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Research paper

Leishmanicidal, antiproteolytic, and mutagenic evaluation of alkyltriazoles and alkylphosphocholines

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ABSTRACT

A series of 16 simple long-chain alkyltriazoles and two novel alkylphosphocholine derivatives containing an azide moiety were evaluated *in vitro* for their leishmanicidal activity against. Among the 18 compounds tested, the eight most active compounds against promastigote forms were selected for further evaluation against amastigote forms. These compounds were also evaluated for their cytotoxicity against murine macrophages and tested as inhibitors of cysteine protease rCPB2.8, an important target for development of antileishmanial drugs. The mutagenicity of some of these compounds was also evaluated in prokaryotic and eukaryotic cells to assess any genetic effects of the leishmanicidal candidates. The compound **4**, an alkylphosphocholine derivative, was found to be the most potent against amastigote forms with an IC_{50} of 3.81 μM, comparable to that of pentamidine (IC_{50} = 6.62 μM) and amphotericin B (IC_{50} = 6.10 μM), two established leishmanicidal drugs. Compound **4** also exhibited the best selectivity index (SI) values of the series, demonstrating low toxicity against macrophages and a cLogP value higher than 5. Among the alkyltriazoles, compounds **13** and **14** were the most active against promastigote and amastigote forms. They were then evaluated for their mutagenicity *in vitro*; the mutagenicity index (MI) values were lower than 2, suggesting that these compounds are not mutagenic.

1. Introduction

Leishmaniasis is a parasitic disease with more than 20 species of the protozoan genus *Leishmania* as the causative agents. It is endemic in 88 tropical and subtropical countries worldwide, with 350 million men, women, and children living at risk [1]. Leishmaniasis displays various clinical manifestations including visceral (VL), cutaneous (CL), and mucocutaneous (MCL) symtpoms, depending on complex interactions between the parasite and host's immune response [2]. This disease is currently difficult to manage, mainly owing to a limited efficient treatments and the prevalence of drug resistance. The first line of drug treatment for all clinical manifestations of leishmaniasis consists of pentavalent antimonial compounds such as sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime). These drugs have been used in the treatment of leishmaniasis for over 50 years but have been found to cause several toxic effects, including myalgia, pancreatitis, cardiac arrhythmias, and hepatitis [3]. Second line drug treatments include amphotericin B and pentamidine; however, these drugs also present potentially serious adverse effects [4]. Though a liposomal formulation of amphotericin B (Ambisome) has been developed to reduce the drug's toxicity, it is a more expensive option and thus can limit therapeutic use in developing countries [3]. Miltefosine, a newer compound of the alkylphospholipid class, is the first FDA-approved oral medicine to treat cutaneous and mucosal leishmaniasis. Efficient oral administration, moderate safety, and low toxicity favour the use of this drug, especially in certain cases such as for the treatment of children. However, its limitations include gastrointestinal toxicity and teratogenic action.

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The development of new, inexpensive, and safe drugs for the treatment of leishmaniasis remains a great challenge for researchers worldwide [5–8].

In recent years, 'click chemistry' or the 'click reaction,' a reaction chemically referred to as the copper-catalysed azide-alkyne cycloaddition or CuAAC, for the efficient synthesis of the 1,2,3-triazole nucleus, has attracted significant attention [9–12]. This heterocycle is easily obtained by the CuAAC reaction [13] and has occupied an important position in medicinal chemistry research [14]. The 1,2,3-triazole ring acts not only as a pharmacophore, but also as a linker between two or more substances through a molecular hybridization strategy. Furthermore, the triazole can act as a bioisostere of the amide group, presenting similar physicochemical properties. The triazole unit acts as a rigid linker, which accommodates the R^1 and R^2 groups (Fig. 1), at a distance of 5.0 Å (for comparison, the same distance on the amide groups is 3.8 Å). In contrast to amides, the triazole ring is stable, since it does not undergo hydrolysis, oxidation, or reduction. The 1,2,3-triazoles have a large dipole moment (5D) and nitrogen atoms on positions 2 and 3 act as weak hydrogen bond acceptors. In most recent years, compounds belonging to several chemical classes and containing 1,2,3-triazoles have been described as potential biological agents, including leishmanicidal drugs [15].

Recently we have synthesized a series of novel, structurally simple, long-chain alkyltriazoles and evaluated their activity against tumour cells [16]. A promising anticancer candidate was found in the series, with cytotoxicity comparable to that of etoposide, a known antitumour agent. In the current work, we have investigated the leishmanicidal activity of two new alkylphosphocholine analogues of miltefosine and 16 long-chain alkyltriazoles obtained via the click approach, which include 14 compounds previously described [16]. The compounds were tested against promastigote and amastigote forms of *Leishmania amazonensis*. Moreover, the cytotoxicity against murine peritoneal macrophages and the mutagenic effect of these compounds were also evaluated. We have also investigated the antiproteolytic activity of these compounds toward *Leishmania mexicana* cysteine protease, r-CPB2.8. Several papain-like cysteine proteases have been identified in different *Leishmania* spp and are thought to be crucial for the survival and infectivity of the parasite in its human host. These enzymes have been involved in successful invasion of host macrophages by promastigotes, subsequent transformation of parasitic forms, and evasion from the host immune system [17,18]. Because of the importance of cysteine proteases in the survival and life cycle of *Leishmania*, they have been targets for the development of antileishmanial drugs [19].

To complete the analyses performed in this study, the mutagenic effects of some compounds were evaluated. This has permitted the identification of potential mutagenic and carcinogenic effects and the study of the mechanisms by which these agents can cause damage and mutations in genetic material [20]. The monitoring of genotoxic effects of carcinogens in humans has been widely utilized for hazard identification or risk assessment purposes [21]. Mutagenic agents can cause severe genetic alterations and cancer at doses much lower than those necessary to display acute toxicity. Therefore, specific exclusion of genotoxic effects is important in products used for human foods or drugs [22].

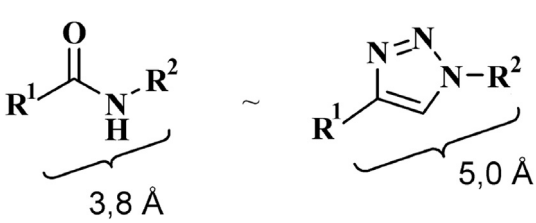


Fig. 1. 1,2,3-triazole: Bioisostere of amide.

2. Methods and materials

2.1. General

Reagents and solvents were purchased as reagent grade PA and used without further purification. All melting points were measured on Fisher-Jonhs and are uncorrected. IR spectra were recorded on Perkin–Elmer *Spectrum One SP-IR Spectrometer*. ^{1}H and ^{13}C NMR spectra were recorded on a *Bruker AVANCE DRX* 200 MHz spectrometer using TMS as an internal standard. The results are presented as chemical shift δ in ppm, number of protons, multiplicity, *J* values in Hertz (Hz), proton position and carbon position. Multiplicities are abbreviated as follows: s (singlet), d (doublet), t (triplet), m (multiplet) and qn (quintet). High resolution mass spectra were recorded on ESI-MS Bruker Daltonics Micro TOF mass spectrometer with electrospray ionization coupled to time-of-flight (Solvent: MeOH). The progress of the reactions was monitored by TLC on Merck silica plates (GF254). Column chromatography was performed over silica gel 60, 70–230 mesh (Merck).

