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Article

Phenylbenzohydrazides Obtained from Isatoic Anhydride Present Anti-Inflammatory Activity *in Vivo* and *in Vitro*

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Abstract: Background: Despite the existence of a wide variety of anti-inflammatory drugs, the vast majority are classified as steroidal or non-steroidal. Both classes with many side effects that limit usage. Thus, the search for new molecules with anti-inflammatory potential is still important. Methods: Five phenylbenzohydrazides were synthetized and evaluated in pre-clinical models of acute inflammation *in vivo* and *in vitro*. Results: Substances significantly reduced cell migration induced by carrageenan. It was also observed an intense inhibition in protein extravasation and cytokines (IL-6, IL-1β, and TNF-α) production as well as nitric oxide reduction. INL-11 demonstrated to be the most potent since the reduction was significant even when compared with dexamethasone. *In vitro* INLs also reduced cytokines and NO production and iNOS activity. INL-11 was the most effective in reducing cell migration *in vitro*. Conclusions: our data suggest that these substances are suitable for further development to new series of compounds that could lead to new hits and future drug prototypes for anti-inflammatory conditions.

Keywords: Phenylbenzohydrazides; isatoic anhydride; inflammation; anti-inflammatory substance; carrageenan-induced inflammation; nitric oxide

1. Introduction

The complexity of regulation of inflammation, and the overlapping between different mediator systems is a complexity presenting challenges to the development of highly effective, tolerable, and safe therapeutics. Inflammatory diseases are often controlled only partly, even by cocktails of substances [1].

The most effective functional group of drugs to treat inflammation are the glucocorticoids. These drugs have the potential to impact on the expression of at least 6,500 genes [2]; their non-steroidal counterpart, non-steroid anti-inflammatory drugs have a narrower, but nonetheless broad impact, in part by virtue of the range of cyclo-oxygenase products that are affected. However, both the efficacy and the adverse effect profile of glucocorticoids are related to their breadth of activity. Thus, the search for new drugs that could act in a different system from NSAID and SAID continues to be an objective to the pharmacologists and the medicinal chemists.

Phenylbenzohydrazides reported in this paper were designed through molecular simplification of the arylidenehydrazinyl-quinazolinones which showed anti-inflammatory activity [3,4]. Different substituents were introduced to phenyl hydrazide moiety aiming to investigate their influence on anti-inflammatory activity. Isatoic anhydride have

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also been tested for being the starting material and presenting structural similarity with molecules previously studied by our group [5,6].

2. Materials and Methods

2.1. Synthesis of INLs

Synthesis of INL-06: A mixture of isatoic anhydride (1 g, 6.13 mmol) and phenyl hydrazine (0.663 g, 6.13 mmol) in ethanol (60 mL) was heated under reflux for 2 hours. Reaction was accomplished by TLC. The resulting solid was filtered and air-dried to furnish the product in 80% yield. Its structure was confirmed by ^{1}H NMR, GC-MS and melting point according to literature. m.p = 170-171 °C (as reported, Shemchuk, 2008), ^{1}H NMR (DMSO-d6, 400 MHz) δ ppm 10.10 (s, 1H, NH), 7.79 (s, 1H, NH), 7.66 (d, 1H, J = 7.8, ArH), 7.22-7.13 (m, 3H, ArH), 6.78 (d, 2H, *J* = 7.7, ArH), 6.75-6.69 (m, 2H, ArH), 6.56 (t, 1H, *J* = 7.8, ArH), 6.39 (s, 2H, NH_2). MS (EI): m/z 227 (24%), m/z 120 (100%), m/z 92 (21%), m/z 121 (8%), m/z 77 (4%).

Synthesis of INL-07, 09, 10 and 11: To a solution of corresponding phenyl hydrazine (6.13 mmol) in ethanol (60 mL) was added NaOH (0.245 g, 6.13 mmol). The mixture was stirred by 5 minutes then isatoic anhydride (1 g, 6.13 mmol) was added. The reaction media was heated under reflux for 2 hours. After this period, the resulting solid was filtered and air-dried to furnish the respective phenylbenzohydrazide. Their structures were confirmed by ^{1}H NMR, GC-MS and melting point according to literature [7-9].

INL-07. Yield 61 %. White solid, mp = 198-199 °C (Anand, 2015) ^{1}H NMR (DMSO-d6, 400 MHz) δ ppm 10.13 (s, 1H), 8.00 (s, 1H), 7.66 (d, 1H, *J* = 7.9Hz), 7.31-7.27 (m, 2H), 6.73 (d, 3H, *J* = 8.9 Hz), 6.54 (t, 1H, *J* = 8.0 Hz), 6.40 (s, 2H). MS (EI): m/z 305 (9%), m/z 307 (9%), m/z 120 (100%), m/z 92 (17%), m/z 121 (12%).

INL-09. Yield 60%. White solid. ^{1}H NMR (DMSO-d6, 400 MHz) δ ppm 10.13 (s, 1H), 7.98 (s, 1H), 7.21-7.16 (m, 3H), 6.78 (d, 2H, *J* = 8.9Hz), (d, 1H, *J* = 9.2 Hz), 6.54 (t, 1H, *J* = 8.0 Hz), 6.39 (s, 2H). MS (EI): m/z 261 (15%), m/z 263 (5%), m/z 120 (100%), m/z 92 (18%), m/z 121 (9%).

