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Acid heteropolysaccharides with potent antileishmanial effects

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ABSTRACT

The present study investigated *in vitro* the effects of sulphated heterorhamnan (Go3), iota-/nu-carrageenans (G3d and EHW-I) and arabinogalactan (ARAGAL) polysaccharides on macrophage activation and inhibition of intracellular amastigotes of *Leishmania (L.) amazonensis*. All the sulphated polysaccharides (Go3, G3d and EHW-I) promoted increased nitric oxide production varying from 71 to 110%. The leishmanicidal activity of all compounds was compared to the inhibition effect of Meglumine Antimoniate at 300 μg/mL (~79%), used as positive control. Inhibition of *Leishmania* (*L.*) *amazonensis* growth was 55% with 5 μg/mL of Go3, 50% and 98% to G3d and EHW-I, respectively at 10 μg/mL, and 88% with 10 μg/mL of ARAGAL. The superoxide anion scavenging activity for the sulphated polysaccharides varied from approximately 30–55% at 10 μg/mL. In conclusion, the results of the present study indicate the promising potential of these polysaccharides for the development of new alternative therapeutic agents against leishmaniasis.

1. Introduction

Leishmaniasis is an anthropozoonosis considered one of world's six major infectious diseases due to its high detection rate and its ability to produce deformities [1]. This disease is caused by infection by protozoan parasites of the genus *Leishmania*, a member of the family *Trypanosomatidae*. It is transmitted to mammals through the bite of female invertebrates of the subfamily *Phlebotominae*, a sandfly [2]. In the mammalian host, parasites live inside macrophage cells and develop several strategies to escape from immune responses [3,4]. The treatment of leishmaniasis is based on pentavalent antimonials (PVAs), which have been used for over six decades [5]. This chemotherapy is known for its serious side effects and the emergence of resistance of the parasites to antimony. Although, the leishmanicidal mechanism exerted by PVAs is not well understood, evidences have suggested that the leishmanicidal effects exhibited by these drugs require, in part, host cell response [6]. Once activated, macrophages produce different cytokines, reactive oxygen and nitrogen species, which are important to microbicidal activity exerted by these cells [7]. Thus, compounds that are able to activate macrophages to produce these and other mediators could present leishmanicidal activity. Therefore, the immunochemotherapy should be considered as an alternative modality for the treatment of leishmaniasis [6,8]. Polysaccharides of various sources and with different structural characteristics have been classified as immunomodulators because they increase the speed or prolong the duration of the specific immune response [9–12]. It has been proposed that polysaccharides can bind to macrophage membrane receptors and activate these cells in various ways, promoting specific responses as antitumor and antimicrobial [9,13,14]. Among the receptors already characterized on macrophages that recognize polysaccharides include Toll-like receptor (TLR); scavenger receptor (SR); complement receptor type 3 (CR3); MR, mannose receptor (MR); mannan binding lectin (MBL) as revised [15]. The binding of polysaccharides at receptors on macrophages can stimulate these cells to trigger different process like enhance of phagocytosis, production of mediators as reactive oxygen and nitrogen species, and different cytokines, which act as effectors in immunological response [9]. In this sense, many studies aiming to evaluate the immunomodulatory potential of polysaccharides have been performed in recent decades.

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In previous study, arabinogalactan (ARAGAL) stimulated the releasing of tumour necrosis factor-α (TNF-α) in mouse and showed a significant effect against Sarcoma 180 (S-180) tumour model [16]. The authors suggested that the antitumor activity of ARAGAL may be due to its immunomodulatory effects, since it increased the number of activated macrophages and superoxide anion production by these cells in mouse treated with the polymer [12]. In macrophages infected with *Leishmania*, this activation may culminate in killing the intracellular parasites. Accordingly, galactomannan (GMPOLY) isolated from lichen *Ramalina celastri* and an arabinomannose (Z-100) extracted from *Mycobacterium tuberculosis* have been classified as macrophage activators exhibiting leishmanicidal effect *in vitro* [17,18]. The use of the chitosan polymer as a vehicle in the preparations of meglumine antimoniate or doxorubicin for experimental leishmaniasis treatment has been investigated and promising results has been observed [19,20]. Recently, mannose-conjugated chitosan nanoparticles loaded with rifampicin, aiming a selective delivery of rifampicin for the treatment of visceral leishmaniasis has been investigated [21]. Sulphated polysaccharides also have been studied as biological response modifiers [22]. Sulphated heterorhamnan (Go3), iota-/nu-carrageenan fraction (G3d) and fraction (EHW-I) exhibited antiviral activity against herpes simplex [23–25].

Considering the need to find alternative treatments for human leishmaniasis, the immunomodulatory effects exhibited by ARAGAL [12,16], the absence of study with sulphated heterorhamnan (Go3), iota-/nu-carrageenan fractions (G3d and EHW-I) on macrophages and *Leishmania*, and the leishmanicidal activity already observed in some polysaccharides [17,26–28], this study investigated the effect of different polysaccharides on macrophage activation and on viability of amastigote forms of *Leishmania (L.) amazonensis in vitro*.

