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Imidazolium salts as an alternative for anti-Leishmania drugs: Oxidative and immunomodulatory activities

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In this study we explored the previously established leishmanicidal activity of a complementary set of 24 imidazolium salts (IS), 1-hexadecylimidazole (C_{16}Im) and 1-hexadecylpyridinium chloride (C_{16}PyrCl) against *Leishmania (Leishmania) amazonensis* and *Leishmania (Leishmania) infantum chagasi*. Promastigotes of *L. amazonensis* and *L. infantum chagasi* were incubated with 0.1 to 100 µM of the compounds and eight of them demonstrated leishmanicidal activity after 48 h – C_{10}MImMeS (IC_{50} *L. amazonensis* = 11.6), C_{16}MImPF$_6$(IC_{50} *L. amazonensis* = 6.9), C_{16}MImBr (IC_{50} *L. amazonensis* = 6), $C_{16}M_2$ImCl (IC_{50} *L. amazonensis* = 4.1), $C_{16}M_4$ImCl (IC_{50} *L. amazonensis* = 1.8), $(C_{10})_2$MImCl (IC_{50} *L. amazonensis* = 1.9), C_{16}Im (IC_{50} *L. amazonensis* = 14.6), and C_{16}PyrCl (IC_{50} *L. amazonensis* = 4).The effect of IS on reactive oxygen species production, mitochondrial membrane potential, membrane integrity and morphological alterations of promastigotes was determined, as well as on *L. amazonensis*-infected macrophages. Their cytotoxicity against macrophages and human erythrocytes was also evaluated. The IS C_{10}MImMeS, C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl, and the compounds C_{16}Im and C_{16}PyrCl killed and inhibited the growth of promastigote forms of *L. amazonensis* and *L. infantum chagasi* in a concentration-dependent manner, contributing to a better understanding of the structure-activity relationship of IS against *Leishmania*. These IS induced ROS production, mitochondrial dysfunction, membrane disruption and morphological alterations in infective forms of *L. amazonensis* and killed intracellular amastigote forms in very low concentrations (IC_{50} amastigotes ≤ 0.3), being potential drug candidates against *L. amazonensis*.

KEYWORDS

imidazolium salt, *L. amazonensis*, *L. infantum chagasi*, oxygen reactive species, leishmanicidal activity

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1 Introduction

Leishmaniasis are endemic in 102 countries or territories with around 1.3 million new cases and 20.000 to 30.000 deaths yearly (1). Brazil is one of the countries with the highest incidence of leishmaniasis (2). *Leishmania (Leishmania) amazonensis* is an important etiological agent of cutaneous, anergic diffuse cutaneous and less commonly mucocutaneous leishmaniasis, especially in South America (3, 4), and *Leishmania (Leishmania) infantum chagasi* causes visceral leishmaniasis, mainly in the Mediterranean Basin, Middle East, Central Asia and South America (4).

Currently, there is no vaccine for leishmaniasis, and their treatment is based on very toxic and poorly tolerated drugs, such as pentavalent antimonials and amphotericin B (5). Azole antifungals presented leishmanicidal activity against some *Leishmania* species (6). Moreover, various biological activities have been described for the cationic equivalent of imidazoles, imidazolium salts (IS), including antimicrobial (7), antifungal (8, 9), larvicidal (10), antitumoral, antioxidant and others (11). The IS present high structural variability and tunable interactions with biomolecules like phospholipids and proteins, especially because their amphiphilic nature (11), becoming potential candidates in medicinal chemistry (12). Recently, our group described for the first time the leishmanicidal activity of IS (13). Within the tested IS, 1-methyl-3-octadecylimidazolium chloride (C_{18}MImCl), 1-hexadecyl-3-methylimidazolium methanesulfonate (C_{16}MImMeS), 1-hexadecyl-3-methylimidazolium chloride (C_{16}MImCl), 1-decyl-3-methylimidazolium chloride (C_{10}MImCl) and 1-hexadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide (C_{16}MImNTf$_2$) were identified as potent agents against both promastigote and amastigote forms of *L. amazonensis*. Here, we further explored this leishmanicidal action with a structurally complementary set of 24 IS, 1-hexadecylimidazole (C_{16}Im) and 1-hexadecylpyridinium chloride (C_{16}PyrCl) (Figure 1) to (i) investigate the structure-activity relationship of these molecules on promastigote and amastigote forms of *L. amazonensis* and promastigote forms of *L. infantum chagasi*, (ii) identify mortality pathways, and (iii) evaluate cytotoxicity.

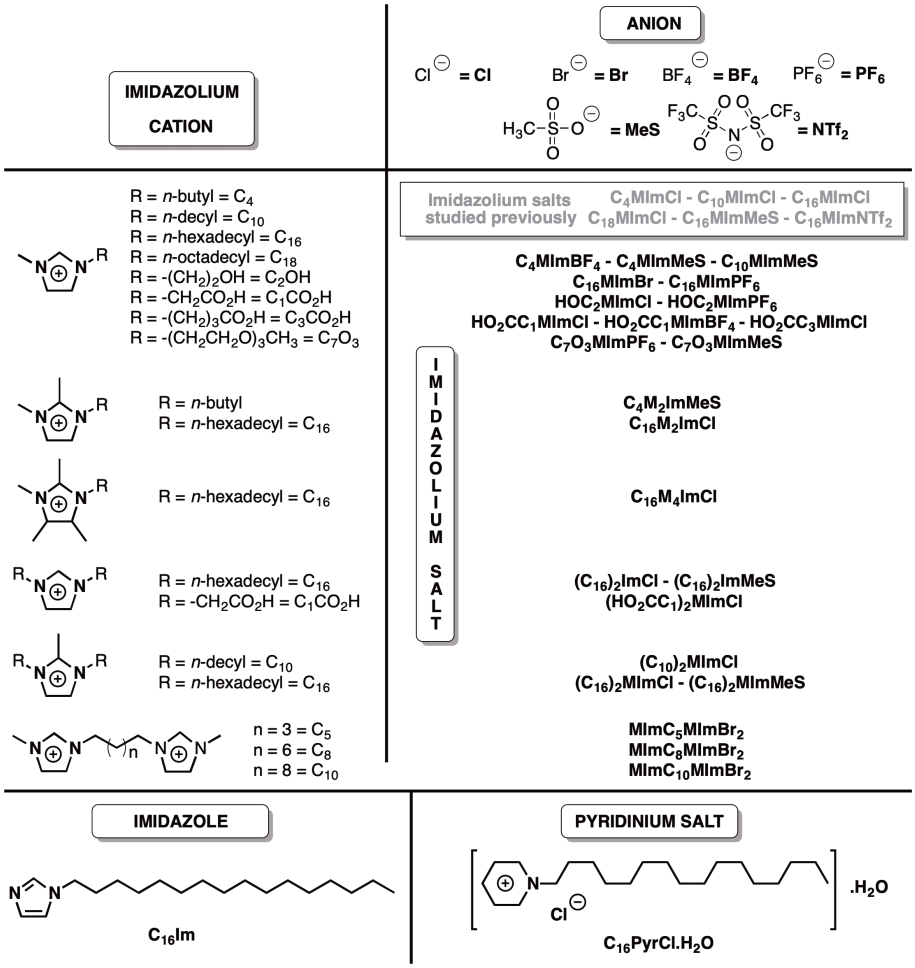

FIGURE 1
Chemical structures of the imidazolium salts, C_{16}Im and C_{16}PyrCl screened for anti-*Leishmania* activity tested in this work, and imidazolium salts studied previously for anti-*Leishmania* activity (ref 13). The compounds were given sample codes that contain information on their chemical structures. For instance, for C_{18}MImCl: (1) C_{18} = *N*-octadecyl substituent; (2) M = *N*-methyl substituent; (3) Im = imidazolium cation; and (4) Cl = chloride anion.

