraldehyde 25% (EMS); potassium ferrocyanide; osmium tetroxide; EPON resin; acetone P.A.; uranyl acetate; lead citrate.

4.2.Chemistry

*4.2.1. Synthesis of L-tryptophan methyl ester (**2**)*

To a suspension of commercial *L*-tryptophan (**1**) (5.00 g, 24.5 mmol) in methanol (50 mL) was added dropwise concentrated sulfuric acid until complete solubilization. The reaction mixture was kept under reflux for 48 hours. The solution was cooled and neutralized with a solution of 5% sodium carbonate, and extracted with ethyl acetate (4 x 30 mL). The organic phase was dried with anhydrous sodium sulfate and after filtration the solvent was removed in a rotary evaporator. The pure product was obtained in 91% yield.

*4.2.2. Synthesis of cis e trans 1-(substituted-phenyl)-3-carbomethoxy-1,2,3,4-tetrahydro-β-carboline (**3a-e**)*

To a solution of *L*-tryptophan methyl ester (**2**) (4.6 mmol) in dichloromethane (20 mL) was added 4.6 mmol of aldehyde [benzaldehyde (**a**), 4-methoxy-benzaldehyde (**b**), 4-fluoro-benzaldeyde (**c**), 2-chloro-benzaldeyde (**d**) and 3-nitro-benzaldehyde (**e**)] and trifluoroacetic acid (9.20 mmol). The reaction mixture was kept at room temperature and its progress monitored by TLC. After complete consumption of starting materials (48 h), the solvent was evaporated, and the crude product solubilized in ethyl acetate and neutralized with a solution of 5% sodium carbonate. The solution was extracted with ethyl acetate (3 x 30 mL). The organic phase was dried with anhydrous sodium sulfate, filtered and the solvent was removed in a rotary evaporator. The solid obtained was washed with methanol, providing a mixture of *cis* and *trans* 1-(substituted-phenyl)-3-carbomethoxy-tetrahydro-*β*-carboline (**3a-e**) in yields in the range 75-85%.

*4.2.3. Synthesis of methyl 1-(substituted-phenyl)-β-carboline-3-carboxylates (**4a-e**)*

To the mixture of *cis* and *trans* 1-(substituted-phenyl)-3-carbomethoxy-1,2,3,4-tetrahydro-*β*-carbolines **3a-e** (6.53 mmol) suspended in xylene (30 mL) was added 20 mmol of sulfur. The mixture was refluxed and the reaction progress monitored by TLC every 12 h. After complete consumption of starting materials (48 h), the solution was cooled and left on ice bath at 0 °C for approximately 1 h. The precipitate formed was filtered and washed with petroleum ether to afford the *β*-carbolines **4a-e** in yields in the range of 85 - 93%.

*4.2.4. Synthesis of 1-(substituted-phenyl)-β-carboline-3-carbohydrazides (**5a-e**)*

To a solution of compounds **4a-e** (3.31 mmol) in ethanol (30 mL) was added hydrazine hydrate 50% (66 mmol). The solution was kept under reflux and the reaction progress monitored by TLC every 12 h. After complete consumption of starting materials (48 h), the solution was cooled and left on ice bath at 0 °C for 1 hour. The precipitate formed was filtered in vacuum and washed with ethanol to afford the compounds **5a-e** in yields in the range of 53-85%.

*4.2.5. Synthesis of of N-(2-aminoethyl)-1-(substituted-phenyl)-β-carboline-3-carboxamides (**6a-e**)*

The compounds **4a-e** (1 mmol) were solubilized in 1 mL of ethylenediamine and the resulting solution was stirred at room temperature. The reaction progress was monitored by TLC every 8 h, and after complete consumption of starting materials (24 h), the solution was treated with 2 mL of distilled water and kept at 0 °C for approximately 1 hour. The precipitates formed were filtered in vacuum and washed with distilled water to furnish the compounds **6a-e** in yields in the range of 60-90%.

*4.2.6. Synthesis of N-(6-aminohexyl)-1-phenyl-β-carboline-3-carboxamide (**7a**)*

To a solution of hexamethylenediamine (0.70 g) in chloroform (2 mL), under reflux, was added a solution of **4a** (0.3 mmol in 0.5 mL of methanol). The solution was kept under reflux, and the reaction progress monitored by TLC every 12 h. After complete consumption of starting materials (36 h), the solution was cooled and treated with 2 mL of distilled water and the precipitate formed was filtered and washed with a mixture of water-ethanol (1:1). The compound **7a** was obtained in 70% yield.

*4.2.7. Procedure for the preparation of β-carboline-4,6-dichloro-1,3,5-triazine derivatives (**8a-d**, **9a-d** and **10a**)*

To a suspension of cyanuric chloride (0.2 mmol) in water-acetonitrile 1:1 (0.5 mL), at 0 °C, was added a suspension of **5a-d** (0.2 mmol), **6a-d** (0.2 mmol) or **7a** (0.2 mmol) in THF (3 mL). The pH was adjusted to 9-10 with sodium hydroxide 1 mol L^{-1}. The reaction mixture was stirred at 0 ºC for 1 hour and treated with distilled water (1 mL). The precipitates formed were filtered and washed with distilled water to afford compounds **8a-d**, **9a-d** or **10a** in yields in the range of 45-98%.

4.2.7.1. *N'-(4,6-dichloro-1,3,5-triazin-2-yl)-1-phenyl-β-carboline-3-carbohydrazide (**8a**):* Yellow crystals; Yield: 50%; M.p. > 350 °C (decomp.); ^{1}H NMR (300 MHz): δ ppm: 7.33 (t, J = 7.3, 1H, H-6), 7.56-7.74 (m, 5H, H-7, H-8, H-3', H-4', H-5'), 8.25 (d, J = 7.2, 2H, H-2', H-6'), 8.44 (d, J = 7.8, 1H, H-5), 8.87 (s, 1 H, H-4), 10.98 (s, 1H, CO-NH), 11.98 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 112.8 (CH, C-8), 114.1 (CH, C-4), 120.4 (CH, C-6), 121.2 (C_0, C-4b), 122.1 (CH, C-5), 128.8 (3CH, C-2', C-4', C-6'), 129.0 (CH, C-3', C-5'), 129.1 (CH, C-7), 129.8 (C_0, C-4a), 134.5 (C_0, C-9a), 137.2 (C_0, C-1'), 138.8 (C_0, C-3), 141.0 (C_0, C-1), 141.6 (C_0, C-8a), 163.9 (C_0, C-2'"), 164.5 (C=O), 169.1 and 170.3 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{21}H_{14}Cl_2N_7O$ $[M+H]^+$ 450.0631, found: 450.0599, error 7.1 ppm..

4.2.7.2. *N'-(4,6-dichloro-1,3,5-triazin-2-yl)-1-(4-methoxyphenyl)-β-carboline-3-carbohydrazide* (**8b**). Yellow crystals; Yield: 98%; M.p. > 350 °C (decomp.); ^{1}H NMR (300 MHz): δ ppm: 3.90 (s, 3H, OC<u>H</u>$_3$), 7.20 (d, J = 8.9, 2H, H-3', H-5'), 7.32 (t, J = 7.5, 1H, H-6), 7.61 (t, J = 7.6, 1H, H-7), 7.72 (d, J = 8.2, 1H, H-8), 8.23 (d, J = 8.9, 2H, H-2', H-6'), 8.43 (d, J = 7.8, 1H, H-5), 8.83 (s, 1 H, H-4), 10.89 (s, 1H, CO-<u>NH</u>), 11.94 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 55.4 (O<u>C</u>H$_3$), 112.8 (CH, C-8), 113.3 (CH, C-4), 114.1 (2CH, C-3', C-5'), 120.3 (CH, C-6), 121.2 (C$_0$, C-4b), 122.0 (CH, C-5), 130.4 (2CH, C-2', C-6'), 129.6 (CH, C-7), 129.5 (C$_0$, C-4a), 128.7 (C$_0$, C-9a), 134.0 (C$_0$, C-1'), 138.5 (C$_0$, C-3), 140.5 (C$_0$, C-1), 141.6 (C$_0$, C-8a), 160.0 (C$_0$, C-4'), 164.5 (C$_0$, C-2'''), 164.5 (C=O), 166.1 and 167.1 (C$_0$, C-4''', C-6'''); HRMS-ESI calcd for $C_{22}H_{16}Cl_2N_7O_2$ $[M+H]^+$ 480.0737, found: 480.0731, error 1.2 ppm.