INL-10. Yield 45%. White solid, mp = 140-142 °C (Yang, 2015). ^{1}H NMR (DMSO-d6, 400 MHz) δ ppm 10.10 (s, 1H), 7.76 (s, 1H), 7.65 (d, 1H, *J* = 7.8 Hz), 7.18 (t, 1H, *J* = 8.4 Hz), 6.99 (t, 2H, *J* = 8.8 Hz), 6.80-6.77 (m, 2H), 6.72 (d, 1H, *J* = 8.2 Hz), 6.54 (t, 1H, *J* = 8.0 Hz), 6.37 (s, 2H). MS (EI): m/z 245 (24%), m/z 246 (4%), m/z 120 (100%), m/z 92 (21%), m/z 121 (8%).

INL-11. Yield 78%. White solid. ^{1}H NMR (DMSO-d6, 400 MHz) δ ppm 10.15 (s, 1H), 7.69 (d, 1H, *J* = 7.9 Hz), 7.18 (t, 1H, *J* = 8.4 Hz), 7.14 (s, 1H), 7.03-6.99 (m, 2H), 6.72 (t, 2H, *J* = 8.6 Hz), 6.66 (t, 1H, *J* = 7.3 Hz), 6.55 (t, 1H, *J* = 8.0 Hz), 6.39 (s, 2H), 2.21 (s, 3H). MS (EI): m/z 241 (29%), m/z 242 (5%), m/z 120 (100%), m/z 92 (17%), m/z 121 (9%).

2.2. Animals

Swiss Webster mice (25-30 g) were kindly donated by Instituto Vital Brazil (Niterói, Rio de Janeiro, Brazil). Mice were maintained in a room with cycle light-dark of 12 h, 22 ± 2 °C to 60% to 80% humidity and with food and water provided ad libitum. Animals were acclimatized to the laboratory conditions for at least 1h before each test on set and were used only once throughout the experiments. All protocols were conducted in accordance with the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals [10] and followed the principles and guidelines adopted by the National Council for the Control of Animal Experimentation (CONCEA), approved by the Ethical Committee for Animal Research (# 31/19 and 34/19). All experimental protocols were performed during light phase. Animal numbers per group was kept at a minimum and at the end of each experiment mice were killed by ketamine/xylazine overdose.

2.3. Drugs, reagents, and treatments

Dexamethasone, L-NMMA (L-NG-monomethyl arginine), Ara-C (cytosine arabinoside), MTT (3-(4,5-dimethyl-ltiazol-2-yl)-2,5-diphenyltetrazole), isatoic anhydride (AIST**C**), phenyl hydrazines, NaOH and lipopolysaccharide were purchased from Sigma

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Aldrich (St. Louis, MO, USA). Ethanol was purchased from Merck Inc. (Brazil). Cytokines kits were purchased from BD Biosciences (EUA), protein kit (Kit Pierce BCA ™ Protein Assay) was purchased from Thermo Fisher Scientific, Inc (MA, EUA).

INLs were dissolved in dimethylsulphoxide (DMSO) to prepare 100 mg/ml stock solutions. For use, solutions were prepared from each stock solution using tween as vehicle. Doses of 1 to 30 mg/kg (final volume of 0.1ml per animal) were administered by gavage and final tween percentage did not exceed 1%. Dexamethasone (7.5 μmol/kg), L-NMMA (100 μM) were used as references drugs. The dose of dexamethasone and L-NMMA was chosen based in previous results obtained by our group when it was calculated the DE_{50} or IC_{50}, the dose/concentration that caused a 50% reduction in the effect in each procedure. The control group was given vehicle (tween). All drugs were diluted just before their use.

2.4. Cell Culture

RAW 264.7 (ATCC # TIB-71) was grown in RPMI medium supplemented with 10% fetal bovine serum (from now on, named as RPMI) and kept in a 5% CO_2 incubator at 37°C. An exchange of RPMI was carried out until cells reached 90% confluence and exponential growth. On the day of assays, cells were collected by scraping bottles and put to adhere in 96- or 12-well culture plates (2 x 10^6 cells/ml).

2.5. In vitro Toxicity Test (Cell Viability)

In 96-well plates, RAW 264.7 cells were put to adhere at 37°C, 5% CO_2. After 30 minutes incubation with INLs (0.1, 1 or 10 μM) LPS (1 μg/ml) was added to some groups. After 24 h incubation (at 37º C, 5% CO_2) supernatant was changed and MTT solution (5 mg/ml, 100 μl/well) was added. After 4 h incubation (at 37°C, 5% CO_2) supernatants were discarded and DMSO (100 μl/well) was added to solubilize the MTT-formazan crystals formed [11]. Absorbance was measured at a wavelength of 570 nm using a Flexstation reader (Molecular Devices, USA). Control groups were composed by cells which received only RMPI plus DMSO.

2.6. In vivo Toxicity Test

Different groups of animals received an oral administration of 100 mg/kg of INLs. After 24 h mice were euthanized with ketamine (50 mg/kg)/xylazine (20 mg/kg). Sample of blood was collected. The femur was removed, the ends were cut, the bone marrow was washed with 1 ml of saline (NaCl 0.9%) and collected. Both samples of blood and bone marrow were submitted to a complete blood hemogram and cell count, respectively, in an automatic cell counter (PocH-100iV Diff, Sysmex).

2.7. Carrageenan-induced inflammation into the subcutaneous air pouch (SAP)

The protocol was based in Romano et al [12] with modifications [13]. A subcutaneous air pouch was induced in mice's back through an injection of 10 ml of sterile air. After 3 days, a new injection of 7 ml of sterile air was performed on the animals' backs. On the 6th day, the animals were oral treated with vehicle, INLs (at different doses) or dexamethasone (7.5 μmol/kg) and after 60 min mice received an injection of saline (NaCl 0.9%) or carrageenan (0.5%, 1 ml) into the SAP. After 24 h the animals were euthanized, and the SAP washed with 1 ml of saline. The exudate was collected for leukocyte count and centrifuged at 1,500 r.p.m., for 10 minutes, 4 °C. The supernatant was collected and stored at -20 °C for several dosages (see below).