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2 Materials and methods

2.1 Imidazolium salts and reference compounds

1,3-Didecyl-2-methylimidazolium chloride ($(C_{10})_2$MImCl) (CJC China JIE Chemical), 1-hexadecylpyridinium chloride monohydrate (C_{16}PyrCl.H_2O) (Sigma-Aldrich), and 1-(2-hydroxyethyl)-3-methylimidazolium chloride (HOC_2MImCl) (IOLITEC) were purchased and used as received.

1-Butyl-3-methylimidazolium methanesulfonate (C_4MImMeS) (14), 1-decyl-3-methylimidazolium methanesulfonate (C_{10}MImMeS) (15), 1-hexadecyl-3-methylimidazolium bromide (C_{16}MImBr) (16), 1-butyl-3-methylimidazolium tetrafluoroborate (C_4MImBF_4) (14), 1,10-bis(methylimidazolium-1-yl) decane bromide (MImC_{10}MImBr_2) (16), 1-triethylene glycol monomethyl ether-3-methylimidazolium hexafluorophosphate (C_7O_3MImPF_6) (15), 1-triethylene glycol monomethyl ether-3-methylimidazolium methanesulfonate (C_7O_3MImMeS) (15), 1-butyl-2,3-dimethylimidazolium methanesulfonate (C_4M_2ImMeS) (17), 1-hexadecylimidazole (C_{16}Im) (18), 1,3-dihexadecylimidazolium chloride ($(C_{16})_2$ImCl) (19), 1-(3-hydroxypropyl)-3-methylimidazolium hexafluorophosphate (HOC_3MImPF_6) (20), 1-carboxymethyl-3-methylimidazolium chloride (HO_2CC_1MImCl) (21), 1,3-bis(carboxymethyl)imidazolium chloride ($(HO_2CC_1)_2$ImCl) (21), 1-carboxymethyl-3-methylimidazolium tetrafluoroborate (HO_2CC_1MImBF_4) (22), 1-carboxypropyl-3-methylimidazolium chloride (HO_2CC_3MImCl) (23), 1-hexadecyl-3-methylimidazolium hexafluorophosphate (C_{16}MImPF_6) (24), 1-hexadecyl-2,3,4,5-tetramethylimidazolium chloride ($C_{16}M_4$ImCl) (25), decyl methanesulfonate (C_{10}MeS) (15), hexadecyl methanesulfonate (C_{16}MeS) (26), butyl methanesulfonate (C_4MeS) (14), 1-hexadecyl-2-methylimidazole (C_{16}MIm) (27), and triethylene glycol monomethyl ether methanesulfonate (C_7O_3MeS) (15) were synthesized according to previously reported procedures. C_4MeS, C_{10}MeS, C_{16}MeS, C_{16}Im, C_{16}MIm, and C_7O_3MeS were used as source for the syntheses of C_4MImMeS, C_4M_2ImMeS, C_{10}MImMeS, $(C_{16})_2$ImCl, C_7O_3MImMeS, 1,3-dihexadecylimidazolium methanesulfonate $(C_{16})_2$ImMeS, 1,3-dihexadecyl-2-methylimidazolium chloride ($(C_{16})_2$MImCl), and 1,3-dihexadecyl-2-methylimidazolium methanesulfonate ($(C_{16})_2$MImMeS). Literature procedures were used as basis for the synthesis of 1-hexadecyl-2,3-dimethylimidazolium chloride ($C_{16}M_2$ImCl) (15), 1,5-bis(methylimidazolium-1-yl) pentane bromide (MImC_5MImBr_2) (16), 1,8-bis(methylimidazolium-1-yl) octane bromide (MImC_8MImBr_2) (16), $(C_{16})_2$ImMeS (15), $(C_{16})_2$MImCl (15) and $(C_{16})_2$MImMeS (15), which are yet unpublished IS. See the Supporting Information for further details. Before use, the IS were dried under vacuum at 60°C for 5 h to remove residual water.

2.2 Parasite culture

Leishmania (L.) amazonensis (MHOM/BR/73/M2269 strain) and *Leishmania* (*L.*) *infantum chagasi* (MHOM/BR/74/PP75 strain) were cultured at 26 °C as previously described elsewhere (28). To avoid the loss of *in vitro* infectivity of promastigotes, samples of parasites were kept cryopreserved in liquid nitrogen soon after isolation of infected animals (BALB/c or hamsters, respectively). All experiments were performed using parasites with few passages of *in vitro* growth.

2.3 Anti-promastigote activity

The effect of IS, C_{16}Im and C_{16}PyrCl on *L. amazonensis* and *L. infantum chagasi* was evaluated as previously described (28). Promastigotes of *L. amazonensis* in the stationary phase were distributed in 96-well microplates (3 x 10^6 cells/well) and incubated with M199 medium or 100 µM (screening concentration) of tested compound (IS, C_{16}Im or C_{16}PyrCl) for 44 h at 26 °C. Subsequently, 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich®, St. Louis, MO, USA) was added and the incubations continued for an additional 4 h at 26 °C. The purple formazan product that was formed by the action of mitochondrial enzymes in living cells was solubilized by the addition of acidic isopropanol, and the absorbance at 570 nm was measured using a microplate reader spectrophotometer EZ Read 400 from Biochrom. The compounds that caused 100% of *L. amazonensis* mortality in the screening test (C_{10}MImMeS, C_{16}MImPF_6, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl, $(C_{10})_2$MImCl, C_{16}Im and C_{16}PyrCl) were selected for additional tests against *L. amazonensis* and *L. infantum chagasi* at concentrations of 0.1 to 100 µM. The IS C_{10}MImCl, C_{16}MImCl and C_{16}MImMeS, tested against *L. amazonensis* in our previous study (13), were tested here against promastigotes of *L. infantum chagasi*. The 50% inhibitory concentration (IC_{50}) value for each compound was determined. The survival rate was calculated according to the formula: OD in treated group/OD of untreated group x 100. The 50% inhibitory concentration (IC_{50}) value for each compound was determined by nonlinear regression analysis using GraphPad Prism 5.03 software. The activity of each compound was compared with control samples incubated with culture medium (M199 medium for *L. amazonensis* and Schneider's medium for *L. infantum chagasi*) containing less than 0.05% of polysorbate 80 (Sigma-Aldrich) or amphotericin B at 5 µM (100% mortality: Sigma-Aldrich) as reference antileishmanial drug.

2.4 Evaluation of leishmania growth

Promastigote forms of *L. amazonensis* or *L. infantum chagasi* in the stationary growth phase were distributed in 12-well microplates at a density of 1 x 10^5 mL^{-1} in medium (control) (M199 medium for *L. amazonensis* and Schneider's medium for *L. infantum chagasi*) or medium plus C_{10}MImMeS, C_{16}MImPF_6, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl, $(C_{10})_2$MImCl, C_{16}Im or C_{16}PyrCl at the IC_{50} found after 48 h of incubation, and the parasite growth at 26 °C was evaluated daily by motility and cell density using a hemocytometer (28).

