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Kavain analogues as potential analgesic agents

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Abstract:
Background: Kavalactones are pharmacologically active compounds present in preparations of the root trunk of *Piper methysticum* Forst, known as kava. This work describes the analgesic activity of some synthesized analogues of synthetic kavain, which is the main component of kava.
Methods: The essays were initially performed against the writhing test in mice, and the most promising compound was analyzed using other classical models of nociception, including formalin-, capsaicin-, glutamate-induced nociception, the hot plate test, and measurement of motor performance.
Results: The results indicated that compound 6-(4-fluorophenyl)-4-methoxy-5,6-dihydropyran-2-one (**2d**) exerts potent and dose-dependent analgesic activity, inhibiting abdominal constrictions caused by acetic acid in mice, and being more active than some reference drugs. It also presented activity in the other models of pain, with the exception of the hot plate test and the measurement of motor performance.
Conclusions: Although compound **2d** exerts antinociceptive activity, the mechanism of action remains uncertain, but it does not involve the opioid system and does not appear to be associated with non-specific effects such as changes in locomotor activity or motor coordination.

Key words:
kavain, lactones, enol ethers, δ-valerolactones, antinociceptive activity

Introduction

The active constituents for the therapeutic actions of *Piper methysticum* (kava) extract consist of a group of structurally related lipophilic derivatives with an aryl-ethylene-α-pyrone skeleton. They are typically 4-methoxy-2-pyrones with a phenyl or styryl substituent at the 6-position [2]. The commercial extracts consist of a mixture of more than 18 different α-pyrones, collectively known as kavapyrones, or kavalactones. The major constituents are (+)-kavain (1.8%), (+)-methy-

sticin (1.2%), desmethoxyangonin (1.0%), (+)-dihydrokavain (0.6%), (+)-dihydromesthysticin (0.5%), tetrahydroxyangonin. Minor constituents included other kavalactones, chalcones and essential oils [9]. Pharmacological studies have revealed that these substances are responsible for the analgesic [1], sedative [10, 22], anticonvulsive [16] and spasmolytic properties [23] of kava. Four lactones from kava (kavain, dihydrokavain, methysticin, and dihydromethysticin) have been found to possess significant analgesic effects in animal studies, probably by non-opiate pathways [18]. Kavain (**1**) appears to be the most effective in surface anesthesia, comparable to cocaine in its strength and duration of action [21]. It was selected as the prototype because of its yield in the plant extract, and because it presented analgesic activity in previous studies [1]. In this work, we have synthesized kavain (**1**) and nine analogues, according to a previous report of an aldolization-lactonization process [3], and evaluated these compounds in models of pain in mice.

Materials and Methods

Drugs

The following drugs and reagents were used: acetylsalicylic acid (ASA), metamizole (MET), capsaicin and glutamate (Sigma Chemical Co., St. Louis, USA); formalin, acetic acid (Vetec, Rio de Janeiro, Brazil) and morphine (Dimorf®), kindly provided by Cristália (Itapira, São Paulo, Brazil). The compounds studied, as well as the reference drugs, were dissolved in Tween 80 (Merck, AG, Darmstadt, Germany), with the exception of the capsaicin, which was dissolved in ethanol, plus 0.9% of NaCl solution. The final concentration of Tween and ethanol did not exceed 5% and did not cause any effect "*per se*".

Animals

The animals used were Swiss male mice (25–30 g), obtained from the University of Vale do Itajaí (Itajaí, Brazil). They were kept in a temperature-controlled environment (23 ± 2°C) with a 12 h light-dark cycle. Food and water were freely available. The allocation of animals into different groups was randomized, and the experiments were carried out under blind conditions. Since some suffering might result from the experiments, the International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues Guidelines [28] were followed, and the experiments were approved by the Animal Ethics Committee of UNIVALI (protocol no. 609/2007 UNIVALI), and all the experiments were conducted according to the principles of the Brazilian College of Animal Experimentation (COBEA).

