Inflammation (© 2018)
DOI: 10.1007/s10753-018-0783-x

ORIGINAL ARTICLE

Salicytamide: a New Anti-inflammatory Designed Drug Candidate

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Abstract— Salicytamide is a new drug developed through molecular modelling and rational drug design by the molecular association of paracetamol and salicylic acid. This study was conducted to assess the acute oral toxicity, antinociceptive, and antioedematogenic properties of salicytamide. Acute toxicity was based on the OECD 423 guidelines. Antinociceptive properties were investigated using the writhing, hot plate and formalin tests in Swiss mice. Antioedematogenic properties were evaluated using the carrageenan-induced paw oedema model and croton oil-induced dermatitis in Wistar rats. Salicytamide did not promote behavioural changes or animal deaths during acute oral toxicity evaluation. Furthermore, salicytamide exhibited peripheral antinociceptive activity as evidenced by the reduction in writhing behaviour (ED50 = 4.95 mg/kg) and licking time in the formalin test's inflammatory phase. Also, salicytamide elicited central antinociceptive activity on both hot plate test and formalin test's neurogenic phase. Additionally, salicytamide was effective in reducing carrageenan or croton oil-induced oedema formation. Overall, we have shown that salicytamide, proposed here as a new NSAID candidate, did not induce oral acute toxicity and elicited both peripheral antinociceptive effects (about 10–25 times more potent than its precursors in the writhing test) and antioedematogenic properties. Salicytamide also presented central antinociceptive activity, which seems to be mediated through opioid-independent mechanisms. These findings reveal salicytamide as a promising antinociceptive/antioedematogenic drug candidate.

KEY WORDS: antinociception; antioedematogenic; paracetamol; acetylsalicylic acid; salicytamide.

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are some of the main therapeutic options used worldwide, being prescribed for a broad range of inflammatory diseases and pain management. Salicylates and *p*-aminophenol derivatives are the most commonly prescribed NSAIDs, either for the easy access to them or for their many clinical indications [1–3].

Published online: 13 April 2018

Salicylates (*e.g.* salicylic acid and acetylsalicylic acid) were introduced into the clinical setting over 100 years ago. Nonetheless, these NSAIDs remain useful tools in the clinical practice, being used as analgesic, antipyretic and antiplatelet agents [4–6]. Derivatives of *p*-aminophenol (*e.g.* paracetamol) were later proposed as safer salicylates-alternatives, becoming the drugs of choice for moderate pain and fever treatment [7–9]. However, the use of both acetylsalicylic acid and paracetamol is often hampered by a number of serious adverse effects. Salicylates often cause gastrointestinal bleeding, renal failure and Reye's syndrome [10–12]. As for paracetamol, its increased consumption leads to the build-up of the metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is known to cause severe hepatocellular injury [13–16].

Drug development studies have emerged as a way to overcome NSAID severe adverse events and increase their therapeutic efficacy. In this context, molecular modelling has been proven to be a useful tool, as it allows rational chemical structure planning, whilst also provides the possibility of predicting the pharmacological and toxicological profiles of a new drug candidate [3]. Recently, a molecular combinatorial approach based on the structural characteristics of salicylic acid and paracetamol leads to the generation of a new compound named salicytamide [17]. Compared to its parent molecules, all theoretical parameters for salicytamide revealed its potential as a more effective and less toxic analgesic and anti-inflammatory agent [17].

In the present work, we set out to investigate the antinociceptive and antioedematogenic effects and toxicity profile of the new NSAID candidate, salicytamide. Here, we report, for the first time, that salicytamide was able to elicit more potent peripheral and central antinociceptive effects, as well as antioedematogenic capacity *in vivo*, when compared to its precursors, whilst also exhibiting lower acute oral toxicity.

MATERIAL AND METHODS

Salicytamide Design and Synthesis

The rational drug design for salicytamide (5-acetamidosalicylic acid) was performed using a molecular association approach between salicylic acid and paracetamol (Fig. 1). Salicytamide was synthesized through the acetylation of 5-amino-salicylic acid. The structural properties of salicytamide have been previously characterized [17].

Drugs and Treatments

Salicytamide was solubilized in saline solution with 5% Tween 80 (Sigma-Aldrich, St. Louis, MO) and administered orally (gavage) 1 h before the beginning of experiments, except for the toxicity test, which started from the time of administration. The doses varied according to the test, but the standardized administration was 0.1 mL/10 g of body weight for mice and 0.1 mL/100 g for rats. Prior to the beginning of experiments, all animals were deprived of food for 6 h.

Acetic acid, formaldehyde (Vetec Chemistry, Rio de Janeiro, Brazil), croton oil and lambda-carrageenan type IV (Sigma-Aldrich, St. Louis, USA) were used as noxious stimuli agents. Morphine sulphate (Cristália Lab, São Paulo, Brazil) was used as standard positive control drug for central antinociceptive effects. Paracetamol and acetylsalicylic acid (Sigma-Aldrich, St. Louis, USA) were used as standard positive control drugs for antinociceptive and anti-inflammatory effects. Naloxone hydrochloride (Cristália Lab, São Paulo, Brazil) was used to investigate the possible involvement of opioid receptors in the analgesic effects observed. A trained and treatment-blinded observer carried out all the evaluations.

