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In vitro binding affinities of a series of flavonoids for μ-opioid receptors. Antinociceptive effect of the synthetic flavonoid 3,3-dibromoflavanone in mice

Josefina Higgs, Cristina Wasowski, Leonardo M. Loscalzo, Mariel Marder*

Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (C1113AAD), 1113 Buenos Aires, Argentina

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ABSTRACT

The pharmacotherapy for the treatment of pain is an active area of investigation. There are effective drugs to treat this problem, but there is also a need to find alternative treatments free of undesirable side effects. In the present work the capacity of a series of flavonoids to bind to the μ opioid receptor was evaluated. The most active compound, 3,3-dibromoflavanone (**31**), a synthetic flavonoid, presented a significant inhibition of the binding of the selective μ opioid ligand [^{3}H]DAMGO, with a *Ki* of 0.846 ± 0.263 μM. Flavanone **31** was further synthesized using a simple and cheap procedure with good yield. Its *in vivo* effects in mice, after acute treatments, were studied using antinociceptive and behavioral assays. It showed no sedative, anxiolytic, motor incoordination effects or inhibition of the gastrointestinal transit in mice at the doses tested. It evidenced antinociceptive activity on the acetic acid-induced nociception, hot plate and formalin tests (at 10 mg/kg and 30 mg/kg). The results showed that the 5-HT$_2$ receptor and the adrenoceptors seem unlikely to be involved in its antinociceptive effects. Naltrexone, a nonselective opioid receptors antagonist, totally blocked compound **31** antinociceptive effects on the hot plate test, but naltrindole (δ opioid antagonist) and nor-binaltorphimine (κ opioid antagonist) did not. These findings demonstrated that 3,3-dibromoflavanone (**31**), at doses that did not interfere with the motor performance, exerted clear dose dependent antinociception when assessed in the chemical and thermal models of nociception in mice and it seems that its action is related to the activation of the μ opioid receptor.

1. Introduction

Pain constitutes a major public health problem and it is the primary reason why people seek medical care and one of the most prevalent conditions that limits productivity and diminishes quality of life. Although adequate pain relief is achieved with the currently available analgesic like opioids or nonsteroidal anti-inflammatory drugs (NSAIDs), some of their serious side effects are major limitations to their routine use in therapy. It is well known that pharmaceutical companies around the world are interested in developing safer and more effective drugs to treat pain (Milano et al., 2008).

The endogenous opioid system is critical for many physiological and behavioral effects. Opioid receptor activation by endogenous and exogenous ligands results in a multitude of effects, which include analgesia, respiratory depression, euphoria, inhibition of gastrointestinal transit, effects on anxiety, etc. (Kieffer et al., 2009). There are three known 'classical' types of opioid receptors, originally defined according to their selectivity for certain kinds of opioids, μ (for morphine), κ (for ketocyclazocine), and δ (first identified in mouse vas deferens). Of them, the μ receptor is thought to be primarily responsible for the mediation of opioid antinociception and tolerance.

Flavonoids form a large family of natural products which are widely distributed in the plant kingdom. In the course of our survey of substances exerting pharmacological effects on the central nervous system (CNS) a range of available flavonoids were explored. Many flavone aglycones were described as ligands for the benzodiazepine binding site (BDZ-bs) of the gamma aminobutyric acid

* Corresponding author. Tel.: +54 11 4964 8289; fax: +54 11 4962 5457.
E-mail addresses: jhiggs@qb.ffyb.uba.ar (J. Higgs), criswasowski@qb.ffyb.uba.ar (C. Wasowski), leoloscalzo@hotmail.com (L.M. Loscalzo), mmarder@qb.ffyb.uba.ar (M. Marder).

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type A ($GABA_A$) receptors in the CNS, mediating anxiolytic effects *in vivo* (Jäger and Lasse, 2011; Wasowski and Marder, 2012). Several flavonoid glycosides, present in plants used as tranquillizers, were found to have CNS depressant effects in mice, but the behavioral effects induced by these compounds do not involved classical $GABA_A$ receptors (Fernández et al., 2006).

Although the mechanisms of action of a variety of biological effects of flavonoids, such as its antioxidant and anti-inflammatory properties have been studied extensively (Harbone and Williams, 2000; Havsteen, 2002; Rathee et al., 2009), little is known about the action of these compounds on the modulation of pain transmission. Some glycosylated flavonoids, such as myricitrin and baicalin, produced systemic antinociception when assessed in chemical models of nociception in mice, but the opioid system seemed unlikely to participate in them (Chou et al., 2003; Meotti et al., 2006). On the other hand, other flavonoid glycosides with analgesic activities were reported as involving an opiate like mechanism, since their activities were reversed by naloxone pre-treatments, an opioid receptor antagonist; such is the case of gossypin (Viswanathan et al., 1984) and the flavone glycoside linarin (Martinez-Vazquez et al., 1996). Furthermore, several hydroxylated derivatives of flavones possess antinociceptive properties that showed a mechanism mediated by opioid receptors (Umamaheswari et al., 2006; Vidyalakshmi et al., 2010).

Previous studies from our laboratory demonstrated that opioid receptors were involved in the sedative and antinociceptive effects of hesperidin, a widely consumed flavanone glycoside (Loscalzo et al., 2008, 2011). Binding assays showed that hesperidin did not bind to the μ opioid receptor, as it was not able to displace the specific binding of [^{3}H]DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin) from synaptosomal membranes of rat's forebrains. Otherwise the aglycone of hesperidin, hesperetin, inhibited the specific binding of this peptide but did not show any *in vivo* activity at the doses tested (Fernández et al., 2006; Guzmán-Gutiérrez and Navarrete, 2009; Loscalzo et al., 2008, 2011; Marder et al., 2003).

In this work the capacities of natural flavonoid glycosides and aglycones, synthetic flavonoids, previously obtained in our laboratory (Marder and Paladini, 2002), and related compounds to bind to μ opioid receptors present in rat's forebrain membranes were evaluated. As 3,3-dibromoflavanone (**31**) emerged as the most active compound of this series, an efficient syntheses of this novel compound and its pharmacological effects in acute treatment in mice were developed.

2. Materials and methods

2.1. Drugs and injection procedures

Morphine hydrochloride was purchased from Gramon, Argentina. Naltrexone hydrochloride, ketanserin tartrate salt, yohimbine hydrochloride, nor-binaltorphimine dihydrochloride, hesperidin (**1**), neohesperidin (**2**), naringin (**3**), rutin (**5**), hesperetin (**8**), naringenin (**9**), flavone (**10**), diosmetin (**11**), quercetin (**12**), apigenin (**13**), chrysin (**14**), 6-methylflavone (**15**), hesperidin methyl chalcone (**33**), cinnamic acid (**34**), caffeic acid (**35**), chromone (**36**), β-naphthoflavone (**39**), ipriflavone (**37**) and α-naphthoflavone (**38**) were obtained from Sigma–Aldrich Chemical Company, USA. [^{3}H]-DAMGO ([D-Ala^2, N-Me-Phe^4, Gly-ol^5] enkephalin) was purchased from PerkinElmer, USA; indomethacin from Montpellier, Argentina and naltrindole hydrochloride from Tocris Bioscience, France. Diosmin (**4**), gossypin (**6**), flavanone (**7**) and chalcone (**32**) were obtained from Extrasynthese, France. Synthetic flavonoids aglycones were previously obtained in our laboratory (Marder and Paladini, 2002). 3,3-Dibromoflavanone (**31**) (Fig. 1) was further synthesized as indicated bellow. Chemical purity of the synthetic flavonoids was above 95%, estimated by us based on analytical HPLC experiments.