Chemistry

The solvents were distilled prior to use, following the standard procedures, and reactions were performed under nitrogen or argon atmosphere. Silica gel 60 F_{254} plates were used to monitor the synthetic transformations, with visualization under UV light, 2% $KMnO_4$ or sulfuric anisaldehyde 2% solutions. Chromatographic purifications were carried out using 70–230 mesh silica gel. Melting points were determined on a System Kofler type WME apparatus, and were uncorrected. Infrared (FT-IR) spectra were recorded with a Perkin Elmer 1600 spectrometer. Nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) were recorded on a Bruker AC-200F spectrometer and performed at 300 MHz and 75 MHz, respectively. Chemical shifts (δ values) are given in parts per million downfield from tetramethylsilane as the internal standard. Mass spectral analyses were performed at the Centre Régional de Mesures Physiques de l'Ouest (CRMPO) in Rennes (France). All the compounds (Fig. 1) have already being described by some of the authors in reference [3].

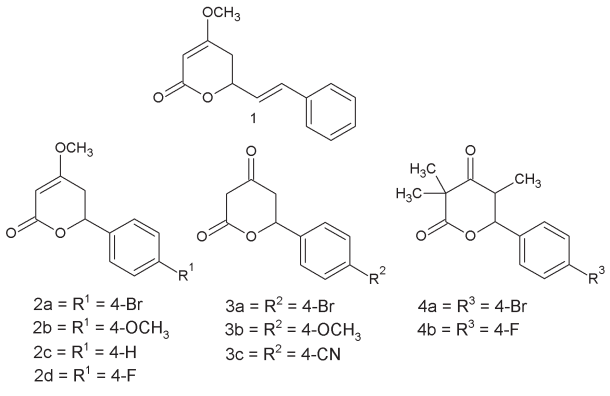

Fig. 1. Structure of kavain and the compounds evaluated

PHARMACOLOGY

Abdominal constriction response caused by injection of acetic acid

Abdominal constrictions induced by intraperitoneal (*ip*) injection of acetic acid (0.6%), consisting of a contraction of the abdominal muscle together with a stretching of the hind limbs, were counted according to the previously described procedures [10]. The animals were pretreated with 10 mg/kg of all analogues from kavain (**1**) or standard drugs *via* the intraperitoneal route, 30 min before acetic acid injection. The control animals received a similar volume of vehicle (Tween 80 and 0.9% NaCl). For compounds **1**, **2a**, **2b**, **2d** and **3c** the abdominal constriction response was analyzed, following pretreatment *via* the *ip* route in three doses (1 to 30 mg/kg) or the standard drugs, but in higher doses (10 to 60 mg/kg) for evaluation of ID_{50}. The enol ether **2d** (6-(4-fluorophenyl)-4-methoxy-5,6-dihydropyran-2-one), which was the most active of the compounds tested, was also analyzed orally at 100 mg/kg. After the challenge, pairs of mice were placed in separate boxes and the number of abdominal constrictions was cumulatively counted over a period of 20 min. Antinociception was expressed as the reduction in the number of abdominal constrictions between the control animals and the mice pretreated with compounds or standard drugs.

Formalin-induced pain

The procedure was similar to that described previously [17, 25]. The observation chamber was a glass cylinder of 20 cm in diameter, with a mirror at a 45° angle to allow clear observation of the animal's paws. For the formalin-induced pain 20 µl of 2.5% formalin (0.92% formaldehyde) was injected under the surface of the right hind paw. Two mice (control and treated) were observed simultaneously from 0 to 30 min following formalin injection. The amount of time spent on licking the injected paw was considered as indicative of pain. The initial nociceptive scores normally peaked 5 min after formalin injection (early phase) and 15–30 min after formalin injection (late phase), representing the tonic and inflammatory pain responses, respectively. Animals were treated with compound **2d** *ip* at 6, 10 and 30 mg/kg, 30 min before formalin injection, respectively. Following intraplantar injection of formalin, animals were immediately placed in a glass cylinder of 20 cm in diameter, and the time spent licking the injected paw (second phase of formalin test) was determined.