2.8. Quantification of proteins and cytokines

To perform the quantification of proteins in the exudate obtained in the BAS experiment, a proper protein dosing kit, the BCA Protein Assay Reagent (Thermo Fisher Scientific, Inc., USA) was used. Then, 5 μl of the sample was incubated with 195 μl of the solution of reagents A and B in an oven at 37 °C for 30 min. The absorbance was measured at

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570 nm and the protein concentration was calculated using a standard curve made from BSA and the results expressed in μg/ml.

Quantification of cytokines was performed in the exudate collected from BAS and in the supernatant of RAW 264.7 cells, using an immunoenzymatic assay method (ELISA) using specific ELISA kits (BD OptEIA™ Set mouse, B&D, USA). For the assay, 96-well plates were incubated with specific capture antibody at 4 °C for 18 h. The plates were then washed (3 times), filled with blocking buffer and incubated at room temperature for 1 h, then they were washed again (3 times) and 25 μl of the specific standard or samples were added. The plates were incubated for 18 h in an oven at 37 °C, after which they were washed (5 times) and incubated with detection antibody plus the enzyme, with subsequent incubation for 1 h, at room temperature. Substrate solution was then added and incubated for 30 min. and protected from light followed by addition of stop solution (H_3PO_4 1N, 12.5 μl/well). The absorbance reading was performed at 450 nm and the concentration of cytokines was performed using a standard curve for each respective cytokine. Values were expressed as pg/ml.

2.9. Quantification of Nitric Oxide (NO) Production

When produced in biological fluids, NO interacts with hemoglobin and decays to nitrate (NO_3^-) and when its production occurs *in vitro* it interacts with oxygen decaying to nitrite (NO_2^-). The protocol for converting nitrate to nitrite was described by Bartholomew [14] with adaptations made by Raymundo et al. [13] and the technique used to measure NO_2 was described by Green et al. [15]. The absorbance reading was done in a microplate reader (FlexStation, Molecular Devices, USA), at 540 nm. The sodium nitrite concentrations were calculated using a standard sodium nitrite curve.

2.10. Cell Migration in vitro

To assess the effect of INLs on cell migration *in vitro*, RAW 264.6 cells were plated at $1x10^6$/well in 12-well plates (in a final volume of 2 ml) and after 3 hours a risk was made in the well with the aid of a tip. The wells were washed with RPMI and 10^{-5} M of Arabinoside (AraC; Sigma-Aldrich, USA) was added to prevent cell proliferation. The cells were treated with INLs (0.1, 1 or 10 μM). Photos were obtained immediately after treatment (0 h) and after 24 h, using an EvosM500 microscope (ThermoFisher). The area was measured with the aid of the ImageJ software. To obtain the results, three independent experiments were carried out.

2.11. Statistical analysis

Each *in vivo* group was composed of 6 to 8 animals selected at random. *In vitro* experiments were repeated at least 3 times on different days and each experimental group was done in triplicate. The results were expressed as mean ± standard deviation and statistical significance was calculated by analysis of variance (ANOVA) followed by the Tukey post-test, using GraphPad Prism 8.2 program. P values less than 0.05 were considered significant.

3. Results

3.1. Chemistry

First, it was investigated optimal conditions to obtain phenylbenzohydrazides (INL-06, 07, 09, 10 and 11) due to the existence of several procedures described for obtaining INL-06 [7-9,17]. With the optimized condition on hand, isatoic anhydride was reacted with different phenyl hydrazines under reflux in ethanol for two hours to afford respective phenylbenzohydrazides (Scheme 1). We consider using sonication to improve our results given its known use in heterogeneous systems [18-20], however all attempts to reduce the reaction time and improve the yields using ultrasound irradiation have not been successful.

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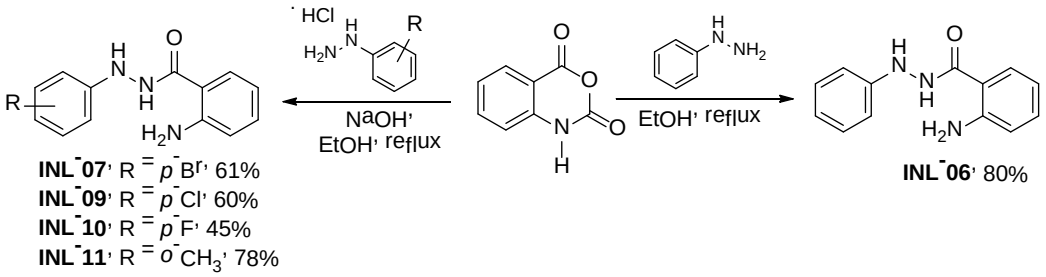

Scheme 1 – Synthesis of phenylbenzohydrazides.

3.2. None of INLs did induce any toxicity after oral administration

The oral administration of each INLs to mice (at 100 µmol/kg dose) did not affect haematological parameters after 7 days. It can be observed in figure 1 that AISTC-01, INL-06, INL-07, INL-10 or INL-11 did not affect the total number of cells in blood or bone marrow. No changes in the number of red blood cells, platelets, haemoglobin, and hematocrit were observed either indicating that all these substances do not present any toxic effect at the dose tested. However, when INL-09 was evaluated, it could be observed a significant reduction in all parameters suggesting a possible toxic effect. In view of these data INL-09 was not further tested in the subsequent models.

