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Convolutamydine A and synthetic analogues have antinociceptive properties in mice

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ABSTRACT

Convolutamydine A, an oxindole that originated from a marine bryozoan, has several biological effects. In this study, we aimed to investigate the antinociceptive effects of convolutamydine A and two new synthetic analogues.

Convolutamydine A and the two analogues were given orally to assess their ability to induce antinociceptive effects. Formalin-induced licking response, acetic acid-induced contortions, and hot plate models were used to characterize the effects of convolutamydine A and its analogues.

Convolutamydine A (4,6-bromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole), compound 1 (3-(2-oxopropyl)-3-hydroxy-2-oxindole), and compound 2 (5-bromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole) caused peripheral antinociceptive and anti-inflammatory effects in the acetic acid-induced contortions and the formalin-induced licking models. Supraspinal effects were also observed in the hot plate model and were similar to those obtained with morphine. The peripheral effects were not mediated by the cholinergic or opioid systems. The antinociceptive effects of convolutamydine A seem to be mediated by all three systems (cholinergic, opioid, and nitric oxide systems), and the mechanism of action of compounds 1 and 2 involved cholinergic and nitric oxide-mediated mechanisms. Convolutamydine A and its analogues (compounds 1 and 2) showed good antinociceptive ability after systemic administration in acute pain models. The antinociceptive action mediated by cholinergic, opioid, and nitric oxide systems could explain why convolutamydine A, compound 1, and compound 2 retained their antinociceptive effects. The doses used were similar to the doses of morphine and were much lower than that of acetylsalicylic acid, the classical analgesic and anti-inflammatory drug.

In conclusion, convolutamydine A and the two analogues demonstrated antinociceptive effects comparable to morphine's effects.

1. Introduction

Pain is a warning condition of the organism against an injury and is sometimes the only symptom of various diseases. Although pain often has a protective function, in many cases, it is a condition that limits productivity and reduces quality of life. Considerable efforts have been made to discover new analgesic agents with increased efficacy and improved side effect profiles. While many pain medications are currently available, there is some concern regarding their safety and side effects, making their clinical use problematic (Jage, 2005; Whittle, 2003). Therefore, the search for new molecules with greater analgesic potency and fewer side effects remains a goal of researchers from universities and the pharmaceutical industry.

Convolutamydine A (Fig. 1) is an oxindole alkaloid isolated in low yields from the Floridian marine bryozoan species *Amathia convoluta*. This compound reduces the differentiation of HL-60 human promyelocytic leukemia cells (Kamano et al., 1995). The promising biological effects have led several research groups to work on the synthesis of convolutamydine A (Cravotto et al., 2006; Garden et al., 1997; Luppi et al., 2006; Malkov et al., 2007) in racemic and chiral form.

Convolutamydine A is a derivative of isatins (Fig. 1), which are heterocyclic compounds with considerable synthetic versatility (Da Silva et al., 2001; Shvekhgeimer, 1996; Silva et al., 2008, 2010, 2011). Isatin and its derivatives have been found to have anticholinesterase, anticonvulsant, antihypertensive, anti-hypoxic, antimicrobial, antineoplastic, antiulcer, antiviral and anti-inflammatory activities (Bhrigu et al., 2010; Da Silva et al., 2001; Hall et al., 2009; Matheus et al., 2007; Peddibhotla, 2009; Silva et al., 2010; Vine et al., 2008; Zapata-Sudo et al., 2007).

Our group has been investigating the possible pharmacological potential of convolutamydine A and synthetic analogues obtained from isatins. The aim of the present study was to investigate the

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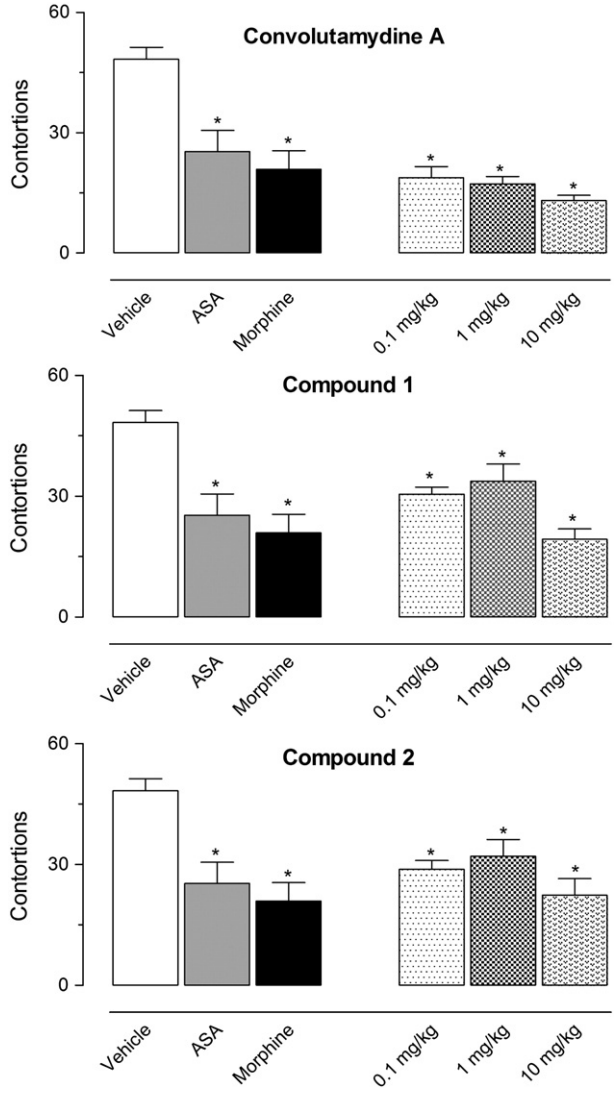


Fig. 1. Effects of convolutamydine A (4,6-dibromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole), compound 1 (3-(2-oxopropyl)-3-hydroxy-2-oxindole), and 2 (5-bromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole) on acetic acid-induced abdominal writhing in mice. Animals were pre-treated with oral administration of different doses of convolutamydine A, compound 1, 2, acetylsalicylic acid (ASA, 200 mg/kg), morphine (5 mg/kg) or vehicle. The results are presented as mean ± S.D. (n = 6–10) of contortions. Statistical significance was calculated by ANOVA followed by Bonferroni's test. *$P<0.05$ when compared to vehicle-treated mice.

antinociceptive effects of two novel analogues of convolutamydine A using chemical (acetic acid-induced contortions and formalin) and thermal (hot plate) models of nociception in mice.

