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6-Chloro-3′-nitroflavone is a Potent Ligand for the Benzodiazepine Binding Site of the $GABA_A$ Receptor Devoid of Intrinsic Activity

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VIOLA, H., C. WOLFMAN, M. MARDER, J. D. GOUTMAN, M. BIANCHIN, C. WASOWSKI, D. J. CALVO, I. IZQUIERDO, A. C. PALADINI AND J. H. MEDINA. *6-Chloro-3′*-nitroflavone is a potent ligand for the benzodiazepine binding site of $GABA_A$ receptor devoid of intrinsic activity. PHARMACOL BIOCHEM BEHAV **65**(2) 313–320, 2000.—6-Chloro-3′-nitroflavone integrates a list of nearly 70 flavone derivatives synthesized in our laboratories. The effects of 6-chloro-3′-nitroflavone on the benzodiazepine binding sites (BDZ-BSs) of the $GABA_A$ receptor were examined in vitro and in vivo. 6-Chloro-3′-nitroflavone inhibited the [^{3}H]flunitrazepam ([^{3}H]FNZ) binding to rat cerebral cortex membranes with a K_i of 6.68 nM and the addition of GABA to extensively washed membranes did not modify its affinity for the BDZ-BSs (GABA-shift = 1. 16 ± 0.12). The binding assays performed in rat striatal and cerebellar brain membranes showed that this compound has similar affinity to different populations of BDZ-BSs. Electrophysiological experiments revealed that 6-chloro-3′-nitroflavone did not affect $GABA_A$-receptors ($GABA_A$-Rs) responses recorded in *Xenopus* oocytes expressing $\alpha_1\beta_2\gamma_{2s}$ subunits, but blocked the potentiation exerted by diazepam (DZ) on GABA-activated chloride currents. In vivo experiments showed that 6-chloro-3′-nitroflavone did not possess anxiolytic, anticonvulsant, sedative, myorelaxant actions in mice or amnestic effects in rats; however, 6-chloro-3′-nitroflavone antagonized diazepam-induced antianxiety action, anticonvulsion, short-term, and long-term amnesia and motor incoordination. These biochemical, electrophysiological, and pharmacological results suggest that 6-chloro-3′-nitroflavone behaves as an antagonist of the BDZ-BSs. © 2000 Elsevier Science Inc.

Flavonoids Benzodiazepine binding site of the $GABA_A$ receptor Antagonist

A large series of compounds involved in the modulation of anxiety, sedation, convulsion, myorelaxation, hypnotic, and amnestic states interact with the benzodiazepine binding site (BDZ-BS) of the $GABA_A$ receptor. The $GABA_A$ receptor complex is a pentameric ionotropic receptor permeable to chloride ions (16). The combinatorial association of the different subunits of this complex gives rise to pharmacological receptor subtypes, unevenly distributed in brain (e.g., type I is predominantly localized in cerebellum, and type II in striatum and hippocampal areas) (22,32,37). The selective interaction with a given receptor type and/or the efficacy level displayed by a compound determines a wide spectrum of pharmacologi-

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cal activities. Some drugs interact with the BDZ-BSs, and possess classical BDZ-like profiles (full or partial agonists) or cause BDZ-opposite actions (full or partial inverse agonists). Other drugs simply have no effects on the binding site, but block the action of different BDZ-BSs ligands (antagonists) (1).

Several BDZ-BSs antagonists have been synthesized. Ro 15-1788 is the most studied compound, and the only agent of this class that is clinically available. Ro 15-1788 is useful in the treatment of hepatic encephalopathy and benzodiazepine overdose, and it has been suggested as an agent that reverses benzodiazepine-related amnesia, epilepsy, sleep and cognitive disorders, and the symptoms of benzodiazepine tolerance and withdrawal (1). However, due its quite short half-life and its anxiogenic effect exhibited in some behavioral tests (8,9,11,31), the development of novel BDZ-BS antagonists as new pharmacological tools for studying the pathophysiology of the BDZ-BS is of relevance.

This work describes the pharmacological profile of 6-chloro-3′-nitroflavone, a potent ligand for the BDZ-BSs with antagonistic properties. This molecule, not chemically related to BDZs, belongs to a series of halo and/or nitro flavonoid derivatives synthesized in our laboratories [for review, see (25,26)]. Our first report demonstrating that naturally occurring flavonoids interact with the BDZ-BSs was included in a study of the active principles of plants used in folkloric medicine as "tranquilizers" (23). Subsequently, we isolated and identified other flavonoids from various plant extracts, also acting through the BDZ-BSs (24,35,36,40). Owing to the moderate affinity (low μM range) for the BDZ-BSs of these natural flavonoids, we attempted to increase their biochemical potency by chemically introducing electronegative groups in their molecules (33). Thus, about seventy substituted flavones were generated by introducing one or more halogen atoms and/or nitro groups in them (19–21). The main findings of the subsequent structure–activity studies were: 1) a pronounced increase in affinity for the BDZ-BSs of some derivatives compared to the natural flavonoids; 2) the existence of favored positions in the flavone nucleus where the substitutions are highly effective, and 3) that some electronegative groups are more effective than others, as measured both in binding studies and in behavioral tests performed in animals (18,38,39).