The enzyme r-CPB2.8 were generously gifted by Dr. Luiz Juliano (Department of Biophysics, Federal University of São Paulo, Brazil), E-64 (1-[[(Ltrans-epoxysuccinyl)-L-leucyl]amino]-4-guanidinobutane), and fluorogenic substrate Z-FR-AMC (carbobenzyloxy-Phe-Arg-(7-amino-4-methylcoumarin) were commercially obtained from Sigma–Aldrich Sigma (St. Louis, USA). Substrate hydrolyses were monitored in a spectrofluorometer F2500 Hitachi using the λ_{Ex} = 380 nm and λ_{Em} = 460 nm as wavelength, respectively, and the enzymatic molar concentrations were estimated by titration according to kinetic parameters [23].

2.2. Synthesis

2.2.1. Synthesis of alkylphosphocholines 3 and 4

The calcium salt of phosphorylcholine **1** (1.0 equiv) was treated with an aqueous solution of oxalic acid (1.1 equiv). The precipitate calcium oxalate was removed by filtration, and the filtrate was titrated with tetra-n-butylammonium hydroxide until pH 9. The resulting solution was evaporated to dryness, and the residue was dissolved in 10 mL of toluene. After evaporating to dryness, the residue was re-dissolved in a further 10 mL toluene, evaporated again and the crude obtained was dried in a vacuum desiccator over P_2O_5 to yield the choline phosphate tetra-n-butylammonium **2**. The compounds **3** and **4** were obtained by classic S_N2 substitution between the compound **2** and methanesulfonate alkylazide compounds (**8a** or **8b**) in acetonitrile at room temperature for 24 h, followed by refluxing for 3 h. The crude product was purified by column chromatography over silica gel, eluting with methanol, to give pure compounds **3** and **4.**

*2.2.1.1. 9-azidenonylphosphocholine **(3)**.* Colour less oil. Yield 19%. IR (ATR): 2928, 2855, 2094, 1223, 1082, 1059, 966, 816, 763, 722. ^{1}H NMR (200 MHz, CD_3OD): 1.27–1.44 (m, 10H), 1.50–1.69 (m, 4H), 3.20 (s, 9H), 3.22–3.33 (m, 2H), 3.85 (qa; J_{H1-P} = 6.0 and J_{H1-H2} = 6.0, 2H), 3.73–3.94 (m, 2H), 4.12–4.32 (m, 2H). ^{13}C NMR (50 MHz, CD_3OD): 25.38, 26.31, 28.40, 28.71, 28.84, 29.08, 29.19, 30.28, 30.42, 50.93, 53.25, 58.57, 63.48, 65.75. ESI-MS: *m/z* $[M+Na]^+$ calcd for $C_{14}H_{31}N_4O_4P$: 373.1981; found *m/z* $[M+Na]^+$: 372.9000.

*2.2.1.2. 12-azidedodecylphosphocholine **(4)**.* Colour less oil. Yield 19%. IR (ATR): 2923, 2852, 2100, 1231, 1084, 1059, 968, 873, 796, 777.

^{1}H NMR (200 MHz, $CDCl_3$): 1.24–1.47 (m, 16H), 1.59–1.69 (m, 4H), 3.23 (s, 9H), 3.30–3.32 (m, 2H), 3.57–3.69 (m, 2H), 3.87 (qa; $J_{H1-P} = 6.0$ e $J_{H1-H2} = 6.0$, 2H), 4.13–4.34 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): 25.44, 26.33, 28.42, 28.78, 28.99, 29.18, 30.45, 50.93, 53.17, 58.59, 61.26, 65.38. ESI-MS: *m/z* $[M+H]^+$ calcd for $C_{17}H_{38}N_4O_4P$: 393.2631; found *m/z* $[M+H]^+$: 393.2773.

2.2.2. Synthesis of 1,2,3-alkyltriazoles 10 and 11

To a stirred solution of the 1,12-dodecanediol (**5**) (1.00 equiv) in 30 mL of toluene HBr 48% (2.00 equiv) was added. The reaction was stirred at 110 °C for 24 h. The solvent was removed under reduced pressure, and the residue was purified by column chromatography over silica gel, eluting with hexane/EtOAc 9:1, to yield pure halo alcohol (**6**). This compound was transformed into their corresponding azidoalcohol (**7**) via classic S_N2 substitution. A stock solution of 0.5 M NaN_3 in DMSO was prepared by stirring the solution for 24 h at room temperature. To a 100 mL round-bottom flask equipped with a magnetic stir bar was added a 0.5 M solution of NaN_3 in DMSO at room temperature. To this solution was added the bromo alcohol (**6**) (1.00 equiv) and the mixture was stirred for 24 h at room temperature. The reaction was quenched with H_2O (50 mL) and stirred until it cooled to room temperature. The mixture was extracted with Et_2O (3 × 30 mL), and the resultant extracts were washed with H_2O (3 × 50 mL) and brine (50 mL). The organic layer was dried (Na_2SO_4) and filtered, and the residue obtained was purified by column chromatography over silica gel, eluting with hexane/EtOAc 9:1, to yield pure alkyl azidoalcohol (**7**). A solution of the azidoalcohol (**7**) (1.00 equiv) in CH_2Cl_2 (50 mL) was cooled to 0 °C. Et_3N (2.00 equiv) and methanesulfonyl chloride (2.00 equiv) were added. The reaction mixture was allowed to warm to room temperature and stirred for additional 24 h. The reaction mixture was poured into crushed ice (70 mL) and was then extracted with methylene chloride (3 × 30 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue obtained was purified by column chromatography over silica gel, eluting with hexane/EtOAc 9:1, to yield pure halo alcohol pure methanesulfonate alkylazide compound (**8**). The azide compound (**8**) (1.00 equiv) was added to a 10 mL round-bottom flask containing 1 mL of dichloromethane, 1 mL of water, $CuSO_4.5H_2O$ (0.08 equiv), sodium ascorbate (0.20 equiv) and pent-4-yn-1-ol (1.00 equiv). The reaction mixture was vigorously stirred at room temperature for 24 h. After completion of the reaction, 5 mL of water were added, followed by extraction with dichloromethane (3 × 8 mL). The resulting organic layer was washed three times with a 25% EDTA solution buffered with NH_4Cl at pH 9.5. The organic layer was dried with Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by column chromatography over silica gel, eluting with dichloromethane: EtOAc (8:2 v/v; 5:5 v/v; 2:8 v/v), EtOAC and EtOAc/MeOH (8:2 v/v; 5:5 v/v; 2:8 v/v), to give pure compound **9**.

To a stirred solution of the compound **9** (1.00 equiv) in 5 mL of acetone was added 4-DMAP (0.40 equiv) and acetic anhydride (2.00 equiv). The reaction system was kept under magnetic stirring at room temperature for 24 h, poured into crushed ice (70 mL) and was then extracted with methylene chloride (3 × 30 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue obtained was purified by column chromatography over silica gel, eluting with hexane/EtOAc 7:3, to yield pure compound **10**.

