

PA-Int5: An isatin-thiosemicarbazone derivative that exhibits anti-nociceptive and anti-inflammatory effects in Swiss mice

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Abstract. Pain and inflammation are symptoms of various diseases, and they can be modulated by different pathways, thus highlighting the importance of investigating the therapeutic effects of novel compounds. Previous studies have shown that isatin-thiosemicarbazone exhibits antitumor, antifungal antibacterial and other biological properties. Based on the wide range of biological effects of these compounds, the aim of the present study was to investigate the central nervous system (CNS) performance, and the anti-nociceptive and anti-inflammatory activity of (Z)-2-(5-nitro-2-oxoindolin-3-ylidene)-N-hydrozinecarbothioamide (PA-Int5) in treated mice. Three doses of PA-Int5 were tested orally (1.0, 2.5 and 5.0 mg/kg) in the nociceptive and inflammatory animal models. Additionally, the potential sedative effects of PA-Int5 (5 mg/kg, oral gavage) were investigated using an open field and rotarod tests, to exclude any possible unspecific effects of the nociceptive assays. Anti-nociceptive activity was assessed using the acetic acid-induced abdominal contortion and formalin tests, whereas anti-inflammatory activity

was assessed using a carrageenan-induced paw edema and zymosan-induced air-pouch models. PA-Int5 (5 mg/kg) induced anti-nociceptive activity in the abdominal contortion model. In the formalin test, PA-Int5 (at 2.5 and 5 mg/kg) reduced nociception in the second phase. At the higher dose tested, PA-Int5 did not affect spontaneous locomotion or motor coordination. The data revealed that at all doses tested, the compound significantly reduced paw edema following carrageenan administration. In the zymosan-induced air-pouch model, PA-Int5 potently inhibited leukocyte migration and protein levels at the site of inflammation. When combined, the results revealed, for the first time, that PA-Int5 exhibited anti-nociceptive and anti-inflammatory activities, and highlights its potential, as well that of other derivatives, as novel candidates for pain relief.

Introduction

Inflammation is a primary process in which the immune system reacts against infectious agents, irritations or other injuries. It is characterized by several events involving increased blood flow, vascular permeability and migration of cells and cytokines to the site of the injury (1). Pain is a sign of inflammation, and it serves as a protective mechanism induced by the unpleasant sensory and emotional experiences associated with tissue damage (2). Current pharmacological therapies for pain relief primarily revolve around anti-inflammatory drugs, and their undesirable effects, such as gastric discomfort, induction of an allergic response and impairment of renal function, highlight the need for increased efforts for the identification of novel therapeutic agents that act safely and efficiently (3,4).

Isatin-thiosemicarbazones compounds have garnered significant interest, due to their versatility of obtainment and

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variability of therapeutic activities. Isatins [1H-indol-2,3-dione] are indole heterocyclic compounds that can regulate the growth, differentiation and death of cells. Isatin derivatives have shown a wide range of biological activities including cytotoxic, antimicrobial, antifungal, tuberculostatic, antiviral, anticonvulsive, antioxidant and anticancer properties (5,6).

Previous studies have shown that isatin-thiosemicarbazone compounds exhibit antiviral properties, particularly against smallpox, Human Immunodeficiency Virus and *Encephalitis japonica* viruses (6,7). Additionally, these compounds exhibit antitumor, antifungal, antibacterial, antimalarial, antileishmanial and larvicide activities (7-10). Thus, considering the myriad of functional activities exhibited, the aim of the present study was to investigate the *in vivo* effects of the isatin-thiosemicarbazone compound (Z)-2-(5-nitro-2-oxoindolin-3-ylidene)-N-phenylhydrazinecarbothioamide (PA-Int5; Fig. 1) on inflammation and nociception in mice.

Materials and methods

Animals. All experimental procedures were approved by the Ethics Committees for Animal Use of the Federal University of Rio Grande do Norte (Brazil) (approval no. 088.007/2018) in compliance with Brazilian law (no. 11.794/2008). Male and female Swiss mice (*Mus musculus*), aged 6-8 weeks, weighing 30±2 g, were bred at the animal facility at the Health Sciences Center (Federal University of Rio Grande do Norte, Brazil) under standard conditions (23±2°C; 12 h light-dark cycle, lights on at 6:00 am) with *ad libitum* access to food and water in plastic cages. Mice were fasted of food for 2 h prior to beginning the tests. A total of 76 male and 71 female Swiss mice were used. After assays, the animals were euthanized by heart exsanguination, after anesthesia with thiopental 100 mg/kg (Tiopentax®, Cristália Prod. Químicos Farmacêuticos) and lidocaine 2% w/v (EMS/SA; Hotolândia/SP) intraperitoneally (ip) in accordance with regulations described by the National Council for Control of Animal Experimentation from 2016 (11).

Drugs and treatments. PA-Int5 ((Z)-2-(5-nitro-2-oxoindolin-3-ylidene)-N-phenyl-hydrozinecarbothioamide) was obtained from the Laboratory of Planning in Medicinal Chemistry from the Federal University of Pernambuco, Brazil. This derivative was synthesized from an isatin and a thiosemicarbazide, using a method similar to the one described by Moraes Gomes (2016) (12), and it was chemically characterized by Nuclear Magnetic Resonance, infrared and elemental analysis (unpublished data).