Animal Welfare and Ethical Statements

Two-month-old Wistar rats (150–200 g), nulliparous females ($n = 14$) and males ($n = 150$), and male albino Swiss mice (25–35 g) ($n = 180$) obtained from the Animal Facility of the Instituto Evandro Chagas (IEC) were used in the experiments. Animals were maintained in a climate-controlled room with a 12-h light/dark cycle (lights on 7:00 a.m.), five animals per cage (41 × 34 × 16 cm), with food and water *ad libitum*. All procedures were approved by the Ethics Committee on Experimental Animals of the Federal University of Pará under license number FAR 001-10-CEPAE/UFPA. The number of animals and the intensity of noxious stimuli were standardized according to the ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983) and the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [18].

Pharmacokinetics

Wistar rats ($n = 5$) were given salicytamide (2.5 mg/kg), anesthetized with ethyl ether [19], and a heparinized blood sample (100 μL) was collected from the ocular plexus at 0, 30, 60, 180 and 1440 min post-salicytamide administration. A high-performance

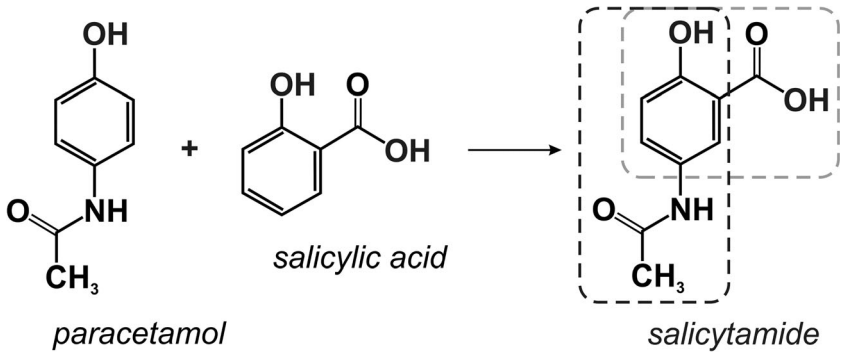

Fig. 1. Salicytamide design, synthesis and chemical structure [17].

liquid chromatography system with fluorescence detection (Flexar; PerkinElmer, USA) was used for the analysis of salicytamide after the liquid-liquid extraction from whole blood with methyl-tert-butyl-ether. The column used was an ODS C18 4.6 mm × 250 mm (Supelco, Inc., Bellefonte, PA). The mobile phase was composed of methanol/acetonitrile/phosphate buffer pH 3 (60:20:20) and set at a flow of 1.0 mL/min. The wavelength of excitation was 311 nm and emission of 449 nm. Salicylic acid was used as internal standard (100 ng/mL). The analytical procedure validated in our laboratory demonstrated that the method was linear over the range from 250 to 3000 ng/m, and the mean within-day and day-to-day coefficients of variation were 7.5 and 12%, respectively. Mean extraction recovery of salicytamide was 90%. The stability of blank blood spiked with salicytamide was noted for 60 days under 4 °C. Data were reported as mean and standard deviation. The main pharmacokinetic parameters of salicytamide derived from a noncompartmental model were the maximum plasma concentration (Cmax), the time to maximum concentration (Tmax), the area under the plasma concentration *vs* time curves (AUC_{0-t} and $AUC_{0-\text{inf.}}$), half-life (T1/2), oral clearance (Cl/F), and the apparent volume of distribution (Vz/F).

Acute Oral Toxicity

Salicytamide acute oral toxicity was assessed in female Wistar rats ($n = 7$ per group) in accordance with the OECD guideline 423 [20]. Animals received salicytamide (2000 and 5000 mg/kg) and were then evaluated for the occurrence of death or behavioural changes at different time-points (0, 0.25, 0.5, 1, 2, 3, 24, 48 and 72 h).

The stimulating action parameters (snout scratching, tremors, exophthalmia, attention, increased respiratory rate, paw licking, tail biting, arousal, spontaneous motor activity, nasal discharge, piloerection, stereotyped movements, escape reaction and convulsions) and depressant action parameters (alienation of the environment, ataxia, catatonia, decreased respiratory rate, decreased motility, decreased corneal reflex, apathy, dyspnoea, response to touch, ptosis, sedation and dorsal tone) were evaluated [21].

Other observed parameters were writhing, diarrhoea, hyperaemia, cyanotic or pallid ears, increased sweating, tail tremors, tearing, aggression, growling and coma. Animals were also monitored for another 11 days to check for the possible occurrence of death [20].

Open-Field Test

To investigate the possible influences of salicytamide on the locomotor activity of animals, Wistar rats ($n = 7$–10 per group) were evaluated in an open field arena (100 × 100 × 40 cm), divided into 25 quadrants, for 5 min. The spontaneous locomotor activity was defined as the number of quadrants crossed (crossed lines). A control group was administered with the vehicle solution used to prepare salicytamide. Treated groups received salicytamide (doses 2.5, 5.0 and 10.0 mg/kg).

Study of the Antinociceptive Properties

Writhing Test

The writhing test allows the evaluation of potential central or peripheral analgesic effects of drugs in mice [22]. Writhing behaviour was induced by intraperitoneal injection of acetic acid (0.6%; 0.1 mL/10 g body weight). Then, the animals ($n = 8$–10/group) were placed in an observation chamber and the number of writhes (repeated movements of rotation of the body contracting the abdominal wall and extension of the hind-paws) were recorded over a 20-min

interval. Treatments were administered 1 h before the injection of acetic acid. The control group received the dissolution vehicle, whereas the experimental groups received increasing doses of salicytamide (1.0, 2.5, 5.0, 10, 50 or 100 mg/kg). Positive control groups received acetylsalicylic acid (100 mg/kg) and paracetamol (127 mg/kg). The antinociceptive activity of salicytamide was defined as its ability to reduce the number of writhes when compared to the control group.