The compound **9** (1.00 equiv) was added to 10 mL of dichlorometane, the solution was cooled to 0 °C, Et_3N (2.00 equiv) and methanesulfonyl chloride (2.00 equiv) were added. The reaction mixture was allowed to warm at room temperature and stirred for additional 24 h. The reaction mixture was poured into crushed ice (70 mL) and was then extracted with methylene chloride (3 × 30 mL). The organic layer was dried (Na_2SO_4), filtered and evaporated under reduced pressure. The residue obtained was purified by column chromatography over silica gel, eluting with hexane/EtOAc 9:1, to yield pure compound **11**.

2.2.2.1. 5-(4-(3-acetoxypropyl)-1H-1,2,3-triazol-1-yl)dodecyl methanesulfonate (**10**).

Yellow oil. Yield 73%. IR(ATR): 2918, 2852, 1730, 1345,1328, 1163, 1036–951, 1244, 835.^{1}H NMR (200 MHz, $CDCl_3$): 1.17–1.52 (m, 18H), 1.63–1.79 (m, 2H), 1.81–1.93 (m, 2H), 2.05 (s, 3H), (2.80, t, $J_{15,16} = 6.0$, 2H), 3.0 (s, 3H), 4.01–4.39 (m, 6H), 7.31 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): 20.84, 21.97, 25.26, 26.34, 28.21, 28.84, 28.96, 29.24, 30.19, 37.21, 49.92, 63.44, 70.14, 120.63, 146.65, 171.01. ESI-MS: *m/z* $[M+H]^+$ calcd for $C_{20}H_{38}N_3O_5$: 0.432.2532; found *m/z* $[M+H]^+$: 432.2540.

2.2.2.2. 5-(4-(3- methanesulfonatepropyl)-1H-1,2,3-triazol-1-yl) dodecyl methanesulfonate (**11**).

White solid. m.p. = 88–90 °C. Yield 56%. IR (ATR): 2916, 2850, 1331, 1165, 1057–953.^{1}H NMR (200 MHz, $CDCl_3$): 1.15–1.39 (m, 16H), 1.58–1.95 (m, 4H), 1.96–2.26 (m, 2H), 2.82 (t, $J_{15,16} = 6.0$, 2H), 2.96 or 2.99 (s, 6H), 4.08–4.38 (m, 6H), 7.35 (s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$): 21.21, 25.24, 26.32, 28.56, 28.83, 28.95, 29.21, 29.24, 30.13, 37.20, 50.19, 69.03,70.24,121.16,145.76. ESI-MS: *m/z* $[M+H]^+$ calcd for $C_{19}H_{38}N_3O_6S_2$: 468.2202; found *m/z* $[M+H]^+$: 468.1846.

2.3. Biological assays

2.3.1. In vitro leishmanicidal activity

2.3.1.1. Leishmanicidal activity against promastigotes.

Promastigotes of *L. amazonensis* (MHOM/BR/71973/M2269) were grown on a 24-well plates in Schneider's Drosophila medium (Sigma, USA) supplemented with 10.0% (v/v) heat-inactivated fetal bovine serum and 1.0% penicillin (10000UI/mL)/streptomycin (10.0 mg/mL) (Sigma, USA). Cells were harvested in the log phase, resuspended in fresh medium, counted in Neubauer's chamber and adjusted to a concentration of 1 × 10^6 cells/mL, using 24-wells plates. Compounds **10**–**25** (Fig. 2) were added to promastigote cultures (1 × 10^6 cells/mL) in the range of 0.10–40.00 μg/mL, solubilized in dimethylsulfoxide (DMSO) (0.6%, v/v in all wells) and incubated at 25 °C. After 72 h of incubation, the surviving parasites were counted in a Neubauer's chamber and compared with controls, with just DMSO in concentration of 0.6% v/v, for the

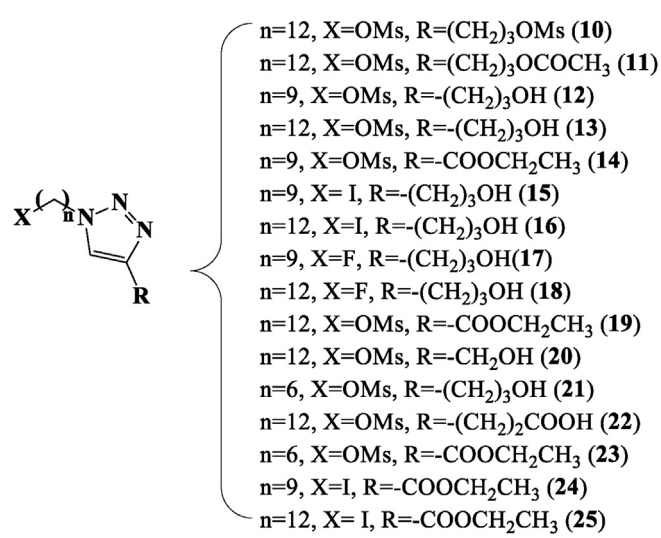


Fig. 2. Alkyltriazoles **12**–**25** obtained by Gontijo [16] and the new compounds **10** and **11**.

determination of 50.0% inhibitory growth concentration (IC_{50}). All tests were performed in triplicate on three different times and amphotericin B (Sigma) was used as the reference drug. Animal procedures were performed with the approval of the Research Ethics Commission from Universidade Federal de Alfenas under number 600/2014 in agreement with the Guidelines for the Care and Use of Laboratory Animals.

2.3.1.2. Leishmanicidal activity against amastigotes. Murine peritoneal macrophages were maintained in RPMI 1640 medium (Sigma, USA) supplemented with 10.0% heat-inactivated fetal bovine serum at 37 °C in 5.0% CO_2 incubator. Cells were cultured in 24-well plates chamber on the glass slides of 13 mm (Nunc, USA) in a 8×10^5 cells density per well and infected with late log-phase promastigotes at a ratio of 10:1 (parasite/macrophage) and incubated at 37 °C in 5.0% CO_2 incubator for 24 h. Non-phagocytosed promastigotes were removed by washing, and compounds **4**, **10, 11, 13, 14**, **16**, **18** and **25** solubilized in DMSO (from 0.10 to 40.00 μg/mL) were administered at the concentration of 0.6% v/v. After 72 h, chamber slides were fixed in absolute methanol, stained with 10.0% Giemsa and examined on an Optical Light Microscope in oil immersion. The percentage of infected cells per well was calculated taking account at least 200 macrophages. The ratio of inhibition (IC_{50} value) was calculated in comparative to the control only with DMSO. All assays were performed in triplicate on three different times using pentamidine (Sigma) and amphotericin B (Sigma) as the reference drugs [16].

