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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

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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

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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.

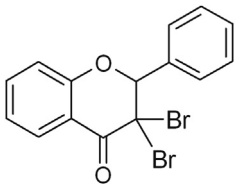

Fig. 1. Molecular structure of 3,3-dibromoflavanone (**31**).

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).