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Anxioselective properties of 6,3′-dinitroflavone, a high-affinity benzodiazepine receptor ligand

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Abstract

6,3′-Dinitroflavone is a synthetic flavone derivative with high affinity for central benzodiazepine receptors that has anxiolytic effects. Here, we describe its biochemical and pharmacological characterization. 6,3′-Dinitroflavone inhibited differentially [^{3}H]flunitrazepam binding to central benzodiazepine receptors in several brain regions, showing a lower K_i value in the cerebellum (central benzodiazepine receptor type I-enriched area), and a higher K_i value in the spinal cord and in the dentate gyrus (central benzodiazepine receptor type II-enriched area). When i.p. injected in mice, 6,3′-dinitroflavone had a potent anxiolytic effect in the elevated plus maze test. This effect was blocked by the specific central benzodiazepine receptor antagonist, Ro 15-1788. 6,3′-Dinitroflavone did not exhibit anticonvulsant or myorelaxant effects in mice or amnestic effects in rats. Moreover, it abolished the myorelaxant effect of diazepam. On the other hand, 6,3′-dinitroflavone possessed a mild sedative action only at doses 100–300-fold greater than the anxiolytic one. Based on these findings, we suggest that 6,3′-dinitroflavone has a benzodiazepine partial agonist profile, with low selectivity for central benzodiazepine receptor types I and II.

Keywords: Benzodiazepine receptor, central; Anxiolysis; Flavonoid; (Partial agonist); Autoradiography

1. Introduction

Previous work from our laboratories has demonstrated that some naturally occurring flavonoids are ligands for the central benzodiazepine receptors, and possess anxiolytic but not depressant effects in rodents (Medina et al., 1990; Wolfman et al., 1994; Viola et al., 1995).

In an attempt to further improve the pharmacological properties of these compounds, we reasoned by analogy to what is known about the benzodiazepines, namely, that the presence of electronegative groups in their molecules is essential for activity (Sternbach, 1978).

In support of this hypothesis, we found that the introduction of a bromine atom in position 6 of flavone increased 14-fold its affinity for the central benzodiazepine receptors (Marder et al., 1996). Similarly, nitration of the same compound in positions 6 and 3′ produced a derivative with still higher affinity for the central benzodiazepine receptors and with a very potent anxiolytic effect when injected i.p. in mice (Marder et al., 1995).

A large body of evidence suggests the existence of two pharmacologically distinct central benzodiazepine receptor subtypes differentially distributed in the brain, which were designated as types I and II. Type I benzodiazepine receptors are probably identical to $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, while type II is a mixture of α_2, α_3 and α_5 subunits, together with β and γ subunits (Wisden et al., 1992). The cerebellum appears to be enriched in type I, the dentate gyrus of the hippocampus and the spinal cord in type II, while the cerebral cortex possesses both types of receptors (Siegharth and Karobath, 1980; Trifiletti et al., 1984). Drugs such as the triazolopyridazine, CL 218,872 (Lippa et al., 1982), and the trimethylimidazolpyridine, zolpidem (Niddam et al., 1987), have higher affinity for type I than for type II. The classical benzodiazepine, diazepam, does

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not distinguish between the two central benzodiazepine receptor types.

We now present experimental evidence that the synthetic flavonoid derivative, 6,3′-dinitroflavone, differentially displaces the binding of [^{3}H]flunitrazepam to various brain regions. We also characterized in detail the pharmacological profile of 6,3′-dinitroflavone when administered acutely to rodents.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 250 g were used for biochemical experiments. Adult male Swiss mice weighing 25–30 g were used for pharmacological assays except for inhibitory avoidance and tail-flick tests which were done in rats. The animals were housed in a controlled environment, with free access to food and water, and were maintained on a 12 h/12 h day/night cycle.

2.2. Radioreceptor binding assays

Displacement curves were performed using [^{3}H]flunitrazepam or [^{3}H]zolpidem as radioligands in washed crude synaptosomal membranes from rat cerebral cortex, cerebellum, hippocampus, striatum or spinal cord. Membranes were prepared according to Medina et al. (1990). Briefly, the brains were rapidly dissected out on ice and the different structures were homogenized in 10 volumes of 0.32 M sucrose and centrifuged at $900 \times g$ for 10 min. The resulting supernatant was centrifuged at $100\,000 \times g$ for 30 min and the pellet washed twice in 25 mM Tris-HCl buffer pH 7.4 at $100\,000 \times g$ for 30 min, and stored at −20°C until used.