In all assays, drug treatments were administered in a volume of 0.5 ml, solubilized in 1% DMSO. All animals were orally pre-treated or post-treated, depending on the assay, according to the specified group: Control, vehicle or PBS (pH=7.2); dexamethasone (2.0 mg/kg); indomethacin (25 mg/kg); codeine (7.5 mg/kg) or PA-Int5 (1, 2.5 or 5 mg/kg).

The drugs used as standards (codeine and indomethacin) were used at doses based on tests that demonstrated both efficacy for the model and safety for animals (13-18). The doses of PA-Int5 were determined according to the protocols of the Organization for Economic Co-operation and Development (protocol nos. 420, 423 and 407) to perform the toxicity tests (19-21).

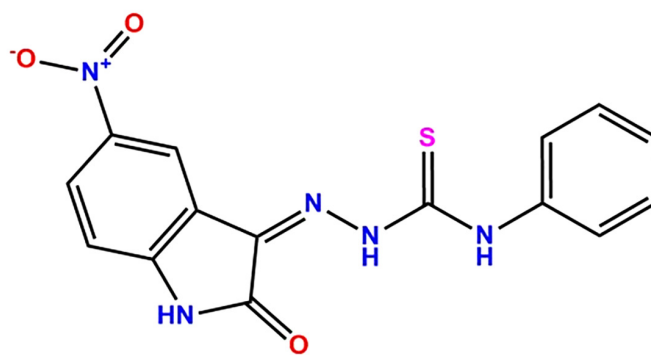


Figure 1. Chemical structure of the isatin-thiosemicarbazone derivative compound (Z)-2-(5-nitro-2-oxoindolin-3-ylidene)-N-phenyl-hydrozinecarbothioamide, termed PA-Int5.

The control group was treated with the control agent via the same route and with an equivalent volume as the treated groups. All drugs used as standards in the tests were commercially purchased from Sigma-Aldrich (Merck KGaA).

Evaluation of central nervous system performance. The same group of mice were treated with PA-Int5 [5 mg/kg, oral gavage (vo)] or 1% DMSO and after 1 h, 24 h or 7 days, they underwent the open field and rotarod tests.

Open field test. The spontaneous locomotor activity of the mouse was measured using the open field test as described previously by Seibenhener and Wooten (22). The apparatus, made of wood covered with impermeable formica, had a black floor, 40x40 cm and black walls 40 cm high. The test room had controlled illumination (dimly-lit condition; <10 lux in the center of the open field). Each mouse was placed in the center of the apparatus, and the following parameters were automatically registered by a video tracking system (Anymaze, Stoelting Co.) for 30 min: Distance moved (in meters) and time spent immobile (in sec). After each evaluation, the area was cleaned with 5% ethanol solution.

Rotarod test. The rotarod apparatus (AVS Projetos, Ribeirão Preto) was composed of a 5-lane rod, which has a 3 cm diameter and was elevated 22 cm from the platform. Before the test, the animals were trained for 2 consecutive sessions of 120 sec, each one on an automated rotarod unit at 10 rpm. During the test session, animals were placed on the rod and sequentially tested at 10 rpm for a maximum of 120 sec with a 2-min rest time between trials. The total time spent on the bar during a 120 sec session was recorded using a stopwatch, and the number of falls during sessions was also recorded (23).

Evaluation of anti-nociceptive activity

Acetic acid-induced abdominal writhing test. First, the mice were allowed to habituate for 20 min in an individual cage, then they were treated orally with vehicle (1% DMSO), indomethacin (25 mg/kg) and PA-Int5 (1, 2.5 or 5 mg/kg). After 1 h, acetic acid (0.6% v/v) was injected intraperitoneally (intraperitoneally; 10 ml/kg body weight), and the frequency of writhing reflexes (as a measure of visceral pain) was counted for 20 min. Writhing reflexes (characterized by the presence of contractions of the abdominal muscles) consist of inward outstretching of the hind limbs, hind paw reflexes and

extension of the whole body (24). The percentage of reduction in the number of abdominal contractions was calculated as follows: $[(C-T)/C] \times 100$, in which C and T are the number of abdominal contractions in the Control and Treatment groups, respectively.

Formalin test. The procedure was performed as previously described by Hunskaar and Hole (25). Mice were individually placed in a glass cone (20 cm in diameter) for 20 min. Following the acclimatization period, formalin (2.5% solubilized in 0.9% NaCl) was injected (ipl) in a volume of 20 μ l into the right hind paw of the mice. Immediately after the formalin injection, animals were placed back into the glass cone and were observed for 30 min. A mirror was placed behind the glass cone to allow an unobstructed view of the formalin-injected paw. The ipl formalin injection induces a biphasic nociceptive response: i) An acute phase of short duration followed by ii) a longer-lasting tonic phase (25). Hence, the evaluation of the nociceptive behavior is divided into two phases. The first 5 min after formalin injection, is considered the first phase followed by a quiescent period of ~10 min, and then the second phase occurs from 15-30 min after injection. A total of 1 h before the formalin injection, the mice were orally treated with vehicle (DMSO 1%), codeine (7.5 mg/kg), indomethacin (25.0 mg/kg) or PA-Int5 (1, 2.5 or 5 mg/kg), and the time (in sec) that animals spent licking, shaking and retracting the injected paw was measured with a chronometer, and was considered to be an indication of ongoing nociception. The percent reduction of paw pain time was calculated using the formula $[(C-T)/C] \times 100$, where C is the paw pain time in the control group and T is the paw pain time in the treatment group (tests and standard).