Hot Plate Test

According to the model proposed by MacDonald et al. [23], applied to evaluate the influence of drugs on central mechanisms of nociception evoked by thermal stimulation, mice ($n = 10$/group) were placed on a hot plate at 50 ± 1 °C (Ugo Basile, model 35,100, Varese, Italy) and the latency to nociception behaviour (paw licking, jumping or shaking) was recorded. The cutoff time was set at 40 s to prevent damage to the feet. The control group received the dissolution vehicle (gavage), and the treatment groups received increasing doses of salicytamide (2.5, 5 and 10 mg/kg), 1 h before experiments began. The positive control groups received morphine (10 mg/kg; s.c.) 30 min before experiments began and acetylsalicylic acid (100 mg/kg; gavage) or paracetamol (127 mg/kg; gavage) an hour before the beginning of experiments.

Formalin Test

The formalin test consists of a biphasic model for the evaluation of influences on central (first phase) and peripheral/inflammatory (second phase) mechanisms of nociception upon administration of formalin as a chemical noxious stimulus [24]. Mice received 20 μL of formalin (1% in saline) into the right hind-paw plantar surface, and the time of licking the affected paw was recorded within two intervals: 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase). The control group received dissolution vehicle (p.o.—gavage), whilst treatment groups received salicytamide (doses 2.5, 5.0 or 10 mg/kg), 1 h prior to the formalin injection. The positive control groups received morphine (10 mg/kg; s.c.) 30 min before the test and acetylsalicylic acid (100 mg/kg; gavage) or paracetamol (127 mg/kg; gavage) 1 h prior to the formalin administration.

The test was repeated with the addition of naloxone (0.4 mg/kg; s.c.), an opioid receptor antagonist, to investigate the possible involvement of opioid receptors in the antinociceptive effects of salicytamide. Naloxone was administered to the treated groups 15 min before the administration of salicytamide.

Study of Anti-inflammatory Properties

Carrageenan-Induced Paw Oedema

Wistar rats ($n = 6$ per group) received a subcutaneous injection of 0.1 mL carrageenan (1%), a potent inducer of inflammatory mediators (histamine, serotonin, kinins, prostaglandins, *etc.*), into the plantar surface of the right hind-paw and the same volume of saline into the left hind-paw [25]. To evaluate effect of salicytamide on the oedematogenic process, the volume of the hind-paws was recorded at different time-points (0, 1, 2, 3, 4 and 5 h) after the carrageenan injection using a digital pachymeter (Kingstool, code 502.150B, São Paulo, Brazil). The volume (mL) of oedema was defined as the difference between right and left hind-paws in each measurement. Control animals received the dissolution vehicle 1 h before the experiment by gavage, whereas the experimental groups received salicytamide (2.5, 5.0 and 10 mg/kg; gavage) and the positive control groups received acetylsalicylic acid (100 mg/kg; gavage), also 1 h before carrageenan administration.

Ear Oedema Induced by Croton Oil

Ear oedema was induced in mice ($n = 8$–10 per group) under anaesthesia (ketamine 72 mg/kg + xylazine 9 mg/kg, i.p.) by applying 20 μL of a solution of croton oil (2.5% acetone, *v*/*v*), a phospholipase A_2 inducer, to the inner surface of the right ear (about 1 cm^2). Acetone was applied to the left ear as a control of the assay [26].

After 6 h, the animals were sacrificed and a section (6 mm) was collected from the point of application in both ears. Oedema was measured by the weight (mg) difference between the sections of the right and left ears.

One hour prior to croton oil application, animals from the control group were treated with the dissolution vehicle (gavage), and treatment groups received salicytamide (2.5, 5 or 10 mg/kg; gavage). Acetylsalicylic acid (100 mg/kg; gavage) was administered to the positive control group.

Data Analyses and Statistical Procedures

Data was submitted to the Kolmogorov–Smirnov test to evaluate normality. Comparisons were performed by one-way analysis of variance (ANOVA) or one-way repeated measures ANOVA, followed by Holm–Sidak test to

compare experimental treatment groups. Linear regression was applied to verify the dose–effect relationship of salicytamide, as well as its median effective dose (ED50). The level of significance was set at $p < 0.05$, and the results are expressed as the mean ± SEM of the analysed parameters. The data was analysed using the SigmaPlot software package (Version 13; Systat Software Inc., London, UK).

RESULTS

Pharmacokinetic Profile

The pharmacokinetic analysis of salicytamide is described in Table 1. In summary, salicytamide presented a half-life of 6 h and its plasmatic peak occurred 1 h after oral administration. Additionally, the apparent volume of distribution of salicytamide was 0.04 (mg)/(ng/mL).

To verify the kinetics of salicytamide over time, blood samples were collected at 0, 30, 60, 180, and 1440 min after salicytamide administration (Table 2). The pharmacokinetic evaluation over time revealed that salicytamide reached a maximum plasmatic concentration of 510 ng/mL 1 h after an oral dose of 2.5 mg/kg. Additionally, even after 1440 min, small amounts of salicytamide (32 ng/mL) could still be detected in the blood samples tested.