For [^{3}H]flunitrazepam (84 Ci/mmol, NEN) displacement curves, various concentrations of 6,3′-dinitroflavone (0.3 nM to 1 μM) were added to 0.3 mg membrane protein suspended in 1 ml of 25 mM Tris-HCl buffer in the presence of 0.6 nM of the radioligand. Protein determination was carried out with Lowry's method (Lowry et al., 1951). Non-specific binding (< 5%) was determined in parallel incubations with 10 μM flunitrazepam (Hoffmann-La Roche). The incubation was carried out at 4°C for 1 h. The assays were terminated by filtration under vacuum through Whatman GF/A glass fiber filters, and two washes with 3 ml each of incubation medium. The filters were dried and counted after the addition of 5 ml 2,5-diphenyl-oxazole-xylene as scintillation fluid.

For [^{3}H]zolpidem (50.8 Ci/mmol, NEN) binding assays we used the technique of Arbilla et al. (1986), slightly modified. In brief, displacement curves were done with 1 nM of the radioligand and 10 μM zolpidem to determine the non-specific binding (< 15%). The samples (0.4 mg protein in 1 ml of 50 mM Tris-HCl buffer, pH 7.4, 120 mM NaCl, 5 mM KCl) were incubated at 4°C for 30 min. The reaction was stopped with 3 ml of the same buffer and 3 washes. The other steps were similar to those in the original technique.

Additional binding studies were performed as described elsewhere: [^{3}H]prazosin binding for α_1-adrenoceptors (Medina et al., 1984), [^{3}H]dihydroalprenolol binding for β-adrenoceptors (Medina et al., 1984), [^{3}H]quinuclinidyl benzylate binding for muscarinic acetylcholine receptors (Jerusalinsky et al., 1983), [^{3}H]muscimol binding for $GABA_A$ receptors (Medina et al., 1983) and [^{3}H]8-hydroxydipropylaminotetralin ([^{3}H]8-OH-DPAT) binding for 5-HT_{1A} receptors (Nénoné et al., 1994), with slight modifications.

2.3. Autoradiographic experiments

Wistar rats were decapitated and the brains rapidly removed. Sagittal sections (15 μm in thickness) were prepared at −20°C using a microtome cryostat. The tissue slices were kept frozen at −70°C until used. For 6,3′-dinitroflavone displacement curves to [^{3}H]flunitrazepam binding (0.65 nM), tissue sections were incubated for 60 min at 4°C in 25 mM Tris-HCl buffer, pH 7.4, in the presence of different concentrations of 6,3′-dinitroflavone (1–600 nM). For non-specific binding we used 10 μM flunitrazepam. The incubation was terminated by rinsing the sections for 2 min in cold buffer. Sections were briefly dipped in cold distilled water and dried rapidly under a stream of cold air (Niddam et al., 1987).

Autoradiograms were generated by apposing the slide-mounted tissue sections to tritium-sensitive film (Hyperfilm Amersham) in a light-proof X-ray cassette at 4°C for 2 weeks.

The optical densities from different brain regions were converted first to radioactive units and then to fmol/mm^2 using the [^{3}H] standards on the film with the aid of a computerized image densitometric analysis system (MCID 4.02). The values were normalized and the K_i values were determined with a computerized program (Graph-Pad Prism) (Bernabeu et al., 1995; Cammarota et al., 1995).

2.4. Pharmacological procedures

2.4.1. Locomotor activity test

An Opto-varimex apparatus was used according to Viola et al. (1994). The apparatus discriminates between total and ambulatory activities. An increase in the number of transitions through the beams reflects augmented locomotor activity. In this and all subsequent tests in mice, animals were i.p. injected with the vehicle, or with 6,3′-dinitroflavone 20 min before the beginning of the tests. In each session, control mice were tested in parallel with those animals receiving drug treatment.