4.2.7.3. *N'-(4,6-dichloro-1,3,5-triazin-2-yl)-1-(4-fluorophenyl)-β-carboline-3-carbohydrazide* (**8c**). Yellow crystals; Yield: 47%; M.p. > 298 °C (decomp.); ^{1}H NMR (300 MHz): δ ppm: 7.32 (td, J = 7.5, 1.1, 1H, H-6), 7.47 (t, J = 8.9, 2H, H-3', H-5'), 7.61 (td, J = 7.6, 1.1, 1H, H-7), 7.71 (d, J = 8.2, 1H, H-8), 8.28 (dd, J = 8.9, 5.5, 2H, H-2', H-6'), 8.42 (d, J = 7.8, 1H, H-5), 8.79 (s, 1 H, H-4), 11.53 (s, 1H, CO-<u>NH</u>), 11.94 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 112.7 (CH, C-8), 112.7 (CH, C-4), 115.5 and 115.8 (2 CH, C-3', C-5'), 120.3 (CH, C-6), 121.2 (C$_0$, C-4b), 122.1 (CH, C-5), 128.7 (CH, C-7), 130.3 (C$_0$, C-4a), 130.7 and 130.8 (2 CH, C-2', C-6'), 130.9 (C$_0$, C-9a), 133.8 (C$_0$, C-1'), 133.8 (C$_0$, C-3), 141.6 (C$_0$, C-1), 141.6 (C$_0$, C-8a), 161.1 (C$_0$, C-4'), 164.3 (C$_0$, C-2'''), 164.3 (C=O); HRMS-ESI calcd for $C_{21}H_{13}Cl_2FN_7O$ $[M+H]^+$ 468.0537, found: 468.0534, error 0.6 ppm.

4.2.7.4. *N'-(4,6-dichloro-1,3,5-triazin-2-yl)-1-(2-chlorophenyl)-β-carboline-3-carbohydrazide* (**8d**). Yellow crystals; Yield: 45%; M.p. > 345 °C (decomp.); ^{1}H NMR (300 MHz): δ ppm: 7.31 (m, 1H, H-6), 7.73 (m, 2H, H-7, H-8), 7.60 (m, 4H, H-3', H-4', H-5', H-6'), 8.43 (d, J = 8.0, 1H, H-5), 8.88 (s, 1 H, H-4), 11.04 (s, 1H, CO-<u>NH</u>), 11.71 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 112.4 (CH, C-8), 114.6 (CH, C-4), 120.2 (CH, C-6), 121.0 (C$_0$, C-4b), 122.3 (CH, C-5), 127.5 (CH, C-7), 128.8 (CH, C-5'), 129.8 (C$_0$, C-4a), 130.7 (C$_0$, C-2'), 132.2 (CH, C-3'), 132.3 (CH, C-6'), 132.6 (C$_0$, C-4'), 135.4 (C$_0$, C-1'), 136.3 (C$_0$, C-3), 136.4 (C$_0$, C-9a), 140.1 (C$_0$, C-1), 141.4 (C$_0$, C-8a); HRMS-ESI calcd for $C_{21}H_{13}Cl_3N_7O$ $[M+H]^+$ 484.0242, found: 484.0214, error 5.8 ppm.

4.2.7.5. *N-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide* (**9a**): White crystals; Yield: 75%; M.p: 146-150 °C; ^{1}H NMR (500 MHz): δ ppm: 3.54 (m, 2H, H-2"), 3.60 (m, 2H, H-1"), 7.32 (ddd, J = 7.9, 7.0, 0.9, 1H, H-6), 7.56-7.70 (m, 5H, H-7, H-8, H-3', H-4', H-5'), 8.13 (dd, J = 8.2, 1.2, 2H, H-2', H-6'), 8.42 (d, J = 8.1, 1H, H-5), 8.83 (s, 1 H, H-4), 8.87 (t, J = 6.2, CONH), 9.25 (t, J = 5.6, 1H, NH-C2"), 11.84 (s, 1H, NH, H-9). ^{13}C NMR (125 MHz): δ ppm: 37.8 (CH_2, C-1"), 40.9 (CH_2, C-2"), 112.7 (CH, C-8), 113.1 (CH, C-4), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.0 (CH, C-5), 128.6 (CH, C-4'), 128.7 (2CH, C-2', C-6'), 128.8 (CH, C-3', C-5'), 128.9 (CH, C-7), 129.8 (C_0, C-4a), 134.2 (C_0, C-9a), 137.5 (C_0, C-1'), 139.7 (C_0, C-3), 140.6 (C_0, C-1), 141.6 (C_0, C-8a), 165.3 (C_0, C-2'"), 165.5 (C=O), 168.4 and 169.4 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{23}H_{18}Cl_2N_7O$ $[M+H]^+$ 478.0944, found: 478.0946, error 0.4 ppm.

4.2.7.6. *N-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}-1-(4-methoxyphenyl)-β-carboline-3-carboxamide (**9b**).* White crystals; Yield: 87%; M.p: 194-196 °C; ^{1}H NMR (500 MHz): δ ppm: 3.54 (m, 2H, H-2"), 3.60 (m, 2H, H-1"), 3.89 (s, 3H, OCH_3), 7.19 (d, J = 8.9, 2H, H-3', H-5'), 7.30 (t, J = 7.9, 1H, H-6), 7.59 (ddd, J = 8.2, 7.1, 1.1, 1H, H-7), 7.69 (d, J = 8.2, 1H, H-8), 8.10 (d, J = 8.9, 2H, H-2', H-6'), 8.39 (d, J = 7.9, 1H, H-5), 8.77 (s, 1 H, H-4), 8.86 (t, J = 6.1, CONH), 9.26 (t, J = 5.6, 1H, NH-C2"), 11.79 (s, 1H, NH, H-9). ^{13}C NMR (125 MHz): δ ppm: 37.8 (CH_2, C-1"), 40.9 (CH_2, C-2"), 55.4 (OCH3), 112.5 (CH, C-8), 112.7 (CH, C-4), 114.2 (2CH, H-3', H-5'), 120.1 (CH, C-6), 121.3 (C_0, C-4b), 122.0 (CH, C-5), 129.6 (C_0, C-4a), 128.5 (CH, C-7), 129.9 (C_0, C-9a), 130. 1 (2CH, C-2', C-6'), 134.0 (C_0, C-1'), 139.5 (C_0, C-3), 140.5 (C_0, C-1), 141.5 (C_0, C-8a), 159.9 (C_0, C-4'), 165.3 (C_0, C-2'"), 165.5 (C=O), 168.4 and 169.4 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{24}H_{20}Cl_2N_7O_2$ $[M+H]^+$ 508.1050, found: 508.1047, error 0.6 ppm.

4.2.7.7. *N-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}-1-(4-fluorophenyl)-β-carboline-3-carboxamide (**9c**).* White crystals; Yield: 65%; M.p: 153-157 °C; ^{1}H NMR (300 MHz): δ ppm: 3.54 (m, 2H, H-2"), 3.60 (m, 2H, H-1"), 3.89 (s, 3H, OCH_3), 7.19 (d, J = 8.9, 2H, H-3', H-5'), 7.30 (t, J = 7.9, 1H, H-6), 7.59 (ddd, J = 8.2, 7.1, 1.1, 1H, H-7), 7.69 (d, J = 8.2, 1H, H-8), 8.10 (d, J = 8.9, 2H, H-2', H-6'), 8.39 (d, J = 7.9, 1H, H-5), 8.77 (s, 1 H, H-4), 8.86 (t, J = 6.1, CONH), 9.26 (t, J = 5.6, 1H, NH-C2"), 11.79 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 37.8 (CH_2, C-1"), 40.9 (CH_2, C-2"), 112.7 (CH, C-8), 113.2 (CH, C-4), 115.5 and 115.8 (2CH, H-3', H-5'), 120.3 (CH,

C-6), 121.2 (C_0, C-4b), 122.1 (CH, C-5), 128.7 (C_0, C-4a), 130.0 (CH, C-7), 131.0 and 131.1 (2 CH, C-2', C-6'), 131.1 (C_0, C-9a), 133.8 (C_0, C-1'), 134.1 (C_0, C-3), 139.6 (C_0, C-1), 141.6 (C_0, C-8a), 165.2 (C_0, C-4'), 165.2 (C_0, C-2'"), 165.5 (C=O), 168.4 and 169.4 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{23}H_{17}Cl_2FN_7O$ $[M+H]^+$ 496.0850, found: 496.0849, error 0.2 ppm.