2.3.1.3. Cytotoxicity evaluation. A suspension of 8×10^5 murine peritoneal macrophages, in RPMI 1640 medium, supplemented with 10.0% heat-inactivated fetal bovine serum and 1.0% penicillin (10000 UI/ml)/streptomycin (10 mg/mL) were added to each well in 24-well plates, on the glass slides of 13 mm. The plates were incubated in a 5.0% CO_2 air mixture at 37 °C to adhesion of the cells. After 24 h, the non-adherent cells were removed by washing with the RPMI 1640 medium. Then, several concentrations of compounds **4**, **10, 11, 13, 14**, **16**, **18, 25** and reference drugs ranging from 0.10 to 40.00 μg/mL in DMSO at the final concentration of 0.6% v/v) were added to the wells containing the cells and the plates were incubated for more 72 h. The non-adherent cells were removed by washing with the RPMI 1640 medium. Afterwards, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was solubilized in PBS (5.0 mg/mL) as solvent. Fifty microliter of this solution was added to RPMI 1640 medium in a final volume of 500.0 uL per well and incubated for 4 h [24]. Then, the medium was removed and 600.0 μL of DMSO was added to each well and homogenized for 15 min. Next, the absorbance of each individual well was calculated at 570 nm according to the following formula (OD represents optical density) using the Equation (1):

$$\%\ \text{inhibition} = \left(\frac{OD_{control} - OD_{compounds}}{OD_{control} \times 100}\right) \quad (1)$$

Each experiment was performed in triplicate on three different occasions, and the percentage of viable cells was calculated taking into account the cell culture control (medium + cells + DMSO 0.6% v/v). The 50.0% cytotoxicity concentrations (CC_{50}) were determined and the selectivity factors (SF) established by the ratio between the values of CC_{50} and IC_{50} for amastigote forms.

2.3.2. Antiproteolytic activity

2.3.2.1. Inhibitory activity against isoform r-CPB2.8. The compounds **6**, **7**, **11**, **13**, **14**, **16**, **18** and **25** were tested for their potential inhibitory r-CPB2.8, inhibitory IC_{50}. The r-CPB2.8 was expressed, purified and activated as previously described [25,26]. The concentration of the enzyme stock solution was determined by active-site titration with human cystatin C, which was a generous gift from M. Abrahamson (University of Lund, Sweden), using Z-Phe-Arg-MCA as substrate. Hydrolysis of the fluorogenic peptide substrate by enzyme was carried out in 0.1 M sodium acetate, pH 5.5 at 37 °C with previous activation of enzymes by 5 mM DTT for 10 min. The substrate hydrolysis was monitored by measuring the fluorescence at $\lambda_{em} = 380$ nm with excitation at $\lambda_{ex} = 460$ nm using a Hitachi F-2500 spectrofluorometer. The IC_{50} values were determined by non-linear regression using the GraFit 5.0 software (Erithacus Software Ltda) using the Equation (2).

$$y = \frac{Range}{1 + \left(\frac{x}{IC_{50}}\right)^s} \quad (2)$$

where *Range* is the fitted uninhibited value, *y* is the enzyme activity, *x* is the inhibitor concentration, and *s* is a slope factor. The equation assumes that *y* falls with increasing *x*.

2.3.3. Mutagenicity studies

2.3.3.1. Cytokinesis-block micronucleus assay (chromosomal mutation analysis). The cytokinesis-block micronucleus assay was performed in CHO–K1 cell line, to assess the potential of the synthetic compounds to induce DNA damage (chromosomal mutations) *in vitro*. The procedures were developed as described by Fenech [27,28], with adaptations. Briefly, the cells were seeded in 24-well plates (2.5×10^5 cells/well) and maintained at 37 °C in a humid atmosphere with 5% CO_2 in culture medium Ham F-12 with serum. After 24 h, the cells were washed twice with PBS, and the treatments were performed in culture media without serum for 3 h. Each treatment was performed in triplicate. The negative control group was treated with culture media without serum, and a positive control group was established with the treatment of the cells with methyl methanesulfonate (MMS-400 μM).

After completing treatments with three concentrations lower than the IC_{50} of the compounds **13** (6.41, 12.82, and 25.63 μM) and **14** (14.51, 29.02 and 58.04 μM) diluted in culture media, the cells were washed twice with PBS, trypsinized and centrifuged for 5 min at 1500 rpm. The pellet was then resuspended in chilled hypotonic solution (1% sodium citrate) together with one drop of 1% formaldehyde and carefully homogenized with a Pasteur pipette. This cell suspension was centrifuged for 5 min at 1500 rpm and resuspended in 2 mL of fixative, methanol/acetic acid (3:1 *v*/*v*). Next, the tubes were centrifuged for 5 min, and the supernatant was discarded; the cell suspension was spread onto slides previously cleaned and covered with a film of chilled distilled water.

At the moment of cytogenetic analysis, the slides were stained with DAPI (4′,6-diamidino-2-phenylindole) diluted in phosphate buffer (0.06 M Na_2HPO_4 and 0.06 M KH_2PO_4, pH 6.8) for 2 min, washed with distilled water and analysed under a fluorescent microscope (Zeiss, Axioscope) with an excitation filter of 365 nm and a barrier filter of 445/450 nm. One thousand cells with a well-preserved cytoplasm were analysed for each treatment in a blind test. Cells containing 1–4 micronuclei were scored. The criterion for the identification of MNs was according to a previous report [28].

2.3.3.2. Nuclear division index (NDI). The influence of the alkyltriazoles **13** and **14** on cell division was assessed by calculating the Nuclear division index (NDI) in CHO–K1 cells. The same slides prepared for the micronucleus assay were used, and 300 cells with a well-preserved cytoplasm were counted using fluorescence microscopy, as described above. The NDI was calculated according Eastmond and Tucker [29]:

$$NDI = (M1 + 2(M2) + 3(M3) + 4(M4))/N$$

where M1–M4 represent the number of cells with one to four nuclei and N is the total number of viable cells scored. The NDI and the proportion of binucleated cells are useful parameters for comparing the mitogenic response of cells and cytostatic effects of agents examined in the assay [27,28].

2.3.3.3. Ames mutagenicity assay (gene mutation analysis). The Ames mutagenicity assay was performed using the micro-suspension procedure modified by Kado et al. [30] and revised by Mortelmans and Zeiger [31]. This protocol is a modification of the traditional Ames test and provided to the assay a higher sensitivity than the original methodology. Overnight culture of *Salmonella typhimurium* strains TA98, TA100, TA97a and TA102 (1×10^9 cells/mL) was concentrated by centrifugation (4000× *g* at 4 °C) for 15 min and resuspended in 0.2 M sodium phosphate buffer pH 7.4. After, 50 μL of each concentrate overnight bacterial culture (1×10^{10} cells/mL) were mixed with 50 μL of 0.2 M sodium phosphate buffer and five concentrations of the compounds in a test tube. Compound **13** was evaluated in concentrations ranging between 150.98 and 700.00 μM and compound **14** was assessed in concentrations between 313.73 and 1454.55 μM. The mixture was incubated at 37 °C for 90 min without shaking. After this period, 2.5 mL molten surface agar (0.6% agar, 0.5% NaCl, 0.5 mM L-histidine, 0.5 mM biotin, pH 7.4, 44 °C) was added to the tube and this mixture was poured into a Petri plate containing minimal agar (1.5% agar, Vogel–Bonner E medium, containing 10% glucose). The Petri plates were incubated at 37 °C for 72 h, and the His + revertant colonies were counted. For tests with metabolic activation, the sodium phosphate buffer was replaced by 50 μL of S9 fraction, before pre-incubation.

To establish negative control, was used dimethyl sulfoxide (DMSO) for all strains. The positive controls used for this assay in the absence and presence of S9 fraction, respectively, were 4-nitro-o-phenylenediamine (NPD) and 2-aminoanthracene (2AA), for TA98 and TA97a; sodium azide (SA) and 2AA for TA100; and mitomycin C (MitC) and 2AA for TA102.