2. Materials and methods

2.1. Animals

All experiments were performed with Swiss mice (20–25 g), from both sexes, obtained from our own animal facility. The animals were maintained in a room with controlled temperature (22 ± 2 °C) and a 12 h light/dark cycle and were given free access to food and water. The animals received only water for 12 h before each experiment to avoid food interference with substance absorption. The experiments were conducted in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983). The number of animals and the intensities of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of drug treatments. The animal care and research protocols (number ICBDFBC-015) were conducted in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethical Committee for Animal Research (Biomedical Science Institute/UFRJ).

2.2. General

Acetylsalicylic acid (ASA), L-nitro arginine methyl ester (L-NAME), and atropine were purchased from Sigma (St. Louis, MO, USA); acetic acid and morphine hydrochloride were purchased from Merck Inc. (Brazil); and naloxone was purchased from Cristália (São Paulo, Brazil). All drugs were dissolved in phosphate buffer saline (PBS) immediately prior to use.

2.3. Synthesis of convolutamydine analogues

A catalytic quantity of diethylamide was added to a suspension of 10 mmol of isatin, 5-bromoisatin or 4,6-dibromoisatin (Table 1) and 20 ml of acetone. The mixture was stirred at room temperature and the reaction progress was followed by thin layer chromatography. After 48 h, the resulting light yellow solid was filtered to produce the product in high yields. Their structures were confirmed by spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR and IR (Garden et al., 1997).

2.4. Administration of drugs, convolutamydine A and analogues

Convolutamydine A, and compounds 1 and 2 were dissolved in dimethyl sulphoxide (DMSO) to prepare a 200 mg/ml stock solution. On the day of the experiments, diluted solutions were prepared from each stock solution using sterile distilled water as the diluent. The substances were administered by oral gavage at doses varying between 0.1 and 30 mg/kg in a final volume of 0.1 ml per animal. Acetylsalicylic acid (ASA, 200 mg/kg) and morphine (5 mg/kg) were used as reference drugs and were administered by oral gavage at the intervals indicated in each protocol. The dose of ASA and morphine was chosen based on previous experiments carried out by our group (Pinheiro et al., 2010) and was that which caused a 50% reduction in each procedure (IC_{50}). The control group was given vehicle (DMSO/water).

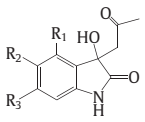
2.5. Acetic acid-induced abdominal writhing

Mice were treated according to Matheus et al. (2005). Briefly, the total number of writhings following intraperitoneal administration of a 2% (v/v) acetic acid solution (AA) was recorded over a period of 20 min, starting 5 min after the AA injection. Mice were pre-treated with convolutamydine A, the analogues, ASA, morphine, or vehicle for 60 min before the administration of AA.

2.6. Formalin test

This procedure was similar to the method described by Gomes et al. (2007). Mice received an injection of 20 μl of formalin (2.5% v/v) into the dorsal surface of the left hind paw. The time that the animal spent licking the injected paw was immediately recorded. The nociceptive and inflammatory response consists of the following two phases: the first phase lasts until 5 min after the formalin injection (first phase, neurogenic pain response), and the second phase occurs 15–30 min after the formalin injection (second phase, inflammatory pain response). The animals were pre-treated with oral doses of convolutamydine A, compound 1, compound 2, vehicle, ASA or morphine for 60 min before the administration of formalin.

Table 1
Structures and yields of convolutamydine A and synthetic analogues obtained from isatins.



Compound	Name	Substituent (R)	Yield (%)
Convolutamydine A	4,6-Dibromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole	$R_1 = Br$, $R_2 = H$, $R_3 = Br$	86
1	3-(2-Oxopropyl)-3-hydroxy-2-oxindole	$R_1 = R_2 = R_3 = H$	80
2	5-Bromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole	$R_1 = H$, $R_2 = Br$, $R_3 = H$	81

2.7. Hot plate test

Mice were tested according to the method described by Sahley and Berntson (1979) and adapted by Matheus et al. (2005). Animals were placed on a hot plate (Insight Equipment, Brazil) set at 55 ± 1 °C. At successive intervals of 30 min after oral administration of convolutamydine A, compound 1, 2, vehicle or morphine, the reaction time was recorded when the animals licked their fore- and hind-paws and jumped. Baseline was considered the mean reaction time obtained at 60 and 30 min before administration of the compounds, vehicle, or morphine and was defined as the normal reaction of the animal to the temperature. When animals were kept on the hot plate for a period of time greater than three times the baseline (cut-off), they were removed to avoid possible damage to the paws. Antinociception was quantified as either the increase in baseline (%) calculated by the formula (reaction time × 100/baseline) − 100 or the area under the curve (AUC) of responses from 30 min after drug administration until the end of the experiment. The following formula based on the trapezoid rule was used to calculate the AUC: AUC = 30 × IB [(min 30) + (min 60) + … + (min 180)/2], where IB is the increase in baseline (in %).

2.8. Assessment of some mechanisms involved in antinociceptive activity

To investigate the participation of the opioid system in the antinociceptive effects of convolutamydine A, compound 1 and 2, mice were pre-treated i.p. with naloxone (1 mg/kg), an opioid receptor antagonist. After 30 min, the animals received oral administration of convolutamydine A, compounds 1 or 2 (10 mg/kg) and were subjected to the methods described above.

Atropine (1 mg/kg, i.p.), a cholinergic receptor antagonist, was administered 30 min before the substances under study (10 mg/kg, p.o.) to assess the possible participation of the cholinergic system in the antinociceptive effects.