Evaluation of anti-inflammatory activity

Carrageenan-induced paw edema model. *In vivo* anti-inflammatory activity was determined using the carrageenan-induced paw edema method in mice as described previously (26) with some modifications. Animals were injected ipl with 50 μ l 1% λ -carrageenan (Sigma-Aldrich; Merck KGaA) or PBS (pH 7.4). A total of 50 μ l is recommended for the formation of edema in the paw, being a more standardized way of evaluating edema in this experimental model, as used in previous studies (27-30).

A total of 30 min later, following the inflammatory challenge, mice were treated by vo with PBS, dexamethasone (2 mg/kg), or PA-Int5 (1, 2.5 or 5 mg/kg). The paw edema was measured after 0 (immediately after treatment), 1, 2, 3 and 4 h (after carrageenan administration) with a digital caliper (Digemess 100.174BL; Digemess Instruments, Ltd.). Paw edema was measured in mm and calculated as the percentage of edema. The following equation was used to obtain the percentage of the respective experimental groups: Percentage edema = [average paw thickness (after 1, 2, 3 or 4 h) - average paw thickness (0 h)]/average paw thickness (0 h).

The area under the time-course curve was also determined using the trapezoidal rule (26,28).

Zymosan-induced air-pouch model. Anti-inflammatory activity was evaluated using the zymosan-induced air pouch model with some modifications (31). Briefly, the Swiss mice received 5 ml sterile air subcutaneously injected into their back. After 3 days, 2.5 ml of sterile air was injected into the cavity, and 6 days after the initial air injection, the animals

were administered a zymosan solution (1 mg/ml) into the air pouch simultaneously with oral administration of PBS or dexamethasone (2 mg/kg), or PA-Int5 (1, 2.5 or 5 mg/kg). A total of 6 h after the treatments, the animals were euthanized and the exudates were harvested from each air pouch with 2 ml PBS. The samples were centrifuged at 200 x g for 10 min at 4°C, the cell pellet was resuspended in 1 ml PBS, and diluted in Turk's solution (Sigma-Aldrich, Merck KGaA) (1:10 v/v). The total number of leukocytes were determined using a Neubauer chamber with the aid of a Nikon ECLIPSE E200[®] microscope (Nikon Corporation) at x40 magnification. The results are expressed as the number of leukocytes per ml. The supernatants were collected for the determination of total proteins.

Determination of total protein concentration. The supernatants were utilized for the determination of total protein concentration using a Bradford assay. A total of 10 μ l of each sample was added to 96-well plates, followed by the addition of 200 μ l Bradford reagent. Results were obtained using a microplate reader (BioTek Instruments, Inc.) at 595 nm and expressed as μ g/ml (32).

Statistical analysis. Data are expressed as the mean \pm standard deviation. Statistical analyses were performed using a one-way ANOVA with a Tukey's post hoc test in GraphPad Prism version 5 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of PA-Int5 on the central nervous system. Due to the absence of *in vivo* information of the effects of PA-Int5, the animals were first assessed with regard to their motor performance, before the evaluation its anti-nociceptive and anti-inflammatory activity.

To exclude any motor impairment induced by the treatment with PA-Int5, the effects of this compound were tested in the open field and rotarod tests. PA-Int5 (5 mg/kg) did not affect the total distance travelled and the time spent immobile in male and female mice in the open field test when they were assessed for 1 h, 24 h and 7 days after treatment (Fig. 2; $P > 0.05$).

The oral administration of PA-Int5 (5 mg/kg) did not impair the motor performance of male and female mice evaluated in the rotarod test, since significant alterations were not observed in the number of falls and in the time spent on the rotating bar when evaluated for 1 h, 24 h and 7 days after treatment (Fig. 3; $P > 0.05$).

Anti-nociceptive effects. As shown in Fig. 4, treatment with indomethacin reduced the acetic acid-induced abdominal contractions by 52.5%. Similar to the positive control, treatment with PA-Int5 reduced the acetic acid-induced abdominal contractions in a dose-dependent manner, displaying a significant reduction in nociceptive behaviors at 2.5 (31%) and 5 mg/kg (34%) compared with the vehicle [F-value (4.18)=31.64; $P < 0.05$].