In this work, we report that the incorporation of a chlorine atom in position 6 and a nitro group in position 3′ of the flavone molecule gives rise to the first compound with high affinity for the BDZ-BSs but devoid of intrinsic activity.

METHOD

Subjects

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 the inhibitory avoidance test, which was carried out in rats. Animals were housed in a controlled environment, with free access to food and water and maintained on a 12L:12D cycle. *Xenopus laevis* oocytes were used for electrophysiological studies. Frogs were purchased from Xenopus One (Ann Arbor, MI, and Nasco, Modesto, CA).

Biochemical Experiments

Displacement curves were performed using [^{3}H]FNZ as radioligand in washed crude synaptosomal membranes from rat cerebral cortex, cerebellum, or striatum. Membrane preparations were carried out according to Medina et al. (24). Briefly, brains were rapidly dissected out on ice and the different structures were homogenized in 10 vol/0.32 M sucrose and centrifuged at 900 × *g* for 10 min. The resulting supernatant was centrifuged at 100,000 × *g* for 30 min, and the pellet washed twice in 25 mM Tris HCl buffer pH 7.4 at 100,000 × *g* for 30 min, and stored at −20°C until used.

For [^{3}H]FNZ (84 Ci/mmol, NEN) displacement curves, different concentrations of 6-chloro-3′-nitroflavone (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 presence of 0.5 nM of the radioligand. Protein determination was carried out by using the Lowry's method. Nonspecific binding (<5%) was determined in parallel incubations with 10 μM FNZ (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. Filters were dried and counted after the addition of 5 ml 2,5-diphenyl-oxazole (PPO)-xylene as scintillation fluid.

We also performed displacement curves using [^{3}H]FNZ as the radioligand in extensively washed crude synaptosomal membranes from rat cerebral cortex in the presence or absence of GABA 100 μM. The GABA shift was calculated as the ratio between the K_i values obtained in the absence versus the presence of GABA (4).

Electrophysiological Experiments

Expression of $GABA_A$-Rs in Xenopus laevis *oocytes.* Plasmids (generously provided by P. Whiting, UK) containing the sequences of the α_1, β_2, or γ_2 subunits of the $GABA_A$-Rs cloned in the vector pCDM8 were used.
$GABA_A$-R α_1, β_2, or γ_2s subunits were propagated in the bacterial strain MC 1061/p3 (InVitroGene) and then plasmidic DNA mini preps were carried out. DNAs were precipitated by addition of ethanol, the pellets were dried and resuspended in water (molecular biology grade, RNAse and DNAse free, Ambion). Solutions of the DNA clones in the proportion 2:2:1 for α_1:β_2:γ_{2s}, respectively, were prepared and used for the expression of functional $GABA_A$-Rs with the above subunits composition.

Follicle-enclosed oocytes (27) were microinjected intranuclearly, using a manual microinjector (Drummond), with 2 ng of $\alpha_1\beta_2\gamma_{2s}$ DNA (2 mg/ml), and stored in Barth's medium (88 mM NaCl; 0.33 mM $Ca(NO_3)_2$; 0.41 mM $CaCl_2$; 1.0 mM KCl; 0.82 mM $MgSO_4$; 2.4 mM $NaHCO_3$; 10 mM HEPES; pH adjusted to 7.4 with NAOH, with 0.1 mg/ml gentamycin). The oocytes were defolliculated 48 h after injection using collagenase (27).

Voltage-clamp recording in Xenopus leavis *oocytes.* Recordings were performed using a two-electrode voltage-clamp amplifier (Axoclamp 2B, Axon Instruments), placing the oocytes in a 0.1-ml chamber continuously perfused (7–10 ml/min) with frog Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7). Membrane current responses were recorded from >30 oocytes taken from three frogs, with the membrane potential held at −70 MV. Uninjected oocytes gave no significant response to GABA (<1 nA). Current traces were collected on a chart recorder (Kipp & Zonen BD 41), or acquired through a TL-1 DMA interface using AXOTAPE (both Axon instruments) and a PC. All the experiments were carried out at room temperature (20–23°C).