Capsaicin-induced pain

The procedure used was similar to that described previously [25]. Animals were placed individually in transparent glass cylinders. Following the adaptation period, 20 µl of capsaicin (1.6 µg/paw) was injected under the skin of the plantar surface of the right hind paw, using a microsyringe. The animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. The animals were *ip* treated with compound **2d** at 1, 3 and 10 mg/kg or saline 1 h before administration of capsaicin. The control animals received a similar volume of 0.9 % NaCl (10 ml/kg, *ip*).

Glutamate-induced nociception

The animals were treated with the compound **2d** *ip* (1, 30 and 60 mg/kg) 30 min before capsaicin injection, respectively. A volume of 20 µl of glutamate solution (30 µmol/ paw), made up in phosphate buffered saline (PBS), was injected intraplantarly under the surface of the right hind paw as described previously [4]. After injection with glutamate, the animals were individually placed in glass cylinders of 20 cm in diameter and observed from 0 to 15 min. The time spent licking and biting the injected paw was timed with a chronometer and considered as indicative of pain.

Hot-plate test

The hot-plate test was used to estimate the latency of responses, according to the method described by Eddy and Leimback [14] with minor modifications. The temperature of the hot-plate was maintained at 55 ± 1°C. The animals (n = 8) were placed on glass funnels on a heated surface, and the time between placing the animals on the hot-plate and the beginning of licking paws or jumping were recorded as latency of response in non-treated animals (saline 10 ml/kg, *ip*), with compound **2d** (10, 30 and 60 mg/kg, *ip*) or morphine (5.5 µmol/kg, *sc*). A test cut-off time of 30 s was chosen to avoid possible tissue damage resulting from the test.

Measurement of motor performance

To exclude possible nonspecific effects of compound **2d** on locomotor activity, mice were treated with **2d** (30 mg/kg, *ip*) and after 30 min, the locomotor activity was assessed in the open-field test, as described previously [7]. The number of squares crossed with all paws ("crossings") in a 6-min session was counted. The control mice received vehicle (10 ml/ kg). The interference of **2d** on motor coordination was also evaluated [13]. This test was performed using a horizontal rotarod device (Letica Scientific Instrument, Barcelona, Spain) set to rotate at a constant speed of 22 rpm. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 60 s. Animals were treated with compound **2d** (30 mg/kg, *ip*) or with the same volume of 0.9% NaCl solution (10 mg/kg, *ip*) 30 min before being tested. The results are expressed as the time (s) the animals remained on the rotarod. The cut-off time used was 60 s.

Statistical analysis

The results are presented as the mean ± SEM except for the mean ID_{50} values (i.e., the dose of drugs or compounds that reduced the algesic responses by 50% in comparison to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The statistical significance between the groups was analyzed by variance followed by Dunnett's multiple comparison tests. The p-values of less than 0.05 were considered as indicative of significance. ID_{50} values were determined by graphical interpolation from individual experiments.

Results and Discussion

Among several reports on the synthesis of different γ-lactones, the aldol reaction is frequently used to prepare these heterocycles, constituting a simple approach and a practical route for preparing this class of compounds. This paper is the continuation of a previous study about the synthesis of kavain (**1**) and several analogues [1, 2]. The nature of the substituent can affect the bioactivity of the compounds, and for this reason, we have introduced substituent groups that could possibly produce different electronic or steric effects of enol ethers (**2a**–**d**), δ-valerolactones (**3a**–**c**) and lactones (**4a**–**b**).

The *ip* treatment of animals demonstrated that all the compounds inhibited the contractions induced by acetic acid, and with the exception of **3b**, the inhibitions were greater or similar to those of the reference drugs (Tab. 1). The results show that compounds **1**, **2a**, **2b** and **3c** caused about 50% inhibition in abdominal constrictions, at a dose of 10 mg/kg. Compound **2d** significantly inhibited the abdominal constrictions, causing 80.5% inhibition, being the most effective compound in this series. This method has been widely used in the analysis of the analgesic activity of different kinds of compounds, including structurally related δ-valerolactones, which showed an interesting profile of antinociceptive action [1].