The concentrations used were based on the bacterial toxicity of each preparation, estimated in a preliminary test. In all subsequent assays, the upper limit of the dose range tested was either the highest non-toxic dose or the lowest toxic dose determined in this preliminary assay. Toxicity was apparent either as a reduction in the number of His + revertants, or as an alteration in the auxotrophic background (i.e. background lawn).

2.4. Statistical analysis

The leishmanicidal activities of compounds were expressed as the concentration that inhibits the growth of 50.0% of protozoan form. Statistical analysis was performed using nonlinear regression to obtain the values of IC_{50} and CC_{50} (cytotoxic concentration for 50.0% of macrophages), followed by variance analyses and Tukey's test. Differences were significant when the p value was lower than 0.05.

Statistical analysis from antiproteolytic activity, cytokinesis-block micronucleus assay and index of nuclear division assays were performed using Grafit 5.0. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Values of $p < 0.05$ were considered statistically significant. Data are expressed as mean ± standard deviation (SD) unless otherwise specified.

For Ames assays, the statistical analysis was performed with the Salanal software, adopting the Bernstein et al. [32] model. The mutagenic index (MI), which is the average number of revertant mutants per plate for treated groups divided by the average number of revertant mutants per plate for the negative control (solvent), was calculated for each concentration.

2.5. Evaluation of lipophilicity by LogP (oct/water)

Lipophilicity values were estimated through theoretical determination of cLogP (oct/wat) by using the ChemDraw Ultra version 11.0 program. Calculated lipophilicity expressed by cLogP (oct/wat) of compounds **4**, **10**, **11**, **13**, **14**, **16**, **18** and **25** are showed on Table 2.

3. Results and discussion

3.1. Synthesis

There are many protocols in the literature for the synthesis of alkylphospholipid analogues [33,34]. The best procedure we had access to was using the calcium salt of phosphorylcholine **1** as starting material (Scheme 1). The phosphate tetra-n-butylammonium **2** was prepared by treatment of **1** with an aqueous solution of oxalic acid followed by titration of a phosphoric acid derivative obtained with tetra-n-butylammonium hydroxide [35]. To obtain the compounds **3** and **4**, a solution of **2** in acetonitrile was treated with alkylating compounds (**8a** or **8b**), producing the alkylphosphocholines **3** and **4**, with 19% yield after purification on silica gel.

The synthesis of **8a** and **8b** compounds is depicted in Scheme 2. The mesylate compounds (**8a**–**b**) were prepared using 1,9-nonanediol or 1,12-dodecanediol as starting materials [16].

Compound **8b** in dichloromethane was treated with a solution of copper sulphate pentahydrate (8 mol%) and sodium ascorbate (20 mol%) in water. The reaction mixture was stirred for 24 h at room temperature, exclusively producing a high yield of the 1,4-disubstituted 1,2,3-triazole **9**. To obtain the alkyltriazoles **10** and **11**, a solution of alkyltriazoles **9** (Scheme 3) in acetone was treated with 4-DMAP and acetic anhydride. The reaction mixture was stirred for 24 h at room temperature, exclusively producing the alkytriazole **11**, with 73% yield. To obtain the compound **10**, a

Table 1
Leishmanicidal activity *in vitro* against promastigote forms of *Leishmania amazonensis* compared to amphotericin B.

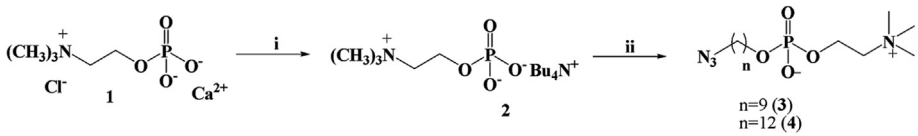
Compounds	Promastigotes[a] IC_{50} (μM)
2	141.20 ± 9.49^{A}
3	136.38 ± 14.65^{A}
4	$25.56 \pm 1.89^{B,C}$
11	4.01 ± 0.38^{H}
10	$30.34 \pm 0.34^{B,C}$
12	82.23 ± 16.55^{D}
13	$28.52 \pm 0.73^{B,C,F}$
14	$37.17 \pm 4.5^{B,F}$
15	$67.68 \pm 8.73^{D,E}$
16	19.26 ± 6.52^{C}
17	81.73 ± 26.18^{D}
18	37.06 ± 0.86^{B}
19	$75.89 \pm 5.26^{D,E}$
20	$51.70 \pm 0.29^{D,F}$
21	229.35 ± 13.19^{G}
22	225.31 ± 42.37^{G}
23	219.49 ± 15.44^{G}
24	$47.64 \pm 1.53^{E,F}$
25	$27.95 \pm 9.62^{B,C}$
Amphotericin B	4.70 ± 0.36^{H}

Same letters = non-statistical difference ($p < 0.05$) by Tukey's test.
[a] Each IC_{50} value represents the mean ± standard deviation of triplicate determined by the software Grafit 5.0.

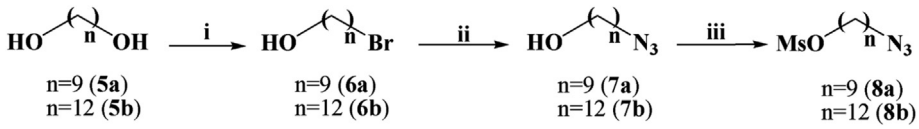
Table 2
Leishmanicidal activity *in vitro* against amastigote forms of *Leishmania amazonensis*, cytotoxicity, selectivity index values, cLogP, and r-CPB2.8 enzyme of *L. (L) mexicana* data of compounds **4**, **10**, **11**, **13**, **14**, **16**, **18,** and **25** compared to pentamidine, amphotericin B, and E64.

Compounds	Amastigotes[a] IC_{50} (μM)	Macrophages[b] CC_{50} (μM)	Selectivity index (SI)*	cLogP[c]	r-CPB2.8[d] IC_{50} (μM)
4	3.81 ± 0.1^{A}	128.62 ± 10.99	33.76	6.90	8.3474 ± 0.5543^{A}
11	56.1 ± 4.3^{D}	26.55 ± 1.06	2.11	2.85	1.0226 ± 0.1761^{B}
10	70.25 ± 5.8^{C}	156.2 + 9.61	2.14	3.54	4.5309 ± 0.2315^{E}
13	14.25 ± 0.92^{B}	163.4 ± 14.85	11.47	3.31	8.1841 ± 0.7048^{A}
14	76.68 ± 4.76^{C}	983.34 ± 37.98	12.82	2.23	0.8571 ± 0.0269^{B}
16	23.67 ± 5.17^{B}	66.15 ± 5.26	2.79	5.50	$1.2220 \pm 0.1005^{B,C}$
18	15.05 ± 4.03^{B}	162.81 ± 16.56	9.83	4.47	2.2605 ± 0.0739^{C}
25	17.67 ± 0.88^{B}	93.35 ± 8.56	5.28	5.68	6.0772 ± 0.7588^{D}
Pentamidine	6.62 ± 0.03^{A}	11.22 ± 1.3	1.69	–	–
Amphotericin B	6.10 ± 1.0^{A}	27.10 ± 2.1	4.44	–	–
E64	–	–	–	–	0.125 ± 0.006

