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Imidazolium salts as innovative agents against *Leishmania amazonensis*

Raísha Costa Martins[a], Gilson Pires Dorneles[b], Vivian Oliveira Nunes Teixeira[b], Ana Maria Antonello[d], Júlia Lacerda Couto[e], Luiz Carlos Rodrigues Júnior[d], Marta Chagas Monteiro[f], Alessandra Peres[c,d,g], Henri Stephan Schrekker[e,*], Pedro Roosevelt Torres Romão[a,b,d,**]

[a] Graduate Program in Biosciences, Federal University of Health Sciences of Porto Alegre, Porto Alegre, RS 90050-170, Brazil
[b] Graduate Program in Health Sciences, Federal University of Health Sciences of Porto Alegre, Porto Alegre, RS 90050-170, Brazil
[c] Graduate Program in Rehabilitation Sciences, Federal University of Health Sciences of Porto Alegre, Porto Alegre, RS 90050-170, Brazil
[d] Laboratory of Cellular and Molecular Immunology, Federal University of Health Sciences of Porto Alegre, Porto Alegre, RS 90050-170, Brazil
[e] Institute of Chemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS 91501-970, Brazil
[f] Graduate Program in Pharmaceutical Science, Graduation Program in Neuroscience and Cellular Biology, Faculty of Pharmacy, Federal University of Pará, Belém, PA, Brazil
[g] Research Center, Methodist University Center IPA, Porto Alegre, RS 90420-060, Brazil

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ABSTRACT

The available chemotherapeutic drugs for the treatment of leishmaniasis present problems relating to efficacy, emergence of parasite resistance, and adverse effects and cost. Azole antifungal drugs have been repurposed for this purpose but the clinical response has been variable. In this sense, this study assessed the leishmanicidal and immunomodulatory activities of azoles-derived imidazolium salts (IS), being the ionic imidazole-derived equivalents: 1-*n*-butyl-3-methylimidazolium chloride (**C_4MImCl**), 1-n-decyl-3-methylimidazolium chloride (**C_{10}MImCl**), 1-*n*-hexadecyl-3-methylimidazolium chloride (**C_{16}MImCl**), 1-*n*-hexadecyl-3-methylimidazolium methanesulfonate (**C_{16}MImMeS**), 1-*n*-hexadecyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (**C_{16}MImNTf$_2$**) and 1-methyl-3-*n*-octadecylimidazolium chloride (**C_{18}MImCl**). Promastigotes of *Leishmania amazonensis* were incubated with IS at concentrations ranging from 0.1 to 100 μM, and the parasite survival was monitored. The effects of IS on reactive oxygen species (ROS) production and mitochondrial membrane potential of promastigotes, as well as on cytotoxicity against peripheral blood mononuclear cells (PBMC) and human erythrocytes were determined. Moreover, the activity of IS against amastigotes and nitric oxide production was also evaluated. The IS inhibited parasite growth and showed potent leishmanicidal activity against promastigotes of *L. amazonensis*. In addition, IS induced mitochondrial dysfunction and ROS production in parasites and presented low cytotoxicity against PBMC and human erythrocytes. Furthermore, at very low concentration (0.5 μM), **C_{18}MImCl**, **C_{16}MImMeS**, **C_{16}MImCl**, **C_{10}MImCl** and **C_{16}MImNTf$_2$** were able to kill intramacrophage parasites in an order of 91.3, 100, 94.4, 95.3 and 35.6%, respectively. These results indicate that IS are promising candidates for the developing of drugs against *L. amazonensis*.

1. Introduction

Leishmaniasis is a parasitic disease transmitted by *Leishmania*-infected sand fly vector that afflict at least 2 million people each year in 98 countries around the world [1]. *Leishmania* causes three main forms of disease – visceral (also known as kala-azar and the most serious form of the disease), cutaneous (the most common), and mucocutaneous leishmaniasis. Brazil is one of the countries with high incidence of leishmaniasis [2]. *Leishmania* (*Leishmania*) *amazonensis* can cause a broad spectrum of diseases, ranging from localized cutaneous leishmaniasis (LCL) to an anergic diffuse cutaneous leishmaniasis (DCL), and less commonly mucocutaneous leishmaniasis (MCL) [3]. Currently, there are no vaccines to prevent human leishmaniasis and drugs available for chemotherapy present severe limitations such as long term of treatment, high toxicities, emergence of parasite resistance and treatment failure [4, 5].

* Corresponding author at: Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, Porto Alegre, RS 91501-970, Brazil.
** Correspondence to: P. R. T. Romão, Universidade Federal de Ciências da Saúde de Porto Alegre, Rua Sarmento Leite 245, Porto Alegre, RS 90050-170, Brazil.
E-mail addresses: henri.schrekker@ufrgs.br (H.S. Schrekker), pedror@ufcspa.edu.br (P.R.T. Romão).