2.4.2. Elevated plus maze test

The test was performed in the same session, inmediately after the locomotor activity measurement (Viola et al., 1994; Wolfman et al., 1994). This test is widely validated for rodents (Pellow et al., 1985; Pellow and File, 1986; Lister, 1987) and possesses several advantages over other tests for measuring anxiety (Dawson and Tricklebank, 1995). A selective increase in the number of entries and in the time spent in the open arms denotes an anxiolytic effect of the drug (Pellow et al., 1985; Pellow and File, 1986). A series of experiments with the injection of the selective central benzodiazepine receptor antagonist, Ro 15-1788, together with 6,3′-dinitroflavone (File and Pellow, 1986), was also carried out.

2.4.3. Holeboard test

The test was performed according to Viola et al. (1994) and Wolfman et al. (1994). The number of head dips and the time spent head dipping were counted for 5 min. A decrease in the value of these parameters reveals sedated behavior (File and Pellow, 1985).

2.4.4. Horizontal wire test

This test was carried out as previously described (Viola et al., 1994, 1995; Wolfman et al., 1994). The test took place after two trials, performed at 5-min intervals. A myorelaxant drug will hamper grasping of the wire by the mice (Bonetti et al., 1982). The effect of 6,3′-dinitroflavone (1 mg/kg) on diazepam-induced myorelaxation was also determined.

2.4.5. Sodium thiopental-induced sleeping time

Sodium thiopental (22 mg/kg) was i.p. injected 15 min after vehicle or 6,3′-dinitroflavone. The disappearance and reappearance of the righting reflex were considered indications of latency and duration of sleep, respectively (Anca et al., 1992).

2.4.6. Seizure testing

The effects of 6,3′-dinitroflavone on pentylenetetrazole-induced convulsions were evaluated according to Medina et al. (1990) with slight modifications. Pentylenetetrazole (200 mg/kg) was administered i.p. to mice 15 min after injection of drug or vehicle. The number of mice showing clonic or tonic-clonic convulsions was determined.

2.4.7. Inhibitory avoidance test

This test was performed according to Izquierdo et al. (1990). The training apparatus was a $50 \times 25 \times 25$ cm acrylic box with a glass front panel and a floor made of parallel 1-mm caliber bronze bars spaced 0.8 mm apart. A 5 cm high, 7 cm wide Formica platform was placed on the extreme left of the box. Rats were placed on the platform and their latency to step-down, placing their four paws on the grid, was measured. On stepping-down they received a 0.35-mA, 2-s scrambled footshock and were withdrawn from the box (training session). The test session was carried out 20 h later and was similar to the training session in all respects except that the footshock was omitted. Test step-down latency (to a ceiling of 180 s) was taken as a measure of retention of inhibitory avoidance (Izquierdo et al., 1990).

2.4.8. Tail-flick test

This test was performed according to Siegfried et al. (1987). Analgesia was assessed with a tail-flick apparatus. Rats were wrapped in a towel and placed on the apparatus; the light source positioned below the tail was focused on a point 2.3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. Light intensity was adjusted so as to obtain a baseline tail-flick latency of 3–6 s. A cut-off time of 10 s was used to prevent tissue damage. Briefly, the general procedure was as follows: a baseline tail-flick latency value was obtained for each animal. Following this, the rats were placed alone in a waiting cage. Tail-flick latency value was measured 1 h after an i.p. injection of vehicle or 6,3′-dinitroflavone.

2.4.9. Drugs

6,3′-Dinitroflavone (Marder et al., 1995) and diazepam (Hoffmann-La Roche) were dissolved in a mixture of dimethyl sulfoxide 20% and ethanol 20%, in distilled water. Ro 15-1788 (Hoffmann-La Roche) was dissolved in propyleneglycol 10% and dimethyl sulfoxide 15% in distilled water. The volume of injection was 0.1 ml/10 g in mice and 0.1 ml/100 g in rats.

2.5. Statistical analyses

The competition curves were analyzed using the GraphPad Prism software. Analysis of variance (ANOVA) was used when results of several treatments in mice were compared. Post-hoc comparisons between individual treatment and controls were made using Dunnett's multiple comparisons test. A χ^2 frequency test was used when required. The non-parametric Mann-Whitney U test was used for inhibitory avoidance and tail-flick latency tests in rats.