Salicytamide Has Low Acute Oral Toxicity in Rats

No deaths were reported up to 14 days after the oral administration of high doses of salicytamide (2000 and 5000 mg/kg). No overt behavioural changes were observed. Additionally, stirring and abdominal movements were only observed at 0.25 and 0.5 h after the administration of salicytamide.

Table 1. Pharmacokinetics Parameters of Salicytamide

Parameter	Unit	Value
T1/2	h	6.07
Tmax	h	1
C max	ng/mL	510
AUC_{0-t}	ng/mL h	4428.5
$AUC_{0-inf.}$	ng/mL h	0.9404
Vz/F	(mg)/(ng/mL)	0.04
Cl/F	(mg)/(ng/mL)/h	0.0053

Maximum plasma concentration (Cmax), time to reach maximum concentration (Tmax), area under the plasma concentration *vs* time curves (AUC_{0-t} and $AUC0-inf.$), half-life (T1/2), oral clearance (Cl/F), apparent volume of distribution Vz/F

Table 2. Concentrations of Salicytamide (ng/mL) in Several Times

Time (min)	Concentration (ng/mL)
0	0
30	240
60	510
180	290
1440	32

Salicytamide Does Not Change the Spontaneous Locomotor Activity

Salicytamide administration (2.5, 5 and 10 mg/kg) did not cause changes in the locomotor activity of animals, subjected to the open-field test, when compared to the control group (Fig. 2).

Study of the Antinociceptive Properties

Salicytamide Is Effective in Reducing Acetic Acid-Induced Writhing

Salicytamide treatment reduced writhing behaviour in a dose-dependent fashion (1 mg/kg—17.68%; 2.5 mg/kg—40.46%; 5 mg/kg—47.33%; 10 mg/kg—60.22%; 50 mg/kg—69.4%; and 100 mg/kg—79%), when compared to the control group (Fig. 3a). Moreover, a linear regression analysis revealed that salicytamide has an ED50 of 4.95 mg/kg, as evidenced in the dose–effect curve (Fig. 3b).

Salicytamide Reduces Neurogenic and Inflammatory Nociception in the Formalin Test

Salicytamide (5.0 and 10 mg/kg) reduced the licking time in both the neurogenic and inflammatory phases of the formalin test when compared to the control group (Fig. 4a). Pre-treatment with naloxone (0.4 mg/kg, s.c.) did not modify the effects of salicytamide (Fig. 4b).

Salicytamide Increases Tolerance to Noxious Stimuli on Hot Plate Paradigm

Salicytamide, at a dose of 10 mg/kg, increased the latency to nociception manifestation during the hot plate test (at 90 min) when compared to control and positive control groups (Fig. 5). The doses of 2.5 and 5 mg/kg did not exhibit antinociceptive activity (data not shown).

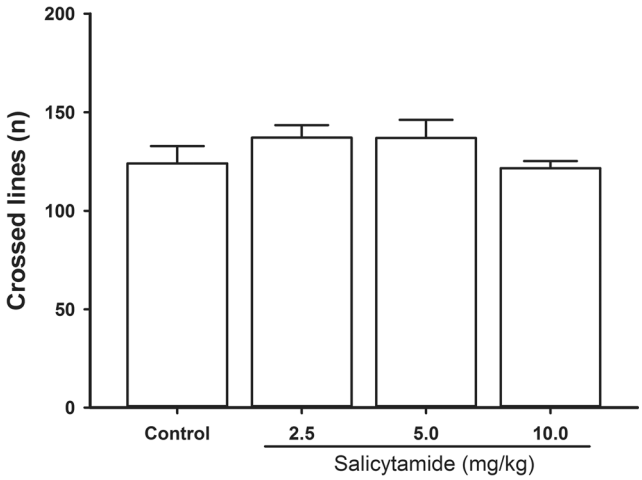

Fig. 2. Effects of salicytamide treatment on the motor performance of male Wistar rats. Groups: control; salicytamide (doses 2.5, 5 and 10 mg/kg). The results are expressed as the mean ± S.E.M. of the total number of crossings in the open-field for 5 min (n = 8–10 animals per group; one-way ANOVA, Holm–Sidak *post-hoc* test).

Study of Anti-inflammatory Properties

Salicytamide Reduces Carrageenan-Induced Paw Oedema in Rats

Salicytamide treatment (2.5, 5.0 and 10 mg/kg) reduced oedematogenic effects of carrageenan, particularly between 2 and 5 h post-carrageenan administration, when compared to the control group. The doses of 2.5 and 5 mg/kg elicited the same antioedematogenic profile (Fig. 6).

Salicytamide Reduces the Ear Oedema Induced by Croton Oil in Rats

All tested doses of salicytamide (2.5, 5.0 and 10 mg/kg) reduced croton oil-induced dermatitis, when compared to the control group. Salicytamide showed very similar effects to its parent drug, as evidenced by the positive control group treated with acetylsalicylic acid (100 mg/kg) (Fig. 7).

DISCUSSION

In the present study, we investigated the pharmacokinetic parameters, toxicity profile, and potential anti-inflammatory and antinociceptive effects of salicytamide. Salicytamide is an innovative designed drug candidate, developed through molecular combinatorial modelling methods based on two classic NSAIDs, paracetamol and acetylsalicylic acid. Here we demonstrate, for the first time, that salicytamide has a long half-life, low acute toxicity and is endowed with potent antioedematogenic and antinociceptive properties, as it regulates inflammatory responses and both peripheral and central nociception mechanisms.