4.2.7.8. *N-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}-1-(2-chlorophenyl)-β-carboline-3-carboxamide (**9d**).* White crystals; Yield: 55%; M.p: 160-164 °C; ^{1}H NMR (300 MHz): δ ppm: 3.54 (m, 2H, H-2"), 3.60 (m, 2H, H-1"), 7.31 (dt, J = 7.9, 4.1, 4.1, 1H, H-6), 7.55-7.72 (m, 6H, H-7, H-8, H-3', H-4', H-5', H-6'), 8.43 (d, J = 7.9, 1H, H-5), 8.73 (t, J = 5.8, CONH), 8.91 (s, 1 H, H-4), 9.21 (t, J = 5.2, 1H, NH-C2"), 11.67 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 37.7 (CH_2, C-1"), 40.9 (CH_2, C-2"), 112.4 (CH, C-8), 113.8 (CH, C-4), 120.1 (CH, C-6), 121.0 (C_0, C-4b), 122.2 (CH, C-5), 127.5 (CH, C-7), 128.7 (CH, C-5'), 128.9 (C_0, C-4a), 129.8 (C_0, C-2'), 130.6 (CH, C-3'), 132.1 (CH, C-6'), 132.7 (C_0, C-4'), 135.2 (C_0, C-1'), 136.4 (C_0, C-3), 139.2 (C_0, C-9a), 139.7 (C_0, C-1), 141.5 (C_0, C-8a), 165.1 (C_0, C-2'"), 165.5 (C=O), 168.3 and 169.3 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{23}H_{17}Cl_3N_7O$ $[M+H]^+$ 512.0555, found: 512.0555, error 0 ppm.

4.2.7.9. *N-{6-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]hexyl}-1-phenyl-β-carboline-3-carboxamide* (**10a**). White crystals; Yield: 74%; M.p: 101-102 °C; ^{1}H NMR (300 MHz): δ ppm: 1.36 (m, 4H, H-3", H-4"), 1.51-1.62 (m, 4H, H-2", H-5"), 3.24-3.29 (m, 2H, H-6"), 3.38-4.42 (m, 2H, H-1"), 7.32 (t, J = 7.1, 1H, H-6), 7.55-7.71 (m, 5H, H-7, H-8, H-3', H-4', H-5'), 8.16 (d, J = 7.0, 2H, H-2', H-6'), 8.41 (d, J = 7.9, 1H, H-5), 8.81 (s, 1H, H-4), 8.68 (t, J = 6.2, CONH), 9.14 (t, J = 5.5, 1H, NH-C2"). ^{13}C NMR (75 MHz): δ ppm: 25.9 (CH_2, C-4"), 26.1 (CH_2, C-3"), 28.1 (CH_2, C-5"), 29.4 (CH_2, C-2"), 38.8 (CH_2, C-1"), 40.7 (CH_2, C-6"), 112.7 (CH, C-8), 112.9 (CH, C-4), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.0 (CH, C-5), 128.6 (CH, C-4'), 128.7 (2CH, C-2', C-6'), 128.8 (CH, C-3', C-5'), 128.9 (CH, C-7), 129.9 (C_0, C-4a), 134.1 (C_0, C-9a), 137.5 (C_0, C-1'), 140.0 (C_0, C-3), 140.5 (C_0, C-1), 141.6 (C_0, C-8a), 164.7 (C_0, C-2'"), 165.1 (C=O), 168.4 and 169.4 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{27}H_{26}Cl_2N_7O$ $[M+H]^+$ 534.1570, found: 534.1565, error 0.9 ppm.

*4.2.8. Procedure for the preparation of β-carboline-4,6-dichloro-1,3,5-triazine derivative (**9e**)*

A suspension of cyanuric chloride (0.2 mmol) and potassium carbonate (0.2 mmol) in anhydrous THF (1 mL) was stirred for 10 minutes at 0 °C, followed by the addition of **6c** (0.2 mmol) suspended in anhydrous THF (2 mL). The reaction mixture was stirred at 0 ºC for 1 hour, treated with 1 mL of distilled water and maintained in ice bath for 1 hour. The precipitate formed was filtered and washed with distilled water providing **9e** in 93 % yield.

4.2.8.1. *N-{2-[(4,6-dichloro-1,3,5-triazin-2-yl)amino]ethyl}-1-(3-nitrophenyl)-β-carboline-3-carboxamide (**9e**).* Yellow crystals; Yield: 93%; M.p: 316-318 °C; ^{1}H NMR (300 MHz): δ ppm: 3.56 (m, 2H, H-2”), 3.63 (m, 2H, H-1”), 7.34 (td, J = 7.4; 1.2, 1H, H-6), 7.60-7.71 (m, 2H, H-7, H-8), 7.94 (t, J = 8.0, 1H, H-5’), 8.39-8.47 (m, 2H, H-5, H-4’), 8.56 (d, J = 7.8, 1H, H-6’), 8.83 (m, 1H, H-2’), 8.91 (s, 1 H, H-4), 8.94 (m, 1H, NH-C2”), 9.25 (t, J = 5.2, 1H, CO-NH), 12.06 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 37.9 (CH_2, C-1"), 40.8 (CH_2, C-2"), 112.6 (CH, C-8), 114.1 (CH, C-4), 120.5 (CH, C-6), 121.2 (C_0, C-4b), 122.3 (CH, C-5), 123.5 (2CH, C-2’, C-4’), 130.4 (C_0, C-4a), 129.0 (CH, C-7), 135.4 (C_0, C-9a), 130.4 (CH, C-5’), 138.2 (C_0, C-1’), 134.5 (CH, C-6’), 138.9 (C_0, C-3), 140.0 (C_0, C-1), 141.7 (C_0, C-8a), 148.3 (C_0, H-3’), 165.1 (C_0, C-2'"), 165.6 (C=O), 168.4 and 169.4 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{23}H_{17}Cl_2N_8O_3$ $[M+H]^+$ 523.0795, found: 523.0791, error 0.8 ppm.

*4.2.9. Procedure general for preparation of (β-carboline)-1,3,5-triazine derivatives **11a-15a**, **16a-d** and **17a-d.***

To a suspension of cyanuric chloride (0.2 mmol) in water-acetonitrile 1:1 (0.5 mL), at 0 °C, was added **5a-d** (0.2 mmol) or **6a-d** (0.2 mmol) suspended in THF (3 mL). The pH was adjusted to 9-10 with sodium hydroxide 1 mol L^{-1}. The reaction mixture was stirred at 0 ºC, for 1 hour followed by addition of 10 equivalents of the appropriate amine. The mixture was kept at 70 °C, and the reaction progress monitored by TLC every 12 h. After complete consumption of starting materials (48 h), the solution was cooled and treated with 2 mL of distilled water. The precipitates formed were filtered, washed with distilled water and recrystallized with ethanol to afford compounds **11a-15a**, **16a-d** and **17a-d** in yields in range of 55-94%.

4.2.9.1. *N-{2-[(4,6-dihydrazinyl-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide (**11a**).* Yellow crystals; Yield: 86%; M.p: 186-189 °C; ^{1}H NMR (500 MHz): δ ppm: 3.42-3.57 (m, 4H, H-1”, H-2”), 4.12 (s*l*, 2H, NH_2), 6.88 (s*l*,

1H, NH-C2”), 7.31 (t, J = 7.3, 1H, H-6), 7.58-7.70 (m, 5H, H-7, H-8, H-3’, H-4’, H-5’), 8.12 (d, J = 7.3, 2H, H-2’, H-6’), 8.41 (d, J = 7.8, 1H, H-5), 8.83 (s, 1 H, H-4), 8.80 (d, J = 5.3, CONH), 11.83 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 38.9 (CH_2, C-1''), 40.4 (CH_2, C-2''), 112.7 (CH, C-8), 113.0 (CH, C-4), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.1 (CH, C-5), 128.6 (CH, C-4’), 128.7 (2CH, C-2’, C-6’), 128.8 (CH, C-3’, C-5’), 129.0 (CH, C-7), 129.9 (C_0, C-4a), 134.2 (C_0, C-9a), 137.5 (C_0, C-1’), 139.8 (C_0, C-3), 140.6 (C_0, C-1), 141.6 (C_0, C-8a), 165.1 ($3C_0$, C-2”’, C-4''', C-6'''), 167.6 (C=O); HRMS-ESI calcd for $C_{23}H_{24}N_{11}O$ $[M+H]^+$ 470.2160, found: 470.2160, error 0 ppm.