L-Nitro arginine methyl ester (L-NAME; 3 mg/kg, i.p.), an inhibitor of nitric oxide synthase, was administered 30 min before convolutamydine A or its analogues (10 mg/kg, p.o.) to evaluate the participation of the nitric oxide system. The choice of the doses of the antagonists or inhibitors and their treatment times were based on previous data described in the literature (Otuki et al., 2005; Tabarelli et al., 2004) and experiments conducted in our laboratory. Dose response curves of each antagonist were previously performed and the dose that reduced to 50% the response of the agonist was chosen for these assays (Pinheiro et al., 2010).

2.9. Reduction of spontaneous activity

The spontaneous activity was evaluated as described in Barros et al. (1991). Mice received oral administrations of convolutamydine A, compound 1 or 2 (at 30 mg/kg, p.o.). Immediately, they were placed individually in an observation chamber whose floor was divided into 50 squares (5 cm × 5 cm). Total numbers of squares by which mouse walked during 5 min were counted.

The effect of compounds on locomotor performance was also tested on the rotarod apparatus as described previously (Godoy et al., 2004). Twenty-four hours before the experiments, all animals were trained in the rotarod (3.7 cm in diameter, 8 r.p.m) until they could remain in the apparatus for 60 s without falling. On the day of the experiment, mice were treated with convolutamydine A, compound 1 or 2, (30 mg/kg, p.o.) and tested in the rotarod from 0.5 up to 3.5 h after their administration. The number of falls from the apparatus was recorded with a stopwatch for up to 240 s.

2.10. Acute toxicity

Acute toxicity was determined following the experimental model described previously (Lorke, 1983). A single oral dose of convolutamydine A, compound 1 or 2 (500 mg/kg) was administered to a group of ten mice (five males and five females). Behavioral parameters observed over a period of 14 days included convulsion, hyperactivity, food and water intake, grooming, loss of righting reflex, increased or decreased respiration, and sedation. After this period animals were killed by cervical dislocation, stomachs were removed and an incision along the greater curvature was made. The number of ulcers (single or multiple erosion, ulcer or perforation) and hyperemia were measured.

2.11. Statistical analysis

Each experimental group consisted of 6–10 mice. The results are presented as mean ± S.D. The area under the curve (AUC) was calculated using Prism Software 5.0 (GraphPad Software, La Jolla, CA, USA). Statistical significance between groups was determined using the application of an analyses of variance (ANOVA) followed by Bonferroni's test. P values less than 0.05 were considered to be significant.

3. Results

3.1. Evaluation of the antinociceptive effects of convolutamydine A and analogues in the acetic acid-induced contortions model

The intraperitoneal injection of acetic acid led to 48.3 ± 3.0 contortions in an interval of 20 min. After pre-treatment of animals with oral doses ranging from 0.1 to 10 mg/kg of convolutamydine A, compound 1 or 2 a significant reduction in the number of contortions induced by the acetic acid was observed (Fig. 2). In comparison, the nonsteroidal anti-inflammatory drug acetylsalicylic acid (ASA) was used in a dose that, in our model, caused 50% inhibition, 200 mg/kg. In the same way, the opioid agonist morphine was used and reduced the nociceptive response by 50%.

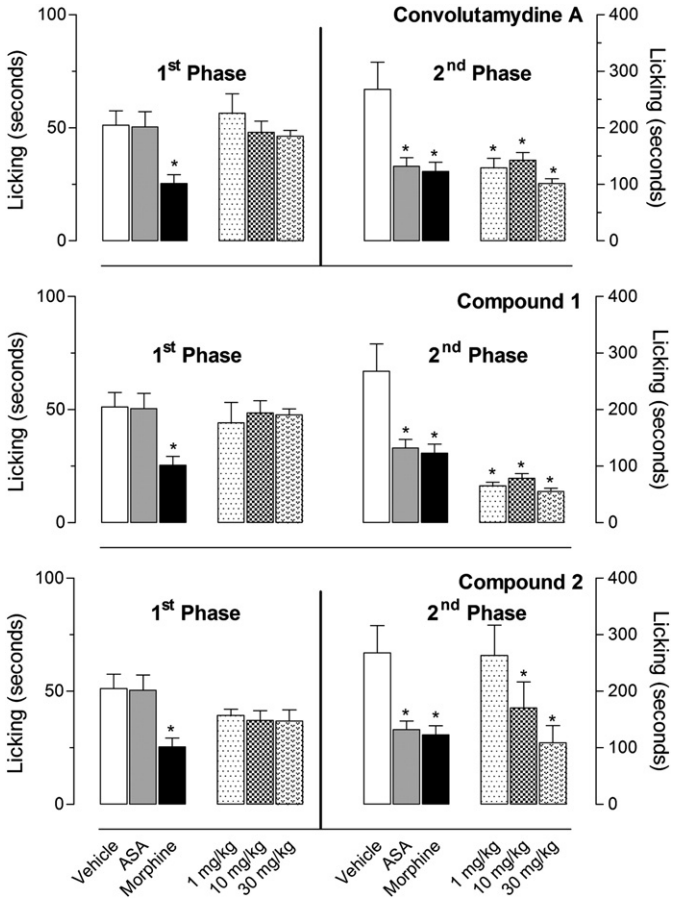


Fig. 2. Effects of convolutamydine A (4,6-dibromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole), compound 1 (3-(2-oxopropyl)-3-hydroxy-2-oxindole), and 2 (5-bromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole) on formalin-induced licking response in mice. Animals were pre-treated with oral administration of different doses of convolutamydine A, compound 1, 2, acetylsalicylic acid (ASA, 200 mg/kg), morphine (5 mg/kg) or vehicle. The results are presented as mean ± S.D. (n = 6–10) of the time that the animal spent licking the formalin-injected paw. Statistical significance was calculated by ANOVA followed by Bonferroni's test. *$P<0.05$ when compared to vehicle-treated mice.