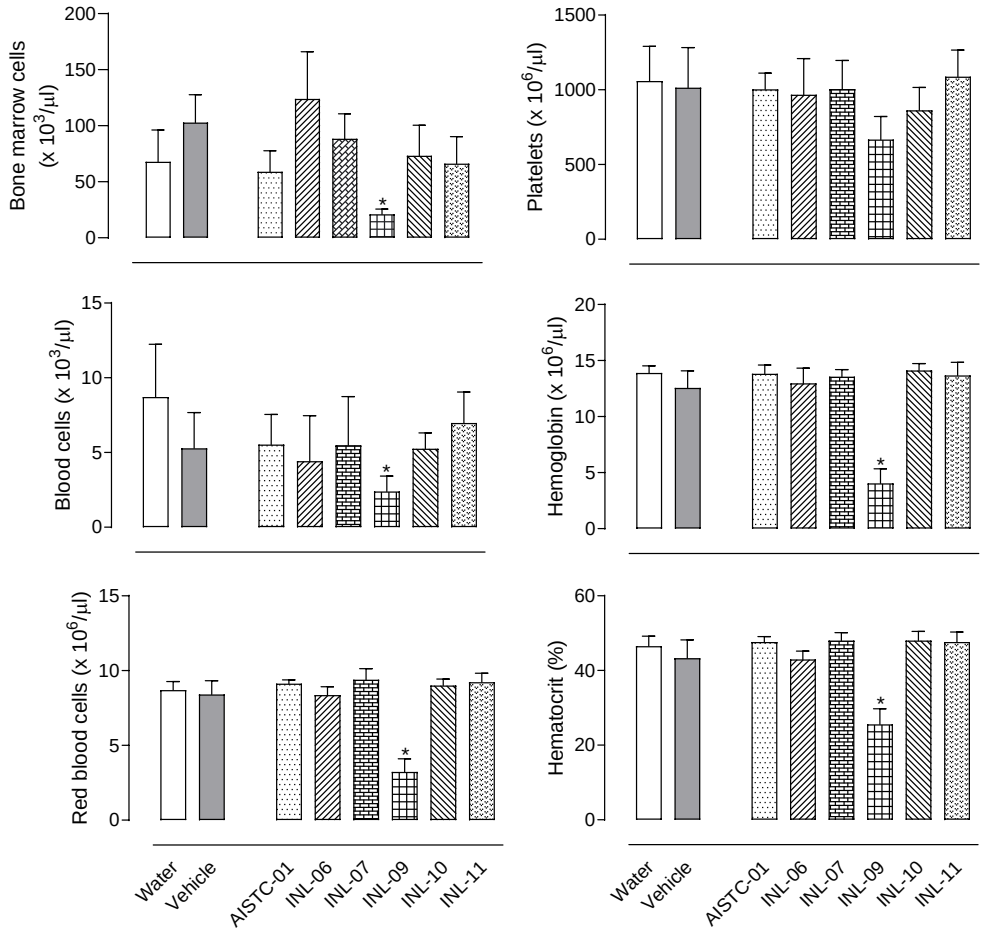

Figure 1 - AISTC-01 and INL-06, -07, -10, and -11 did not present *in vivo* toxic effects. Substances were orally administered (at 100 µmol/kg) to mice. After 7 days mice were sacrificed and blood, and bone marrow were collected to measurements. Data are expressed as media ± standard error (n=7). Statistical analyses were done using ANOVA following Newman-Kauls as post-test. # $p < 0.01$ when comparing the INL-09-treated group with vehicle-treated group.

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3.3. Leukocytes migration and protein extravasation were inhibited by INLs

The first step was to evaluate if INLs could present an effect against cell migration *in vivo*. In this regard, mice were pretreated with different doses of each INL and 1 hour after carrageenan was injected in the subcutaneous air pouch (SAP). The pretreatment with dexamethasone (a steroidal anti-inflammatory drug) inhibited in almost 50% the number of leukocytes that migrated to the SAP. AISTC as well as INL-11 significantly reduced cell migration at all doses used (10, 30 and 100 μmol/kg) while INL-06, INL-07, and INL-10 did present effects at 30 and 100 μmol/kg. It is interesting to note that effects observed were comparable to that with the positive control group, dexamethasone, even when the drug was intraperitoneally administered. In the same assay we decided to quantify the protein extravasated to the exudate as an indicative of increase in vascular permeability. AISTC and INLs also reduced protein accumulated in exudate, with all doses used. It is important to highlight that INL-10 and INL-11 significantly reduced protein extravasated even when comparing with dexamethasone-treated group (Figure 2).