4.2.9.2. *N-{2[(4,6-bis(cyclohexylamino)-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide (**12a**)*. White crystals; Yield: 74%; M.p: 201-202 °C; ^{1}H NMR (500 MHz): δ ppm: 1.07-1.82 (m, 10H, H-2””, H-3””, H-4””, H-5””, H-6””), 3.54-3.67 (m, 5H, H-1”, H-2”, H-1””), 7.32 (m, 1H, H-6), 7.55-7.70 (m, 4H, H-7, H-3’, H-4’, H-5’), 7.69 (d, J = 8.2, 1H, H-8), 8.10 (s*l*, 2H, H-2’, H-6’), 8.41 (d, J = 7.9, 1H, H-5), 8.82 (s, 1H, H-4), 8.73 (s*l*, CONH), 11.84 (s, 1H, NH, H-9). ^{13}C NMR (125 MHz): δ ppm: 25.0 ($2CH_2$, C-3””, C-5””), 25.4 (CH_2, C-4””), 32.9 ($2CH_2$, C-2””, C-6””), 39.4 (CH_2, C-1''), 41.4 (CH_2, C-2''), 48.6 (CH, C-1””), 112.7 (CH, C-8), 112.9 (CH, C-4), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.0 (CH, C-5), 128.7 (3CH, C-2’, C-4’, C-6’), 128.9 (3CH, C-7, C-3’, C-5’), 129.9 (C_0, C-4a), 134.2 (C_0, C-9a), 137.5 (C_0, C-1’), 139.8 (C_0, C-3), 140.6 (C_0, C-1), 141.6 (C_0, C-8a), 165.0 (C_0, C-2”’), 165.0 (C=O), 166.0 (C_0, C-4''', C-6'''). HRMS-ESI calcd for $C_{35}H_{42}N_9O$ $[M+H]^+$ 604.3507, found: 604.3496, error 1.8 ppm.

4.2.9.3. *N-{2-[(4,6-bis(benzylamino)-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide (**13a**)*. White crystals; Yield: 65%; M.p: 144-145 °C; ^{1}H NMR (300 MHz): δ ppm: 3.43-3.52 (m, 4H, H-1”, H-2”), 6.63-6.94 (m, 1H, NHC2”’), 7.21-7.35 (m, 6H, H-6, H-2””, H-3””, H-4””, H-5””, H-6””), 7.53-7.71 (m, 5H, H-7, H-8, H-3’, H-4’, H-5’), 8.11 (d, J = 7.2, 2H, H-2’, H-6’), 8.42 (d, J = 7.9, 1H, H-5), 8.84 (s, 1H, H-4), 8.71-8.74 (m, CONH), 11.84 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 39.7 (CH_2, C-1''), 40.2 (CH_2, C-2''), 43.0 and 43.3 ($2CH_2$, CH_2-Ph), 112.7 (CH, C-8), 113.0 (CH, C-4), 120.2 (CH, C-6), 121.3 (C_0, C-4b), 122.1 (CH, C-5), 126.4 (CH, C-2””, C-6””), 126.9 (CH, C-4””), 127.4 (CH, C-1””), 128.0 (CH, C-3””, C-5””), 128.7 (3CH, C-2’, C-4’, C-6’), 128.9 (C-3’, C-5’), 129.0 (CH, C-7), 129.9 (C_0, C-4a), 134.2 (C_0, C-9a), 137.5 (C_0, C-1’), 139.8 (C_0, C-3), 140.7 (C_0, C-1), 141.6 (C_0, C-8a), 165.1

(C=O), 165.9 (C_0, C-2'''), 166.0 (C_0, C-4''', C-6'''); HRMS-ESI calcd for $C_{37}H_{34}N_9O$ $[M+H]^+$ 620.2881, found: 620.2859, error 3.5 ppm.

4.2.9.4. *N-{2-[(4,6-bis(4-methylpiperazin-1-yl)-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide (**14a**)*. Beige crystals; Yield: 55%; M.p: 158-160 °C; ^{1}H NMR (300 MHz): δ ppm: 2.00-2.27 (m, 10H, H-3'''', H-5'''', 2C$\underline{H_3}$), 3.44-3.63 (m, 8H, H-1'', H-2'', H-2'''', H-6''''), 6.88 (t, J = 5.1, 1H, $\underline{NH}$C2'''), 7.32 (t, J = 7.3, 1H, H-6), 7.56-7.71 (m, 5H, H-7, H-8, H-3', H-4', H-5'), 8.07 (d, J = 6.7, 2H, H-2', H-6'), 8.41 (d, J = 7.8, 1H, H-5), 8.82 (s, 1H, H-4), 8.74 (t, J = 5.5, CO$\underline{NH}$), 11.84 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 39.4 (CH_2, C-1''), 40.1 (CH_2, C-2''), 42.4 (CH_2, C-2'''', H-6''''), 45.7 ($\underline{C}H_3$), 45.9 ($\underline{C}H_3$), 54.3 (CH_2, C-3''''), 54.5 (CH_2, C-5''''), 112.7 (CH, C-8), 113.0 (CH, C-4), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.0 (CH, C-5), 128.7 (3CH, C-2', C-4', C-6'), 128.8 (C-3', C-5'), 128.9 (CH, C-7), 129.9 (C_0, C-4a), 134.2 (C_0, C-9a), 137.5 (C_0, C-1'), 139.8 (C_0, C-3), 140.5 (C_0, C-1), 141.6 (C_0, C-8a), 164.6 (C_0, C-4''', C-6'''), 165.0 (C=O), 166.1 (C_0, C-2'''). HRMS-ESI calcd for $C_{33}H_{40}N_{11}O$ $[M+H]^+$ 606.3412, found: 606.3409, error 0.5 ppm.

4.2.9.5. *N-{2-[(4,6-dimorpholino-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide (**15a**)*. Beige crystals; Yield: 84%; M.p: 193-194 °C; ^{1}H NMR (500 MHz): δ ppm: 3.50-3.60 (m, 12H, H-1'', H-2'', H-2'''', H-3'''', H-5'''', H-6''''), 6.97 (t, J = 5.4, 1H, $\underline{NH}$C2'''), 7.31 (t, J = 7.5, 1H, H-6), 7.55-7.64 (m, 4H, H-7, H-3', H-4', H-5'), 7.69 (d, J = 8.1, 1H, H-8), 8.10 (d, J = 7.2, 2H, H-2', H-6'), 8.41 (d, J = 7.8, 1H, H-5), 8.83 (s, 1H, H-4), 8.76 (t, J = 5.8, CO$\underline{NH}$), 11.83 (s, 1H, NH, H-9). ^{13}C NMR (125 MHz): δ ppm: 39.4 (CH_2, C-1''), 40.1 (CH_2, C-2''), 43.1 (CH_2, C-2''''), 43.2 (CH_2, C-6''''), 66.0 (CH_2, C-3''''), 66.1 (CH_2, C-5''''), 112.7 (CH, C-8), 113.0 (CH, C-4), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.0 (CH, C-5), 128.6 (CH, C-4'), 128.7 (2CH, C-2', C-6'), 128.8 (C-3', C-5'), 128.9 (CH, C-7), 129.9 (C_0, C-4a), 134.2 (C_0, C-9a), 137.5 (C_0, C-1'), 139.8 (C_0, C-3), 140.5 (C_0, C-1), 141.6 (C_0, C-8a), 164.7 (C_0, C-4''', C-6'''), 165.0 (C=O), 166.0 (C_0, C-2'''). HRMS-ESI calcd for $C_{31}H_{34}N_9O_3$ $[M+H]^+$ 580.2779, found: 580.2761, error 3.1 ppm.