3. Results

3.1. Biochemical studies

6,3′-Dinitroflavone had different potencies for displacing [^{3}H]flunitrazepam binding in various central nervous system (CNS) regions. It was more potent in the cerebellum, with a K_i ($\pm$S.E.M.) value of 17.2 ± 1.9 nM ($n = 6$); least potent in the striatum and spinal cord, with K_i values of 44.1 ± 4.9 nM ($n = 7$) and 48.1 ± 5.5 nM ($n = 4$),

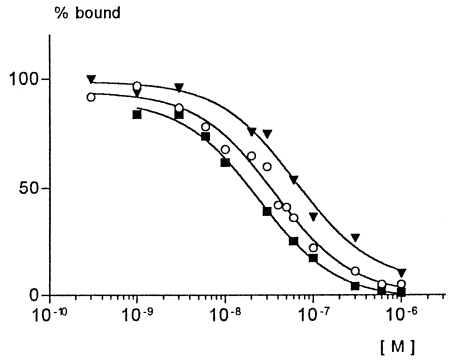

Fig. 1. Competition by 6,3′-dinitroflavone for [^{3}H]flunitrazepam binding to extensively washed crude synaptosomal membranes from various rat brain regions. Membranes from cerebellum (■), cerebral cortex (○) and spinal cord (▼) were prepared as described in Section 2. Data are from a representative experiment replicated 6–8 times.

respectively; and intermediate in potency in the cerebral cortex (25.9 ± 3.1 nM, $n = 8$) and hippocampus (36.1 ± 5.0 nM, $n = 4$). Fig. 1 shows representative displacement curves of [^{3}H]flunitrazepam binding to cerebellar, cerebral cortical and spinal cord membranes by 6,3′-dinitroflavone (9–14 different concentrations). In contrast, the non-selective central benzodiazepine receptor agonist, diazepam, displaced with similar K_i the [^{3}H]flunitrazepam binding to all the brain regions studied (~ 7 nM).

When the radioligand was [^{3}H]zolpidem, a well known central benzodiazepine receptor agonist that recognizes preferentially type I (Arbilla et al., 1986), 6,3′-dinitroflavone showed similar K_i values in synaptosomal membranes from cerebral cortex, striatum and cerebellum (17.2 ± 3.7 nM, $n = 5$; 21 ± 1 nM, $n = 2$ and 17 ± 1.5 nM, $n = 3$, respectively).

6,3′-Dinitroflavone (20 μM) did not displace the binding of [^{3}H]quinuclinidyl benzilate, [^{3}H]muscimol, [^{3}H]8-OH-DPAT, [^{3}H]prazosin or [^{3}H]dihydroalprenolol to muscarinic acetylcholine, $GABA_A$ and 5-HT_{1A} receptors, and α_1- and β-adrenoceptors, respectively (data not shown).

Similar regional variations in the potency of 6,3′-dinitroflavone to displace [^{3}H]flunitrazepam binding were observed in autoradiographic experiments (Fig. 2). The maximal inhibitory effect was observed in the cerebellum ($K_i = 17.2 \pm 2.3$ nM, $n = 3$) followed by parietal cortex ($K_i = 30.1 \pm 2.6$ nM, $n = 3$), striatum ($K_i = 53.7 \pm 7.3$ nM, $n = 3$) and dentate gyrus ($K_i = 82.2 \pm 7.6$ nM, $n = 3$). Therefore, 6,3′-dinitroflavone is 5 times more potent to displace [^{3}H]flunitrazepam binding from the cerebellum than from the dentate gyrus.

3.2. Pharmacological experiments

3.2.1. Effect of 6,3′-dinitroflavone on ambulatory locomotor activity

Fig. 3 shows that the i.p. administration of 6,3′-dinitroflavone (up to 3 mg/kg) had no effect on spontaneous