Salicytamide Presents Longer Half-Life Than Paracetamol and Acetylsalicylic Acid

Salicytamide pharmacokinetic profile was assessed by HPLC assay after a single oral dose. Our data demonstrated that salicytamide half-life (mean of 6 h) was higher when compared to its parent drugs, salicylates and paracetamol. In fact, acetylsalicylic acid has a very low half-life (around 0.25 h), because the drug is converted promptly to salicylic acid, which has only a 2-h-long half-life. Similarly, paracetamol has a half-life of 2.5 h [27]. This particular feature of salicytamide may favour treatment adherence, as it reduces the frequency for drug intake throughout the day.

Salicytamide Is Safer Than Paracetamol and Acetylsalicylic Acid

No deaths or significant behavioural changes were observed after rats received high doses of salicytamide (2000 or 5000 mg/kg), revealing its low toxicity. In fact, salicytamide seems to have a much safer therapeutic index when compared to its prototype drugs acetylsalicylic acid and paracetamol, which have oral LD_{50} of 1500 and 1944 mg/kg, respectively [26]. Additionally, salicytamide did not induce motor function impairment, as no changes on animals' spontaneous locomotor

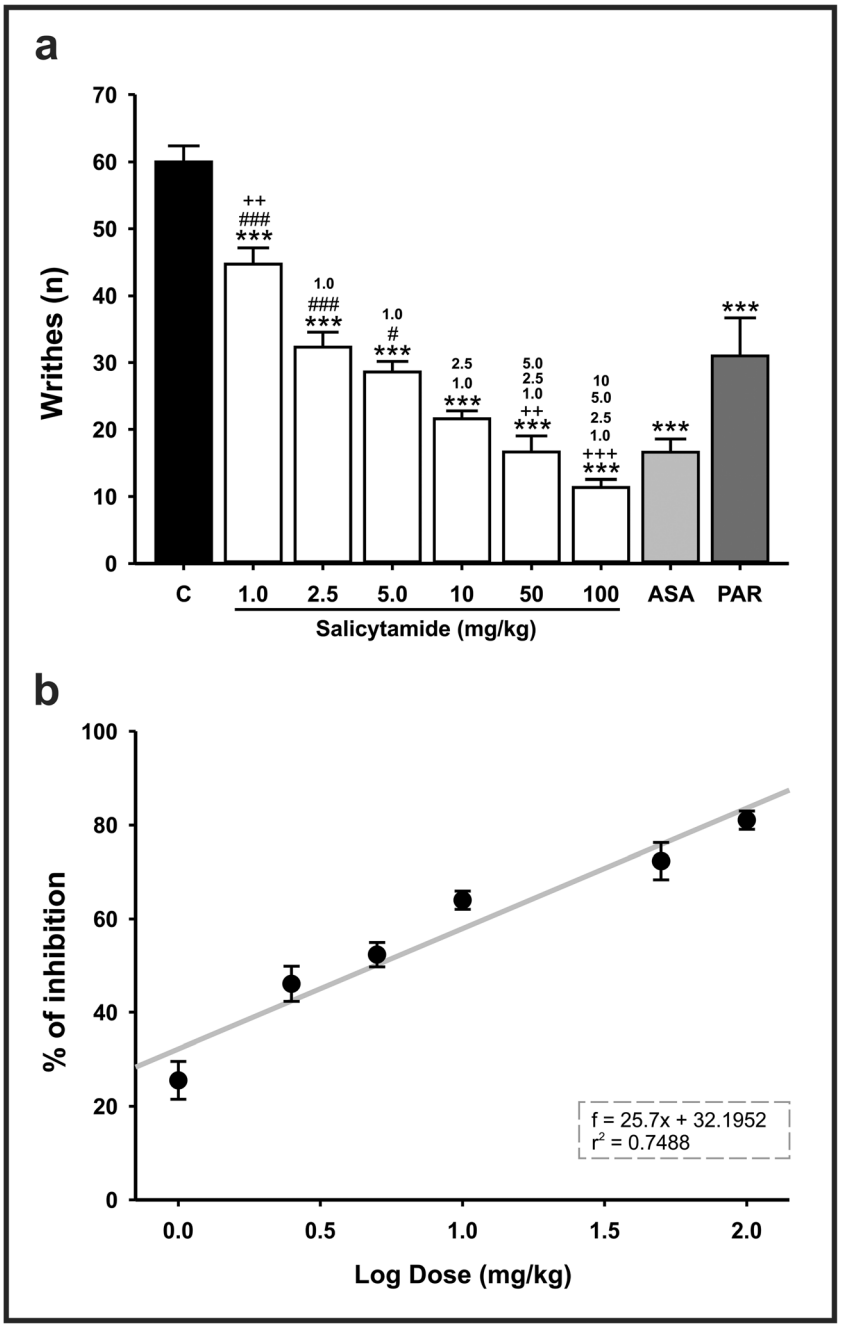

Fig. 3. Effects of salicytamide treatment in mice subjected to the acetic acid-induced writhing test (**a**). **b** The dose–effect curve of salicytamide, as well as ED50. Groups: Control; Salicytamide (doses 1, 2.5, 5, 10, 50 and 100 mg/kg); paracetamol 127 mg/kg (PAR); acetylsalicylic acid 100 mg/kg (ASA). The results are expressed as the mean ± S.E.M. of the number of writhes within 20 min. $^{***}p < 0.001$ when compared to control group; $^{\#\#\#}p < 0.001$ when compared to ASA group; $^{\#}p < 0.05$ when compared to ASA group; $^{+++}p < 0.001$ when compared to PAR group; $^{++}p < 0.01$ when compared to PAR group; $^{1.0,\ 2.5,\ 5.0,\ 10}p < 0.05$ when respectively compared to salicytamide group (doses 1.0, 2.5, 5.0, 10 mg/kg) ($n = 8$–10 animals per group; one-way ANOVA, followed by Holm–Sidak test and linear regression).