4.2.9.6. *N-{2-[(4,6-bis(isopropylamino)-1,3,5-triazin-2-yl)amino]ethyl}-1-phenyl-β-carboline-3-carboxamide (**16a**)*. White crystals; Yield: 94%; M.p: 200-201 °C; ^{1}H NMR (500 MHz): δ ppm: 1.05 (s*l*, 12 H, $\underline{CH_3}$), 3.45-3.55 (m, 4H, H-1'', H-2''), 4.02 (dq, J = 13.0, 6.7, 2H, $\underline{CH}CH_3$), 6.06-6.64 (m, 3H, $\underline{NH}$), 7.31 (t, J = 7.4, 1H, H-6), 7.55-7.70

(m, 5H, H-7, H-8, H-3', H-4', H-5'), 8.11 (d, J = 7.0, 2H, H-2', H-6'), 8.41 (d, J = 7.9, 1H, H-5), 8.82 (s, 1 H, H-4), 8.74 (t, J = 5.6, CONH), 11.83 (s, 1H, NH, H-9). ^{13}C NMR (125 MHz): δ ppm: 22.7 (CH$\underline{C}H_3$), 39.8 (CH_2, C-1"), 39.9 (CH_2, C-2"), 40.1 ($\underline{C}HCH_3$), 112.9 (CH, C-8), 112.7 (CH, C-4), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.0 (CH, C-5), 128.6 (CH, C-4'), 128.7 (2CH, C-2', C-6'), 128.8 (2CH, C-3', C-5'), 128.9 (CH, C-7), 129.9 (C_0, C-4a), 134.2 (C_0, C-9a), 137.5 (C_0, C-1'), 139.8 (C_0, C-3), 140.6 (C_0, C-1), 141.6 (C_0, C-8a), 164.9 (C_0, C-2"'), 165.0 ($2C_0$, C-4"', C-6"'), 165.9 (C=O); HRMS-ESI calcd for $C_{29}H_{34}N_9O$ $[M+H]^+$ 524.2881, found: 524.2868, error 2.5 ppm.

4.2.9.7. *N-{2-[(4,6-bis(isopropylamino)-1,3,5-triazin-2-yl)amino]ethyl}-1-(4-methoxyphenyl)-β-carboline-3-carboxamide (**16b**).* White crystals; Yield: 75 %; M.p: 262-264 °C; ^{1}H NMR (300 MHz): δ ppm: 1.05 (s*l*, 12H, CH$\underline{CH_3}$), 3.55 (m, 4H, H-1", H-2"), 3.89 (s, 3H, O$\underline{CH_3}$), 4.03 (m, 2H, $\underline{CH}CH_3$), 7.18 (d, J = 8.4, 2H, H-3', H-5'), 7.30 (t, J = 7.3, 1H, H-6), 7.58 (t, J = 7.3, 1H, H-7), 7.69 (m, 1H, H-8), 8.07 (d, J = 8.1, 2H, H-2', H-6'), 8.38 (d, J = 7.7, 1H, H-5), 8.77 (s, 1 H, H-4), 8.73 (s*l*, CO$\underline{NH}$), 11.79 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 22.7 (CH$\underline{CH_3}$), 38.6 (CH_2, C-1"), 41.2 ($\underline{CH}CH_3$), 41.7 (CH_2, C-2"), 55.4 (O$\underline{C}$H3), 112.4 (CH, C-8), 112.7 (CH, C-4), 114.3 (2CH, H-3', H-5'), 120.2 (CH, C-6), 121.3 (C_0, C-4b), 122.0 (CH, C-5), 128.5 (C_0, C-9a), 129.7 (C_0, C-4a), 130.0 (CH, C-7), 130. 1 (2CH, C-2', C-6'), 134.0 (C_0, C-1'), 139.6 (C_0, C-3), 140.5 (C_0, C-1), 141.5 (C_0, C-8a), 160.0 (C_0, C-4'), 165.0 (C_0, C-2"'), 165.1 (C=O), 166.0 (C_0, C-4"', C-6"'); HRMS-ESI calcd for $C_{30}H_{36}N_9O_2$ $[M+H]^+$ 554.2986, found: 554.2990, error 0.7 ppm.

4.2.9.8. *N-{2-[(4,6-bis(isopropylamino)-1,3,5-triazin-2-yl)amino]ethyl}-1-(4-fluorophenyl)-β-carboline-3-carboxamide (**16c**).* White crystals; Yield: 48 %; M.p: 190-195 °C; ^{1}H NMR (500 MHz): δ ppm: 1.05 (d, J = 6.90, 12H, CH$\underline{CH_3}$), 3.30-3.60 (m, 4H, H-1", H-2"), 4.03 (dd, J = 13.5; 6.8, 2H, $\underline{CH}CH_3$), 7.46 (t, J = 8.8, 2H, H-3', H-5'), 7.32 (t, J = 7.5, 1H, H-6), 7.60 (t, J = 7.5, 1H, H-7), 7.68 (d, J = 8.2, 1H, H-8), 8.15 (s*l*, 2H, H-2', H-6'), 8.42 (d, J = 7.8, 1H, H-5), 8.73 (s*l*, CO$\underline{NH}$), 8.82 (s, 1 H, H-4), 11.86 (s*l*, 1H, NH, H-9). ^{13}C NMR (125 MHz): δ ppm: 22.6 (CH$\underline{CH_3}$), 39.3 (CH_2, C-1"), 40.9 (CH_2, C-2"), 42.1 ($\underline{CH}CH_3$), 112.6 (CH, C-8), 113.0 (CH, C-4), 115.6 (CH, H-3') 115.8 (CH, H-5'), 120.2 (CH, C-6), 121.2 (C_0, C-4b), 122.1 (CH, C-5), 128.7 (C_0, C-9a), 130.0 (C_0, C-4a), 130.8 (CH, C-2'), 130.9 (CH, C-6'), 133.9 (CH, C-7), 134.1 (C_0, C-1'), 139.5 (C_0, C-3), 139.7 (C_0, C-1), 141.6 (C_0, C-8a), 161.6 (C_0, C-4'), 163.6 (C=O),

164.9 (C_0, C-2''', C-4''', C-6'''); HRMS-ESI calcd for $C_{29}H_{33}FN_9O$ $[M+H]^+$ 542.2787, found: 542.2802, error 2.8 ppm.

4.2.9.9. *N-{2-[(4,6-bis(isopropylamino)-1,3,5-triazin-2-yl)amino]ethyl}-1-(2-chlorophenyl)-β-carboline-3-carboxamide (**16d**).* White crystals; Yield: 69 %; M.p: 152-157 °C; ^{1}H NMR (300 MHz): δ ppm: 1.05 (d, J = 4.3, 12H, CH$\underline{CH_3}$), 3.38-3.52 (m, 4H, H-1", H-2"), 4.01 (d, J = 6.0, 2H, $\underline{CH}CH_3$), 7.31 (ddd, J = 7.9; 4.9; 3.1, 1H, H-6), 7.56-7.71 (m, 7H, H-7, H-8, H-3', H-4', H-5', H-6', NH), 8.42 (d, J = 7.8, 1H, H-5), 8.62 (s*l*, CO$\underline{NH}$), 8.90 (s, 1 H, H-4), 11.66 (s*l*, 1H, NH, H-9). HRMS-ESI calcd for $C_{29}H_{33}ClN_9O$ $[M+H]^+$ 558.2491, found: 558.2486, error 0.9 ppm.

4.2.9.10. *N'-[4,6-bis(isopropylamino)-1,3,5-triazin-2-yl]-1-phenyl-β-carboline-3-carbohydrazide (**17a**).* White crystals; Yield: 49 %; M.p: 204-206 °C; ^{1}H NMR (300 MHz): δ ppm: 1.12 (s*l*, 12H, $\underline{CH_3}$), 4.05 (s*l*, 2H, $\underline{CH}CH_3$), 6.58 (s*l*, 2H, $\underline{NH}$), 7.33 (t, J = 7.2, 1H, H-6), 7.59-7.73 (m, 5H, H-7, H-8, H-3', H-4', H-5'), 8.25 (s*l*, H-2', H-6'), 8.45 (d, J = 7.6, 1H, H-5), 8.84 (s, 1 H, H-4), 10.17 (s, CO$\underline{NH}$), 11.91 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 22.5 (CH$\underline{C}H_3$), 40.7 ($\underline{C}HCH_3$), 112.7 (CH, C-8), 113.4 (CH, C-4), 120.3 (CH, C-6), 121.2 (C_0, C-4b), 122.1 (CH, C-5), 128.7 (CH, C-4'), 128.8 (4CH, C-2', C-3', C-5', C-6'), 129.0 (CH, C-7), 129.8 (C_0, C-4a), 134.2 (C_0, C-9a), 137.4 (C_0, C-1'), 139.3 (C_0, C-3), 140.6 (C_0, C-1), 141.5 (C_0, C-8a), 164.0 (C=O), 165.0 (3C_0, C-2"', C-4''', C-6'''); HRMS-ESI calcd for $C_{27}H_{30}N_9O$ $[M+H]^+$ 496.2568, found: 496.2553, error 3.0 ppm.