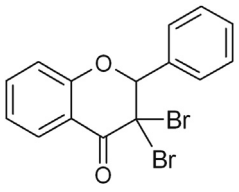


Fig. 1. Molecular structure of 3,3-dibromoflavanone (**31**).

Compound **31** was dissolved by the sequential addition of: dimethylsulfoxide up to a final concentration of 5%, a solution of 0.25% Tween 80 up to a final concentration of 20%, and saline to complete 100% volume. Morphine, naltrexone, ketanserin, naltrindole, nor-binaltorphimine and indomethacin were dissolved in saline solution.

The rodents were intraperitoneally (i.p.) injected 20 min or 30 min before performing the pharmacological tests, as indicated. In each session, a control group receiving only vehicle was tested in parallel with those animals receiving drug treatment. Vehicle control mice showed no significant differences in any of the tests assayed compared to mice treated with saline (data not shown).

The volume of i.p. injections was 0.15 mL/30 g of body weight.

2.2. Animals

Adult male Swiss mice weighing 25–30 g were used in the pharmacological assays and adult male rats (200–300 g) Wistar strain for biochemical studies, both were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires. For behavioral assays mice were housed in groups of five in a controlled environment (20–23 °C), with free access to food and water and maintained on a 12 h/12 h day/night cycle, light on at 06:00 AM. Housing, handling, and experimental procedures complied with the recommendations set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and the Institutional Committee for the Care and Use of Laboratory Animals, University of Buenos Aires, Argentina. All efforts were taken in order to minimize animal suffering. The number of animals used was the minimum number consistent with obtaining significant data. The animals were randomly assigned to any treatment groups and were used only once. The behavioral tests were evaluated by experimenters who were kept unaware of the treatment administered and were performed between 10:00 AM and 2:00 PM.

2.3. Chemistry

The synthesis of compound **31** was performed as follows: to a solution of flavanone (2.2 mmol) in CH_3Cl (10 mL), at 25 °C, an excess of bromine (7.8 mmol) was added dropwise. The mixture was stirred for 2 h at 50 °C. After cooling, the reaction mixture was washed with two 10 mL portions of a saturated aqueous solution of $Na_2S_2O_5$ and then with water, dried over Na_2SO_4 and concentrated to dryness in a rotary evaporator (De Diesbach and Kramer, 1945).

The reaction product was analyzed by TLC on silica gel on polyester sheets, with 254 nm fluorescent indicator (Sigma, USA) and by HPLC performed using C18 reversed phase Vydac columns (The Separation Group, Hesperia, CAL, USA) and elutions carried out with a lineal gradient of 30–80% ACN in water, in 30 min, at a flow rate of 1 mL/min.

The reaction product was then recrystalized twice from ethanol to water and used for identification and assay (yield 66%). EIMS were measured in a Shimadzu QP-1000 quadrupole mass spectrometer. NMR spectra were recorded in a Bruker AMX 400 spectrometer. Reagents and solvents were of analytical reagent grade and were purchased from Sigma–Aldrich and Fluka.

3,3-dibromoflavanone (**31**) (Fig. 1) white crystals from ethanol-water. ^{1}H NMR ($CDCl_3$, 300 MHz) δ_H 8.09 (dd, J = 1.4 Hz, 6.7 Hz, H-5), 7.76 (m, H-2′, H-6′), 7.61 (td, J = 1.8 Hz, 7.4 Hz, H-7), 7.49 (m, H-3′, H-4′, H-5′), 7.20 (t, J = 7.5 Hz, H-6), 7.11 (d, J = 8.5 Hz, H-8), 5.30 (s, H-2). ^{13}C NMR ($CDCl_3$, 300 Mhz) δ_C 86.3 (C-2), 69.4 (C-3), 180.7 (C-4), 115.6 (C-4a), 123.3 (C-5), 129.6 (C-6), 137.0 (C-7), 117.9 (C-8), 160.0 (C-8a), 133.5 (C-1′), 129.5 (C-2′/C-6′), 127.7 (C-3′/C-5′), 129.9 (C-4′). EIMS *m/z* 380/382/384 (rel. 1/2/1) M^+, 301/303 (rel. 1/1), 260/262/264 (rel. 1/2/1), 221, 120.

2.4. Biochemical assay ([^{3}H]-DAMGO binding assay)

A crude membrane fraction was prepared from male Wistar rat forebrains as previously described (Ioja et al., 2007) with small modifications. Rats were humanely killed by decapitation and the brains without cerebellum were rapidly removed and washed several times in ice-cold 50 mM Tris–HCl buffer pH 7.4. Afterward they were homogenized in 10 volumes/weight of 0.32 M sucrose at 0 °C and the homogenate was centrifuged at 900 × *g* for 10 min at 4 °C. The supernatant was decanted and centrifuged at 100,000 × *g* for 30 min at 4 °C, the resulting pellets were resuspended in 30 volumes/weight of 50 mM Tris–HCl buffer pH 7.4 and incubated at 37 °C for 30 min to remove any endogenous opioid peptides. The last centrifugation step was repeated under the same conditions as described above and the final pellets were resuspended in 10 volumes/weight of the same buffer and stored at −80 °C until use. Protein concentration was determined by the method of Bradford using bovine serum albumin as standard (Bradford, 1976).

For the binding assay, membranes were thawed and suspended in 50 mM Tris–HCl pH 7.4 to a final protein concentration of 0.25–0.35 mg/mL. The incubation was carried out at 25 °C for 1 h in a final volume of 1 mL of membrane suspension (in duplicate) in the presence of the sample assayed and with 1 nM of [^{3}H]DAMGO (56.8 Ci/mmol). Non specific binding was determined in parallel incubations in the presence of 10 μM naltrexone. In the screening assays each compound was tested at 300 μM in triplicate. In the competition assays, the incubations were done with a range of concentrations of compounds **8**, **9**, **10**, **12**, **15**, **31** and **32**. Naltrexone was used as positive control in concentrations between 0.01 and 30 nM. The assays were terminated by filtration under vacuum through Whatman GF/A glassfiber filters and three washes with 3 mL each of incubation medium. Filters were counted after addition of Optiphase "Hisafe" 3 (Wallac, Turku, Finland) liquid scintillation cocktail.

2.5. Pharmacological studies

2.5.1. Antinociceptive assays

2.5.1.1. Writhing test. The writhing test is a visceral pain model widely used for the evaluation of peripheral antinociceptive activity of new agents. The test was carried out according to the method described earlier (Koster et al., 1959); 0.75% acetic acid aqueous solution was injected i.p. (0.2 mL/30 g body weight) and the animals were placed in a glass observation chamber (15 cm × 15 cm × 30 cm). The number of writhing responses (abdominal cramps) was counted for 15 min after 5 min of the injection of the acetic acid solution.

The writhing test was performed 30 min after the i.p. injections of 3 mg/kg, 10 mg/kg and 30 mg/kg of compound **31**, morphine (6 mg/kg) or vehicle.

2.5.1.2. Hot plate test. The hot plate assay is one of the most commonly used tests for determining the antinociceptive efficacy of experimental drugs in rodents (Carter, 1991). It is suitable for the study of central drugs by assessing the acute pain sensitivity to a thermal stimulus. Each mouse was placed into a transparent beaker made of Plexiglass with a height of 18 cm and a diameter of 10 cm to avoid the animals escaping from the plate which temperature was set at 55 °C ± 0.1 °C by using a thermoregulated water-circulating pump (Schreiber et al., 1999).