For the more effective compounds **2d**, **2a**, **2b**, **1**, **3c**, the ID_{50} values were evaluated in this model in order to estimate their potencies. They produced dose-related and significant inhibition of acetic acid-induced abdominal constrictions, with the following respective ID_{50} values: 18.1 (15.3–21.2), 44.2 (34.9–56.0), 57.7 (36.5–91.2), 68.2 (57.4–81.3) and 87.4 (72.1–106.5) μmol/kg (Tab. 2). The following order of potency was observed: **2d** > **2a** > **2b** > **1** = **3c**, being 8.1, 3.3, 2.6, 2.1 and 1.6 times more potent, respectively, than the drugs used as reference.

Tab. 1. Analgesic activity of kavain-like analogues and reference drugs against acetic acid-induced abdominal constrictions in mice at 10 mg/kg, given intraperitoneally

Compounds	Inhibition (%)
1	49.0 ± 2.8**
2a	49.8 ± 3.0**
2b	49.3 ± 2.5**
2c	36.8 ± 3.3*
2d	80.5 ± 2.9**
3a	37.6 ± 3.8*
3b	Inactive
3c	49.7 ± 1.6**
4a	46.3 ± 3.4**
4b	32.5 ± 2.8*
ASA	35.0 ± 2.0*
MET	33.0 ± 1.0**

Each group represents the mean ± SEM of 5–6 experiments; * $p < 0.05$ and ** $p < 0.01$, compared with respective control values

Tab. 2. Comparison of ID_{50} values of selected compounds more effective and acetylsalicylic acid (ASA) and metamizole (MET), used as reference drugs in model of abdominal constriction response caused by injection of acetic acid

Compounds	ID_{50} (mg/kg)	ID_{50} (μmol/kg)	MI ± SEM (%)
3c	18.8 (15.5 – 22.9)	87.4 (72.1 – 106.5)	65.4 ± 2.3**
1	15.71 (13.2 – 18.7)	68.2 (57.4 – 81.3)	77.2 ± 2.5**
2a	12.5 (9.9 – 15.8)	44.2 (34.9 – 56.0)	61.4 ± 1.9**
2b	13.5 (8.2 – 21.3)	57.7 (36.5 – 91.2)	72.0 ± 2.5**
2d	4.0 (3.4 – 4.7)	18.1 (15.3 – 21.2)	80.5 ± 2.9**
ASA	24.0 (13.1 – 43.7)	133.0 (73.0 – 243.0)	83.0 ± 1.4**
MET	54.0 (29.3 – 98.6)	162.0 (88.0 – 96.0)	54.0 ± 2.0**

Each group represents the mean ± SEM of 6–8 animals; ** $p < 0.01$, compared with respective control values, MI = maximal inhibition

The most effective compounds were those of the enol ether derivatives containing substituent on the phenyl ring, being more active than kavain (**1**). These results suggest the involvement of the *para*-substitution on the phenyl ring, as well as the importance of the spacer between the phenyl ring and the lactone-enol ether ring. Compound **3c**, with an oxo group instead of an enol ether group on the lactone ring, was the weakest of all, but more active than the reference drugs, justifying further studies of this series of compounds.

Among the enol ether derivatives, it can be observed that compound **2d**, which has a fluorine atom at position 4 of the phenyl ring, was the most effective and the most potent of all the series evaluated. In a previous study, we evaluated the antinociceptive activity of some δ-valerolactones [1], with similar structures to the compounds presented here, and the most active compound of the series was also the derivative containing fluorine atom at position 4 of phenyl ring.

It is known that the introduction of fluorine in the lactones is of great interest to the synthesis because the fluorination of natural products produces a significant improvement in its pharmacological activities [15]. Organic fluorine compounds have caused a profound impact on the development of bioactive substances for the modern pharmaceuticals market. It is estimated that up to 20% of pharmaceuticals prescribed or administered in the clinic contain a fluorine atom, and 30% of the leading 30 blockbuster drugs by sales contain a fluorine atom. The element generally finds its way into the organic framework during lead optimization studies, particularly as a strategy for blocking the metabolism, for example by hydroxylation enzymes, to increase lipophilicity (logP) or to raise the pharmacokinetic profile [24].