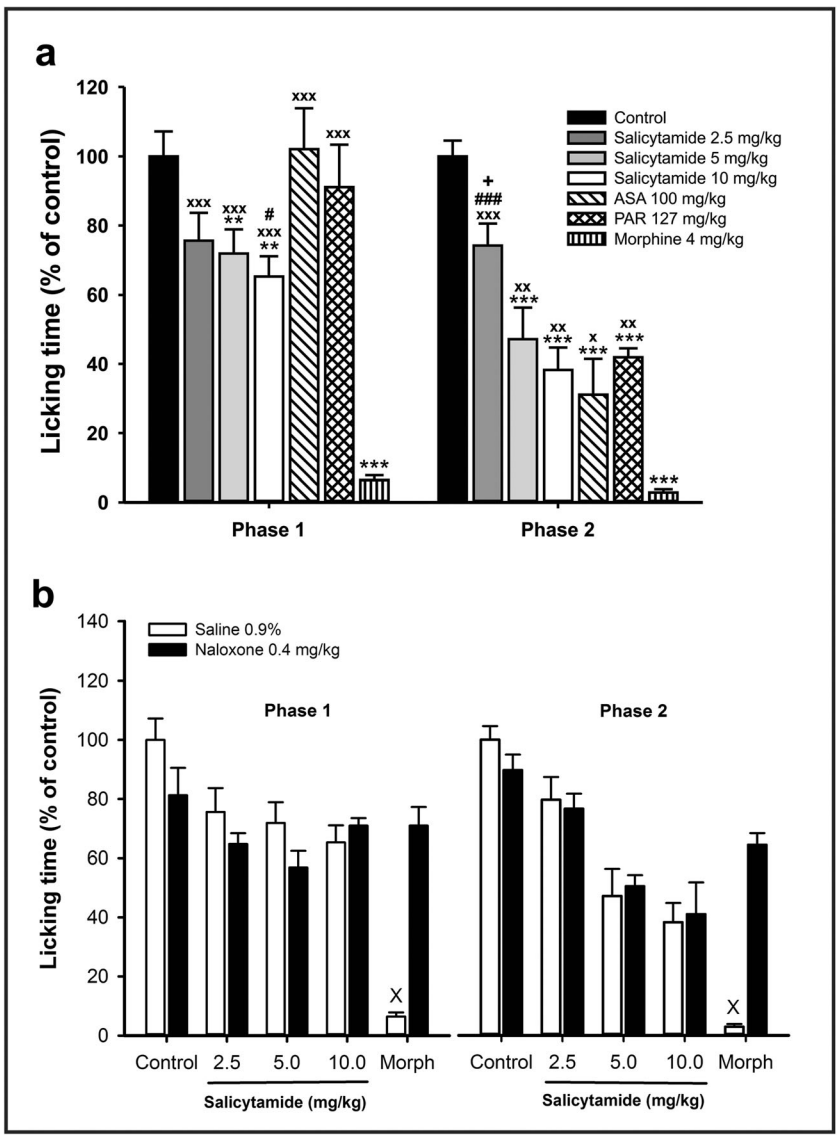

Fig. 4. Effects of salicytamide treatment in mice subjected to the formalin test (**a**). Influence of saline 0.9% or naloxone 0.4 mg/kg pre-treatment on salicytamide effects (**b**). Groups: control; salicytamide (doses 2.5, 5 and 10 mg/kg); paracetamol 127 mg/kg (PAR); acetylsalicylic acid 100 mg/kg (ASA) and morphine 10 mg/kg, s.c. (Morph). The results are expressed as the mean ± S.E.M. of the licking time (s) in the first (0–5 min–neurogenic pain) and second phases (15–30 min—inflammatory pain) of the test. $^{*}p < 0.05$ when compared to control group; $^{**}p < 0.01$ when compared to control group; $^{***}p < 0.001$ when compared to control group; $^{\#}p < 0.05$ when compared to PAR group; $^{\#\#\#}p < 0.001$ when compared to ASA group; $^{xx}p < 0.01$ when compared to morphine group; $^{xxx}p < 0.001$ when compared to morphine group; $^{x}p < 0.05$ when compared to naloxone group ($n = 7$–10 animals per group; one-way repeated measures ANOVA, followed by Holm–Sidak post-hoc test).

activity were observed in the open-field test. Taken together, these results highlight that the molecular combinatorial modelling approach used to create salicytamide was very effective in reducing toxicity.