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Nowadays, the design and development of new antileishmanial agents based on azoles compounds has been considered extensively [6]. In fact, azole antifungals have been used for the treatment of leishmaniasis [7, 8], and act in the ergosterol biosynthetic pathway through the inhibition of the cytochrome P450-dependent enzyme sterol 14α-demethylase, a validated antiparasitic drug target, present in fungi and trypanosomatid parasites including *Leishmania* [9]. Over recent years several biological activities of imidazolium salts (IS), ionic derivatives of imidazoles, including antioxidant [10], neuroprotective [11], antifibrogenic [12], antibacterial [13], larvicidal [14] and antifungal activities [15, 16] have been described. To the best of our knowledge, this is the first study reporting the activity of IS against both promastigote (infective stage transmitted by sand fly vector) and amastigote (replicating intracellular) forms of parasites from genus *Leishmania*. Our data exhibit that 1-*n*-decyl-3-methylimidazolium chloride (**C_{10}MImCl**), 1-*n*-Hexadecylimidazolium chloride (**C_{16}MImCl**), 1-methyl-3-*n*-octadecylimidazolium chloride (**C_{18}MImCl**), 1-*n*-Hexadecylimidazolium methanesulfonate (**C_{16}MImMeS**) and 1-*n*-hexadecyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (**C_{16}MImNTf$_2$**) present potent activity against both promastigote and amastigote forms of *L. amazonensis*.

2. Materials and methods

2.1. Imidazolium salts

The IS (Fig. 1) 1-*n*-butyl-3-methylimidazolium chloride (**C_4MImCl**) and 1-*n*-decyl-3-methylimidazolium chloride (**C_{10}MImCl**) were purchased at Sigma-Aldrich. 1-*n*-Hexadecylimidazolium chloride (**C_{16}MImCl**) and 1-methyl-3-*n*-octadecylimidazolium chloride (**C_{18}MImCl**) were purchased at CJC CHINA JIE CHEMICAL. 1-*n*-Hexadecylimidazolium methanesulfonate (**C_{16}MImMeS**) and 1-*n*-hexadecyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (**C_{16}MImNTf$_2$**) were synthesized according to previously reported procedures [17, 18].

2.2. Leishmania (Leishmania) amazonensis culture

Leishmania (*Leishmania*) *amazonensis* (IFLA/BR/67/PH8) was routinely isolated from draining popliteal lymph node of infected BALB/c mice and maintained as promastigotes. The cultures were maintained in M199 medium supplemented with 10% FBS (fetal bovine serum), 2% human urine, 100 μM adenine, 7.7 mM hemin, 0.0001% biotin, 40 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.4), 50 unit/mL penicillin, and 50 μg/mL streptomycin (Sigma-Aldrich®, St. Louis, MO, USA). Cultures were incubated at 26 °C with cells densities ranging between 5×10^5 and 3×10^7 parasites/mL [19]. All experimental procedures were performed in accordance with the guidelines of the National Institute of Health and the Brazilian Society for Science on Laboratory Animals, with the approval of the local Ethics Committee from Federal University of Health Sciences of Porto Alegre (process number 505/17).

2.3. Effects of IS on growth of L. amazonensis promastigotes

Promastigote forms of *L. amazonensis* in the stationary growth phase (day 6 of culture) were distributed in 12-well microplates at a density of 1×10^5/ mL in M199 medium (control) or M199 plus IS at 5 μM. *L. amazonensis* growth was determined daily by motility and cell density using a hemocytometer [20].

2.4. Activity of IS against promastigotes of L. amazonensis

The direct cytotoxic effect of IS on *L. amazonensis* was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [21]. For this purpose, promastigote forms of *L. amazonensis* in the stationary phase (day 6 of culture) were distributed in 96-well microplates (3×10^6 cells/well) and incubated with M199 medium in the presence of IS **C_4MImCl**, **C_{10}MImCl**, **C_{16}MImCl**, **C_{16}MImMeS**, **C_{16}MImNTf$_2$** or **C_{18}MImCl** in different concentrations (0.1 to 100 μM) for 44 h at 26 °C. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich®, St. Louis, MO, USA) 0,5 mg/mL 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 survival rate was calculated according to the formula: OD in treated group/OD of untreated group × 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 M199 medium (100% of viability). Amphotericin B (Sigma, USA) at 5 μM as used a standard antileishmanial drug (100% of mortality).

2.5. Evaluation of cell membrane integrity

L. amazonensis promastigote (3×10^6) in the logarithmic growth phase (day 4 of culture) were either untreated, treated with IS (at 5 or

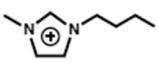
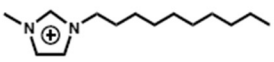
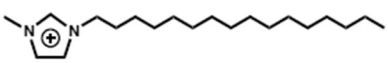
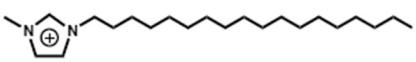
Cation	Anion		
	$Cl^{\ominus}$	$H_3C-S(=O)_2-O^{\ominus}$	$F_3C-S(=O)_2-N^{\ominus}-S(=O)_2-CF_3$
	C_4MImCl	-	-
	C_{10}MImCl	-	-
	C_{16}MImCl	C_{16}MImMeS	C_{16}MImNTf$_2$
	C_{18}MImCl	-	-

Fig. 1. Chemical structures of the IS screened for anti-*L. amazonensis* activity.