The results of the writhing test demonstrated a reaction in mice, described as a typical model of inflammatory pain, a model that has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new medicinal agents [8]. This method shows good sensitivity, as it allows for the effects of weak analgesics, but shows poor specificity, given that the abdominal writhing response can be suppressed by muscle relaxants and other drugs, leaving scope for misinterpretation of the results [18].

Moreover, kava extracts have been used to treat anxiety due to their psychoactive properties, causing alterations in cerebral functions involving various mechanisms such as GABA binding, inhibition of noradrenaline uptake, and binding to sodium ion channel receptor sites [5, 6].

Therefore, in order to evaluate the possible non-specific muscle relaxant or sedative effects of compound **2d**, mice were tested in the open-field, rotarod and hot plate tests. The results showed that compound **2d** does not affect the locomotor activity of the animals in the open-field test at 30 mg/kg. In contrast, mice treated with morphine had a significantly higher number of crossings (Fig. 2A); while under the same conditions, **2d** also did not alter motor coordination in the rotarod test (Fig. 2B). This result demonstrates that compound **2d** was not associated with non-specific effects such as changes in locomotor activity or motor coordination.

In the hot-plate test, even increasing the dose (from 10 at 60 mg/kg) was not effective in abolishing pain in a non-opioid manner, as shown by the lack of effects in the hot-plate test (Fig. 2C). A significant difference was observed in the mice treated with morphine, the positive-control group. Furthermore, the compound **2d** does not seem to interfere with the sensitivity and nociceptive threshold in healthy mice, a frequent characteristic of clinical analgesics such as morphine.