Salicytamide Reduces Inflammatory Nociception More Effectively Than Paracetamol and Acetylsalicylic Acid

Salicytamide was effective in reducing nociceptive behaviours in both the acetic acid-induced writhing test

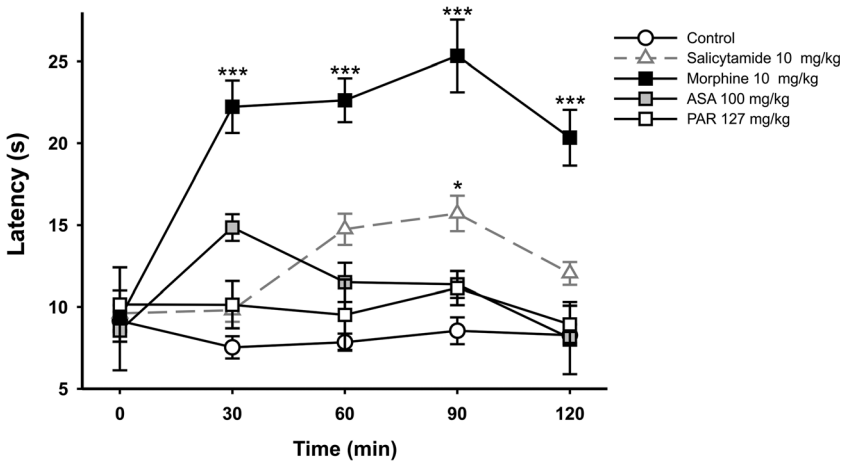

Fig. 5. Effects of salicytamide treatment in mice subjected to the hot plate test. Groups: control; salicytamide (dose 10 mg/kg); paracetamol 127 mg/kg (PAR); acetylsalicylic acid 100 mg/kg (ASA) and morphine 10 mg/kg (s.c.). The results are expressed as the mean ± S.E.M. of the latency (s) for mice to show nociceptive behaviour at 50 °C. *$p < 0.05$ when compared to control group; ***$p < 0.001$ when compared to control group ($n = 8$–10 animals per group; one-way repeated measures ANOVA, Holm–Sidak post-hoc test).

and during formalin's test second phase. Salicytamide antinociceptive effects were clearly dose-dependent and most evident in the writhing test. Acetic acid and formalin are well-known chemical noxious stimuli capable of inducing inflammatory mechanisms, as they induce activation of pro-inflammatory enzymes (*e.g.* cyclooxygenases and lipoxygenases) and consequent production and release of inflammatory mediators (*e.g.* prostaglandins, leukotrienes, substance P, bioactive amines, interleukins, TNF) [28, 29]. Therefore, salicytamide antinociceptive effects seem to be related to its capacity to mitigate the pro-inflammatory effects of acetic acid and formalin, possibly through shared pharmacological mechanisms with paracetamol and acetylsalicylic acid.

In fact, both acetylsalicylic acid and paracetamol have been reported to inhibit acetic acid-induced nociception, with ED50 values of 67.5 mg/kg [30] and 125 mg/kg [30–32], respectively. In the same paradigm, salicytamide was found to have an ED50 of

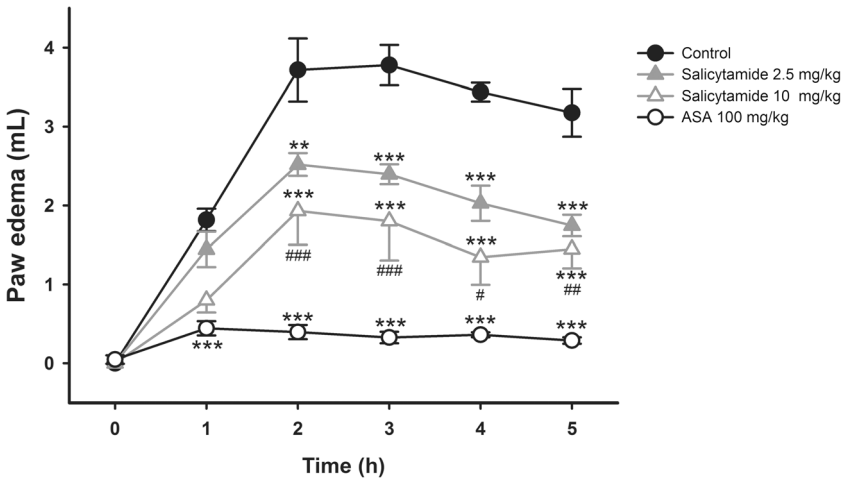

Fig. 6. Effects of salicytamide treatment in rats subjected to the carrageenan-induced paw oedema test. Groups: control; salicytamide (doses 2.5 and 10 mg/kg); acetylsalicylic acid 100 mg/kg (ASA). The results are expressed as the mean ± S.E.M. of the changes in the rats' paws volume in a 5-h period, using a digital pachymeter (mL). ***$p < 0.001$ when compared to control group. $^{\#}p < 0.05$ when compared to ASA group; $^{\#\#\#}p < 0.001$ when compared to ASA group ($n = 4$–6 animals per group; one-way repeated measures ANOVA, Holm–Sidak post-hoc test).