2.5 Field emission scanning electron microscopy

To evaluate parasite ultrastructural alterations by scanning electron microscopy (SEM), promastigotes of *L. amazonensis* in the stationary phase were distributed in 96-well microplates (3 x 10^6 cells/

well) and incubated at 26 °C with M199, M199 containing IS at the IC_{50} or amphotericin B at 0.4 µM (IC_{50}) for 6 h. The parasites were washed with phosphate buffered saline (PBS) and fixed with 25% glutaraldehyde in 0.2 M phosphate buffer pH 7.4. After being fixed three times with the same buffer, the parasites were adhered to glass slides previously coated with 0.1% aqueous poly-L-lysine for 30 min at room temperature. Subsequently, the parasites were washed three times with 0.2 M phosphate buffer pH 7.4 and post-fixed in a solution of 2% OsO_4 with 0.2 M phosphate buffer (1:1) for 45 min at room temperature. All samples were dehydrated in a graded series of acetone in water from 30% to 100%, critical point dried using CO_2, mounted on metal stubs, and coated with gold for observation in a field emission scanning electron microscope (Inspect F50, FEI) (29). In addition, the IS C_{16}MImMes and C_{16}MImCl, tested in our previous study (13), were also analyzed.

2.6 Evaluation of cell membrane integrity

L. amazonensis promastigotes (2 x 10^5) in the logarithmic growth phase were distributed in 96-well microplates and either untreated, treated with IS (2-fold IC_{50} for 48 h), hydrogen peroxide (H_2O_2) 2 mM or amphotericin B (0.4 µM) as positive control. After 48 h at 26° C, the parasites were harvested and washed twice with PBS, resuspended and incubated with 100 µL of propidium iodide (PI – Sigma–Aldrich) 50 µg/mL at 26°C for 15 min. Following, each sample was completed with 400 µL of PBS and a total of 20,000 events were acquired and analyzed on BD FACScalibur flow cytometer and CellQuest Pro software. PI fluorescence intensity was quantified as the percentage of the fluorescence compared with untreated promastigotes (30). Results were obtained from two independent experiments performed in triplicate.

2.7 Determination of the mitochondrial membrane potential (ΔΨm)

Mitochondrial membrane potential (ΔΨm) was quantified using the fluorescent dye rhodamine 123 (Rh 123, Sigma-Aldrich) (29). Briefly, promastigote forms of *L. amazonensis* (2 x 10^5) in the logarithmic growth phase were incubated with M199 medium (6 h), medium plus IS (2-fold IC_{50} – 6 h), amphotericin B (0.4 µM - 6 h) or H_2O_2 (2 mM - 1 h) in 96-well microplates, at 26°C. These tested concentrations and time were chosen based on previous results with other IS (13). Then, cells were washed in PBS and incubated with 500 µL of Rh 123 (1 µg/mL) for 10 min at 37 °C. After being washed, they were resuspended in 0.5 mL of PBS and the analysis was performed using a BD FACSCalibur (Becton–Dickinson®, Rutherford, NJ, USA) flow cytometer and CellQuest® Pro software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA), using the blue argon-ion 488 nm laser with the FL1 filter channel. A total of 30,000 events were acquired in the region that corresponded to the parasites. Histograms were build using the CellQuest Pro software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

2.8 Detection of reactive oxygen species production

ROS generation was quantified using the dye 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich) and flow cytometer analysis (13, 30, 31). Briefly, promastigote forms of *L. amazonensis* (2 x 10^5/well) in the logarithmic growth phase were incubated with M199 medium (6 h), M199 plus IS (2-fold IC_{50} - 6 h), amphotericin B (0.4 µM - 6 h) or H_2O_2 (2 mM – 1 h) in 96-well microplates, at 26°C. Then, cells were washed with PBS and incubated with 0.5 mL of 10 µM DCF-DA for 30 min at room temperature. After being washed with PBS, cells were resuspended in 0.5 mL PBS and analysed using a BD FACSCalibur (Becton–Dickinson, Rutherford, NJ, USA) flow cytometer and CellQuest Pro software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA) using the blue argon-ion 488 nm laser with the FL1 filter channel. A total of 30,000 events were acquired in the region that corresponded to living parasite.

2.9 Intramacrophage anti-amastigote activity

The anti-amastigote assay was performed as previously described (13). Macrophages RAW 264.7 cell line (ATCC) were cultured in DMEM plus 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 µg/mL) until reaching 90% confluence, when were distributed into 96-well microplates at a concentration of 2 x 10^5 cells/well in the presence of DMEM. After 12-16 h at 37°C in a humidified atmosphere of 5% CO_2, non-adherent cells were removed, and adherent macrophages were infected with promastigotes of *L. amazonensis* (10 parasites/cell) in the stationary phase, and 4 h later were washed with PBS to remove parasites and incubated with DMEM (control), lipopolysaccharide (LPS) (10 ng/mL) plus interferon-γ (IFN-γ (1 ng/mL) as positive control, or IS, C_{16}Im or C_{16}PyrCl at concentrations of 0.1, 1 µM or 5 µM for 48 h. After, cells were incubated with 100 µL of 0.01% (w/v) SDS solution in serum-free M199 medium at 37°C for 20 min. Then, cells were supplemented with M199 30% FBS and cultured at 26°C for 7 days to determine the number of promastigotes recovered, once only viable amastigotes are capable to differentiate to motile promastigotes. The leishmanicidal activity of macrophages was assessed by determining the number of viable parasites (4 replicates) using a hemocytometer.

2.10 Macrophage viability and hemolytic assay

RAW cells were incubated or not with IS, C_{16}Im or C_{16}PyrCl (5 to 200 µM) for 48 h at 37 °C and *in vitro* cytotoxicity determined by MTT assay (30). Triton X-100 1% was used as positive control. The hemolytic assay was performed according to the procedure used by (13). Briefly, red blood cells solution 1% (v/v) obtained from healthy voluntary donors (n=3) (project approved by the research ethical committee from the Universidade Federal de Ciências da Saúde de

Porto Alegre - CAAE 63282416.6.0000.5345) were distributed into 96-well microplate and incubated with different IS, C_{16}Im or C_{16}PyrCl (1 to 100 µM). The microplate was incubated at 37°C under agitation (90 rpm) for 60 min. Then, the suspension was centrifuged at 3,000 rpm for 5 min, and the absorbance of the supernatant was measured at 540 nm using a spectrophotometer EZ Read 400 from Biochrom. Triton X-100 1% and PBS were used as positive and negative control, respectively. The 50% cytotoxicity concentration (CC_{50}) and 50% of red blood cell hemolysis (RBC_{50}) were determined by nonlinear regression analysis. The selectivity index (SI) (CC_{50} or RBC_{50}/IC_{50} against *L. amazonensis*) were determined for each compound.

2.11 Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM) from five replicates in each three independent experiments. Kolmogorov-Smirnov test was applied to verify normality. Comparisons were performed by the One-way ANOVA test followed by Bonferroni's post-test, Two-way ANOVA repeated measures test followed by Bonferroni's post-test. Differences were considered statistically significant when $p < 0.05$. All statistical tests were performed using GraphPad Prism 6.01 software.

3 Results

3.1 Imidazolium salts

The results regarding the syntheses of IS are reported in the Supplementary materials, together with ^{1}H NMR spectra of the synthesized compounds, and ^{13}C NMR and FTIR spectra for the novel IS.