Drugs delivered by perfusion took about 6 ± 1 s to exert their effects. BDZs were added 30 s before GABA. The same protocol was used for testing 6-chloro-3′-nitroflavone. For the

experiments where the 6-chloro-3′-nitroflavone was assayed together with diazepam (DZ), flavonoid was delivered 30 s before the BDZ addition started.

Behavioral Experiments

Elevated plus-maze test. The test (performed in the same session immediately after the locomotor activity assay) (36,40) is widely validated for rodents (17,30), and possesses several advantages over other tests for measuring anxiety (6). A selective increase in the number of entries in the open arms and/or the time spent in the open arms reveals an anxiolytic effect of the drug (30). The IP injections were administered 20 min before the test.

Seizure testing. The effects of 6-chloro-3′-nitroflavone on pentylenetetrazole (PTZ)-induced convulsions were evaluated according to Medina et al. (24), with slight modifications. PTZ (80 mg/kg) is administered IP to mice 15 min after injection of drug or vehicle. The number of mice presenting clonic–tonic convulsions is determined.

Inhibitory avoidance test. This test was performed according to Izquierdo et al. (15). The training apparatus is a 50 × 25 × 25 cm acrylic box with a frontal glass 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 is placed on the left extreme of the box. Rats are placed on the platform, and their latency to step down placing their four paws on the grid is measured. Upon stepping down they receive a 0.35-mA, 2-s duration scrambled footshock, and are withdrawn from the box (training session). The test session is carried out 1.5 h and 24 h later, and is similar to the training session in all respects except that the footshock is omitted. The step-down latency (to a ceiling of 180 s) was taken as a measure of retention of inhibitory avoidance (15). The rats were IP injected with vehicle, 6-chloro-3′-nitroflavone, DZ, or 6-chloro-3′-nitroflavone+DZ immediately before the training session.

Rotarod test. Male mice Swiss (25–30 g) were placed on a horizontal metal rod (diameter 3 cm) rotating at a rate of 10 rpm, 27 cm above the bench. The mice were submitted to a training session, and those that fell less than three times in 2 min were selected. In the test session, performed 24 h later, the number of failings during 3 min were measured, 15 min after the IP injection of vehicle, 6-chloro-3′-nitroflavone, DZ, or 6-chloro-3′-nitroflavone+DZ.

Locomotor activity test. An Opto-varimex^R apparatus was used according to Viola et al. (36). 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 following tests in mice, animals were IP injected with the vehicle (control) or with 6-chloro-3′-nitroflavone 20 min before the beginning of the tests. In each session, control mice were tested in parallel with those animals receiving drug treatment.

Holeboard test. The test was performed according to Viola et al. (36) and Wolfman et al. (40). The number of head dips and the time spent head dipping were counted during 5 min. A decrease in these parameters reveals a sedative behavior (10). The IP injections were performed 20 min before testing.

Horizontal wire test. This test was carried out as previously described (35,36,40). The test takes place after two trials, performed at 5-min intervals. A myorelaxant drug impairs mice to grasp the wire (3). The IP injections were made after trials, 20 min before testing.

Drugs Solutions and Injection Procedures

The synthesis of 6-chloro-3′-nitroflavone (Fig. 1) was carried out in our lab (20). 6-Chloro-3′-nitroflavone and DZ (Hoffmann–La Roche) were dissolved in dimethylsulfoxide 20%, ethanol 10%, in distilled water. The volume of IP injections was 0.1 ml/10 g in mice and 0.1 ml/100 g in rats. The antagonistic effects of 6-chloro-3′-nitroflavone were tested performing coadministrations of DZ plus 6-chloro-3′-nitroflavone.

Statistical Analyses

The competition curves were analyzed using the GraphPad Prism software. ANOVA was used when several mice treatments were compared. Post hoc comparisons between individual treatments and controls were made using Dunnett's

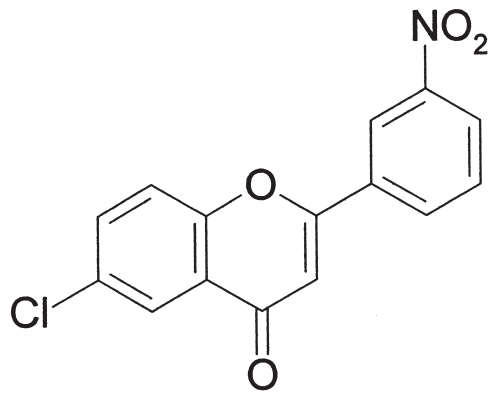


FIG 1. 6-Chloro-3′-nitroflavone.