4.2.9.11. *N'-[4,6-bis(isopropylamino)-1,3,5-triazin-2-yl]-1-(4-methoxyphenyl)-β-carboline-3-carbohydrazide (**17b**).* White crystals; Yield: 98 %; M.p: 182-183 °C; ^{1}H NMR (500 MHz): δ ppm: 0.86-1.13 (m, 12H, CH$\underline{CH_3}$), 3.90 (s, 3H, O$\underline{CH_3}$), 4.04-4.09 (m, 2H, $\underline{CH}CH_3$), 7.19 (d, J = 8.9, 2H, H-3', H-5'), 7.32 (t, J = 7.4, 1H, H-6), 7.60 (td, J = 7.7; 1.1, 1H, H-7), 7.71 (d, J = 8.2, 1H, H-8), 8.23 (d, J = 6.4, 2H, H-2', H-6'), 8.42 (d, J = 7.9, 1H, H-5), 8.79 (s, 1 H, H-4), 10.15 (s, CO$\underline{NH}$), 11.85 (s, 1H, NH, H-9). ^{13}C NMR (125 MHz): δ ppm: 22.5 (CH$\underline{C}H_3$), 41.6 ($\underline{C}HCH_3$), 55.4 (O$\underline{C}$H3), 112.7 (CH, C-8), 112.9 (CH, C-4), 114.1 (2CH, H-3', H-5'), 120.2 (CH, C-6), 121.3 (C_0, C-4b), 122.0 (CH, C-5), 129.6 (C_0, C-4a), 128.5 (CH, C-7), 129.8 (C_0, C-9a), 130.2 (2CH, C-2', C-6'), 134.0 (C_0, C-1'), 139.1 (C_0, C-3), 140.6 (C_0, C-1), 141.5 (C_0, C-8a), 160.0 (C_0, C-4'), 164.1 (C_0, C-2''', C-4"', C-6'"), 165.0 (C=O); HRMS-ESI calcd for $C_{28}H_{32}N_9O_2$ $[M+H]^+$ 526.2673, found: 526.2639, error 6.5 ppm.

4.2.9.12. *N'-[4,6-bis(isopropylamino)-1,3,5-triazin-2-yl]-1-(4-fluorophenyl)-β-carboline-3-carbohydrazide (**17c**).* White crystals; Yield: 60 %; M.p: 176-180 °C; ^{1}H NMR (300 MHz): δ ppm: 1.03-1.11 (m, 12H, CHC$\underline{H}_3$), 4.04 (s*l*, 2H, C$\underline{H}$CH$_3$), 6.57 (s*l*, 2H, NH), 7.32 (td, 7.2, 1.0, 1H, H-6), 7.46 (t, J = 8.9, 2H, H-3', H-5'), 7.61 (td, J = 7.7; 1.1, 1H, H-7), 7.71 (d, J = 8.2, 1H, H-8), 8.32 (s*l*, 2H, H-2', H-6'), 8.44 (d, J = 7.9, 1H, H-5), 8.84 (s, 1 H, H-4), 10.22 (s, CO$\underline{NH}$), 11.92 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 22.6 (CHC$\underline{H}_3$), 41.3 (C$\underline{H}$CH$_3$), 112.7 (CH, C-8), 113.5 (CH, C-4), 115.5 and 115.7 (2CH, C-3', C-5'), 120.3 (CH, C-6), 121.2 (C$_0$, C-4b), 122.1 (CH, C-5), 128.7 (C$_0$, C-4a), 129.9 (CH, C-7), 131.1 and 131.2 (2 CH, C-2', C-6'), 133.8 (C$_0$, C-9a), 134.1 (C$_0$, C-1'), 139.3 (C$_0$, C-3), 139.6 (C$_0$, C-1), 141.5 (C$_0$, C-8a), 161.0 (C$_0$, C-4'), 164.1 (C$_0$, C-2'"), 164.3 (C=O), 165.0 (2 C$_0$, C-4"', C-6'"); HRMS-ESI calcd for $C_{27}H_{29}FN_9O$ $[M+H]^+$ 514.2474, found: 514.2460, error 2.7 ppm.

4.2.9.13. *N'-[4,6-bis(isopropylamino)-1,3,5-triazin-2-yl]-1-(2-chlorophenyl)-β-carboline-3-carbohydrazide (**17d**).* White crystals; Yield: 56 %; M.p: 177-181 °C; ^{1}H NMR (300 MHz): δ ppm: 1.08 (s*l*, 12H, CHC$\underline{H}_3$), 4.04 (s*l*, 2H, C$\underline{H}$CH$_3$), 6.56 (s*l*, 1H, NH), 7.32 (ddd, J = 7.9; 4.8; 3.2, 1H, H-6), 7.59-7.74 (m, 4H, H-3', H-4', H-5', H-6'), 7.71-7.74 (m, 2H, H-7, H-8), 8.45 (d, J = 7.9, 1H, H-5), 8.91 (s, 1 H, H-4), 9.91 (s*l*, CO$\underline{NH}$), 11.73 (s, 1H, NH, H-9). ^{13}C NMR (75 MHz): δ ppm: 22.5 (CHC$\underline{H}_3$), 41.2 (C$\underline{H}$CH$_3$), 112.4 (CH, C-8), 114.2 (CH, C-4), 120.2 (CH, C-6), 121.1 (C$_0$, C-4b), 122.3 (CH, C-5), 127.5 (CH, C-7), 128.8 (CH, C-5'), 129.8 (C$_0$, C-4a), 130.7 (C$_0$, C-2'), 132.2 (CH, C-3'), 132.5 (CH, C-6'), 132.5 (C$_0$, C-4'), 135.3 (C$_0$, C-1'), 136.3 (C$_0$, C-3), 138.8 (C$_0$, C-9a), 139.8 (C$_0$, C-1), 141.4 (C$_0$, C-8a), 163.8 (C=O), 164.9 (2 C$_0$, C-4"', C-6'"), 167.3 (C$_0$, C-2'"); HRMS-ESI calcd for $C_{27}H_{29}ClN_9O$ $[M+H]^+$ 530.2178, found: 530.2162, error 3.0 ppm.

*4.2.10. Procedure for the preparation of β-carboline-4,6-bis(isopropylamino)-1,3,5-triazine derivative (**16e**)*

A suspension of cyanuric chloride (0.2 mmol) and potassium carbonate (0.2 mmol) in anhydrous THF (1 mL) was stirred for 10 minutes at 0 °C, followed by the addition of **6e** (0.2 mmol) suspended in anhydrous THF (2 mL). The reaction mixture was stirred at 0 ºC for 1 hour, followed by addition of 10 equivalents of isopropylamine. The mixture was kept at 70 °C, and the reaction progress monitored by TLC every 12 h. After complete consumption of starting materials (48 h), the solution was cooled and

treated with 2 mL of distilled water. The precipitate formed was filtered and washed with distilled water, providing **16e** in 40% yield.

4.2.10.1. *N-{2-[(4,6-bis(isopropylamino)-1,3,5-triazin-2-yl)amino]ethyl}-1-(3-nitrophenyl)-β-carboline-3-carboxamide (**16e**).* Yellow crystals; M.p. > 350 °C; Yield: 40 %; ^{1}H NMR (300 MHz): δ ppm: 1.10 (dd, J = 7.8; 6.7, 12H, CH$\underline{CH_3}$), 2.78 (t, J = 6.1, 2H, H-2"), 3.41 (q, J = 6.1, 2H, H-1"), 3.94 - 4.05 (m, 2H, $\underline{CH}CH_3$), 7.34 (m, 1.2, 1H, H-6), 7.60-7.70 (m, 3H, H-7, H-8, NH), 7.94 (t, J = 8.0, 1H, H-5'), 8.39-8.46 (m, 2H, H-5, H-4'), 8.58 (d, J = 7.8, 1H, H-6'), 8.80 – 8.86 (m, 2H, H-2', NH), 8.91 (s, 1 H, H-4). ^{13}C NMR (75 MHz): δ ppm: 22.0 ($\underline{C}H_3CH$), 41.2 (CH_2, C-1"), 41.6 (CH_2, C-2"), 42.0 ($\underline{C}HCH_3$), 112.6 (CH, C-8), 113.9 (CH, C-4), 120.4 (CH, C-6), 121.2 (C_0, C-4b), 122.2 (CH, C-5), 123.5 (CH, C-2'), 123.6 (CH, C-4'), 129.0 (C_0, C-4a), 130.4 (CH, C-7), 130.4 (C_0, C-9a), 134.4 (CH, C-5'), 135.4 (C_0, C-1'), 138.1 (CH, C-6'), 139.0 (C_0, C-3), 140.1 (C_0, C-1), 141.7 (C_0, C-8a), 148.3 (C_0, H-3'), 164.5 (C_0, C-2'"), 164.5 (C=O), 164.7 (C_0, C-4'", C-6'"); HRMS-ESI calcd for $C_{29}H_{33}N_{10}O_3$ $[M+H]^+$ 569.2732, found: 569.2702, error 5.3 ppm.