3.2 Leishmanicidal activity of the compounds on promastigotes of *L. amazonensis* and *L. infantum chagasi*

Six of the 24 IS tested (Figure 1) were able to kill promastigotes of *L. amazonensis* in a concentration-dependent manner (Figure S38A: Leishmanicidal activity of imidazolium salts, C_{16}Im and C_{16}PyrCl (0.1 to 100 µM) on promastigote forms of *L. amazonensis*, determined at 48 h incubation using MTT assay). C_{10}MImMeS, C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl at 5 µM caused a significant decrease in the viability after 48 h, resulting in 60, 37, 42, 85, 95 and 97% of mortality, respectively. The neutral imidazole C_{16}Im and the pyridinium salt C_{16}PyrCl at concentrations ≥ 10 µM and ≥ 5 µM caused significant parasite mortality, respectively (Figure S38A). The IC_{50} values of C_{10}MImMeS, C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl on promastigotes of *L. amazonensis* were calculated to be 11.6, 6.9, 6.0, 4.1, 1.8 and 1.9 µM, respectively (Table 1). Although C_{10}MImMeS, C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl also presented leishmanicidal activity against *L. infantum chagasi* (Figure S38B: Leishmanicidal activity of imidazolium salts, C_{16}Im and C_{16}PyrCl (0.1 to 100 µM) on promastigote forms of *L. infantum chagasi*, determined at 48 h incubation using MTT assay), the IC_{50} values were in general higher than against *L. amazonensis* (Table 1). In contrast, the IC_{50} values for C_{16}Im and C_{16}PyrCl were lower in the case of *L. infantum chagasi*. To reinforce the higher activity of IS towards *L. amazonensis* we tested the effect of IS with the best activity against *L. amazonensis* in our previous study (13) in promastigotes of *L. infantum chagasi*. The IS C_{10}MImCl, C_{16}MImCl and C_{16}MImMeS presented lower IC_{50} values for *L. amazonensis* (2.31, 1.23 and 0.75 µM, respectively) (13) when compared to those values found for *L. infantum chagasi* (IC_{50} = 114.9 µM, 9.9 µM and 10.8 µM, respectively; data not shown). As expected, amphotericin B at 5 µM caused 100% mortality of *L. amazonensis* and *L. infantum chagasi*.

TABLE 1 Leishmanicidal activity, macrophage toxicity and hemolytic effect of compounds.

Compounds	IC_{50}[a] (CI 95%[b])	IC_{50}[c] (CI 95%)	IC_{50}[d] (CI 95%)	CC_{50}[e] (CI 95%)	RBC_{50}[f] (CI 95%)	SI[g]	SI[h]	SI[i]	SI[j]
C_{10}MImMeS	11.6 (10.5-12.9)	0.3 (0.2-0.4)	> 100	76.9 (63.8-92.8)	> 100	6.6	> 8.6	256	333
C_{16}MImPF$_6$	6.9 (6.4-7.3)	0.3 (0.2-0.3)	26.3 (20.1-34.5)	16.8 (14.1-20.1)	24.8 (22.9-26.8)	2.4	3.6	56	82.7
C_{16}MImBr	6 (5.6-6.3)	0.2 (0.1-0.2)	29.7 (25.1-35.1)	16 (12.1-21.3)	22.8 (21.7-24)	2.7	3.8	80	114
$C_{16}M_2$ImCl	4.1 (3.9-4.3)	0.2 (0.1-0.2)	15.4 (13.2-17.9)	19.8 (18.1-21.7)	21.6 (20.8-22.5)	4.8	5.3	99	108
$C_{16}M_4$ImCl	1.8 (1,7-1.9)	0.2 (0.1-0.3)	14.9 (12.7-17.5)	17.8 (16.3-19.5)	15.6 (14.5-16.8)	10	8.7	89	78
$(C_{10})_2$MImCl	1.9 (1.7-2)	0.2 (0.1-0.2)	16.5 (14.5-18.8)	16.4 (14.4-18.8)	20.9 (20-21.9)	8.6	11	82	105
C_{16}Im	14.6 (12.6.-17.1)	0.2 (0.1-0.2)	8.3 (4.4-15.5)	54.9 (39.9-75.6)	> 100	3.8	> 6.8	275	> 500
C_{16}PyrCl	4.0 (2.0-7.9)	0.2 (0.2-0.3)	2.6 (1.4-4.6)	6.3 (4.8-8.1)	21.3 (19.2-23.7)	1.6	5.3	31.5	107

Data are presented on µM.
[a]IC_{50}: concentration of compound that causes 50% of L. amazonensis promastigotes mortality.
[b]CI 95%: 95% confidence interval.
[c]IC_{50}: concentration of compound that causes 50% of L. amazonensis amastigotes mortality.
[d]IC_{50}: concentration of compound that causes 50% of L. infantum chagasi promastigotes mortality.
[e]CC_{50}: concentration of compound that causes 50% of macrophage mortality.
[f]RBC_{50}: concentration of compound that causes 50% of red blood cell hemolysis.
[g]SI: selectivity index, calculated as ratio of CC_{50} for macrophage/IC_{50} for L. amazonensis promastigotes.
[h]SI: selectivity index, calculated as ratio of CC_{50} for RBC hemolysis/IC_{50} for L. amazonensis promastigotes.
[i]SI: selectivity index, calculated as ratio of CC_{50} for macrophage/IC_{50} for L. amazonensis amastigotes.
[j]SI: selectivity index, calculated as ratio of CC_{50} for RBC hemolysis/IC_{50} for L. amazonensis amastigotes.

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3.3 Imidazolium salts, C_{16}Im and C_{16}PyrCl inhibit the growth of promastigotes of *L. amazonensis* and *L. infantum chagasi*

When promastigotes of *L. amazonensis* or *L. infantum chagasi* were treated with IS, C_{16}Im or C_{16}PyrCl at IC_{50} concentrations, a complete inhibition of the parasites growth was observed (Figures 2A, B).

3.4 Imidazolium salts increase reactive oxygen species and induce mitochondrial membrane potential (ΔΨm) alterations in *L. amazonensis*

The treatment of promastigotes of *L. amazonensis* with IS at 2-fold IC_{50} values led to a significant increase in ROS production at 6 h of incubation (Figures 3A, B). C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl caused significant ΔΨm depolarization (loss of Rh 123 fluorescence), while C_{10}MImMeS induced ΔΨm hyperpolarization (increase in Rh 123 fluorescence) (Figures 3C, D). Hydrogen peroxide at 2 mM or amphotericin B at 0.4 μM also induced significant elevation in ROS production and mitochondrial depolarization.

3.5 Imidazolium salts induce loss of plasma membrane integrity in *L. amazonensis*

When parasites were treated with IS at 2-fold IC_{50} values for 48 h, a significant increase in propidium iodide labeling was observed for all IS tested, indicating that the IS interfere with membrane integrity causing cell death (Figures 4A, B). Besides, as illustrated in Figure 4C, incubation with $C_{16}M_4$ImCl at 1.8 μM (IC_{50}) for 48 h led to total lysis of the parasites.