The time (in seconds) between the placement of the animal and the first response: hind paw licking/fanning or jumping was measured as latency. A 45 s cut-off was used to prevent tissue damage.

This test was performed 30 min after the i.p. injection of 3 mg/kg, 10 mg/kg and 30 mg/kg of compound **31**, morphine (6 mg/kg) or vehicle. A time course of the effect of compound **31** at 30 mg/kg was also determined.

2.5.1.3. Formalin test. The formalin test measures the response to a long-lasting nociceptive stimulus and thus may have a closer resemblance to clinical pain (Abbott et al., 1982; Murray et al., 1988) due to its tonic nature. It is not only used as a tonic pain model but also as an inflammatory pain model.

Animals were first habituated for 10 min in an observation chamber made of transparent acrylic. 20 μl of 1% formalin solution, made up in phosphate buffered solution (pH 7.4), were injected into the dorsal surface of the right hind paw of the mice with a microsyringe and a 30-gauge needle, 30 min after the i.p. administration of compound **31** (10 mg/kg and 30 mg/kg), morphine (6 mg/kg), indomethacin (10 mg/kg) or vehicle. Immediately after the formalin injection animals were placed individually in the chamber; beneath the floor, a mirror was mounted at a 45° angle to allow clear observation of the animal's paws. The amount of time the animal spent licking the injected paw was considered as indicative of pain. The nociceptive response was recorded in two phases: first or early phase (5 min after formalin injection) and second or late phase (15–50 min after formalin injection).

The percentages of the reduction of the licking time were calculated as:

$$\%\ \text{reduction of licking time} : 100\% - (A \times 100\%/B)$$

A: licking time of mice injected with compound **31**

B: licking time of control mice

2.5.2. Behavioral studies

2.5.2.1. Hole board assay. This assay was conducted in a walled black Plexiglass arena with a floor of 60 cm × 60 cm and 30 cm high walls, with four centered and equally spaced holes in the floor, 2 cm in diameter each as previously described (Fernández et al., 2006) and illuminated by an indirect and dimly light. Each animal was placed in the center of the hole board and allowed to freely explore the apparatus for 5 min and the number of head dips, the time spent head dipping and the number of rearings were measured. The test was performed 20 min after the i.p. injection of 1 mg/kg, 10 mg/kg and 30 mg/kg of compound **31** or vehicle.

2.5.2.2. Locomotor activity. The spontaneous locomotor activity was automatically measured as previously described (Fernández et al., 2006) and was expressed as total light beam counts per 5 min. The test was performed 20 min after the i.p. injection of 1 mg/kg, 10 mg/kg and 30 mg/kg of compound **31** or vehicle.

2.5.2.3. Elevated plus maze test. This test has been described as a simple method for assessing anxiety responses in rodents. The elevated plus maze set-up consisted of a maze of two open arms, 25 cm × 5 cm, crossed by two closed arms of the same dimensions, with free access to all arms from the crossing point. The closed arms had walls 15 cm high all around. The maze was suspended 50 cm from the room floor. Mice were placed on the central part of the cross facing an open arm. The number of entries and the time spent going into open arms were counted during 5 min under red dim light. The total exploratory activity (number of entries in both arms) was also determined (Lister, 1987).

The test was performed 20 min after the i.p. injection of 3 mg/kg, 10 mg/kg and 30 mg/kg of compound **31** or vehicle.

2.5.2.4. Inverted screen test. The inverted screen test was used to assess the motor toxicity of the drug (Coughenour et al., 1977). Mice were placed on a 13 cm × 13 cm wire mesh screen elevated 40 cm above the ground. After slowly inverting the screen through an angle of 180° the mice were tested for their ability to climb to the top. Mice were i.p. injected with vehicle or compound **31** (30 mg/kg) 30 min before the assay. Mice not climbing to the top (all four paws on upper surface) were counted as failures.

2.5.3. Blockade experiments

The involvement of various brain receptors on 3,3-dibromoflavanone (**31**) antinociceptive effects in mice were investigated by measuring its blockade with different specific antagonists in the hot plate test.

Several doses of each antagonist were tested to find the maximal dose devoid of intrinsic action to be used in the blockade experiments and were based on our previous studies (Loscalzo et al., 2008) and other literature data (Kaur et al., 2005; Jürgensen et al., 2005).

To assess the participation of the opioid system, mice were pretreated with naltrexone (5 mg/kg, i.p., a nonselective opioid receptor antagonist), naltrindole (5 mg/kg, i.p., a potent and highly selective δ opioid receptor antagonist) or nor-binaltorphimine (10 mg/kg, subcutaneously, s.c., a potent and highly selective κ opioid receptor antagonist), and after 20 min or 24 hr (for nor-binaltorphimine), the animals received an injection of compound **31** (30 mg/kg, i.p.) or vehicle. The hot plate assay was performed 30 min later.

To examine the possible participation of the serotonin receptors and the adrenoceptors on the antinociceptive effect of compound **31**, mice were pretreated with ketanserin (0.5 mg/kg, i.p., a selective antagonist for the 5-HT$_{2A/C}$ receptor), prazosin (0.2 mg/kg, i.p., an α_1-adrenoceptor antagonist) or yohimbine (2 mg/kg, i.p., an α_2-adrenoceptor antagonist). After 20 min, the animals received an injection of compound **31** (30 mg/kg) or vehicle and mice were evaluated on the hot plate assay 30 min later.

2.5.4. Gastrointestinal transit

Gastrointestinal transit was assessed using the charcoal meal test (Manara et al., 1986; Schulz et al., 1979). Mice were fasted, with water available ad libitum, for 18 h before the experiments. Thirty min after the i.p. injection of compound **31** (30 mg/kg), morphine (6 mg/kg), or vehicle a suspension of a charcoal meal (10% vegetable charcoal in 5% arabic gum) was orally administered at a volume of 0.25 mL/mouse. Thirty minutes after administration of the charcoal meal animals were sacrificed by cervical dislocation and their stomach and small intestine were carefully removed. Both the length of the small intestine from pyloric sphincter to the ileocoecal junction and the farthest distance to which the charcoal meal had traveled were measured. For each animal, the gastrointestinal transit was calculated as the percentage of distance traveled by the charcoal meal relative to the total length of the small intestine. The inhibition of gastrointestinal transit (%) was calculated as: Inhibition of gastrointestinal transit (%) = [(vehicle gastrointestinal transit-drug gastrointestinal transit)/(vehicle gastrointestinal transit)] × 100.

2.6. Statistical analyses

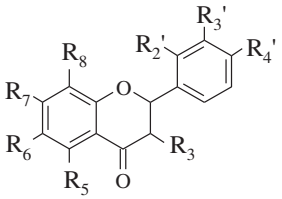
The effects of compounds in mice were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparisons between treatments and vehicle were made using Dunnett multiple comparison test. The blockade experiments were analyzed by two-way ANOVA (pre-treatment vs. treatment) and post-hoc comparison was made using Bonferroni post test. When a significant interaction between pre-treatment and treatment was observed, subsequent one-way ANOVAs and Newman–Keuls Multiple Comparison post-hoc test was applied.

Data from the inverted screen tests were analyzed with Chi-square test and unpaired *t*-test. The time course of the effect of compound **31** in the hot plate test was analyzed using unpaired *t*-test.