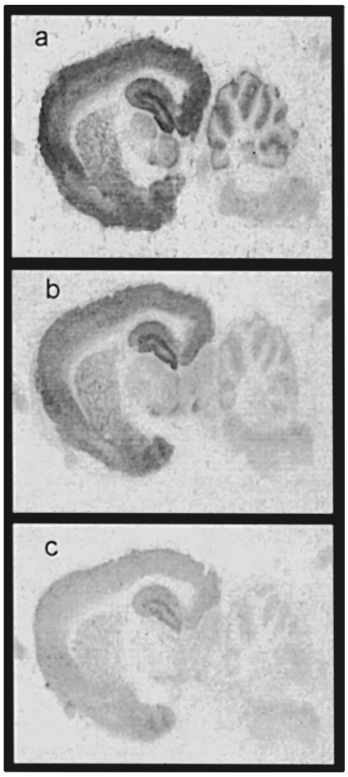

Fig. 2. Bright-field photographic images of autoradiograms generated by 0.65 nM [^{3}H]flunitrazepam exposed to [^{3}H] Hyperfilm for 14 days. Sagittal sections were incubated in the absence (a), or in the presence of 20 nM (b) or 60 nM (c) of 6,3′-dinitroflavone. In these photographs, dark regions represent areas of high receptor densities. Note the considerable decrease in cerebellar binding in comparison with that obtained in the dentate gyrus of the hippocampus.

ambulatory locomotion; at 10 mg/kg (the highest dose tested) there was a 55% reduction in locomotion ($F(9,152) = 4.52$, $P < 0.01$). A slight increase in this parameter was observed at 0.3 mg/kg ($P < 0.05$).

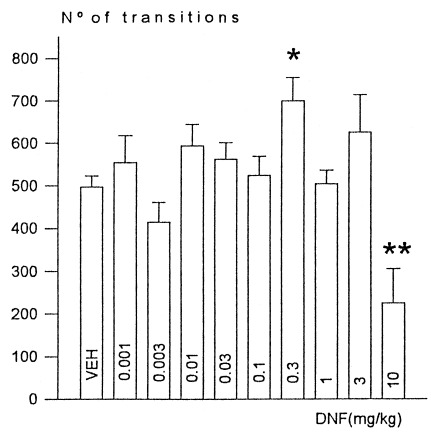

Fig. 3. Ambulatory locomotor activity counts during a 5-min test session in an Opto-varimex apparatus 15 min after an i.p. injection of vehicle (VEH) or 6,3′-dinitroflavone (DNF, 0.001–10 mg/kg as detailed inside the bars). Data are expressed as means ± S.E.M. Number of animals in the experimental groups ranged between 10 and 16. * $P < 0.05$, ** $P < 0.01$, significantly different from controls (Dunnett's multiple comparison test after ANOVA).

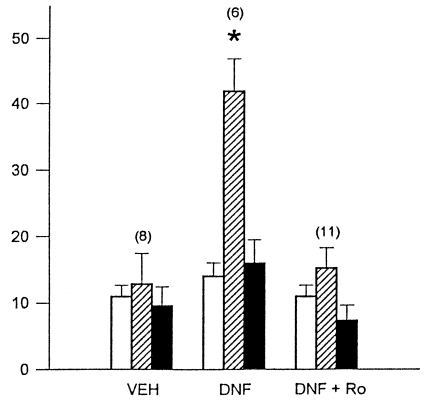

Fig. 4. Mean ± S.E.M. of total entries (open bars), percentage of open arm entries (hatched bars) and percentage of time spent in open arms (closed bars) by mice given a 5-min session in the elevated plus maze 20 min after i.p. injection of vehicle (VEH), 6,3′-dinitroflavone (DNF, 30 μg/kg) or 6,3′-dinitroflavone (30 μg/kg) + Ro 15-1788 (1 mg/kg) (DNF + Ro). * $P < 0.01$, significantly different from controls (Dunnett's *t*-test after ANOVA). Number of animals per group is shown in parentheses.

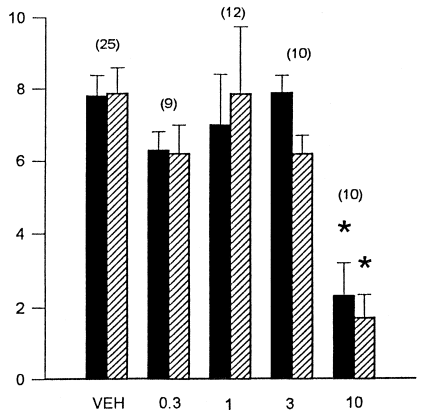

Fig. 5. Mean ± S.E.M. number of head dips (closed bars) and time (in seconds) spent head dipping (hatched bars) of mice given a 5-min session in the hole board test 20 min after an i.p. injection of vehicle (VEH) or 6,3′-dinitroflavone (0.3–10 mg/kg). * $P < 0.01$, significantly different from controls (Dunnett's *t*-test after ANOVA). Number of animals per group is shown in parentheses.