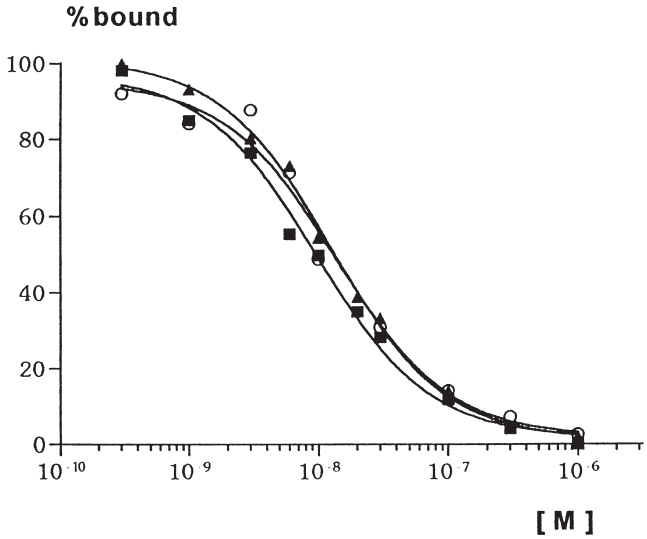


FIG 2. Displacement curves generated for the 6-chloro-3′-nitroflavone competition of [^{3}H]FNZ binding to washed crude synaptosomal membranes from various rat brain regions. Membranes from cerebellum (○), cerebral cortex (■), and striatum (▲) were prepared as described in the Method section. Data are from representative experiments replicated three to six times.

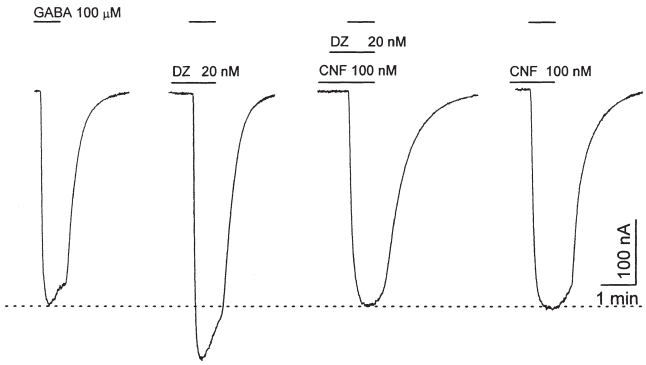


FIG 3. Effect of 6-chloro-3′-nitroflavone (CNF) on the DZ-induced potentiation of $GABA_A$-Rs ($\alpha_1\beta_2\gamma_2$s) expressed in *Xenopus* oocytes. Macroscopic ionic currents (Cl^-) were recorded using a two-electrodes voltage clamp. Membrane potential was held at −70 mV and inward currents denoted as downward deflections of the trace. GABA responses were elicited by the application of GABA 100 μM (upper bars) for all the records in the figure. DZ was delivered 30 s before GABA and both removed at the same time. 6-Chloro-3′-nitroflavone alone was applied 30 s before GABA, or 30 s before DZ when coapplied with DZ (6-chloro-3′-nitroflavone + DZ).

multiple comparisons test. Chi-square frequency test was used when required. Nonparametric Mann–Whitney *U*-test was used for the analysis of inhibitory avoidance and rotarod results.

RESULTS

Biochemical Experiments

6-Chloro-3′-nitroflavone had similar potency in displacing [^{3}H]FNZ binding in various CNS regions (Fig. 2). The K_i values obtained in cerebral cortex, cerebellum, or striatum were 6.68 ± 1.77, 7.56 ± 1.78, and 8.27 ± 1.27 nM, respectively (mean ± SEM, three to six independent determinations). No differences were found in the K_i values obtained in similar experiments performed with extensively washed cerebral cortical membranes incubated in the presence or in the absence of GABA, yielding a GABA shift of 1.16 ± 0.12 (n = 5).

As already shown for other flavone derivatives with halogen atom and/or nitro groups in position 6 and 3′ [see (26,38,39)], 6-chloro-3′-nitroflavone appears to be a selective BDZ-BS ligand because it did not displace (10 μM) [^{3}H]AMPA, [^{3}H]8-OH-DPAT, [^{3}H[Ketanserine, and [^{3}H]CCPA bindings to AMPA glutamate, 5-HT_{1A}, 5-HT_{2A}, and A_1 receptors.

Electrophysiological Experiments

Recombinant $\alpha_1\beta_2\gamma_2$s $GABA_A$-Rs from rat were expressed in *Xenopus* oocytes, and their responses recorded. Different concentrations of GABA (1 μM–1 mM) in the perfusion chamber elicited ionic currents carried by Cl^-, which were antagonized by bicuculline methyl-bromide or picrotoxin, and potentiated by different BDZs, alphaxalone, or pentobarbital.