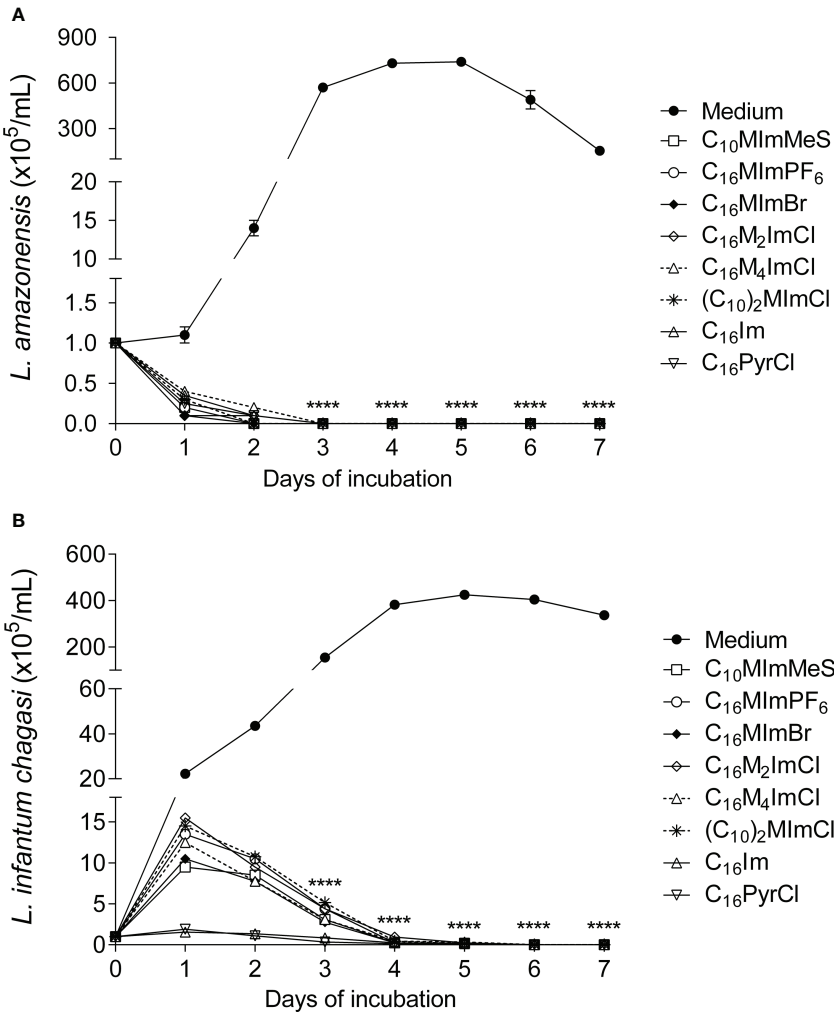

FIGURE 2
Growth kinetics of *L. amazonensis* and *L. infantum chagasi* promastigotes treated with the different compounds. *L. amazonensis* **(A)** *or L. infantum chagasi* **(B)** promastigotes in the stationary phase (1×10^5 cells/mL) were incubated with medium as control (M199 to *L. amazonensis* and Schneider's medium to *L. infantum chagasi*) or medium plus compounds at IC_{50} values (for 48 h incubation), at 26°C, and the growth was determined daily using a hemocytometer. Data are expressed as means ± SEM (n = 5) and are representative of three independent experiments. The Two-way ANOVA repeated measures test followed by the Bonferroni's test were applied. ****p < 0.0001.

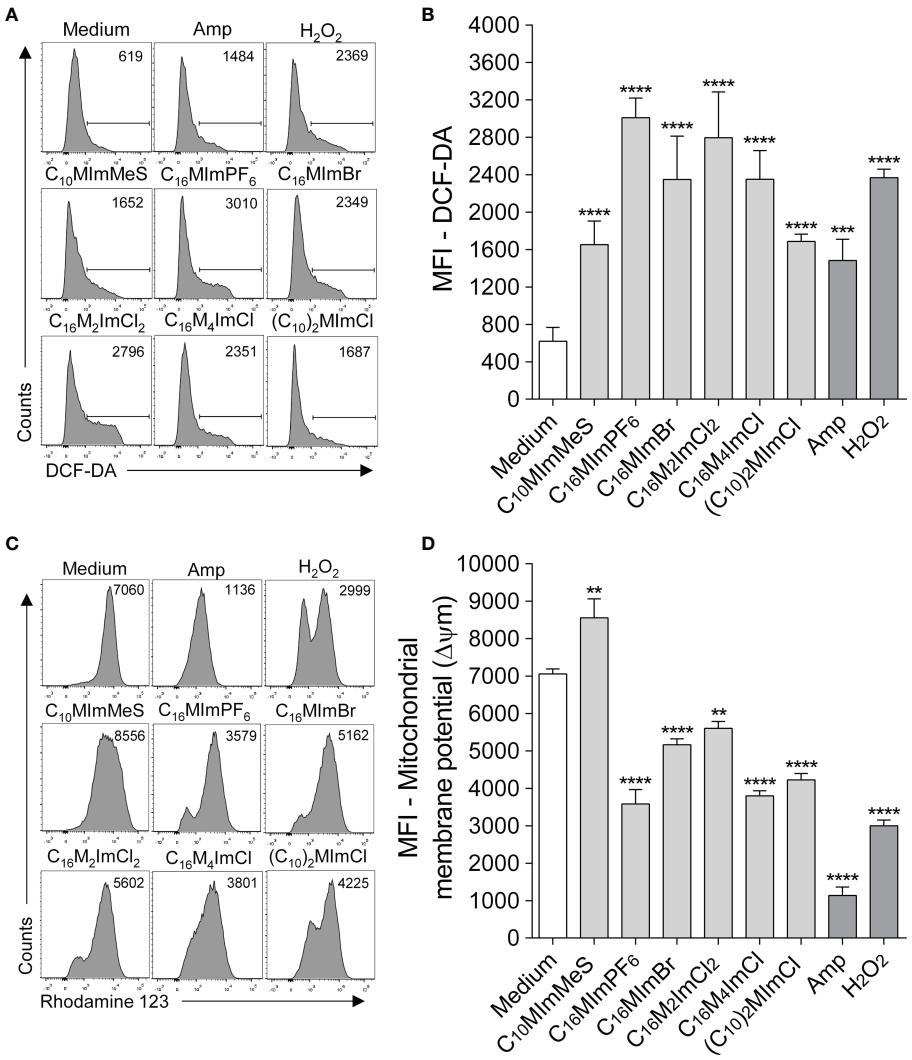

FIGURE 3
Determination of intracellular ROS **(A, B)** and mitochondrial membrane potential (ΔΨm) **(C, D)** of *L. amazonensis* promastigotes incubated with imidazolium salts. Promastigote forms in the logarithmic phase (2 x 10^5 cells/well) were incubated or not with 2-fold IC_{50} value of IS at 26°C for 6 h. M199 was used as negative control, and amphotericin B (0.4 μM) and H_2O_2 (2 mM) as positive controls. Data are expressed as means ± SEM and are representative of three independent experiments. The One-way ANOVA test followed by the Bonferroni's test were applied. **p < 0.01, ***p < 0.001 and ****p < 0.0001 when compare with the medium group.

3.6 Imidazolium salts cause morphological alterations on *L. amazonensis* promastigotes

As illustrated in Figure 5, when promastigotes were treated with IS at IC_{50} values for 6 h, the parasites showed striking morphological alterations. While untreated parasites exhibited elongated shape, integral flagellum and membrane integrity (Figures 5A, B), promastigotes treated with amphotericin B at 0.4 μM (Figures 5C, D), as well as with C_{10}MImMeS, C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl or $(C_{10})_2$MImCl (Figures 5E–J) evidenced rounded shape, membrane damage, flagellum disruption or twisted, and cell aggregation. In addition, C_{16}MImMeS and C_{16}MImCl that killed *L. amazonensis* in our previous study (13) also caused similar morphological alterations in promastigotes of *L. amazonensis* (Figures 5K, L).