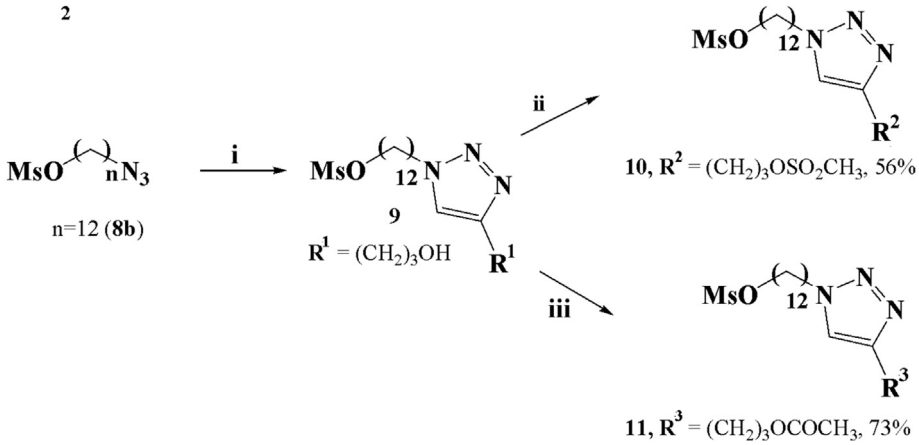
nd = not determined.
*SI = ratio $CC_{50(Macrophages)}/IC_{50(Amastigotes)}$.
Same letters = non-statistical difference ($p < 0.01$) by Tukey's test.
[a] Concentration for decrease of 50% infected macrophages in treated vs. non-treated wells.
[b] Cytotoxicity concentration for 50% macrophages.
[c] Calculated lipophilicity expressed as cLogP (oct/wat) by using ChemDraw Ultra program version 11.0.
[d] Each IC_{50} value represents the mean ± standard deviation of triplicate determined by the software Grafit 5.0.



Scheme 1. Reagents and conditions: (i) Oxalic acid, tetra-n-butylammonium hydroxide, pH 9; (ii) methanesulfonate alkylazide (**8a** or **8b**), acetonitrile, rt., 24 h and reflux, 3 h; 19%.



Scheme 2. Reagents and conditions: (i) HBr (48%), toluene, 110 °C, 24 h, 65–87%; (ii) NaN_3, DMSO, rt, 24 h, 48–85%; (iii) CH_2Cl_2, methanesulfonyl chloride, triethylamine, rt, 24 h, 37–90%.



Scheme 3. Reagents and conditions: (i) NaAsc (20 mol%), $CuSO_4 \bullet 5H_2O$ (8 mol%), pent-4-yn-1-ol, CH_2Cl_2:H_2O (1:1), rt., 24 h, 46–93%; (ii) 4-DMAP, acetic anhydride, acetone, rt., 24 h, 73%; (iii) CH_2Cl_2, methanesulfonyl chloride, triethylamine, rt, 24 h, 56%.

solution of alkyltriazoles **9** in dichloromethane was treated with mesyl chloride and triethylamine. The reaction mixture was stirred for 24 h at room temperature, producing the alkytriazole **10**, with 56% yield.

The triazoles **12**–**25** were obtained as previously described by Gontijo et al. (2014) [16].

All synthesized compounds were initially evaluated *in vitro* for their antileishmanial activities against promastigote forms of *Leishmania amazonensis*. The compounds showed IC_{50} values ranging from 4.01 ± 0.38 μM (**11**) to 229.35 ± 13.19 (**21**). Only eight compounds (**4**, **10**, **11**, **13**, **14**, **16**, **18,** and **25)** exhibited inhibitory activities up to the limit of ten times less than amphotericin B's IC_{50} (Table 1).

The eight compounds that were found to be more active against promastigote forms were also tested against the non-motile amastigote forms of *Leishmania amazonensis.* The IC_{50} values of the active alkyltriazole compounds (**10**, **11**, **13**, **14**, **16**, **18,** and **25**) and of alkylphosphocholine derivative **4**, pentamidine, and amphotericin B, are listed in Table 2. The cytotoxic effects of all compounds against murine peritoneal macrophages (CC_{50}) were also evaluated to determine the Selectivity Index (SI).

Similar to previous observations [35], the leishmanicidal activity of the compounds against promastigotes was substantially different from the activity detected against amastigotes. This confirms that it is not appropriate to extrapolate the activities obtained for one form of the parasite to the other. The modulation of cell-mediated response and the cellular and biochemical pathways of amastigotes differ considerably from those of promastigotes, suggesting that the chemotherapeutic potential of anti-leishmanial drugs depend on their action against amastigotes.

Among the compounds tested, the phospholipid derivative **4** was the most potent against amastigote forms of *Leishmania amazonensis*, with an IC_{50} of 3.81 μM. Its IC_{50} shows that its activity is comparable to that of the control drugs pentamidine (IC_{50} = 6.62 μM) and amphotericin B (IC_{50} = 6.10 μM). Additionally, this compound was found to be the least toxic to human macrophages, with a CC_{50} of 128.62 μM and folds more selective (SI = 33.76) than the standard drugs pentamidine (CC_{50} = 11.22 μM, SI = 1.69) and amphotericin B (CC_{50} = 27.10 μM, SI = 4.44). Considering the SI, expressed as the ratio of cytotoxicity (CC_{50}) and antileishmanial potency (IC_{50}), the compound **4** exhibited the best biological profile. This result is very important for developing novel

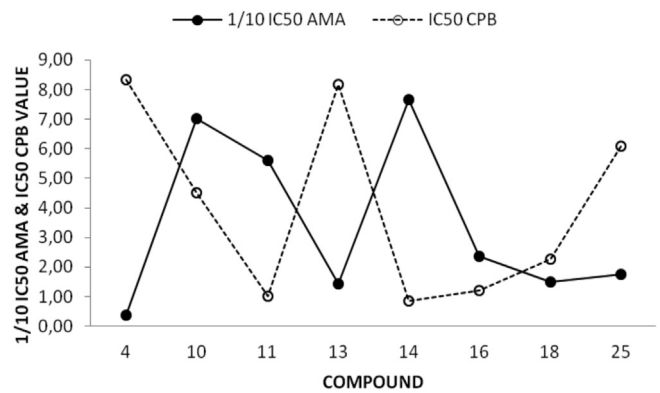


Fig. 3. Relation between leishmanicidal effect against amastigote forms (1/10 IC50 AMA) and antiproteolytic activity for cysteine protease r-CPB2.8 (IC50 CPB) for compounds **4**, **10, 11, 13, 14**, **16**, **18,** and **25**.

phospholipid antileishmanial drugs. A large number of studies have reported the synthesis and biological screening of several ether phospholipids [36–42] derivatives for their *in vitro* leishmanicidal activity and citotoxicity. Avlonitis et al. [36] described the synthesis of a series of ring-substituted ether phospholipids and the evaluation of antileishmanial activity of the new compounds against the promastigote forms of *Leishmania donovani* and *Leishmania infantum*. Among the 37 compounds tested, 14 were more potent than miltefosine (IC_{50} 22.56 μM) against *L. donovani*, but only two with IC_{50} lower than 3.81 μM. Cogghi et al. [42] also obtained 18 analogues of ether-linked phospholipids, but all compounds exhibited IC_{50} values higher than miltefosine against amastigote forms of *L. donovani*. As observed by many authors [36–38], the length of alkyl chain on alkylphospholipid derivatives demonstrates to be important for the activity of this class of compounds. This could explain the higher activity of **4** (n = 12) in comparison with compound **3** (n = 9) against both promastigote and amastigote stages of *L. amazonensis* in the present study.