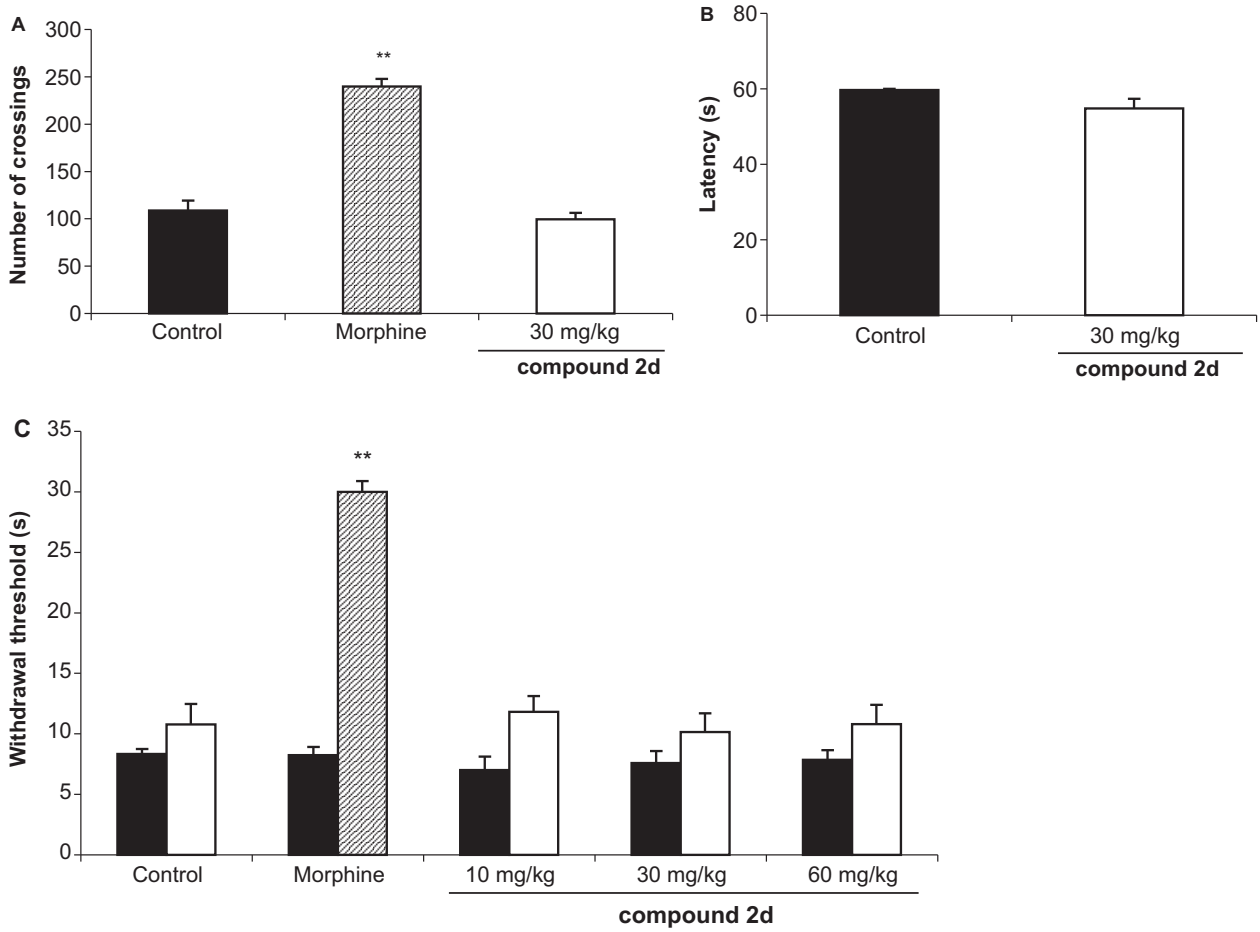

Fig. 2. Effects of systemic **2d** in the open-field (**A**), rotarod (**B**) and hot plate (**C**) tests in mice. Each column represents the mean ± SEM of six experimental values. ** $p < 0.01$, in comparison with the corresponding control values

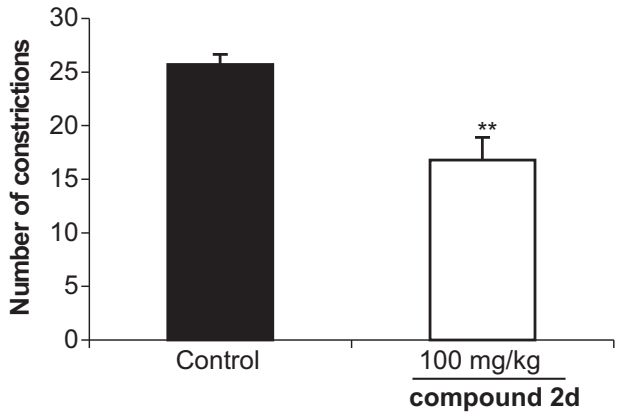

Fig. 3. Effect on acetic acid-induced pain test in mice, of compound **2d**, administrated orally. Each column represents the mean ± SEM of six experimental values; ** $p < 0.01$, in comparison with the corresponding control values

These results prompted us to select compound **2d** for further analyses in different pain models. Additionally, it was evaluated again in the model of writhing induced by acetic acid, but administered orally, demonstrating a moderate absorption by this route, with inhibition of 34.6% of writhing compared to the control group (Fig. 3).

The fluorinated lactone **2d** was also analyzed and compared with the standard drugs in the formalin-induced pain test. The results showed that compound **2d** caused significant and dose-related antinociception when administered by the *ip* route against the neurogenic (first phase), with maximum inhibitory activity of 74.5% at a dose of 30 mg/kg and with an ID_{50} value of 52.0 (49.3 to 54.9) μmol/kg. Compound **2d** was more potent than the reference drugs, since ASA was inactive in this first phase of pain induced by formalin, and MET showed an ID_{50} of 154.5 (99.9 to

Tab. 3. Comparison of the analgesic effect of compound **2d** with acetylsalicylic acid (ASA) and metamizole (MET)