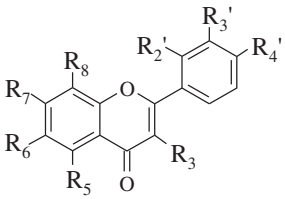
For the competition binding, data were analyzed by nonlinear regression fit to one site of specific bound vs radioligand concentration. *K*i values were calculated using the Cheng-Prusoff/Chou equation: $Ki = IC_{50}/[1 + (L/Kd)]$, where *K*i refers to the inhibition constant of the unlabeled ligand, IC_{50} is the concentration of unlabeled ligand required to reach half-maximal binding, *K*d refers to the equilibrium dissociation constant of the radioactive ligand and *L* refers to the concentration of radioactive ligand.

A P value < 0.05 was considered statistically significant. All data were expressed as mean ± S.E.M. and analyzed with GraphPad Prism 5.00 software.

Table 1
Molecular structures and activities on the μ-opioid receptor binding assay of the investigated compounds. .



Flavanone (A)



Flavone (B)

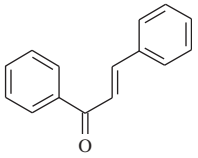
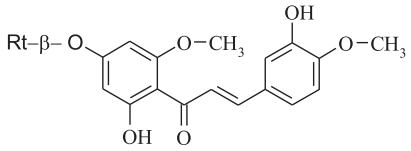
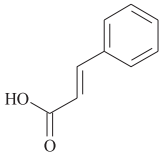
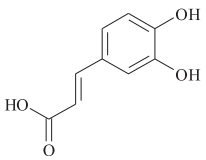
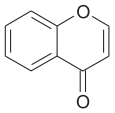
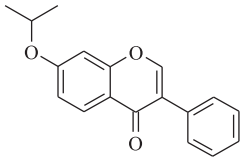
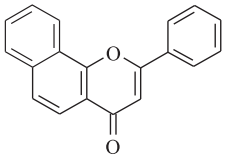
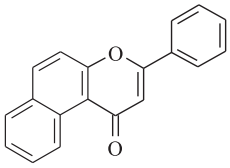
	Compound	Moiety	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	Binding inhibition[a]
A) Flavonoid glycosides											
1	Hesperidin	A	H	OH	H	*O*-β-Rt	H	H	OH	OCH_3	–[b]
2	Neohesperidin	A	H	OH	H	*O*-β-Nh	H	H	OH	OCH_3	–
3	Naringin	A	H	OH	H	*O*-β-Nh	H	H	H	OH	–
4	Diosmin	B	H	OH	H	*O*-β-Rt	H	H	OH	OCH_3	+
5	Rutin	B	*O*-β-Rt	OH	H	OH	H	H	OH	OH	+
6	Gossypin	B	OH	OH	H	OH	O-β-Glc	H	OH	OH	+
B) Flavonoid aglycones											
7	Flavanone	A	H	H	H	H	H	H	H	H	++
8	Hesperetin	A	H	OH	H	OH	H	H	OH	OCH_3	+++[b]
9	Naringenin	A	H	OH	H	OH	H	H	H	OH	+++
10	Flavone	B	H	H	H	H	H	H	H	H	+++
11	Diosmetin	B	H	OH	H	OH	H	H	OH	OCH_3	++
12	Quercetin	B	OH	OH	H	OH	H	H	OH	OH	+++
13	Apigenin	B	H	OH	H	OH	H	H	H	OH	+++
14	Chrysin	B	H	OH	H	OH	H	H	H	H	++
15	6-Methylflavone	B	H	H	CH_3	H	H	H	H	H	+++
C) Synthetic flavonoids											
16	6-Nitroflavone	B	H	H	NO_2	H	H	H	H	H	–
17	6-Fluorflavone	B	H	H	F	H	H	H	H	H	+
18	6-Chloroflavone	B	H	H	Cl	H	H	H	H	H	–
19	6,3′-Dimethylflavone	B	H	H	CH_3	H	H	H	CH_3	H	+
20	6-Methyl-3′-bromoflavone	B	H	H	CH_3	H	H	H	Br	H	–
21	2′-Nitroflavone	B	H	H	H	H	H	NO_2	H	H	+
22	3′-Nitroflavone	B	H	H	H	H	H	H	NO_2	H	–
23	3′-Chloroflavone	B	H	H	H	H	H	H	Cl	H	+
24	3′-Bromoflavone	B	H	H	H	H	H	H	Br	H	+
25	3′-Methylflavone	B	H	H	H	H	H	H	CH_3	H	++
26	4′-Nitroflavone	B	H	H	H	H	H	H	H	NO_2	–
27	4′-Fluoroflavone	B	H	H	H	H	H	H	H	F	–
28	4′-Bromoflavone	B	H	H	H	H	H	H	H	Br	–
29	3-Bromo-3′-nitroflavone	B	Br	H	H	H	H	H	NO_2	H	–
30	3,6-Dibromoflavone	B	Br	H	Br	H	H	H	H	H	–
31	3,3-Dibromoflavanone	A	Br_2	H	H	H	H	H	H	H	++++
D) Related compounds											
32	Chalcone										+++
33	Hesperidin methyl chalcone										–
34	Cinnamic acid										–
35	Caffeic acid										–

Table 1 (*continued*)

	Compound	Moiety	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	Binding inhibition[a]
36	Chromone										–
37	Ipriflavone										–
38	α-Naphthoflavone										–
39	β-Naphthoflavone										–

[a] Capacity of the compounds, at 300 μM, to inhibit the binding of [^{3}H]-DAMGO to the μ-opioid receptor indicated as: inhibition > 90% (++++); inhibition 70–90% (+++); inhibition 40–70% (++); inhibition 20–40% (+) and inhibition <20% (−). Sugar moieties: glucose (Glc), neohesperidose (*O*-α-L Rhamnosyl-(1 → 2)-glucose, Nh), rutinose (*O*-α-L Rhamnosyl-(1 → 6)-glucose, Rt).
[b] Loscalzo et al., 2011.

3. Results

3.1. Effects of flavonoids on [^{3}H]-DAMGO binding

The capacity of the compounds, tested at 300 μM, to inhibit the binding of [^{3}H]-DAMGO to the μ-opioid receptor is shown in Table 1.

The results pointed out that compounds **7**–**15**, **25** and **32** exhibited low to medium activity, meanwhile compound **31** showed the highest activity in the binding assay. Competition binding assays and *K*i values (mean ± S.E.M.) of these compounds are shown in Fig. 2 and Table 2, respectively. In all cases, data obtained were best fitted to one site binding hyperbola.

3.2. Antinociceptive effects of flavanone ***31*** in the writhing, hot plate and formalin tests

The effect of compound **31** in the writhing and the hot plate tests are shown in Figs. 3 and 4, respectively. ANOVA indicated a significant effect on the number of writhes [$F(3,33) = 4.739$, $P = 0.0080$, Fig. 3] and on the latency time in the hot plate test [$F(3,47) = 5.174$, $P = 0.0038$, Fig. 4A]. Comparisons between the vehicle control group and experimental groups (Dunnett's procedure) indicated that compound **31** decreased the number of writhes at 10 mg/kg and 30 mg/kg ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 3) and increased the latency time in the hot plate test at 10 mg/kg and 30 mg/kg ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 4A). Alternatively, mice i.p. injected with morphine (6 mg/kg), a classical μ opioid receptor ligand used as a reference compound, showed no abdominal cramps in the writhing test and displayed a high latency time in the hot plate assay (latency time = 42.6 s ± 4.2 s). The time course of the effect of compound **31** at 30 mg/kg in the hot plate test is shown in Fig. 4B. A significant antinociceptive effect could be achieved at 5 min (unpaired *t*-test, $P = 0.0392$), 20 min ($P = 0.0002$), 30 min ($P = 0.0004$) and until 60 min ($P = 0.0004$), without reaching control values until 120 min ($P = 0.0743$).