3.7 Cytotoxicity of imidazolium salts on mammalian cells

In vitro cytotoxicity of imidazolium salts, C_{16}Im and C_{16}PyrCl (5 to 200 μM) towards macrophages, determined by MTT assay

In relation to the effects of IS on macrophages, a concentration-dependent cytotoxicity was found (Figure S39A: *In vitro* cytotoxicity of imidazolium salts, C_{16}Im and C_{16}PyrCl [5 to 200 μM] towards macrophages, determined by MTT assay). All IS at 10 μM kept more than 90% of the macrophages alive after 48 h of incubation, and C_{10}MImMeS maintained this condition up to 50 μM. Besides that, $C_{16}M_4$ImCl, $(C_{10})_2$MImCl and C_{10}MImMeS at concentrations of 5.6-, 5.3- and 4.3-fold IC_{50} for *L. amazonensis* kept 90, 94 and 89% of macrophages viability, respectively. $C_{16}M_4$ImCl and $(C_{10})_2$MImCl presented the best selectivity indexes (10 and 8.6, respectively)

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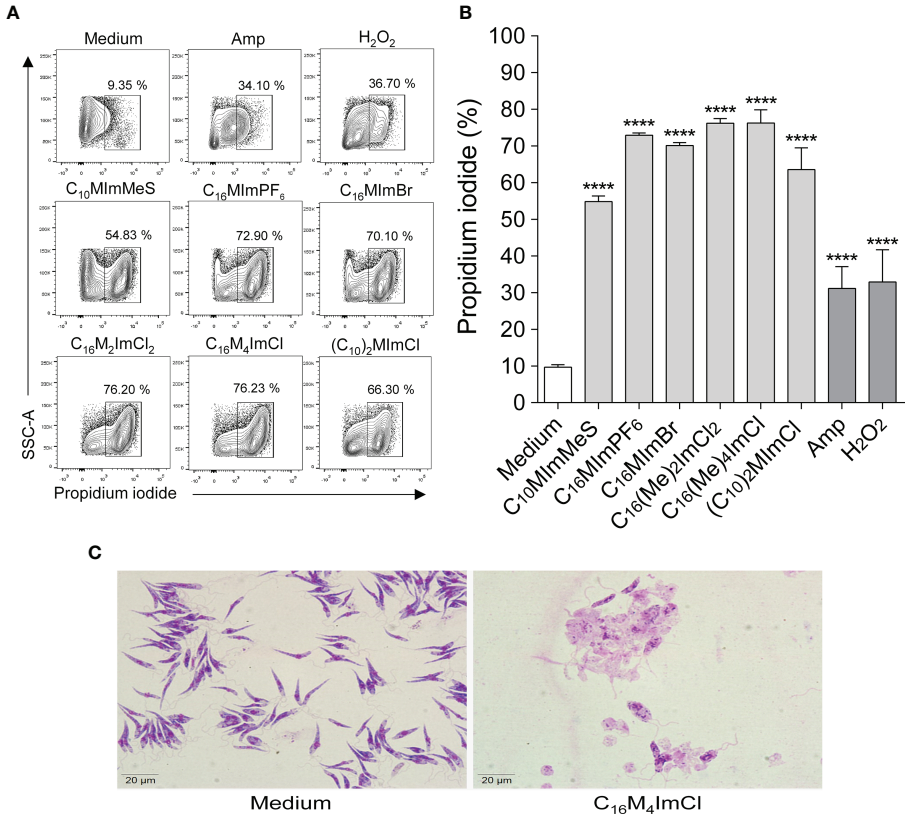

FIGURE 4
Determination of membrane integrity of *L. amazonensis* promastigotes treated with imidazolium salts **(A, B)**. Promastigote forms in the logarithmic phase (2 x 10^5 cells/well) were incubated or not with 2-fold IC_{50} value of IS for 48 h at 26°C. M199 was used as negative control, and amphotericin B (0.4 µM) or H_2O_2 (2 mM) as positive controls. Data are expressed as means ± SEM (n = 5) and are representative of three independent experiments. The One-way ANOVA test followed by the Bonferroni's test were applied. ****p < 0.0001 when compare with the medium group. **(C)** *L. amazonensis* integrity after 48 h of exposure to $C_{16}M_4ImCl$ at 1.8 µM or M199 (panoptic stain – 1000 × magnification).

(Table 1). Moreover, human erythrocytes incubated with IS at 10 µM demonstrated hemolysis up to 8% and, as well as for macrophages, C_{10}MImMeS was the less toxic IS, without significant hemolysis up to 40 µM (Figure S39B: *In vitro* cytotoxicity of imidazolium salts, C_{16}Im and C_{16}PyrCl (1 to 100 µM) towards human red blood cells, determined by hemolysis rates at 540 nm). $C_{16}M_4ImCl$ and $(C_{10})_2$MImCl also presented the best SI when considering hemolysis – 8.7 and 11, respectively (Table 1). The neutral imidazole C_{16}Im presented lower cytotoxicity against both macrophages and erythrocytes, however it presented low SI (Table 1). The pyridinium salt C_{16}PyrCl presented the higher toxicity and lowest SI among the tested compounds (Table 1).

3.8 Imidazolium salts reduce the intracellular survival of *L. amazonensis* on macrophages

As *Leishmania* can survive inside macrophages, we evaluate whether IS, C_{16}Im and C_{16}PyrCl stimulate the leishmanicidal activity of macrophages. Our data showed that all compounds at 1 µM were able to reduce amastigotes survival in 86% to 93% (Figure 6).

4 Discussion

C_4MImMeS, C_{10}MImMeS, C_{16}MImBr, C_4MImBF_4, MImC_{10}MImBr_2, C_7O_3MImPF_6, C_7O_3MImMeS, C_4M_2ImMeS, $(C_{16})_2$ImCl, HOC_3MImPF_6, HO_2CC_1MImCl, $(HO_2CC_1)_2$ImCl, HO_2CC_1MImBF_4, HO_2CC_3MImCl, C_{16}MImPF_6, $C_{16}M_4$ImCl, $C_{16}M_2$ImCl, MImC_5MImBr_2, MImC_8MImBr_2, $(C_{16})_2$ImMeS, $(C_{16})_2$MmCl, and $(C_{16})_2$MImMeS were synthesized, which along with the commercially available $(C_{10})_2$MImCl, and HOC_2MImCl constituted the group of 24 IS tested (Figure 1). The spectral data of C_4MImMeS (14), C_{10}MImMeS (15), C_{16}MImBr (16), C_4MImBF_4 (14), MImC_{10}MImBr_2 (16), C_7O_3MImPF_6 (15), C_7O_3MImMeS (15), C_4M_2ImMeS (17), C_{16}Im (18), $(C_{16})_2$ImCl (19), HOC_3MImPF_6 (20), HO_2CC_1MImCl (21), $(HO_2CC_1)_2$ImCl (21), HO_2CC_1MImBF_4 (22), HO_2CC_3MImCl (23), C_{16}MImPF_6 (24), $C_{16}M_4$ImCl (25), C_{10}MeS (15), C_{16}MeS (26), C_4MeS (14), C_{16}MIm (27), and C_7O_3MeS (15) are in agreement with those previously reported. For the new IS $C_{16}M_2$ImCl, MImC_5MImBr_2, MImC_8MImBr_2, $(C_{16})_2$ImMeS, $(C_{16})_2$MmCl, and $(C_{16})_2$MImMeS, the NMR, FTIR and MS data support their structures and purity.

To the best of our knowledge, this is the first study reporting the leishmanicidal and immunomodulatory activities for C_{10}MImMeS,