2. Materials and methods

2.1. Material

Phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides (LPS), *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES), ferricytochrome *c*, superoxide dismutase (SOD) (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (MTT), sulphanilamide, and naphthyl ethylenediamine were obtained from Sigma Chemical Co. (St Louis, MO). Tissue culture materials were supplied by Corning or Nunc A/S. Eagle's minimum essential medium (MEM) and foetal bovine serum were obtained from Cultilab (Brazil), bacteriological agar, bacteriological peptone and beef extract from Himedia and Glucantime® (Meglumine antimoniate) from Aventis. The PMA was dissolved in Me_2SO and stored at −20 °C as a 1 mg/mL stock solution. All the other reagents were commercial products of the highest purity available.

2.2. Polysaccharides

The arabinogalactan (ARAGAL) used in this study was isolated from *Anadenanthera colubrina* and characterized [29], the heteropolysaccharide has a (1,3)-linked β-D-Galp main-chain and many different side-chains. Sulphated heterorhamnan Go3 (26.1% sulphate) was extracted from the green seaweed *Gayralia oxysperma* [23], the backbone is constituted by 3- and 2-linked rhamnosyl units with ratio (1.0:0.8) and side chains containing 2-sulphated glucuronic and galacturonic acids. The iota-/nu-carrageenan fraction, G3d (29.4% sulphate) was extracted from the red seaweed *Gymnogongrus griffithsiae*, as previously described [24]. This carrageenan is composed of iota (70%), nu- (17%) and kappa (13%) diads [25]. Ground red seaweed *Eucheuma denticulatum* was subjected to aqueous extraction at 25 °C for 3 h. After centrifugation, the pellet was extracted with water at 80 °C for 3 h to yield the EHW-I fraction (31% sulphate). The ^{1}H NMR analysis of EHW-I showed signals at 5.33 and 5.52 ppm, which correspond, respectively, to H1 of 3,6-anhydro-α-D-galactopyranose 2-sulphate and H1 of α-D-galactopyranose 2,6-disulphate [30]. These results indicated that EHW-I contains a hybrid iota-/nu-carrageenan (ratio 77:23), as previously reported for the carrageenan-producing *E. denticulatum* red seaweed [31]. The ^{1}H NMR analysis spectrum of EHW-I is included in the supplementary data.

The polysaccharides were dissolved in ultrapure water, sterilized by filtration through a 0.22 μm membrane, and their concentrations determined by the phenol–sulphuric acid method [32]. For the purpose of control in case of an eventual LPS contamination, samples of polysaccharides were analyzed by GC–MS [33], but showed no signal of the presence of LPS (data not shown).

2.3. Leishmania culture

Leishmania (Leishmania) amazonensis (designated MHOM/BR/73/M2269) promastigotes were cultured at 26–28 °C in Evans' modified Tobie's medium (EMTM), and in the exponential phase transferred and maintained in MEM medium supplemented with 10% heat-inactivated foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin.

2.4. Peritoneal macrophage preparation

Swiss mice (6–8 weeks old) provided by biotery of Biological Campus of Federal University of Paraná have received a standard laboratory diet (Purine) were used to obtain peritoneal macrophages. All the legal recommendations of Brazilian legislation (Law No. 6.638,05 Nov. 1979) for animal handling procedures for scientific research were approved by the Animal Ethics Committee. The peritoneal macrophages were collected by infusing the donors' peritoneal cavity with 8–10 mL of chilled sterile phosphate-buffered saline (PBS). The cells were plated in a culture medium (minimum essential medium-MEM, 5% foetal bovine serum and antibiotics) or HBSS in 24 or 96-well plates (5×10^5 cells/well). After 1–2 h of incubation at 37 °C under 5% CO_2 in a humidified incubator, non-adherent cells were removed by washing twice with PBS at 37 °C.

2.5. Cytotoxicity assays

Adherent macrophages in 96-well plates were incubated for 48 h in the absence (control) or presence of ARAGAL, Go3, G3d and EHW-I at various concentrations (5–100 μg/mL). Cell viability was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT), as previously described [34].

2.6. Measurement of superoxide anion production

Adherent macrophages in 96-well plates were incubated in a standard reaction mixture consisting of HBSS containing ferricytochrome c (80 μM) in the presence or absence of PMA (1 μg/mL). Go3, G3d and EHW-I (25–100 μg/mL) were added to the standard reaction mixture. Controls were prepared without polysaccharides, and with adequate amounts of DMSO (solvent of PMA). Absorbance was measured at 550 nm after 2 h and the amount of superoxide anion released was calculated as previously demonstrated [35]. The concentration of reduced cytochrome c was determined using the molar extinction coefficient = 2.1×10^4 M^{-1} cm^{-1}. The results are expressed in μmol of reduced cytochrome c per 5×10^5 cells.