Fig. 5 illustrates the effects of the standard analgesic drugs, indomethacin and codeine, on ongoing nociception induced by formalin. Animals that received the injection with formalin displayed a typical biphasic nociceptive response. Pre-treatment with codeine (7.5 mg/kg) significantly attenuated the amount

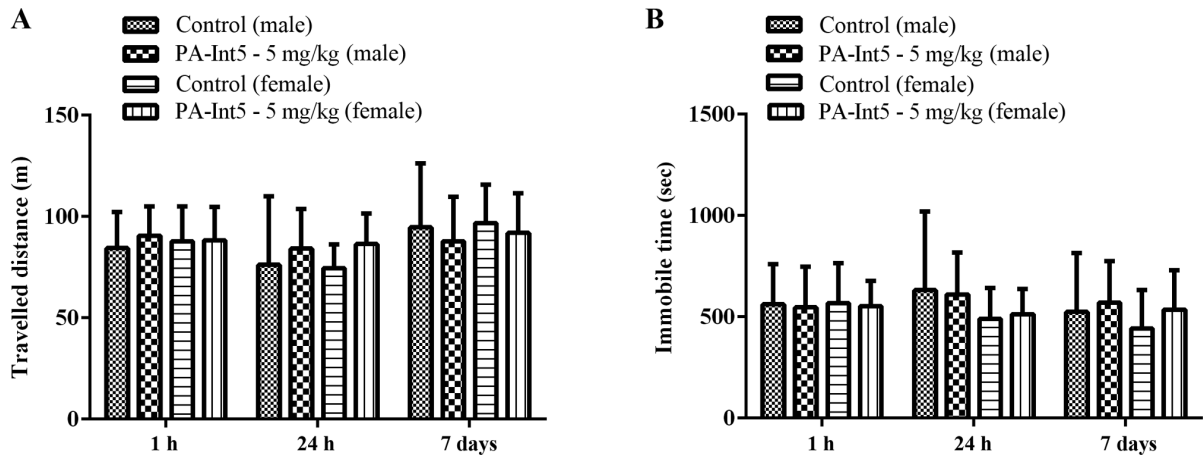


Figure 2. Central nervous system performance of male and female mice injected with 5 mg/kg PA-Int5 via oral gavage, assessed using the open field test 1 h, 24 h and 7 days after treatment. (A) Distance travelled in meters. (B) Time spent immobile in sec. Data are presented as the mean \pm standard deviation of 8 mice/group.

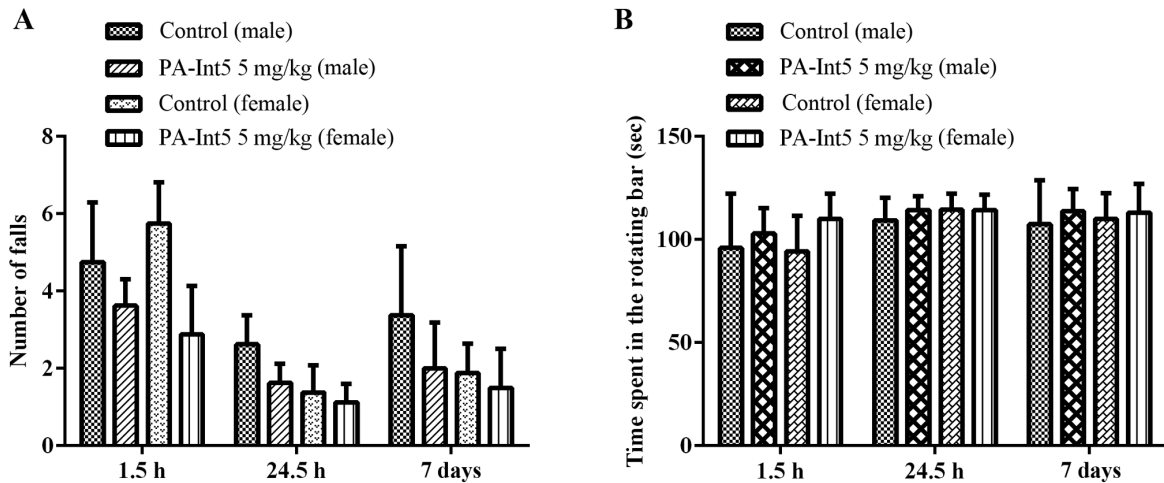


Figure 3. Central nervous system performance of male and female mice injected 5 mg/kg PA-Int5 via oral gavage, assessed using the rotarod test 1.5 h, 24.5 h and 7 days after treatment. (A) Number of falls from the rotating bar. (B) Time spent on the rotating bar during the 2-min session. Data are presented as the mean \pm standard deviation of 8 mice/group.

of time mice exhibited nociceptive behavior for in both phases of the formalin test. The inhibitory effect exerted by the opioid in the nociceptive behaviors amounted to 75 and 65% of the controls for phases 1 and 2, respectively (Fig. 5). Moreover, animals treated with indomethacin (25 mg/kg) displayed a statistically significant reduction in nociceptive behavior only during phase 2. The inhibitory effect of indomethacin was 55% that of the control (Fig. 5). In the first phase of the formalin test, treatment with PA-Int5 did not evoke any anti-nociceptive effects (Fig. 5A; $P > 0.05$). However, during the second phase, oral administration of 2.5 and 5 mg/kg PA-Int5 significantly reduced the time spent exhibiting nociceptive behaviors (92.7 and 93.2% compared with the control; Fig. 5B; F-value (5.18)=74.90; $P < 0.05$).