3.2.2. Effect of 6,3′-dinitroflavone in the elevated plus maze test

Previous experiments from our laboratory had demonstrated that i.p. injection of low doses (1–30 μg/kg) of 6,3′-dinitroflavone in mice had an anxiolytic effect as measured in this test (Marder et al., 1995). Confirming these results, the i.p. administration of an anxiolytic dose of 6,3′-dinitroflavone (30 μg/kg) increased the percentage of entries in the open arms ($F(2,22) = 13.37$, $P < 0.01$; Dunnett test after ANOVA) (Fig. 4). No differences were observed in the total arm entries ($F(2,22) = 0.83$, $P > 0.05$). This anxiolytic effect was blocked by the injection of Ro 15-1788 (1 mg/kg) (Fig. 4). Experiments run in parallel with diazepam revealed that this well known anxiolytic drug produced an increase in the percentage of open arm entries only at doses 10–100 times higher (vehicle = 22.9 ± 2.0%; diazepam 0.3 mg/kg = 35.0 ± 4.5%, $P < 0.05$; Fig. 4 and Marder et al., 1995).

3.2.3. Effect of 6,3′-dinitroflavone in the holeboard test

The performance of mice injected with vehicle or 6,3′-dinitroflavone in the holeboard test is shown in Fig. 5. As can be seen, doses up to 3 mg/kg did not change the number of head dips and the time spent head dipping. Only at the high dose of 10 mg/kg (300-times higher than the anxiolytic dose used in the present study), did 6,3′-dinitroflavone decrease both parameters ($F(4,61)$ (head dips) = 6.04; $F(4,61)$ (time) = 4.63, $P < 0.01$; Dunnett comparison test after ANOVA). Diazepam had similar effects when injected at 1 mg/kg (head dips, vehicle = 8.5 ± 1.2; diazepam 1 mg/kg = 1.2 ± 0.5, $P < 0.001$).

3.2.4. Effect of 6,3′-dinitroflavone in the horizontal wire test

6,3′-Dinitroflavone, at doses up to 10 mg/kg, did not affect the percentage of mice grasping the wire (Fig. 6). On the other hand, diazepam (1 mg/kg) produced a marked myorelaxant effect (8 animals out of 10, χ^2 (0.05, 1) = 22.64, $P < 0.001$, χ^2 test with respect to control) (Fig. 6). This myorelaxant action was counteracted by co-administration with 6,3′-dinitroflavone (1 mg/kg; 2 animals out of 15) (Fig. 6).

3.2.5. Effect of 6,3′-dinitroflavone on pentylenetetrazole-induced convulsions

6,3′-Dinitroflavone, in a wide range of doses (30 μg/kg–6 mg/kg), did not prevent the seizures induced by 200 mg/kg pentylenetetrazole in mice (Table 1). In contrast, diazepam (0.3–3 mg/kg) showed anticonvulsant activity ($P < 0.001$, χ^2 test) (Table 1).

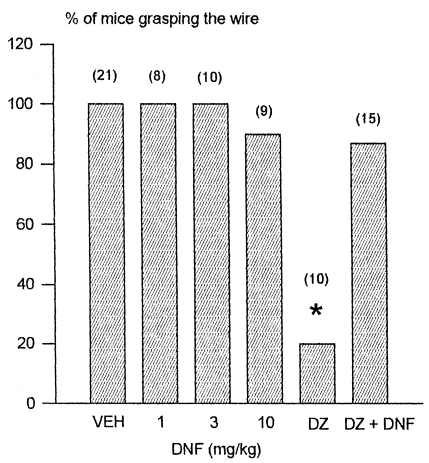

Fig. 6. Performance of mice in the horizontal wire test after an i.p. injection of vehicle (VEH), 6,3′-dinitroflavone (DNF, 1–10 mg/kg), diazepam (DZ, 1 mg/kg) or 6,3′-dinitroflavone (1 mg/kg) + diazepam (1 mg/kg). The test session took place after two trials, executed after a 5-min interval (see Section 2). * $P < 0.001$, χ^2 frequency test versus control value. Number of animals per group is shown in parentheses.