Among the alkyltriazole compounds obtained, the compound **13** was the more potent against amastigote forms of *L. amazonensis* (14.25 μM, SI = 11.47), followed by compound **18** (15.05 μM, SI = 9.83), and compound **25** (17.67 μM, SI = 5.28) (Table 2). For compounds **13, 18** and **25**, the length of carbon long-chain is the

Table 3
Absence of mutagenicity in *Salmonella typhimurium* strains, without (−S9) and with (+S9) metabolic activation. Data are shown as revertants/plate, standard deviation, and mutagenicity index (in parentheses), after treatment with five different concentrations of compound **13**.

Treatment (μM/plate)	TA98		TA100		TA97a		TA102	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0[a]	41.00 ± 8.54	34.33 ± 3.06	164.33 ± 23.43	181.33 ± 17.04	135.50 ± 3.54	128.67 ± 4.51	313.33 ± 23.86	272.67 ± 42.34
150.98	40.67 ± 5.03 (1.0)	39.33 ± 8.33 (1.1)	174.67 ± 18.82 (1.1)	162.67 ± 13.50 (0.9)	175.50 ± 10.61 (1.3)*	119.33 ± 23.86 (0.9)	353.33 ± 17.93 (1.1)	307.33 ± 12.06 (1.1)
296.15	47.33 ± 5.03 (1.2)	38.33 ± 7.02 (1.1)	210.50 ± 7.78 (1.3)	163.00 ± 8.72 (0.9)	151.00 ± 9.54 (1.1)	112.33 ± 27.23 (0.9)	267.33 ± 18.15 (0.9)	354.67 ± 37.87 (1.3)
435.85	46.67 ± 9.29 (1.1)	39.00 ± 4.00 (1.1)	201.67 ± 33.56 (1.2)	126.00 ± 14.53 (0.7)	157.00 ± 8.00 (1.2)*	114.33 ± 10. 69 (0.9)	278.00 ± 8.00 (0.9)	312.00 ± 21.07 (1.1)
570.37	55.33 ± 5.86 (1.3)	29.00 ± 9.54 (0.8)	207.67 ± 23.29 (1.3)	141.00 ± 7.55 (0.8)	124.00 ± 10.82 (0.9)	132.33 ± 17. 01 (1.0)	298.67 ± 26.03 (1.0)	388.67 ± 21.50 (1.4)*
700.00	53.67 ± 5.51 (1.3)	40.33 ± 40.33 (1.2)	164.50 ± 2.12 (1.0)	159.00 ± 31.48 (0.9)	141.00 ± 4.24 (1.1)	109.00 ± 13.45 (0.8)	306.00 ± 25.06 (1.0)	376.33 ± 3.21 (1.4)*
Ctrol+	1350.00 ± 413.14[b]	812.00 ± 149.29[c]	2808.00 ± 344.43[d]	1019.00 ± 144.65[c]	1101.33 ± 169.77[b]	803.33 ± 164.08[c]	1828.67 ± 583.01[e]	2512.00 ± 325.00[c]

*Representation of a significant response ($P \leq 0.01$).
[a] Negative control (DMSO − 10 μL).
[b] NPD (10 μg/plate).
[c] 2AA (5 μg/plate).
[d] SA (5 μg/plate).
[e] MitC (0.5 μg/plate).

Table 4
Absence of mutagenicity in *Salmonella typhimurium* strains, without (−S9) and with (+S9) metabolic activation. Data are shown as Revertants/plate, standard deviation and mutagenicity index (in parentheses), after treatment with five different concentrations of compound **14**.

Treatment (μM/plate)	TA98		TA100		TA97a		TA102	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0[a]	41.00 ± 8.54	34.33 ± 3.06	164.33 ± 23.43	181.33 ± 17.04	117.33 ± 5.86	128.66 ± 4.51	295.33 ± 11.24	272.67 ± 42.34
313.73	61.67 ± 5.77 (1.5)	34.00 ± 6.56 (1.0)	193.00 ± 37.40 (1.2)	167.67 ± 27.02 (0.9)	151.00 ± 6.56 (1.3)**	102.67 ± 4.73 (0.8)	347.33 ± 41.00 (1.2)	376.00 ± 9.17 (1.4)*
616.38	50.67 ± 5.77 (1.2)	28.00 ± 2.65 (0.8)	200.00 ± 36.77 (1.3)	173.50 ± 19.09 (1.0)	192.00 ± 7.55 (1.6)**	114.33 ± 26.58 (0.9)	311.33 ± 59.00 (1.1)	446.33 ± 12.66 (1.7)**
905.66	47.67 ± 3.51 (1.2)	33.33 ± 5.86 (1.0)	197.00 ± 0 (1.2)	177.50 ± 28.99 (1.0)	207.00 ± 7.02 (1.8)**	117.33 ± 6.11(0.9)	258.67 ± 40.07 (0.9)	364.33 ± 34.27 (1.3)
1185.19	63.00 ± 15.72 (1.5)	27.33 ± 2.08 (0.8)	217.00 ± 31.11 (1.3)	157.33 ± 13.58 (0.9)	155.00 ± 17.09 (1.3)	95.00 ± 5.29 (0.7)	364.00 ± 37.04 (1.2)	389.33 ± 14.29 (1.4)*
1454.55	56.67 ± 6.11 (1.4)	26.67 ± 9.45 (0.8)	192.33 ± 8.39 (1.2)	168.00 ± 14.18 (0.9)	159.33 ± 15.04 (1.4)*	105.67 ± 5.13 (0.8)	331.00 ± 13.61 (1.1)	326.00 ± 22.52 (1.2)
Crtol+	1350.00 ± 413.14[b]	812.00 ± 149.29[c]	2808.00 ± 344.43[d]	1019.00 ± 144.65[b]	924.00 ± 104.23[c]	803.33 ± 164.08[d]	2714.50 ± 284.96[e]	2512.67 ± 325.00[c]

**Representation of a significant response ($P \leq 0.01$).
*Representation of a significant response ($P \leq 0.05$).
[a] Negative control (DMSO − 10 μL).
[b] NPD (10 μg/plate).
[c] 2AA (5 μg/plate).
[d] SA (5 μg/plate).
[e] MitC (0.5 μg/plate).