3.2. Evaluation of the antinociceptive effects of convolutamydine A and its analogues in the formalin model

To confirm the antinociceptive effects of convolutamydine A and its analogues, we evaluated these substances using the formalin model. The injection of formalin (2.5%) leads to a biphasic licking response of the injected paw. The first phase lasts until 5 min after injection, and the second phase occurs between 15 and 30 min after formalin injection. As shown in Fig. 3, none of the three substances (convolutamydine A, compound 1 or 2) significantly reduced the time that the animal spent licking the formalin-injected paw during the first phase. In contrast, pre-treatment of animals with any of the three compounds inhibited the second phase of the response to formalin. More pronounced effects were observed with convolutamydine A and compound 1. Even at the lowest dose of 0.1 mg/kg, convolutamydine A and compound 1 reduced the total licking time by 53.1% and 59.9%, respectively (276.0 ± 46.0 s in the control group versus 113.3 ± 12.9 s and 129.4 ± 16.4 s in the groups treated with convolutamydine A or compound 1, respectively). In the second phase of the formalin model, a dose of 200 mg/kg of ASA reduced the total licking time by 52.9% (276.0 ± 46.0 s in the control group versus 132.5 ± 6.9 s in the ASA-treated group). Comparing the results with convolutamydine A, compound 1, and compound 2 with the ED_{50} calculated to ASA (200 mg/kg) we can observe that convolutamydine A and the compound 1 and 2 were 2000, 1667, and 15 times more potent than the ED_{50} dose of ASA, respectively.

3.3. Evaluation of the antinociceptive effects of convolutamydine A and analogues in the hot plate model

We used the hot plate model to evaluate the supraspinal antinociceptive effects of the substances. As can be observed in Fig. 4 (left graphs), at the lowest dose of 1 mg/kg, any of the three substances significantly increased the baseline. On the basis of these results, we decided to increase the doses to 30 mg/kg. To compare the results obtained with morphine (at it ED_{50}) and the substances, we plotted the results as a graph of area under the curve. Compounds 1 and 2 (at a dose of 10 mg/kg) reached area under the curve (AUC) values similar to those obtained with morphine (5 mg/kg). Surprisingly, at this same dose, convolutamydine A showed an effect that was almost 1.5 times higher than the effect observed with the dose of morphine corresponding to its ED_{50} (Fig. 4, right graphs). As we expected, ASA did not present any antinociceptive effect and was not used for comparisons with our compounds (data not shown).

3.4. Evaluation of the mechanism of action of convolutamydine A and its analogues

As all substances demonstrated an antinociceptive effect in the analgesia models, next we tried to elucidate the mechanism by which convolutamydine A and its analogues exert out their activities. We decided to evaluate the involvement of the opioid, cholinergic, and nitric oxide systems on the antinociceptive effects by pre-treating mice with inhibitors of each system and evaluating the antinociceptive effects of each compound in all three models.

It could be observed that none of the antagonists (atropine and naloxone) and nitric oxide synthase inhibitor (L-NAME) demonstrated any antinociceptive effects *per se* in all models used. Neither atropine (muscarinic antagonist, 1 mg/kg) nor naloxone (opioid antagonist, 1 mg/kg) given 30 min beforehand was able to significantly reverse the antinociception caused by any of the three substances in the acetic acid-induced contortions and formalin-induced licking models. When L-NAME (3 mg/kg) was administered 30 min prior to the substances, it reversed the convolutamydine A activity in both models (Figs. 4 and 5). Atropine and L-NAME reversed the antinociceptive effect of convolutamydine A, compound 1, and compound 2 in the hot plate model, while naloxone reversed only the antinociceptive effect of convolutamydine A (Fig. 6).

3.5. Assessment of side effects and acute toxicity

Neither convolutamydine A nor its analogues (30 mg/kg, p.o.) had any significant effect on motor performance or spontaneous activity (Table 2); at 0.5, 2 or 3.5 h after administration in mice. When animals were administered convolutamydine A, compound 1 or 2 at doses of 500 mg/kg, p.o., the death rates were 6.3%, 5.4%, and 3.1%, respectively. Also, none of the compounds evaluated (convolutamydine A, compound 1 and 2) altered any of the parameters observed during the toxicity assays. There were no alterations in normal activity, such as food and water intake, grooming, and loss of righting reflex. We did not observe alterations on righting reflex or respiration and no ulcers were observed in stomachs after 14 days (data not shown).

4. Discussion

The antinociceptive effects of convolutamydine A and two analogues (compounds 1 and 2) have been demonstrated for the first time. The oral administration of the three substances produced a marked antinociception in several models of pain.