On the other hand, in the first phase of the formalin test, compound **31** at 3 mg/kg, 10 mg/kg and 30 mg/kg inhibited formalin induced pain by 29.8%, 60.5% and 45.2% respectively [$F(4,39) = 4.774$, $P = 0.0035$, Fig. 5A]. Mice receiving compound **31** at 10 mg/kg and 30 mg/kg had significantly minor licking time than control mice ($P < 0.01$, $P < 0.05$, respectively, Dunnett's procedure) (Fig. 5A). Indomethacin, the positive control drug, showed no effect in this phase, as expected.

In the second phase the i.p. administration of 3 mg/kg, 10 mg/kg and 30 mg/kg of compound **31** produced a reduction of the licking time of 34.1%, 70.4% and 78.4%, respectively [$F(4,39) = 5.386$, $P = 0.0017$, Fig. 5B]. The comparison between the vehicle control group and experimental groups by the Dunnett's test indicated that flavanone **31** at 10 mg/kg and 30 mg/kg ($P < 0.01$) and indomethacin at 10 mg/kg ($P < 0.05$), significantly reduced the licking time of mice (Fig. 5B).

Mice i.p. injected with morphine (6 mg/kg) did not lick appreciably their paws in any phase of this assay.

3.3. Behavioral effects of compound ***31*** in the hole board and the locomotor activity tests

The effects of compound **31** in the hole board and locomotor activity tests are shown in Fig. 6. For compound **31** i.p. injected at 1 mg/kg, 10 mg/kg and 30 mg/kg, ANOVA indicated that there is no significant effect on the number of mouse rearings, the number of head dips, the time spent head dipping and the locomotor activity counts; so it failed to show any effect in these assays, at the doses tested.

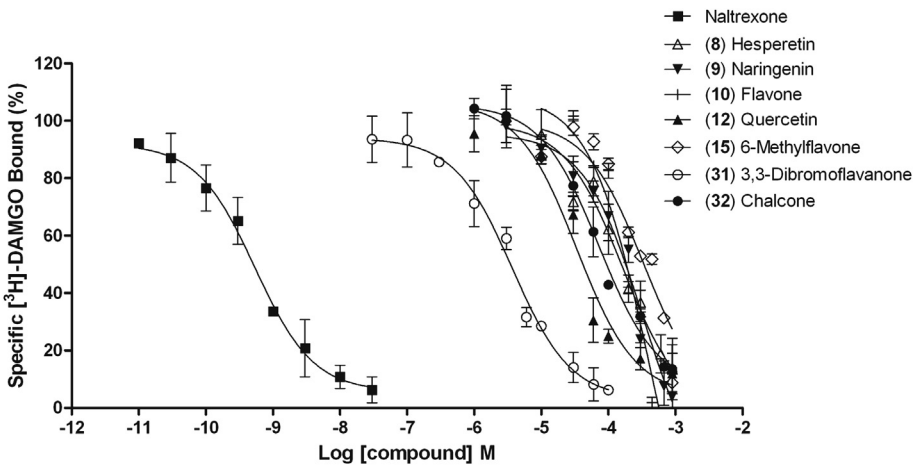


Fig. 2. Competition curves of naltrexone (■) and compounds **8** (△), **9** (▼), **10** (+), **12** (▲), **15** (◇), **31** (○) and **32** (●) for [^{3}H]-DAMGO binding to Wistar rat forebrain membranes. Each point represents the mean ± S.E.M., expressed as a percentage of [^{3}H]-DAMGO bound, of two to four independent experiments performed in duplicate.

3.4. Effect of compound *31* in the inverted screen test

Compound **31** at 30 mg/kg, the higher dose used in all the pharmacological tests performed, did not alter the motor coordination performance of mice in the inverted screen assay, as the number of mice that climb to the top of the screen (Chi-Square = 0.00001; $P = 1$) (Fig. 7A) and the time mice spent climbing (unpaired t-test, $P = 0.4429$) (Fig. 7B) did not have significant differences with the control group.

3.5. Effects of flavanone *31* in the plus maze test

ANOVA of the results obtained for compound **31** (3 mg/kg, 10 mg/kg and 30 mg/kg) did not yield statistically significant differences in the percentage of open arm entries, time spent in open arms and in the number of total arm entries in the plus maze test (data not shown).

3.6. Analysis of the possible mechanism of the antinociceptive action of compound *31*

As shown in Table 3, pre-treatment of the animals with ketanserin (0.5 mg/kg, i.p., a selective antagonist for the 5-HT$_{2A/C}$ receptor), prazosin (0.2 mg/kg, i.p., an α_1-adrenoceptor antagonist) and yohimbine (2 mg/kg, i.p., an α_2-adrenoceptor antagonist) given 20 min before the injection of compound **31** (30 mg/kg, i.p.) failed in to revert the antinociceptive effect of this compound in the hot plate test. The treatment of these antagonists did not evoke any response by themselves in this test.

The pre-treatment with naltrexone (a nonselective opioid receptor antagonist, 5 mg/kg, i.p.) given 20 min before the injection of compound **31** (30 mg/kg, i.p.) caused an inhibition of the analgesic effect induced by this flavanone in the hot plate test. In turn, naltrindole (a potent and highly selective δ opioid receptor antagonist, 5 mg/kg, i.p.), given 20 min before the administration of compound **31** (30 mg/kg, i.p.) and nor-binaltorphimine (a potent and highly selective κ opioid receptor antagonist, 10 mg/kg, s.c.), given 24 h before the administration of the flavonoid, did not antagonize the antinociceptive action in the hot plate test.

3.7. Effect of a single administration of morphine or compound *31* on gastrointestinal transit

Compound **31,** at 30 mg/kg, did not modify the gastrointestinal transit of mice compared with vehicle treated mice (mean ± S.E.M.: 62.5% ± 4.5% and 62.1% ± 3.6%, respectively). On the other hand,

Table 2
Binding affinity of selected natural and synthetic flavonoids for the μ-opioid receptors present in rat forebrain membranes.

	Compound	*Ki* (μM)[a]
8	Hesperetin	39.04 ± 1.32[b]
9	Naringenin	98.42 ± 15.59
10	Flavone	120.20 ± 16.66
12	Quercetin	11.32 ± 2.26
15	6-Methylflavone	106.50 ± 13.85
31	3,3-Dibromoflavanone	0.846 ± 0.263
32	Chalcone	28.21 ± 9.97

[a] *Ki* ± standard error of the mean values are means of 2–4 independent determinations and estimate the inhibition of [^{3}H]-DAMGO binding to rat forebrain membranes as described under Materials and methods. Naltrexone, used as a control, gave a *Ki* value of 0.21 ± 0.01 nM.
[b] Loscalzo et al., 2011.

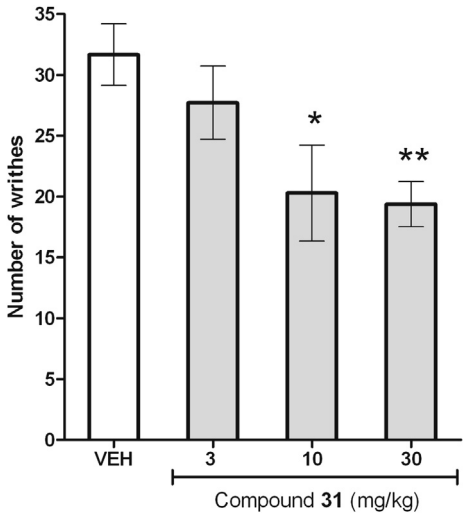


Fig. 3. Effect of the i.p. injection of compound **31** on the acetic acid induced writhing in mice. Results are expressed as mean ± S.E.M of the number of writhes. The writhing test was performed 30 min after the i.p. injection of vehicle (VEH) or compound **31**. $^*P < 0.05$, $^{**}P < 0.01$ significantly different from VEH; Dunnett's multiple comparison test after one-way ANOVA. Number of animals per group ranged between 7 and 12.