4.3. Antileishmanial activity

4.3.1. Parasites and cell culture

Leishmania amazonensis promastigotes were maintained at 25 °C in Warren medium and axenic amastigotes forms were maintained at 25 °C in Schneider's medium, supplemented with 10% FBS. J774-A1 macrophages were maintained at 37 °C under 5% CO_2 atmosphere in RPMI 1640 medium (pH 7.2) supplemented with 10% FBS.

4.3.2. Antiprotozoal activity

The effects of synthesized compounds were evaluated in promastigotes and axenic amastigotes forms of *L. amazonensis* in log phase of growth (48 h and 72 h, respectively) at concentration of 1 x 10^{-6} cells mL^{-1}. The inoculants were introduced into sterile 96-well micro plates containing increasing concentrations of compounds **8a-d**, **9a-e**, **10a-15a**, **16a-d** and **17a-d**. After incubation for 72 h at 25 °C and 32 °C for promastigotes and axenic amastigotes forms, was added 50 µL of solution of XTT/ PMS (0.5 and 0.3 mg/mL) in the absence of light. After 4 h, the absorbance was read in a spectrophotometer at 450 nm. The concentration that decreased 50% (IC_{50}) of the

absorbance values compared with the negative control was determined by regression analysis of the data. To **16b** also was determined the concentration that decreased 90% (IC_{90}) of the absorbance values. Miltefosine was used as positive control.

4.3.3. Cytotoxicity assay

The cytotoxicity was evaluated in J774-A1 macrophages. To a suspension of macrophages in log phase growth (72 h) at concentration of 5 x 10^{-5} cells mL^{-1} was cultured in RPMI 1640 medium supplemented with 10% FBS, were introduced into sterile 96-well micro plates and incubated for 24 h at 37 °C and 5 % of CO_2 tension. After this period, the supernatant was removed and increasing concentrations of the substances were added. After 48 h of incubation under the same conditions mentioned above, the cells were washed with phosphate buffered saline 0.01 M and 50 µL of MTT (2 mg mL^{-1}) was added to each well and incubated at absence of light at 25 °C. After 4 h, 150 µL of DMSO was added in order to disrupt the cells and solubilize purple formazan crystals. The absorbance was read at 570 nm in microplate reader (Biotek Power Wave XS spectrofluorometer). The concentration that decreased 50% (CC_{50}) of the absorbance value compared with the negative control was determined by regression analysis of the data.

4.3.4. Electron Microscopy

Promastigotes (1 x 10^6 parasites/mL) were treated with IC_{50} value of **16b** and incubated for 24, 48 and 72 h at 25 °C. The cells were fixed with 0.1 M cacodylate buffer buffer and 2.5% of glutaraldehyde. For the ultrastructural analysis, promastigotes fixed were post-fixed with 1% OsO_4 and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer for 1 h. After that, the samples were dehydrated in acetone and included in EPON resin. Ultrathin sections of 60 nm were obtained and contrasted with uranyl acetate and lead citrate. The samples were observed in Microscopy Jeol JM 1400 TEM. For scanning electron microscopy (SEM), promastigotes were placed on a glass support with poly-L-lysin, dehydrated with ethanol and submitted at critical point (replacement of ethanol by CO_2). After that, the samples were metalized with gold and observed in FEI Quanta 250 SEM.

4.3.5. Oxidative stress analysis

Promastigotes (1 x 10^6 parasites/mL) were pre-incubated or no with *N*-acetyl-cysteine (NAC) (10 μM) for 3 h and after that, treated with IC_{50} and IC_{90} and incubated as previously described. After 24 h of incubation, the cells were washed in PBS and loaded with 10 μM H_2DCFDA for 45 min. The fluorescence was determined in a Victor X3 spectrofluorometer at λ_{ex} of 488 nm and λ_{em} of 530 nm [33].

4.3.6. Storage-lipid bodies analysis

Promastigotes (1 x 10^6 parasites/mL) were treated with IC_{50} and IC_{90} and incubated as previously described. After 24 h of incubation, the cells were washed in PBS and loaded with 10 μg/mL Nile red for 30 min. The fluorescence was determined in a Victor X3 spectrofluorometer at λ_{ex} of 485 nm and λ_{em} of 535 nm [21].

4.3.7. Statistical analysis

The data shown in the tables and graphs are expressed as the mean ± standard deviation of at least three independent experiments. The statistical analysis was realized using GraphPad Prism 6.0 software. The samples were analyzed using one-way analysis of variance (ANOVA), and the Tukey *post hoc* test was used to compare means when appropriate. Values of p of ≤ 0.05 were considered statistically significant.

Acknowledgments

This work was supported by Fundação Araucária (Brazil, PR) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ). We thank CAPES for fellowship (Baréa P) and to Complexo de Centrais de Apoio à Pesquisa of Universidade Estadual de Maringá (COMCAP-UEM) for the facilities.

Appendix A. Supplementary data

Supplementary data related to this article can be found at.

References

[1] World Health Organization, Global Health Observatory (GHO) data: Leishmaniasis: Situations and trends. http://www.who.int/gho/neglected_diseases/leishmaniasis/en/, 2017 (accesed 29 August 2017).

[2] R. Reithinger, J. Dujardin, H. Louzir, C. Pirmez, B. Alexander, S. Brooker, **Cutaneous leishmaniases**, 9 (2007), pp. 581-596. http://dx.doi.org/10.1016/S1473-3099(07)70209-8

[3] S. L. Croft, G.H. Coombs, **Leishmaniasis- current chemotherapy and recent advances in the search for novel drugs**, Trends. Parasitol., 19 (2003), pp. 502-508.

[4] A. Ponte-Sucre, F. Gamarro, J. Dujardin, M.P. Barret, R. López-Vélez, R. García-Hernández, A.W. Pontain, R. Mwenechanya, B. Papadopoulou, **Drug resistance and treatment failure in leishmaniasis: A 21st century challenge**, PLoS Negl Trop Dis., 12 (2017), pp. 1-24. https://doi.org/ 10.1371/journal.pntd.0006052

[5] P. Mitropoulos, P. Konidas, M. Durkin-Konidas, **New World cutaneous leishmaniasis: Updated review of current and future diagnosis and treatment.** J. Am. Acad. Dermatol. (63) 2010, pp. 309-322.

[6] F. Modabber, **Leishmaniasis vaccines: past, present and future**, Int. J. Antimicrob. Agents, 36 (2010) pp. 58-61. http://dx.doi.org/10.1016/j.ijantimicag.2010.06.024.

[7] H. Hussain, A. Al-Harrasi, A. Al-Rawahi, I.R. Green, S. Gibbons, **Fuitful decade for antileishmanial compounds from 2002 to late 2011**, *Chem. Rev*., 114 (2014), pp. 10369-10428.

[8] R. Cao, W. Peng, Z. Wang, A. Xu, ***β*-carboline alkaloids: biochemical and pharmacological functions**. Curr. Med. Chem., 14 (2007), pp. 479-500.

[9] A.E. Laine, C. Lood, A.M.P. Koskinen, **Pharmacological importance of optically active tetrahydro-*β*-carbolines and synthetic approaches to create the C1 stereocenter**, Molecules, 19 (2014), pp. 1544-1567.

[10] S.S. Chauhan, S. Pandey, R. Shivahare, K. Ramalingam, S. Krishna, P. Vishwakarma, M.I. Siddiqi, S. Gupta, N. Goyal, P.M.S. Chauhan, **Novel *β*-carboline–quinazolinone hybrid as an inhibitor of Leishmania donovani trypanothione reductase: Synthesis, molecular docking and bioevaluation**. Med. Chem. Commun., 6 (2015), pp. 351-356.

[11] V.M. Gohil, K.G. Brahmbhatt, P.M. Loiseau, K.K. Bhutani, **Synthesis and anti-leishmanial activity of 1-aryl-*β*-carboline derivatives against Leishmania donovani**, Bioorg Med Chem Lett., 22 (2012), pp. 3905-3907.