DZ (20–30 nM), as expected, potentiated $GABA_A$ responses. Using a GABA concentration of 100 μM the enhancing effect of DZ was of about 25% (+24.8 ± 0.7%, n = 3), while when using GABA 30 μM, the DZ-induced potentiation was 90% (+89.7 ± 10.4%, n = 4). These effects were totally surmontable by an equivalent concentration of RO 15-1788 (data not shown).

6-Chloro-3′-nitroflavone produced similar effects to those observed with RO 15-1788. In fact, flavonoid applications (100 and 300 nM) completely inhibited the potentiation of the GABA (100 μM) responses after a brief exposure to DZ (20 nM) (Fig.3). At a lower GABA concentration (30 μM), DZ

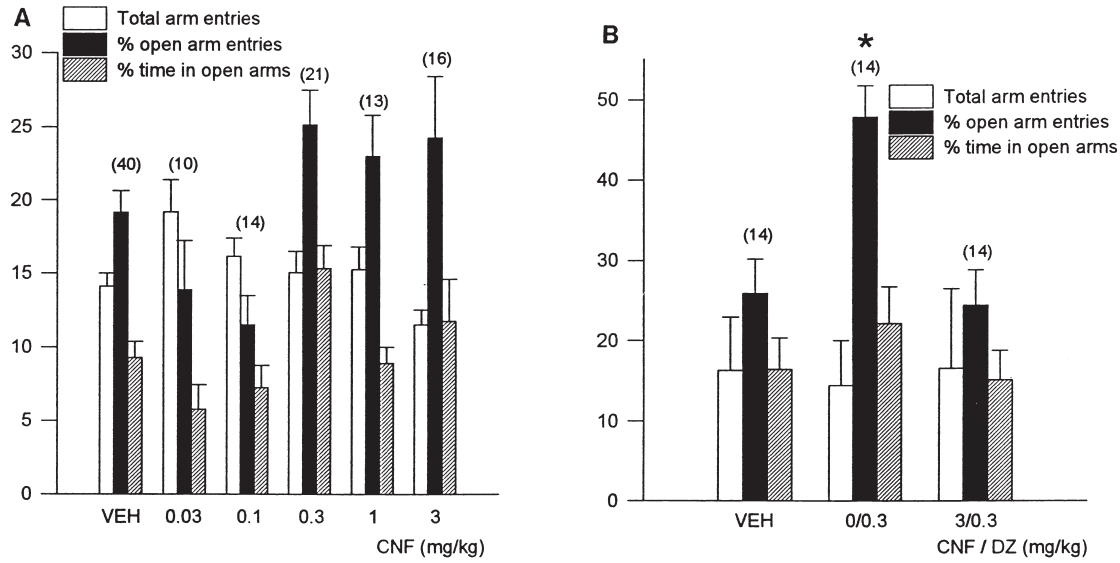
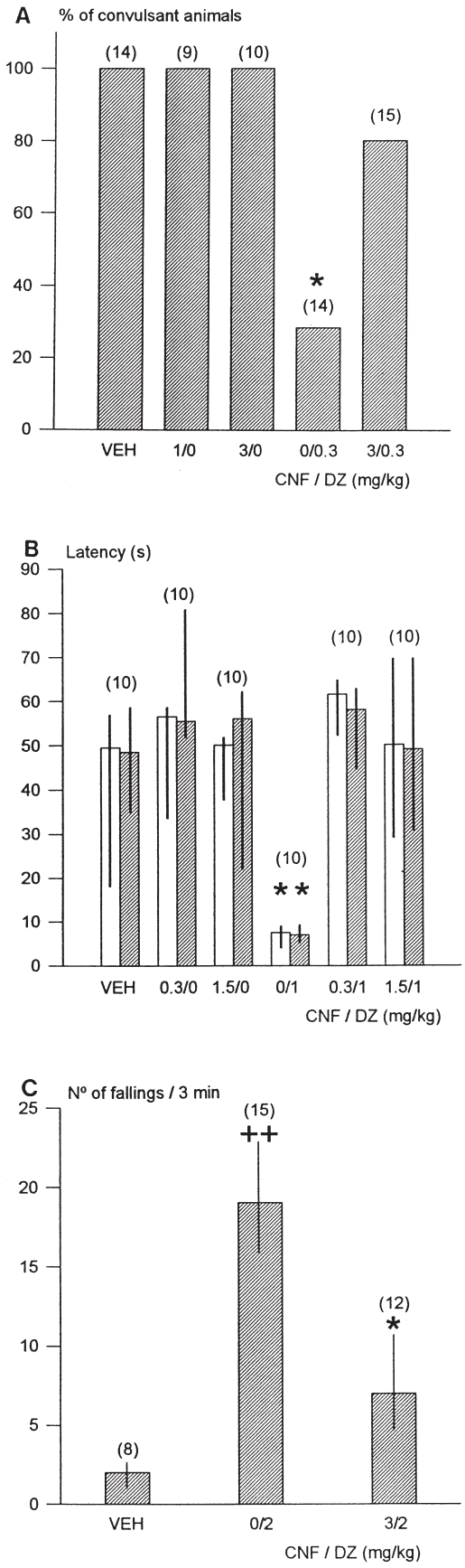