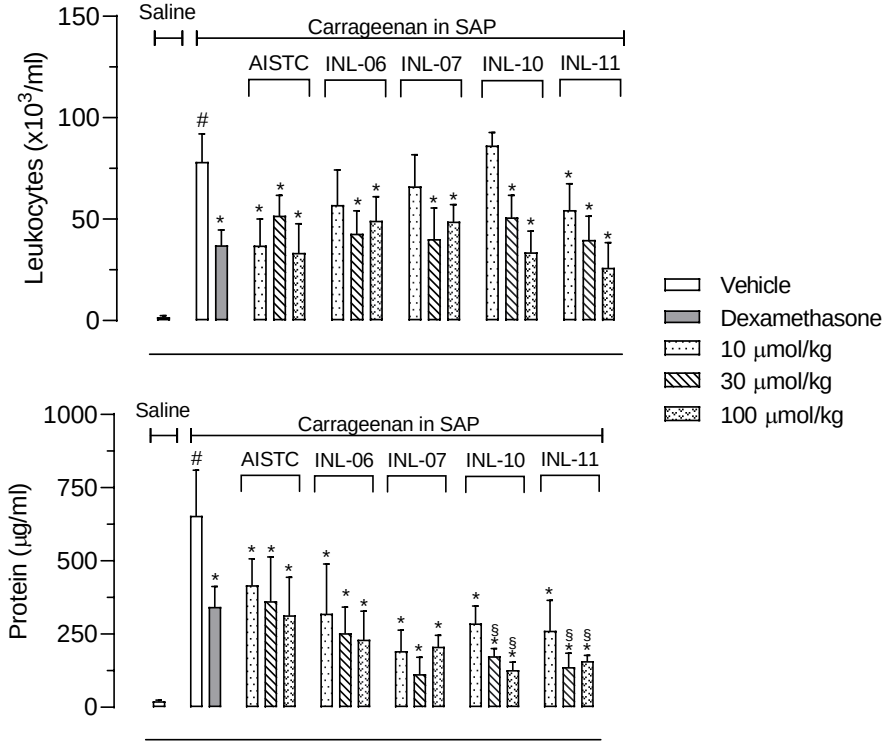

Figure 2 - AISTC and INLs did not reduce leukocyte migration and protein extravasated. Mice were pretreated with vehicle, AISTC, INL-06, INL-07, INL-10 or INL-11 (10, 30 and 100 μmol/kg, oral) or dexamethasone (7.5 μmol/kg, i.p.) 1 hour before carrageenan (0.5%, w/v) injection into the subcutaneous air pouch. Exudates were collected 24 hours after carrageenan injection. Results are expressed as media ± standard error (n=5-7). Statistical analyses were done using ANOVA following Newman-Kauls as post-test. # $p < 0.01$ when comparing the vehicle-treated group that received carrageenan in SAP with the vehicle-treated group that received saline in SAP and * $p < 0.01$ when comparing the dexamethasone, AISTC or INLs pretreated groups that received carrageenan in SAP with the vehicle-treated group that received carrageenan in SAP. & $p < 0.005$ when comparing AISTC or INLs pretreated groups that received carrageenan in SAP with the dexamethasone-treated group that received carrageenan in SAP.

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3.4. INLs inhibited inflammatory mediators produced in the subcutaneous air pouch

Next, we decided to quantify the production of cytokines and nitric oxide (NO) extravasated after carrageenan injection into the SAP. Figure 3 shows that IL-6 production was reduced only after pretreatment of mice with the higher dose of INL-10, whereas INL-11 presented a significant effect with all doses tested. In the other hand neither AISTC nor INL-6 and INL-7 affected the production of this cytokine.

When IL-1β was measured it could be observed that AISTC as well as all INLs significantly inhibited the cytokine production. The effects obtained after pre-treatment of mice with INL-06, INL-07, INL-10 or INL-11 were comparable to that observed with dexamethasone.

The most significant effect was regarding to TNF-α production. Although all substances significantly reduced the cytokine levels, it was observed an important difference between them. AISTC, INL-06 and INL-07 only presented a mild and significant effect with the two higher doses (30 and 100 μmol/kg). The most important effect was obtained with INL-11. This substance almost completely abolished the TNF-α production even when the lowest dose (10 μmol/kg) was used. In these groups the TNF-α production was similar to that quantified in negative control group (mice that received saline in the SAP).

We also measured nitric oxide (NO) accumulated in the exudate of the SAP. It is interesting to note that similarly to that observed with data to TNF-α. Although, AISTC, INL-06, INL-07, and INL-10 did reduce NO production, the effects of INL-11 were more pronounced. INL-11 almost completely abolished NO production (Figure 3).