Anti-inflammatory effects. Fig. 6 shows the effects of treatment with PA-Int5 and dexamethasone in the carrageenan-induced paw edema in mice. The injection of carrageenan in the paw significantly increased the edema compared with the control group during the observation period. The administration of

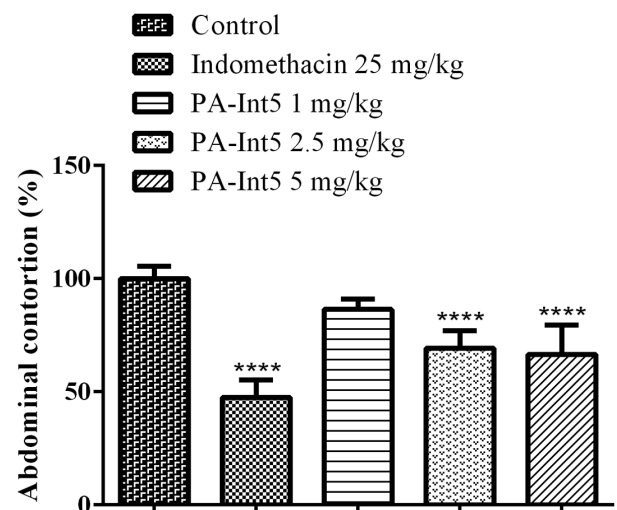


Figure 4. Anti-nociceptive effects of indomethacin (25 mg/kg, vo) and PA-Int5 (1, 2.5 and 5 mg/kg, vo) assessed in the acetic acid-induced abdominal contortions in mice. **** $P < 0.001$ vs. control. Data are presented as the mean \pm standard deviation of 5 mice/group. vo, oral gavage.

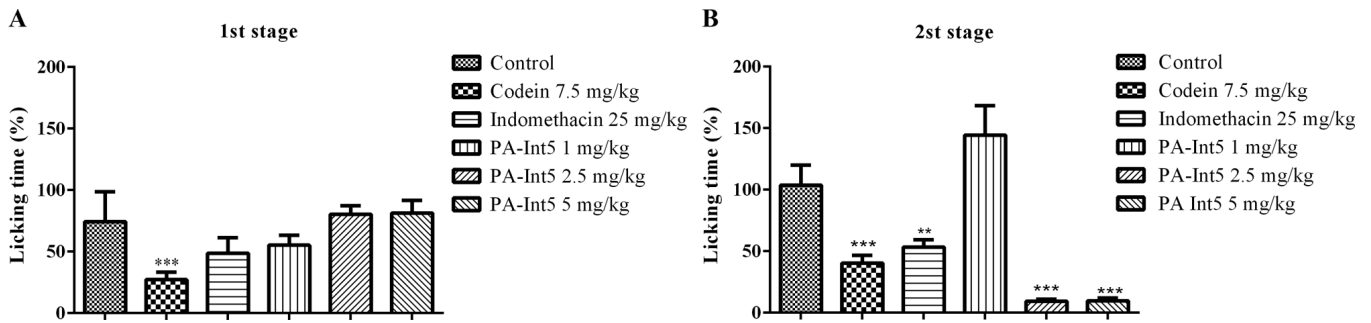


Figure 5. Anti-nociceptive effects of indomethacin (25 mg/kg, vo), codeine (7.5 mg/kg, vo), and PA-Int5 (1, 2.5 and 5 mg/kg, vo) assessed in the formalin test in mice. (A) Percentage of time spent expressing nociceptive behaviors in the first (0-5 min). (B) Second phase (15-30 min) of ongoing nociception induced by 2.5% formalin (20 μ l/paw). **P<0.01; ***P<0.001 vs. control. Data are presented as the mean \pm standard deviation of 5 mice/group. vo, oral gavage.