FIG 4. (A) Mean ± SEM of the parameters registered in a 5-min session in the elevated plus-maze 20 min after IP injection of vehicle (VEH) or 6-chloro-3′-nitroflavone (CNF, 0.03–1 mg/kg) in mice, $p > 0.05$, Dunnett's test compared to vehicle. Number of animals per group is shown in parentheses. (B) Antagonist effect of 6-chloro-3′-nitroflavone on anxiolysis produced by DZ. Mean ± SEM of the parameters registered in a 5-min session in the elevated plus-maze 20 min after IP injection of vehicle (VEH), DZ 0.3 mg/kg or 6-chloro-3′-nitroflavone 3 mg/kg plus DZ 0.3 mg/kg (DZ + CNF) in mice. *$p < 0.01$, significantly different (Tukey–Kramer multiple comparison test). Number of animals per group is shown in parentheses.



produced a larger potentiating effect (about 90%); in this condition, 6-chloro-3′-nitroflavone (300 nM) was able to block up to a 25% of the DZ effect (data not shown). The antagonism of DZ action by 6-chloro-3′-nitroflavone was reversible after washing the oocytes with Ringer's solution.

6-Chloro-3′-nitroflavone alone was unable to produce significative effects up to a concentration of 300 nM.

Behavioral Experiments

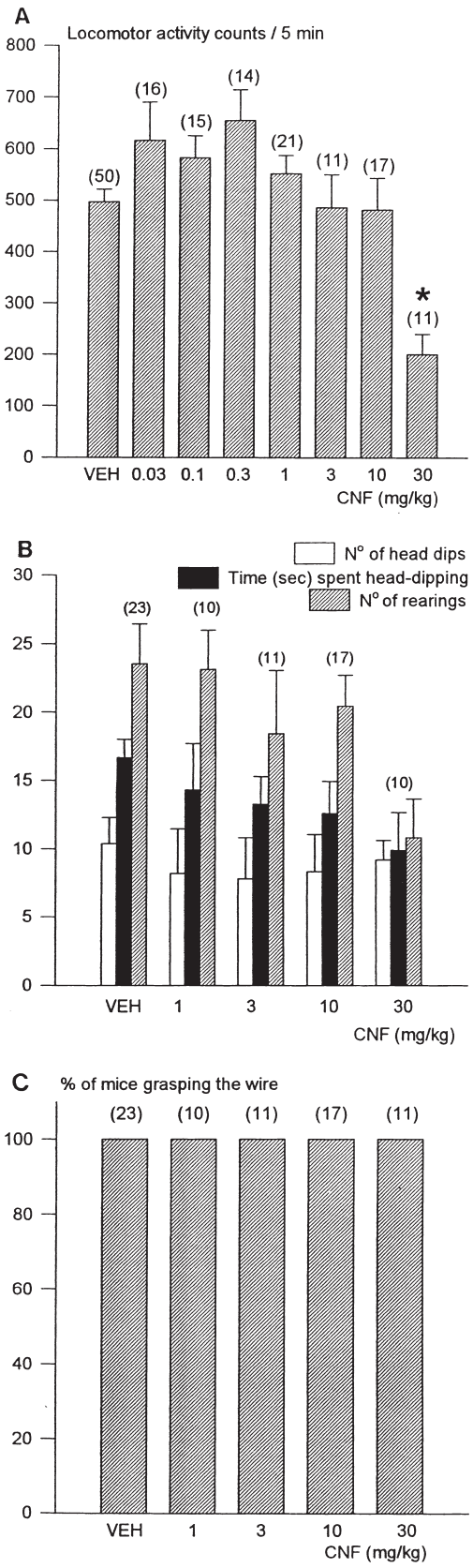
Effect of 6-chloro-3′-nitroflavone on the elevated plus-maze test. 6-Chloro-3′-nitroflavone (0.03–3 mg/kg) was IP injected in mice and no significant anxiolytic effect was detected in the elevated plus-maze test (Fig. 4). Experiments run in parallel using 0.3 mg/kg DZ, revealed that this well-known anxiolytic drug produced an increase in the percentage of open-arm entries ($p < 0.01$, Tukey–Kramer multiple comparison test) (Fig. 4B). The anxiolytic action of DZ (0.3 mg/kg) was blocked by the coadministration of 3 mg/kg of 6-chloro-3′-nitroflavone (Fig. 4B).