Table 1
Effect of diazepam and 6,3′-dinitroflavone on pentylenetetrazole-induced seizures

	n [a]	% Mice convulsing
VEH + PTZ	25	96
DZ 0.3 mg/kg	6	33.3 [b]
DZ 1.0 mg/kg	12	42 [b]
DZ 3.0 mg/kg	9	0 [b]
DNF 0.03 mg/kg	6	100
DNF 0.1 mg/kg	12	75
DNF 0.3 mg/kg	15	80
DNF 1.0 mg/kg	13	92.4
DNF 3.0 mg/kg	5	100
DNF 6.0 mg/kg	7	100

Mice were i.p. injected with 200 mg/kg of pentylenetetrazole 15 min after i.p. administration of vehicle (VEH), diazepam (DZ) or 6,3′-dinitroflavone (DNF). Data are expressed as the percentage of mice presenting clonic or tonic-clonic seizures. [a] Number of animals per group. [b] $P < 0.001$, χ^2 test.

3.2.6. Effect of 6,3′-dinitroflavone on thiopental-induced sleeping time

In mice, the i.p. administration of 3 mg/kg 6,3′-dinitroflavone augmented sleeping time (vehicle = 190 s (11/478, $n = 16$); 6,3′-dinitroflavone 3 mg/kg = 1277 s (248/1800, $n = 12$); median (interquartile range) $P < 0.05$, Dunn's multiple comparison test after Kruskall-Wallis (KW = 6,93)). When injected at a lower dose (1 mg/kg), 6,3′-dinitroflavone did not change the sleeping time (207 s (20/600, $n = 7$)). No changes were observed in latency to sleep at any dose tested.

3.2.7. Effect of 6,3′-dinitroflavone on inhibitory avoidance and tail-flick tests

In rats, the i.p. administration of 6,3′-dinitroflavone (100 μg/kg) had no effect, either pre- or post-training, on the test session performance of inhibitory avoidance (Fig. 7). Furthermore, 6,3′-dinitroflavone at the same dose, did not alter tail-flick latency (vehicle = 3.2 s (2.8/4.3, $n = 11$); 6,3′-dinitroflavone 100 μg/kg = 3.5 s (2.7/4.2, $n = 11$) (median (interquartile range) $P > 0.05$, Mann-Whitney U test).

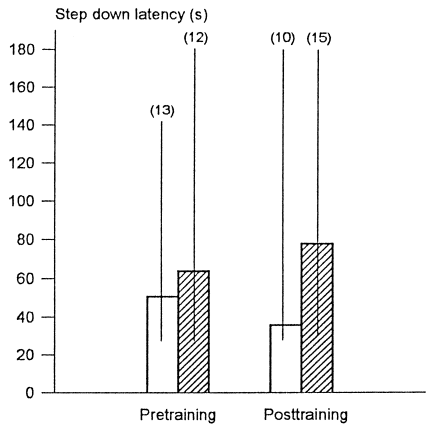

Fig. 7. Effect of pre- and post-training i.p. administration of vehicle (open bars) or 100 μg/kg of 6,3′-dinitroflavone (hatched bars) on memory for an inhibitory avoidance test in rats. The ordinate represents the step-down latency (in seconds) in the test session. Data are expressed as medians (interquartile range). Number of animals per group is shown in parentheses.

4. Discussion

The main finding of the present study was that 6,3′-dinitroflavone has anxioselective properties and probably acts at the central benzodiazepine receptor as a partial agonist with low selectivity for central benzodiazepine receptor types I and II.