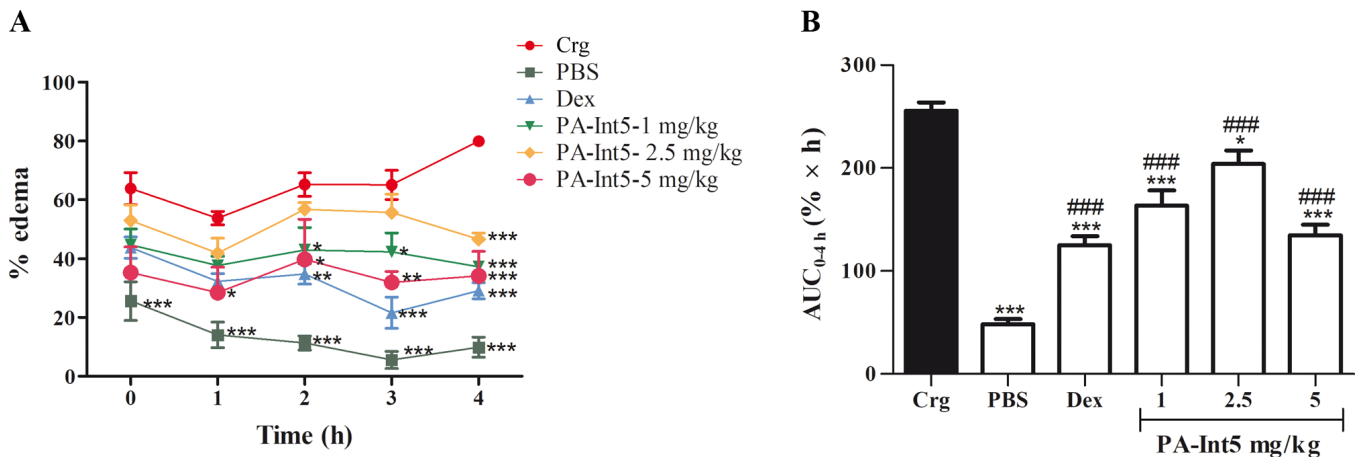


Figure 6. Anti-edematogenic effects of Dex (2 mg/kg, vo) and PA-Int5 (1, 2.5 and 5 mg/kg, vo) in the Crg-induced paw edema in mice. (A) Percentage of paw edema measured after 0, 1, 2, 3 and 4 h following intraplantar injection of Crg. (B) Area under the curve for each drug treatment. Data are presented as mean \pm standard deviation of 5 mice/group. ###P<0.001 vs. PBS group; *P<0.05, **P<0.01, ***P<0.001 vs. carrageenan group. Dex, dexamethasone; vo, oral gavage; Crg, carrageenan.

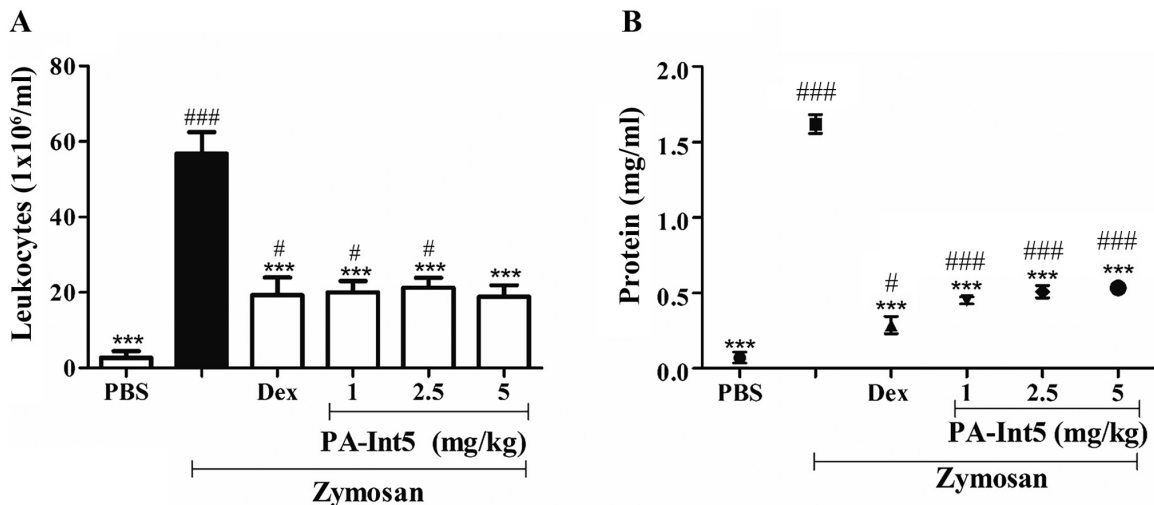


Figure 7. Anti-inflammatory effects of Dex (2 mg/kg, vo) and PA-Int5 (1, 2.5 and 5 mg/kg, vo) in the zymosan-induced air-pouch model. (A) Total leukocyte counts collected in the mouse dorsal cavity 6 h after dexamethasone or PA-Int5 administration. (B) Protein levels measured in the exudates collected from the dorsal cavity in mice. Data are presented as mean \pm standard deviation of 5 mice/group. #P<0.05, ###P<0.001 vs. PBS group; ***P<0.001 vs. zymosan group. Dex, dexamethasone; vo, oral gavage.

dexamethasone significantly reduced paw edema compared with the carrageenan group. The oral administration of

PA-Int5, at a dose of 1 mg/kg significantly inhibited the formation of edema from 2 h after treatment when compared with

Table I. Anti-inflammatory activity of the PA-Int5 in the zymosan-induced air pouch model.

Group	Dose, mg/kg	Cell migration, $1 \times 10^6/\text{ml}$	Inhibition, %
Zymosan 1 mg/ml	-	56.750±5.732	-
Dexamethasone	2	18.375±4.695 ^a	65
PA-Int5	1	20.000±3.048 ^a	64
PA-Int5	2.5	21.250±2.618 ^a	62
PA-Int5	5	24.500±3.060 ^a	66

^aP<0.001 vs. saline-treated group. Data are presented as the mean ± standard deviation. n=5.

the carrageenan group; 2.5 mg/kg showed significant inhibition only after 4 h; and the most effective dose was 5 mg/kg, which inhibited edema after only 1 h (Fig. 6A). When evaluated, the progression of the edema after the 4 h of observation, calculated through the area under the curve of edema kinetics using the trapezoidal rule (26,28), showed a significant decrease in all doses of PA-Int5 tested when compared with the carrageenan group [Fig. 6B; F-value (5.21)=43.17; P<0.05]. Doses of 1, 2.5 and 5 mg/kg PA-Int5 inhibited the formation of edema by 36, 20.2 and 47.3%, respectively.