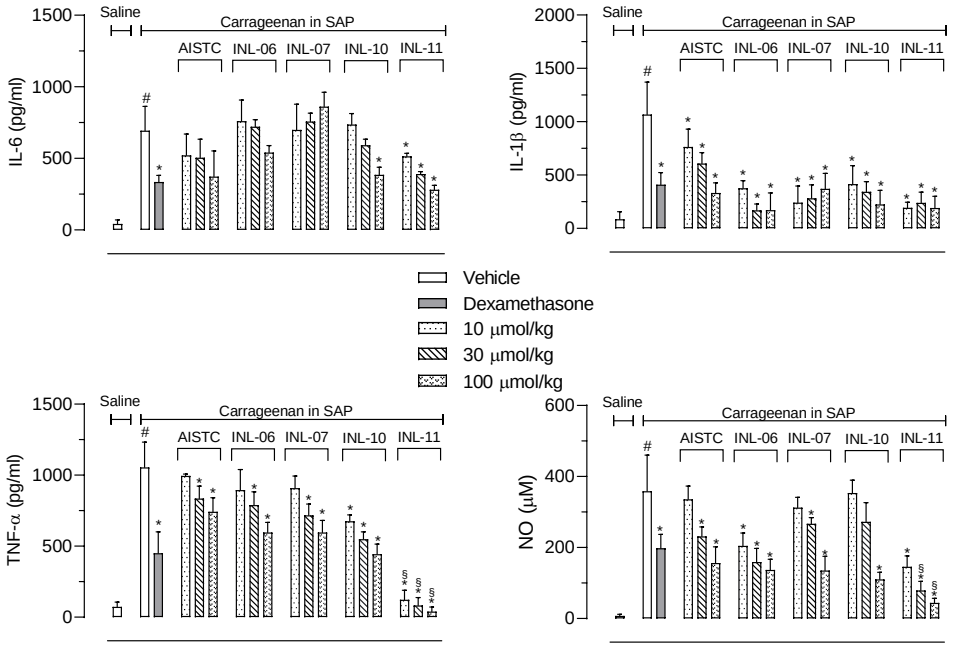

Figure 3 - AISTC and INLs reduced inflammatory mediators' production. Mice were pretreated with vehicle, AISTC, INL-06, INL-07, INL-10 or INL-11 (10, 30 and 100 μmol/kg, oral) or dexamethasone (7.5 μmol/kg, i.p.) 1 hour before carrageenan (0.5%, w/v) injection into the subcutaneous air pouch. Exudates were collected 24 hours after carrageenan injection. Results are expressed as media ± standard error (n=5-7). Statistical analyses were done using ANOVA following Newman-Kauls as post-test. # $p < 0.001$ when comparing the vehicle-treated group that received carrageenan in SAP with the vehicle-treated group that received saline in SAP and * $p < 0.05$ when comparing the dexamethasone-, AISTC- or INLs-pretreated groups that received carrageenan in SAP with the vehicle-treated group that received carrageenan in SAP. & $p < 0.001$ when comparing AISTC or INLs pretreated groups that received carrageenan in SAP with the dexamethasone-treated group that received carrageenan in SAP.

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3.5. AISTC and INLs reduced inflammatory mediators' production and cell migration in vitro

As INLs presented significant effects when administered orally to mice we decided to further investigate if these substances could present effects when incubated *in vitro*. For this purpose, RAW 264.A1 cell line was incubated with INLs, at different concentrations, and activated with lipopolysaccharide (LPS, 1 μg/ml). After 24 hours cell supernatants were collected to several dosages. Figure 4 shows that non-activated cells produce 4.6 ± 1.4 μM of NO. After activation with LPS cells increased in 10.58-fold NO production reaching 48.7 ± 6.5 μM of the mediator. The inhibitor of the inducible nitric oxide synthase, L-NMMA, reduced NO production in 62% (18.3 ± 6.7 μM). Despite AISTC did not affect NO production by cells, all INLs did significantly reduce the levels of NO. While INL-07, INL-10 and INL-11 inhibited only at 30 μM concentration, INL-06 presented significant effect with the two higher concentrations used (10 and 30 μM).

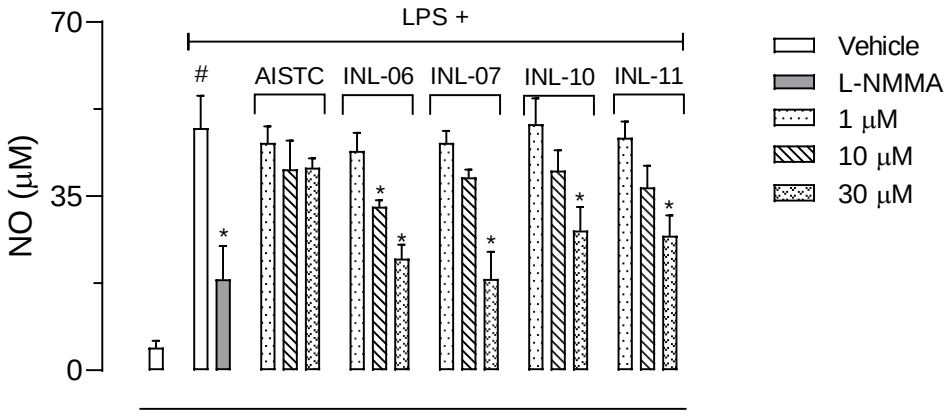

Figure 4 - INLs inhibited nitric oxide production (NO). RAW 264.A1 cells were incubated with different concentrations of AISTC or INLs and after 1 hour received lipopolysaccharide (LPS, 1 μg/ml). After 24 hours the supernatants were collected for NO measurement. Results are expressed as media ± standard error (n=5). Statistical analyses were done using ANOVA following Newman-Kauls as post-test. # $p < 0.001$ when comparing with the vehicle-treated group, and * $p < 0.01$ when comparing AISTC - or INLs-groups + LPS with LPS-treated groups.

The data obtained so far are suggestive that AISTC and INLs can inhibit NO production, however we cannot conclude if this effect is due to inhibition in inducible nitric oxide synthase (iNOS) enzyme expression, its activity, or a direct NO-scavenger effect of each substance.

Trying to elucidate these possibilities we first incubated LPS-activated cells with the substances and after 8 hours of activation, a period where protein synthesis of iNOS was finished and the enzyme begins it activity, each substance was added. After 24 hours of activation the supernatants were collected, and NO was measured. Results showed in figure 5 demonstrated that INLs affected the NO production when added 8 hours post-LPS activation suggesting that these substances can influence enzyme activity.

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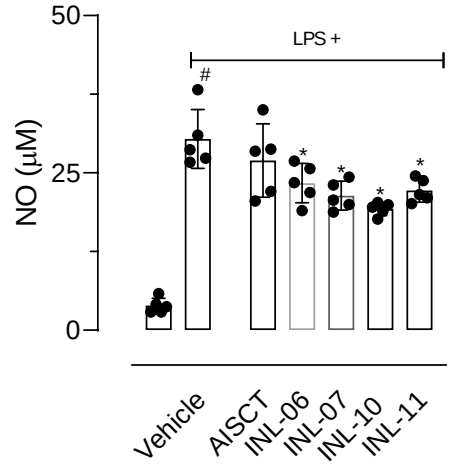

Figure 5: Effect of AISTC and INLs in inducible nitric oxide synthase enzyme activity. RAW 264.7 cells were incubated with vehicle or lipopolysaccharide (LPS, 1 μg/ml) and after 8 hours received AISTC or INLs (10 μM). Twenty-four hours after LPS activation NO accumulated in supernatant was measured. Results are expressed as media ± SD (n=5-7). Statistical significance was calculated in GraphPad Prism 8.0 (San Diego, CA, USA) using analyses of variance (ANOVA) followed by Tukey post-test with $p < 0.01$ (#) when comparing LPS-activated cells treated with vehicle and non-activated cells treated with vehicle. (*) when comparing LPS-activated cells treated with AISTC or INLs and LPS-activated cells treated with vehicle.