Pharmacological and biochemical findings suggest the existence of several central benzodiazepine receptor types (Siegharth and Karobath, 1980; Trifiletti et al., 1984; Niddam et al., 1987; Wisden et al., 1992; Mohler et al., 1995; McKernan and Whiting, 1996). Type I is the most abundant in the brain. The cerebellum is primarily enriched in this type (Niddam et al., 1987); the hippocampus (Arbilla et al., 1986) and cerebral cortex contain mixed amounts of types I and II (Trifiletti et al., 1984), whereas type II is predominant in a few brain areas such as striatum, spinal cord, dentate gyrus and olfactory bulb (Watanabe et al., 1985; Niddam et al., 1987; Pritchett et al., 1989; McKernan and Whiting, 1996). Using crude synaptosomal membranes (Fig. 1), we found the lowest 6,3′-dinitroflavone inhibition constants for [^{3}H]flunitrazepam binding in cerebellum, followed by cerebral cortex > hippocampus > striatum ~ spinal cord. In autoradiographic experiments the rank order of potency was cerebellum > parietal cortex > striatum > dentate gyrus (see Results). These findings could have been the results of different affinities of 6,3′-dinitroflavone for the central benzodiazepine receptor types and their relative densities in each brain region. Because of its high potency, 6,3′-dinitroflavone probably binds selectively with very high affinity (K_i value in the picomolar range) to a $GABA_A$/benzodiazepine receptor complex constituting only a few percent of all $GABA_A$/benzodiazepine receptor complexes in the brain, e.g. $\alpha_5\beta_3\gamma_2$ (McKernan and Whiting, 1996), and almost obscured by the large amount of [^{3}H]flunitrazepam binding sites with K_i values in the 17–48 nM range.

In those areas with mixed populations of types I and II (e.g. cerebral cortex and hippocampus) the resultant K_i values may reflect the average affinity for central benzodiazepine receptor types I and II. In contrast, we found two well-defined binding sites in cortical membranes when we used the preferentially central benzodiazepine receptor type I agonist, CL 218,872 (high-affinity K_i value = 10 nM, low-affinity K_i value = 1.2 μM). In addition, when a selective central benzodiazepine receptor type I ligand, [^{3}H]zolpidem (Arbilla et al., 1986) was used, 6,3′-dinitroflavone had similar K_i values for cerebral cortex, striatum and cerebellum. These affinities are consistent with the nature of the cerebellar binding site. 6,3′-Di-

nitroflavone is a selective ligand for the central benzodiazepine receptors because it did not displace the binding of specific [^{3}H]radioligands to α_1- and β-adrenoceptors and muscarinic acetylcholine, $GABA_A$ or $5\text{-}HT_{1A}$ receptors.

Confirming and extending recent findings indicating that very low doses of 6,3′-dinitroflavone (1–30 μg/kg) have a potent anxiolytic action as measured in the elevated plus maze (Marder et al., 1995), we found that the 6,3′-dinitroflavone-induced increase in open arms exploration observed in the present study was blocked by the administration of the selective benzodiazepine receptor antagonist, Ro 15-1788 (Fig. 4). 6,3′-Dinitroflavone had no anticonvulsant (up to 6 mg/kg), myorelaxant (up to 10 mg/kg), amnestic or analgesic effects.

On the other hand, 6,3′-dinitroflavone possesses a slight depressant action at high doses (100–300-times higher than those producing anxiolytic effects) as shown by the thiopental sleeping time potentiation and by both a reduction in the locomotor ambulatory activity and a decrease in holeboard exploration (Figs. 3 and 5) (File, 1985). Therefore, 6,3′-dinitroflavone can reduce anxiety at doses well below those causing sedation.

6,3′-Dinitroflavone is a 30-fold more potent anxiolytic than diazepam and required a 10-fold higher dose to produce similar sedative effects. This dissociated behavioural profile may be explained by partial agonism at the central benzodiazepine receptors, which results in a reduced efficacy related to the maximal action of the compound at receptor saturation (Potokar and Nutt, 1994). In support of this contention, we demonstrated that 6,3′-dinitroflavone was able to reverse the myorelaxant effect of the full agonist, diazepam (Fig. 6).

Due to its selective pharmacological profile and low intrinsic efficacy, and its potential to induce fewer side-effects, 6,3′-dinitroflavone may represent an improved therapeutic tool for the treatment of anxiety.

In conclusion, 6,3′-dinitroflavone is a specific and high-affinity benzodiazepine receptor ligand that exhibits mild regional differences in its potency to displace [^{3}H]flunitrazepam binding to central benzodiazepine receptors. It has an anxioselective action in mice and prevents the muscle-relaxant effect of a full benzodiazepine receptor agonist. Based on these properties, we would like to postulate that 6,3′-dinitroflavone behaves as a partial agonist of the central benzodiazepine receptors, with a low selectivity for types I and II.

Further studies are necessary to determine the pharmacological profile of 6,3′-dinitroflavone in chronically treated animals. This type of experiment, together with those evaluating the effects of acute oral administration, are being carried out currently.

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