same (n = 12), which suggest the importance of this structural feature for activity (compare **12** versus **13**, for example). Additionally, the substituent in the position 4 of triazole nucleus also contains only three carbon atoms. A few studies have described the synthesis and leishmanicidal activity of triazoles compounds in the last decade. Corrales et al. [39] synthesized a series of novel 6-thiopurine derivatives containing 1,2,3-triazoles and evaluated the leishmanicidal activity against promastigote forms of *L. amazonensis*. Neither compound was more active than amphotericin B, used as control. Ferreira et al. [40] also synthesized and evaluated new triazole compounds carrying either the carbaldehyde or the difluoromethylene functionalities against promastigote forms of *L. amazonensis*. Neither compound was more active than pentamidine, used as control. Tahghighi et al. [41] synthesized 5-(5-nitrofuran-2-yl)-1,3,4-thiadiazol-2-amines containing triazole moieties to examine the leishmanicidal potency. The anti-promastigote activity against *Leihsmania major* ranged between 12.2 μM and 107.7 μM. In sum, compared to these examples, the structurally simple long-chain alkyltriazoles described in our work are attractive because they are easy to synthesize and may

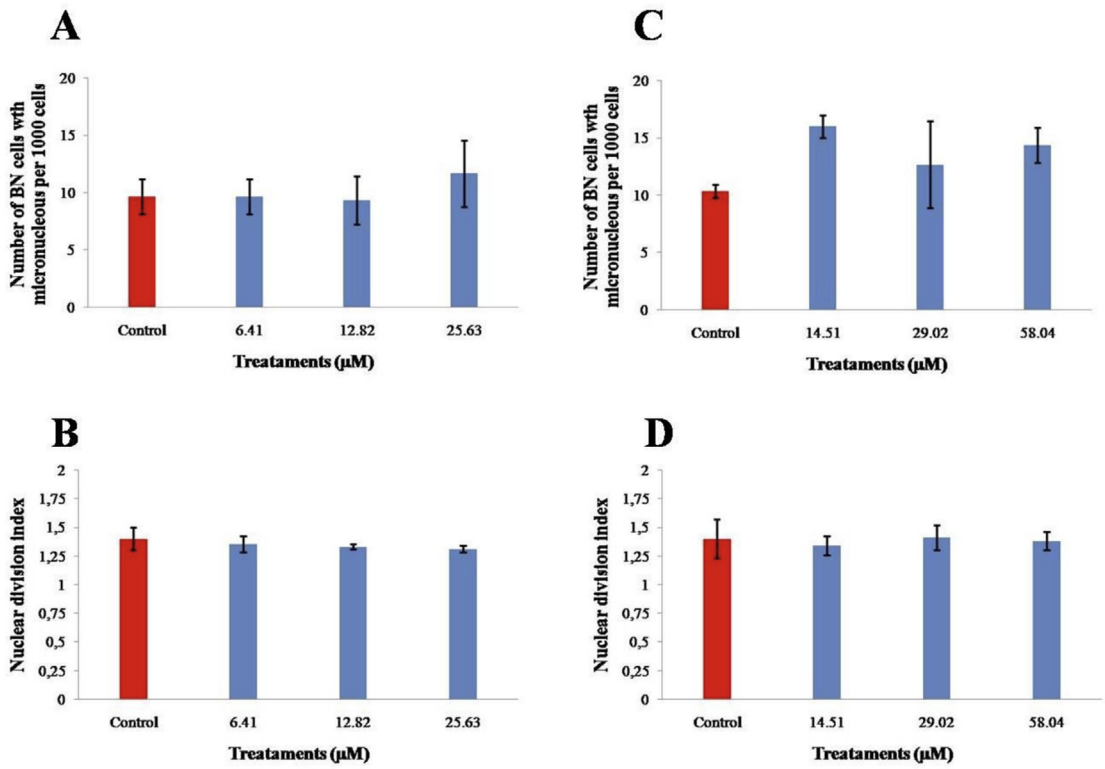


Fig. 4. Results obtained in cytokinesis-block micronucleus assay performed with CHO−K1 cells treated with different concentrations of compounds **13** and **14**.

represent a new and promising scaffold for the development of new leishmanicidal substances.

In order to investigate a possible mechanism of action, the antiproteolytic activity of compounds **4**, **10, 11, 13, 14**, **16**, **18,** and **25** against the *L. (L) mexicana* cysteine protease r-CPB2.8 was evaluated. The results are shown in Table 2 and Fig. 3. The compound **14** was observed to be the most potent inhibitor with an IC_{50} value of 0.8571 μM; however, it was almost seven-fold less active than the standard compound E64 (IC_{50} = 0.125 μM). We observed that almost all compounds exhibited an inverse correlation between anti-amastigote and antiproteolytic activities as shown in Fig. 3, since a smaller IC_{50} enzyme inhibition value consistently correlates with a greater IC_{50} anti-amastigote action. This led to the speculation that the compounds' proteolytic action is not essential to avoid macrophage infection by amastigote forms.

According to Abad-Zapatero and Metz [43], potent compounds do not necessarily result in promising drugs and other fundamental parameters can be related to optimal pharmacological properties. As observed in Table 2, compounds **4**, **13,** and **14** presented the best SI values of the series. Though compound **4** exhibits potent activity against amastigotes, its cLogP value is higher than 5, indicating high lipophilicity. Hann [44] affirms that compounds with this characteristic are correlated with poor oral drug-like properties, have more difficulty being excreted, and tend to be more toxic. For instance, in a series of compounds, increased microsomal clearance and pharmacological promiscuity are often associated with higher cLogP values, while limited cell permeation and absorption are linked with low lipophilicity. To achieve a compromise between absorption and first-pass clearance, a cLogP value between 2 and 3 is often considered optimal in an oral drug program [45]. Because of this, only compounds **13** and **14**, which presented promising properties as leishmanicidal agents having SI values higher than 10 and cLogP values between 2 and 3, were evaluated for their mutagenicity *in vitro* by employing the Ames assay (gene mutation analysis) and the micronucleus assay (chromosomal mutation analysis). As observed in Tables 3 and 4 and Fig. 4, these compounds did not induce DNA damage in the biological systems used in this study. The Ames Microsuspension Test verifies that, in some conditions, compounds **13** and **14** increased the frequency of revertant colonies in comparison with the negative control. However, all the mutagenicity index (MI) values were lower than 2, which show that these compounds were not mutagenic.

As observed, the micronucleus frequency in 1000 binucleated cells treated with all concentrations of compounds **13** and **14** was not statistically higher than the values identified in the control group, indicating absence of mutagenic activity at this concentration (shown in A and C, respectively). B and D show the influence of treatments in the nuclear division process (compounds **13** and **14**, respectively). There were no significant differences observed in the Nuclear division index (NDI) values when compared with that of the control group.

Evaluation of mutagenicity for compounds **13** and **14** is also important because, as affirmed in a previous work [16], these compounds were effective against HeLa (human uterine tumour) and RKO (human colon tumour) cell lines, with pro-apoptotic effect induced by the compound **13** in RKO [16].

4. Conclusions

In summary, a series of alkyltriazoles and two new alkylphosphocholines were evaluated for their *in vitro* inhibitory activity against the *Leishmania* parasite. Several target compounds exhibited moderate anti-leishmanial activity against the promastigote forms of *Leishmania amazonensis*. The most cytotoxic compound against promastigotes was found to be the compound **11** and, against amastigote form, the compound **4**. Considering the lipophilicity aspect of the assessed compounds, compound **13** presented the best characteristics, which were low IC_{50} in promastigotes and amastigotes, low toxicity to murine macrophages (high Selectivity Index), and absence of mutagenicity. These compounds could represent a promising template for developing a new class of leishmanicidal agents, and deserve further investigation of derived scaffolds.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.06.005.

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