	Formalin test		Capsaicin test	Glutamate test
	First phase ID_{50} (μmol/kg, *ip*)	Second phase ID_{50} (μmol/kg, *ip*)	ID_{50} (μmol/kg, *ip*)	ID_{50} (μmol/kg, *ip*)
2d	52.03 (49.28–54.91)[b] (MI[a] = 74.5%)	84.64 (78.60–91.17)[b] (MI[a] = 62.8 %)	23.0 (20.6–25.7)[b] (MI[a] = 77.0%)	160.8 (133.0–194.3)[b] (MI[a] = 85.3%)
ASA	Inactive	123.0 (77.0–209.0)[b] (MI[a] = 88.0%)	NT	NT
MET	154.5 (99.9–238.8)[b] (MI[a] = 74 ± 2%)	263.7 (234.3–296.9)[b] (MI[a] = 91 ± 1%)	207.6 (179.5–240.0)[b] (MI[a] = 70 ± 8%)	9.0 (6.8–11.9)[b] (MI[a] = 100%)

NT – not tested; [a] maximal inhibition; [b] 95% confidence limit. Each group represents the mean of 6–8 animals

238.8) μmol/kg. In the second phase of formalin-induced pain, compound **2d** caused 62.8% of inhibition at 30 mg/kg, with an ID_{50} value of 84.6 (78.6 to 91.2) μmol/kg, being also more potent than the reference drugs ASA and MET, which presented calculated ID_{50} values of 123.0 (77.0–209.0) μmol/kg and 263.7 (234.3–296.9) μmol/kg, respectively (Tab. 3).

It has been reported that nitric oxide inhibitors and both NMDA and non-NMDA receptor antagonists, among other drugs, are able to inhibit the acetic acid- and formalin-induced nociceptive response [12, 19]. Therefore, the activity observed could be due to both the inhibition of NOS and the blockade of NMDA receptors.

Furthermore, compound **2d** exhibited considerable antinociceptive activity in the capsaicin test, providing more direct evidence of the antinociceptive effect of this compound on neurogenic pain. The results in Table 3 show that compound **2d** produced significant and dose-dependent inhibition of capsaicin-induced neurogenic pain, with ID_{50} value of 23.0 (20.6–25.7) μmol/kg and maximum inhibition of 77.0%. It was about 9-fold more active than MET, for which the ID_{50} value was 207.6 (179.5–240.0) μmol/kg.

Although in this test the activity of **2d** was very similar to that of the first phase of the formalin test, it was twice as potent in inhibiting the neurogenic pain caused by capsaicin when compared to formalin. Also, it was about 9 times more active than MET. It has been proposed that the capsaicin-induced nociception is brought about by activation of the capsaicin receptor, also known as the vanilloid receptor (VR), termed VR subtype 1 (VR1), a ligand-gated non-selective cation channel in primary sensory neurons [9]. Studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids, nitric oxide and pro-inflammatory mediators in the periphery, and transmits nociceptive information to the spinal cord [20, 26]. Our results provide evidence that compound **2d** exerts a pronounced antinociception in chemical capsaicin-induced pain.

As can be observed in Table 3, compound **2d** administrated *ip* produced dose-dependent inhibition in the glutamate induced-nociception, with a mean ID_{50} value of 160.8 (133.0–194.3) μmol/kg and inhibition of 85.4%. MET presented in this test ID_{50} value of 9.0 (6.8–11.9) μmol/kg, with maximum inhibition of 100.0%. The nociceptive response induced by glutamate appears to involve peripheral, spinal and supraspinal sites of action, and is heavily mediated by both NMDA and non-NMDA receptors as well as by the release of nitric oxide or by some nitric oxide-related substance [27].

In summary, our results extend previous studies conducted by our group regarding the antinociceptive activity of δ-valerolactones, indicating that compound **2d** was more potent than some clinically used drugs. Compound **2d** exerts antinociceptive action in the chemical models of nociception (acetic acid, formalin, capsaicin and glutamate tests) in mice at a dose that does not interfere with motor performance. The precise mechanism of action should be further investigated. However, the opioid system seems unlikely to participate in the antinociception caused by this compound. The results suggest that compound **2d** represents a new promising candidate for future analgesic drugs.

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