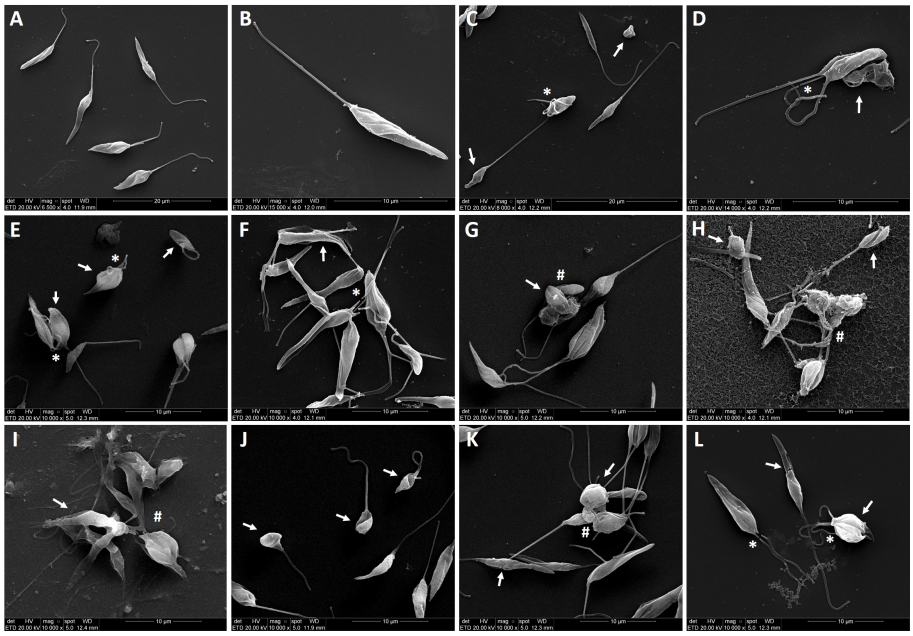

FIGURE 5
Scanning electron micrographs of *L. amazonensis* promastigotes treated or not with imidazolium salts at IC_{50} values for 6 h. Untreated parasites showing the typical elongated shape (parasite body and anterior flagella) **(A, B)**. Parasites treatment with amphotericin B at 0.4 μM **(C, D)**, C_{10}MImMeS at 11.6 μM **(E)**, C_{16}MImPF$_6$ at 6.9 μM **(F)**, C_{16}MImBr at 6 μM **(G)**, $C_{16}M_2$ImCl at 4.1 μM **(H)**, $C_{16}M_4$ImCl at 1.8 μM **(I)**, $(C_{10})_2$MImCl at 1.9 μM **(J)**, C_{16}MImMeS at 0.75 μM **(K)** and C_{16}MImCl at 1.23 μM **(L)**. The images show alterations in promastigote body shape or disruption of plasma membrane (arrows), flagellum loss (asterisks), and aggregation of cells (hashes). Bars: **(A, C)** represent 20 μm, **(B)** and **(D–L)** represent 10 μm.

C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl (Figure 1) on two *Leishmania* species. Here, it was demonstrated that depending on the concentration, IS were able to kill infective forms of *L. amazonensis* and *L. infantum chagasi* (Figure S38).

In a previous study, we demonstrated that IS are able to kill both promastigote and amastigote forms of *L. amazonensis* (13). Here, we investigated a structurally complementary set of mono- (*N*-substituent = methyl; *N'*-substituent = *n*-butyl, *n*-decyl, *n*-hexadecyl, 2-hydroxyethyl, methylcarboxylic acid, propylcarboxylic acid, methoxycarbonylmethyl, triethylene glycol monomethyl ether; C_2-substituent = H, methyl; C_4-substituent = H, methyl; C_5-substituent = H, methyl – *N,N'-substituents* = *n*-decyl, *n*-hexadecyl, methylcarboxylic acid; C_2-substituent = H, methyl) and dicationic (*N*-substituent = methyl; alkane bridge = pentane, octane, decane) IS with

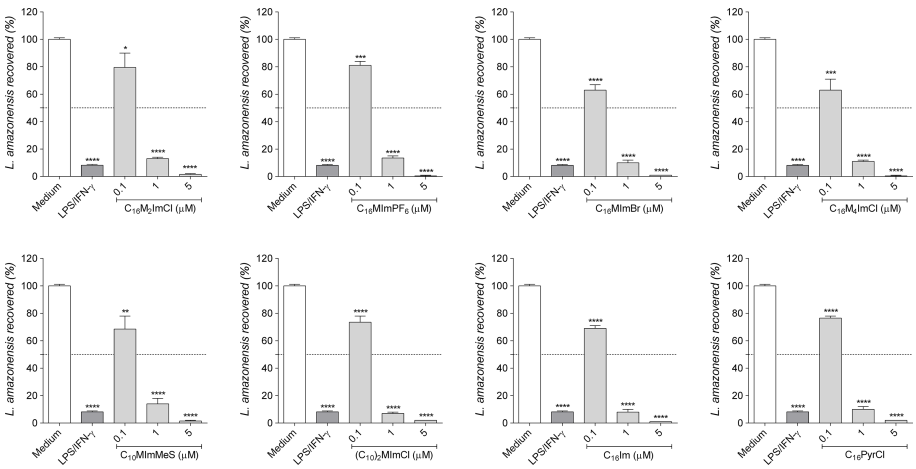

FIGURE 6
Intramacrophage anti-amastigote activity of compounds. RAW cells (2×10^5 cells/well) were infected with promastigotes of *L. amazonensis* (10 parasites/cell) in the stationary phase (day 5 of culture) and incubated with DMEM (control), lipopolysaccharide (LPS; 10 ng/mL) plus interferon-γ (IFN-γ; 1 ng/mL) as positive control, or IS, C_{16}Im or C_{16}PyrCl at concentrations of 0.1 μM, 1 μM or 5 μM for 48 h. After, cells were lysed, supplemented with M199 30% FBS and cultured at 26°C for 7 days to determine the number of promastigotes recovered using a hemocytometer. Data are expressed as means ± SEM (n = 5). The One-way ANOVA test followed by the Bonferroni's test were applied. *p < 0.05, ** p < 0.01, ***p < 0.001 and ****p < 0.0001 when compared with the medium group (100% parasite recovered).

different anions, as well as the neutral C_{16}Im and the pyridinium salt C_{16}PyrCl (Figure 1) against *L. amazonensis* and *L. infantum chagasi*. This provided the perspective to compare the chemical structures and extend the comprehension about activity of this class of compounds against *Leishmania*. Significant differences in leishmancidal activity of IS against *L. amazonensis* were observed (Table 1). $(C_{10})_2$MImCl and $C_{16}M_4$ImCl presented the highest activity (lowest IC_{50} values): $(C_{10})_2$MImCl = $C_{16}M_4$ImCl > $C_{16}M_2$ImCl > C_{16}MImBr > C_{16}MImPF$_6$ > C_{10}MImMes. Corroborating with scientific literature (10, 11, 13), the monocationic methylimidazolium salts with longer *N*-alkyl chain-lengths are closely related to higher leishmanicidal activities. In this sense, we point out that C_{16}MImMeS showed the higher leishmanicidal activity (IC_{50} = 0.75 μM) (13) compared to C_{10}MImMeS (IC_{50} = 11.6 μM) and C_4MImMeS (without activity at 100 μM) tested here. On the other hand, shorter *N*-alkyl chain-lengths afford lower lipophilicity to the molecule and consequently less cytotoxic effects (32), as demonstrated by C_{10}MImMeS which showed lower toxicity towards macrophages (CC_{50} = 76.9 μM) and erythrocytes (RBC_{50} > 100 μM) when compared to C_{16}MImMeS (CC_{50} = 11.5 μM and RBC_{50} = 36.9 μM) (13).

The *N,N'*dialkyl substituted IS with *n*-decyl and *n*-hexadecyl groups (Figure 1) showed that, independent of the anions present in the *n*-hexadecyl-based IS (MeS, Cl or Br), only $(C_{10})_2$MImCl presented leishmanicidal activity (IC_{50} = 1.9 μM), evidencing a higher leishmanicidal effect than C_{10}MImCl – tested in the previous study of our group (IC_{50} = 2.31 μM) (13). Another study demonstrated that $(C_{10})_2$MImCl also inhibits bacterial (*Escherichia coli* and *Staphylococcus aureus*) and fungal (*Candida albicans* and *Candida tropicalis*) growth *in vitro* (33), and the addition of two hydrophobic alkyl chains to the imidazolium ring also contributed greatly to improve the activity of IS against tumor-derived cell lines (34). This reaffirms the potential balance between hydrophilicity and lipophilicity, which is essential for the antimicrobial activity of IS (7).