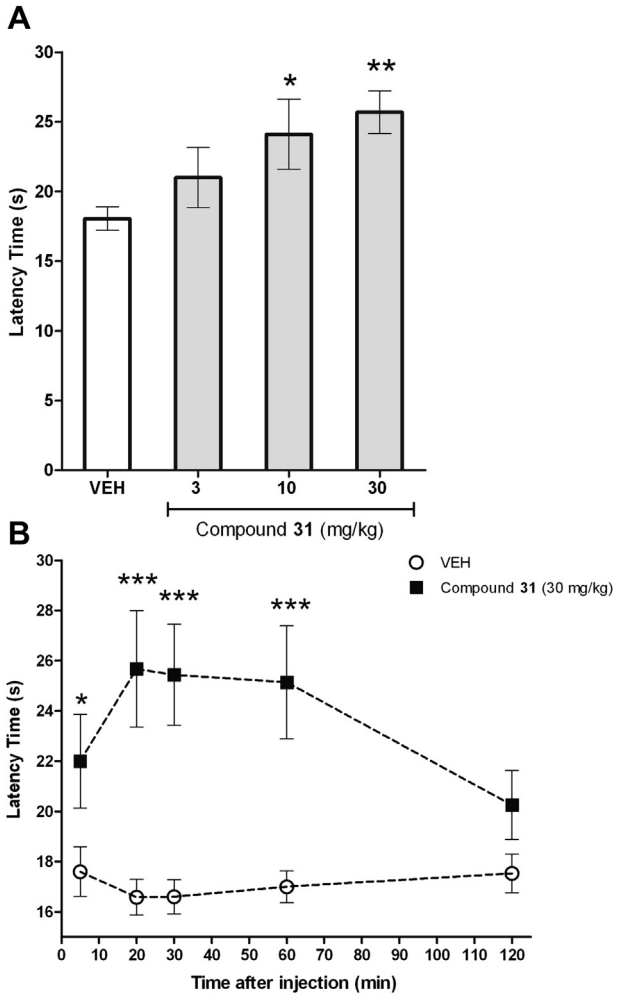


Fig. 4. Effect of flavanone **31** on the hot plate test in mice. **A**) Mean ± S.E.M. of the reaction time of mice in the hot plate test, 30 min after an i.p. injection of vehicle (VEH) or increasing doses of compound **31**. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from VEH; Dunnett multiple comparison test after ANOVA (n = 8–17 mice/group). **B**) Mean ± S.E.M. of the time course of the effect of compound **31** at the dose of 30 mg/kg. $^{*}P < 0.05$, $^{***}P < 0.001$ significantly different from the corresponding VEH; unpaired t-test (n = 6–15 mice/group).

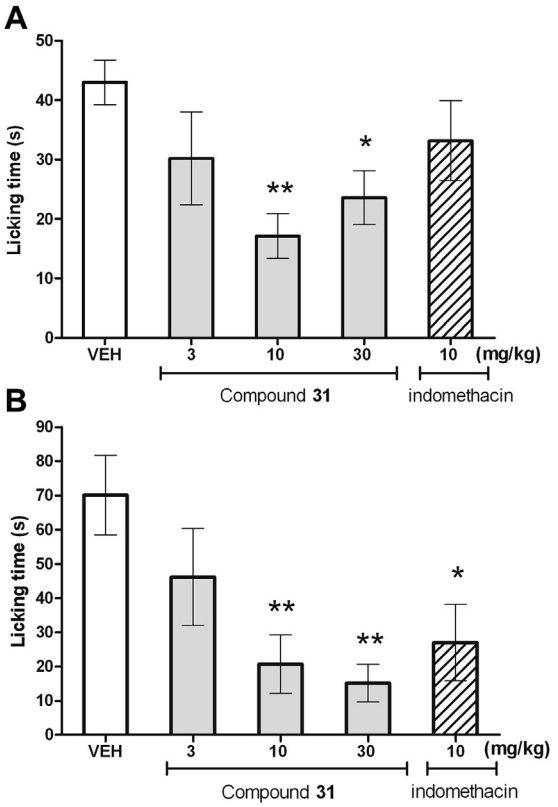


Fig. 5. Antinociceptive activity of compound **31** on paw licking during (A) first and (B) second phase of the formalin test. Vehicle (VEH) and flavanone **31** were i.p. administered 30 min prior the injection of formalin in the dorsal surface of the right hind paw. The licking time was measured during a period of 5 min (A) or 15–50 min (B) after the formalin injection. Indomethacin (10 mg/kg, i.p.) was used as reference drug. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from VEH; Dunnett multiple comparison test after ANOVA (n = 6–13 mice/group).

morphine 6 mg/kg decreased gastrointestinal transit (mean ± S.E.M.: 24.6% ± 3.2%; $^{***}P < 0.0001$, significantly different from vehicle; Dunnett multiple comparison test after ANOVA [$F(2,17) = 32.25$, $P < 0.0001$], n = 6 mice/group), which means an inhibition of 60.4%.

4. Discussion

In the present work the capacity of a series of flavonoids to bind to the μ opioid receptor was evaluated. We found that flavonoid glycosides seem unlikely to bind to μ opioid receptors. On the contrary, several natural aglycones (hesperetin, naringenin, flavone, quercetin, 6-methylflavone, and chalcone) were able to displace the binding of the specific μ opioid agonist [^{3}H]DAMGO with *K*i values ranging from 10 μM to 100 μM. These results point out that the presence of a sugar moiety in the flavonoid glycosides appears to interfere for the interaction with the receptor. The most active compound 3,3-dibromoflavanone (**31**), a synthetic flavonoid from our library of compounds, presented the highest inhibition of the binding of the selective tritiated ligand [^{3}H]DAMGO to μ opioid receptors present in rat forebrain membranes, with a *K*i value of 0.846 ± 0.263 μM.

Recently, several flavonoid aglycones from *Vitex agnus-castus* L., were found to bind to both μ and δ opioid receptors in a dose-dependent manner. Casticin, a flavanone, was found to have the highest affinity for μ opioid receptors with a *K*i value of 1.14 ± 0.17 μM. Meanwhile, for apigenin a *K*i value of 16.2 ± 3.16 μM was reported (Webster et al., 2011).

An efficient synthesis of compound **31** was developed, the procedure employed was simply, easy and cheap, and the yield obtained was appropriate. Then, the *in vivo* effects of flavanone **31**, after acute treatments, were studied using behavioral and anti-nociceptive assays. The antinociceptive tests are based on an inhibition of a nocifensive response to a normally painful stimulus, in which opioid receptors are involved, as they have a fundamental participation in pain transmission.

The sequence of the μ opioid receptors is highly conserved among human, rat and mouse. Also, μ and δ site localizations in the mouse are very similar to those described in the rat (Moskowitz and Goodmans, 1984). It has been demonstrated that the μ opioid receptor ligand DAMGO showed similar specificity in competition binding studies in whole brain homogenate in rat and mouse. There was, also, no difference between the mouse and rat in the density and affinity of DAMGO sites (Yoburn et al., 1991). For practical reasons, we decided to perform the binding studies using rat forebrains. Meanwhile, mice were used for the pharmacological assays.