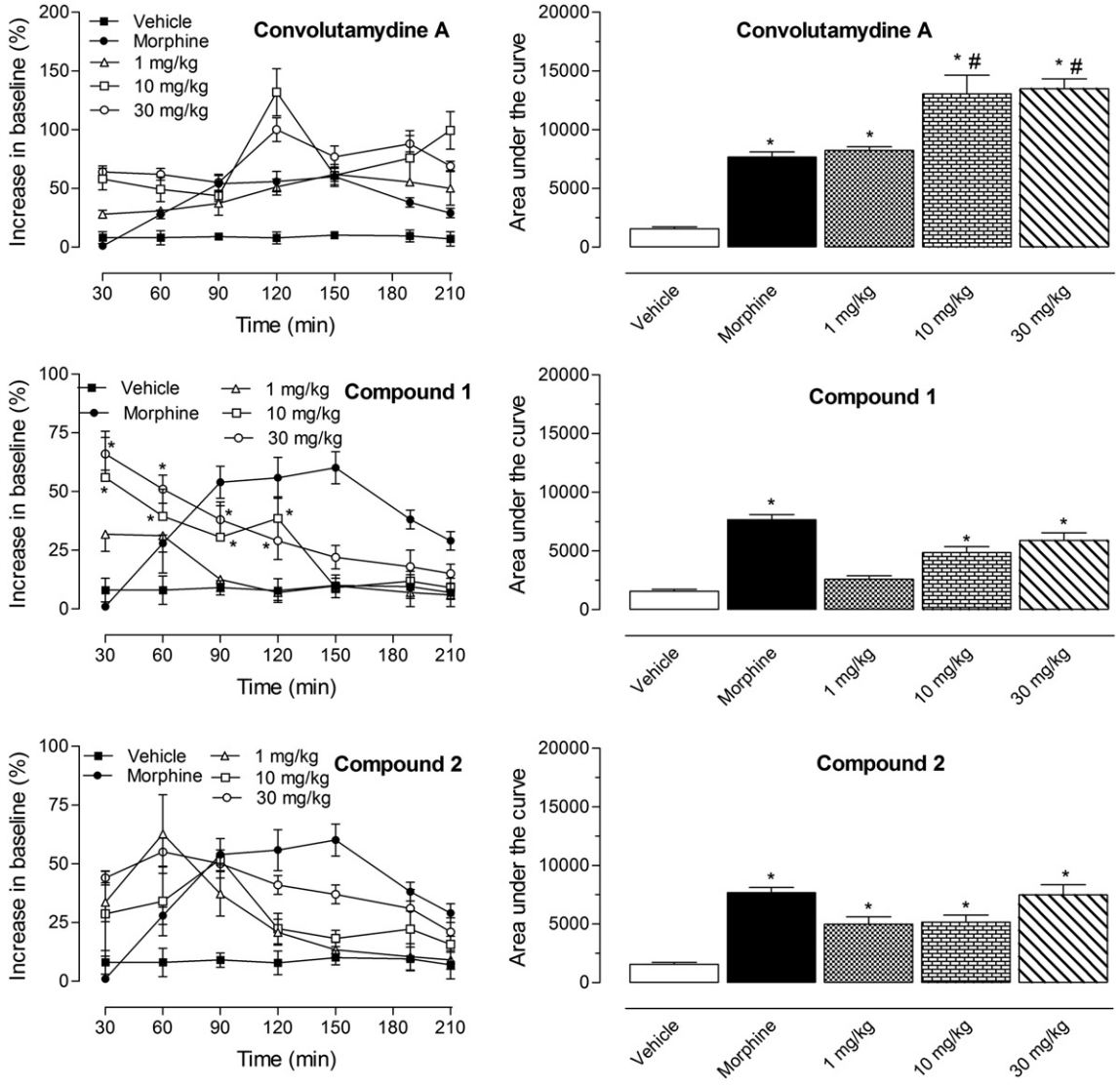


Fig. 3. Effects convolutamydine A (4,6-dibromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole), compound 1 (3-(2-oxopropyl)-3-hydroxy-2-oxindole), and 2 (5-bromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole) in the hot plate model. Animals were pre-treated with oral administration of different doses of convolutamydine A, compound 1, 2, morphine (5 mg/kg) or vehicle. The results are presented as mean ± S.D. (n = 6–10) of the increase in baseline levels (left graphs) or area under the curve (right graphs) calculated by Prism Software 5.0. Statistical significance was calculated by ANOVA followed by Bonferroni's test. * indicates $p<0.05$ when comparing treated mice to the vehicle-treated group; # indicates $p<0.05$ when comparing treated mice with the morphine-treated group.

The acetic acid-induced contortion is a model of visceral inflammatory nociception that is used for the evaluation of analgesic and/or anti-inflammatory drugs (Tjølsen et al., 1991, 1992). The injection of acetic acid induces an inflammatory process that leads to the release of inflammatory mediators in the abdominal cavity with subsequent activation of nociceptors (Collier et al., 1968). Local tissue injury prompts the release of chemical mediators (potassium, hydrogen ions, ATP, and bradykinin) and inflammatory mediators (for example, PGE2) from inflammatory cells. These substances directly activate nerve endings and trigger the release of algesic mediators (for example, histamine, serotonin (5-HT), nerve growth factor (NGF), and prostanoids) from other cells and afferent nerves (Kennedy and Leff, 1995; Purcell and Atterwill, 1995; Tracey and Walker, 1995). This sensitizes the endings of afferent nerve terminals resulting in an increased response to painful stimuli (Bueno and Fioramonti, 2002). Our results demonstrated that convolutamydine A and its two analogues, compounds 1 and 2, significantly reduced the number of contortions. These results may be a result of a reduction in the production of inflammatory mediators that were liberated in the peritoneal cavity or a blockage in the pain transmission through pain fibers. Using this test, it is not possible to ascertain whether the effects of convolutamydine A, compound 1 or 2 were due to anti-inflammatory or central effects.

In an attempt to determine the possible effects of convolutamydine A and its analogues, we decide to test their effects on the formalin-induced licking response. This model is commonly employed as a model of acute and tonic peripheral pains. The intraplantar injection of formalin induces a peripheral pain response and is used to test for neurogenic and inflammatory pain. Traditionally, the first phase (or neurogenic phase) corresponds to acute neurogenic pain due to direct stimulation of nociceptors by formalin. Phase 2 corresponds to inflammatory pain mediated by a combination of peripheral input and spinal cord sensitization and that can be inhibited by nonsteroidal anti-inflammatory drugs (Hunskaar and Hole, 1987; Tjølsen et al., 1991). Drugs that act primarily as central analgesics inhibit both phases while peripherally acting drugs inhibit only the second phase (Rosland et al., 1990; Shibata et al., 1989). It is also known that the first phase of the formalin-induced licking response is due to the activation of the nociceptors by bradykinin, serotonin, and histamine (Chapman and