Effect of 6-chloro-3′-nitroflavone on pentylenetetrazole-induced convulsions. 6-Chloro-3′-nitroflavone (1 or 3 mg/kg), did not prevent seizures induced by 80 mg/kg PTZ in mice (Fig. 4A). In contrast, DZ (0.3 mg/kg) showed anticonvulsant activity ($p < 0.001$, χ^2 test) (Fig. 5A). The coadministration of 6-chloro-3′-nitroflavone (3 mg/kg) with DZ (0.3 mg/kg) antagonized the anticonvulsant action of DZ (Fig. 5A).

Effect of 6-chloro-3′-nitrolavone on learning. In rats, pretraining IP administration of 0.3 or 1.5 mg/kg of 6-chloro-3′-nitroflavone had no effect on the step-down latency registered in the inhibitory avoidance test session performed either at 1.5 or 24 h after training (Fig. 5B). In this paradigm, 1 mg/kg DZ produced an amnestic effect ($p < 0.02$, Mann–Whitney U-test) that was antagonized by the administration of 6-chloro-3′-nitroflavone in doses of 0.3 or 1.5 mg/kg. (Fig. 5B).

Effect of 6-chloro-3′-nitroflavone on the rotarod test. 6-Chloro-3′-nitroflavone (3 mg/kg) did not impair the motor coordination measured in the rotarod test (2.25 ± 1.25 vs. vehicle = 1.17 ± 0.36). However 6-chloro-3′-nitroflavone (3 mg/kg) antagonized the motor incoordination produced by the IP injection of 2 mg/kg of DZ (Fig. 5C) ($p < 0.05$, Dunn's Multiple Comparison test).

FIG 5. (A) Antagonist effect of 6-chloro-3′-nitroflavone on anticonvulsion induced by DZ. The mice were IP injected with vehicle (VEH), 6-chloro-3′-nitroflavone (CNF, 1–3 mg/kg), DZ (0.3 mg/kg), or 6-chloro-3′-nitroflavone 3 mg/kg plus DZ 0.3 mg/kg, 15 min before the IP administration of pentylenetetrazole 80 mg/kg. The occurrence of clonic–tonic seizures was registered. The data are expressed as percentage of convulsant animals. *$p < 0.001$, chi-square test. Number of animals per group is shown in parentheses. (B) Effect of pretraining IP administration of vehicle (VEH), 6-chloro-3′-nitroflavone (CNF, 0.3 or 1.5 mg/kg), DZ (1 mg/kg), or 6-chloro-3′-nitroflavone (0.3 or 1.5 mg/kg) plus DZ (1 mg/kg) on memory of an inhibitory avoidance test. The ordinate represents the step-down latency (in seconds) of the test session performed at 1.5 h (open bars) and 24 h (hatched bars). Data are expressed as medians (interquartile range). *$p < 0.02$, Mann–Whitney U, two-tailed test. Number of animals per group is shown in parentheses. (C) Antagonistic effect of 6-chloro-3′-nitroflavone on the motor incoordination produced by DZ. Medians (interquartile range) of number of failings registered during 3 min in the rotarod test, 15 min after the IP injection of vehicle (VEH), DZ (2 mg/kg), or DZ (2 mg/kg) plus 6-chloro-3′-nitroflavone (CNF, 3 mg/kg). *$p < 0.05$, significantly different from diazepam; ++$p < 0.001$, significantly different from vehicle, Dunn's Multiple Comparison test. Number of animals per group is shown in parenthesis.



Effect of 6-chloro-3′-nitroflavone on the ambulatory locomotor activity. Figure 6A shows that the IP administration of 6-chloro-3′-nitroflavone (0.03–10 mg/kg) had no effect on spontaneous ambulatory locomotion. Only at 30 mg/kg (the highest dose tested), there was a 60% reduction in the locomotion ($p < 0.01$, Dunnett multiple comparison test).

Effect of 6-chloro-3′-nitroflavone on the holeboard test. Performance of mice injected with vehicle or 6-chloro-3′-nitroflavone in the holeboard test is shown in Fig. 6B. As can be seen, doses up to 30 mg/kg did not change the number of head dips, the time spent head dipping, or the number of rearings.

Effect of 6-chloro-3′-nitroflavone on the horizontal-wire test. 6-Chloro-3′-nitroflavone, at doses up to 30 mg/kg, did not affect the percentage of mice grasping the wire (Fig. 6C), indicating that 6-chloro-3′-nitroflavone does not produce myorelaxant effects.