[12] S. Manda, S.I. Khan, S.K. Jain, S. Mohammed, B. Tekwani, I.A. Khan, R.A.

Vishwakarma, S.B. Bharate, **Synthesis, antileishmanial and antitrypanosomal activities of N-substituted tetrahydro-*β*-carbolines**, Bioorg Med Chem Lett., 24 (2014), pp. 3247-3250.

[13] A. Gellis, A. Dumètre, G. Lanzada, S. Hutter, E. Ollivier, P. Vanelle, N. Azas, **Preparation and antiprotozoal evaluation of promising *β*-carboline alkaloids,** N. *Biomed Pharmacother*., 66 (2012), pp. 339-347.

[14] A. Kumar, S.B. Katiyar, S. Gupta, P.M.S. Chauhan, **Synthesis of new substituted triazino tetrahydroisoquinolines and *β*-carbolines as novel antileishmanial agents**. *Eur. J. Med. Chem.*, 41 (2006), pp. 106-113.

[15] P. Ashok, S. Chander, A. Tejería, L. García-Calvo, R. Balana-Fouce, S. Murugesan, **Synthesis and anti-leishmanial evaluation of 1-phenyl-2,3,4,9-tetrahydro-1H-*β*-carboline derivatives against *Leishmania infantum***, *Eur. J. Med. Chem.*, 123 (2016), pp. 814-821.

[16] P. Ashok, S. Chander, L.M.C. Chow, I.L.K. Wong, R.P. Singh, P.N. Jha, M. Sankaranarayanan, **Synthesis and in-vitro anti-leishmanial activity of (4-arylpiperazin-1-yl)(1-(thiophen-2-yl)-9H-pyrido[3,4-b]indol-3-yl)methanone** derivatives, Bioorg. Chem., 70 (2017), pp. 100–106.

[17] H. Volpato, V.C. Desoti, J. Cogo, M.R. Panice, M.H. Sarragiotto, S.O. Silva, T. Ueda-Nakamura, C.V. Nakamura **The effects of *N*-butyl-1-(4-dimethylamino)phenyl-1,2,3,4-tetrahydro-*β*-carboline-3-carboxamide against *Leishmania amazonensis* are mediated by mitochondrial dysfunction.** *Evid. Based. Complement. Alternat. Med.*, (2013), pp. 1-7.

[18] C.M.B.L. Silva, F.P. Garcia, J.H. da Silva Rodrigues, C.V. Nakamura, T. Ueda-Nakamura, E. Meyer, A.L.T. Gois Ruiz, M.A. Foglio, J.E. de Carvalho, W.F. da Costa, M.H. Sarragiotto, **Synthesis, antitumor, antitrypanosomal and antileishmanial activities of benzo[4,5]canthin-6-ones bearing the N′-(substituted benzylidene)-carbohydrazide and *N*-alkylcarboxamide groups at C-2**, Chem. Pharm. Bull. 60 (2012), pp. 1372–1379.

[19] L.T.D. Tonin, M.R. Panice, C.V. Nakamura, K.J.P. Rocha, A.O. Dos Santos, T. Ueda-Nakamura, W. Ferreira Da Costa, M.H. Sarragiotto, **Antitrypanosomal and antileishmanial activities of novel *N*-alkyl-(1-phenylsubstituted-*β*-carboline)-3-carboxamides,** Biomed. Pharmacother., 64 (2010), pp. 386-389.

[20] R.B. Pedroso, L.T.D. Tonin, T. Ueda-nakamura, B.P. Dias filho, M.H. Sarragiotto, C.V. Nakamura, ***β*-carboline-3-carboxamide derivatives as**

promising antileishmanial agentes, Ann Trop Med Parasitol, 105 (2011), pp. 549-557.

[21] T.F. Stefanello, M.R. Panice, T. Ueda-Nakamura, M.H. Sarragiotto, R. Auzély-Velty, C.V. Nakamura, ***N*-butyl-[1-(4-methoxy)phenyl-9*H*-*β*-carboline]-3-carboxamide prevents cytokinesis in *Leishmania amazonensis***, Antimicrob. Agents and Chemother., 58 (2014), pp. 7112-7120.

[22] E.A. Mendes, V.C. Desoti, S.O. Silva, T. Ueda-nakamura, B.P. Dias Filho, S.F. Yamada-Ogatta, M.H. Sarragiotto MH, C.V. Nakamura, **C5 induces different cell death patways in promastigotes of *Leishmania amazonensis***, Chem Biol Interact., 256 (2016), pp. 16-24.

[23] P. Singla, V. Luxami, K. Paul, **Triazine as a promising scaffold for its versatile biological behavior**, *Eur J Med Chem*., 102 (2015), pp. 39-57.

[24] N.Sunduru, A. Agarwal, S.B. Katiyar, Nishi, N. Goyal, S. Gupta S, P.M.S. Chauhan, **Synthesis of 2,4,6-trisubstituted pyrimidine and triazine heterocycles as antileishmanial agents**, Bioorg. Med. Chem., 14 (2006), pp. 7706-7715.

[25] R.G. Booth, C.D. Selassie, C. Hansch, D.V. Santi, **Quantitative Structure-Activity Relationship of triazine-antifolate inhibition of Leishmania Dihydrofolate Reductase and Cell Growth**, J. Med. Chem., 30 (1987), pp. 1218-1224.

[26] N. Sunduru, Nishi, S. Palne, P.M.S. Chauhan, S. Gupta, **Synthesis and antileishmanial activity of novel 2,4,6-trisubstituted pyrimidines and 1,3,5-triazines**, Eur J Med Chem., 44 (2009), pp. 2473-2481.

[27] L. Gupta, N. Sunduru, A. Verma, S. Srivastava, S. Gupta, N. Goyal, P.M.S. Chauhan, **Synthesis and biological evaluation of new [1,2,4]triazino[5,6-b]indol-3-ylthio-1,3,5-triazines and [1,2,4]triazino[5,6-b]indol-3-ylthio-pyrimidines against *Leishmania donovani***, *Eur J Med Chem*., 45 (2010), pp. 2359-2365.

[28] K. Chauhan, M. Sharma, R. Shivahare, U. Debnath, S. Gupta, Y.S. Prabhakar, P.M.S. Chauhan, **Discovery of triazine mimetics as potent antileishmanial agents**, *ACS Med. Chem. Lett*., 4 (2013), pp. 1108-1113.

[29] G. Blotny, **Recent applications of 2,4,6-trichloro-1,3,5-triazine and its derivatives in organic synthesis**, *Tetrahedron*., 62 (2006), pp. 9507-9522.

[30] V. Kaplun, J. Cogo, D.P. Sangi, T. Ueda-Nakamura, A.G. Corrêa, C.V. Nakamura, ***In vitro* and *in vivo* activity of 2,3-diarylsubstituted quinoxaline**

derivatives in *Leishmania amazonensis*, Antimicrob. Agents Chemother 60 (2016), pp. 02582-15. http://doi:10.1128/AAC.02582-15.

[31] S.T. Macedo-Silva, J.A. Urbina, W. Souza, J.C.F. Rodrigues, ***In vitro* Activity of the Antifungal Azoles Itraconazole and Posaconazole against *Leishmania amazonensis***, Plos One, (2013), pp. e83247. http://doi:10.1371/journal.pone.0083247.

[32] S.J. Lee, J. Zhang, A.M.K. Choi, H.P. Kim, **Mitochondrial dysfunction induces formation lipid droplets as a generalized response to stress**. Oxid Med Cell Longev (2013), pp. 327167. http://dx.doi.org/10.1155/2013/327167.

[33] A.K. Shukla, S. Patra, V.K. Dubey VK, **Iridoid glucosides from *Nyctanthes arbortristis* result in increased reactive oxygen species and cellular redox homeostasis imbalance in *Leishmania* parasite**, Eur. J. Med. Chem., 54 (2012), pp. 49–58. http://dx.doi.org/10.1016/j.ejmech.2012.04.034.

HIGHLIGHTS

Novel hybrids β-carboline-1,3,5-triazine were assayed for antileishmanial activity.

Hybrids **9d**, **9e**, **16a** and **16b** were strongly active against *Leishmania amazonensis.*

Hybrid 1**6b** showed low toxicity, and potent activity to amastigotes and promastigotes.

Compound **16b** caused alterations in cell division cycle, and led to protozoan cell death.