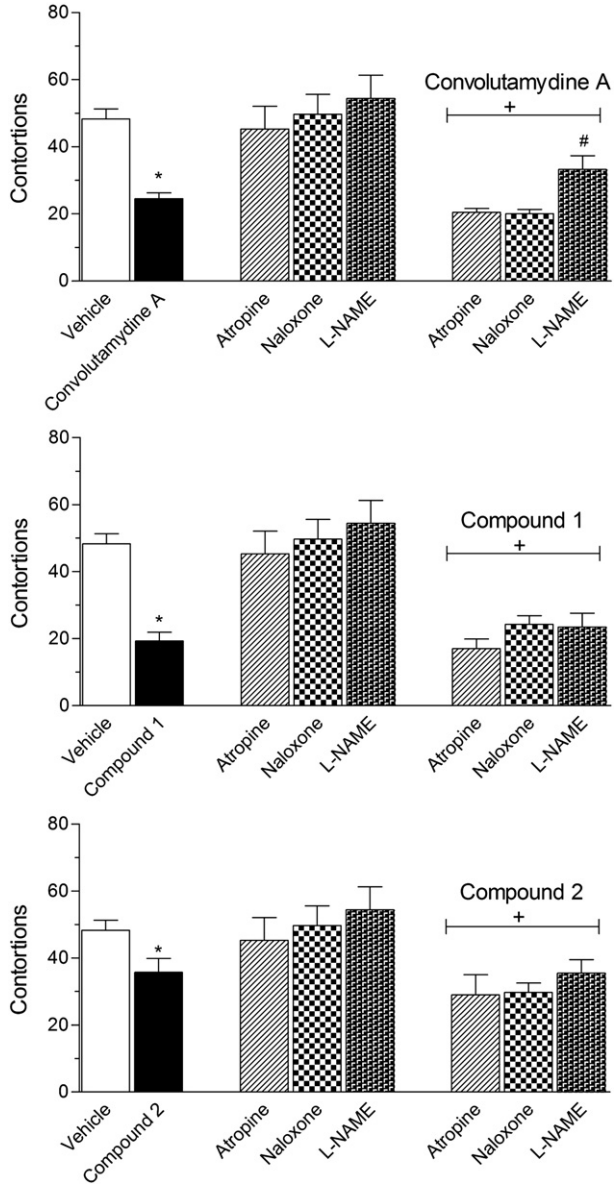


Fig. 4. Effects of different antagonists on the antinociceptive activity of convolutamydine A (4,6-dibromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole), compound 1 (3-(2-oxopropyl)-3-hydroxy-2-oxindole), and 2 (5-bromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole) in the acetic acid-induced contortion model. Animals were pre-treated with naloxone (1 mg/kg, i.p.), atropine (1 mg/kg, i.p.) or L-NAME (3 mg/kg, i.p.) 30 min prior to oral administration of convolutamydine A, compound 1, or 2 (10 mg/kg). The results are presented as mean ± S.D. (n = 6–10) of contortions. Statistical significance was calculated by ANOVA followed by Bonferroni's test. * indicates $p<0.05$ when comparing compound-treated mice to the vehicle-treated group; # indicates $p<0.05$ when comparing antagonist pre-treated mice with the compound-treated group.

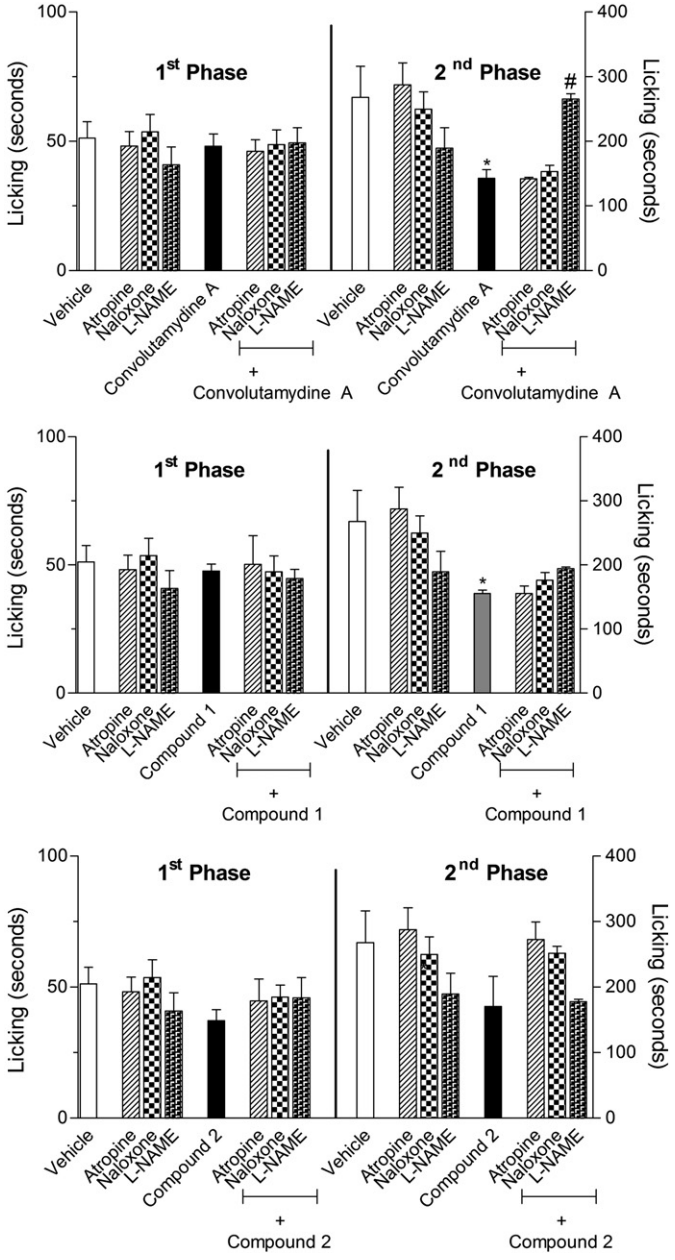


Fig. 5. Effects of different antagonists on the antinociceptive activity of convolutamydine A (4,6-dibromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole), compound 1 (3-(2-oxopropyl)-3-hydroxy-2-oxindole), and 2 (5-bromo-3-(2- oxopropyl)-3-hydroxy-2-oxindole) in the formalin-induced licking response. Animals were pre-treated with naloxone (1 mg/kg, i.p.), atropine (1 mg/kg, i.p.), or L-NAME (3 mg/kg, i.p.) 30 min prior to oral administration of convolutamydine A, compound 1, or 2 (10 mg/kg). Results are presented as mean ± S.D. (n = 6-10) of time that the animal spent licking the formalin-injected paw. * indicates $p<0.05$ when comparing compound-treated mice to the vehicle-treated group; # indicates $p<0.05$ when comparing antagonist pre-treated mice with the compound-treated group.