DISCUSSION

Recently, many flavone derivatives that recognize specifically and selectively the BDZ-BSs were synthesized in our laboratories (20,21,26). 6-Chloro-3′-nitroflavone and other flavone derivatives with halogen atoms and/or nitro groups in positions 6 and 3′ were found to be the most potent BDZ-BSs ligands. (19,26,34,39). Previous pharmacological studies demonstrated that 6,3′-dinitroflavone and 6-bromo-3′-nitroflavone behave as partial agonists of the BDZ-BSs (38,39). The present findings suggest that the incorporation of chlorine atom in position 6 gives rise to a compound (6-chloro-3′-nitroflavone) with a different pharmacological profile, endorsing the assumption that the nature of the substitution in position 6 is important to establish the pharmacological properties of these compounds.

Ai et al. (2) have also shown that 6-methylflavone is a competitive BDZ-BSs ligand with a GABA shift close to unity, suggesting that this flavonoid has an antagonistic profile in vitro. Unfortunately, no in vivo data was reported.

The main finding of the present study is that 6-chloro-3′-nitroflavone is the first flavonoid with pharmacological properties of a BDZ-BSs antagonist, both in in vitro and in in vivo experiments. This conclusion is based on the following set of data: (a) 6-chloro-3′-nitroflavone is a potent, nonselective, ligand for different populations of BDZ-BSs, whose GABA-shift is close to unity. The GABA shift is used as a biochemical index to establish the intrinsic activity of ligands interacting with the BDZ site of the $GABA_A$-Rs complex. This effect is due to the allosteric interactions induced by GABA on the BDZ-BSs (2,4,7). (b) 6-Chloro-3′-nitroflavone has no significant effect on the $GABA_A$-R mediated Cl^- currents, but is able to antagonize DZ-induced potentiation of these $GABA_A$ responses. (c) 6-Chloro-3′-nitroflavone is devoid of anxi-

FIG 6. (A) Ambulatory locomotor activity counts during a 5-min test session in a Opto-varimex^R apparatus 15 min after an IP injection of vehicle (VEH) or 6-chloro-3′-nitroflavone (CNF, 0.03–30 mg/kg). Data are expressed as mean ± SEM. Number of animals per group is shown in parentheses. *$p < 0.01$, significantly different from vehicle, Dunnett's *t*-test after ANOVA. (B) Mean ± SEM of the parameters registered in a 5-min session in the holeboard test 20 min after an IP injection of vehicle (VEH) or 6-chloro-3′-nitroflavone (CNF, 1–30 mg/kg) in mice. $p > 0.05$, ANOVA. Number of animals per group is shown in parentheses. (C) Performance of mice in the horizontal wire test after an IP injection of vehicle (VEH), 6-chloro-3′-nitroflavone (CNF, 1–30 mg/kg). The session test took place after two trials, executed after a 5-min interval (see the Method section). Number of animals per group is shown in parentheses.

olytic, anticonvulsant, sedative, myorelaxant, or amnestic actions. (d) Although 6-chloro-3′-nitroflavone is pharmacologically inert at the BDZ-BSs, it antagonizes several effects provoked by DZ, such as anxiolysis, anticonvulsion, the loss of memory, and the motor incoordination.

This pharmacological characterization led us to postulate 6-chloro-3′-nitroflavone as an antagonist of the BDZ-BSs, devoid of effects on the $GABA_A$–chloride channel complex but antagonizing those of agonists.

The pharmacological characterization of 6-chloro-3′-nitroflavone resembles that reported for Ro 15-1788, the most studied BDZ-BSs antagonist (3,14). In their pioneering work, Haefely and his co-workers concluded that Ro 15-1788 is devoid of BDZ-like activity, almost devoid of intrinsic actions, and is a very potent and selective antagonist of the pharmacological effects of BDZs. There are many evidences of the antagonistic role of Ro 15-1788 both in anxiolytic (3,31) and in anxiogenic states (8,9,28) evaluated in plus-maze, social interaction, two-way active avoidance, or conflict tests in rodents.

In addition, Ro 15-1788 blocks the amnestic action of BDZs measured in a passive avoidance paradigm, the same utilized in the present study, or in a discriminative learning test in rodents (3,5) and in human memory tests (12), and antagonizes the anticonvulsant effect of DZ (3,13,29).

Several reports, however, were coincident in demonstrating the anxiogenic effects of Ro 15-1788, generally at high doses (8,9,11,31). Unlike Ro 15-1788, no intrinsic action was found for 6-chloro-3′-nitroflavone in the behavioral experiments included in this study. Based on biochemical and electrophysiological experiments and behavioral tests performed in rodents, we provide evidence revealing that 6-chloro-3′-nitroflavone is a pure BDZ-BSs antagonist.

We have already reported that natural and synthetic flavone derivatives are BDZ-BSs ligands (26). This family of compounds exhibits a wide spectrum of pharmacological profiles ranging from full agonists (19), partial agonists (36,38–40) to the novel antagonist described in this article, 6-chloro-3′-nitroflavone.

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