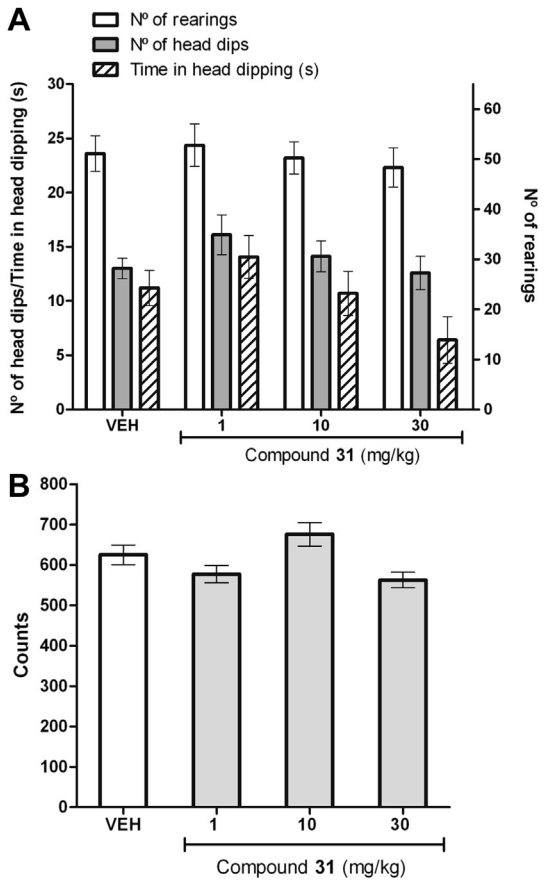


Fig. 6. Effect of the i.p. injection of compound **31** in the hole board and locomotor activity tests in mice. Results are expressed as mean ± S.E.M. of (A) the hole board parameters and (B) spontaneous locomotor activity counts; registered in 5 min sessions, 20 min after an i.p. injection of vehicle (VEH) or compound 31 ($n = 7–12$ mice/group).

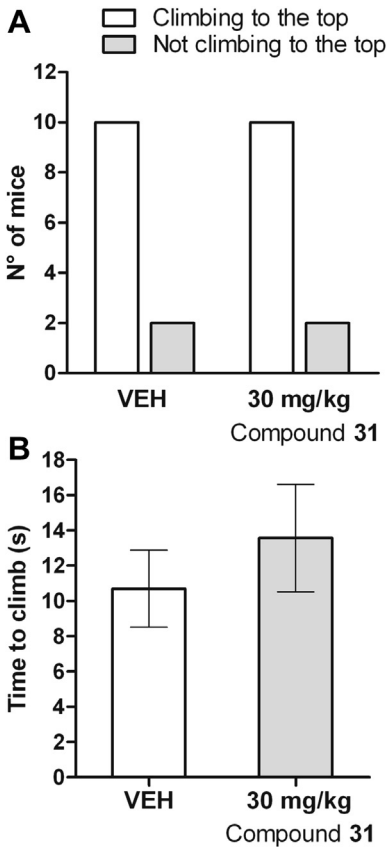


Fig. 7. Effect of the i.p. injection of compound **31** in the inverted screen test in mice. Results are expressed as mean ± S.E.M. of (A) number of mice which succeed in performing the test and (B) time spent climbing; registered 30 min after an i.p. injection of vehicle (VEH) or compound **31** ($n = 7–10$ mice/group).

It is necessary to apply several tests, with differences regarding stimulus quality, intensity and duration, to obtain a complete picture of the antinociceptive properties of a drug (Tjølsen, 1992; Vidyalakshmi et al., 2010). Nociceptive tests use electrical, thermal, mechanical, or chemical stimuli. They can be of short duration representing the phasic pain or of long duration stimuli representing the tonic pain (Le Bars et al., 2001).

In the hot plate test short thermal stimulus is employed and the behavioral responses measured are considered to be supraspinally integrated responses, so it is suitable for evaluation of centrally but not of peripherally acting antinociceptive drugs. Compounds exhibiting good antinociceptive effect in this method may be considered as potent analgesics (Bannon and Malmberg, 2007; Vidyalakshmi et al., 2010). Compound **31** significantly increased the latency time in the hot plate test at 10 mg/kg and 30 mg/kg. The antinociceptive effect reached its maximum at 20 min after drug injection, remained relatively stable until 60 min and slowly decreased after this time without reaching control values until 120 min.

Different irritating chemical agents can be used as nociceptive stimuli to assess pain and preclinically evaluate analgesic drugs, such as acetic acid and formalin (Barrot, 2012). In the writhing test the irritating agents, usually acetic acid, are administered i.p. inducing a stereotyped behavior characterized by abdominal contractions evidencing visceral pain (Le Bars et al., 2001). This test has poor specificity because the abdominal writhing response may be suppressed by muscle relaxants and other drugs, leaving scope for the misinterpretation of results (Jürgensen et al., 2005). This can be avoided by complementing the test with other models of nociception and with motor toxicity tests, as the inverted screen test, where compound **31** showed no motor impairment patterns at the higher dose it produced complete suppression of the writhing response.

The formalin induced nociception assay measures the ability of a substance to attenuate moderate continuous pain generated by injured tissue. The early and late phases of formalin nociception are considered to represent neurogenic and inflammatory pain behavior, respectively. The first phase is acute and represents the direct chemical stimulation of nociceptors, whereas the second phase is tonic and appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and central sensitization. Centrally acting drugs inhibit both phases of pain, while peripheral acting drugs such as acetylsalicylic acid or indomethacin, only inhibit the second phase (Bannon and Malmberg, 2007; Tjølsen, 1992). The i.p. administration of 10 mg/kg and 30 mg/kg of compound **31** reduced significantly the licking time in both phases of the formalin test.

In summary, the present study demonstrates that systemic (i.p.) administration of 3,3-dibromoflavanone (**31**) elicits a dose dependent inhibition of the nociceptive behavioral response in mice submitted to chemical and thermal pain inducing stimuli and demonstrates the central action of this drug and its effect on visceral pain.

To further investigate the mechanism of action of compound **31** *in vivo* blockade experiments of receptors which are involved in the mechanism of pain were performed. The hot plate test was used as

Table 3
Effect of different antagonists pretreatments on the antinociceptive activity of 3,3-dibromoflavanone in mice measured on the hot plate assay.