Dickenson, 1992; De Campos et al., 1996; Parada et al., 2001). The absence of inhibitory effects from convolutamydine A, compound 1, and 2 in this phase could be explained by the hypothesis that these compounds did not directly interact with the nociceptors but only with the inflammatory receptors. Similar to non-opiate analgesics, we have found that the compounds under study decreased the inflammatory phase. This observation suggests a possible inhibition of inflammatory mediators released in the mouse paw and also corroborates with the inhibitory effect of the compounds on the acetic acid-induced writhing response. The fact that compound 2 (at 1 mg/kg) did not reduce the second phase of formalin response but demonstrated a significant effect on acetic acid-induced contortions might be explained by the compound having easier access to the peritoneal cavity. It could be possible that a 1 mg/kg dose has a small amount of substance in such a way that only a minimal quantity of the compound can affect the paw tissue and thus not inhibiting the formalin response.

We also investigated the effects of convolutamydine A and it analogues in the hot plate test. The paws of mice and rats are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws (Eddy and Leimbach, 1953). The time until these responses occur is prolonged after administration of supraspinal acting analgesics, whereas peripheral analgesics of the acetylsalicylic acid or phenylacetic acid type do not generally affect these responses. The hot plate test has been used by many investigators and has been found to be

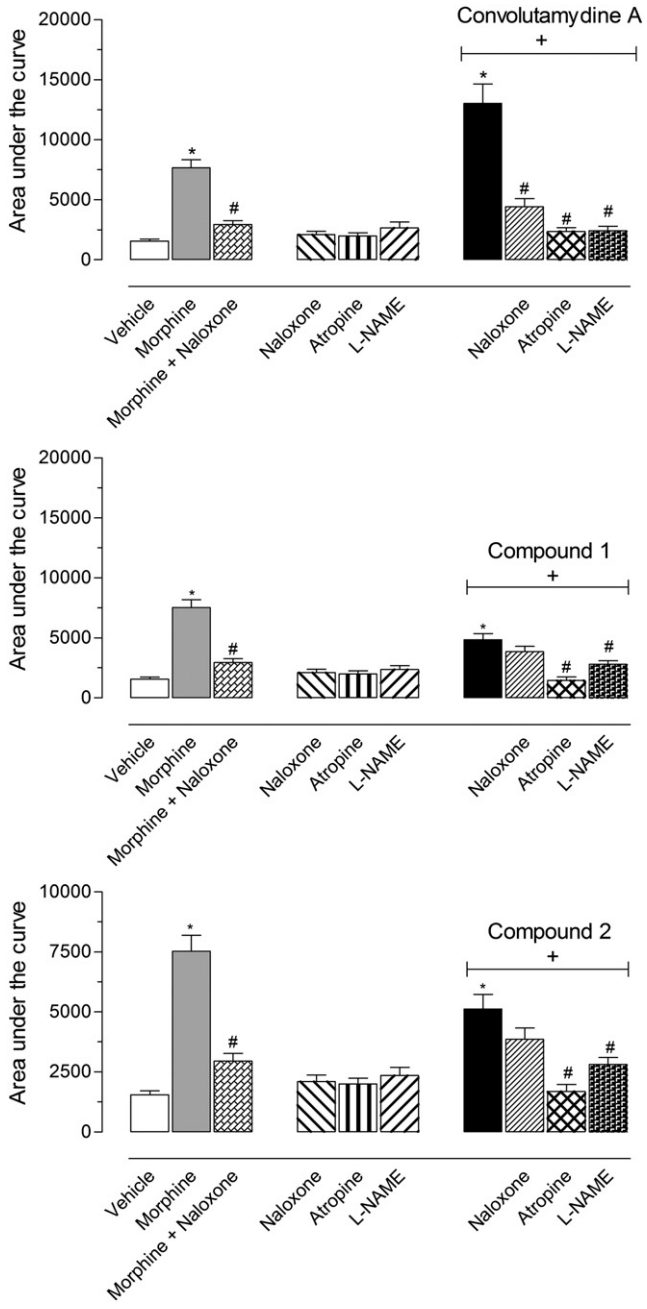


Fig. 6. Effects of different antagonists on the antinociceptive activity of convolutamydine A (4,6-dibromo-3-(2-oxopropyl)-3-hydroxy-2-oxindole), compound 1 (3-(2-oxopropyl)-3-hydroxy-2-oxindole), and 2 (5-bromo-3-(2- oxopropyl)-3-hydroxy-2-oxindole) in the hot plate model. Animals were pre-treated with naloxone (1 mg/kg, i.p.), atropine (1 mg/kg, i.p.) or L-NAME (3 mg/kg, i.p.) 30 min prior to oral administration of convolutamydine A, compound 1, or 2 (10 mg/kg). The results are presented as mean ± S.D. (n = 6–10) of the area under the curve calculated by Prism Software 5.0. Statistical significance was calculated by ANOVA followed by Bonferroni's test. * indicates $p<0.05$ when comparing compound-treated mice to the vehicle-treated group; # indicates $p<0.05$ when comparing antagonist pre-treated mice with the compound-treated group.

suitable for evaluation of centrally but not of peripherally acting analgesics (Tjølsen et al., 1991; Zimer et al., 1986).

The present study clearly demonstrates that all three substances induced antinociceptive effects in the hot plate model. Characteristic differences occurred in the time course and maximal effects of the antinociceptive action of convolutamydine A and it analogues. A rapid onset with an early maximum effect is characteristic of the time course of action of opioid agonists (*e.g.*, morphine), which mediate analgesia

Table 2
Effects of convolutamydine A, compound 1 and 2 on spontaneous activity and motor performance of mice.