We also measured cytokines produced by RAW 264.7 cells after activation with LPS. Our data demonstrated that LPS activation resulted in high levels of IL-1β, IL-6 and TNF-α production after 24 hours (1,065 ± 55.8; 559.3 ± 76.7; 3,399 ± 414.1 pg/ml, respectively). When cells were preincubated with different concentrations of each INL it could be noted that none of the substances affected IL-1β production. On the Other hand, all INLs significantly reduced IL-6 production. The higher concentration of INL-07, INL-10 and INL-11 drastically reduced the levels of IL-6 even when comparing with the positive control group (activated cells preincubated with dexamethasone). The same three INLs also reduced the TNF-α production. Worth mentioning that all three concentrations used (1, 10 and 30 μM) significantly inhibited the cytokine and the higher concentration (Figure 6).

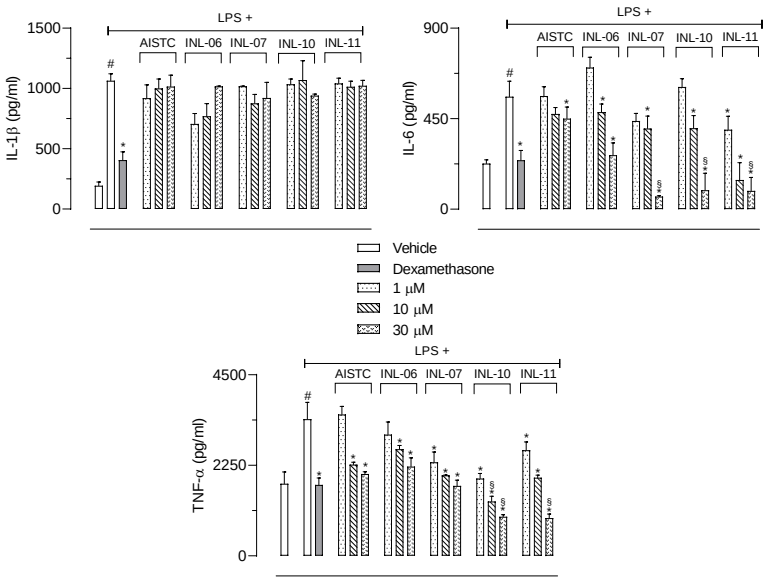

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Figure 6 - INLs inhibited cytokines production in vitro. RAW 264.7 cells were incubated with different concentrations of AISTC or INLs and after 1 hour received lipopolysaccharide (1 μg/ml). After 24 hours the supernatants were collected for IL-1β, IL-6 or TNF-α measurement. Results are expressed as media ± standard error (n=5). Statistical analyses were obtained using ANOVA following Newman-Kauls as post-test. # $p < 0.001$ when comparing with the vehicle-treated group, and * $p < 0.01$ when comparing AISTC- or INLs-groups + LPS with LPS-treated groups.

3.6. INLs reduced cell migration in vitro

Since our data suggest that INLs reduce cell migration into the subcutaneous air pouch after injection of an inflammatory agent we decided to test if the substances could present a similar effect *in vitro*. For this purpose, we used the wound healing model. In this model data acquisition indicate the cell-free area after 24 hours of incubation with INLs and LPS. Our results indicate that INL-11 was the substance that most inhibited cell migration. After 24 hours in INL-11-treated cells the percentage of area remaining free of cells 55% of the total. All Other INLs also reduced/inhibited cell migration and AISTC was the substance with the lowest ability to inhibit cell migration (Figure 7).

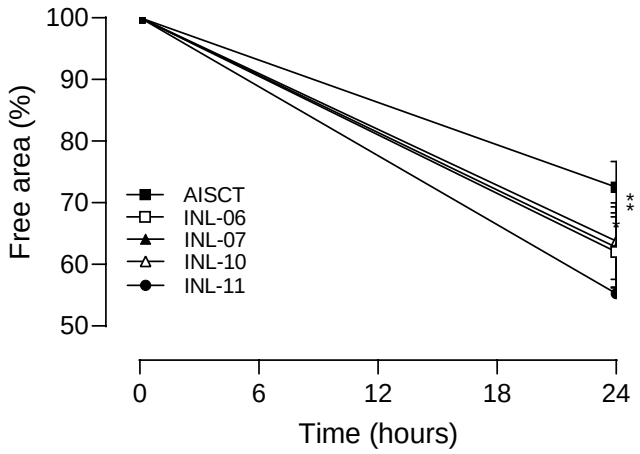

Figure 7 - INLs inhibited RAW 264.7 cells migration *in vitro*. In a confluent cell monolayer, a healing was done with a tip. After 24 hours the healing capacity of cells was quantified by measuring the cell-free area using a microscope. Results are expressed as media ± standard error (n=5). Statistical analyses were done using ANOVA following Newman-Kauls as post-test. # $p < 0.01$ when comparing each group at 24 hours with their respective group at 0 hour.

4. Discussion

Non-steroidal anti-inflammatory drugs are one of the most prescribed classes of drugs worldwide and are used to treat pain, acute inflammation, and are also used to treat chronic diseases such as osteoarthritis, rheumatoid arthritis, and musculoskeletal disorders [21]. However, the drugs of this class currently available on the market cause many adverse effects that harm the patient's life, leading to limitation of use. Thus, searching for new drugs with better therapeutic potential are a goal of several groups. Our data suggest for the first time, the phenylhydrazide molecules INL-10 and INL-11 as anti-inflammatory substances with potential to further studies.