Pretreatment	Treatment	Time before measure (min)	Latency time (s)[a]
Vehicle	Vehicle	50	17.9 ± 0.7
Vehicle	Compound **31** (30 mg/kg)	50	25.1 ± 1.1***
Yohimbine (2 mg/kg)	Vehicle	50	19.6 ± 1.4
Yohimbine (2 mg/kg)	Compound **31** (30 mg/kg)	50	23.2 ± 2.4*
Ketanserin (0.5 mg/kg)	Vehicle	50	20.0 ± 2.0
Ketanserin (0.5 mg/kg)	Compound **31** (30 mg/kg)	50	24.4 ± 2.2**
Prazosin (0.2 mg/kg)	Vehicle	50	18.0 ± 2.1
Prazosin (0.2 mg/kg)	Compound **31** (30 mg/kg)	50	25.0 ± 2.0**
Naltrindole (5 mg/kg)	Vehicle	50	20.5 ± 1.2
Naltrindole (5 mg/kg)	Compound **31** (30 mg/kg)	50	23.2 ± 0.5*
Naltrexone (5 mg/kg)	Vehicle	50	20.0 ± 1.6
Naltrexone (5 mg/kg)	Compound **31** (30 mg/kg)	50	18.3 ± 1.4++
Vehicle (s.c.)	Vehicle (i.p.)	1470	17.8 ± 0.6
Vehicle (s.c.)	Compound **31** (30 mg/kg, i.p.)	1470	25.0 ± 1.3***
Nor-binaltorphimine (10 mg/kg, s.c.)	Vehicle (i.p.)	1470	20.0 ± 1.5
Nor-binaltorphimine (10 mg/kg, s.c.)	Compound **31** (30 mg/kg, i.p.)	1470	26.7 ± 2.3***

Number of animals per group ranged between 6 and 22. The symbols denote significance levels: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different from vehicle–vehicle (two-way ANOVA, Bonferroni post-hoc test); $^{++}P < 0.01$ significantly different from vehicle-compound **31** (one-way ANOVA followed by Newman–Keuls test).
[a] Results are expressed as mean ± S.E.M. of the latency time (s). Mice were pretreated with vehicle, yohimbine, ketanserin, prazosin, naltrexone, naltrindole or nor-binaltorphimine. The hot plate test was performed 30 min after the i.p. injection of compound **31** or vehicle.

it distinguished the strong central antinociceptive actions of μ opioids and α_2-adrenoceptor agonists from the weak, predominantly peripheral actions of the NSAIDs, which were ineffective in this paradigm (Le Bars et al., 2001). Antinociceptive properties of κ opioid agonists, α_1-adrenoceptor agonists and serotonin agonists could also be detected in this model (Millan, 1994). Moreover, in advantage to the formalin test it is a faster and easier method to determine the supraspinal response.

Adrenergic receptors appear to play an important role in the modulation of pain and their activation can generate notable analgesic effects (Carroll et al., 2007). Central α_2-adrenoceptors mediate sedative-hypnotic, analgesic, hypotensive and anxiolytic responses (Lakhlani et al., 1997). Some data indicate that all three α_2-adrenoceptor subtypes are implicated in the regulation of pain perception in the mouse (Philipp et al., 2002) and an involvement of α_1-adrenoceptors in nociceptive responses was described (Tanoue et al., 2002). Prazosin and yohimbine are selective antagonists of α_1 and α_2-adrenoceptors, respectively, but they do not differ between receptor subtypes (Bourin et al., 2005; Yue et al., 2007). Both antagonists, prazosin and yohimbine, were ineffective to reverse the antinociceptive action of flavanone **31** in the hot plate test at the doses tested, demonstrating that α_1 and α_2 adrenoceptors were not involved in the antinociceptive activity of this drug.

The serotonergic system is involved in the regulation of many physiological and behavioral functions, including mood, sleep and appetite (Schloss and Williams, 1998). The multiple 5-HT receptor types within the spinal cord appear to fulfill different roles in the control of nociception (Millan, 1994). Several pieces of evidence point that 5-HT_2 are involved in the modulation of pain perception, acting at spinal and supraspinal level (Ormazábal et al., 1999). The subtypes 5-HT_{2A} and 5-HT_{2C} predominate in the CNS. The pretreatment of mice with ketanserin, a 5-$HT_{2A/C}$ receptor antagonist, did not reverse the antinociceptive action of compound **31** in the hot plate test at the doses tested, evidencing that 5-$HT_{2A/C}$ receptors are not implicated in this action.

To study the implication of the opioid receptors in the antinociceptive effect of compound **31**, the antagonists naltrexone, nor-binaltorphimine and naltrindole were used. Naltrexone is considered a nonselective antagonist of opioid receptors; it has higher affinity for μ opioid receptors, then for κ and even less for δ opioid receptors (Peng et al., 2007; Takemori and Portoghese, 1984). The selective κ and δ opioid receptor antagonists, nor-binaltorphimine and naltrindole, respectively, were used to investigate the possible participation of these receptor subtypes on flavanone **31** action. Only naltrexone could block the antinociceptive action of 3,3-dibromoflavanone, reinforcing the involvement of the μ opioid receptors in its mechanism of action. In turn, naltrexone completely abolished the antinociceptive action caused by injection of morphine (6 mg/kg, i.p.) (Loscalzo et al., 2011).

Efficacy is normally the primary outcome in the evaluation of analgesics; however, it is equally important to consider that opioid receptor agonists are not devoid of unpleasant side effects. For example morphine is considered the “gold standard” analgesic, but effective analgesic doses can produce sedation, constipation, dependence and addiction. Previous rodent studies have also assessed the influence of morphine on locomotion (Neubert et al., 2007). The effect of compound **31** on general mice behavior was evaluated using the hole board test, which provides measurements of the exploratory behavior of mice, and it is sensitive to depressant effects of a variety of compounds which act by different mechanism of actions (File and Pellow, 1985). The locomotor activity test was also performed as it is a commonly used assay to evaluate potential sedative effects of compounds (Vogel et al., 2002). In both assays, the i.p. administration of the flavanone **31** did not reduce the exploration and ambulatory activity of the animals, discarding that compound **31** presents sedative effects in the CNS at the doses tested. Constipation is a common morphine undesired side effect that can appear even after a single dose (Kromer, 1988). In this work we found that, unlike morphine, a single administration of the maximal dose tested for compound **31** (30 mg/kg) was not able to produce an inhibition of gastrointestinal transit on the charcoal meal test.

Patients with chronic pain frequently have comorbid anxiety disorders. As some flavanone derivatives, such as 6-bromoflavanone and 5-methoxy-6,8-dibromoflavanone have shown anxiolytic activity (Ognibene et al., 2008), we decided to evaluate the possible anxiolytic activity of compound **31** in the plus maze test. Flavanone **31** did not show anxiolytic activity in mice at the doses tested. It was suggested that substitution at position 6 of the flavanone nucleus with a bromine atom is essential for the compounds to manifest elevated anxiolytic properties (Ognibene et al., 2008).

A structure activity relationship (SAR) was developed with the basic flavone nucleus and many monohydroxy and monomethoxy

derivatives. It was demonstrated that the flavone nucleus *per se* has an inherent analgesic action; the C_2–C_3 double bond in the flavone nucleus appears to be essential for the analgesic effect and substitution of different groups (hydroxyl or methoxyl) at different positions in the flavone nucleus altered the analgesic potency of flavone (Thirugnanasambantham et al., 1993). Moreover, the flavonoid core was studied as a potential new scaffold for the development of opioid receptor ligands and it was observed that the stereochemistry of the C2 and C3 positions, and its substitution, is important for antagonist activity and selectivity (Katavic et al., 2007). Some dihydroxy flavones (5,3′-, 7,3′-, 2′,3′-, 2′,4′-, 3,3′-, 5,6-, 3,7 and 6,3′- dihydroxy flavones) with antinociceptive activity have been already synthesized. These flavones produced dose related antinociception through mechanisms that involve an interaction with opioid and GABAergic systems (Vidyalakshmi et al., 2010). In the present work we demonstrated that 3,3-dibromoflavanone (**31**), despite having C_2–C_3 single bond in its structure, presents antinociceptive activity.

5. Conclusion

The results of the present study provided convincing evidence that i.p. administration of 3,3-dibromoflavanone (**31**), at doses that do not interfere with the motor performance and the gastrointestinal transit, exerted clear dose dependent antinociception when assessed in the chemical and thermal models of nociception in mice and it seems that its action is related to the activation of the μ opioid system.

It could be suggested that flavanone derivatives can afford opioid ligands emerging as novel analgesics without motor side effects.

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