In the zymosan-induced air-pouch model, treatment with zymosan significantly increased leukocyte migration compared with the PBS group. PA-Int5, at all doses tested, significantly inhibited the migration of leukocytes into the dorsal cavity, when compared with the zymosan group, as shown in Fig. 7A and Table I. Concomitantly, it was shown that there was a reduction in protein concentration at this site (Fig. 7B). Treatment with dexamethasone reduced cell migration by 65% compared with zymosan. The oral administration of PA-Int5 (1, 2.5 and 5 mg/kg) significantly inhibited leukocyte migration to the dorsal cavity by 64, 62 and 66%, respectively [Fig. 7A; F-value (5.19)=22.41; P<0.05]. Concerning the amount of protein measured in the exudates of the dorsal cavity, it was observed that there was a significant increase in this parameter when treated with zymosan. By contrast, there was a significant reduction in protein extravasation to the inflammation site when treated with dexamethasone and all doses of PA-Int5 [Fig. 7B; F-value (5.17)=177.6; P<0.05]. PA-Int5 (1, 2.5 or 5 mg/kg) treatment reduced protein exudation by 72, 68 and 67%, respectively, compared with the zymosan-treated mice.

Discussion

The present study is the first to show that an isatin-thiosemicarbazone compound exhibited potential anti-inflammatory and anti-nociceptive effects in mice. Toxicity studies performed in our research group have shown that PA-Int5 is safe at the doses tested (unpublished data). Considering these promising biological properties reported here for PA-Int5, systematic studies for assessing its safety and efficacy are recommended as the next steps.

Pain is produced by several mechanisms, and it can be classified as physiological, inflammatory or neuropathic (33).

In this sense, different animal models of nociception were used to mimic the different causes of pain: Chemical (formalin test and acetic-acid induced writhing reflexes) and inflammatory (carrageenan-induced paw edema and zymosan-induced air-pouch). The results showed that PA-Int5 exhibited potent anti-nociceptive and anti-inflammatory effects at the doses tested in all models of nociception and inflammation in mice.

The anti-nociceptive activity was assessed based on acetic acid-induced abdominal contortion is a sensitive assay used for the evaluation of peripheral-acting analgesics clinically used in the relief of inflammatory and visceral pain (34). Acetic acid induces nociception due to the local inflammatory response resulting from the release of arachidonic acid from resident peritoneal cells, in addition to the release of TNF- α and prostaglandin E2 that sensitizes nociceptive fibers (34). Non-steroidal anti-inflammatory drugs, such as indomethacin, are effective in inhibiting prostaglandin synthesis (35); this drug was used as positive control here. Similar to indomethacin, treatment with the isatin-thiosemicarbazone derivative compound significantly reduced abdominal contortions induced by acetic acid, suggesting that Pa-Int5 exhibited analgesic activity.

The formalin test is one of the most widely used methods to investigate anti-nociceptive activity (25,36-38). Formalin sensitizes the peripheral nerve endings, inducing nociceptive effects (3). The formalin test was divided into two phases. The initial phase, which is termed the neurogenic phase, begins immediately after formalin injection and persists for up to 5 min. During the early stage, substance P and bradykinin mediate the elicitation of pain (25). The second phase, termed the inflammatory phase, begins 15-20 min after formalin injection and persists for up to 30 min. During this stage, histamine, serotonin, prostaglandins and bradykinin are involved in the induction of the inflammation and the elicitation of pain (25). Opioids are effective in both phases of the test, whereas non-steroidal anti-inflammatory drugs and steroids are only effective in the second phase (3,16,25). When designing the experimental conditions, two positive-control drugs were used: Codeine (7.5 mg/kg), an analgesic opioid, and indomethacin (25 mg/kg), a non-selective COX inhibitor. The opioid agonist reduced formalin-induced nociceptive behaviors during the first and second phases, whereas indomethacin was effective only during the second phase. Similar to the COX inhibitor positive-control, PA-Int5, at the higher doses, promoted pain relief only during the second phase in the formalin test. Such anti-nociceptive effects suggest inhibition of synthesis or release of arachidonic acid and its metabolites, including prostaglandins and leukotrienes (3,39,40).

The effect of PA-Int5 on open-field and rotarod tests were investigated to exclude non-specific behaviors, such as sedation or psychomotor agitation, which may influence the interpretation of anti-nociceptive actions (41). Open-field testing is one of the most popular types of behavioral tests used to evaluate spontaneous locomotor activity in rodents (41,42). Locomotion is the most universal and conserved form of movement, which involves mechanisms controlled by the CNS (41). The rotarod test is a widely used method in preclinical research, as it evaluates motor coordination and it can detect physical deficiencies caused by pharmacological agents, such as muscle relaxants and CNS depressants (41).

Motor coordination is a complex behavior that reflects muscle strength, balance and standard gait, and is influenced by the effects of benzodiazepines or barbiturates (41,42). The results of the present study showed that the administration of PA-Int5 (5 mg/kg, vo) did not affect locomotion or motor coordination. Thus, this compound did not promote alterations that compromise the interpretation of biological activities that are based on motor performance.