Our data demonstrated that AISTC as well as INLs reduced in a dose-dependent manner the migration of leukocyte into the SAP. In this model, at 24 hours-post carrageenan injection, there are a high number of neutrophils accumulated in the exudate. Our results agree with literature demonstrating that cellular reactions in inflammatory responses are seen within hours, with neutrophils being the first cells attracted to the site of injury [22-24]. Although neutrophils are the first line cells in the process, they also

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contribute to inflammation by releasing inflammatory mediators, including those that attract macrophages to the site of inflammation [23,25].

The inflammatory processes evoked by carrageenan also involves an increase in vascular permeability. Among diverse inflammatory mediators that can induce vascular permeability, it is well known that nitric oxide (NO) and PGE2 are major factors involved in the pathogenesis of many inflammation-associated diseases. The resultant is an increase in protein leakage and the accumulation of NO and cytokines in the exudate [26]. INLs also drastically reduced the increase in vascular permeability observed as an increase in protein leakage to the exudate. The effect observed was even more than the positive control group, dexamethasone.

As TNF-NO and COX2-PGE2 pathways are considered the two main pathways of inflammatory processes, and they are blocked by inhibitors of iNOS (corticosteroids) and COX (NSAID), respectively [12,27] it could be expected that INLs could inhibit one of these mediators thus reducing the inflammatory phenomena. Several cytokines, particularly TNF-α, IL-1ß are known to play key roles in the induction and perpetuation of inflammation. TNF-α is a cytokine that plays a key role in the innate immune response and is associated with reduced cell migration and exudation [28]. IL-1ß promotes the expression of adhesion molecules, leukocyte migration and increased vascular permeability, indicating that it acts as an important pro-inflammatory mediator [29]. In this regard we assess the effect of the substances under study in their ability in reducing the levels of those cytokines accumulated in the inflammatory exudates. When comparing all compounds tested it could be noted that INL-10 and INL-11 were the most potent in inhibiting production of all cytokines. Moreover, INL-11 almost completely abolished TNF-α production.

The inhibition of leukocyte migration observed in our study could also be related to a decrease in IL-1ß and TNF-α levels. These results are suggestive that the substances may be acting as modulators of the immune system by decreasing cell migration, exudation, and the production of immunomodulatory cytokines. Another possibility to explain the reduction in cytokines production could be a direct inhibition in leukocyte number migrating to SAP. Thus, a parallel reduction in cell number could consequently reduce cytokines produced. To exclude this hypothesis, we further used a cell culture of macrophages (RAW 264.7 cell line) to evaluate the direct effect of the substances against the cells. Similarly to that observed *in vivo*, INL-10 and INL-11 presented an inhibitory effect higher than the positive control group. In this system NO inhibition was observed only with higher concentrations. These results corroborate those observe *in vivo* suggesting that INLs reduce cell activity and not only cell motility and migratory capacity.

Salim et al [28] described that after 8 hours of activation with LPS there is a peak in the expression of the iNOS enzyme and that, despite this, the production of NO remains for 12 hours. So, in a tentative to evaluate if INLs could present a direct effect in the enzyme we evaluated the effect of INLs on NO production after 8 hours of LPS activation. Data obtained showed that INLs affected NO production. These results suggest that the action of inhibiting the production of NO caused by INLs can happen through the reduction of the activity of this enzyme.

After obtaining positive results in reducing the production of inflammatory mediators *in vitro*, we chose to evaluate whether incubation with AISTC and INLs would influence the process of cell migration. Our assay is advantageous because it mimics the process of cell migration *in vivo*, in addition to being considered a simple and inexpensive technique to analyze this process [30]. INL-10 and INL-11 inhibited in at least 50% the migratory capacity of macrophage. This result can be explained, at least in part, by the role of INLs in the inhibition of NO.

According to Maa et al. [31] macrophages activated with LPS initiate the synthesis of a series of mediators, including the iNOS enzyme. The NO produced actively participates in the SRC-FAK cascade (steroid receptor co-activator - focal adhesion kinase). Studies indicate that this cascade is linked to the process of macrophage mobility, influencing their migratory capacity [31,32]. By understanding the importance of the SRC-FAK cascade in

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the migration process and how this cascade is highly dependent on NO, our hypothesis is that the effect caused by INLs to reduce cell migration could be due to the reduction caused in the production of NO.

It was observed that when the substituents were slightly electron-withdrawing halogens as bromine and chlorine (INL-07 and INL-09, respectively) the anti-inflammatory activity had an increase. The introduction of methyl group (INL-11) an electron-donating group increased activity. We described herein an improvement compared to literature in which there was no need for long reaction times [3,8,16,17]. Reactions were carried out catalyst free [3,7] without the use of non-volatile solvents such as DMF [7,33] and using an equimolar amount of substrate and nucleophile [3,8] thus improving the synthesis of these molecules.

5. Conclusions

Taken together our data suggest that the phenylbenzohydrazides synthetized present a significant anti-inflammatory effect *in vivo* as well as *in vitro* indicating that these compounds could be useful hits for the search of new analogues of new anti-inflammatory drugs.

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Institutional Review Board Statement: The animal study protocol was approved by Animal Experimentation Ethics Committee of the Health Sciences Center of UFRJ, Rio de Janeiro (# 31/19 and 34/19).

Informed Consent Statement: Not applicable.

Data Availability Statement: all data can be obtained directly with authors.

Sample Availability: Samples of the compounds (AISTC, INL-06, INL-07, INL-10 and INL-11) are available from the authors.

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