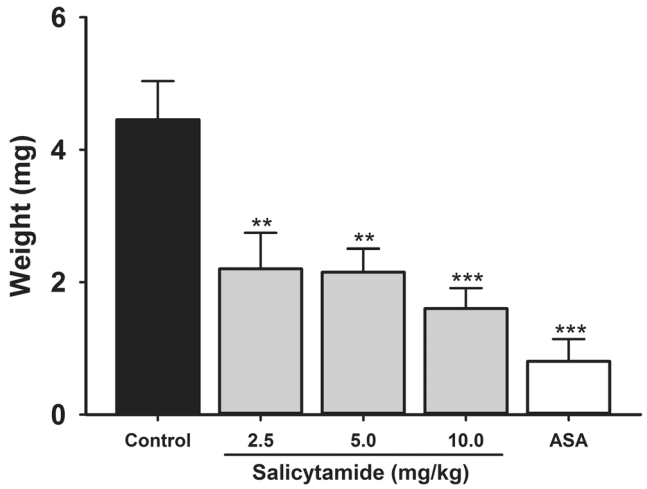

Fig. 7. Effects of salicytamide treatment in rats subjected to the croton oil-induced ear oedema test. Groups: control; salicytamide (doses 2.5, 5 and 10 mg/kg); acetylsalicylic acid 100 mg/kg (ASA). The results are expressed as the mean ± S.E.M. of the weight difference of plugs from both rats' ears, 6-h post-treatment with irritant or acetone (6 mm). $^{**}p < 0.01$ when compared to control group; $^{***}p < 0.001$ when compared to control group ($n = 8$–10 animals per group; one-way ANOVA, Holm–Sidak post-hoc test).

4.95 mg/kg, which makes it 14 and 25 times more potent than acetylsalicylic acid and paracetamol, respectively. Additionally, salicytamide decreased up to 52 and 62% of the formalin-induced nociception at the doses of 5 and 10 mg/kg, respectively. Meanwhile, acetylsalicylic acid and paracetamol have only been reported to elicit similar effects at much higher doses, 100 and 50 mg/kg, respectively [33–35].

Salicytamide Interferes with Central Mechanisms of Nociception

The hot plate test and the first phase of the formalin test are often employed in the screening of compounds with possible central antinociceptive effects, as they mediate direct stimulation of nociceptive terminals (C and Aδ fibers) with subsequent engagement of the central nervous system in the processing of nociceptive signals [29, 36]. In line with this, we found that salicytamide increased latency to nociception during the hot plate test, but only after 90 min of its administration and at the highest dose tested (10 mg/kg). Moreover, salicytamide antinociceptive effects were also observed during the formalin's test neurogenic phase (first), even at the lowest dose tested (2.5 mg/kg).

To investigate the possible participation of opioid receptors in the central antinociceptive effects of salicytamide, animals were treated with naloxone, prior to the formalin test. Naloxone did not interfere with the antinociceptive effects of salicytamide during the neurogenic phase of the formalin test, therefore excluding the involvement of opioid receptors. Actually, both acetylsalicylic acid and paracetamol were previously reported to mediate central antinociceptive effects [34, 37–39], which were linked to the regulation of serotoninergic and cannabinoid receptors, respectively. Therefore, considering the structural similarities between salicytamide and its parent drugs (acetylsalicylic acid and paracetamol), additional studies on the underlying pharmacological mechanisms for the central antinociceptive effects of salicytamide must be performed.

Salicytamide Is Effective in Inhibition of the Inflammatory Oedema Formation

The carrageenan-induced paw oedema presents a well-characterized temporal relationship with the underlying molecular mechanisms triggered for oedema formation, where the first phase (0–90 min) is marked by increased histaminergic and serotonergic activities, the second (90–150 min) by the release of cytokines, and the third (150–300 min) by the action of prostaglandins [40]. The antioedematogenic effects of salicytamide (2.5, 5.0 and 10 mg/kg) were observed within 2 h, second phase, post-carrageenan administration, remaining until the end of the third phase. Such effect suggests that salicytamide may inhibit both the release of pro-inflammatory cytokines,

and prostaglandin synthesis. However, additional studies should be conducted to confirm this hypothesis.

Salicytamide was also effective in preventing the ear oedema induced by croton oil, a model marked by the activation of phospholipase A2 and consequent increase in arachidonic acid synthesis, leukotrienes prostaglandins [41], which reinforces the findings observed on the carrageenan model. As expected, paracetamol failed to have any anti-oedematogenic effects [42]. On the other hand, acetylsalicylic acid (60–300 mg/kg) is known to prevent oedema formation in both models [43, 44]. Of interest, salicytamide promoted antioedematogenic effects at doses that are 10 times lower than the ones usually used for acetylsalicylic acid (100 mg/kg).

>In summary, the present study validates the use of rational drug design and combinatorial molecular modelling approaches for the synthesis of a new, more effective and safer NSAID drug candidate, salicytamide. Here we report, for the first time, that salicytamide has better pharmacokinetic profile, significantly reduced acute oral toxicity and increased antinociceptive and antioedematogenic properties, when compared to its parent drugs acetylsalicylic acid and paracetamol. Thus far, we have shown that the anti-inflammatory mechanisms of salicytamide rely on the inhibition of both pro-inflammatory cytokines release and prostaglandins synthesis. Additionally, our tests excluded the participation of opioid receptors in the central antinociceptive effects of salicytamide. Nonetheless, the exact mechanisms through which salicytamide exerts its effects remain unclear, and therefore, more studies remain necessary.

ACKNOWLEDGMENTS

KMMG and SCC were supported by a Brazilian Government/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) fellowship. RSB is supported by a research fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Funding information We would like to thank the Pró-Reitoria de Pesquisa e Pós-Graduação (PROPESP/UFPA) for providing financial support (PAPQ).

COMPLIANCE WITH ETHICAL STANDARDS

All procedures were approved by the Ethics Committee on Experimental Animals of the Federal University of Pará under license number FAR 001-10-CEPAE/UFPA. The number of animals and the intensity of noxious stimuli were standardized according to the ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983) and the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [18].

Conflict of Interest. The authors declare that they have no conflict of interest.

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