The effects of treatment with drugs on inflammatory components in the present study were evaluated using a carrageenan and zymosan-induced air-pouch model. These tests induce acute and not immune inflammation, that is well-validated, and widely reproduced in numerous studies (43,44). Signs of inflammation (edema, hyperalgesia, erythema) become visible shortly after subcutaneous injection of such stimuli through the activation of pro-inflammatory agents (43).

Carrageenan-mediated inflammation is mediated by three mechanisms in the production of edema: The primary mechanism is mediated by histamine, the secondary mechanism is kinin-mediated, and the tertiary mechanism is characterized by the local synthesis of prostaglandins and their metabolites, and it is the tertiary mechanism where the majority of anti-inflammatory drugs exert their effects (43,45). In the present study, the glucocorticoid dexamethasone was used to attenuate vasodilation, arachidonic acid metabolism synthesis and leukocyte migration (40,43,46).

The results of the present study indicated that PA-Int5, at the highest dose, exhibited anti-edematogenic activity with reduction in paw edema to an equivalent degree of that observed with dexamethasone, acting on the first signs of inflammation. It was possible to observe biases inherent to animals in the evaluation of these activities, such as the differentiated absorption of the sample by the individuals of each group, and the different anti-inflammatory responses produced by different individuals from the same experimental group, reflecting on the anti-edematogenic activity, over 4 h, in the other doses. Even so, the data were consistent with regard to the activity of the compound.

The zymosan-induced air-pouch model employs zymosan (glucan) as an anti-inflammatory agent, which is a cell wall extract of yeast (43). Its mechanism of action involves the activation of the inflammatory process via the complement system, thus inducing lysosomal enzymes, prostaglandins and leukotriene release (47). The primary findings showed that a significant decrease in leukocyte migration and protein levels measured at the site of the inflammation were observed in animals treated with dexamethasone and PA-Int5. These results suggest that PA-Int5 exerts its anti-inflammatory action using a mechanism similar to that of glucocorticoids (48), which would contribute to the inhibition of polymorphonuclear cell migration as well as reduction of proteins and other inflammatory components at the site of the inflammation.

Structural changes of compounds has been extensively used and studied in attempts to improve their biological activities. Thus, several studies on the structure-activity relationship can provide security in the inferences produced in this work. The structural changes in the molecules can alter the binding sites and pharmacophoric groups, favoring improved activity and lower toxicity, via the substitution with electron donor groups that have improved anti-inflammatory activity, as observed in this work (5,49-51).

Data from the literature suggest that the anti-inflammatory action of PA-Int5 can be attributed to the presence of electron donor groups in its structure at the para position of the isatin ring; such action may increase the anti-inflammatory and antioxidant activities (51). Additionally, as reported by Jarapula *et al* (51), the presence of a halogen atom in the isatin ring serves a key role in anti-inflammatory activity. It has also been reported that the substitution at the C5 and C7 positions of the isatin ring results in higher anti-inflammatory activity than other chain substitutions (51). When combined, the potent *in vivo* anti-inflammatory activity of PA-Int5 is, at least in part, justified by its chemical structure. Furthermore, comparing PA-Int5 with other isatin-thiosemicarbazone derivatives, the presence of a nitro group (NO₂) serves an important role in the increase in biological activities (5,12). Additionally, the presence of different substituents at the C5 position of the isatin structure and the phenyl ring at N4 in the thiosemicarbazone showed a reduction or elimination of the toxic potential (5,7,12), thus increasing the interest in the study of this compound.

Another important point related to the chemical characteristics of these substituents is the hydrophilic radicals that favor lower toxicity of the compounds (5,44-46). However, further *in vivo* studies are required to investigate the specific underlying mechanisms.

The present study has some limitations. One of these is related to the mechanisms of action of the compound that are not yet molecularly defined. Another limitation is the lack of histological analysis of the paws from animals submitted to the paw edema model. However, to minimize this limitation, other methodologies were used for the evaluation of the anti-inflammatory parameters of PA-Int5, and through them, the promising effects of the studied compound were inferred.

In summary, the present study revealed potent *in vivo* anti-inflammatory and anti-nociceptive actions of PA-Int5, an isatin-thiosemicarbazone compound. The anti-nociceptive activity was observed in visceral inflammatory pain and in moderate inflammatory pain models induced by formalin, whereas the anti-inflammatory activity was shown by its anti-edematogenic actions and reduction of leukocyte migration and protein levels to the site of the inflammation. Further studies are required to investigate the mechanisms by which PA-int5 induces these biological activities as well as for assessing the safety and efficacy of the compound, and are currently being performed in our lab.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TMAML, AGDF, MDFFP and ECG conceived and designed the study. AGDF, LLDSFRD, JPR, MPDCAF, PATDMG, VGDMS and AAF performed the experiments. AGDF, MJBDMR, MGDPR, ACLL, MDFFP, ECG and TMAML wrote and edited the manuscript. TMAML, AGDF, LLDSFRD, MDFFP, MJBDMR, MGDPR, ACLL and ECG analyzed and interpreted the data. LLDSFRD, JPR and TMAML confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Ethics Committees for Animal Use of the Federal University of Rio Grande do Norte (Brazil) (approval no. 088.007/2018) in compliance with Brazilian law (no. 11.794/2008).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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