The introduction of methyl groups on the C_2-, C_4- and C_5-positions of the imidazolium ring influences the *L. amazonensis* mortality (Table 1). When comparing the activities of $C_{16}M_4$ImCl (IC_{50} = 1.8 μM) and $C_{16}M_2$ImCl (IC_{50} = 4.1 μM), the additional two methyl groups reduced 2.3-fold the IC_{50} value for *L. amazonensis* mortality. However, C_{16}MImCl presented the lowest IC_{50} value among these three compounds (IC_{50} = 1.23 μM) (13). This H-methyl substituent alteration in the imidazolium ring does not seem to interfere to the same extend on *L. infantum chagasi* mortality, according to the IS $C_{16}M_4$ImCl (IC_{50} = 14.9 μM) and $C_{16}M_2$ImCl (IC_{50} = 15.4 μM).

From results of this study and our previous work (13), it is possible to compare the effect of different IS anions on *L. amazonensis* mortality. The IS C_{16}MImMeS (IC_{50} = 0.75 μM), C_{16}MImCl (IC_{50} = 1.23 μM) and C_{16}MImNTf$_2$ (IC_{50} = 1.64 μM) demonstrated higher capacities to kill *L. amazonensis* than C_{16}MImBr (IC_{50} = 6.0 μM) and C_{16}MImPF$_6$ (IC_{50} = 6.9 μM), reaffirming the hypothesis that the IS anion also has an important but secondary effect on activity against *Leishmania* (13). For the C_{16}MIm-based IS, the established order of anion-dependent inhibition was MeS > Cl > NTf$_2$ > Br > PF$_6$. As observed for *L. amazonensis* mortality with C_{16}MImBr and C_{16}MImPF$_6$, Br and PF$_6$ anions did not trigger differences in *L. infantum chagasi* mortality – C_{16}MImPF$_6$ (IC_{50} = 26.3 μM) and C_{16}MImBr (IC_{50} = 29.7 μM). A decrease in anion volume has been related to an increasing fungal mortality, evidencing the IS with MeS and chloride anions as the most promising antifungals (9). This was also observed for anti-leishmanial activity of the IS.

Considering the highest leishmanial activity achieved with C_{16}MImMeS, we tested the effect of its neutral imidazole equivalent C_{16}Im on promastigote forms of *Leishmania*. This study showed that C_{16}Im was not so effective to kill *L. amazonensis* as C_{16}MImMeS. Furthermore, this compound presented lower toxicity than all other tested compounds, except C_{10}MImMeS (Table 1). In contrast, C_{16}Im showed a higher activity than the IS tested in this work against *L. infantum chagasi*.

Compounds containing cationic pyridinium heterocycles have concerned great awareness as prospective chemotherapeutic agents, such as anticancer, analgesic, antimicrobial and antiviral (35). Based on this, the effect of C_{16}PyrCl on *Leishmania* mortality was tested, and this compound showed being effective against *L. amazonensis* (IC_{50} = 4.0 μM) (Figure S38A and Table 1), but being more toxic toward macrophages than IS (Figure S39A and Table 1). In our previous study we tested the structurally related IS C_{16}MImCl, which showed higher leishmanicidal activity and lower macrophage and red blood cell cytotoxicity than C_{16}PyrCl (13). C_{16}PyrCl also demonstrated high capacity to kill *L. infantum chagasi* (IC_{50} = 2.6 μM) (Figure S38B). Although this compound is commonly used as antiseptic (mouthwash), its high toxicity against macrophages turns it into a weak candidate for drug development.

To elucidate possible mechanisms of action of IS on *Leishmania* parasites, membrane and mitochondrial dysfunctions, ROS generation and morphological changes in response to IS were investigated. In *Leishmania*, mitochondria are a drug target (36), since this unique organelle is the main site of ROS production, and excessive ROS may lead to oxidative stress, which may be one of the major contributors to cell death by IS (37, 38). In addition, the maintenance of an appropriate mitochondrial membrane potential ($\Delta\Psi$m) is essential for *Leishmania* survival (36, 37). Here, we demonstrate that IS with leishmanicidal activity caused an increase in ROS production (Figures 4A, B) and $\Delta\Psi$m alterations (Figures 34C, D) in *L. amazonensis*. C_{10}MImMeS triggered mitochondrial membrane hyperpolarization, while C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl caused depolarization of mitochondrial membrane. Both hyperpolarization and depolarization can result in promastigotes death (29), and depolarization can be preceded by a transient hyperpolarization as the last attempt by cells to avoid death (39). Moreover, all six IS presenting leishmanicidal activity caused plasma membrane alterations (Figures 4A, B), indicating cell death involving necrosis (40).

In this regard, the scanning electron microscopy analyses revealed that the treatment with IS induced swelling and overall rounding, cell aggregation, membrane rupture, besides lower parasite length and flagellum damage (Figure 5), corroborating with evidences of the action of IS on *L. amazonensis* (13). In addition to the biochemical alterations, IS inhibited the *in vitro* growth of promastigotes forms of *L. amazonensis* (Figure 2A) and *L. infantum chagasi* (Figure 2B). This effect could be related to the ergosterol availability. Similarly to fungi, ergosterol in *Leishmania* is essential for the organization of membrane domains, cell growth and survival. In a previous study it was demonstrated that C_{16}MImCl reduced the total ergosterol

content of clinical isolates of yeasts, suggesting that IS inhibit the ergosterol biosynthesis (8). Although C_{16}MImCl and other IS presented potent leishmanicidal activity, the activity of enzyme involved in ergosterol synthesis (lanosterol 14α-demethylase) or ergosterol content were not evaluated in this study.

IS, C_{16}Im and C_{16}PyrCl at 1 μM reduced the amastigote survival of *L. amazonensis* in about 90% (Figure 6), with an IC_{50} between 0.2 to 0.3 and higher SI (31.5 to 275) than those calculated for promastigotes, indicating a higher selectivity for amastigotes. These data are in agreement with our previous data showing the better selectivity of IS for amastigotes (13).

For the first time, the IS C_{10}MImMeS, C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl, and $(C_{10})_2$MImCl have been identified as potent agents against *L. amazonensis*. These compounds induced ROS generation, membrane, and mitochondrial dysfunction in parasites. The results reaffirm the hypothesis that IS with longer *N*-alkyl chain-lengths are related with higher biological activities, and on a secondary basis IS with MeS and chloride anions are the most promising antileishmanial agents. As a limitation of this study, we mentioned that we did not test the effects of these IS on antimony-resistant *Leishmania* spp. lines, or their *in vivo* effects.

5 Conclusions

The IS C_{10}MImMeS, C_{16}MImPF$_6$, C_{16}MImBr, $C_{16}M_2$ImCl, $C_{16}M_4$ImCl and $(C_{10})_2$MImCl, and the compounds C_{16}Im and C_{16}PyrCl killed and inhibited the growth of promastigote forms of *L. amazonensis* and *L. infantum chagasi* in a concentration-dependent manner. Moreover, induced ROS production, mitochondrial dysfunction, membrane disruption and morphological alterations in infective forms of *L. amazonensis* and were able to kill intracellular amastigote forms in a very low concentration, being worthy of further *in vivo* studies as potential antileishmanial drugs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization PR, HS, FB. Methodology: FB, TF, MS, PF, VS, DP, JK, ES. Software: TF, FB, LJ. Validation: FB, TF. Formal analysis: FB, LJ. Investigation: FB, ED, TF. Resources: PR, HS, MM. Data curation: FB, PR. Writing—original draft preparation: KN-L, FB, PR, TF. Writing—review and editing: MM. PR, HS. Visualization: AP, LJ, MM, ED. Supervision: PR, HS, MM. Project administration: PR, HS. Funding acquisition: PR, HS, MM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1096312/full#supplementary-material

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