Spontaneous activity	Hour after treatment			
	0.5	1	2	3.5
Vehicle	55 ± 6.8	62.4 ± 7.1	59.1 ± 6.7	53.9 ± 6.4
Convolutamydine A	60.8 ± 9.7	65.7 ± 9.9	52.4 ± 6.9	56.7 ± 8.1
Compound 1	51.9 ± 7.7	58.3 ± 8.6	55.4 ± 10.1	60.3 ± 7.2
Compound 2	66.1 ± 11.6	61.7 ± 9.3	54.6 ± 7.8	49.8 ± 8.1
Locomotor performance	0.5	1	2	3.5
Vehicle	12 ± 1.3	15.4 ± 3.3	14.7 ± 2.8	19.7 ± 2.6
Convolutamydine A	8.9 ± 3.1	12.7 ± 3.1	13.9 ± 4.2	15.3 ± 4.8
Compound 1	9.9 ± 1.8	14.7 ± 2.1	16.7 ± 2.9	17.6 ± 2.7
Compound 2	11.4 ± 3.1	16.7 ± 1.9	15.5 ± 2.4	16.8 ± 1.9

via opioid receptors under both normal and inflammatory conditions (Aceto et al., 1997). The administration of convolutamydine A, compound 1 or compound 2 produced a time course of action in the hot plate test that was similar to morphine. One possible explanation for the rapid onset of action might be the solubility of the substances, which allows them to rapidly reach the brain.

Pain sensation can be divided into four components: transduction, transmission, modulation and perception. Several studies have shown that systems of neurotransmitters such as oxidonitrergic, opioid, cholinergic, adrenergic, and others may act in different ways during the pain transmission process, interfering with one of its components and making them a very interesting and complex phenomenon (Xie et al., 2009). A natural or synthetic substance with analgesic properties can then interfere with one of these components to produce analgesia. Its mechanism of action may involve neurotransmitter systems such as those listed above. In this regard, we decided to evaluate the antinociceptive mechanisms of action of convolutamydine A, and compounds 1, and 2, pre-treating animals with some drugs that interfere with those systems. Our results showed that the L-arginine–nitric oxide pathway is involved in the antinociception caused by convolutamydine A but does not participate in the effects caused by compounds 1 and 2. These results are consistent with other studies that show participation of the L-arginine–nitric oxide–cGMP system in the antinociceptive effects produced by several drugs during peripheral inflammation (Brito et al., 2006; Lorenzetti and Ferreira, 1996; Pol, 2007) and in several models of nociception (Riedel and Neeck, 2001).

Among the neurotransmitter systems involved in pain, the opioid system is one of the most important. In the four components of pain, this system participates in both the perception and modulation of the process (Bruehl et al., 2009). Moreover an analgesic agent whose therapeutic effect is the same as that of morphine, but does not have the same side effects has not been found. To evaluate the participation of the opioid system, mice were pre-treated with naloxone, an opioid antagonist. Our data demonstrated that the activation of the opioid pathway is probably not involved in the antinociceptive effect of convolutamydine A, or compounds 1 and 2 because naloxone significantly reversed morphine, but not convolutamydine A, compound 1 and 2 antinociception.

Several pieces of evidence demonstrate that the cholinergic system has therapeutic potential for some clinical pain states. Moreover, acetylcholine mediates its analgesic effects through the nicotinic and muscarinic receptors (Jones and Dunlop, 2007). Cholinergic neurons in the spinal cord have been reported to produce an antinociceptive effect (Hood et al., 1995; Iwamoto and Marion, 1993; Schechtmann et al., 2008; Tanabe et al., 2005; Yaksh et al., 1985). For instance, the muscarinic M3 receptor is involved in formalin-induced nociception (Honda et al., 2000), and the muscarinic M1 receptor is involved in the antinociceptive effect caused by the intrathecal injection of clonidine in mice (Honda et al., 2002). Muscarinic acetylcholine receptors

(mAChRs) have been widely reported as pharmacologic targets for pain treatment. Most of them focused on central nervous system mAChR (Schechtmann et al., 2008). Recent evidence suggests that activation of mAChRs present on peripheral nociceptors can also suppress the transmission of pain impulses (Bernardini et al., 2001a, 2001b). Our results show that the antinociception of convolutamydine A and the compounds 1, and 2 was blocked by atropine in the hot plate model, making a possible connection between the antinociceptive effect of convolutamydine A, compound 1 and 2 and mAChR. As we know, nicotinic and muscarinic receptor agonists are associated with typical acetylcholine-like effects, such as reduced motor activity, hypothermia, tremor, incoordination, polysialia, and bradycardia (Barocelli et al., 2001; Decker et al., 1994). Consequently, we assessed the effect of convolutamydine A, compound 1 and 2 on motor coordination and spontaneous activity in animals. The results show that neither substance affected their behavior, nor did it change animals' gross behavior in acute toxicity tests. This indicates that the antinociceptive effect of convolutamydine A and its analogues (compounds 1 and 2) being due to any degree of motor impairment or sedation is improbable.

Our results demonstrated that convolutamydine A and its analogues (compounds 1 and 2) develop significant antinociceptive activity in all three models of antinociception. Some differences occurred between the models and the compounds. It seems that the presence of two bromide radicals (in positions 4 and 6 of convolutamydine A) confers a better antinociceptive effect. The absence of this radical (in compound 1) or the presence of one bromide in another position (in compound 2) seems to reduce the biological effect, but without a significant difference between both compounds. One hypothesis could be that the presence of two bromide radicals could confer to the convolutamydine structure more spatial stability, preventing its rotation and maybe the reduction on activity.

5. Conclusion

To the best of our knowledge it is the first work describing the antinociceptive effect of convolutamydine A and two new analogues. All substances were able to produce an oral antinociceptive effect in acute pain models in mice. Further investigations are necessary to clarify the exact mechanism of action of these substances. Nevertheless, the results of the present study suggest that these bioactive compounds could be of some interest as a prototype for new the synthesis of new analgesic drugs.

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